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TOWARDS A 3D CULTURE OF OVARIAN FOLLICLES: Transcriptome- and molecular-based discovery of cumulus cell markers of mouse oocyte quality

PhD Thesis by
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*"**Ognuno è un genio.***

Ma se si giudica un pesce dalla sua abilità
di arrampicarsi sugli alberi,
lui passerà l'intera vita a credersi stupido."
Albert Einstein

Abstract

L'infertilità è una patologia del sistema riproduttivo, definita come inabilità al concepimento, che si stima riguardare circa il 15% delle coppie in età fertile. Inoltre, l'infertilità rappresenta uno dei principali effetti collaterali delle terapie di cura del cancro, quali la radio e chemioterapia, note per il loro effetto gonadotossico e responsabili dell'insorgenza di decadimento prematuro della funzione ovarica; circa un terzo delle pazienti oncologiche così trattate (~100.000/anno in Italia and ~1.000.000/anno negli Stati Uniti), infatti, perde la propria capacità riproduttiva. Ad oggi, la principale strategia di preservazione della fertilità è la crioconservazione di biopsie di tessuto ovarico o di singoli follicoli immaturi che possono, in un secondo momento, essere recuperati e coltivati *in vitro* fino al completamento della loro maturazione. Nonostante i numerosi sistemi di coltura messi a punto ed ottimizzati e i notevoli passi avanti compiuti recentemente, le rese di maturazione di questi follicoli immaturi rimangono piuttosto basse, così come sono tuttora poco efficienti le attuali tecniche di fecondazione *in vitro* (FIV): solo circa il 30% dei trasferimenti embrionali ha un esito positivo.

Il lavoro svolto durante il mio dottorato si inserisce in questo contesto in quanto parte di un progetto multidisciplinare a lungo termine, chiamato "Bioingegnerizzazione del follicolo ovarico", il cui principale obiettivo è la progettazione e messa a punto di un sistema tridimensionale (3D) di coltura *in vitro* di follicoli ovarici. Tale sistema prevede il mantenimento dell'organizzazione 3D del follicolo durante l'intero processo maturativo, mimando nel miglior modo possibile le fisiologiche condizioni di crescita di queste cellule in vivo, con lo scopo di ricreare artificialmente il microambiente ovarico.
A tal proposito, i due principali scopi della mia tesi sono stati i seguenti:

1. *L'identificazione di marcatori della competenza allo sviluppo di oociti murini nella porzione somatica del follicolo, attraverso l'utilizzo di strumenti molecolari e bioinformatici.*
   L'oogenesi è un processo che porta alla maturazione di un elevato numero di follicoli, di cui la maggior parte è di bassa qualità e solo un numero ristretto è competente allo sviluppo. Quando oociti antrali maturi di mammifero sono marcati utilizzando un fluorocromo che metta in risalto l'organizzazione cromatinica, possono essere facilmente classificati in: oociti che presentano un anello di eterocromatina attorno al nucleolo (SN) ed oociti che presentano una cromatina più lassa nel nucleo (NSN). Se maturati fino allo stadio di metafase II (MII) e fecondati *in vitro*, solo

oociti di tipo SN danno origine ad embrioni in grado di sostenere lo sviluppo fino a termine, mentre embrioni derivati da oociti NSN si bloccano sempre allo stadio di due cellule. Benché sia possibile utilizzare il fluorocromo a concentrazione vitale, una classificazione di questo tipo non può essere utilizzata nella pratica clinica, in quanto prevede il trattamento diretto del gamete con una sostanza potenzialmente dannosa. Con lo scopo di ricercare un marcatore molecolare ma non invasivo che distingua i due tipi di oociti, ho analizzato gruppi delle cellule somatiche del cumulo (CCs) che li circondano. In primo luogo, grazie ad una collaborazione con i gruppi del Prof. Riccardo Bellazzi (Università degli Studi di Pavia, Italia) e del Prof. James Adjaye (Università di Düsseldorf, Germania), ho confrontato l'espressione genica delle due popolazioni di cellule a livello dell'intero trascrittoma attraverso un'analisi con microarrays. Questa analisi ha mostrato un simile profilo di espressione genica ed ha permesso di identificare una lista di 422 geni differenzialmente espressi, la quasi totalità dei quali (412) down-regolati in CCs appartenenti ad oociti non competenti allo sviluppo e solo 10 up-regolati. Successivamente, una serie di analisi bioinformatiche ha caratterizzato questi geni e fatto emergere una lista di 27 trascritti significativamente coinvolti nella funzionalità ovarica e del follicolo. A conferma dei risultati così ottenuti, mi sono focalizzata su 5 geni specifici delle CCs (*Has2*, *Ptgs2*, *Ptx3*, *Tnfaip6* and *Amh*) dei quali ho analizzato l'espressione tramite qRT-PCR. Tra le sequenze studiate, *Amh* si è distinta per essere la sequenza più differenzialmente espressa, mostrando un'espressione 4 volte maggiore in CCs attorno ad oociti non competenti allo sviluppo. Tale risultato è stato confermato anche a livello proteico da uno studio dell'espressione di AMH tramite immunofuorescenza. Inoltre, siccome lo scopo di un eventuale marcatore è il suo utilizzo diagnostico nella selezione degli oociti di miglior qualità da sottoporre a FIV, ho proseguito il mio studio analizzando l'espressione di Amh a livello del singolo cumulo ooforo, confermando i risultati precedentemente ottenuti. La ricerca di marcatori è stata estesa allo studio di due geni e delle rispettive proteine, noti per il loro ruolo fondamentale durante la follicologenesi: Lhr ed Fshr. Per quanto riguarda i trascritti, solo Lhr ha mostrato differenze statisticamente significative, con una maggiore espressione in CCs circondanti oociti di bassa qualità. Lo studio delle proteine, invece, ha rivelato una differenza di espressione sia di FSHR che LHR, entrambe maggiormente espresse in CCs di oociti non competenti e quindi potenziali marcatori della competenza allo sviluppo.

2. *La messa a punto di un protocollo per la coltura in 3D di follicoli ovarici immaturi: un microambiente che mimi al meglio le naturali condizioni di maturazione presenti all'interno dell'ovario.*

Durante tutta la follicologenesi, la cellula germinale e le cellule somatiche che la circondano sono strettamente connesse le une alle altre, in uno stato di continua comunicazione bidirezionale, fondamentale affinché entrambe le componenti maturino correttamente a formare la complessa struttura 3D del follicolo antrale preovulatorio. Recentemente, l'attenzione dei ricercatori si è spostata verso lo sviluppo di nuove tecniche di coltura in 3D che stanno mostrando risultanti incoraggianti, sebbene ancora preliminari. Dopo un'attenta analisi di tutti i biomateriali ad oggi utilizzati nel tentativo di mimare la ricca matrice extracellulare dell'ovario, ho selezionato l'alginato di sodio, un polisaccaride naturale già ampiamente utilizzato per la coltura cellulare e per scopi medici. Un'analisi di diverse concentrazioni di polimero (comprese tra 0,25% e 2%) e di differenti soluzioni cross-linkanti (Ba^{2+}, Mg^{2+}, Ca^{2+}) ha permesso di selezionare la combinazione di 0,25% di alginato di sodio e 50 mM di $CaCl_2$ per i successivi esperimenti di coltura. Il passaggio più difficoltoso dell'intera procedura di preparazione della matrice e coltura dei follicoli ovarici è rappresentato dalla tecnica di incapsulamento delle cellule all'interno del biomateriale. Un primo tentativo è stata la creazione di capsule, che possono essere ottenute facendo gocciolare la soluzione cross-linkante contenente i follicoli immaturi direttamente all'interno della soluzione di polimero. Così facendo, solo la superficie esterna della goccia polimerizza a formare una matrice, mentre all'interno rimane un core liquido. Le strutture così ottenute risultavano, però, eccessivamente grandi rispetto ai piccoli follicoli incapsulati, avendo un diametro di circa 0,5 cm. Ho, quindi, cambiato strategia e provato a creare un unico letto di matrice che ricoprisse l'intera superficie della piastra Petri per la coltura dei follicoli. Per poter creare uno strato sottile, ho sviluppato un sistema di nebulizzazione in grado di nebulizzare microgocce di soluzione cross-linkante direttamente sulla superficie della soluzione di alginato di sodio liquido posta sul fondo della piastra Petri. Nonostante la facilità nell'ottenere il sottile strato di matrice, l'inserzione dei follicoli al suo interno, senza il loro danneggiamento ma assicurandosi che rimanessero intrappolati nel biomateriale, risultava molto difficoltosa.

Infine, ho ottimizzato una tecnica per l'incapsulamento del singolo follicolo in piccole sferette di alginato di calcio, ottenute facendo gocciolare la soluzione di polimero contenente le cellule all'interno della soluzione cross-linkante. Le sferette ottenute con questa metodologia risultano nettamente più piccole rispetto alle capsule ottenute con la tecnica inversa (circa 2 mm di diametro), ma altrettanto facili da maneggiare utilizzando una piccola spatola a cucchiaio. Grazie ad una collaborazione con il gruppo della Professoressa Conti (Università degli Studi di Pavia, Italia), la matrice è stata testata chimicamente per la sua capacità di resistere a stress osmotico e cambi di pH e temperatura,

risultando stabile e costante nella sua struttura. Inoltre, dati preliminari mostrano una sopravvivenza dei follicoli incapsulati e la loro crescita mantenendo integra la struttura tridimensionale fino a 6-7 giorni di coltura. Sono ora in corso esperimenti mirati all'ottimizzazione del protocollo di coltura stesso, al fine di ottenere la completa maturazione di follicoli immaturi fino allo stadio antrale preovulatorio.
Sto, quindi, studiando l'effetto della co-coltura di oociti e cellule del cumulo sull'acquisizione della competenza allo sviluppo di oociti murini, applicando il modello SN-NSN ed analizzando in entrambi i tipi cellulari specifici trascritti, col fine di determinare l'eventuale ruolo di SN-CCs e NSN-CCs nel promuovere la corretta maturazione degli oociti allo stadio di MII.

Abstract

Infertility is a pathology of the reproductive system, defined as the inability to conceive, estimated to affect about 15% of couples in their reproductive age. In addition to this pathological condition, infertility may arise as a major side effect of chemo or radio therapy, as a consequence of premature ovarian failure. About one-third of patients treated for cancer (~100.000/year in Italy and ~1.000.000/year in USA) lose their reproductive capability and, at present, the main strategy to preserve their fertility potential is the cryopreservation of either biopsies of ovarian tissue or isolated immature follicles. Subsequently, these could be retrieved and grown *in vitro* to complete maturation. Despite all the novel culture systems developed and the progresses made, the maturation rates of such immature follicles are still very low, as much as the efficiency of current *in vitro* fertilization (IVF) techniques: only ~30% of transferred embryos results in pregnancy.

To this regard, the work I performed during my PhD is part of a long-term, multidisciplinary project, named "Bioengineering the ovarian follicle", whose main objective is the delineation of a system for three-dimensional (3D) *in vitro* culture of ovarian follicles. This system should maintain the 3D organization of follicles during the whole maturation process and best mimic their natural growth conditions, in order to recreate artificially the ovarian microenvironment.

In the pursuit of this goal, two were the main specific aims of my thesis:

1. *The identification, by using molecular and bioinformatics tools, of markers of mouse oocyte developmental competence within the somatic compartment of the follicle.*

   Oogenesis is a process that leads to the maturation of high numbers of developmentally incompetent follicles among a low number of follicles of good quality. The hunt for markers that allow follicles selection is open. When mammalian fully-grown oocytes are stained with a fluorochrome that highlights their chromatin organization,

   they can easily be sorted into a group with a chromatin ring surrounding their nucleolus (SN) and another that lacks of this ring (NSN). When *in vitro* matured to the metaphase II (MII) and fertilized, whilst SN oocytes may develop to term, NSN oocytes never develop beyond the two-cell stage.

   In the need to find an objective, but non-invasive, marker able to discriminate between these two types of oocytes, I analysed pools of their

surrounding cumulus cells (CCs). First, thanks to a collaboration with the groups of Prof. Riccardo Bellazzi (University of Pavia, Italy) and Prof. James Adjaye (University of Düsseldorf, Germany) I compared the whole transcriptome of the two cell populations by microarrays, that highlighted a similar transcriptional profile, with only 422 genes differentially expressed, of which 412 down- and 10 up-regulated in CCs surrounding NSN developmentally incompetent oocytes. A series of bioinformatics analyses characterized this differentially expressed genes and brought up a list of 27 transcripts significantly involved in the follicle function. To confirm the results obtained, I focused my attention on 5 CC-related genes (*Has2*, *Ptgs2*, *Ptx3*, *Tnfaip6* and *Amh*) and analysed their expression by qRT-PCR. Among all the sequences studied, *Amh* stood up as the most differentially expressed, being 4-fold more expressed in CCs surrounding incompetent oocytes. This differential expression was further confirmed at the protein level, by immunofluorescence analyses. Furthermore, since the aim of a non-invasive marker would be its use as a diagnostic tool to select the best quality oocytes for IVF, I analysed *Amh* expression also at the level of a single cumulus oophorus, confirming my previous results.

To extend my search to other non-invasive markers, I focused on two genes and their respective proteins, known for their fundamental role in the follicle maturation process: Lhr and Fshr. Only *Lhr* transcript showed a different level of expression, higher in CCs of incompetent oocytes. Instead, both proteins were differentially expressed and, thus, resulted as good markers of developmental competence. Altogether, these works highlighted a short list of putative markers potentially capable of distinguishing between developmentally competent and incompetent follicles.

2. *The setting of a protocol for 3D follicle culture: a microenvironment that best mimic the natural maturation conditions found within the ovary.*

Throughout folliculogenesis, germ and somatic cells are tightly connected within the follicle in a state of constant bidirectional communication, which is necessary for both the components to correctly develop into a big, complex 3D structure. The endeavour of culturing ovarian follicles in a 3D environment has been attempted only recently and the results, although encouraging are still meagre. With this in mind, I set up to begin my work. After having examined all the biomaterials that have been used so far to mimic the rich extracellular matrix of the ovary, I focused on calcium alginate, a natural polysaccharide widely used for biological and medical purposes. I tested different polymer concentrations (ranging from 0.25% to 2%) and cross-linking molecules (Ba^{2+}, Mg^{2+}, Ca^{2+}) and choose a combination of 0.25% sodium alginate with 50 mM $CaCl_2$.

The most tricky passage was the encapsulation of the follicles. First, I tried to produce capsules by dripping the cross-linking solution containing the follicle into the polymer solution. So doing, only the surface of the drop polymerized and the core remained liquid, however, the structures obtained were far too big (~0.5 cm in diameter).

Then, I changed strategy and attempted to create a bed of matrix on the bottom of a Petri dish for follicles embedding. To obtain a thin layer, I set up a system for the nebulization of the cross-linking solution directly onto the liquid sodium alginate. In this case, the difficult step was the insertion of follicles into the matrix in a way that avoided the damaging of cells on one side and secured their entrapping within the alginate bed.

Finally, I optimized single follicle encapsulation into calcium alginate beads, by letting the liquid sodium alginate solution containing the follicle drip directly into the cross-linking solution. Beads were much smaller (~2 mm in diameter) than capsules and easy to handle with a spoon-shaped spatula. Thanks to a collaboration with the group of Prof. Conti (University of Pavia, Italy), the matrix obtained has been chemically tested for the capability to resist to osmotic, pH and temperature stresses and resulted stable and constant in its structure. Preliminary data show that encapsulated follicles are able to grow up to 6-7 days within the biomaterial, maintaining their 3D structure and expanding in all directions. Ongoing experiments are now trying to optimize the culture conditions that allow the complete follicle maturation until the antral preovulatory stage.

To this purpose, I am currently studying the effect of oocyte-cumulus cells co-culture on developmental competence of mouse oocytes by applying the SN-NSN model. In more detail, I analyse the expression of specific known oocyte- and CC-markers to investigate the role of SN-CCs and NSN-CCs in promoting the correct oocyte maturation to the MII stage.

Contents

Chapter 1

The medical issue: infertility

Infertility is a pathology of the reproductive system defined as the inability to conceive after 12 months of regular unprotected sexual intercourse. The European Society of Human Reproduction and Embryology (ESHRE) estimates that about 15% of couples in western countries experiences some form of infertility problem during its reproductive lifetime. A percentage of these cases are of genetic or physiological origin in women, being caused either by a pathology or by detrimental lifestyle factors such as smoking, high body-weight and stress, as well as increasing age.
Moreover, a growing number of women every year is diagnosed with cancer (~1.000.000/year in USA; ~100.000/year in Italy) and treated with chemo or radio therapy. These cytotoxic therapies are known to have a detrimental effect on the reproductive function, acting both on the ovarian reserve and on uterine vascularization. Indeed, about one third of oncologic patients develops premature ovarian failure (POF), which results in infertility and premature menopause. Given that a high percentage of these patients are children, teens and young women, fertility preservation programs have been developed and the main preservation strategies are: IVF followed by embryo freezing, direct oocyte freezing or cryopreservation of ovarian tissue biopsies prior to chemotherapy. In this case, at some point, immature follicles contained in the biopsies could be retrieved and *in vitro* grown up to the MII stage, to be *in vitro* fertilized. Up to date, a number of novel culture systems has been proposed, but the maturation rates of very immature follicles are still quite low and an efficient protocol for human primordial follicle growth is still lacking.

Assisted reproductive technology (ART) is the set of methodologies used to achieve pregnancy by artificial means, including *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI). Treatments for assisted reproduction are increasingly required and, since the first successful *in vitro* fertilization was performed on humans in 1978 (Steptoe and Edwards, 1978), around 5 million babies have been conceived worldwide.
One of the main strategies that are in focus at present is single embryo transfer, that would limit superovulation and consequently avoid the related health problems both of women and of the babies (Allen and Reardon 2005;

Market-Velker et al. 2010). In addition, this technique would decrease multiple births that are commonly associated with IVF. Despite the advances and significant improvements made in recent years, the success rates of IVF cycles are still rather low, with only the 30% of embryo transfers results in a pregnancy. Many are the variables that come into play and condition the success of the procedure, but the main protagonist is undoubtedly the oocyte, whose role is fundamental not only in fertilization, but also in the development of the deriving new organism (Mtango et al., 2008). The identification of biological markers predictive of developmental potential of the oocytes retrieved from patients would allow the selection of the highest quality oocytes. Clearly, the possibility of selecting the gametes with the highest developmental potential prior to fertilization would significantly increase the chances of a successful pregnancy.

Chapter 2

Introduction

2.1. The story of the female gamete

The ultimate function of the female gonad is to produce mature fertilizable eggs. This arduous task is accomplished through a complicated and intricate process that requires close coordination and interaction between developing germ and somatic cells in the ovary. In addition, mammalian ovaries sustain growth and specialization of somatic follicle cells and the production of sex hormones, which are necessary for the development of sexual characters and the maintenance of pregnancy.

Murine gestation lasts 21 days and sexual differentiation of the bipotential gonad lasts from embryonic day 12.5 (E12.5) to E16.5 (Menke et al., 2003). Primordial germ cells (PGCs) are the progenitors of female and male gametes and differentiate from the proximal epiblast guided by signals from extra-embryonic bone morphogenetic proteins (BMPs) (Ying et al., 2001; Ying and Zhao, 2001). They appear in the embryonic ectoderm on 7.5 day *post coitum* (dpc) and they first migrate to the wall of the yolk sac and then reach the genital ridge of the developing gonad on 10-11.5 dpc, when they are still morphologically indistinguishable (Hirshfield, 1991; Gomperts et al., 1994; Molyneaux et al., 2004) (Fig. 1). Here, about 25.000 PGCs mitotically divide into approximately 85.000 oogonia.

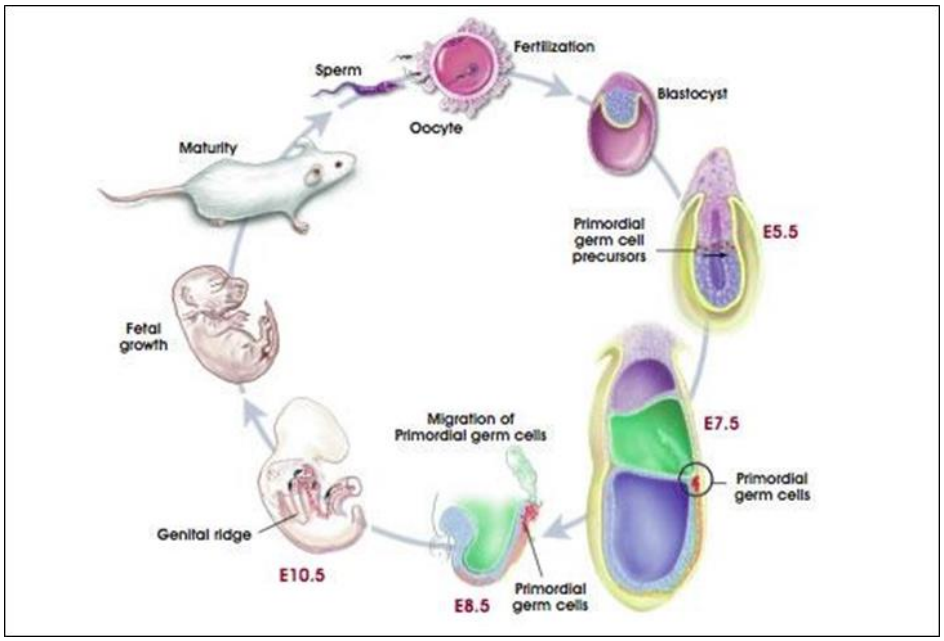

Figure 1. Mouse developmental cycle. Precursors of PGCs are first visible from E5.5 and start colonizing the genital ridge on E10.5 (from http://stemcells.nih.gov/info/scireport/appendixa.asp).

Starting from 12.5 dpc, by means of unknown factors, these cells enter meiosis and transform into oocytes, a process that lasts until 16.5 dpc, when all the oocytes have gone through meiotic prophase I and arrested at the diplotene stage.

The dividing germ cells are closely associated by intracellular cytoplasmic bridges and form clusters, the so-called germ cell nests or cysts, due to an incomplete cytokinesis at the end of cell division (Pepling and Spradling 1998). Only a fraction of all the cells contained in these nests manage to survive, whereas two-thirds act as nurse cells helping oocytes in their development and die in a wave of programmed perinatal loss. Just after birth, cysts are broken down both by the progressive death of many germ cells and by the invasion of follicle cells precursors (Pepling and Spradling, 2001; Tingen et al., 2009). At this time, oocytes become surrounded by a single layer of squamous follicle cells, with a basement membrane defining each follicle.

Life begins with a fixed number of primordial follicles, but only a few hundreds develop to produce a completely mature oocyte to be ovulated; indeed, the 99.9% of the initial endowment of oocytes degenerates through a process called atresia.

2.2. Mouse folliculogenesis

As sexual maturity is reached, groups of immature follicles periodically take the long way towards maturation under the influence of hormonal stimuli. This process is called folliculogenesis, it takes place in the ovary and leads to the complete maturation of the oocyte within three weeks. It occurs through different steps:

- a recruitment of primordial non growing-follicles from the pool;
- a later development of primordial follicles to primary and secondary preantral follicles;
- the finale formation of preovulatory follicles enclosing completely mature oocytes ready to leave the ovary through ovulation.

As follicles are recruited to grow, they start increasing in size by both proliferation of granulosa cells and growth of the oocyte in diameter and undergo a series of morphological and molecular changes, detailed below (Fig. 2).

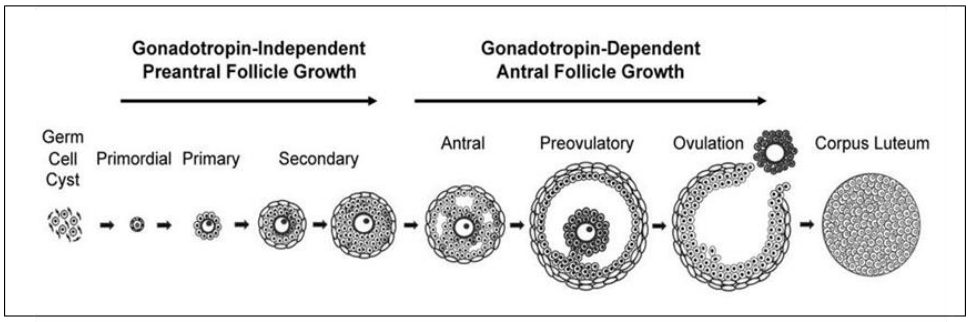

Figure 2. Folliculogenesis. Representation of the different stages of folliculogenesis that follicles encounter during their maturation (modified from Edson et al., 2009).

- A **primordial** follicle forms when germ cell cysts brake down and a small non growing-oocyte, 10-20 µm in diameter, becomes surrounded by a single layer of flat squamous somatic cells, the precursors of granulosa cells. Thousands of primordial follicles are present at the time of birth, but most of them (around 99.9%) will undergo degeneration without entering the growing phase (Fig. 2).
- As folliculogenesis begins, a **primary** oocyte starts to grow and to produce the component of the zona pellucida (ZP), which will assemble into a glycoprotein membrane that surrounds the oocyte itself. Meanwhile, follicle cells that surround the germ cell become cuboidal and initiate their proliferation. An additional layer of somatic cells, theca cells, begins to organize outside the basement membrane (Fig. 2).

- Once the oocyte is surrounded by 2-4 layers of granulosa cells, it becomes **secondary**, **preantral**. The ZP continues to be laid down and the oocyte starts to produce mRNAs and proteins required for fertilization and early embryonic development. At this stage cavities filled with follicular fluid start to form within granulosa cells (Fig. 2).
- The mature **tertiary**, **antral** follicle is made of an the oocyte having a diameter of 70-80 μm, surrounded by 3-6 layers of cumulus cells (about 1.500 cells). The small cavities aggregate to form a unique follicle antrum determining the separation of granulosa cells into two different and specialized cell populations: *mural granulosa cells*, that form the follicle wall, and *cumulus cells*, strictly connected to the oocyte (Fig. 2). During ovulation the oocyte resumes meiosis and complete the first meiotic division, with the extrusion of the first polar body (PB), while cumulus undergo cumulus expansion. This process is also called mucification: cells start to secrete hyaluronic acid, which becomes hydrated and forms a sticky matrix that weaken the tight junctions between cells. This is extremely important for enabling ovulation, indeed its suppression greatly decreases the ovulation rate (Chen et al., 1993; Hess et al., 1999; Eppig 2001).

At the end of the three-week growth, only of a fraction of the primordial follicle pool manage to complete its development and is ovulated; most of the starting small immature follicles die through apoptosis, in a process called atresia (Krysko et al., 2008).

2.3. The mouse antral follicle

The antral follicle is a follicle at the final stage of its maturation. It is composed by an oocyte arrested at prophase of meiosis I, surrounded by about 1500 cumulus cells (CCs) tightly connected to each other and to the oocyte itself, to form a structure called cumulus-oocyte complex (COC) (Fig. 3). This is located in a big antral cavity filled with follicular fluid containing a variety of molecules that mediate communication between different parts of the structure. Other specialized follicle cells, mural ganulosa cells, cover the internal portion of the follicle. Moreover, a basal lamina composed by extracellular matrix separates this structure from a third outer layer of thecal cells (Fig. 3). Granulosa and theca cells have important steroidogenic functions.

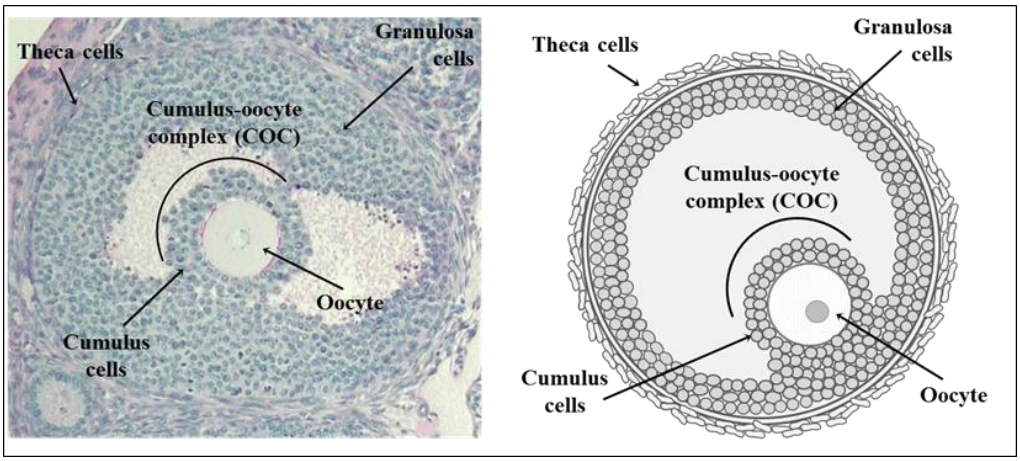

Figure 3. Murine antral follicle. On the left, a section of a murine ovary displaying a preovulatory antral follicle. On the right, a schematic representation of the same follicle. Arrows indicate the different components: oocyte, cumulus cells, granulosa cells, basal lamina and the outer layer of theca cells.

The correct development and maturation of the oocyte occurs within this complex multilayered structure, where the germ and somatic components are tightly connected to each other, through transzonal projections (TZPs) and gap junctions (GJ). TZPs are cytoplasmic extensions of cumulus cells that reach the oocyte cytoplasm by passing through the ZP; they are numerous at the beginning of folliculogenesis and they decrease while reaching the antral stage (Albertini et al., 2001). GJs, instead, are membrane hemichannels that physically connects the cytoplasms of two adjacent cells, allowing the free passage of ions, glucose metabolites and small molecules. Among the connexins expressed in the ovary, two are needed during follicle maturation: connexin43, connecting granulosa cells, responsible for their proliferation during the first growth phase, and connexin37, that connects the oocyte to cumulus cells and is essential to antral follicle formation (for a review see Gershon et al., 2008).

The close association among the different components is crucial since follicles cannot form without an oocyte and the oocyte itself develops competence to undergo meiosis and be fertilized under the influence of surrounding granulosa cells (Brower and Schultz, 1982; Eppig 1991; Kidder and Mhawi, 2002; Matzuk et al., 2002; Gilchrist et al., 2004; Su et al., 2009). By means of this connection, each component carries out its precise function: CCs are responsible for metabolic processes such as glycolysis, amino acid uptake and cholesterol synthesis in place of the oocyte (reviewed by Huang and Wells, 2010 and Sutton-McDowall et al., 2010) which, in turn, regulates follicle development (Eppig et al., 2002) and somatic cells metabolism and proliferation (Gilchrist et al., 2008, Su et al., 2009). Therefore, interruption or blockage of this intimate coupling can have detrimental effects on oocyte growth and acquisition of the developmental competence (Carabatsos et al., 2000; Modina et al., 2001),

as confirmed by the use of gap junctions inhibitors (Fagbohun and Downs, 1991; Downs, 1995, 2001) . Moreover, follicle cells are crucial in the maintenance of oocyte meiotic arrest by providing cGMP via GJs, which prevents the degradation of cAMP and the progression of oocytes from prophase to metaphase (Norris et al., 2009).

Other factors with meiosis-inducing capability could be secreted by the cumulus oophorus and be central in promoting oocyte maturation. Despite the well-known critical role played by follicle cells throughout oocyte maturation, their detailed function and mechanisms of action are poorly understood. A list of potential paracrine substances has been proposed, including the meiosis-activating sterol (MAS, Byskov et al., 1997; Downs et al., 2001), pyruvate (Downs, 2001), progesterone (Yamashita et al., 2003), nitric oxide (Bu et al., 2003), Leydig insulin-like 3 (Kawamura et al., 2004), androgens (Gill et al., 2004) and members of the epidermal growth factor family (Tsafriri et al., 1989; Ashkenazi et al., 2005), but the results obtained so far still remain unclear.

The production of a fully-grown antral follicle and its ovulation are regulated by a multitude of factors, mostly belonging to the transforming growth factor-β (TGF-β) superfamily. Some have a stimulatory role, others exert the opposite function. They can be of somatic origin, such as the anti-Müllerian hormone (AMH), which inhibits the recruitment of primordial follicles from the resting pool (Durlinger et al., 1999), or they are expressed by the oocyte, such as bone morphogenetic protein 15 (BMP-15) and growth differentiation factor 9 (GDF-9), which are positive regulators of preantral follicle growth (for a review see Knight and Glister, 2006).

Another fundamental role is played by hormones, especially steroids (estrogens and androgens) and gonadotrophins. Their action is mediated both by intrinsic signals provided by multiple families of paracrine factors as well as by direct cell-cell communication via GJ channels.

Hormonal influence on follicle growth starts as follicles reach the preantral stage, when they become responsive to follicle stimulating hormone (FSH), whose action is mediated via the surrounding follicle cells (Roche, 1996; Lei et al., 2010). By binding to its receptor (FSHR), FSH stimulates granulosa cells proliferation, estradiol production and prevent granulosa cell apoptosis and atresia. Moreover, it promotes LH receptors expression as the follicle reach the antral stage, preparing the complex to respond to the LH surge and be ovulated.

2.3.1. The production of a mature, fertilizable egg

The production of a completely mature egg, able to be fertilized and to sustain the first phases of embryo development is an extremely complex

and difficult process that begins during oogenesis. Cyclically, as folliculogenesis progresses, a selective process takes place and most of the growing follicles are lost through atresia. Those that survive and develop to antral preovulatory stage undergo a last maturation step, called ovulation. An increase in estradiol level induces the concomitant suppression of pituitary FSH secretion and stimulation of LH production, that results in the LH surge. Preovulatory follicles respond to this surge through the LH receptors (LHCGRs) and this triggers a cascade of events that lead to oocyte meiotic resumption (Hillier, 1994) and important morphological and functional differentiative processes within follicle cells.

As for the oocyte, LHCGRs activation on the surface of granulosa cells induces the GJs to close, with a consequent decrease in cGMP: altogether, this leads to a reduction in the entry of cGMP into the oocyte, that lowers cAMP levels and causes resumption of the meiotic cell cycle. The oocyte progresses through the last phases of the first meiotic division, with the help of CCs that might influence correct meiotic spindle positioning (Barrett and Albertini, 2010), it extrudes the first PB and arrests at metaphase of the second meiotic division (Fig. 4), that will be completed upon fertilization.

At the same time, CCs undergo cumulus expansion or mucification, an extensive rearrangement of the cytoskeleton through the assembly of actin microfilaments and the induction of a series of cumulus-related genes, mostly by means of GDF9 action. It stimulates hyaluronan synthase 2 (HAS2) to produce a highly hydrated sticky matrix of hyaluronic acid (HA) around cells, which separate them, transforming the tightly packed cell mass into an expanded cumulus oophorus (Fig. 4) (Tsafriri et al., 1999).

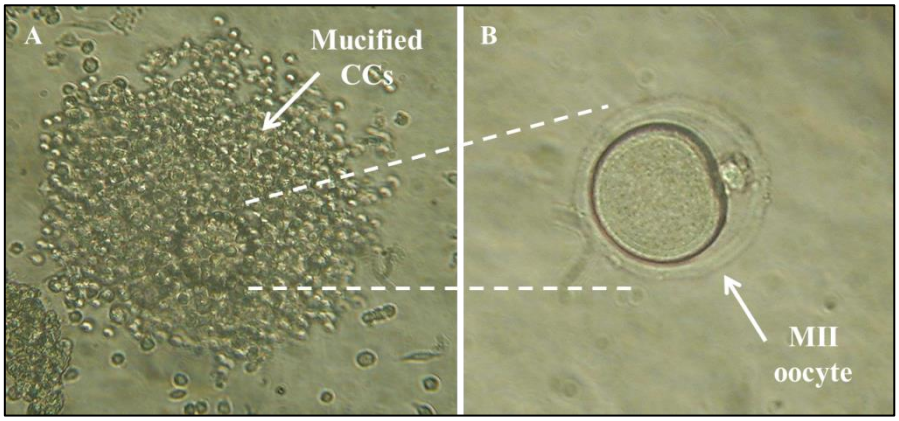

Figure 4. Murine metaphase II follicle and oocyte. A, ovulated follicle with an expanded cumulus oophorus. B, MII ovulated oocyte.

Moreover, it induces the expression of several transcripts involved in the stabilization and maintenance of this matrix (Fig. 5): pentraxin3 (*Ptx3*), that protects the matrix against the action of proteolytic enzymes during

extrusion and in the oviduct (Varani et al., 2002), tumor necrosis factor α-induced protein 6 (*Tnfaip6*), that helps organizing hyaluronan strands, and prostaglandin endoperoxide synthase 2 (*Ptgs2*) (Lim et al., 1997).

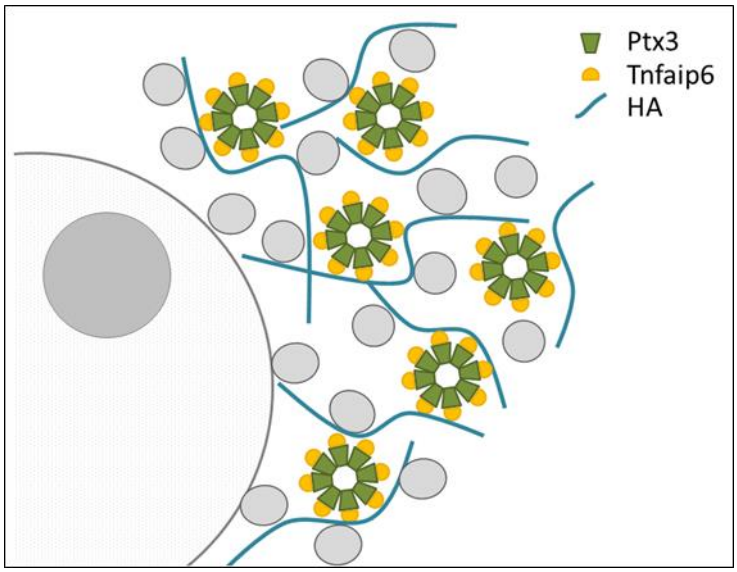

Figure 5. Schematic detail of the expanded matrix. The oocyte and CCs are entrapped and surrounded by a sticky HA matrix, stabilized and protected by several factors, at the time of ovulation.

This mucified and expanded matrix has the essential role of binding the oocyte and the CCs together, thus protecting the oocyte from proteolytic and mechanical stresses of ovulation and, in a second time, allowing sperm binding, penetration and fertilization.
Concurrently, mural granulosa cells induce the degradation of peri-follicular matrix and lead to the formation of a rupture site in the follicle wall, thus facilitating the extrusion of the mature egg (Chappel et al., 1991). Soon after ovulation, follicle cells remaining within the ovary undergo a terminal differentiative process, called luteinisation, and give rise to the *corpus luteum*, a temporary structure with blood vessels crossing the basement membrane. Upon egg fertilization, this structure has an endocrine function, actively producing hormones that are necessary for pregnancy maintenance. On the contrary, if the egg remains unfertilized, it degenerates to form a mass of fibrous scar tissue.

2.3.2. The model: SN and NSN oocytes

Since entrance into the meiotic process during embryonic life, oocytes maintain their arrest at the dictyate stage of prophase I until the end of the growth process, when they response to the ovulation stimulus and resume meiosis. In the meantime, as the oocyte is growing, numerous epigenetic changes take places, including not only DNA methylation and histone

acetylation, but also chromatin organisation and nuclear architecture. This dynamic process is known as “epigenetic maturation” and is finalized to organise the genome for early embryonic development. All the epigenetic modifications are equally important and have a key role in the acquisition of the oocyte developmental competence, as they are responsible for the regulation of gene expression during the first phases of preimplantation development.

Regarding chromatin organisation, an extensive remodelling occurs throughout folliculogenesis: within primordial and primary oocytes, the chromatin is finely dispersed and centromeres and chromocenters appear to be located mainly at the periphery of the nucleus (Garagna et al., 2004). As small, immature oocytes reach a diameter of 40-50 μm, in a small proportion of them, most of the centromeres and chromocenters start clustering around the nucleolus and form a ring of heterochromatin. Thus, at this stage, two main chromatin conformations can be distinguished:

- one in which the chromatin is more diffused, characterized by a few spots dispersed within the nucleus; these oocytes are named non surrounded nucleolus (NSN) (Fig. 6);
- the other displays the typical ring of heterochromatin around the nucleolus and those oocytes are named surrounded nucleolus (SN) (Mattson et al., 1990; Debey et al., 1993; Zuccotti et al., 1995, 1998, 2005) (Fig. 6).

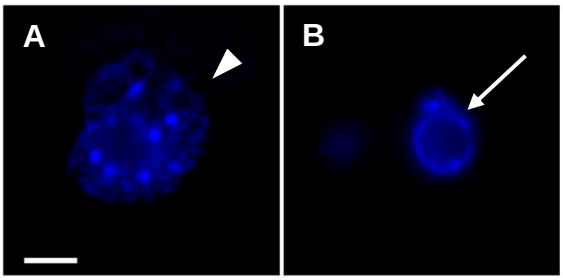

Figure 6. Hoechst-stained chromatin within the nucleus of a NSN and a SN oocyte. The ring of Hoechst-positive chromatin surrounding the nucleolus is clearly visible in the SN oocyte (B, arrow), while it is lacking in the NSN oocyte (A, arrowhead). Bar, 10 μm.

The proportion of these two nuclear architectures changes during the different phases of folliculogenesis: the SN configuration is first found in only 5% of 40-50 μm oocytes, but this percentage increases until it reaches about 50% of the whole pool of antral oocytes (70-80 μm in diameter). Moreover, a dramatic change in the proportion is also seen with aging. In 2-week-old females only the 5.9% of the few oocytes that have reached the antral compartment displays a SN configuration, but when the first ovulation occurs, SN and NSN oocytes are found in equal numbers.

Interestingly, this balance could be perturbed by injecting mice with PMSG: priming causes a considerable increase in the proportion of SN oocytes. Instead, in natural conditions, the equal SN and NSN number is maintained until 6 weeks of age; after that, the percentage of SN keeps on increasing until 90% (Zuccotti et al., 1995).
The different nuclear architecture described underscores a different transcriptional activity. Namely, the NSN class has a high transcriptional activity, having a less condensed chromatin, while SN oocytes are basically transcriptionally silent (Debey et al., 1993; Bouniol-Baly et al., 1999; Christians et al., 1999; Liu et al., 2002; Miyara et al., 2003). Altogether, these differences in chromatin organization and gene expression reflect a different developmental potential: only SN oocytes, upon fertilization, may successfully reach the blastocyst stage and full term development, whilst NSN oocytes are unable to develop *in vitro* beyond the two-cell stage, (Zuccotti et al., 1998, 2002; Inoue et al., 2007).
The chromatin configurations in germinal vesicle (GV) oocytes defining the typical SN and NSN organisations have been found in many mammals, showing some slight differences depending on the species: in rats (Mandl et al., 1952), pigs (Crozet, 1983; Sun et al., 2004), monkeys (Lefevre et al., 1989), rabbits (Wang et al., 2009), bovine (Liu et al., 2006), sheep (Russo et al., 2007), horses (Hinrichs et al., 2005), dogs (Lee et al., 2008), goats (Sui et al., 2005) and humans (Parfenov et al., 1989; Combelles et al., 2002).

2.3.2.1. Oocyte developmental competence

Developmental competence refers to the ability of an oocyte to resume meiosis, be fertilized and sustain the first phases of embryo development. As previously described, the two different chromatin organizations found within the antral compartment are known to be associated with a different developmental potential. Experimental evidences show that both SN and NSN oocytes are able to resume meiosis, but whilst almost all SN oocytes successfully progress towards the metaphase II (MII) stage, only about half of NSN oocytes manage to reach this stage and become available for fertilization (Zuccotti et al., 1998). Since ZP and oolemma compositions are independent on the chromatin organization of the cell, fertilization occurs with about the same rate in the two oocyte types, but differences arise during the progression through early embryo development. Namely, upon *in vitro* fertilization, NSN oocytes fail to go beyond the two-cell stage, whereas SN oocytes have a much higher potential and are able to complete embryo development to term (Zuccotti et al., 1998).
During early development, numerous variables come into play and are crucial in defining the fate of the embryo. The major one is the expression

of maternal-effect genes and proteins, which is rather different in SN and NSN oocytes. These genes start to be transcribed as the oocyte reach the preantral stage and their transcripts are stored in the cell to sustain the pre-implantation development until the time of zygotic genome activation (ZGA) which, in the mouse, occurs at the 2-cell stage. ZGA is an essential step during embryo development, indeed it seems to be the main cause of embryo loss in human IVF, since any alteration can have a detrimental effect on development.

Among the 27 principal maternal-effect genes identified so far (Li et al., 2010), Oct4 seems to be fundamental in regulating early development (Zuccotti et al., 2008, 2009), being the central factor of a large transcriptional network involved in the maintenance of pluripotency and self-renewal in embryonic stem cells and in the inner cell mass. Oct4 (also named Pou5f1) is a nuclear transcription factor that regulates the expression of a number of developmental genes through chromatin remodelling (for a review see Cavaleri et al., 2005). Experimental evidences show that, in mouse embryonic stem cells, it influences the transcription of genes involved in the maintenance of pluripotency, such as Stella, Foxj2 and Nanog, which are part of a cluster located at the Nanog locus (Levasseur et al., 2008). It is expressed in oogonia and in oocytes since the primordial stage, then it is down-regulated until follicles are recruited to start folliculogenesis (Pesce et al., 1998). At that time, Oct4 is again up-regulated, but only in those oocytes that have a SN configuration (Monti et al., 2009; Zuccotti et al., 2009); in these cells it is expressed throughout their growth and maturation to the MII^{SN}, until the 2-cell stage embryo. An opposite pattern of expression is found in NSN oocytes: in this case it is always down-regulated, suggesting that these oocytes may be destined to be eliminated or recruited later during folliculogenesis. Oct4 protein regulates the expression of Stella and their expression profiles overlap each other; it is, therefore, expressed in SN oocytes and down-regulated in NSN ones. The main role of Stella is to protect the maternal genome and some paternal imprinted genes against demethylation at the very early stages of development, so its absence is not compatible with preimplantation embryonic development (Payer et al., 2003; Nakamura et al., 2007). Differently, Foxj2 is down-regulated in developmentally competent oocytes and is up-regulated in the mouse zygote, leading to a developmental arrest at the 2-cell stage (Martin-de-Lara et al., 2008). Therefore, Oct4 gene product might not be sufficient by itself, but it plays a key and central role in defining oocyte developmental competence.

Since the expression of maternal-effect genes is stage-specific, it is finely regulated by several transcription factors. One of these oocyte- derived transcription factors, known to play a regulatory role in early embryonic development, is the newborn ovary homeobox (NOBOX). NOBOX protein

was first found to be expressed in oocytes throughout follicle maturation, from the primordial to the antral stage (Suzumori et al., 2002). Its important role during folliculogenesis was further confirmed by studies conducted on female mice lacking NOBOX, which turned out to be infertile due to atrophic ovaries and the absence of mature oocytes (Rajkovic et al., 2004). Moreover, its expression is directly correlated to that of several genes involved in oogenesis, which resulted strongly down-regulated in Nobox-/- females, including Oct4, Gdf9, Bmp15 (Rajkovic et al., 2004) and Sall4 (Choi et al., 2007). Recently, Nobox expression profile during folliculogenesis has been detailed both at the mRNA and at the protein level (Belli et al., 2013). A significantly higher number of transcripts was found in NSN compared to SN oocytes of 41-50 μm, at the antral stage and in MII. Similarly, NOBOX protein is detected in both SN and NSN oocytes up to 51-60 μm in diameter, then it persists only within developmentally incompetent oocytes, whereas it becomes gradually undetectable in developmentally competent oocytes; this different expression pattern is clearly maintained in MII eggs. Because of this marked difference between the two types of oocytes, NOBOX, as well as Oct4 and Stella, can be proposed as putative marker of the mouse female gamete developmental competence.

Chapter 3

State of the Art

3.1. Markers of the oocyte developmental competence

Correct embryonic development to full-term is strictly dependent on the quality of the gametes that have contributed to the formation of the zygote. In this process, the egg plays a central role, especially during the first phases of preimplantation development, which rely almost entirely on its contents. As described above, a certain amount of the ovulated eggs are developmentally incompetent, especially in the case of ovary hormonal stimulation, which somehow suppresses the thorough natural selection occurring during follicle maturation *in vivo* (Swain and Pool, 2008). Thus, the possibility of identifying those eggs with the highest developmental potential would significantly improve the chances of pregnancy and full-term development. In this direction, many efforts have been made in the past 10 years to find markers of oocyte quality and most of the work has focused on the analysis of the female gamete.

3.1.1. Morphological markers

The easiest and most intuitive type of selection is based on the evaluation of a number of morphological features that have been reported in some studies as indicative of the oocyte quality. As for IVF cycles, where the presence of the CCs impedes the direct evaluation of the gamete, the size and expansion of the cumulus and corona radiata itself was identified as a potential indicator of the embryo fate, being related to the *in vitro* developmental potential as well as blastocyst quality (Lin et al., 2003). Instead, in the case of ICSI cycles, the egg is denuded prior to sperm injection, allowing the operator to directly evaluate its morphology. Among the features analysed, the absence of the MII spindle formation (Wang et al., 2001; Moon et al. 2003; Rienzi et al., 2003) or its shifting relative to the first PB have been related to reduced rates of fertilization (Rienzi et al.,

2003). Moreover, by means of a simple observation with polarization light microscopy, another number of morphological characteristics have been tested. A ZP showing an abnormal shape was linked to a bad-quality oocyte, while its thickness was positively correlated to higher embryo development (Høst et al., 2002; Shen et al., 2005) and increased blastocyst rates (Rama Raju et al., 2007). Regarding oocyte cytoplasm, the presence of vacuoles, inclusions (Xia, 1997; Otsuki et al., 2007; Balaban et al., 2008), central granulations (Balaban et al., 2008; Ebner et al., 2008; Rienzi et al., 2008) or smooth endoplasmic reticulum clusters (Otsuki et al., 2004) were recognized as potential markers of low embryo quality, as well as a bigger size of the perivitelline space (Chamayou et al., 2006). A disproportionate first PB was identified as a sign of decreased fertilization, cleavage rates and compromised embryo development (Rienzi et al., 2008; Navarro et al., 2009), whereas ovoid shaped-oocytes have been associated to delays in *in vitro* culture (Ebner et al., 2008). Nowadays, the morphological assessment of the oocyte is routinely performed in the clinical practice but, though easy and quick to be made, it is subjected to a bias depending on the operator experience and the time point of the observation. Even though it is known that heavy deformities can compromise the developmental potential of the deriving embryos, there is a concern regarding the predictive value of these parameters. Moreover, the high heterogeneity of approaches and diversity of outcome parameters used in the different studies make a direct comparison of all the results obtained so far impossible. Therefore, whether or not oocytes displaying one or more of these abnormal features are less competent than a "normal" one is still under debate.

Morphological selection is also routinely applied to embryos, especially in the case of single embryo transfer, which is to be preferred to reduce the risk of multiple gestation pregnancies. In this context, one possible selective strategy to avoid the transfer of those embryos destined to arrest their development at early stages could be the extension of culture period up to the blastocyst stage. The use of such approach in combination to other diagnostic tools has been proposed, but is still under discussion. Recently, new imaging and time-lapse methodologies are starting to be applied for morphological assessment of embryos before their transfer. These techniques allow the direct observation of the very beginning of embryo development during its maturation inside the incubator, without perturbing the culture conditions. Additionally, the lack of information deriving from the simple cells observation at defined time points is overcome by the continuous monitoring of the whole dynamic development process (Mio and Maeda, 2008). Complex algorithms for the analysis of images and time-lapses have been developed to link different aspects of early development and its kinetics to the quality of embryos and their ability to

implant. Among the predictive parameters identified so far, early pronuclei disappearance and first cleavage appear to be related to high developmental potential ad blastomeres number, while synchronous appearance of nuclei after the first cell division is linked to pregnancy success (Lemmen et al., 2008). As for kinetic aspects of cell division, success in reaching the blastocyst stage could be predict by examining, before embryonic genome activation occurs: timing of the first cytokinesis, time occurring between first and second mitosis and synchronicity in appearance of the third and fourth cells (Wong et al., 2010, Cruz et al., 2012). As well, embryo cleavage from 2- to 3-cells occurring in less than 5 hours has been associated with a low implantation potential (Rubio et al., 2012). These new and promising methodologies are on the way from research to clinical use, as they proved to be non-invasive and advantageous in improving current embryo selection procedures.

In combination to the evaluation of embryo morphology, other parameters have been proposed as potential indicators of embryo health and quality, such as autophagic activity. A very recent study showed a strong correlation between high autophagic activity and higher blastocysts and full-term development rates (Tsukamoto et al., 2014).

3.1.2. Molecular markers

As discussed before, many transcripts and proteins expressed by the oocyte have already been identified as markers of developmental competence, but their analysis through PCR or immunofluorescence would clearly be impossible, as it implies the sacrifice of the gamete. In the need to find an objective, but still non-invasive kind of selection for choosing the ideal oocyte for fertilization and embryo transfer, various studies have identified potential biomarkers by using proteomics and metabolomics for the study of follicle or embryo microenvironment (Royere et al., 2009). In addition, CCs are regarded as a possible source of markers. Firstly, they are closely connected to the oocyte with which they share the same follicular environment since the beginning of follicle growth, so they might somehow reflect the general condition of the gamete that they enclose. Also, their analysis would be completely non-invasive since in the clinics they are discarded as waste products.

Human CCs protein expression was investigated by two-dimensional polyacrylamide gel electrophoresis (Hamamah et al., 2006), mass spectrometry and Western blotting (McReynolds et al., 2012), but without identifying links to specific protein expression and developmental competence. Recently, a very sensitive protein microarray-based technique, called reverse phase protein array, was optimised for protein detection and

quantification at the level of human single CCs, demonstrating its potential future application in the search for new biomarkers (Puard et al., 2013).
Instead, there is a number of studies, both in humans and in animal models, that through the use of quantitative RT-PCR and protein investigation showed a significant correlation between the expression of specific CCs genes and the developmental competence of the eggs that they surround. Namely, a higher number of *BCL2L11*, *FSHR*, *GREM1*, *HAS2*, *LHR*, *PCK1*, *PR*, *PTGS2*, *PTX3*, *SDC4* and *VCAN* transcripts has been associated to improved developmental ability and competence of human oocytes (McKenzie et al., 2004; Zhang et al., 2005; Cillo et al., 2007; Assou et al., 2008; Gebhardt et al., 2011; Wathlet et al., 2011; Maman et al., 2012; Ekart et al., 2013). Similarly, in the bovine, the expression of *CDX3*, *FSHR*, *GREM1*, *HAS2*, *LHR*, *PTGS2*, *PTX3*, and (Calder et al., 2003; Assidi et al., 2008), was positively correlated to good preimplantation development and pregnancy outcome. On the other hand, a higher expression of *BDNF*, *CCND2*, *CTNND1*, *CXCR4*, *GPX3* and *NFIB* was found in human CCs related to oocytes with poor developmental potential, giving rise to poor-quality embryos (van Montfoort et al., 2008; Assou et al., 2008; Anderson et al., 2009). The same correlation was found for cysteine proteinases cathepsin B, S, K and Z) in the bovine (Bettegowda et al., 2008). As for the mouse, *Alcam*, *Grem1*, *Has2*, *Ptgs1*, *Ptgs2*, *Sdc4*, and *Vcan* expression in oocytes with different maturity was associated to the MII oocyte potential for blastocyst formation (Adriaenssens et al., 2011).
CCs gene expression could also be predictive of aneuploidy risk in the oocyte enclosed. A microarray study showed a tendency to a global lower transcriptional activity in CCs surrounding aneuploid oocytes, with *SPSB2* and *TP53I3* being more expressed in CCs associated with healthy oocytes (Fragouli et al., 2012). Human mural granulosa cells, as well as CCs, have been tested in the search of molecular markers leading to the identification of a list of five potential follicular gene markers associated to a positive pregnancy outcome: 3*βHSD1*, *CDC42*, *CYP19A1*, *FDX1* and *SERPINE2* (Hamel et al., 2008).

3.2. *In vitro* follicle culture

In vitro follicle culture techniques are all the methodologies that allow ovarian follicles at different maturity stages to complete their maturation, become ready to be fertilized and to support embryo development outside the ovary. Thus, the main goal of *in vitro* follicle culture is to create an "artificial" environment that, in place of the ovarian cortex where follicles grow *in vivo*, is able to physically and physiologically sustain follicle development. Within the ovary, the female gamete matures inside a

complex multicellular structure that, during folliculogenesis, undergo a series of morphological and molecular changes, leading to the formation of the preovulatory follicle. As described before, since the very beginning of folliculogenesis, the germ and somatic components of the follicle are intimately connected to each other, in a state of continuous bidirectional exchange of molecules and information. Each cell type carries out a specific function and is essential for the health of the entire structure; indeed, naked oocytes are not able to correctly complete their maturation and, even though they spontaneously resume meiosis, the developmental capacity of the resulting MII oocyte is poor. A proper system for *in vitro* follicle culture should, therefore, ensure the maintenance of the follicle integrity during the whole growth.

Historically, the first studies on follicle culture were performed using conventional two-dimensional (2D) culture systems, which envisage the culture of cells inside microdrops of maturation medium, either covered by mineral oil or not, inside multi-well plates, onto membranes enriched with extracellular matrix proteins or collagen impregnated gels. The application of these methodologies to murine oocytes led to the obtainment of mature, fertilizable eggs out of primary (Eppig and O'Brien, 1996) and pre-antral (Eppig and Schroeder, 1989; Cortvrindt et al., 1996) oocytes. However, even though 2D culture systems have proved to be suitable for murine follicle growth, those belonging to other species, above all human ones, require much longer culture periods, in which these systems might not be able to properly sustain follicle development. Specifically, as the follicle grows in the liquid medium on a 2D surface and increases both in diameter and in complexity, it gets in contact with the solid support onto which it attaches. This allows follicle cells to spread on the surface and grow on it, thus the follicle loses its spatial configuration, flattens and rupture, losing its 3D architecture (Fig. 7).

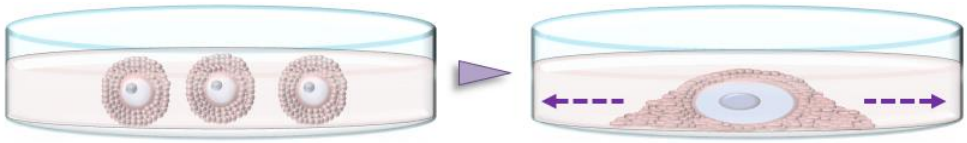

Figure 7. Representation of follicle flattening onto the Petri dish. Immature follicles growing *in vitro* get in contact with the solid support, onto which they attach, flatten and rupture, losing their 3D architecture.

Since losing cell-to-cell contact would avoid cellular communication and have a detrimental effect on follicle development, the attention moved to a different approach for follicle culture, that aim at maintaining the 3D architecture and integrity of the complex.

3.2.1. Tissue engineering and three-dimensional follicle culture

Cellular functionality *in vivo* is the result of a combination of factors, including soluble molecules (i.e. growth factors, hormones) and the environment, in terms of physical forces and structures acting on the cells in culture (i.e. extracellular matrix, ECM). Globally, the set of all these stimuli to which the cell is subjected is called structural context. It is a fundamental concept of tissue engineering and it is of particular interest in cellular biology since most of the factors regulating cellular metabolism and physiology are anchored to structural elements, such as receptors and junction proteins on the cell membrane, which are all in structural continuity with one another. Indeed, a cell is a tensegrity system that acquires its stability by distributing and balancing tensional and compressional mechanical forces to the whole structure, thus maintaining cellular shape and strength. In a tensegrity system, when an increase in the stressing force is applied to a single element, this input is evenly transmitted and distributed to the entire structure. As there is a structural continuity between all cellular elements, varying the mechanical and geometrical properties of the cytoskeleton would affect biochemical reactions and even gene expression. This means that remodelling cellular structures gives the cell inputs on how to behave. Structural context is of particular relevance in the culture of ovarian follicles, because the follicle itself can be considered as a small organ where oocyte, follicle cells and a network of small capillaries form a complex multi-layered unit that can be isolated from the ovary and grown *in vitro*. Over the last few years, much work has been done in the effort to set up a culture system that most accurately mimics the ovarian microenvironment, and a number of different techniques has been developed to this end.

Three-dimensional follicle culture systems have been developed to sustain follicle maturation, allowing the spherical growth of the follicle, with the surrounding follicle cells expanding in all directions (Fig. 8).

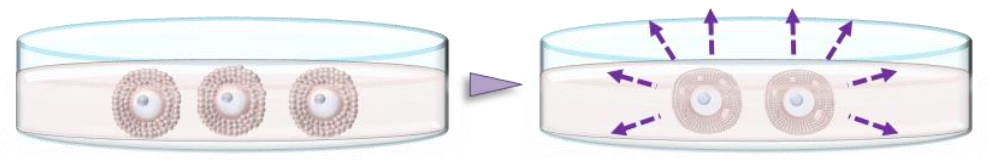

Figure 8. Representation of follicle growth into a 3D culture system. Immature follicles growing *in vitro* in a 3D culture system maintain their shape and structure, with follicle cell proliferating and expanding in all directions.

Various techniques are used, based on different principles, but the common aim is to impede follicles attachment onto the rigid support, thus avoiding the rupture of the whole structure.

3.2.1.1. Matrix-based systems

The most part of culture systems designed for 3D ovarian follicle growth involves the use of a biomaterial in the form of a matrix. Such matrices try to reproduce the ECM-rich cortical region of the ovary, where follicles grow *in vivo*. By "entrapping" cells in such matrices, they are free to grow spherically, without facing the hindrance of a rigid material. Moreover, trophic factors produced and released by granulosa cells are maintained in the vicinity of the oocyte, instead of being dispersed in the culture medium, thus conserving the nourishing role of these cells. Many different biomaterial are commonly used in the laboratory for cell culture and most of them has been tested also for the culture of ovarian follicles. They can be classified basing on their origin into natural or artificial:

- Natural biomaterials are plainly biocompatible and bioactive, having the capability of making contacts with cells in culture and they also spontaneously enclose endogenous factors that enhance cellular functions. On the other hand, being extracted by tissues or plants, even by the laboratory itself, variability between batches can occur leading to possible differences among results. Also, their composition is standard, so mechanical and physical properties are difficult to be modified;
- Synthetic polymers, instead, have a standard composition and are predictable in their action/degradation properties. They can be designed and have the properties that best fit the researcher needs, but they are inert and function as mere scaffolds for cell culture.

The matrices that are commonly used for cell culture include agar/agarose, sodium alginate, hyaluronic acid, collagen, fibrin, Matrigel as natural polymers and polyethylene glycol (PEG), polyvinyl alcohol (PVA), polylactic acid (PLA), polyglycolic acid (PGA), as synthetic materials. These can also be found in combination to one or more ECM proteins (laminin, fibronectin, collagen) for the enhancement of cell growth, differentiation, and quality of the oocyte.

In spite of the advances made so far in this field, there still is the need to understand which is the most biocompatible material onto which focus the attention, with the best physical and chemical properties, evaluating the toxicity, permeability, viscosity and the handiness. Also, a fine tuning of polymer concentrations and culture conditions has to be considered.

Calcium alginate

Alginate is a natural polysaccharide composed by β-D-mannuronic and α-L-guluronic acid units, which can be isolated from brown algae. It displays a series of positive properties that led to its use in a variety of cell culture techniques. It is highly biocompatible and permeable and one of the main advantages is its handiness: under mild conditions, positive bivalent ions can form interchain ionic bridges that cause aqueous alginate solutions to form gels and encapsulate cells (Fig. 9). Calcium alginate is mostly used in the form of small round shaped beads, that spontaneously form when the cell-alginate solution gets in contact with the cross-linking solution.

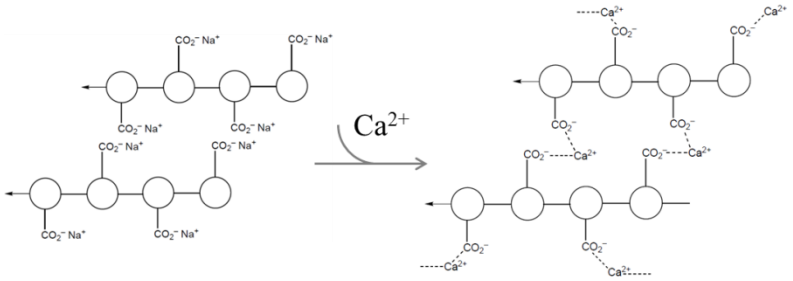

Figure 9. Alginate cross-linking. When a positive bivalent ion is added to sodium alginate solution, the ion cross-links two carboxyl groups of guluronic units of two alginate molecules.

Calcium alginate has been used for the culture of a variety of cell types, including human (Sidhu et al., 2012) and murine (Maguire et al., 2006) embryonic stem cells, induced pluripotent stem cells (Wei et al., 2012), and fibroblasts (Bohari et al., 2011). Although still in its early days, it is the most commonly used biomaterial for 3D ovarian follicle culture, as it allows the single encapsulation and growth of immature follicles in a 3D environment. To date, different protocols have been proposed, involving the use of a matrix made up of alginate alone (Pangas et al., 2003; Heise et al., 2005; Xu et al., 2006; West et al., 2007; Shikanov et al., 2009; Parrish et al., 2011; Tagler et al., 2012; King et al., 2013) or combined with fibrin (Shikanov et al., 2009; Jin et al., 2010) or ECM proteins (Kreeger et al., 2006). Various groups have managed to culture two- (100-130 μm in diameter) or multi-layered (150-180 μm in diameter) secondary follicles and the first attempt to obtain mature murine follicles was made by Pangas and colleagues (2003). They obtained granulosa-oocyte complexes whose oocytes were able to reach the MII stage in the 40% of cases, proving that culture within alginate not only sustains follicle growth, but also allows the acquisition of meiotic competence. Later on, the first attempt to obtain even live and fertile pups has been achieved by Xu and colleagues in 2006. In this work, they encapsulated single multi-layered secondary follicles into

beads of 1.5% alginate and matured them in αMEM medium at 37°C, for 8 days, until the antral stage. The COCs obtained were enzymatically freed from the matrix by treating beads with alginate lyase and the antral oocytes denuded and cultured for further 16 hr: 70.9% ± 9.9% of the initial pool reached the MII stage. *In vitro* fertilized MII eggs resulted into a mean of 68.2% ± 14.5% single-cell embryos. Upon transfer of 20 embryos, pseudopregnant females gave birth to 4 live and fertile pups. Despite the still low fertilization and delivery rates, this work proved for the first time that, within this culture system, follicles are sustained in their growth, are able to maintain their 3D architecture, growing in diameter and acquiring all the morphological features characteristic of the *in vivo* grown antral follicle. Moreover, follicle cells proliferate, differentiate and actively secrete hormones, while oocytes acquire both the meiotic and the developmental competence, being able to resume meiosis and be fertilized. Nonetheless, maturation and fertilization rates were much lower compared to the *in vivo* grown control. To deepen functional aspects leading to this lower developmental competence of *in vitro* grown oocytes, Mainigi and colleagues (2011) cultured early secondary follicles into 0.25% calcium alginate up to the antral stage and studied both encapsulated oocytes and the quality of the resulting MII eggs. Again, alginate proved to sustain both follicle growth, maintaining the 3D structure, and oocyte maturation with MII rates similar to those obtained with canonical 2D systems. However, only 19% of these oocytes managed to be fertilized and 5% developed up the blastocyst stage, suggesting that their developmental competence could be seriously compromised. This might be partially attributed to higher rates of disorganized and misaligned meiotic spindles and an uneven distribution of cortical granules. The use of alginate combined to ECM proteins, such as fibrin in an interpenetrating fibrin-alginate matrix, has been proposed as a possible improvement to the overall procedure. Indeed, even though the rates of follicle growth and survival did not increase, the rates of meiotically competent oocytes able to be fertilized and develop to the two-cell stage resulted significantly higher than those obtained by using alginate alone (Shikanov et al., 2009; Jin et al., 2010).

Besides the rodents, alginate-based culture systems has been tested with follicles from a variety of other species, including nonhuman primate (Xu et al., 2009a, 2010, 2011a, 2011b; Hornick et al., 2012) and human (Amorim et al., 2009; Xu et al., 2009b). Rhesus monkey multilayered secondary follicles have been cultured within alginate for up to 30 days, demonstrating the capability of this matrix to successfully support follicle growth and maintenance of follicle cells steroidogenic functions (Xu et al., 2009a). Also, different polymer concentrations were tested and 0.5% proved to be optimal in terms of follicle survival and growth rates, a higher concentration compared to that used for the mouse (0.25%), suggesting that

physical environment should be tuned basing on the different species studied (Xu et al., 2009a). An even higher concentration was proposed by Hornick and colleagues that, after a comparison of different alginate ratios, found that 2% is the one which best supports primate follicle growth (Hornick et al., 2013). Also, the addition of various substances (i.e. gonadotrophins, insulin, fetuin, oxygen) at specific doses proved to be useful in optimising the whole culture protocol (Xu et al., 2010, 2011b). As for human small preantral follicles, alginate hydrogel demonstrated to sustain their growth and maintain their viability (Amorim et al., 2009) and steroidogenic function (Xu et al., 2009b). Human ovarian tissue has also been cultured on 3D macroporous alginate scaffolds, reporting a higher proportion of developing follicles and a lower rate of atretic ones compared to other matrices (Kedem et al., 2011). A two-step culturing technique envisaging a first culture step of ovarian tissue onto such alginate scaffolds has thus been proposed as the optimal strategy for the culture of human follicles.

All the studies described above aimed at recreating the ovary microenvironment by providing physical support to growing follicles. Nonetheless, the natural endocrine and paracrine interactions between different follicle cell types is another aspect that should be taken into account. The importance of preserving the natural spatial arrangement and intercellular communication of granulosa and theca cells during culture was recently highlighted by Sittadjody and colleagues (2013). Among the different settings tested for encapsulation, the one giving the best results in terms of cell survival and estradiol, progesterone, activin and inhibin production was a multilayer alginate microcapsule. Such structure is composed by an inner layer of encapsulated granulosa cell, enveloped by an outer layer of encapsulated theca cells, thus mimicking follicle cells native organization. Therefore, the exact recapitulation of follicle structure would maximize the steroidogenic role of follicle cells approaching their natural condition and functionality. The preservation of beneficial steroidogenic effects of granulosa and theca cells during follicle culture implies the maintenance of the three-layer stratification of oocyte-granulosa-theca cells, in the recreation of a proper artificial ovary. In this direction, Krotz and colleagues (2010) succeeded in recreating ovarian tissue by using an agar honeycomb-shaped mould as structural support for theca and granulosa cells seeding and culture. Upon co-culture of three human COCs within opening of this seeded mould, one out of three oocytes managed to reach the MII stage. Additional studies on a larger sample and the assessment of hormone production are needed, but this methodology could allow a continuous culture of follicles from the primordial up to the antral stage for the production of high quality MII eggs. The production and further grafting of artificial ovarian tissue would especially be

beneficial for fertility preservation after cancer treatments. A preliminary study revealed that murine ovarian cells encapsulated within an alginate-matrigel matrix, when grafted into recipient mice, are able to survive and proliferate. Also, the transplanted matrix allows vascularization, is intrinsically degradable and only elicits a low inflammatory response (Vanacker et al., 2012).

Collagen

The first biomaterial to be used for cell culture is collagen, an abundant component of ECM, with a typical triple helix structure that confers to the molecule high flexibility and elasticity. Its first use dates back in the late '80s, both for 2D and 3D follicle culture (Eppig and Schroeder, 1989; Torrance et al., 1989). Through the years, several protocols have been developed for the use of collagen as a matrix, in combination to other ECM proteins (i.e. fibronectin and laminin) and RGD (arginine-glycine-aspartate) sequences or as microdrops. For what concerns murine follicles, all the protocols proposed proved to maintain follicle viability and sustain growth, somatic cells proliferation and oocyte maturation and acquisition of developmental competence. Recently, primary and early secondary murine follicles cultured within a collagen matrix not only completed their maturation, but also developed to term when subjected to *in vitro* fertilization (Mochida et al., 2013). This novel culture system is made of three principal steps: a first culture for 9 days in a collagen gel, a further culture for 8 days onto a collagen-coated membrane (Transwell-COL) and a last maturation to MII. Upon fertilization, 3.7% of 4-cell embryos transferred to pseudopregnant females developed into live and fertile pups. Despite the still low developmental rates, this study confirmed the possibility of achieving live births by growing very immature follicles within a matrix in a multi-step protocol.
As for other species, follicles of pig (Hirao et al., 1994, Shen et al., 1998), buffalo (Sharma et al., 2009) and cow (Yamamoto et al., 1999) have also been cultured in a 3D collagen matrix, with bovine early antral follicles giving rise to birth of one live calf after 14 days of culture embedded within a collagen gel. Human follicles, instead, have only been cultured in canonical 2D systems (for a review Telfer and McLaughlin, 2012) until the first attempt in 1999, when Abir and colleagues used a collagen gel to embed and culture primordial/primary follicles from fresh or frozen-thawed ovarian tissue. More than 24 hours of culture under this conditions seemed to be detrimental for follicle organization, however follicle cells and overall follicle size showed a significant increase (Abir et at., 1999). Besides intact follicles, denuded oocytes (DOs) embedded in a 3D collagen

gel, with or without their autologous cumulus cells, showed rates of maturation to MII similar to those of control GV oocytes, but with a marked increase in MAPK activity (Combelles et al., 2005).
In the attempt to recreate the 3D cell organization, as discussed above, rigid scaffolds with porous surfaces could also be used, as demonstrated by Ma and colleagues (2007). In this study, somatic cells were isolated from antral follicles, transferred into a demineralized bone matrix and cultured for 24 hours. Subsequently, isolated DOs were added to the scaffold and co-cultured for additional 14 hours. In the comparison with microdrops and 2D co-culture, this system showed to better support oocytes maturation and further development to the blastocyst stage. Moreover, lower levels of MPF and ZP hardening and a more natural cortical granules distribution where observed in oocytes co-cultured within the demineralized bone matrix. Globally, all the studies described above have brought up the advantages and drawbacks of a collagen matrix and its use for 3D *in vitro* follicle culture. Despite all the positive results achieved and the starting points highlighted for further studies, a few disadvantages limit the use and successive test of such biomaterial. First, the lack in standardization in matrix preparation could be a possible source of high variability; then collagenase treatment for follicles recovery could be detrimental for cells and the overall follicle structure and a non-physiological temperature is required for maintenance of collagen in a liquid status for cell encapsulation. These are the main reasons why the attention of tissue engineers moved towards a handier biomaterial, such as calcium alginate.

Other matrices

Several biomaterials, other than calcium alginate, are routinely used in the laboratory practice as scaffolds for cell culture, in pharmacology as vehicle for active ingredients or in medicine, for specific therapies.
One of the most used is Matrigel, a complex of ECM proteins extracted from Engelbreth-Holm-Swarm mouse sarcoma, mainly composed of laminin, collagen type IV, heparin sulfate proteoglycan, entactin and growth factors, which *in vitro* mimics the function of basal lamina (Zhu et al., 2012). Already extensively used in a variety of cell cultures, it has also been tested for embedding and growth of follicles from different species. A combination of Matrigel and activin-a showed to sustain murine follicle growth and survival, preserving their structure for up to 7 days in culture (Oktem and Oktay 2007). Consistent with these findings, also human ovarian tissues (Hovatta et al., 1997; 1999) and isolated follicles (Xu et al., 2009b) have been successfully cultured, showing growth, development and antrum formation.

Another promising biomaterial is hyaluronic acid, a glycosaminoglycan abundantly present in connective tissues. Displaying positive plasticity and viscosity properties, it has been tested and used for *in vitro* culture of numerous cell types and tissues. Also, its optical transparency when turned into a hydrogel permits a direct observation of encapsulated cells and its manageability allows moulding into different functional shapes that can be tuned on the base of cell type under study. For these reasons, it was recently tested in the culture of murine ovarian follicles (Desai et al., 2012). Follicles embedded in cylindrical beads preserved their 3D architecture, oocytes acquired meiotic competence, estradiol was secreted, and antrum formed in mature antral follicles. Nevertheless, low maturation rates were recorded, suggesting that these are preliminary data and the whole maturation protocol still needs improvements.

Among other matrices, agar is commonly used in cell biology laboratories, but has been less tested in the culture of ovarian follicles because of its polymerization dynamics, as it requires jumps in temperature to form a matrix and this might be detrimental for encapsulated follicles. Despite preliminary positive results obtained by culturing caprine preantral follicles in culture wells coated with 0.6% agar gel (Huanmin and Yong, 2000), agar matrix gave poor results in terms of oocyte maturation in the comparison with other systems for the culture of murine early preantral follicles (Mousset-Simeón et al., 2005). However, it proved to preserve the viability of cat and dog follicles cultured within ovarian cortical slices on an agarose gel block (Fujihara et al., 2012).

All the matrices so far discussed are of natural origin and are widely used for their numerous advantages and their innate biocompatibility. Despite the progresses made, difficulty in regulating the composition of natural matrices still represents a major limitation to their use. This is especially true in the field of developmental biology, as variability represents the major limitation for further use of a protocol in the clinical practice, where standardization of each delicate passage is strictly required. To overcome such limitation, tissue engineers started to design new synthetic biomaterials whose chemical and physical properties could be tuned according to the need and with an always standard composition (for a review see Tibbitt et al., 2009).

Among these newly synthesized polymers, polyethylene glycol (PEG) is the only one tested for *in vitro* culture of ovarian follicles, mainly for its degradation properties. Indeed, along culture, cells spontaneously secrete proteases that slowly degrades the surrounding matrix, thus allowing the follicle to expand in all directions without being submitted to high compressive forces that can limit its growth. Preliminary experiments showed that culture within this matrix enabled the survival of murine immature secondary follicles, which matured and acquired the normal

appearance of antral follicles in 10 days (Shikanov et al., 2011). Another biomaterial displaying similar characteristics of spontaneous degradation is Polyvinyl alcohol (PVA) (Alves et al., 2012). It has never been tested for follicle cells so far, but its promising properties make it a good candidate for future studies.

3.2.1.2. Non-Matrix systems

Along with 3D culture systems that aim at maintaining the native three-dimensionality of follicles by recreating ovarian cortex, other culture systems pursue the same goal applying other principles. For instance, all these other methodologies are based on movement to avoid follicle attachment onto the Petri dish by impeding it to get in contact with the culture support. The simplest technique implies culture of single follicles within inverted medium microdrops. By so doing, cells lie at the bottom of the drop, at the medium/gas interface where gas and oxygen exchange is maximized. When compared to the canonical upright culture system, it proved to better sustain murine preantral follicles growth, somatic cells proliferation and estradiol secretion along 6 days of culture (Wycherley et al. 2004). A similar trend resulted from the culture of primary marsupial follicles, which reached the antral stage and showed size and morphological features typical of *in vivo* matured follicles (Nation and Selwood, 2009). Other systems, such as orbiting test tubes and rotating wall vessels, prevent cell-plate contact by moving culture medium, thus maintaining follicles suspended within the liquid medium (Rowghani et al. 2004; Heise et al. 2005, 2009). Rat preantral follicles cultured in orbiting test tubes showed relatively high survival and growth rates, whereas those cultured within rotating wall vessels were abundantly damaged (Rowghani et al. 2004). This could be the result of an excessive shear stress to which follicles are subjected, that could trigger follicle cells detachment and thus lead to follicle rupture and injury.

3.2.2. Oocyte-cumulus cells co-culture

As detailed above, the oocyte and its surrounding small somatic cells are physically closely connected to each other since the very beginning of folliculogenesis. Thousands of gap junctions and transzonal projections put into direct contact the cytoplasm of close cells so that a continuous exchange of small signalling molecules occurs among all the cells. There are evidences that interruption of this exchange is detrimental for the whole follicle and above all for the oocyte, as it limits its developmental capability. Even though the crucial role played by this communication in promoting the correct development and maturation of oocytes is well

known, what remains to be clarified is the mechanism of action of the specific molecules involved (i.e. meiosis-activating sterol, pyruvate, nitric oxide, progesterone). Oocyte-cumulus cells co-culture experiments could contribute to shed light on the puzzling results obtained so far. Indications that cumulus cells can somehow condition the culture medium and stimulate murine DOs to resume meiosis (Downs and Mastropolo, 1994; Xia et al., 1994; Byskov et al.,1997; Downs, 2001; Fan et al., 2004) suggest that these cells might produce and secrete one or more diffusible paracrine factors with meiosis-inducing activity. To deepen the knowledge on these signalling mechanisms, DOs have been co-cultured with COCs, autologous or other species CCs, even in combination with conditioned medium to unravel the mechanisms involved in the paracrine communication and to understand to what extent somatic cells contribute to follicle growth.

A first study conducted with cow DOs co-cultured with their own follicle cells revealed that the sole presence of dispersed CCs within the same drop of medium was not sufficient to improve neither developmental capability nor blastocyst rate (Luciano et al., 2005). Instead, co-culture with intact COCs during both IVM and IVF proved to be beneficial to the development of DOs, improving cleavage and blastocyst rates (Luciano et al., 2005). Altogether, these results indicate that direct cell-to-cell contact is not strictly necessary for CCs to play their central role in helping the oocyte to correctly complete its maturation process. As for the mouse, results obtained are slightly different. Both dissociated CCs and intact COCs demonstrated a stimulatory effect on meiosis resumption of GV DOs, CCs displaying a higher meiosis-inducing effect, probably due to the release of pyruvate within the culture medium, with an additional beneficial effect arising from culture in a medium previously conditioned with COCs (Downs et al., 2006). Despite the faint effect of co-culture on DOs maturation, fertilization and blastocyst development, co-culture with dispersed CCs, on a CC-monolayer or with COCs proved to completely restore the developmental capability of corona-enclosed oocytes, in a way that is directly dependent on CCs density and age, gonadotropin within the medium and the length of *in vivo* priming (Ge et al., 2008). A further improvement to co-culture on a CC-monolayer could arise from the addition of both cysteamine and cystine to the medium, which proved to increase developmental capability of murine DOs to a level similar to that of control COCs (Zhou et al., 2010).

However, since denuding oocytes is a perturbation of the *in vivo* growing conditions, the effects observed by studying DOs might not directly reflect the paracrine effects occurring in the native environment. Unravelling the mechanisms that regulate oocyte growth would certainly contribute to understand how the oocyte acquires its developmental competence and clarify why only one of the two major classes of oocytes present in the

ovary becomes competent to undergo fertilization and embryo development to term.

Chapter 4

Results

4.1. Cumulus cell markers of the oocyte developmental competence highlighted by microarrays analysis

With the aim of comparing the whole transcriptomic profile of cumulus cells surrounding antral SN (SN-CCs) and NSN (NSN-CCs) oocytes, I first performed a microarrays analysis. A total of 25697 probes (from now on named genes) were analysed with an Illumina microarray chip and genes were considered differentially expressed when their fold-change was > 2.0, with a *p* value < 0.01. Of the total sequences analysed, 47 resulted differentially regulated in the comparison between NSN-CCs *vs.* SN-CCs; no genes were exclusively expressed in one of the two types of CCs and, interestingly, almost all (46, 97.9%) were down-regulated, with a single gene (Amh) up-regulated in CCs surrounding non competent oocytes (Table 1S). Later, to confirm the trend to a global down-regulation in the transcriptional profile of NSN-CCs that I first observed, I set less stringent conditions, lowering the fold-change cut-off to > 1.3, but maintaining a *p* value of < 0.01. With this new setting, I came up with a list of 422 differentially regulated genes in the comparison between NSN-CCs *vs.* SN-CCs (see data deposited in GEO, accession number GSE46906). Again, the most part (412, 97.6%) was down-regulated in NSN-CCs, with the exception of 10 up-regulated genes, among which Amh still was the most highly up-regulated one.

Thereafter, a series of bioinformatics and bibliographic analysis were performed to give a meaning to the differentially regulated sequences, starting from a Gene Ontology (GO) enrichment analysis, performed with the tool provided by the data mining and bioinformatics software Orange (http://orange.biolab.si/). This highlighted 11 major biological processes characterizing 247 of the total differentially expressed genes (Table 1; Fig. 10, A), while the remaining 175 were non annotated sequences with unknown functions. The main processes recognized are: signal transduction (72 genes, 29.5%), nucleic acid metabolism (28, 11.5%), protein metabolism (26, 10.7%),

cell death (22, 9%), cell adhesion (20, 8.2 %), reproduction (17, 7%), cell differentiation (15, 6.1%), transmembrane transport (13, 5.3%), development (12, 5%), lipid metabolism (11, 4.5%), and cell cycle (8, 3.3%) (Fig. 10, B). The most abundant category was the 'Signal transduction' process, comprising around 30% of genes, all of which down-regulated. Out of 10 total up-regulated genes, only 7 were annotated and attributed to 'Nucleic acid metabolism' (Giyd2 and Hspa1a), 'Cell death' (Lgmn), 'Cell differentiation' (Erdr1 and Vim) and 'Reproduction' (Cited2 and Amh) processes. Remarkably, 7% and 5% of genes resulted somehow involved in reproduction and development, respectively.

The second step of the analysis consisted in the exploration of the literature public database PubMed, in order to bring up differentially expressed genes that were specifically involved in the ovarian and follicle function. To this regard, I looked for associations between a list of MeSH terms describing the ovarian function (Table 2S) and the list of 422 differentially regulated genes. At least one link was found for only 303 out of 422 sequences, whereas the remaining 119 could not be found in the interrogated database, probably being non annotated sequences. Of these 303, 26 genes obtained the highest score, and Amh and Has2 stood up at the top of the list among the most associated to the chosen key MeSH terms. Indeed, they are both well known for their fundamental role during folliculogenesis. Globally, these analyses highlighted a deep involvement of the genes in follicle function, suggesting that this list could be an optimal starting point for further, more functional studies aiming at deepening our understanding of the intimate relationship occurring between the female gamete and its companion CCs.

Table 1. Annotated differentially expressed genes. List of the differentially regulated genes within each of the 11 main biological processes identified by the GO analysis. Green, down-regulated genes; red, up-regulated genes.

Gene symbol	Pathway	N. of genes
Agrp, Ahrr, Arhgap15, Bcar3, Cabp4, Ccbp2, Cenpj, Clec4a3, Cx3cr1, Dclk2, Dnaja1, Edg2, Emr1, Enpp2, F10, Ffar3, Fgf15, Fgf5, Freq, Gabrb2, Gem, Gnat3, Gpr182, Gpr85, Grb7, Htr4, Ifnz, Irs1, Kremen2, Lag3, Ms4a10, Npffr2, Olfr104, Olfr1040, Olfr1105, Olfr1208, Olfr1219, Olfr1347, Olfr1383, Olfr1424, Olfr244, Olfr323, Olfr725, Olfr915, Omp, Oscar, Pdyn, Plcg2, Ppp1r1a, Ppp1r1b, Pth2, Ptpn6, Rab9b, Rassf9, Rgma, Rgs6, Rgs9, Rgs9bp, Rin1, Rl1, Rsu1, Sag, Shc4, Tmod2, Trem1, Treml2, Trpc5, Trpm3, V1rh5, Vmn2r42, Zap70, Zcrhav1	**Signal transduction**	72
Batf2, Chd1l, Cux2, Dhfr, Dis3, Ell3, Fhit, Giyd2, Gtf2h5, Gtf3c6, Hmbox1, Hspa1a, Il33, Kif18a, Larp6, Mef2b, Nono, Orc4l, P42pop, Rbm35a, Sp5, Tasp1, Tuba4a, Utp15, Zfp1, Zfp526, Zfp786, Zscan2	**Nucleic acid metabolism**	28
493444A2Rik, Agbl1, Alg11, B3gnt8, Cct6b, Cpeb2, Ctrl, Dnajc4, Dph5, EG623661, Eif3h, Eif4e, Fbxo44, Hipk4, Klhl13, Mrpl15, Mrpl39, Otub2, Rpl22l1, Senp6, Sh3d19, St3gal1, St8sia4, Tor2a, Usp45, Wnk2	**Protein metabolism**	26
Aif1, Alox12, Bik, Bnipl, C5ar1, Cacybp, Casp1, Casp4, Dapk2, Grid2, Isg20l1, Lgmn, Moap1, Nuak2, Pdcl3, Srgn, Tnfrsf4, Tnfsf13b, Tns4, Tpt1, Ube2z, Ywhae	**Cell death**	22
A930038C07Rik, Azgp1, Cdh1, Cdh11, Cdh13, Cdh3, Cldn3, Cml4, Col12a1, Col3a1, Ctnna3, Lypd3, Mia1, Pcdhb20, Pcdhb4, Serpini1, Spon2, Tecta, Utrn, Zan	**Cell adhesion**	20
Amh, Cited2, Dazl, Fgf17, Fkbp6, Ggnbp1, Ghrh, Gnrhr, Hsf2, Mtl5, Pgr, Plau, Sox8, Spag6, Tcp11, Theg, Vmo1	**Reproduction**	17
AI428936, Abca5, Dixdc1, Dlk2, Erdr1,Fig4, Gdf10, Irf8, Lhx3, Lrg1, Neu2, Nkx2-3, Plk4, Rpgr, Vim	**Cell differentiation**	15
Abcc2, Atp5e, Cox7b, Fxyd1, Gjb1, Hvcn1, Kcns1, Kctd7, Ranbp2, Slc25a13, Slc29a4, Slc39a1, Timm22	**Transmembrane transport**	13
Actl6b, Amn, Bin3, Cdx1, Cftr, Des, Ebf1, Eln, Fnbp1, Hydin, Myl6b, Prr15	**Development**	12
Alox5ap, Cpt1c, Cyp4f14, Elovl2, Fabp5, Idi1, Ihpk3, Ndufab1, Pigw, Pnliprp2, Ptgds	**Lipid metabolism**	11
Cdkn2a, Gbx2, Gm606, Il13, Pttg1, Tnfrsf9, Txnrd1, Wfdc1	**Cell cycle**	8

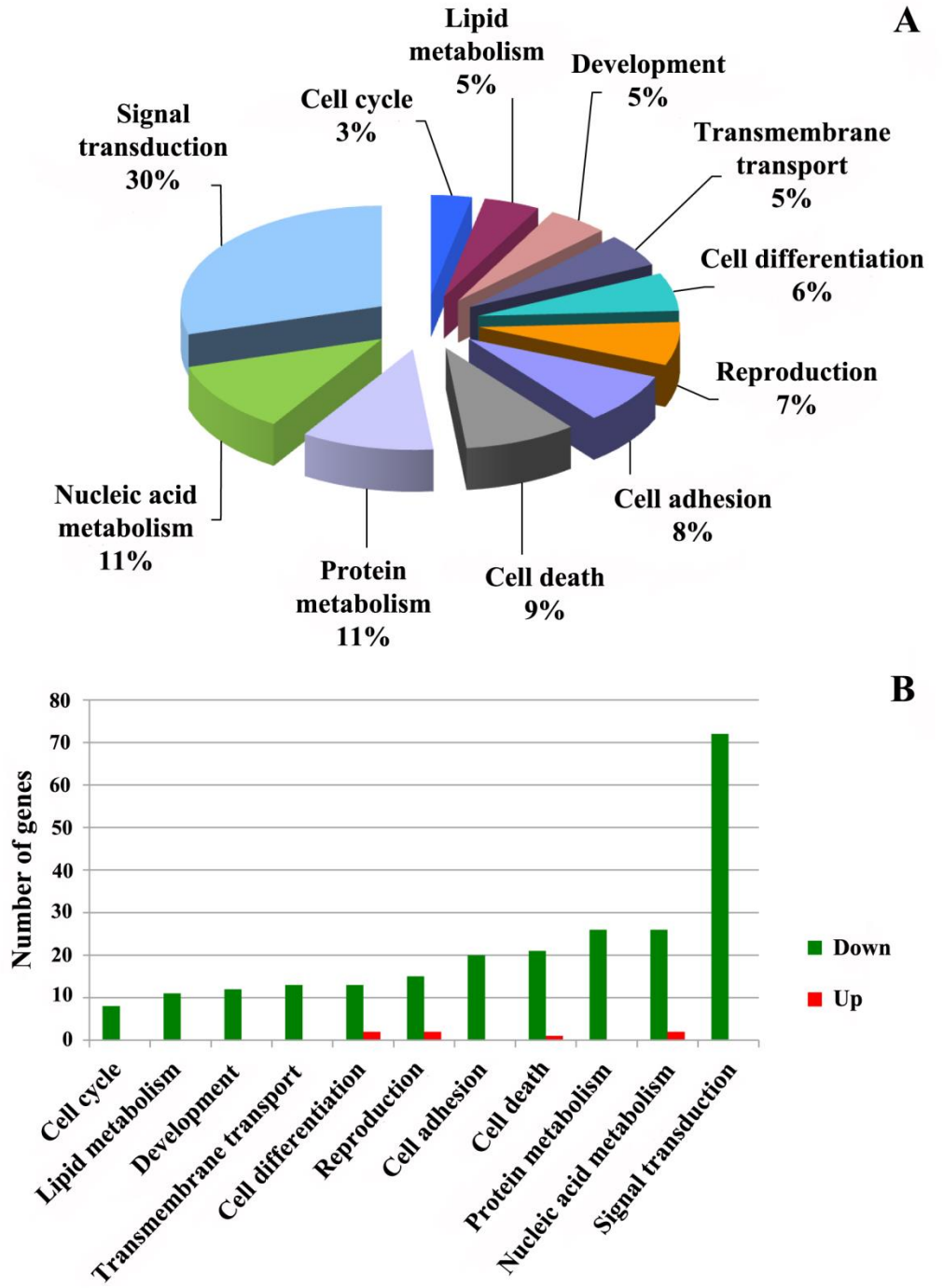

Figure 10. GO analysis of the differentially regulated genes. (A) The 11 major biological processes characterizing annotated genes; (B) The distribution of up- and down-regulated genes within each of the identified biological process.

A last and more functional analysis was made to determine whether and how proteins encoded by the differentially expressed genes interact with each other. I, therefore, searched for pathways and inter-relationships between genes by interrogating the MeSH and STRING repositories with Orange. This analysis resulted in a broad gene network consisting of 142 genes (Fig. 11, A), with the gene Pttg1 (Pituitary tumor-transforming gene 1) being that with the highest

number of neighbours. From this, a sub-network centred on Pttg1 could be isolated, where the gene is related to its two more proximal neighbours (Figure 11 B). Pttg1 is an oncogene involved in tumor development and metastasis (for a review see Panguluri et al., 2008) by inducing epithelial to mesenchymal transition (EMT) though the regulation of TGFβ expression in ovarian cancer cells (Shah and Kakar, 2011). It was already identified as a differentially regulated gene in a previous study performed on cow follicles. A microarrays analysis showed its down-regulation in the comparison between the expression profile of CCs isolated from immature *vs.* mature cow follicles (Ghanem et al., 2007). This result is consistent with the down-regulation observed in NSN-CCs, suggesting that the lower expression of this gene might be related to an inferior maturity of follicles containing NSN oocytes. The biological role of the gene network obtained is yet unclear but, again, it is a fundamental and rich starting point for more functional analyses that will contribute to shed light onto biological and biochemical pathways acting within the ovarian follicle and, more specifically, within follicle cells.

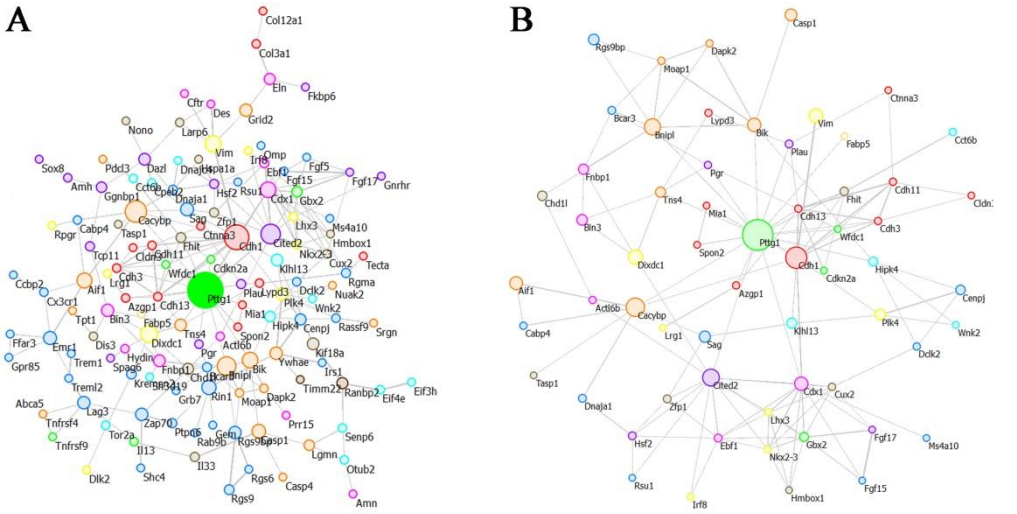

Figure 11. Annotation-based gene networks. Each annotated gene corresponds to a node and its colour relates to the main GO biological process that characterizes it. The size of nodes is proportional to Betweenness Centrality parameter and line width increases as annotation relationship become stronger. (A) The global Pttg1 network made of 142 genes. (B) Pttg1-centered gene sub-network, where it is represented with its two more proximal neighbours.

4.2. The analysis of five cumulus cell-related genes for the identification of the developmental competence of the enclosed oocyte

Starting from the results obtained by analysing the whole transcriptional profile of cumulus cells, I focused my attention onto a list of five cumulus cell-related genes. Among these, Amh and Has2 were brought up by microarrays, being highly differentially expressed and significantly involved in follicle function. Actually, Amh (Anti Müllerian hormone) acts on both primordial follicle recruitment, having an inhibitory role on FSH stimulus, and dominant follicle selection in mice (Visser et al., 2005). It was the most up-regulated, displaying a 2.34-fold higher expression in the comparison between CCs surrounding developmentally incompetent *vs.* competent oocytes. Likewise, Has2 (Hyaluronan synthase 2) is responsible for the production of hyaluronic acid during cumulus expansion just prior to ovulation and resulted 1.38-fold down-regulated in NSN-CCs. Previous studies in different species found a positive correlation between its expression and developmental competence and capability of human and cow oocytes.

I further analysed the expression of both genes by qRT-PCR in confirmation to the microarrays results and I added to the analysis three other CC-related genes known to have a key role during cumulus expansion, whose function is correlated to that of Has2: Ptx3 (Pentraxin 3), Tnfaip6 (Tumor necrosis factor alpha-induced protein 6) and Ptgs2 (Prostaglandin-endoperoxide synthase 2), acting in the stabilisation of the matrix during cumulus mucification and its protection against proteases.

Results were obtained from pools of about 40 COCs and confirmed the trend to a global gene down-regulation in NSN-CCs compared to SN-CCs previously observed. Specifically, among the four genes involved in cumulus expansion, Has2 showed the highest down-regulation (-2.62-fold; p=0.029), followed by Tnfaip6 (-1.51-fold; p=0.008); Ptx3 (-1.41-fold; p<0.001) and Ptgs2 (-1.34-fold; p=0.007) (Figure 12). In line with what previously published, the expression of mouse Has2, Tnfaip6, Ptx3 and Ptgs2 resulted lower in CCs enveloping developmentally incompetent *vs.* competent oocytes (McKenzie et al., 2004; Zhang et al., 2005; Cillo et al., 2007; Assidi et al., 2008; Gebhardt et al., 2011). Their expression profile is indicative of a general immaturity/lack of competence of antral follicles containing an NSN incompetent oocyte.

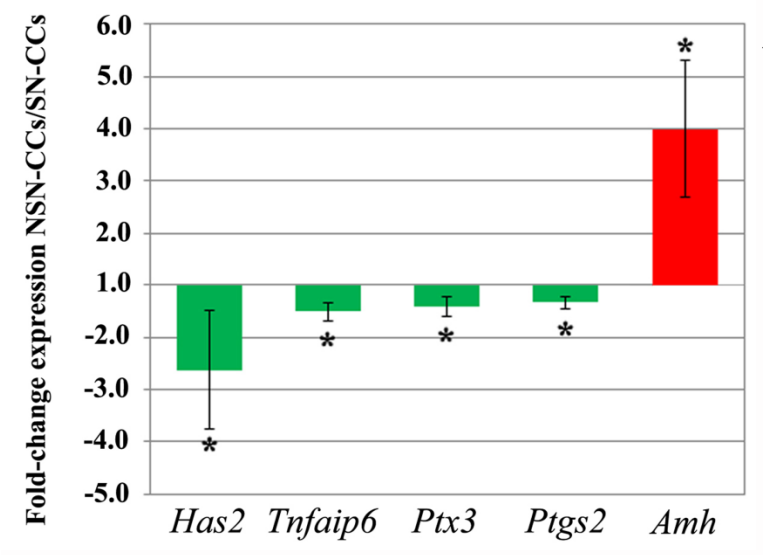

Figure 12. Fold-change expression of cumuls cell-related genes. qRT-PCR fold-change expression based on the reference value of SN-CCs. Green, down-regulated genes; red, up-regulated genes. Bars, S.D.; *, *p* value <0.05.

As for *Amh* transcript, again the qRT-PCR analysis underlined the high up-regulation in NSN-CCs *vs.* SN-CCs previously observed with microarrays data. With a more sensitive technique, this difference was even higher, showing a 4-fold up-regulation ($p<0.05$) (Fig. 12). A further confirmation came from an immunofluorescence analysis performed on dispersed NSN-CCs and SN-CCs: AMH protein resulted more abundant in NSN-CCs, displaying a clear and very bright fluorescence, especially at the periphery of CCs. SN-CCs, instead, only exhibited a weak signal (Fig. 13).

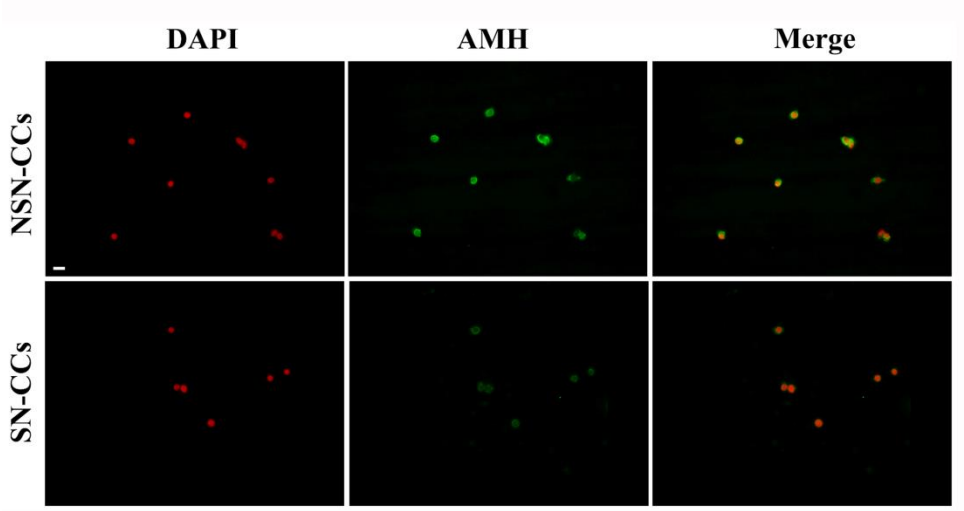

Figure 13. Immunofluorescence analysis of AMH protein expression in NSN-CCs and SN-CCs. In green, immunostaining of AMH on CCs surface; in red, counterstaining of nuclei with DAPI; bar, 5 μm.

All the results described above have been obtained by carrying out experiments on tens of thousands CCs collected from pools of COCs. The analyses performed allowed the identification of a selected short list of putative cumulus cell markers of the oocyte developmental competence. Nevertheless, to be diagnostic of a single oocyte health and quality, a marker should be tested and analysed on CCs of a single cumulus oophorus. Also, in order not to compromise oocyte health, this analysis should be carried out easily and quickly. With this in mind, I focused my attention on *Amh* transcript, as it gave the best results in terms of different expression between NSN-CCs and SN-CCs. Moreover, as it is well known that oocytes maturing enveloped by their surrounding CCs within the follicle microenvironment have better chances to acquire developmental competence, I tested whether the differential expression of Amh was maintained also between matured MII follicles. I, therefore, set up a new protocol for the isolation, manipulation and culture of single antral and *in vitro* matured COCs. Upon isolation from the ovary, a part of antral COCs was immediately classified into SN and NSN and further analysed by qRT-PCR, while in the meantime another group was *in vitro* matured for further 15 hr until the MII stage.

Regarding antral COCs, the analysis of gene expression is highly complicated by the reduction in cell number compared to previous experiments; indeed, each GV oocyte is enclosed by a limited number of CCs, about 1500 per oocyte. In addition, being these cells highly specialized and oocytes almost completely mature, CCs only have a very low proliferative rate, as demonstrated by flow cytometry analysis, and produce very few transcripts. To overcome the limit of such a small amount of RNA, each passage of the protocol was optimised: a very sensitive kit for RNA isolation involving the addition of a carrier tRNA was used to maximize the RNA yield. In addition, the conditions of amplification were finely tuned and preliminary results from the analysis of *Amh* expression on single antral COCs confirmed those obtained with pooled CCs, showing an even higher 8.1-fold up-regulation in NSN-CCs *vs.* SN-CCs ($p<0.05$). Although in line with what previously observed, variability when dealing with a single cumulus was very high, suggesting the need to further the analysis by increasing the sample number.

About *in vitro* matured follicles, at the end of maturation MII oocytes could clearly not be classified anymore on the base of their chromatin organization. Instead, an a posteriori analysis of their competence was done by examining the expression of NOBOX. The immunofluorescence analysis on MII oocytes revealed the presence of at least three levels of expression corresponding to three different classes of cells: some oocytes displayed a clear and bright signal spread in the whole ooplasm (Fig. 14, A); others completely lacked the signal (Fig. 14, G) and a third class exhibited intermediate patterns of expression, having a detectable but weak signal (Fig. 14, D).

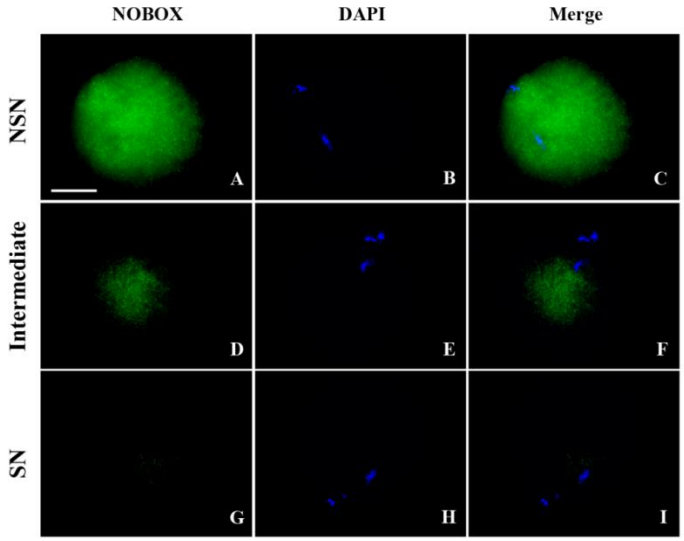

Figure 14. Expression of NOBOX protein in *in vitro* matured MII oocytes. In green, NOBOX immunostaining in oocyte cytoplasm (A,D,G); in blue, counterstaining of nuclei with DAPI (B,E,H); bar, 30 μm.

To conform the analysis with that of antral CCs, I only selected the outer patterns of expression (i. e., intense fluorescence and total lack of signal), in order to perform an *a posteriori* classification comparable to that done with GV oocytes. On the base of this sorting I collected single *in vitro* matured cumuli for further analyses. Again, evidences of a differential expression of *Amh* transcript characterizing SN and NSN oocytes were present, but the very high variability detected made the results not clear; further analyses are still needed.

4.3. Lhr and Fshr are differentially expressed in NSN-CCs compared to SN-CCs

In the search for new non-invasive follicular markers I focused my attention also on two genes and their related proteins, known to play a crucial role during folliculogenesis and ovulation: Fshr and Lhr, the gonadotrophin receptors expressed by follicle cells and responsible for the response to hormonal stimuli. I analysed both the mRNA expression through qRT-PCR and protein expression by immunofluoresce.

qRT-PCR analysis revealed a higher number of *Lhr* transcripts in NSN-CCs compared to SN-CCs in all the four independent experiments conducted, with a mean fold-change ± S.D. of 2.23 ± 0.8 (p=0.029) (Fig. 15). Instead, *Fshr* expression did not result significantly different, with -1.092 ± 0.167 fold-change (p>0.05).

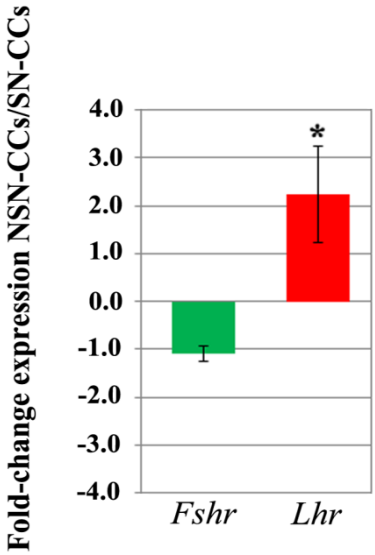

Figure 15. Fold-change expression of *Fshr* and *Lhr*. qRT-PCR fold-change expression of NON-CCs *vs.* SN-CCs. Green, down-regulated gene; red, up-regulated gene. Bars, S.D.; *, *p* value <0.05.

Next, I examined the presence of LHR and FSHR proteins on the surface of cumulus cells by performing an immunofluorescence analysis. Here, the fluorescent signal of both FSHR and LHR antibodies was visibly clearly higher on the surface of NSN-CCs than on that of SN-CCs (Fig. 16).

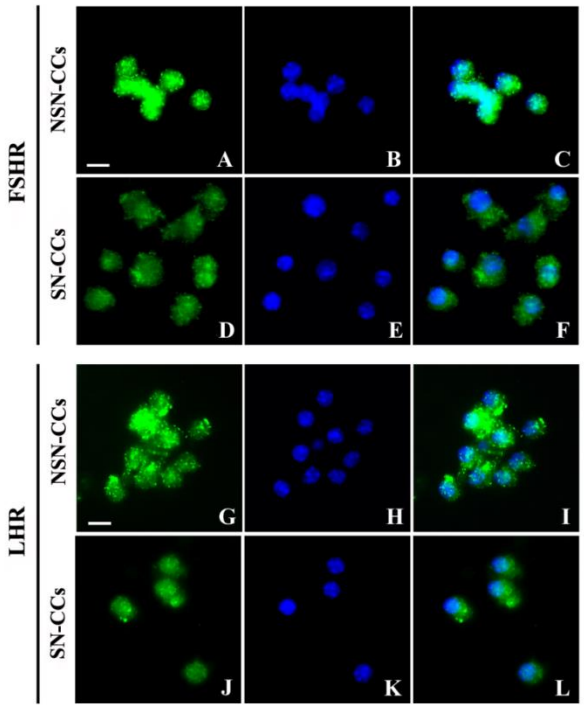

Figure 16. Immunofluorescence analysis of FSHR and LHR protein expression in NSN-CCs and SN-CCs. In green, immunostaining of FSH and LH receptors on the surface of CCs; in blue, counterstaining of nuclei with DAPI; bar, 8 μm.

In the attempt to make a sort of quantification of the fluorescent signal, CCs were classified into four different groups on the base of the brightness of fluorescence displayed. Namely, they were classified into: "none-weak", "medium", "high" and "very high" (see Materials and Methods for details, Fig. 25). In the case of FSHR, more than 500 NSN-CCs or SN-CCs were analysed and, of these, a significantly lower number of NSN-CCs compared to SN-CCs was sorted into the "none-weak" class (p=0.035) (Table 2). Also, the frequency of NSN-CCs displaying a "very high" fluorescence was significantly higher compared to that of SN-CCs (p=0.038). The classes in between, corresponding to "medium" or "high" fluorescent signal, comprised all the remaining CCs, without showing any significant difference (p>0.05).

Table 2: Frequency mean of the four different fluorescence intensity groups for FSHR and LHR antibodies. Data shown as the mean ± S.D. *p=0.035, **p=0.038, $^{\S}p$<0.001, $^{\S\S}p$=0.024 in the comparison between NSN-CCs and SN-CCs.

		N. of CCs analysed	Mean percentage ± S.D.			
			None-weak	Medium	High	Very high
FSHR	NSN-CCs	587	15.7 ± 9.0*	30.8 ± 17.7	29.6 ± 12.3	29.9 ± 15.0**
	SN-CCs	798	40.5 ± 16.4	46.7 ± 10.3	10.6 ± 13.9	2.3 ± 4.6
LHR	NSN-CCs	668	5.1 ± 6.3§	42.1 ± 14.7	33.8 ± 20.6	12.9 ± 7.9§§
	SN-CCs	737	29.0 ± 1.6	54.7 ± 12.3	21.2 ± 7.3	0.8 ± 1.5

As for LHR antibody, the analysis led to a similar result, with a lower frequency of NSN-CCs showing a "none-weak" fluorescence intensity signal (p<0.001) and a higher number of NSN-CCs displaying a "very high" signal (p=0.024). Then, I calculated the mean fluorescence value of a single NSN-CC *vs.* SN-CC by attributing an arbitrary numerical value to cells belonging to each class. Namely, cells belonging to the "none-weak" class were attributed the value 0, cells falling in the class "medium" were considered 1 and cells in the classes "high" and "very high" were given a value of 2 or 3, respectively. This confirmed the previous results, with a fold-change of 2.3 (p=0.041) and 1.7 (p=0.002) for FSHR and LHR, respectively, both up-regulated in NSN-CCs compared to SN-CCs.

The nuclei counterstaining with DAPI revealed the occurrence of cell populations with a different nucleus size, suggesting a possible association with different cell cycle stages. A more detailed analysis through flow cytometry highlighted the presence of two subpopulations, by analysing physical parameters defining the cell internal complexity and granulosity (side scatter, SSD) and dimension (forward scatter, FSC), one of which was characterized by a smaller size (Fig. 17). The two populations were spotted

within both SN-CCs and NSN-CCs, without showing statistical differences (p>0.05).

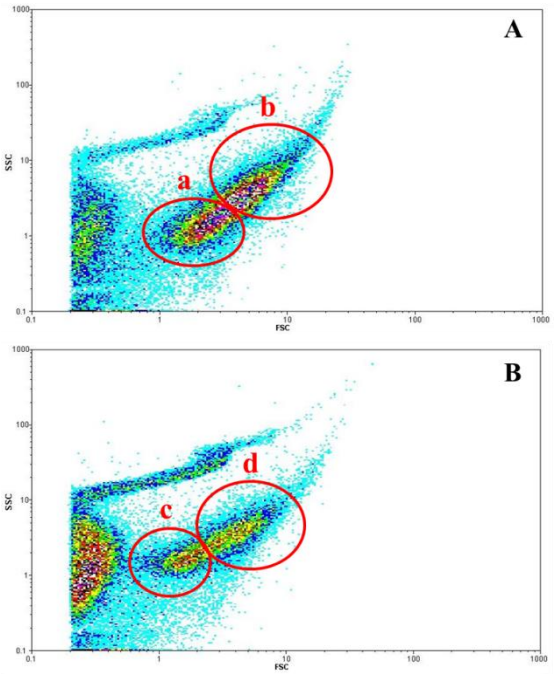

Figure 17. Cell subpopulations of SN-CCs (A) and NSN-CCs (B). In a and c are circled smaller cell, compared to those circled in b and d red circles.

To examine the possibility of different distributions of cells within cell cycle phases, I performed a flow cytometry analysis. When sorted with a flow cytometer, most of both NSN-CCs and SN-CCs were found in G0/G1 and no significant differences were found in the comparison between the two cell populations (p>0.05). Interestingly, both NSN-CCs and SN-CCs showed a low but again not statistically different, S phase (p>0.05) (Fig. 18).

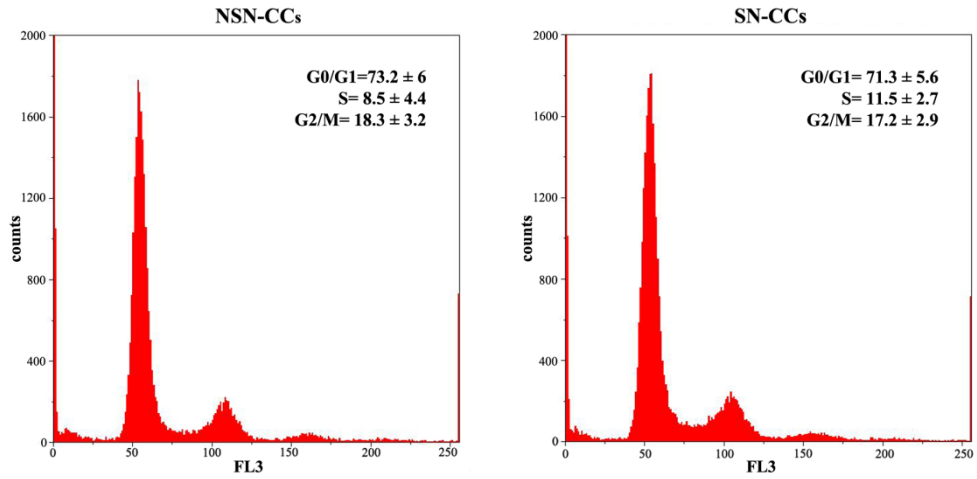

Figure 18. Distribution of cells within the cell cycle phases. In both NSN-CCs and SN-CCs most of the cells are in G0/G1 phase and only a small fraction are in S phase.

4.4. A culture system for preantral follicles encapsulation and maturation into a calcium alginate matrix

In order to obtain a matrix that recreates *in vitro* the ECM-rich cortex of the ovary, I used different concentrations of sodium alginate combined with diverse cross-linking agents and tested various techniques.

Sodium alginate only needs to get in contact with a ion possessing two positive charges to undergo mild gelification and, potentially, all ions with these characteristics could be applied. I therefore tested Ca^{2+}, Ba^{2+} and Mg^{2+}: all of them were able to trigger rapid alginate polymerization but, when attempting to culture cells within the respective matrices, only Ca^{2+} proved to sustain cell viability. The other two, instead, led to the formation of a matrix that exerted an action somehow detrimental on the cells in culture. After deciding to use Ca^{2+}, I tried to limit its potential effect as a cellular messenger by lowering its concentration down to the lowest that allowed the rapid obtainment of a stable and easy-to-handle matrix: 50 mM.

In the attempt to set up a protocol for 3D *in vitro* follicle culture, the most difficult step of the whole procedure is the encapsulation of cells within the matrix. Three are the main techniques tested:

a) Alginate capsules. Capsules are small structures composed by a solid surface with an empty or liquid core. They can be obtained by letting the cross-linking solution containing follicles drip directly into the polymer solution, so that as soon as the drop gets in contact with the polymer, its surface polymerizes and cells remain entrapped within the core. Polymerization only takes a few minutes and the capsules obtained are easily handled with a spoon-shaped spatula (Fig. 19). However, they have a diameter of about 0.5 cm, much bigger than the diameter of the entrapped small immature follicles and this might somehow interfere with nourishment and waste products exchange between capsule core and the environment.

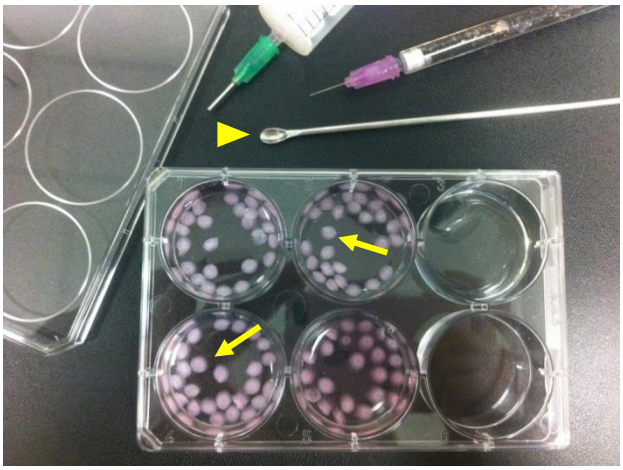

Figure 19. Multi-well dish with alginate capsules rinsed in a buffer solution. The capsules have a diameter of about 0.5 cm (arrows) and can be moved from the rinsing buffer solution to the culture medium with a spoon-shaped spatula (arrowhead).

b) Alginate bed. The second strategy envisages the formation of a thin bed of matrix covering the bottom of a Petri dish, in which follicles are embedded. I developed a system for the nebulization of small drops of cross-linking solution onto a thin layer of liquid alginate (1 ml spread on the bottom of a 3 mm plastic Petri dish). In order to obtain a smooth, homogeneous and evenly distributed matrix, the drops must have a very small diameter, around 5 µm, and in about 20 minutes of nebulization the matrix is completely polymerized. For the use of this alginate bed, the biggest limit is the difficulty in entrapping cells within the polymerized biomaterial, as keeping cells inside liquid alginate at room temperature for the whole nebulization procedure would damage follicles. I tried different options, such as mechanically inserting cells in the middle of the bed with a micropipette or lay follicles on a first layer of matrix and entrap them by adding a second layer on the top. However, none of these strategies ensures a reliable embedding without a damage to the cells.

c) Alginate beads. Beads are small spherical structures obtained through polymerization of the whole drop of liquid alginate solution. For the production of such beads I used the inverse of the technique proposed for the production of capsules: the cell-alginate solution drips from the tip of a Pasteur micropipette directly into the cross-linker solution. In this way, the small drop of liquid alginate completely polymerizes in about 2 minutes, not all the beads obtained have a perfectly round shape, but they have a smaller diameter compared to that of capsules (about 2 mm) and are as well easy to handle with a spatula (Fig. 20).

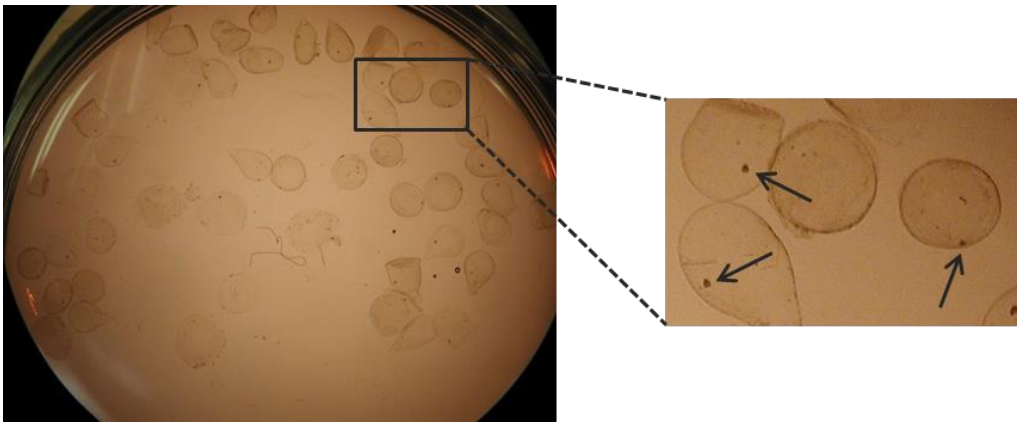

Figure 20. Petri dish containing small preantral follicles encapsulated within calcium alginate beads. On the left, alginate beads containing immature preantral follicles in culture medium. On the right, detail of a few beads and their encapsulated follicles (arrows).

Tests of these different encapsulation procedures led to the choice of calcium alginate beads as tool for 3D follicle culture. The further step was the tuning of polymer concentration, which needed to be high enough to produce a stable matrix, but low enough to let follicles grow and expand in all directions. Concordant with what found in the literature, I started with 2% calcium alginate and lowered the concentration to 0.25%.

In collaboration with the group of Prof. Conti (University of Pavia, Italy), the matrix of 0.25% calcium alginate has been chemically tested for its resistance to osmotic, pH and temperature stresses and resulted stable and constant in its structure.

Preliminary culture experiments showed that encapsulated follicles grew and maintained their 3D structure up to 6-7 days within the biomaterial (Fig. 21 2, d-g). In the 2D control, instead, follicles get in contact with the bottom of the Petri dish as soon as the second/third day of culture and at day 6 they were almost all flattened and attached to the support, losing their shape and cell-to-cell contacts (Fig. 21 1, a-c). With respect to the control, immature follicles retained their spherical shape, follicle cells proliferating in all directions and maintained a healthy morphology for a longer period.

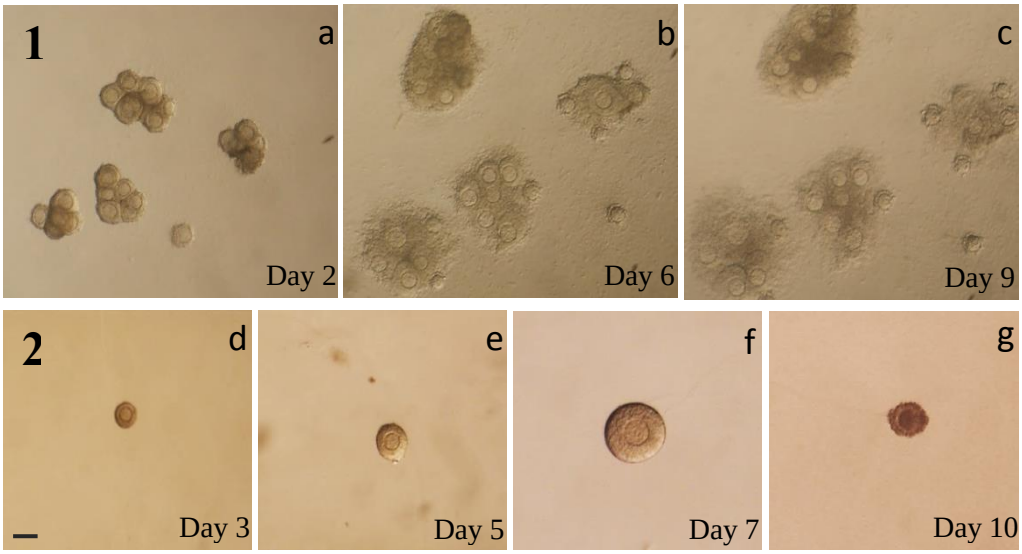

Figure 21. Representative photographs of immature preantral follicles. Follicles cultured in a 2D system (a-c) lose their shape and flatten onto the Petri dish. Within 0.25% calcium alginate (d-g), instead, they maintain their 3D architecture up to 7 days. Bar, 150 µm.

Nevertheless, in both culture systems, no follicles managed to grow up to the antral stage, develop an antrum and complete their maturation process. Therefore, ongoing experiments are now trying to optimize the culture conditions that allow the complete follicle maturation until the antral preovulatory stage.

4.5. Murine oocyte-cumulus cells co-culture

Antral COCs were isolated from the ovary, cumuli were broken apart and oocytes at the germinal vesicle stage were classified into SN and NSN. On the base of their chromatin organization, oocytes were collected and cultured *in vitro* with the same number of cumuli, either of the same (i.e. SN oocytes with SN-CCs), or of the opposite type (i.e. SN oocytes with NSN-CCs) (Fig. 22). At the end of the culture period, maturation of oocytes was assessed by evaluating the presence of the first PB.

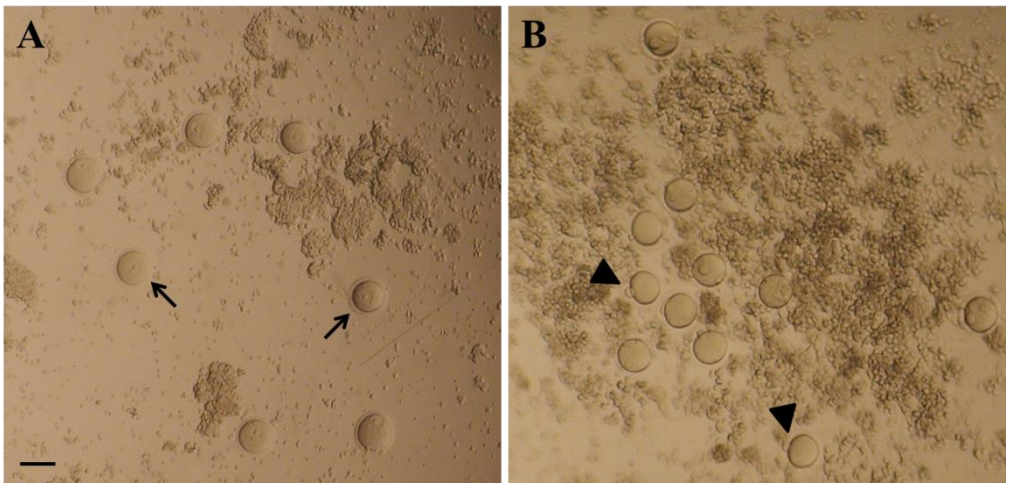

Figure 22. Oocyte-cumulus cells co-culture. Upon isolation, GV oocytes (arrows) are classified into SN and NSN and cultured on the same number of cumuli (A); after 15 hr *in vitro* maturation, oocyte are the MII stage (arrowheads) and CCs have visibly proliferated and produced a sticky HA matrix (B). Bar, 80 µm.

The effect of co-culture with dispersed CCs did not seem to influence the rates of oocyte maturation to MII. Namely, more than 90% of SN oocytes successfully matured regardless of the CC type present within the same drop of medium. Likewise, about 64% NSN oocytes completed the first meiotic division when cultured with their own CCs, compared to about 52% of matured NSN oocytes. Even though no significant differences were observed between maturation rates, probably because of the high variability among experiments, the quality of MII oocytes obtained might be highly influenced by the presence of SN-CCs releasing factors and molecules beneficial to oocytes in culture. Therefore, in the short future, qualitative studies will be performed on co-cultured MII oocytes and expanded CCs to evaluate the quality of cells obtained by evaluating the expression of specific markers of the oocyte developmental competence.

Chapter 5

Discussion

Specific cumulus cell-related genes are putative markers of the oocyte developmental competence

A high quality of the female gamete is essential to a successful development of the zygote into a healthy embryo able to grow to term. In our and other mammalian species not all the oocytes that complete their maturation are of the same quality; instead, part of them is defined as developmental incompetent, being unable to be fertilized or to sustain early preimplantation development. The possibility of identifying and selecting those eggs that are developmentally competent would be of particular relevance in the clinic, where focusing on high quality gametes would significantly improve the overall rates of pregnancy and full-term development. The identification of the optimal selective protocol has been the main aim of many studies in recent years but, up to now, no universal and objective procedure has been described. The main difficulty encountered is the invasiveness of methodologies required to test the proposed markers, as most of them imply the manipulation of the gamete. To avoid the risks associated to a direct examination of the oocyte, follicle cells have been extensively studied, as their analysis would be entirely non-invasive. The results obtained by previous investigations conducted on various animal species, including human, are so far puzzled and not widely accepted, mainly because of the unevenness among the end points adopted, protocols employed and the influence of different hormonal treatments. Moreover, in all these studies the quality of gametes was only inferred *a posteriori* basing on different parameters, with the risk of introducing high variability among the results.

The possibility of knowing in advance the real developmental competence of the gametes under study is the major novelty of my work. Knowing *a priori* oocytes quality by classifying antral oocytes into developmentally competent (SN) and incompetent (NSN), I was able to exactly establish a correlation between CCs gene and protein expression and oocyte developmental competence. A first microarrays analysis of their entire transcriptome highlighted a similar transcriptional activity, differing only for the expression

of 422 genes, almost entirely (97.6%) down-regulated in NSN-CCs. An additional, more sensitive analysis through qRT-PCR confirmed this trend to a lower gene expression identifying a list of four CC-related down-regulated (Has2, Ptx3, Tnfaip6 and Ptgs2) and one up-regulated (Amh) gene in NSN-CCs *vs.* SN-CCs. Among these, the gene with the highest differential expression was Amh, being 4-fold up-regulated in CCs surrounding developmentally incompetent NSN oocytes, a difference that was maintained at the protein level. Interestingly, a concordant expression pattern resulted from the analysis of AMH in human follicles, where the gene was found highly expressed in CCs of preovulatory follicles enclosing premature or atretic oocytes (Kedem-Dickman et al., 2012).

To further test the robustness of this putative marker and to verify whether it could be used as a diagnostic tool to define the developmental capability of oocytes selected for IVF, I set up a protocol to analyse its expression at the level of a single antral COC. Isolation, manipulation and gene expression analysis of a single follicle were optimized for the examination of a small amount of cells and RNA. Moreover, clinical procedures require the whole selective process to be safe and non-invasive, but also easy and rapid, as marker analysis should be performed while eggs are in the incubator under controlled conditions before their *in vitro* fertilization. Among the different protocols tested, a very sensitive RNeasy Micro Kit was used for total RNA extraction in order to maximize yields and purity in the shortest time. As a whole, from follicles retrieval to molecular analysis of *Amh* expression, the total procedure only requires 5-6 hours and previous data obtained analysing pools of hundreds follicles were confirmed also at the level of a single COC. Therefore, *Amh* emerges as a valid marker for the selection of the best quality antral oocyte, even though further studies are needed to confirm the preliminary data obtained so far.
Evidences suggest that an intact cumulus oophorus is of central importance for proper maturation of the enclosed oocyte, resulting in a better quality egg. Hence, a further optimization to the proposed selective approach would be to isolate intact antral COCs from the ovary, let them *in vitro* mature without separating the two components and remove CCs for molecular analysis only when oocytes have completed the first meiotic division. Unfortunately, the use of matured and expanded CCs encountered an additional difficulty due to an even lower content of RNA. When oocytes reach the MII stage, the about 1500-2000 surrounding CCs have proliferated, are already terminally differentiated and their transcriptional activity is extremely low. Moreover, partial RNA degradation occurs and a fraction of these cells undergoes apoptosis. The amount of material available for molecular investigation is, thus, limited and sometimes not sufficient to be detected and amplified through qRT-PCR. Another source of variability among the results obtained is

the *a posteriori* classification of oocytes developmental potential, based on immunofluorescence detection of NOBOX protein in *in vitro* matured MII eggs. Even though the CCs analysed for *Amh* expression were those surrounding oocytes that displayed a clear NOBOX expression profile, many oocytes had a wide range of intermediate signals and fell in a third category, which might represent cells that are switching from NSN to SN configuration. Despite having some clear evidences that a differential expression of *Amh* is still present in expanded CCs of MII^{NSN} and MII^{SN} oocytes, the analysis does not seem to be reproducible yet and the setting up of a more effective protocol is still a work in progress.

Still with the aim of identifying follicle cell markers of the oocyte developmental competence, I focused my attention onto two hormonal receptors, whose function is particularly relevant during folliculogenesis and ovulation: Fshr and Lhr. Their expression profile, both at the mRNA and protein level, has been previously described in many mammalian species, but mostly in granulosa cells, while little is known regarding CCs surrounding fully-grown antral oocytes. By using the same experimental approach described above, I found a significant 2.23-fold up-regulation of *Lhr* transcript in NSN-CCs compared to SN-CCs, while *Fshr* didn't result differentially regulated in the two cell types. Instead, when comparing the expression of their corresponding proteins by immunofluorescence, they both showed a brighter and higher expression in NSN-CCs *vs.* SN-CCs. Such a discrepancy between the expression of the mRNA and that of the protein might be due to some mRNA instability, rates of degradation and synthesis or post-translational modifications since protein translation is a highly regulated process.
This expression profile is consistent with what shown previously in the mouse and other species. Namely, in CCs surrounding antral oocytes isolated from unprimed mouse females, both transcripts resulted undetectable (Fu et al. 2007). Similar results were found when analysing the same cell type in the cow (Calder et al. 2003; Simoes et al. 2012), sheep (Tisdall et al. 1995, McNatty et al. 2000, Logan et al. 2002; Cotterill et al. 2012) and pig (Shimada et al. 2003), where Fshr and Lhr mRNAs were scarcely detectable. However, *Lhr* displayed a marked up-regulation when analysed in CCs from COCs cultured in the presence of FSH, suggesting a stimulatory role of this hormone both *in vivo* and *in vitro* (Shimada et al., 2003). Regarding the association between these receptors expression and oocyte developmental competence, high levels of Lhr gene were detected in CCs belonging to COCs with a better meiotic competence in the cow (Calder et al. 2003; Simoes et al. 2012). Likewise, a higher expression was associated to an increased developmental potential to the blastocyst stage in human CCs (Yang et al. 2005; Maman et al. 2012; Papamentzelopoulou et al. 2012). These results are not consistent with

what discovered in this study and the cause might be found in the differences occurring between monovulatory (human and cow) and pluriovulatory (mouse) species or in the various hormonal treatments to which females are subjected before cells retrieval.
As for Fshr and Lhr proteins, the expression of both receptors has been studied principally in theca and granulosa cells isolated from growing follicles in the mouse (Choi et al. 2011), rat (Bukovsky et al. 2003; Armenti et al. 2008), squirrel (Li et al. 2012), pig and human (Bukovsky et al. 2003). Instead, less is known about their expression in CCs of fully-grown antral follicles. Hence, this is the first study where FSHR and LHR pattern of expression is described in murine CCs enveloping antral preovulatory oocytes and where a link is proposed between their expression and different developmental capabilities of the egg. Regarding mRNA analysis, only qRT-PCR measurement of *Lhr* expression allows the discrimination between CCs enveloping developmentally competent or incompetent oocytes. About protein expression, the methodology described still needs improvement, leading to the loss of a high number of CCs and being at the moment not applicable to the single COC. Nevertheless, both FSHR and LHR immunofluorescence detection might help in distinguishing between the two CC types, as they are both significantly more expressed in CCs surrounding poor quality oocytes unable to develop beyond the two-cell stage.
Therefore, these two potential biomarkers add to the list of putative markers previously found and described. The availability of such valuable non-invasive markers able to identify those eggs that have the highest developmental potential would represent a strong support to the morphological evaluations nowadays routinely performed in most IVF clinics.

The road towards the artificial ovary

In vitro follicle culture has been extensively studied in recent years for its huge potential in the field of assisted reproductive technologies. The possibility to isolate and grow *in vitro* immature follicles from cryopreserved ovarian biopsies would allow the preservation of fertility for those patients that have undergone gonadotoxic treatments for the cure of cancer or autoimmune diseases. Recent years have seen an enormous breakthrough, but still the maturation rates of present technologies are quite low and the quality of the gametes obtained is far from being optimal, as required by human IVF clinics. Within the ovary, each ovarian follicle can be considered as a small organ *per se*, with its own blood vessel network surround its periphery. For its peculiar characteristics it can be isolated from the ovarian cortex and grown in an artificial environment. Up to now, most of the work has been performed by using the canonical 2-dimensional systems, where cells are cultured in drops of maturation medium on a rigid support. Despite the huge achievements

obtained with these methodologies, including the birth of live offspring, such procedures are not suitable for long-term cultures. Their major limit is the loss of follicle 3D architecture occurring as soon as cells get in contact with the rigid culture plate. The need to maintain follicle three-dimensionality and, consequently, intercellular contacts and communications, is the driving force that led to the development of 3D systems for *in vitro* follicle culture.
Various biocompatible matrices, either of natural or synthetic origin, have been developed and tested with a series of cell types, including ovarian follicles of many mammalian species. Some proved to be suitable for follicle encapsulation and growth, leading to promising preliminary results. Among these, calcium alginate is now centralizing most of the attention, thanks to its many positive properties and handiness. Huge improvements have already been done to the protocols described in the literature, but still the manipulation and encapsulation of immature follicles remains a delicate and tricky passage that might compromise the entire culture. With this in mind I tried to set up a new protocol for an easy and quick encapsulation of single murine immature preantral follicles within beads of 0.25% calcium alginate. The results obtained, though preliminary, are encouraging.

Three-dimensional *in vitro* follicle culture within a biomaterial has highlighted the necessity of reproducing the structural context of ovarian cortex as a whole, including not only chemical but also and especially physical stimuli that act on cells *in vivo*. Among these, shear stress, compressive and tensional forces and the geometrical features of the culture microenvironment may give inputs to cells altering their behaviour, activity and globally their tensegrity state. Effects of these forces on growing encapsulated ovarian follicles have never been investigated, but their discovery and description would certainly improve our understanding of cells interactions and allow further optimization of actual culture techniques.

Chapter 6

Materials and Methods

6.1. Bioinformatics analyses

6.1.1. Microarrays

Messenger RNAs for microarrays analysis were extracted with the RNeasy Mini Kit (Qiagen) from NSN-CCs and SN-CCs, following the manufacturer's instructions and the total mRNA was quality checked by Nanodrop analysis (Nanodrop Technologies, Wilmington, DE, USA). In order to have enough RNA for the analysis, a two-round linear amplification with Illumina TotalPrep RNA Amplification Kit (Ambion, Austin, TX, United States) was performed with 400 ng mRNA, which is a complete system for generating biotin-labelled cRNA for hybridization with Illumina Sentrix arrays.
For each sample of the two biological replicates, 1.5 µg cRNA was hybridized onto Illumina mouse-8 BeadChip version 3. Samples were washed, stained with Cy3-streptavidin and then scanned. All these passages have been performed on the Illumina Bead-Station 500 platform (Illumina, San Diego, CA, United States) with reagents and following the protocols supplied by the manufacturer.

6.1.2. Microarrays data analysis

All basic expression data analysis was carried out using the manufacturer's software BeadStudio 1.0. Raw data were background-subtracted and normalised using the "rank invariant" algorithm. Normalized data were, then, filtered for significant expression basing on negative control beads. Selection for differentially expressed genes was performed on the basis of arbitrary thresholds for fold changes plus statistical significance, computed through a permutation-based test.
The Gene Ontology (GO) enrichment analysis was performed using the tools provided by the data mining and bioinformatics software Orange

(http://orange.biolab.si/). Pathways and networks were obtained using Orange and David (http://david.abcc.ncifcrf.gov/).

6.1.3. Mesh annotation analysis

In order to bring out the genes mostly involved in the ovarian and follicle function among all the differentially expressed transcripts, we performed a literature-based search (PubMed), exploiting the NCBI Web Services. Specifically, we choose a list of MeSH annotation terms characterizing folliculogenesis (i.e. Cumulus Cells, Embryonic Development, Fertilization, Gonadotropins, Granulosa Cells, Growth and Development, Hormones, Oocytes, Oogenesis, Ovarian Follicle, Ovulation, FSH receptors, LH receptors, Reproduction, Zygote) and assigned it to each of the differentially regulated genes. Then, using a text mining technique, called TF-IDF (Nuzzo et al., 2010), to rank the lists of terms according to their relevance we obtained a subset of MeSH terms specifically associated to each gene. The information obtained from the literature was further combined with expert knowledge on the domain. From the chosen keywords related to folliculogenesis listed above, we identified the corresponding terms in the MeSH database and obtained a set of basic key annotations (MESH_B, Table 2S). In addition, including the subordinate of the starting key terms, which are the more specialized child terms in the MeSH hierarchy, a further list was obtained (MESH_BC). Likewise, also parents terms of all the annotations in MESH_B, were added to get a third subset of key annotations (MESH_BCP).

To evaluate the real involvement of each regulated gene in the domain of interest, a score was developed basing on the number of its related key terms for each set of key annotations. A first sub-score indicated for each gene the proportion of related MeSH terms from the complete list. A second one estimated the proportion of terms within the 10% top-relevant ones according to TF-IDF. A final score was calculated by applying a weighted sum of these two sub-scores, with a higher weight assigned to the second one in order to focus onto the most relevant terms instead of the entire list of annotations. A permutation strategy was used to assess the statistical significance for each gene and each score; genes were considered significantly associated to folliculugenesis keyword when their p values were ≤ 0.05.

Moreover, to bring to light possible functional relationships among the proteins encoded by the differentially regulated genes, MeSH terms were combined with information on protein associations provided by the STRING database (http://string-db.org). A gene network was generated, connecting regulated genes with a MeSH annotation similarity or a STRING confidence score higher than the 0.6 cut-off value. In order to

quantify the importance of the nodes in the network, we calculated a topological index known as Betweenness Centrality (Yu et al., 2007), indicating the central genes characterized by high connectivity.

6.2. Biomaterials for *in vitro* follicle culture

6.2.1. Preparation of sodium alginate matrix

Sodium alginate (Pronova, NovaMatrix, USA) was dissolved at a final concentration of 0.25% in PBS 1X Ca^{2+}- and Mg^{2+}-free overnight and maintained at 4°C until use.

6.2.2. Encapsulation and culture of preantral follicles

As preantral follicles were isolated, they were pipetted into drops of αMEM. In the meanwhile, each well of a 96-well plate was filled with 200 µl 50 mM $CaCl_2$. Follicles were washed into sodium alginate solution, singularly aspired into a glass mouth-controlled Pasteur micropipette in a total volume of 2-3 µl and dripped into wells filled with $CaCl_2$. After 5 minutes beads were formed (Fig. 23), retrieved by using a micro spoon, washed and incubated for 11 days into αMEM Glutamax supplemented with 5% fbs, 10 mU/ml rFSH, ITS, Pen/Strep. Half of the medium was replaced every 4 days and the last day of culture it was changed with fresh medium with the addition of 1.2 IU/ml r-hCG and 4 ng/ml rEGF.

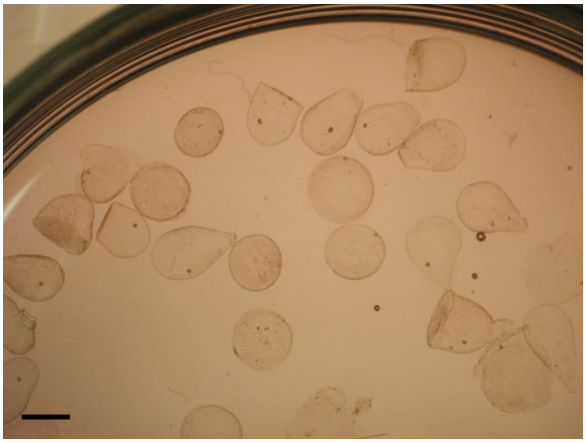

Figure 23. Examples of beads made of 0.25% calcium alginate. Bar, 2 mm.

6.3. Animals, cells and molecular analyses

6.3.1. Animals, reagents and hormonal treatment

Depending on the study, B6C3-F1 and CD1 female mice 12-14 days or 4-6 weeks old were used. All the animals were purchased from Charles River (Como, Italy) and maintained under temperature- and humidity- controlled conditions (22°C of temperature and a dark/light cycle of 10/14 hr) in the department animal facility. For the experiments, they were sacrificed by cervical dislocation, in strict accordance with the protocol approved by our University and the European (n. 86/609/CEE) and Italian (n. 116/92, 8/94) legislation. The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of Pavia (Protocol Number: 1-2010). For antral follicles isolation, adult females were hormonally treated by intraperitoneal injection with 3.75 or 10 IU pregnant mare serum gonadotrophin (PMSG) 48 hr before sacrifice, in order to synchronize ovarian cycles.

6.3.2. Media for follicle isolation, manipulation and culture

M2 medium (Fulton and Whittingham, 1978; Table 3) was used for COCs isolation and CCs collection. Single COCs were matured in α-MEM medium (Sigma-Aldrich, M4526) with the addition of 5% of fetal bovine serum (fbs), 2 mM glutamine (Life Technologies), 5 mM taurine (Sigma-Aldrich), 26 μg/ml pyruvate, 50 mU/ml rFSH (Merk Millipore, Milan, Italy) and 10 ng/ml EGF (Sigma-Aldrich).
Oocytes and CCs were isolated and co-cultured in αMEM-Glutamax (Life Technologies, 32561-029) supplemented with 5% fbs, 1 mg/ml fetuin, 26 μg/ml pyruvate, Pen/Strep, 50 mU/ml rFSH, 5 ng/ml EGF. Instead, small preantral follicles were cultured in αMEM-Glutamax with the addition of 5% fbs, ITS (5μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenium; Sigma-Aldrich), 10 mU/ml rFSH. Half medium was replaced every other day.
All the maturation media were prepared and equilibrated at 37°C, 5% CO_2 overnight the day before the experiment.

Table 3. Recipe for the preparation of isolation medium.

Reagent	M2
NaCl	5.534 g
KCl	0.356 g
CaCl2	0.189 g
CaCl2•2H2O	
MgSO4	
MgCl2•6H2O	
MgSO4•7H2O	0.293 g
KH2PO4	0.162 g
NaH2PO4•12 H2O	
NaHCO3	0.336 g
Phenol Red	0.010 g
Hepes	5.004 g
D-glucose	1.000 g
Sodium Pyruvate	0.036 g
Sodium Lactate	3.9 ml
EDTA	1.0 ml
Penicillin/Streptomycin	1x104 U
BSA	4 g

6.3.3. Isolation of preantral and antral follicles

Ovaries of prepubertal (12-14 day-old) CD1 female mice were placed in a glass dish in buffered αMEM and punctured with a sterile needle under a stereo-micoscope. By using a mouth-controlled sterile Pasteur micropipette, early preantral follicles of 110-130 µm in diameter were collected and rinsed in maturation medium.

The same procedure was used for the mechanical isolation of COCs from antral preovulatory follicles: ovaries of 4-6 week-old females were punctured on their surface in M2 medium and multi-layered complexes (Fig. 24) were released and collected. With a Pasteur micropipette, they were singularly transferred into 7 µl droplets of fresh M2 medium where CCs and their enclosed oocytes were separated by gently pipetting the follicle in and out.

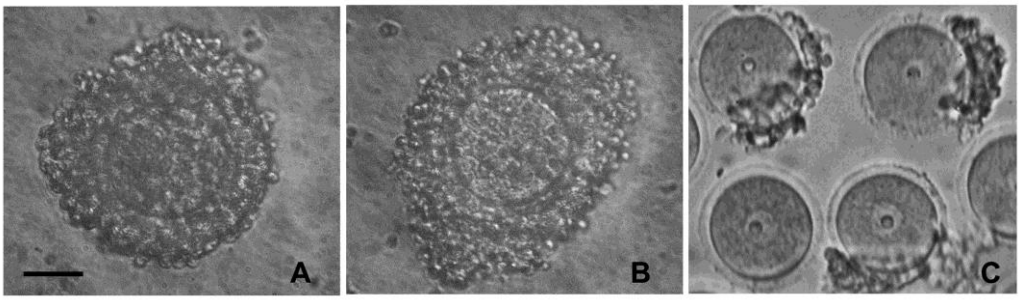

Figure 24. Examples of COCs isolated and used in this study. Only cumulus cells isolated from antral oocytes surrounded by >2 layers of follicle cells were used (A, B). C, examples of GV antral oocytes excluded because only partially surrounded by cumulus cells. Bar, 40 µm.

6.3.4. Maturation of single antral follicles

For the analysis of *Amh* expression on the single mature cumulus, COCs were singularly *in vitro* matured until the stage of MII. After the isolation, each COC was washed in maturation medium, pipetted in a 4 µl drop of αMEM supplemented with 2 mM glutamine, 5 mM taurine, 5% fbs, 26 µg/ml pyruvate, 50 mU/ml rFSH and 10 ng/ml EGF under mineral oil and incubated at 37°C, 5% CO_2, 5% O_2, 90% N_2 for 15h.

6.3.5. Classification of antral oocytes

After COCs isolation into single drops of medium, oocytes were mechanically freed from their surrounding CCs and singularly transferred into 3.5 µl droplets of M2 medium with 0.05 µg/ml Hoechst 33342, that specifically binds to the DNA AT-rich regions. After a 12 min incubation, oocytes were observed with a AX70 microscope (Olympus, Japan) at 10X of magnification under UV fluorescence light and classified into SN or NSN, on the base of their chromatin organization (Fig. 6). Upon classification, CCs surrounding the two types of oocyte were collected singularly or in pools of about 40 cumuli into 1.5 ml Eppendorf tubes. 300 µl 1X PBS were added to each sample and they were centrifuged at 1.500 rpm for 5 min and the supernatant eliminated; pellets were snap-frozen and stored for following analyses.

6.3.6. Antral oocyte-cumulus cells co-culture

COCs were isolated in αMEM-Glutamax buffered with HEPES and put into single drops of fresh medium as previously described. Cumuli were broken up, oocytes were stained with 0.5 µg/ml Hoechst 33342 and classified into SN and NSN. After classification, DOs were washed twice into maturation medium and matured together with CCs into drops of 2 µl medium per oocyte in

four experimental conditions: SN-DOs with SN-CCs, SN-DOs with NSN-CCs, NSN-DOs with NSN-CCs and NSN-DOs with SN-CCs.
Cells were cultured for 15 hr at 37°C in a humidified atmosphere of 5% CO_2 in air. At the end of the culture period, CCs were collected in groups of 20 cumuli and MII^{NSN} or MII^{SN} oocytes were collected in groups of 10. Cells were snap-frozen in liquid nitrogen and stored at -80°C until RNA extraction.

6.3.7. Total RNA extraction, Retro-Transcription and Real-Time PCR

For the analysis of pools of cumuli or MII oocytes, RNA was extracted from cell pellets with the RNeasy Mini Kit (Quiagen) following the manufacturer's instructions. Briefly: the pellet was carefully loosened by gently flicking the tube, RLT buffer was added and samples were vortexed and homogenized to disrupt the cells. After the addition of 1 volume of 70% ethanol, the whole lysate was transferred to an appropriate spin column and centrifuged at ≥ 10.000 rpm for 15 seconds. To avoid genomic DNA contamination, on-column DNase digestion was performed by adding DNase directly onto the spin column membrane for 15 min. The enzyme was washed away through washing with RW1 buffer and the spin column membrane was washed by adding RPE buffer. The RNA was, then, eluted with RNase-free water.
The RNA from antral or mature single cumuli and pools of 10 MII oocytes, instead, was extracted with the RNeasy Micro Kit (Quiagen) following the same procedure but with the addition of tRNA as a carrier to improve extraction yields.
The total RNA extracted was retro-transcribed into cDNA using the following mix of reagents (all purchased from Applied Biosystems) (Tab. 4).

Table 4. PCR mix components for the retro-transcription.

	Final concentration/sample
Buffer 10X (GeneAmp®)	1 X
MgCl2 (GeneAmp®, 5 mM)	1 mM
dNTPmix (GeneAmp®, 10 mM each)	2 mM
Oligo d(T)16 (GeneAmp®, 50 µM)	2.5 µM
RNase inhibitor (GeneAmp®, 20 U/µl)	20 U
MuLV reverse transcriptase (GeneAmp®, 50 U/µl)	50 U

17.0 µl reaction mix were added to 3 µl RNA to reach a total volume of 20 µl for each sample. The reaction of amplification was performed by using an Applied Biosystems GeneAmp 9700® thermocycler and the conditions used for the retro-transcription of RNA extracted from pools of cumuli are the following:

25°C 10 min
42°C 15 min
99°C 5 min

Instead, a longer amplification program was used to retro-transcribe the RNA extracted from single cumuli:

25°C 10 min
42°C 60 min
99°C 5 min

At the end, samples were kept at 4°C. The cDNAs obtained were then amplified through real-time PCR using the following PCR mixture (Tab. 5).

Table 5. PCR mix components for the amplification.

	Final concentration/sample
Master mix (Eurogenter, 4 mM)	2 mM
Primer forward (Diatech, 10 mM)	0.2 mM
Primer reverse (Diatech, 10 mM)	0.2 mM

15.0 µl reaction mix were added to 5 µl cDNA of each sample. The reaction was performed on a Rotorgene 6000 thermocycler with different amplification programs, depending on the genes amplified. For the amplification of cDNA from pools of CCs, the following conditions were used:

- 95°C 5 min

- 95°C 10 sec
- 60°C 15 sec
- 72°C 20 sec

} 40 cycles

The amplification of cDNA obtained from single cumuli was done using the following conditions:

- 95°C 5 min

- 95°C 10 sec
- 61°C 15 sec
- 72°C 10 sec

} 45 cycles

Behind are the conditions used for the amplification of cDNA obtained from pools of MII oocytes:

- 95°C 5 min

- 95°C 10 sec
- 62.5°C 20 sec
- 72°C 20 sec

} 35 cycles

Table 4 lists all the primers used for PCR amplification, which were all designed using the program Primer3.

Table 6. List of primer sequences used for qRT-PCR. F, forward primer; R, reverse primer.

Primer	Sequence	Amplicon length (bp)
***Fshr* F**	5'-TTGGGGACCTGGAGAAAATAG-3'	95
***Fshr* R**	5'-AATTTCATGCAAGTTGGGTAGG-3'	
***Lhr* F**	5'-AAGCGAATAACGAGACGCTTT-3'	96
***Lhr* R**	5'-GGAGTGTCTTGGGTGAACAGA-3'	
***Has2* F**	5'-AAGACCCTATGGTTGGAGGTG-3'	95
***Has2* R**	5'-CATCCAGTATCTCACGCTGCT-3'	
***Ptx3* F**	5'-TGGACAACGAAATAGACAATGG-3'	95
***Ptx3* R**	5'-GATGAACAGCTTGTCCCACTC-3'	
***Ptgs2* F**	5'-GAGTGGGGTGATGAGCAACTA-3'	96
***Ptgs2* R**	5'-GCTCAGGTGTTGCACGTAGTC-3'	
***Tnfaip6* F**	5'-CGGGTATCATCGATTATGGAA-3'	100
***Tnfaip6* R**	5'-TCTGTGAAGACACCACCACAC-3'	
***Amh* F**	5'-AGCAGGCCCTGTTAGTGCTAT-3'	94
***Amh* R**	5'-CGAGTAGGGCAGAGGTTCTGT-3'	
***Nobox* F**	5'- AAAGACCCGAACCCTGTACC-3'	100
***Nobox* R**	5'-GGAAATCTCATGGCGTTTGT-3'	
Dnmt3l F	5'-GTGCGGGTACTGAGCCTTTTTAGA-3'	120
Dnmt3l R	5'-CGACATTTGTGACATCTTCCACGTA-3'	
Luciferase F	5'-AGTCGATGTACACGTTCGTCA-3'	
Luciferase R	5'-CAGTGCAATTGTTTTGTCACG-3'	
***Actb* F**	5'-CGCGAGCACAGCTTCTTTGC-3'	90
***Actb* R**	5'-GACGACCAGGCGAGCGATAT-3'	

Real-time PCR was performed on cDNA from at least three independent experiments. Also, for what concerns CCs, in order to perform a quantitative analysis, a reference gene was chosen basing on its expression in 100 SN-CCs and NSN-CCs: among the sequences tested, Actb resulted that with the most constant expression. As for the normalization of the results of MII oocytes, 1 picogram Luciferase control RNA (Promega, Milano, Italy) was added to each sample and co-retro-transcibed with target RNA as an exogenous marker.

6.3.8. Immunocytochemistry and image analysis

- ***Analysis of CCs:***

For immunocytochemistry analyses, SN-CCs and NSN-CCs were collected in 1.5 ml Eppendorf tubes, diluted in 80 µl fresh M2 medium and cytospun onto a glass slide with the following protocol: a first centrifuge at 1000 rpm for 10 min, another centrifuge at 2000 rpm for 10 min and a last one at 5000 rpm for 15 min. The exceeding medium was removed and cells were fixed with 4% paraformaldehyde in 1X PBS for 20 min. To eliminate any residue of fixative, a quick washing was performed with PBS and cells were incubated overnight at 4°C with primary antibody: anti-FSH receptor monoclonal antibody (Santa Cruz Biotechnology; SC-25828; diluted 1:50 in PBS), rabbit anti-LH receptor monoclonal antibody (Santa Cruz Biotechnology; SC-13935; diluted 1:50 in PBS) or goat polyclonal AMH antibody (C-20) (Santa Cruz Biotechnology; SC-6886; 1:100 in PBS). Anti FSHR and anti-LHR were removed by washing cells 3 times for 10 min each with 1X PBS whereas anti-AMH was eliminated by washing cells with 0.1% Tween PBS for 10 min.

Anti-FSHR and anti-LHR were revealed by incubating cells for 1 hr at 37°C with the secondary antibody Alexa Fluor488-conjugated goat anti-rabbit IgG (Molecular Probes) diluted 1:200 in 1X PBS. Samples were, then, washed in 3 changes of 1X PBS for 10 min each.

Anti-AMH was revealed by incubating cells for 1 hr at 37°C with Alexa 555-conjugated donkey anti-goat antibody (1:500 in 0.1% Tween PBS; Invitrogen, Italy). At the end of incubation, cells were washed three times for 10 min each with 0.1% Tween PBS.

In both cases nuclei were counterstained with DAPI (0.2 µg/ml in PBS) for 10 min and Glass slides were further mounted in Vectashield (Vector) and examined with an Olympus BX60 epifluorescence microscope equipped with single-bandpass filters for DAPI and Alexa555.

Data were obtained from three independent experiments and negative controls were treated with the same procedure, except for the incubation with the primary antibody. Digital images were obtained with an Olympus DP72 digital camera and processed using Adobe Photoshop software.

- ***Analysis of MII oocytes:***

After 15 hr of culture, MII oocytes were singularly fixed in 4% paraformaldehyde (PFA) in PBS for 30 min at 4°C. At the end of fixation, cells were washed in PBS 3 times for 5 min and permeabilized with 0.5% triton X-100 in PBS for 15 min at 4°C. To block non-specific sites, they were treated with blocking reagent (0.1% PBT + 1% BSA) for 1h at 37°C. Then, oocytes were incubated with rabbit anti-NOBOX monoclonal antibody (Abcam; AB41521) diluted 1:500 in PBS for 1 hr at 37°C and

washed 3 times for 15 min in PBT. The primary antibody was revealed by incubating cells with the secondary antibody Alexa Fluor488-conjugated goat anti-rabbit IgG (Molecular Probes) diluted 1:500 in PBT for 45 min at 37°C. After 3 washing steps of 15 min each in PBT, oocytes were counterstained using 0.2 µg/ml DAPI for 5 min at RT. At the end, oocytes were mounted on a glass slide using Vectashield (Vector) and analysed as described above.

Cumulus cells and MII oocytes were observed with an Olympus Provis epifluorescence microscope equipped with single-bandpass filters for DAPI and Alexa488. Digital images were obtained by using a cooled-coupled Photometrix CH-350 controlled by the IPLab software, and processed using ImageJ 3.0 and Adobe Photoshop software.
For what concerns the analysis of FSHR and LHR expression in SN- and NSN-CCs, signal intensity was classified into 4 classes on the basis of the fluorescence: none-weak, medium, high and very high, corresponding to the maximal fluorescence recorded (Fig. 25).

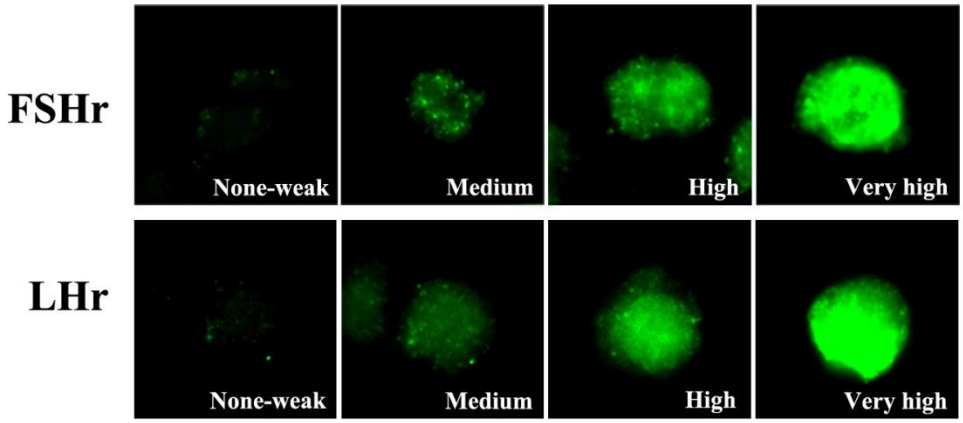

Figure 25. Signal intensity of CCs FSHR and LHR immunostaining. Basing on the fluorescence intensity, CCs were classified into four classes: none- weak, medium, high and very high. Bar, 8 µm.

6.3.9. Flow cytometry

Following COCs isolation, pools of about 40 cumuli of SN-CCs and NSN-CCs were stained with 500 µl propidium iodide (50 µg/ml) containing 1 µg/ml RNase A. To analyse the frequency distribution of cells within the cell cycle phases, a Partec PAS II (Munster, Germany) flow cytometer equipped with dual excitation system (argon ion laser and HBO 100W arc lamp) was used. Cells form three independent experiments were analysed.

6.3.10. Statistical analysis

At least three replicates have been independently performed for all the experiments reported. Values are expressed as the mean ± S.D. and data coming from the different replicates were analysed using either the Student's t-test (for parametric data of immunocytochemistry or flow cytometry), or the Mann Whitney rank sum test (for nonparametric data of real-time qPCR,). Differences were considered statistically significant when $p < 0.05$.

Additional tables

Table 1S is the list of 47 differentially regulated genes in NSN-CCs *vs.* SN-CCs resulting from the microarrays analysis firstly performed by setting a fold-change cut-off of >2.0 and a *p* value <0.01. Red highlights up-regulated genes, green down-regulated ones. The enlarged list of 422 differentially expressed genes obtained by lowering the fold-change cut-off to >1.3 is deposited in GEO, accession number GSE46906.

Table 2S shows the lists of mesh terms related to folliculogenesis and ovarian follicle function. MESH_B comprises the basic chosen keywords. MESH_BC is the enlarged list of terms obtained by adding specialized child terms subordinate of the starting key terms. MESH_BCP is an additional list comprising also the parents terms of all the annotations in MESH_B.

Table 1S. List of differentially regulated genes in the comparison between NSN-CCs *vs.* SN-CCs. In green: down-regulated genes; in red: up-regulated genes.

SYMBOL (TargetID)	SEARCH_KEY	CHROMOSOME	DEFINITION	SYNONYMS	SOURCE_REFERENCE_ID	ENTREZ_GENE_ID	SN.AVG_Signal	NSN.AVG_Signal	Fold-change
Amh	ILMN_223758	10	Mus musculus anti-Mullerian hormone (Amh), mRNA.	MIS	NM_007445.2	11705	873,083	4407,988	2,336
Omp	ILMN_184266	7	Mus musculus olfactory marker protein (Omp), mRNA.	?	NM_011010.2	18378	145,861	36,416	-2,002
Kctd7	ILMN_218245	?	Mus musculus potassium channel tetramerisation domain containing 7 (Kctd7), mRNA.	4932409E18; 9430010P06Rik	NM_172509.3	212919	132,439	33,002	-2,005
Ctrl	ILMN_216616	8	Mus musculus chymotrypsin-like (Ctrl), mRNA.	Ctra-1; 1810004D15Rik; Ctra1; 0910001G08Rik; mFLJ00366; AV005227; FLJ00366	NM_023182.2	109660	149,171	37,086	-2,008
Gm606	ILMN_228173	16	Mus musculus gene model 606, (NCBI) (Gm606), mRNA.	Gm1778	NM_001013761.1	239789	138,297	34,345	-2,01
Dux	ILMN_237306	?	Mus musculus double homeobox (Dux), mRNA.	Dux4	NM_001081954.1	672911	139,915	34,708	-2,011
Rab9b	ILMN_210400	X	Mus musculus RAB9B, member RAS oncogene family (Rab9b), mRNA.	MGC130383; 9330195C02Rik	NM_176971.1	319642	141,192	35,004	-2,012
AI595366	ILMN_253609	?	Mus musculus expressed sequence AI595366 (AI595366), mRNA.	?	NM_001033042.3	432779	137,715	34,09	-2,014
Bik	ILMN_220051	15	Mus musculus BCL2-interacting killer (Bik), mRNA.	Nbk; Blk; Biklk	NM_007546.2	12124	153,179	37,849	-2,017
Ifi47	ILMN_214867	11	Mus musculus interferon gamma inducible protein 47 (Ifi47), mRNA.	47kDa; Iipg4; Iigp4; Olfr56; IRG-47	NM_008330.1	15953	150,482	37,144	-2,018
A530099J19Rik	ILMN_221280	13	Mus musculus RIKEN cDNA A530099J19 gene (A530099J19Rik), mRNA.	?	NM_175688.3	319293	134,621	33,191	-2,02
Ifit2	ILMN_216122	19	Mus musculus interferon-induced protein with tetratricopeptide repeats 2 (Ifit2), mRNA.	Ifi54; AV302338	NM_008332.2	15958	141,959	34,922	-2,023
Zfp526	ILMN_188423	7	Mus musculus zinc finger protein 526 (Zfp526), mRNA.	D030024H03Rik; MGC106217	NM_175436.2	210172	142,69	35,101	-2,023
Pcdhb4	ILMN_221098	18	Mus musculus protocadherin beta 4 (Pcdhb4), mRNA.	Pcdhb5A; PcdhbD	NM_053129.3	93875	132,145	32,463	-2,025
Krtap5-5	ILMN_257190	7	Mus musculus keratin associated protein 5-5 (Krtap5-5), mRNA. XM_925516	AI586182; MGC130543; BB149782; A030001H12Rik	NM_001037822.1	114666	149,148	36,462	-2,032
Olfr1208	ILMN_212778	2	Mus musculus olfactory receptor 1208 (Olfr1208), mRNA.	MOR225-4	NM_146778.1	258774	134,492	32,895	-2,032
Ltbp2	ILMN_193948	?	?	?	scl42970.1.243_7	?	137,981	33,577	-2,039
Gtf3c6	ILMN_209771	?	Mus musculus general transcription factor IIIC, polypeptide 6, alpha (Gtf3c6), mRNA.	2410016F19Rik; AU019813	NM_026113.4	67371	132,876	32,204	-2,045
Myh8	ILMN_214541	11	Mus musculus myosin, heavy polypeptide 8, skeletal muscle, perinatal (Myh8), mRNA.	KIAA4211; 4832426G23Rik; MyHC-pn; mKIAA4211; Myhsp; AI327267; Myhs-p	NM_177369.3	17885	133,667	32,276	-2,05
Ppp1r1a	ILMN_211515	15	Mus musculus protein phosphatase 1, regulatory (inhibitor) subunit 1A (Ppp1r1a), mRNA.	0610038N18Rik; I-1	NM_021391.3	58200	152,589	36,665	-2,057
0610012D14Rik	ILMN_213393	7	Mus musculus RIKEN cDNA 0610012D14 gene (0610012D14Rik), mRNA.	?	NM_026690.1	68352	133,787	32,123	-2,058
Mfap5	ILMN_224138	6	Mus musculus microfibrillar associated protein 5 (Mfap5), mRNA.	MAGP-2	NM_015776.2	50530	134,387	32,219	-2,06
Fgf17	ILMN_213696	14	Mus musculus fibroblast growth factor 17 (Fgf17), mRNA.	?	NM_008004.2	14171	157,533	37,436	-2,073

Mtl5	ILMN_210081	19	Mus musculus metallothionein-like 5, testis-specific (tesmin) (Mtl5), transcript variant 1, mRNA.	tesmin	NM_001039657.1	17771	138,621	32,822	-2,078
Fkbp6	ILMN_257529	5	Mus musculus FK506 binding protein 6 (Fkbp6), mRNA.	36kDa; D5Ertd724e; AU017274; 1700008G22Rik	NM_033571.1	94244	141,509	33,491	-2,079
Pdyn	ILMN_223702	2	Mus musculus prodynorphin (Pdyn), mRNA.	Dyn	NM_018863.2	18610	158,336	37,447	-2,08
V1ri8	ILMN_193013	13	Mus musculus vomeronasal 1 receptor, I8 (V1ri8), mRNA.	?	NM_145846.1	252908	154,521	36,177	-2,095
LOC100045628	ILMN_217413	?	PREDICTED: Mus musculus similar to X-linked PEST-containing transporter (LOC100045628), misc RNA.	?	XR_031850.1	100045628	141,473	33,097	-2,096
Rftn2	ILMN_186841	1	Mus musculus raftlin family member 2 (Rftn2), mRNA. XM_920263 XM_986521 XM_986556 XM_986596 XM_986637 XM_986673	3222401M22Rik; KIAA0084; 2700010E02Rik	NM_028713.1	74013	137,482	32,055	-2,101
Cyp4f14	ILMN_223369	17	Mus musculus cytochrome P450, family 4, subfamily f, polypeptide 14 (Cyp4f14), mRNA.	AW108534; 1300014O15Rik	NM_022434.1	64385	179,903	41,819	-2,105
Krt82	ILMN_210328	15	Mus musculus keratin 82 (Krt82), mRNA.	Krt2-20; MGC124293	NM_053249.2	114566	161,317	37,489	-2,105
Casp1	ILMN_211039	9	Mus musculus caspase 1 (Casp1), mRNA.	Il1bc; ICE	NM_009807.2	12362	151,187	35,12	-2,106
Pdcl3	ILMN_212554	1	Mus musculus phosducin-like 3 (Pdcl3), mRNA.	1110061A19Rik; Viaf1; C80025	NM_026850.2	68833	186,673	43,279	-2,109
P42pop	ILMN_209787	7	Mus musculus Myb protein P42POP (P42pop), mRNA.	?	NM_145579.2	232934	143,899	33,047	-2,122
5730593F17Rik	ILMN_223419	11	Mus musculus RIKEN cDNA 5730593F17 gene (5730593F17Rik), mRNA.	RP23-67E18.1; AW548096; FAM117A	NM_172543.2	215512	157,698	36,115	-2,126
Srgn	ILMN_220490	10	Mus musculus serglycin (Srgn), mRNA.	Sgc; Prg; Prg1	NM_011157.2	19073	143,66	32,859	-2,128
Ptgds	ILMN_221328	?	?	?	scl19558.7.10_4	?	168,167	38,379	-2,131
Fam123a	ILMN_191854	14	Mus musculus family with sequence similarity 123, member A (Fam123a), mRNA.	?	NM_028113.2	72125	180,902	41,096	-2,138
F10	ILMN_217890	?	?	?	scl34064.10_3	?	167,247	37,759	-2,147
Hrc	ILMN_221655	7	Mus musculus histidine rich calcium binding protein (Hrc), mRNA.	?	NM_010473.2	15464	182,806	40,838	-2,162
Mrpl39	ILMN_217952	?	Mus musculus mitochondrial ribosomal protein L39 (Mrpl39), nuclear gene encoding mitochondrial protein, mRNA.	C21orf8; MRP-L5; ORF22; Rpml5	NM_017404.3	27393	233,222	51,187	-2,188
Myl1	ILMN_218373	1	Mus musculus myosin, light polypeptide 1 (Myl1), mRNA.	Mylf; AI325107; MLC1f; MLC3f	NM_021285.1	17901	167,729	36,612	-2,196
2010308M01Rik	ILMN_215290	?	?	?	scl072121.12_86	?	156,564	34,06	-2,201
Papln	ILMN_219458	12	Mus musculus papilin, proteoglycan-like sulfated glycoprotein (Papln), mRNA.	E030033C16Rik	NM_130887.1	170721	157,836	33,584	-2,233
Vmo1	ILMN_253414	11	Mus musculus vitelline membrane outer layer 1 homolog (chicken) (Vmo1), mRNA.	RP23-122P1.11; Gm741	NM_001013607.1	327956	174,69	34,396	-2,344
Ihpk3	ILMN_209954	17	Mus musculus inositol hexaphosphate kinase 3 (Ihpk3), mRNA.	D830007E07Rik	NM_173027.2	271424	179,241	33,053	-2,439
Siglech	ILMN_210414	7	Mus musculus sialic acid binding Ig-like lectin H (Siglech), mRNA.	MGC106898; Siglec-H; 6430529G09Rik	NM_178706.2	233274	185,003	34,008	-2,444

Table 2S. The three lists of mesh terms chosen to describe folliculogenesis and the follicle function. MESH_B: basic keywords. MESH_BC: MESH_B annotations plus their child terms. MESH_BCP: initially selected key terms plus their parent terms.

Key Mesh_B	Key MESH_BC	Key MESH_BCP
Cumulus Cells	Aging	Adnexa Uteri
Embryonic Development	Anovulation	Aging
Fertilization	Calcification, Physiologic	Anovulation
Gonadotropins	Cell Lineage	Calcification, Physiologic
Granulosa Cells	Chorionic Gonadotropin	Cell Differentiation
Growth and Development	Coitus	Cell Lineage
Hormones	Cumulus Cells	Chorionic Gonadotropin
Oocytes	Ejaculation	Coitus
Oogenesis	Embryo Implantation	Cumulus Cells
Ovarian Follicle	Embryonic and Fetal Development	Ejaculation
Ovulation	Embryonic Development	Embryo Implantation
Receptors, FSH	Embryonic Induction	Embryonic and Fetal Development
Receptors, LH	Epithelial-Mesenchymal Transition	Embryonic Development
Reproduction	Fertilization	Embryonic Induction
Zygote	Follicular Fluid	Epithelial-Mesenchymal Transition
	Gametogenesis	Fertilization
	Gastrulation	Fertilization
	Gonadotropins	Follicular Fluid
	Gonadotropins, Equine	Gametogenesis
	Gonadotropins, Pituitary	Gastrulation
	Granulosa Cells	Germ Cells
	Growth	Gonadotrophs
	Growth and Development	Gonadotropins
	Hormones	Gonadotropins, Equine
	Human Development	Gonadotropins, Pituitary
	Insemination	Granulosa Cells
	Luteinization	Growth
	Luteolysis	Growth and Development
	Morphogenesis	Hormones
	Neurulation	Human Development
	Oocytes	Insemination
	Oogenesis	Luteinization
	Orgasm	Luteolysis
	Ovarian Follicle	Morphogenesis
	Oviposition	Neurulation
	Ovulation	Oocytes

	Ovulation Inhibition	OOgenesis
	Ovum Transport	Orgasm
	Penile Erection	Ovarian Follicle
	Pollination	Ovary
	Pregnancy	Oviposition
	Receptors, FSH	Ovulation
	Receptors, LH	Ovulation Inhibition
	Reproduction	Ovum
	Reproduction, Asexual	Ovum Transport
	Reproductive Behavior	Penile Erection
	Self-Fertilization	Peptide Hormones
	Sexual Development	Physiological Phenomena
	Sperm Capacitation	Physiological Processes
	Sperm Transport	Placental Hormones
	Sperm-Ovum Interactions	Pollination
	Superovulation	Pregnancy
	Theca Cells	Pregnancy Proteins
	Twinning, Monozygotic	Receptors, FSH
	Vitellogenesis	Receptors, G-Protein-Coupled
	Zygote	Receptors, Gonadotropin
		Receptors, LH
		Receptors, Neuropeptide
		Reproduction
		Reproduction, Asexual
		Reproductive Behavior
		Reproductive Physiological Phenomena
		Reproductive Physiological Processes
		Self-Fertilization
		Sexual Development
		Sperm Capacitation
		Sperm Transport
		Sperm-Ovum Interactions
		Superovulation
		Theca Cells
		Twinning, Monozygotic
		Vitellogenesis
		Zygote

References

Abir R, Roizman P, Fisch B, Nitke S, Okon E, Orvieto R, Ben Rafael Z: **Pilot study of isolated early human follicles cultured in collagen gels for 24 hours**. *Hum Reprod* 1999, **14**(5):1299-1301.

Adriaenssens T, Segers I, Wathlet S, Smitz J: **The cumulus cell gene expression profile of oocytes with different nuclear maturity and potential for blastocyst formation**. *J Assist Reprod Genet* 2011, **28**(1):31-40.

Albertini DF, Combelles CM, Benecchi E, Carabatsos MJ: **Cellular basis for paracrine regulation of ovarian follicle development**. *Reproduction* 2001, **121**(5):647-653.

Allen C, Reardon W: **Assisted reproduction technology and defects of genomic imprinting**. *BJOG* 2005, **112**(12):1589-1594.

Alves MH, Young CJ, Bozzetto K, Poole-Warren LA, Martens PJ: **Degradable, click poly(vinyl alcohol) hydrogels: characterization of degradation and cellular compatibility**. *Biomed Mater* 2012, **7**(2):024106.

Amorim CA, Van Langendonckt A, David A, Dolmans MM, Donnez J: **Survival of human pre-antral follicles after cryopreservation of ovarian tissue, follicular isolation and in vitro culture in a calcium alginate matrix.** *Hum Reprod* 2009, **24**(1):92-99.

Anderson RA, Sciorio R, Kinnell H, Bayne RA, Thong KJ, de Sousa PA, Pickering S: **Cumulus gene expression as a predictor of human oocyte fertilisation, embryo development and competence to establish a pregnancy**. *Reproduction* 2009, **138**(4):629-637.

Armenti AE, Zama AM, Passantino L, Uzumcu M: **Developmental methoxychlor exposure affects multiple reproductive parameters and ovarian folliculogenesis and gene expression in adult rats**. *Toxicol Appl Pharmacol* 2008, **233**(2):286-296.

Ashkenazi H, Cao X, Motola S, Popliker M, Conti M, Tsafriri A: **Epidermal growth factor family members: endogenous mediators of the ovulatory response**. *Endocrinology* 2005, **146**(1):77-84.

Assidi M, Dufort I, Ali A, Hamel M, Algriany O, Dielemann S, Sirard MA: **Identification of potential markers of oocyte competence expressed in bovine cumulus cells matured with follicle-stimulating hormone and/or phorbol myristate acetate in vitro**. *Biol Reprod* 2008, **79**(2):209-222.

Assou S, Haouzi D, Mahmoud K, Aouacheria A, Guillemin Y, Pantesco V, Rème T, Dechaud H, De Vos J, Hamamah S: **A non-invasive test for assessing embryo potential by gene expression profiles of human cumulus cells: a proof of concept study**. *Mol Hum Reprod* 2008, **14**(12):711-719.

Balaban B, Ata B, Isiklar A, Yakin K, Urman B: **Severe cytoplasmic abnormalities of the oocyte decrease cryosurvival and subsequent**

embryonic development of cryopreserved embryos. *Hum Reprod* 2008, **23**(8):1778-1785.

Barrett SL, Albertini DF: **Cumulus cell contact during oocyte maturation in mice regulates meiotic spindle positioning and enhances developmental competence**. *J Assist Reprod Genet* 2010, **27**(1):29-39.

Belli M, Cimadomo D, Merico V, Redi CA, Garagna S, Zuccotti M: **The NOBOX protein becomes undetectable in developmentally competent antral and ovulated oocytes**. *Int J Dev Biol* 2013, **57**(1):35-39.

Bettegowda A, Patel OV, Lee KB, Park KE, Salem M, Yao J, Ireland JJ, Smith GW: **Identification of novel bovine cumulus cell molecular markers predictive of oocyte competence: functional and diagnostic implications**. *Biol Reprod* 2008, **79**(2):301-309.

Bohari SP, Hukins DW, Grover LM: **Effect of calcium alginate concentration on viability and proliferation of encapsulated fibroblasts**. *Biomed Mater Eng* 2011, **21**(3):159-170.

Bouniol-Baly C, Hamraoui L, Guibert J, Beaujean N, Szöllösi MS, Debey P: **Differential transcriptional activity associated with chromatin configuration in fully grown mouse germinal vesicle oocytes**. *Biol Reprod* 1999, **60**(3):580-587.

Brower PT, Schultz RM: **Intercellular communication between granulosa cells and mouse oocytes: existence and possible nutritional role during oocyte growth**. *Dev Biol* 1982, **90**(1):144-153.

Bu S, Xia G, Tao Y, Lei L, Zhou B: **Dual effects of nitric oxide on meiotic maturation of mouse cumulus cell-enclosed oocytes in vitro**. *Mol Cell Endocrinol* 2003, **207**(1-2):21-30.

Bukovsky A, Indrapichate K, Fujiwara H, Cekanova M, Ayala ME, Dominguez R, Caudle MR, Wimalsena J, Elder RF, Copas P *et al*: **Multiple luteinizing hormone receptor (LHR) protein variants, interspecies reactivity of anti-LHR mAb clone 3B5, subcellular localization of LHR in human placenta, pelvic floor and brain, and possible role for LHR in the development of abnormal pregnancy, pelvic floor disorders and Alzheimer's disease**. *Reprod Biol Endocrinol* 2003, **1**:46.

Byskov AG, Yding Andersen C, Hossaini A, Guoliang X: **Cumulus cells of oocyte-cumulus complexes secrete a meiosis-activating substance when stimulated with FSH**. *Mol Reprod Dev* 1997, **46**(3):296-305.

Calder MD, Caveney AN, Smith LC, Watson AJ: **Responsiveness of bovine cumulus-oocyte-complexes (COC) to porcine and recombinant human FSH, and the effect of COC quality on gonadotropin receptor and Cx43 marker gene mRNAs during maturation in vitro**. *Reprod Biol Endocrinol* 2003, **1**:14.

Carabatsos MJ, Sellitto C, Goodenough DA, Albertini DF: **Oocyte-granulosa cell heterologous gap junctions are required for the coordination of nuclear and cytoplasmic meiotic competence**. *Dev Biol* 2000, **226**(2):167-179.

Cavaleri F, Scholer H: **Molecular facets of pluripotency. In: Lanza R, Gearhart J, Hogan B Melton D, Pedersen R, Thompson J, West M Stem Cells.** *Elsevier Academic Press* 2005, Amsterdam, pp. 27–44.

Chamayou S, Ragolia C, Alecci C, Storaci G, Maglia E, Russo E, Guglielmino A: **Meiotic spindle presence and oocyte morphology do not predict clinical ICSI outcomes: a study of 967 transferred embryos**. *Reprod Biomed Online* 2006, **13**(5):661-667.

Chappel SC, Howles C: **Reevaluation of the roles of luteinizing hormone and follicle-stimulating hormone in the ovulatory process**. *Hum Reprod* 1991, **6**(9):1206-1212.

Chen L, Russell PT, Larsen WJ: **Functional significance of cumulus expansion in the mouse: roles for the preovulatory synthesis of hyaluronic acid within the cumulus mass**. *Mol Reprod Dev* 1993, **34**(1):87-93.

Choi JH, Kim GA, Park JH, Song GH, Park JW, Kim DY, Lim JM: **Generation of viable embryos and embryonic stem cell-like cells from cultured primary follicles in mice**. *Biol Reprod* 2011, **85**(4):744-754.

Choi Y, Qin Y, Berger MF, Ballow DJ, Bulyk ML, Rajkovic A: **Microarray analyses of newborn mouse ovaries lacking Nobox**. *Biol Reprod* 2007, **77**(2):312-319.

Christians E, Boiani M, Garagna S, Dessy C, Redi CA, Renard JP, Zuccotti M: **Gene expression and chromatin organization during mouse oocyte growth**. *Dev Biol* 1999, **207**(1):76-85.

Cillo F, Brevini TA, Antonini S, Paffoni A, Ragni G, Gandolfi F: **Association between human oocyte developmental competence and expression levels of some cumulus genes**. *Reproduction* 2007, **134**(5):645-650.

Combelles CM, Cekleniak NA, Racowsky C, Albertini DF: **Assessment of nuclear and cytoplasmic maturation in in-vitro matured human oocytes**. *Hum Reprod* 2002, **17**(4):1006-1016.

Combelles CM, Fissore RA, Albertini DF, Racowsky C: **In vitro maturation of human oocytes and cumulus cells using a co-culture three-dimensional collagen gel system**. *Hum Reprod* 2005, **20**(5):1349-1358.

Cortvrindt R, Smitz J, Van Steirteghem AC: **In-vitro maturation, fertilization and embryo development of immature oocytes from early preantral follicles from prepuberal mice in a simplified culture system**. *Hum Reprod* 1996, **11**(12):2656-2666.

Cotterill M, Catt SL, Picton HM: **Characterisation of the cellular and molecular responses of ovine oocytes and their supporting somatic cells to pre-ovulatory levels of LH and FSH during in vitro maturation**. *Reproduction* 2012, **144**(2):195-207.

Crozet N: **Effects of actinomycin D and cycloheximide on the nucleolar ultrastructure of porcine oocytes**. *Biol Cell* 1983, **48**(1):25-29.

Cruz M, Garrido N, Herrero J, Pérez-Cano I, Muñoz M, Meseguer M: **Timing of cell division in human cleavage-stage embryos is linked with blastocyst**

formation and quality. *Reprod Biomed Online* 2012, **25**(4):371-381.

Debey P, Szöllösi MS, Szöllösi D, Vautier D, Girousse A, Besombes D: **Competent mouse oocytes isolated from antral follicles exhibit different chromatin organization and follow different maturation dynamics**. *Mol Reprod Dev* 1993, **36**(1):59-74.

Desai N, Abdelhafez F, Calabro A, Falcone T: **Three dimensional culture of fresh and vitrified mouse pre-antral follicles in a hyaluronan-based hydrogel: a preliminary investigation of a novel biomaterial for in vitro follicle maturation**. *Reprod Biol Endocrinol* 2012, **10**(1):29.

Downs SM: **The influence of glucose, cumulus cells, and metabolic coupling on ATP levels and meiotic control in the isolated mouse oocyte**. *Dev Biol* 1995, **167**(2):502-512.

Downs SM: **A gap-junction-mediated signal, rather than an external paracrine factor, predominates during meiotic induction in isolated mouse oocytes**. *Zygote* 2001, **9**(1):71-82.

Downs SM, Gilles R, Vanderhoef C, Humpherson PG, Leese HJ: **Differential response of cumulus cell-enclosed and denuded mouse oocytes in a meiotic induction model system**. *Mol Reprod Dev* 2006, **73**(3):379-389.

Downs SM, Mastropolo AM: **The participation of energy substrates in the control of meiotic maturation in murine oocytes**. *Dev Biol* 1994, **162**(1):154-168.

Downs SM, Ruan B, Schroepfer GJ: **Meiosis-activating sterol and the maturation of isolated mouse oocytes**. *Biol Reprod* 2001, **64**(1):80-89.

Durlinger AL, Kramer P, Karels B, de Jong FH, Uilenbroek JT, Grootegoed JA, Themmen AP: **Control of primordial follicle recruitment by anti-Müllerian hormone in the mouse ovary**. *Endocrinology* 1999, **140**(12):5789-5796.

Ebner T, Moser M, Shebl O, Sommerguber M, Tews G: **Prognosis of oocytes showing aggregation of smooth endoplasmic reticulum**. *Reprod Biomed Online* 2008, **16**(1):113-118.

Edson MA, Nagaraja AK, Matzuk MM: **The mammalian ovary from genesis to revelation**. *Endocr Rev* 2009, **30**(6):624-712.

Ekart J, McNatty K, Hutton J, Pitman J: **Ranking and selection of MII oocytes in human ICSI cycles using gene expression levels from associated cumulus cells**. *Hum Reprod* 2013, **28**(11):2930-2942.

Eppig JJ: **Intercommunication between mammalian oocytes and companion somatic cells**. *Bioessays* 1991, **13**(11):569-574.

Eppig JJ: **Oocyte control of ovarian follicular development and function in mammals**. *Reproduction* 2001, **122**(6):829-838.

Eppig JJ, O'Brien MJ: **Development in vitro of mouse oocytes from primordial follicles**. *Biol Reprod* 1996, **54**(1):197-207.

Eppig JJ, Schroeder AC: **Capacity of mouse oocytes from preantral follicles**

to undergo embryogenesis and development to live young after growth, maturation, and fertilization in vitro. *Biol Reprod* 1989, **41**(2):268-276.

Eppig JJ, Wigglesworth K, Pendola FL: **The mammalian oocyte orchestrates the rate of ovarian follicular development**. *Proc Natl Acad Sci U S A* 2002, **99**(5):2890-2894.

Fagbohun CF, Downs SM: **Metabolic coupling and ligand-stimulated meiotic maturation in the mouse oocyte-cumulus cell complex**. *Biol Reprod* 1991, **45**(6):851-859.

Fan HY, Huo LJ, Chen DY, Schatten H, Sun QY: **Protein kinase C and mitogen-activated protein kinase cascade in mouse cumulus cells: cross talk and effect on meiotic resumption of oocyte**. *Biol Reprod* 2004, **70**(4):1178-1187.

Fragouli E, Wells D, Iager AE, Kayisli UA, Patrizio P: **Alteration of gene expression in human cumulus cells as a potential indicator of oocyte aneuploidy**. *Hum Reprod* 2012, **27**(8):2559-2568.

Fu M, Chen X, Yan J, Lei L, Jin S, Yang J, Song X, Zhang M, Xia G: **Luteinizing hormone receptors expression in cumulus cells closely related to mouse oocyte meiotic maturation**. *Front Biosci* 2007, **12**:1804-1813.

Fujihara M, Comizzoli P, Wildt DE, Songsasen N: **Cat and dog primordial follicles enclosed in ovarian cortex sustain viability after in vitro culture on agarose gel in a protein-free medium**. *Reprod Domest Anim* 2012, **47 Suppl 6**:102-108.

Fulton BP, Whittingham DG: **Activation of mammalian oocytes by intracellular injection of calcium**. *Nature* 1978, **273**(5658):149-151.

Garagna S, Merico V, Sebastiano V, Monti M, Orlandini G, Gatti R, Scandroglio R, Redi CA, Zuccotti M: **Three-dimensional localization and dynamics of centromeres in mouse oocytes during folliculogenesis**. *J Mol Histol* 2004, **35**(6):631-638.

Ge L, Han D, Lan GC, Zhou P, Liu Y, Zhang X, Sui HS, Tan JH: **Factors affecting the in vitro action of cumulus cells on the maturing mouse oocytes**. *Mol Reprod Dev* 2008, **75**(1):136-142.

Gebhardt KM, Feil DK, Dunning KR, Lane M, Russell DL: **Human cumulus cell gene expression as a biomarker of pregnancy outcome after single embryo transfer**. *Fertil Steril* 2011, **96**(1):47-52.e42.

Gershon E, Plaks V, Dekel N: **Gap junctions in the ovary: expression, localization and function**. *Mol Cell Endocrinol* 2008, **282**(1-2):18-25.

Ghanem N, Hölker M, Rings F, Jennen D, Tholen E, Sirard MA, Torner H, Kanitz W, Schellander K, Tesfaye D: **Alterations in transcript abundance of bovine oocytes recovered at growth and dominance phases of the first follicular wave**. *BMC Dev Biol* 2007, **7**:90.

Gilchrist RB, Lane M, Thompson JG: **Oocyte-secreted factors: regulators of cumulus cell function and oocyte quality**. *Hum Reprod Update* 2008, **14**(2):159-177.

Gilchrist RB, Ritter LJ, Armstrong DT: **Oocyte-somatic cell interactions during follicle development in mammals**. *Anim Reprod Sci* 2004, **82-83**:431-446.

Gill A, Jamnongjit M, Hammes SR: **Androgens promote maturation and signaling in mouse oocytes independent of transcription: a release of inhibition model for mammalian oocyte meiosis**. *Mol Endocrinol* 2004, **18**(1):97-104.

Gomperts M, Wylie C, Heasman J: **Primordial germ cell migration**. *Ciba Found Symp* 1994, **182**:121-134; discussion 134-129.

Hamamah S, Matha V, Berthenet C, Anahory T, Loup V, Dechaud H, Hedon B, Fernandez A, Lamb N: **Comparative protein expression profiling in human cumulus cells in relation to oocyte fertilization and ovarian stimulation protocol**. *Reprod Biomed Online* 2006, **13**(6):807-814.

Hamel M, Dufort I, Robert C, Gravel C, Leveille MC, Leader A, Sirard MA: **Identification of differentially expressed markers in human follicular cells associated with competent oocytes**. *Hum Reprod* 2008, **23**(5):1118-1127.

Heise MK, Koepsel R, McGee EA, Russell AJ: **Dynamic oxygen enhances oocyte maturation in long-term follicle culture**. *Tissue Eng Part C Methods* 2009, **15**(3):323-332.

Heise M, Koepsel R, Russell AJ, McGee EA: **Calcium alginate microencapsulation of ovarian follicles impacts FSH delivery and follicle morphology**. *Reprod Biol Endocrinol* 2005, **3**:47.

Hess KA, Chen L, Larsen WJ: **Inter-alpha-inhibitor binding to hyaluronan in the cumulus extracellular matrix is required for optimal ovulation and development of mouse oocytes**. *Biol Reprod* 1999, **61**(2):436-443.

Hillier SG: **Current concepts of the roles of follicle stimulating hormone and luteinizing hormone in folliculogenesis**. *Hum Reprod* 1994, **9**(2):188-191.

Hinrichs K, Choi YH, Love LB, Varner DD, Love CC, Walckenaer BE: **Chromatin configuration within the germinal vesicle of horse oocytes: changes post mortem and relationship to meiotic and developmental competence**. *Biol Reprod* 2005, **72**(5):1142-1150.

Hirao Y, Nagai T, Kubo M, Miyano T, Miyake M, Kato S: **In vitro growth and maturation of pig oocytes**. *J Reprod Fertil* 1994, **100**(2):333-339.

Hirshfield AN: **Development of follicles in the mammalian ovary**. *Int Rev Cytol* 1991, **124**:43-101.

Hornick JE, Duncan FE, Shea LD, Woodruff TK: **Multiple follicle culture supports primary follicle growth through paracrine-acting signals**. *Reproduction* 2013, **145**(1):19-32.

Hovatta O, Silye R, Abir R, Krausz T, Winston RM: **Extracellular matrix improves survival of both stored and fresh human primordial and primary ovarian follicles in long-term culture**. *Hum Reprod* 1997, **12**(5):1032-1036.

Hovatta O, Wright C, Krausz T, Hardy K, Winston RM: **Human primordial, primary and secondary ovarian follicles in long-term culture: effect of partial isolation**. *Hum Reprod* 1999, **14**(10):2519-2524.

Huang Z, Wells D: **The human oocyte and cumulus cells relationship: new insights from the cumulus cell transcriptome**. *Mol Hum Reprod* 2010, **16**(10):715-725.

Huanmin Z, Yong Z: **In vitro development of caprine ovarian preantral follicles**. *Theriogenology* 2000, **54**(4):641-650.

Høst E, Gabrielsen A, Lindenberg S, Smidt-Jensen S: **Apoptosis in human cumulus cells in relation to zona pellucida thickness variation, maturation stage, and cleavage of the corresponding oocyte after intracytoplasmic sperm injection**. *Fertil Steril* 2002, **77**(3):511-515.

Inoue A, Akiyama T, Nagata M, Aoki F: **The perivitelline space-forming capacity of mouse oocytes is associated with meiotic competence**. *J Reprod Dev* 2007, **53**(5):1043-1052.

Jin SY, Lei L, Shikanov A, Shea LD, Woodruff TK: **A novel two-step strategy for in vitro culture of early-stage ovarian follicles in the mouse**. *Fertil Steril* 2010, **93**(8):2633-2639.

Kawamura K, Kumagai J, Sudo S, Chun SY, Pisarska M, Morita H, Toppari J, Fu P, Wade JD, Bathgate RA, Hsueh AJ: **Paracrine regulation of mammalian oocyte maturation and male germ cell survival.** *Proc Natl Acad Sci U S A* 2004, **101**(19):7323-7328.

Kedem A, Hourvitz A, Fisch B, Shachar M, Cohen S, Ben-Haroush A, Dor J, Freud E, Felz C, Abir R: **Alginate scaffold for organ culture of cryopreserved-thawed human ovarian cortical follicles**. *J Assist Reprod Genet* 2011, **28**(9):761-769.

Kedem-Dickman A, Maman E, Yung Y, Yerushalmi GM, Hemi R, Hanochi M, Dor J, Hourvitz A: **Anti-Müllerian hormone is highly expressed and secreted from cumulus granulosa cells of stimulated preovulatory immature and atretic oocytes**. *Reprod Biomed Online* 2012, **24**(5):540-546.

Kidder GM, Mhawi AA: **Gap junctions and ovarian folliculogenesis**. *Reproduction* 2002, **123**(5):613-620.

King SM, Modi DA, Eddie SL, Burdette JE: **Insulin and insulin-like growth factor signaling increases proliferation and hyperplasia of the ovarian surface epithelium and decreases follicular integrity through upregulation of the PI3-kinase pathway**. *J Ovarian Res* 2013, **6**(1):12.

Knight PG, Glister C: **TGF-beta superfamily members and ovarian follicle development**. *Reproduction* 2006, **132**(2):191-206.

Kreeger PK, Deck JW, Woodruff TK, Shea LD: **The in vitro regulation of ovarian follicle development using alginate-extracellular matrix gels**. *Biomaterials* 2006, **27**(5):714-723.

Krotz SP, Robins JC, Ferruccio TM, Moore R, Steinhoff MM, Morgan JR, Carson S: **In vitro maturation of oocytes via the pre-fabricated self-**

assembled artificial human ovary. *J Assist Reprod Genet* 2010, **27**(12):743-750.

Krysko DV, Diez-Fraile A, Criel G, Svistunov AA, Vandenabeele P, D'Herde K: **Life and death of female gametes during oogenesis and folliculogenesis**. *Apoptosis* 2008, **13**(9):1065-1087.

Lee E, Kim JH, Park SM, Jeong YI, Lee JY, Park SW, Choi J, Kim HS, Jeong YW, Kim S *et al*: **The analysis of chromatin remodeling and the staining for DNA methylation and histone acetylation do not provide definitive indicators of the developmental ability of inter-species cloned embryos**. *Anim Reprod Sci* 2008, **105**(3-4):438-450.

Lefèvre B, Gougeon A, Nomé F, Testart J: **In vivo changes in oocyte germinal vesicle related to follicular quality and size at mid-follicular phase during stimulated cycles in the cynomolgus monkey**. *Reprod Nutr Dev* 1989, **29**(5):523-531.

Lei L, Jin S, Mayo KE, Woodruff TK: **The interactions between the stimulatory effect of follicle-stimulating hormone and the inhibitory effect of estrogen on mouse primordial folliculogenesis**. *Biol Reprod* 2010, **82**(1):13-22.

Lemmen JG, Agerholm I, Ziebe S: **Kinetic markers of human embryo quality using time-lapse recordings of IVF/ICSI-fertilized oocytes**. *Reprod Biomed Online* 2008, **17**(3):385-391.

Levasseur DN, Wang J, Dorschner MO, Stamatoyannopoulos JA, Orkin SH: **Oct4 dependence of chromatin structure within the extended Nanog locus in ES cells**. *Genes Dev* 2008, **22**(5):575-580.

Li L, Zheng P, Dean J: **Maternal control of early mouse development**. *Development* 2010, **137**(6):859-870.

Li X, Zhang H, Sheng X, Li B, Zhou J, Xu M, Weng Q, Watanabe G, Taya K: **Immunoreactivities of androgen receptor, estrogen receptors, p450arom, p450c17 proteins in wild ground squirrels ovaries during the nonbreeding and breeding seasons**. *J Ovarian Res* 2012, **5**(1):26.

Lim H, Paria BC, Das SK, Dinchuk JE, Langenbach R, Trzaskos JM, Dey SK: **Multiple female reproductive failures in cyclooxygenase 2-deficient mice**. *Cell* 1997, **91**(2):197-208.

Lin YC, Chang SY, Lan KC, Huang HW, Chang CY, Tsai MY, Kung FT, Huang FJ: **Human oocyte maturity in vivo determines the outcome of blastocyst development in vitro**. *J Assist Reprod Genet* 2003, **20**(12):506-512.

Liu H, Aoki F: **Transcriptional activity associated with meiotic competence in fully grown mouse GV oocytes**. *Zygote* 2002, **10**(4):327-332.

Liu Y, Sui HS, Wang HL, Yuan JH, Luo MJ, Xia P, Tan JH: **Germinal vesicle chromatin configurations of bovine oocytes**. *Microsc Res Tech* 2006, **69**(10):799-807.

Logan KA, Juengel JL, McNatty KP: **Onset of steroidogenic enzyme gene**

expression during ovarian follicular development in sheep. *Biol Reprod* 2002, **66**(4):906-916.

Luciano AM, Lodde V, Beretta MS, Colleoni S, Lauria A, Modina S: **Developmental capability of denuded bovine oocyte in a co-culture system with intact cumulus-oocyte complexes: role of cumulus cells, cyclic adenosine 3',5'-monophosphate, and glutathione**. *Mol Reprod Dev* 2005, **71**(3):389-397.

Ma S, Lin H, Miao Y, Liu X, Wang B, Dai J: **The effect of three-dimensional demineralized bone matrix on in vitro cumulus-free oocyte maturation**. *Biomaterials* 2007, **28**(21):3198-3207.

Maguire T, Novik E, Schloss R, Yarmush M: **Alginate-PLL microencapsulation: effect on the differentiation of embryonic stem cells into hepatocytes**. *Biotechnol Bioeng* 2006, **93**(3):581-591.

Mainigi MA, Ord T, Schultz RM: **Meiotic and developmental competence in mice are compromised following follicle development in vitro using an alginate-based culture system**. *Biol Reprod* 2011, **85**(2):269-276.

Maman E, Yung Y, Kedem A, Yerushalmi GM, Konopnicki S, Cohen B, Dor J, Hourvitz A: **High expression of luteinizing hormone receptors messenger RNA by human cumulus granulosa cells is in correlation with decreased fertilization**. *Fertil Steril* 2012, **97**(3):592-598.

Mandl AM, Zuckerman S: **The growth of the oocyte and follicle in the adult rat**. *J Endocrinol* 1952, **8**(2):126-132.

Market-Velker BA, Zhang L, Magri LS, Bonvissuto AC, Mann MR: **Dual effects of superovulation: loss of maternal and paternal imprinted methylation in a dose-dependent manner**. *Hum Mol Genet* 2010, **19**(1):36-51.

Martín-de-Lara F, Sánchez-Aparicio P, Arias de la Fuente C, Rey-Campos J: **Biological effects of FoxJ2 over-expression**. *Transgenic Res* 2008, **17**(6):1131-1141.

Mattson BA, Albertini DF: **Oogenesis: chromatin and microtubule dynamics during meiotic prophase**. *Mol Reprod Dev* 1990, **25**(4):374-383.

Matzuk MM, Burns KH, Viveiros MM, Eppig JJ: **Intercellular communication in the mammalian ovary: oocytes carry the conversation**. *Science* 2002, **296**(5576):2178-2180.

McKenzie LJ, Pangas SA, Carson SA, Kovanci E, Cisneros P, Buster JE, Amato P, Matzuk MM: **Human cumulus granulosa cell gene expression: a predictor of fertilization and embryo selection in women undergoing IVF**. *Hum Reprod* 2004, **19**(12):2869-2874.

McNatty KP, Fidler AE, Juengel JL, Quirke LD, Smith PR, Heath DA, Lundy T, O'Connell A, Tisdall DJ: **Growth and paracrine factors regulating follicular formation and cellular function**. *Mol Cell Endocrinol* 2000, **163**(1-2):11-20.

McReynolds S, Dzieciatkowska M, McCallie BR, Mitchell SD, Stevens J,

Hansen K, Schoolcraft WB, Katz-Jaffe MG: **Impact of maternal aging on the molecular signature of human cumulus cells**. *Fertil Steril* 2012, **98**(6):1574-1580.e1575.

Menke DB, Koubova J, Page DC: **Sexual differentiation of germ cells in XX mouse gonads occurs in an anterior-to-posterior wave**. *Dev Biol* 2003, **262**(2):303-312.

Mio Y, Maeda K: **Time-lapse cinematography of dynamic changes occurring during in vitro development of human embryos**. *Am J Obstet Gynecol* 2008, **199**(6):660.e661-665.

Miyara F, Migne C, Dumont-Hassan M, Le Meur A, Cohen-Bacrie P, Aubriot FX, Glissant A, Nathan C, Douard S, Stanovici A *et al*: **Chromatin configuration and transcriptional control in human and mouse oocytes**. *Mol Reprod Dev* 2003, **64**(4):458-470.

Mochida N, Akatani-Hasegawa A, Saka K, Ogino M, Hosoda Y, Wada R, Sawai H, Shibahara H: **Live births from isolated primary/early secondary follicles following a multistep culture without organ culture in mice**. *Reproduction* 2013, **146**(1):37-47.

Modina S, Luciano AM, Vassena R, Baraldi-Scesi L, Lauria A, Gandolfi F: **Oocyte developmental competence after in vitro maturation depends on the persistence of cumulus-oocyte comunications which are linked to the intracellular concentration of cAMP**. *Ital J Anat Embryol* 2001, **106**(2 Suppl 2):241-248.

Molyneaux K, Wylie C: **Primordial germ cell migration**. *Int J Dev Biol* 2004, **48**(5-6):537-544.

Monti M, Redi C: **Oogenesis specific genes (Nobox, Oct4, Bmp15, Gdf9, Oogenesin1 and Oogenesin2) are differentially expressed during natural and gonadotropin-induced mouse follicular development**. *Mol Reprod Dev* 2009, **76**(10):994-1003.

Moon JH, Hyun CS, Lee SW, Son WY, Yoon SH, Lim JH: **Visualization of the metaphase II meiotic spindle in living human oocytes using the Polscope enables the prediction of embryonic developmental competence after ICSI**. *Hum Reprod* 2003, **18**(4):817-820.

Mousset-Simeón N, Jouannet P, Le Cointre L, Coussieu C, Poirot C: **Comparison of three in vitro culture systems for maturation of early preantral mouse ovarian follicles**. *Zygote* 2005, **13**(2):167-175.

Mtango NR, Potireddy S, Latham KE: **Oocyte quality and maternal control of development**. *Int Rev Cell Mol Biol* 2008, **268**:223-290.

Nakamura T, Arai Y, Umehara H, Masuhara M, Kimura T, Taniguchi H, Sekimoto T, Ikawa M, Yoneda Y, Okabe M *et al*: **PGC7/Stella protects against DNA demethylation in early embryogenesis**. *Nat Cell Biol* 2007, **9**(1):64-71.

Nation A, Selwood L: **The production of mature oocytes from adult ovaries following primary follicle culture in a marsupial**. *Reproduction* 2009, **138**(2):247-255.

Navarro PA, de Araújo MM, de Araújo CM, Rocha M, dos Reis R, Martins W: **Relationship between first polar body morphology before intracytoplasmic sperm injection and fertilization rate, cleavage rate, and embryo quality**. *Int J Gynaecol Obstet* 2009, **104**(3):226-229.

Norris RP, Ratzan WJ, Freudzon M, Mehlmann LM, Krall J, Movsesian MA, Wang H, Ke H, Nikolaev VO, Jaffe LA: **Cyclic GMP from the surrounding somatic cells regulates cyclic AMP and meiosis in the mouse oocyte**. *Development* 2009, **136**(11):1869-1878.

Nuzzo A, Mulas F, Gabetta M, Arbustini E, Zupan B, Larizza C, Bellazzi R: **Text Mining approaches for automated literature knowledge extraction and representation**. *Stud Health Technol Inform* 2010, **160**(Pt 2):954-958.

Oktem O, Oktay K: **The role of extracellular matrix and activin-A in in vitro growth and survival of murine preantral follicles**. *Reprod Sci* 2007, **14**(4):358-366.

Otsuki J, Nagai Y, Chiba K: **Lipofuscin bodies in human oocytes as an indicator of oocyte quality**. *J Assist Reprod Genet* 2007, **24**(7):263-270.

Otsuki J, Okada A, Morimoto K, Nagai Y, Kubo H: **The relationship between pregnancy outcome and smooth endoplasmic reticulum clusters in MII human oocytes**. *Hum Reprod* 2004, **19**(7):1591-1597.

Pangas SA, Saudye H, Shea LD, Woodruff TK: **Novel approach for the three-dimensional culture of granulosa cell-oocyte complexes**. *Tissue Eng* 2003, **9**(5):1013-1021.

Panguluri SK, Yeakel C, Kakar SS: **PTTG: an important target gene for ovarian cancer therapy**. *J Ovarian Res* 2008, **1**(1):6.

Papamentzelopoulou M, Mavrogianni D, Partsinevelos GA, Marinopoulos S, Dinopoulou V, Theofanakis C, Anagnostou E, Loutradis D: **LH receptor gene expression in cumulus cells in women entering an ART program**. *J Assist Reprod Genet* 2012, **29**(5):409-416.

Parfenov V, Potchukalina G, Dudina L, Kostyuchek D, Gruzova M: **Human antral follicles: oocyte nucleus and the karyosphere formation (electron microscopic and autoradiographic data)**. *Gamete Res* 1989, **22**(2):219-231.

Parrish EM, Siletz A, Xu M, Woodruff TK, Shea LD: **Gene expression in mouse ovarian follicle development in vivo versus an ex vivo alginate culture system**. *Reproduction* 2011, **142**(2):309-318.

Payer B, Saitou M, Barton SC, Thresher R, Dixon JP, Zahn D, Colledge WH, Carlton MB, Nakano T, Surani MA: **Stella is a maternal effect gene required for normal early development in mice**. *Curr Biol* 2003, **13**(23):2110-2117.

Pepling ME, Spradling AC: **Female mouse germ cells form synchronously dividing cysts**. *Development* 1998, **125**(17):3323-3328.

Pepling ME, Spradling AC: **Mouse ovarian germ cell cysts undergo programmed breakdown to form primordial follicles**. *Dev Biol* 2001, **234**(2):339-351.

Pesce M, Wang X, Wolgemuth DJ, Schöler H: **Differential expression of the Oct-4 transcription factor during mouse germ cell differentiation**. *Mech Dev* 1998, **71**(1-2):89-98.

Puard V, Tranchant T, Cadoret V, Gauthier C, Reiter E, Guerif F, Royere D: **Semi-quantitative measurement of specific proteins in human cumulus cells using reverse phase protein array**. *Reprod Biol Endocrinol* 2013, **11**:100.

Rajkovic A, Pangas SA, Ballow D, Suzumori N, Matzuk MM: **NOBOX deficiency disrupts early folliculogenesis and oocyte-specific gene expression**. *Science* 2004, **305**(5687):1157-1159.

Rama Raju GA, Prakash GJ, Krishna KM, Madan K: **Meiotic spindle and zona pellucida characteristics as predictors of embryonic development: a preliminary study using PolScope imaging**. *Reprod Biomed Online* 2007, **14**(2):166-174.

Rienzi L, Ubaldi FM, Iacobelli M, Minasi MG, Romano S, Ferrero S, Sapienza F, Baroni E, Litwicka K, Greco E: **Significance of metaphase II human oocyte morphology on ICSI outcome**. *Fertil Steril* 2008, **90**(5):1692-1700.

Rienzi L, Ubaldi F, Martinez F, Iacobelli M, Minasi MG, Ferrero S, Tesarik J, Greco E: **Relationship between meiotic spindle location with regard to the polar body position and oocyte developmental potential after ICSI**. *Hum Reprod* 2003, **18**(6):1289-1293.

Roche JF: **Control and regulation of folliculogenesis--a symposium in perspective**. *Rev Reprod* 1996, **1**(1):19-27.

Rowghani NM, Heise MK, McKeel D, McGee EA, Koepsel RR, Russell AJ: **Maintenance of morphology and growth of ovarian follicles in suspension culture**. *Tissue Eng* 2004, **10**(3-4):545-552.

Royère D, Feuerstein P, Cadoret V, Puard V, Uzbekova S, Dalbies-Tran R, Teusan R, Houlgatte R, Labas V, Guérif F: **[Non invasive assessment of embryo quality: proteomics, metabolomics and oocyte-cumulus dialogue]**. *Gynecol Obstet Fertil* 2009, **37**(11-12):917-920.

Rubio I, Kuhlmann R, Agerholm I, Kirk J, Herrero J, Escribá MJ, Bellver J, Meseguer M: **Limited implantation success of direct-cleaved human zygotes: a time-lapse study**. *Fertil Steril* 2012, **98**(6):1458-1463.

Russo V, Martelli A, Berardinelli P, Di Giacinto O, Bernabò N, Fantasia D, Mattioli M, Barboni B: **Modifications in chromatin morphology and organization during sheep oogenesis**. *Microsc Res Tech* 2007, **70**(8):733-744.

Shah PP, Kakar SS: **Pituitary tumor transforming gene induces epithelial to mesenchymal transition by regulation of Twist, Snail, Slug, and E-cadherin**. *Cancer Lett* 2011, **311**(1):66-76.

Sharma GT, Dubey PK, Meur SK: **Survival and developmental competence of buffalo preantral follicles using three-dimensional collagen gel culture system**. *Anim Reprod Sci* 2009, **114**(1-3):115-124.

Shen X, Miyano T, Kato S: **Promotion of follicular antrum formation by pig oocytes in vitro**. *Zygote* 1998, **6**(1):47-54.

Shen Y, Stalf T, Mehnert C, Eichenlaub-Ritter U, Tinneberg HR: **High magnitude of light retardation by the zona pellucida is associated with conception cycles**. *Hum Reprod* 2005, **20**(6):1596-1606.

Shikanov A, Xu M, Woodruff TK, Shea LD: **Interpenetrating fibrin-alginate matrices for in vitro ovarian follicle development**. *Biomaterials* 2009, **30**(29):5476-5485.

Shikanov A, Xu M, Woodruff TK, Shea LD: **A method for ovarian follicle encapsulation and culture in a proteolytically degradable 3 dimensional system**. *J Vis Exp* 2011(49).

Shimada M, Nishibori M, Isobe N, Kawano N, Terada T: **Luteinizing hormone receptor formation in cumulus cells surrounding porcine oocytes and its role during meiotic maturation of porcine oocytes**. *Biol Reprod* 2003, **68**(4):1142-1149.

Sidhu K, Kim J, Chayosumrit M, Dean S, Sachdev P: **Alginate microcapsule as a 3D platform for propagation and differentiation of human embryonic stem cells (hESC) to different lineages**. *J Vis Exp* 2012(61).

Simões RA, Satrapa RA, Rosa FS, Piagentini M, Castilho AC, Ereno RL, Trinca LA, Nogueira MF, Buratini J, Barros CM: **Ovulation rate and its relationship with follicle diameter and gene expression of the LH receptor (LHR) in Nelore cows**. *Theriogenology* 2012, **77**(1):139-147.

Sittadjody S, Saul JM, Joo S, Yoo JJ, Atala A, Opara EC: **Engineered multilayer ovarian tissue that secretes sex steroids and peptide hormones in response to gonadotropins**. *Biomaterials* 2013, **34**(10):2412-2420.

Steptoe PC, Edwards RG: **Birth after the reimplantation of a human embryo**. *Lancet* 1978, **2**(8085):366.

Su YQ, Sugiura K, Eppig JJ: **Mouse oocyte control of granulosa cell development and function: paracrine regulation of cumulus cell metabolism**. *Semin Reprod Med* 2009, **27**(1):32-42.

Sui HS, Liu Y, Miao DQ, Yuan JH, Qiao TW, Luo MJ, Tan JH: **Configurations of germinal vesicle (GV) chromatin in the goat differ from those of other species**. *Mol Reprod Dev* 2005, **71**(2):227-236.

Sun XS, Liu Y, Yue KZ, Ma SF, Tan JH: **Changes in germinal vesicle (GV) chromatin configurations during growth and maturation of porcine oocytes**. *Mol Reprod Dev* 2004, **69**(2):228-234.

Sutton-McDowall ML, Gilchrist RB, Thompson JG: **The pivotal role of glucose metabolism in determining oocyte developmental competence**. *Reproduction* 2010, **139**(4):685-695.

Suzumori N, Yan C, Matzuk MM, Rajkovic A: **Nobox is a homeobox-encoding gene preferentially expressed in primordial and growing oocytes**. *Mech Dev* 2002, **111**(1-2):137-141.

Swain JE, Pool TB: **ART failure: oocyte contributions to unsuccessful**

fertilization. *Hum Reprod Update* 2008, **14**(5):431-446.

Tagler D, Tu T, Smith RM, Anderson NR, Tingen CM, Woodruff TK, Shea LD: **Embryonic fibroblasts enable the culture of primary ovarian follicles within alginate hydrogels**. *Tissue Eng Part A* 2012, **18**(11-12):1229-1238.

Telfer EE, McLaughlin M: **Strategies to support human oocyte development in vitro**. *Int J Dev Biol* 2012, **56**(10-12):901-907.

Tibbitt MW, Anseth KS: **Hydrogels as extracellular matrix mimics for 3D cell culture**. *Biotechnol Bioeng* 2009, **103**(4):655-663.

Tingen C, Kim A, Woodruff TK: **The primordial pool of follicles and nest breakdown in mammalian ovaries**. *Mol Hum Reprod* 2009, **15**(12):795-803.

Tisdall DJ, Watanabe K, Hudson NL, Smith P, McNatty KP: **FSH receptor gene expression during ovarian follicle development in sheep**. *J Mol Endocrinol* 1995, **15**(3):273-281.

Torrance C, Telfer E, Gosden RG: **Quantitative study of the development of isolated mouse pre-antral follicles in collagen gel culture**. *J Reprod Fertil* 1989, **87**(1):367-374.

Tsafriri A, Reich R: **Molecular aspects of mammalian ovulation**. *Exp Clin Endocrinol Diabetes* 1999, **107**(1):1-11.

Tsafriri A, Vale W, Hsueh AJ: **Effects of transforming growth factors and inhibin-related proteins on rat preovulatory graafian follicles in vitro**. *Endocrinology* 1989, **125**(4):1857-1862.

Tsukamoto S, Hara T, Yamamoto A, Kito S, Minami N, Kubota T, Sato K, Kokubo T: **Fluorescence-based visualization of autophagic activity predicts mouse embryo viability**. *Sci Rep* 2014, **4**:4533.

van Montfoort AP, Geraedts JP, Dumoulin JC, Stassen AP, Evers JL, Ayoubi TA: **Differential gene expression in cumulus cells as a prognostic indicator of embryo viability: a microarray analysis**. *Mol Hum Reprod* 2008, **14**(3):157-168.

Vanacker J, Luyckx V, Dolmans MM, Des Rieux A, Jaeger J, Van Langendonckt A, Donnez J, Amorim CA: **Transplantation of an alginate-matrigel matrix containing isolated ovarian cells: first step in developing a biodegradable scaffold to transplant isolated preantral follicles and ovarian cells**. *Biomaterials* 2012, **33**(26):6079-6085.

Varani S, Elvin JA, Yan C, DeMayo J, DeMayo FJ, Horton HF, Byrne MC, Matzuk MM: **Knockout of pentraxin 3, a downstream target of growth differentiation factor-9, causes female subfertility**. *Mol Endocrinol* 2002, **16**(6):1154-1167.

Visser JA, Themmen AP: **Anti-Müllerian hormone and folliculogenesis**. *Mol Cell Endocrinol* 2005, **234**(1-2):81-86.

Wang HL, Sui HS, Liu Y, Miao DQ, Lu JH, Liang B, Tan JH: **Dynamic changes of germinal vesicle chromatin configuration and transcriptional activity during maturation of rabbit follicles**. *Fertil Steril* 2009, **91**(4 Suppl):1589-1594.

Wang WH, Meng L, Hackett RJ, Keefe DL: **Developmental ability of human oocytes with or without birefringent spindles imaged by Polscope before insemination**. *Hum Reprod* 2001, **16**(7):1464-1468.

Wathlet S, Adriaenssens T, Segers I, Verheyen G, Van de Velde H, Coucke W, Ron El R, Devroey P, Smitz J: **Cumulus cell gene expression predicts better cleavage-stage embryo or blastocyst development and pregnancy for ICSI patients**. *Hum Reprod* 2011, **26**(5):1035-1051.

Wei Y, Zeng W, Wan R, Wang J, Zhou Q, Qiu S, Singh SR: **Chondrogenic differentiation of induced pluripotent stem cells from osteoarthritic chondrocytes in alginate matrix**. *Eur Cell Mater* 2012, **23**:1-12.

West ER, Xu M, Woodruff TK, Shea LD: **Physical properties of alginate hydrogels and their effects on in vitro follicle development**. *Biomaterials* 2007, **28**(30):4439-4448.

Wong CC, Loewke KE, Bossert NL, Behr B, De Jonge CJ, Baer TM, Reijo Pera RA: **Non-invasive imaging of human embryos before embryonic genome activation predicts development to the blastocyst stage**. *Nat Biotechnol* 2010, **28**(10):1115-1121.

Wycherley G, Downey D, Kane MT, Hynes AC: **A novel follicle culture system markedly increases follicle volume, cell number and oestradiol secretion**. *Reproduction* 2004, **127**(6):669-677.

Xia G, Byskov AG, Andersen CY: **Cumulus cells secrete a meiosis-inducing substance by stimulation with forskolin and dibutyric cyclic adenosine monophosphate**. *Mol Reprod Dev* 1994, **39**(1):17-24.

Xia P: **Intracytoplasmic sperm injection: correlation of oocyte grade based on polar body, perivitelline space and cytoplasmic inclusions with fertilization rate and embryo quality**. *Hum Reprod* 1997, **12**(8):1750-1755.

Xu J, Bernuci MP, Lawson MS, Yeoman RR, Fisher TE, Zelinski MB, Stouffer RL: **Survival, growth, and maturation of secondary follicles from prepubertal, young, and older adult rhesus monkeys during encapsulated three-dimensional culture: effects of gonadotropins and insulin**. *Reproduction* 2010, **140**(5):685-697.

Xu J, Lawson MS, Yeoman RR, Pau KY, Barrett SL, Zelinski MB, Stouffer RL: **Secondary follicle growth and oocyte maturation during encapsulated three-dimensional culture in rhesus monkeys: effects of gonadotrophins, oxygen and fetuin**. *Hum Reprod* 2011b, **26**(5):1061-1072.

Xu M, Barrett SL, West-Farrell E, Kondapalli LA, Kiesewetter SE, Shea LD, Woodruff TK: **In vitro grown human ovarian follicles from cancer patients support oocyte growth**. *Hum Reprod* 2009b, **24**(10):2531-2540.

Xu M, Fazleabas AT, Shikanov A, Jackson E, Barrett SL, Hirshfeld-Cytron J, Kiesewetter SE, Shea LD, Woodruff TK: **In vitro oocyte maturation and preantral follicle culture from the luteal-phase baboon ovary produce mature oocytes**. *Biol Reprod* 2011, **84**(4):689-697.

Xu M, Kreeger PK, Shea LD, Woodruff TK: **Tissue-engineered follicles produce live, fertile offspring**. *Tissue Eng* 2006, **12**(10):2739-2746.

Xu M, West-Farrell ER, Stouffer RL, Shea LD, Woodruff TK, Zelinski MB: **Encapsulated three-dimensional culture supports development of nonhuman primate secondary follicles**. *Biol Reprod* 2009a, **81**(3):587-594.

Yamamoto K, Otoi T, Koyama N, Horikita N, Tachikawa S, Miyano T: **Development to live young from bovine small oocytes after growth, maturation and fertilization in vitro**. *Theriogenology* 1999, **52**(1):81-89.

Yamashita Y, Shimada M, Okazaki T, Maeda T, Terada T: **Production of progesterone from de novo-synthesized cholesterol in cumulus cells and its physiological role during meiotic resumption of porcine oocytes**. *Biol Reprod* 2003, **68**(4):1193-1198.

Yang SH, Son WY, Yoon SH, Ko Y, Lim JH: **Correlation between in vitro maturation and expression of LH receptor in cumulus cells of the oocytes collected from PCOS patients in HCG-primed IVM cycles**. *Hum Reprod* 2005, **20**(8):2097-2103.

Ying Y, Qi X, Zhao GQ: **Induction of primordial germ cells from murine epiblasts by synergistic action of BMP4 and BMP8B signaling pathways**. *Proc Natl Acad Sci U S A* 2001, **98**(14):7858-7862.

Ying Y, Zhao GQ: **Cooperation of endoderm-derived BMP2 and extraembryonic ectoderm-derived BMP4 in primordial germ cell generation in the mouse**. *Dev Biol* 2001, **232**(2):484-492.

Yu H, Kim PM, Sprecher E, Trifonov V, Gerstein M: **The importance of bottlenecks in protein networks: correlation with gene essentiality and expression dynamics**. *PLoS Comput Biol* 2007, **3**(4):e59.

Zhang X, Jafari N, Barnes RB, Confino E, Milad M, Kazer RR: **Studies of gene expression in human cumulus cells indicate pentraxin 3 as a possible marker for oocyte quality**. *Fertil Steril* 2005, **83 Suppl 1**:1169-1179.

Zhou P, Wu YG, Wei DL, Li Q, Wang G, Zhang J, Luo MJ, Tan JH: **Mouse cumulus-denuded oocytes restore developmental capacity completely when matured with optimal supplementation of cysteamine, cystine, and cumulus cells**. *Biol Reprod* 2010, **82**(4):759-768.

Zhu HY, Wang JX, Tong XM, Xu WH, Jiang LY, Jing XY, Yang LY, Zhou F, Zhang SY: **Three-dimensional cultures of human endometrial cells on Matrigel mimic in vivo morphology**. *Chin Med J (Engl)* 2012, **125**(5):863-868.

Zuccotti M, Garagna S, Merico V, Monti M, Alberto Redi C: **Chromatin organisation and nuclear architecture in growing mouse oocytes**. *Mol Cell Endocrinol* 2005, **234**(1-2):11-17.

Zuccotti M, Giorgi Rossi P, Martinez A, Garagna S, Forabosco A, Redi CA: **Meiotic and developmental competence of mouse antral oocytes**. *Biol Reprod* 1998, **58**(3):700-704.

Zuccotti M, Merico V, Redi CA, Bellazzi R, Adjaye J, Garagna S: **Role of Oct-4 during acquisition of developmental competence in mouse oocyte**. *Reprod Biomed Online* 2009, **19 Suppl 3**:57-62.

Zuccotti M, Merico V, Sacchi L, Bellone M, Brink TC, Bellazzi R, Stefanelli M, Redi CA, Garagna S, Adjaye J: **Maternal Oct-4 is a potential key regulator of the developmental competence of mouse oocytes**. *BMC Dev Biol* 2008, **8**:97.

Zuccotti M, Piccinelli A, Giorgi Rossi P, Garagna S, Redi CA: **Chromatin organization during mouse oocyte growth**. *Mol Reprod Dev* 1995, **41**(4):479-485.

Zuccotti M, Ponce RH, Boiani M, Guizzardi S, Govoni P, Scandroglio R, Garagna S, Redi CA: **The analysis of chromatin organisation allows selection of mouse antral oocytes competent for development to blastocyst.** *Zygote* 2002, **10**(1):73-78.

List of publications

Belli M*, Vigone G*, Merico V, Redi CA, Zuccotti M, Garagna S (2012) **Towards a 3D culture of mouse ovarian follicles.** *Int J Dev Biol*, 56(10-12):931-7. *, equal contributors.

Vigone G*, Merico V*, Prigione A*, Mulas F, Sacchi L, Gabetta M, Bellazzi R, Redi CA, Mazzini G, Adjaye J, Garagna S, Zuccotti M (2013) **Transcriptome based identification of mouse cumulus cell markers that predict the developmental competence of their enclosed antral oocytes.** *BMC Genomics*, 14:380. *, equal contributors.

Vigone G*, Merico V*, Redi CA, Mazzini G, Garagna S, Zuccotti M (2014) **FSH and LH receptors are differentially expressed in cumulus cells surrounding developmentally competent and incompetent mouse fully grown antral oocytes.** *Reprod Fertil Dev*, doi: 10.1071/RD13251. *, equal contributors.

Belli M, Vigone G, Merico V, Redi CA, Garagna S, Zuccotti M (2014) **Time-lapse dynamics of the mouse oocyte chromatin organisation during meiotic resumption.** *Biomed Res Int*, doi: 10.1155/2014/207357.

Zuccotti M, Merico V, Rebuzzini P, Belli M, Vigone G, Redi CA, Mulas F, Fassina L, Wruck W, Adjaye J, Bellazzi R, Garagna S. **Engineering the ovarian follicle.** *A review in progress.*