UNIVERSITA' DEGLI STUDI DI PARMA

DOTTORATO DI RICERCA IN

MEDICINA MOLECOLARE

CICLO XXX

Female infertility: *in vitro* maturation of antral oocytes upon a feeder layer of selected cumulus cells improves developmental competence

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Anno Accademico 2017/2018

L'espressione più eccitante da ascoltare nella scienza, quella che annuncia le più grandi scoperte, non è "Eureka" ma "Che strano…".

(Isaac Asimov)

ABSTRACT

The World Health Organization (WHO) estimates that infertility affects about 20% of couples in western countries. In addition, one third of women undergoing oncological treatments develops premature ovarian failure as a consequence of aggressive chemo- or radio- therapies. In numbers, 280.000/year in USA (American Cancer Society; cancer.org) and 58.000/year in Italy (Associazione Italiana di Oncologia Medica; registri-tumori.it). The isolation and cryopreservation of denuded antral germinal vesicle oocytes (DOs) has been seen, in recent years, as a strategy for preserving fertility of these patients, particularly when DOs are *in vitro* matured to metaphase II (MII) in the presence of a feeder layer of cumulus cells (FL-CCs).

To this regard, the work I carried out during my PhD was focused on the development of a new miniaturized culture system based on DOs maturation upon a FL of selected CCs.

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

1. *To test the hypothesis that improvements could be introduced by a selection of CCs prior to the preparation of the FL.*

In this study we classified CCs based on their association with developmentally competent (SN) or incompetent (NSN) mouse fully-grown antral oocytes and then, developed a miniaturized system, a micro-Insert 4 well plate, to obtain a FL of SN-CCs or NSN-CCs.

We show, for the first time, that maturation of DOs upon FL-SN-CCs significantly better contributes to the acquisition of oocytes meiotic and developmental competence, with a developmental rate to blastocyst (26.9%) equal to that obtained after the maturation of intact cumulus oocyte complexes (28%). Instead, DOs matured in the absence of CCs (NO-FL) or upon FL-NSN-CCs undergo meiotic and developmental failure, with embryos arresting either at the 4-cell or morula stage.

2. *The identification of the time-lapse interval, during the germinal vesicle-to-metaphase II transition, in which the SN-FL-CCs mainly influences the acquisition of oocyte developmental competence.*

To this end, I cultured DOs under three different experimental conditions:

1. without FL-SN-CCs for 3 hr and upon FL-SN-CCs for further 12 hr;
2. without FL-SN-CCs for 6 hr and upon FL-SN-CCs for further 9 hr;
3. upon an SN-FL-CCs for 6 hours and then without FL-SN-CCs for further 9 hr.

When DOs were cultured following protocol 1, the progression to the 2- and 4-cell stages remained similar to that obtained when DOs were cultured upon FL-SN-CCs for the whole 15 hr, suggesting a correct oocyte-to-embryo transition. Instead, the frequency of development to blastocyst decreased significantly, indicating an alteration to key developmental processes.

Furthermore, when the initial culture in the absence of CCs was prolonged to 6 hr, none of the embryos reached the blastocyst.

Even more detrimental was the absence of FL-SN-CCs during the latest 9 hr culture, with a complete developmental arrest at the 2-cell stage.

Overall, the results of these three sets of experiments suggest the presence of an essential paracrine factor/s released from FL-SN-CCs, likely into the culture medium, whose lack is detrimental to the acquisition of a proper developmental competence.

In conclusion, the main finding of my study is the demonstration of a positive contribution to the acquisition of the oocyte meiotic and developmental competence of SN-CCs. Lack of this support, either in the absence of CCs (NO-FL) or in the presence of NSN-CCs, results in meiotic and developmental failure. Furthermore, our results set the bases to further improve the protocols of antral oocytes maturation upon a feeder layer of CCs and to unravel the molecules involved in the cross-talk between the gamete and its companion cumulus cells during the germinal vesicle (GV)-to-MII transition.

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1. INTRODUCTION

1.1 The health issue: women infertility

Infertility is a pathology of reproductive system characterized by the failure to achieve a pregnancy after 12 months or more of regular unprotected sexual intercourses. The World Health Organization (WHO) estimates that infertility affects about 20% of couples in western countries.

Infertility could have a genetic or physiological origin in women i.e., polycystic ovary syndrome (PCOS) (Diamond *et al.,* 2017), endometriosis (Juneau *et al.,* 2017), cervical mucus defects (Check *et al.,* 2006) or tubal damage (for review see Mårdh, 2004) or could be associated to lifestyle factors i.e., smoking (Vanegas *et al.,* 2017), high body-weight (Backonja *et al.,* 2017) and stress (Chow *et al.,* 2016). Furthermore, women diagnosed with cancer [(in numbers: 280.000/year in USA (American Cancer Society; cancer.org) and 58.000/year in Italy (Associazione Italiana di Oncologia Medica; registri-tumori.it)] that eradicate their disease have to deal with reproductive system damages caused by the aggressive therapies to fight cancer (Levine *et al.,* 2015, Trivers *et al.,* 2014). Indeed, one third of them develops premature ovarian failure (POF).

Given the high percentage of POF patients, many fertility preservation programs have been developed and, among them, the cryopreservation of DOs has been seen as the best strategy, especially when they are *in vitro* matured to metaphase II in the presence of CCs (for a review see Lee *et al.,* 2014).

Unfortunately, the developmental potential of such DOs is still low (Fasano *et al.,* 2012) leading to only 20-30% the success rate of *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI), the Assisted Reproductive Technologies (ART) used for the purpose of human reproduction.

The first *in vitro* fertilization was performed on humans by Dr. Robert Edwards and Dr. Patrick Steptoe (Steptoe and Edwards, 1978) and, until now, around 5 million baby have been born thanks to these procedures. Despite attempts have been made to improve the ART efficiency, only a meagre number of embryos is able to overcome the implantation and develop to term.

1.2 Origin and evolution of female gamete

Primordial germ cells (PGCs), precursors of sperm and oocytes, become identifiable as a cluster of 40 cells at the base of the allantois on day 7 *post coitum* (dpc) (Wear *et al.,* 2016). On 10-11.5 dpc, they migrate to the yolk sack reaching the genital ridge of the primitive gonad (Molyneaux *et al.,* 2004). During migration and genital ridge colonization, the behavior of XX and XY germ cell is identical. It is only when specific genes are activated that germ cells commit to a male or female fate (McLaren *et al.,* 1997).
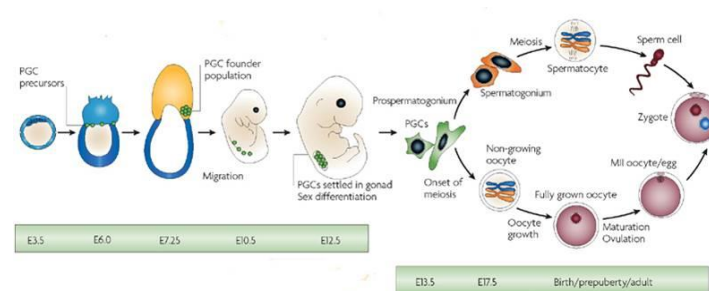
Figure 1. Mouse developmental cycle. Precursor of PGCs colonize the genital ridge of the developing gonad on 10-11.5 dpc. (Sasaki *et al.,* 2008).

In the male genital ridge the expression of gene *Sry* (Koopman *et al.,* 1991) triggers the differentiation of sex cords into the seminiferous tubules and, the tubules epithelium into the Sertoli cells; whereas in the female, lack of *Sry* allows the expression of genes [i.e., *Rspo1*, *Wnt4* and *Foxl2* (Liu *et al.,* 2010)] responsible of PGCs proliferation into about 85.000 oogonia (Saitou *et al.,* 2012). From 13.5 dpc (Amhle and Dean, 2002) the latter enter into meiosis and stop at the diplotene stage of prophase I. At this time, a single layer of squamous somatic cells, precursor of granulosa cells (GCs), surrounds the oocytes giving origin to primordial follicles.

At birth, around one million of those primordial follicles are present in the ovaries; however, only few hundreds of them will complete the maturation process, whereas the majority will degenerates through an apoptotic process called atresia.

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1.3 Mammalian folliculogenesis and gamete selection

Folliculogenesis is an arduous biological process that leads, through a complicated and intricate interaction between the somatic and germ cell components, the formation of a mature oocyte which could be fertilized (Figure 2).

Starting with puberty in humans, and sexual maturity in other mammals, the hypophysis releases the follicle stimulating hormone (FSH), whose action leads to the recruitment of a pool of primordial follicles.

As folliculogenesis begins, oocytes start to grow in dimension and to produce the components of zona pellucida (ZP), a glycoprotein membrane required for fertilization and embryo pre-implantation development. Meanwhile, granulosa cells differentiate toward a more cuboidal shape and start to proliferate until the oocyte is surrounded by almost 2-4 layers of cells, forming a secondary pre-antral follicle. At this point, among granulosa cells, some cavities containing follicular fluid, rich in metabolites and hormones (Hennet and Combelles, 2012), start to form. Gradually, the small cavities aggregate to form a unique antrum and the follicle enters in the antral pre-ovulatory stage. In this phase, the oocyte reaches 70-80 µm in dimension and complete the first meiotic division.

Cumulus Oocyte Complex (COC)

Figure 2. Mouse folliculogenesis
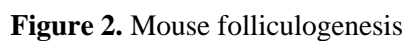
The latter will be surround by 3-6 layers of follicle cells divided, by follicle antrum, in two populations: mural granulosa cells (mGCs), that form the follicle wall, and cumulus cells (CCs), strictly connected to the oocyte (Figure 2). Then, as a results asymmetric meiotic division the gamete will extrude the first polar body (PB-I) remaining arrested in the metaphase of the second meiosis until fertilization.

The whole process of folliculogenesis is regulated by exogenous and endogenous hormonal control. During pre-ovulatory follicular development, the action of FSH drives the induction on granulosa cells of luteinizing hormone (LH) receptors. Few follicles, in poly-ovulatory species i.e., mouse and, only one in mono-ovulatory species i.e., human, will express a number of LH receptors sufficient to be ovulated (Zeleznick *et al.,* 2004; Hennet and Combelles, 2012). Whether or not follicles will reach ovulation depend on the frequency and amplitude of LH pulses induced in the pituitary (Scaramuzzi and Campbell, 1990; Hennet and Combelles, 2012). Those follicles receptiveness to LH start to produce estradiol, leading to a drop of FSH concentration and a degeneration of the rest of the antral follicles.

Then, the increased pressure in the antrum will cause the rupture of the follicle and the expulsion of the oocyte together with its corona radiata (Cumulus-Oocyte Complex, COC). Through the fallopian tube fimbriae, COCs will migrate to the oviduct, where fertilization will eventually occur. In the meantime, in the ovarian compartment, what remain of the ruptured follicle forms the corpus luteum, an endocrine organ essential for the embryo implantation in the uterus. Corpus luteum produces progesterone leading to a very strong thickening of the endometrial tissue and an inhibition of FSH secretion preventing a new wave of follicles recruitment. If the oocyte is fertilized, the corpus luteum continues to produce progesterone until the placenta will be able to start its own production; whereas if not, this organ degenerates leading to a fall of progesterone and an increasing surge of FSH concentration.

1.4 Oocyte-cumulus cells communication during the female gamete growth

As described above, the antral follicle, at the last stage of its maturation, is characterized by the presence of an antrum cavity, containing steroids, proteins, enzymes and electrolytes, which determine the separation of granulosa cells into mural granulosa and cumulus cells (Hennet and Combelles, 2012). The latter are strictly connected to the oocyte leading to the formation of a structure called COC (Figure 3A, B).
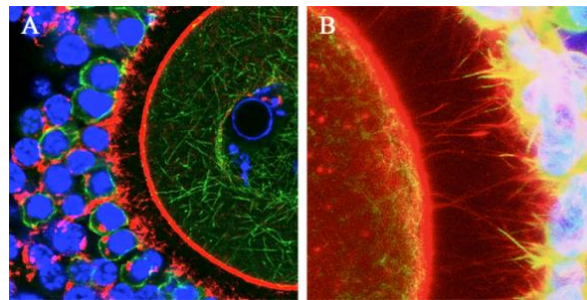
Figure 3. A) Confocal microscopy image of a mouse Cumulus Oocyte Complex (COC); B) three-dimensional reconstruction of human Cumulus Oocyte Complex (COC) (Coticchio *et al.,* 2012).

The ability of an oocyte to correctly sustain its progression to MII depends on the sophisticated bi-directional communication occurring among itself and the companion cumulus cells. During the whole folliculogenesis, the germ and somatic components are tightly connected to each other through cytoplasmic trans-zonal projections (TZPs), prominent CCs extensions that terminate on the oocyte plasma membrane forming gap junctions (GJs).

GJs are membrane hemichannels composed of connexins (Hervè *et al.,* 2007; Gershon *et al.,* 2008) that physically join the cytoplasms of two adjacent cells allowing the free passage of ions, glucose metabolites and small molecules (Li and Albertini, 2013; Hennet and Combelles, 2012). Through gap junctions cumulus cells support the metabolic demands of oocyte during its growth and maturation carrying out precise functions: CCs are responsible of glycolysis, amino acid uptake and cholesterol synthesis process (Biggers *et al.,* 1967; Gilchrist *et al.,* 2008; Hennet and Combelles 2012). In fact, cumulus cells convert glucose into pyruvate and send it to the oocyte (for a review see

Gilchrist *et al.,* 2008; Su *et al.,* 2009), satisfy its necessity of cholesterol (Su *et al.,* 2009; Huang and Wells, 2010; Sutton-McDowall *et al.,* 2010) and regulate the meiotic arrest by providing cAMP (Norris *et al.,* 2009; Downs *et al.,* 2001). The close association among the two components is crucial for the oocyte growth since its interruption causes detrimental effects on the acquisition of the gamete developmental competence (Carabatsos *et al.,* 2000; Modina *et al.,* 2001, Downs, 1995, 2001).

Many of the cumulus cell functions, upright mentioned, are coordinated or stimulated by its own oocyte. Indeed, it has been demonstrated that the oocyte release soluble growth factors (oocyte secreted factors, OSFs) which act on the companion cumulus cells regulating their behavior. Among them, growth-differentiation factor 9 (*GDF9*) and bone morphogenetic protein 15 (*BMP15*) are the most abundant in the mammalian oocytes (Gilchrist *et al.,* 2008; 2011). Since their expression is essential for the oocyte maturation and ovulation (Ying *et al.,* 2000; Moore *et al.,* 2004; Pangas and Matzuk, 2005; Su *et al.,* 2008), mutations ore deletion of these genes is associated with the pathogenesis of ovarian dysfunctions (Dong *et al.,* 1996; Galloway *et al.,* 2000; Gilchrist *et al.,* 2004, 2008; Shimasaki *et al.,* 2004; Juengel and McNatty, 2005; McNatty *et al.,* 2007; Su *et al.,* 2009).

OSFs, through activation of SMAD 2/3 pathway, coordinate CCs proliferation and cumulus expansion and, in response to that, CCs express their own genes i.e., *Amh*, *Inhibin*, *Activin* involved in the oocyte growth process (Knight *et al.,* 2003; Gilchrist *et al.,* 2008). The expression of OSFs is regulated via negative feedback loop by a CCs paracrine factor, KIT ligand (KL), which binds its c-KIT receptor on the oocyte surface and suppress the expression of *GDF-9* and *BMP-15* (Zuccotti *et al.,* 2011). The c-KIT-KIT ligand interactions modulate the ability of the oocyte to undergo both nuclear and cytoplasmic maturation in the late antral stage (Driancourt *et al.,* 2000). Also, *interleukin-1* and *interleukin-6* have been recognized as paracrine molecules involved in determining the follicle and embryo development success rate in mouse (Makekshah *et al.,* 2006). However, although many are the molecules known to be an active players of dialog between CCs and the oocyte, the majority of paracrine factors involved in that communication is still unknown.

1.5 From the oocyte to the embryo

At the end of folliculogenesis, LH binding triggers the GJs coupling interruption and oocyte meiotic resumption (Hillier, 1994). At this point, the gamete progresses in the meiosis and blocks at the state of metaphase II with the expulsion of PB-I. The COC released from the ovary is swept by the fimbriae into the oviduct, a region closed to the ovary, where fertilization will occur. Although the process of fertilization has been widely studied, many events still need to be clarified. Some evidences suggest that mammalian sperm could be driven to the egg by chemoattractant gradients that originates from the COC allowing the sperm binding on the oocyte zona pellucida (Cohen-Dayag *et al.,* 1995; Eisenbach *et al.,* 2004; Sun *et al.,* 2005; Gakamsky *et al.,* 2008; Armon *et al.,* 2014) (Figure 4).
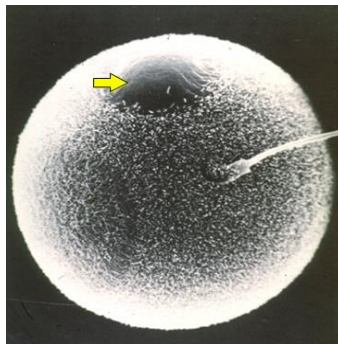
Figure 4. Mouse sperm binding of oocyte's pellucid zone. Notice the oocyte's first polar body extrusion (colored arrow).

Zona pellucida (ZP), composed of different glycoproteins, is an elastic structure which surround and protect the gamete. Since the ZP membrane regulates the gamete maturation, fertilization and preimplantation development (Wassarman *et al.,* 2008; Gao *et al.,* 2017), defect in its formation leads to an infertile condition (Rankin *et al.,* 2000; Wassarman *et al.,* 2008). In mammals, sperm b1,4-galactosyltransferase-I protein binds the zona pellucida ZP3 oligosaccharide chains (Wassarman *et al.,* 2008) activating the molecular events of species-specific sperm penetration. Indeed, once the acrosome reaction occurs with the exocytosis of cortical granules, ZP changes its structure preventing other sperm to enter (Yanagimachi, 1994).

Sperm cells require to undergo to a capacitation process, which *in vivo* occurs during the route through the female genital tract, to became competent to fertilize (Yanagimaci *et al.,* 1963; Stival *et al.,* 2016). The first attempt to induce sperm capacitation dates back to 1963 when Yanagimachi and Chang discovered that, under the right culture conditions, events that lead to the sperm capacitation completion (i.e., removal of glycoproteins, seminal plasma proteins and cholesterol) could be achieved *in vitro*. Once the sperm is capacitated, it fusion with the egg lead to the rupture of two envelopes and the condensation of chromatine in chromosomes which orientation in a meiotic spindle allows the 2-cell stage embryo formation. At this point, in mouse, the mammalian genome is activated and the preimplantation development (Li *et al.,* 2010) will progress through 2-cell, 4-cell, 8-cell, morula up to blastocyst (Figure 5, 6).
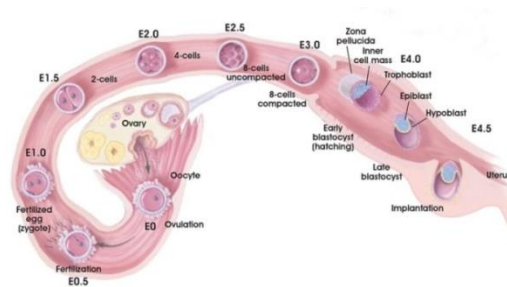
Figure 5. Schematic illustration of embryo pre-implantation development *in vivo* (Winslow *et al.,* 2011).

Figure 6. Bright field images of A) fertilized oocyte, B) 2-cell embryo, C) 4-cell embryo, D) morula and E) blastocyst.

During the 8-cell to morula stage transition the embryo blastomeres undergo a spectacular change in the behavior. They maximize their contact with each other forming a compact ball of cells stabilized by tight junctions. Indeed, they

start to differentiate in two populations: an outer cell layer, that form Trophectoderm (TE) and an internal cell layer, that form Inner Cell Mass (ICM). The trophoblast cells will give origin to the chorion and the embryonic portion of the placenta, whereas the ICM will give rise to the embryo. A complex balance of molecular factors trigger the cell to be part of one of the two cell lines. The expression of *Cdx2* gene initiates cells to form the trophectoderm, whereas the up-regulation of *Oct4* and *Nanog* together with *Sox2* leads to the ICM formation (Strumpf *et al.,* 2005; Jedrusik *et al.,* 2008).

1.6 The model: SN and NSN oocytes

During folliculogenesis, numerous epigenetic changes take places, including DNA methylation and chromatin organization (Kageyama *et al.,* 2007).

Within the antral compartment of the mouse ovary two different type of oocytes have been identified on the basis of their chromatin conformation. In fact, when GV antral oocytes are stained with the supravital fluorochrome Hoechst 33342, they can be classified as Surrounded Nucleolus (SN) oocytes if their chromatin is highly condensed in a ring of Hoechst-positive chromatin around the nucleolus (Figure 7A) or as Non Surrounded Nucleolus (NSN) oocytes, if they lack of a ring surrounding the nucleolus and their chromatin is dispersed in the nucleus (Figure 7B).

Oocytes that show these two different patterns of chromatin organization have been found in mice (Zuccotti *et al.,* 1995, 2005; Inoue *et al.,* 2008) , rats (Mandl and Zuckerman, 1952), monkeys (Lefèvre *at al.,* 1989), pigs (Crozet *et al.,* 1983) and also humans (Parfenov *et al.,* 1989).

The proportion of SN and NSN oocytes varies on the basis of the different phases of ovarian folliculogenesis. Initially, all the oocytes maintain the NSN-type chromatin configuration but, as the oocytes continue to grow, some of them shifts to the SN-, while others remain in the NSN-configuration (Zuccotti *et al.,* 1995).

Moreover, interestingly, the percentage of SN-type oocytes population is associated with the mouse age. 2-week-old untreated females display only 5.9% of SN oocytes in the antral compartment compared to 88.2% in 5-6 week old mice (Zuccotti *et al.,* 1995).
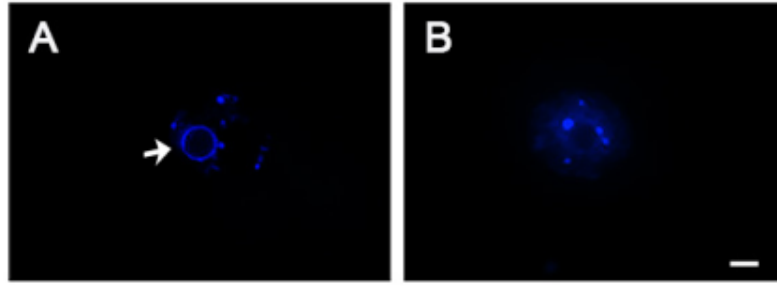
Figure 7. Mouse antral oocyte nucleolus stained with Hoechst 33342. A) Surrounded Nucleolus oocyte (SN); B) Non-Surrounded Nucleolus oocyte (NSN). Scale bar: 5 μm.

The differences described above have a huge biological relevance since only SN oocytes may successfully reach the blastocyst stage whereas NSN oocytes always block at the two-cell stage (Zuccotti *et al.,* 1998, 2002; Inoue *et al.,* 2008). Furthermore, they differ in their transcriptional activity: SN oocytes produce all classes of RNA, while NSN oocytes remain almost inactive (Debey *et al.,* 1993; Bouniol-Baly *et al.,* 1999).

The analysis of the whole transcriptome of the two classes of oocytes have pointed out the existence of significant differences in the expression of those genes known to be fundamental for the embryo pre-implantation development. In more details, the comparison between incompetent MII-NSN *vs.* competent MII-SN oocytes highlights a group of 380 genes differentially expressed, 303 up-regulated and 77 down-regulated in MII-NSN oocytes. Also, the "maternal-effect gene" *Oct4* appears to be down-regulated in incompetent *vs.* competent oocytes, together with its encoded protein. (Zuccotti *et al.,* 2008). The lack of such *Oct4* transcripts could be one of the reason of the inability of NSN oocyte, once fertilized, to beyond the 2-cell stage.

Oct4 is a nuclear transcription factor that regulates the expression of a great number of developmental genes involved in mouse embryo development and in self-renewal of embryonic stem cells (ESCs) (Stewart *et al.,* 2000; Pesce *et al.,* 2000; Cavaleri *et al.,* 2005; Zuccotti *et al.,* 2011). In mouse embryonic stem cells, *Oct4* controls 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; Zuccotti *et al.,*

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2012). Among them, *Stella*, a transcription factor extremely important for oocyte maturation, is highly down-regulated in MII-NSN *vs.* MII-SN oocytes (Zuccotti *et al.,* 2008; 2009a, b).

On the contrary, NOBOX protein, correlated to the poor oocyte developmental potential, is up-regulated in non-developmentally (NSN) *vs.* developmentally competent (SN) oocytes (Belli *et al.,* 2013): whilst NOBOX protein can be found in NSN during the whole growth, in SN oocytes its expression starts to be down-regulated from follicle class of 61-70 μm in diameter and continues to be undetectable until the last stage of its maturation.

All these studies puzzled together suggest that the SN/NSN model could be used as a source of possible markers of the oocyte developmental competence.

1.7 Oocyte developmental competence

During its growth, the oocyte becomes competent to undergo two aspects of maturation: cytoplasmic and nuclear. Both are essential for the formation of an oocyte having the capacity to be fertilized and support the full-term development. The bulk of the gamete growth requires changes in its chromatin nucleus together with the re-organization of many cytoplasmic organelles i.e., cytoskeleton, cortical granules and mitochondria.

As LH pre-ovulatory surge occurs, the oocyte nucleus undergoes to germinal vesicle breakdown (GVBD), progresses through meiosis I and arrests at metaphase II on the second meiotic division. During the metaphase II stage each pair of sister chromatids aligns on meiotic spindle with sister centromeres oriented to opposite poles. Then, the oocyte blocks its progression at metaphase II with the expulsion of the PB-I as a results of an unequal cell division (Figure 8).
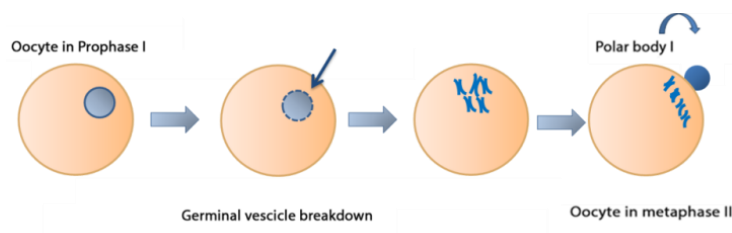
Figure 8. Meiotic gamete progression from GV-to-MII stage.

The oocyte capability to resume meiosis is strictly connected with its developmental competence.

A recent study shows differences in the timing of GV-to-MII transition between competent and incompetent oocyte. Whilst developmentally incompetent oocytes spend a long time at the diplotene stage before undergoing GVBD, developmentally competent oocytes reach more rapidly the MI stage (Belli *et al.,* 2014). According with these results, Higaki and colleagues (2017) demonstrated that early germinal vesicle breakdown is a predictor of high preimplantation developmental competence; oocytes that undergo GVBD earlier display a cleavage and blastocyst formation rates almost double when compared with those with a late GVBD.

Meanwhile the oocyte is completing its GV-to-MII transition, numerous morphological, biochemical and molecular modifications take place in its cytoplasm. Through a rearrangements of the oocyte cytoskeleton, the microtubules drive the process of chromosome and organelle movement (Sun and Schatten, 2006), whereas the microfilaments regulate chromosome migration, cortical spindle anchorage and first polar body emission (Mao *et al.,* 2014). Moreover, cortical granules, start to move to the periphery of the oocyte in a process that would lead to a selection of specie-specific sperm penetration through their exocytosis.

Also mitochondria play an extremely important role during the maturation process. Initially, they aggregated only around the GV, but as the oocyte progress in its development they move away from the perinuclear region and in a mature MII oocyte occupy most of the gamete volume (Dumollard *et al.,* 2006).

However, a drawback of the *in vitro* maturation system is still the incapacity to maintain a correct synchrony between the nuclear and cytoplasmic events in place of the oocyte. Thus, several groups have been tried to improve the oocytes developmental potential by temporarily inhibiting the spontaneous meiotic maturation occurring *in vitro* meanwhile cytoplasmic maturation takes place (Vanhoutte *et al.,* 2009; Li *et al.,* 2012; Guimarães *et al.,* 2016).

1.8 Female fertility preservation: role of cumulus cells on oocyte cryopreservation and *in vitro* maturation

The increased number of women developing defects in their reproductive function as a results of cancer therapies, raises the need to find fertility preservation strategies that precede chemo and radiotherapy treatments.

Nowadays, freezing immature oocytes is one of the possible fertility preservation options in patients with cancer. In this case, at some point, such retrieved immature oocytes can be thawed out and *in vitro* grown up to MII stage, to be fertilized. Since protocols for cryopreserving intact follicles are still inadequate and not enough robust, cumulus cells are usually removed from the gamete prior to cryopreservation in human (Chan Woo Park *et al.,* 2016, Fasano *et al.,* 2012, Zhang *et al.,* 2011), mouse (Endoh *et al.,* 2007, Eroglu *et al.,* 2009) and bovine (Luciano *et al.,* 2009).

Some studies sustain that the survival rate of thawed oocytes (Kuwayama *et al.,* 2005; Nikseresht *et al.,* 2015) is increased when they were frozen together with their companion CCs. In fact the somatic component alleviates the dramatic changes of osmotic pressure occurring during the gamete freezing (Jin *et al.,* 2012) and protects cytoplasmic factors essential for the meiotic spindle reassembly (Tharasanit *et al.,* 2009; Zhou *et al.,* 2016).

However, even if cumulus cells are demonstrated to be helpful for the oocyte survival, at this time, technically, it is not possible to submit both the two cell types to a single cryopreservation protocol, since they dramatically differ in size and permeability.

To this regard, structural evaluation of vitrified bovine (Fuku *et al.,* 1995) and mouse (Ruppert-Lingham *et al.,* 2003) COCs revealed a disruption of the intercellular communications between the oocyte and it companion cumulus cells after follicles freezing. Therefore, some researchers support the hypothesis that the presence of cumulus cells is detrimental for the oocytes vitrification process.

They affirm that the thickness of cumulus cells layers could interfere with the cryoprotectant passage through ooplasm and acts as potential sites of ice crystals formation (Mandelbaum *et al.,* 1988, Gasparrini *et al.,* 2007). Bogoglio and collegues (2007) reported a higher survival and meiotic

maturation rate in immature oocytes cryopreserved without cumulus cells compared to those frozen together with its own cumulus-oophorus. Whilst it is not clear if cumulus cells could improve or not the gamete cryopreservation, it is widely known that their presence is absolutely required for the oocyte IVM. Indeed, the gamete fundamentally depends on its helper somatic cells to perform many of the functions that support the preimplantation embryo development.

If the cryopreservation of cumulus-free oocytes remains the best option available, the oocyte development potential could be improved by re-establishing the cumulus-oocyte interaction in a co-culture system.

In the attempt to recreate the dialog occurring among the somatic and germ cell components, DOs have been co-cultured upon a feeder layer of cumulus cells (Combelles *et al.,* 2005; Ge *et al.,* 2008a, b; Vanhoutte *et al.,* 2009; Zhou *et al.,* 2010; Zhao *et al.,* 2014), together with COCs (Luciano *et al.,* 2005), with autologous or other species CCs (Zhao *et al.,* 2014) even in combination with conditioned medium (Downs *et al.,* 2006; Ge *et al.,* 2008b). Among these strategies, the CCs feeder layer has been seen as the ideal culture system to both improve the oocyte developmental potential and unravel the molecules involved in the cross-talk between the gamete and its companion cumulus cells during the GV-to-MII transition.

1.9 Germinal vesicle oocytes culture upon a feeder layer of cumulus cells

In vitro oocyte culture upon a FL-CCs is the system that better sustains the oocyte maturation, fertilization and the subsequent full term development (Ge *et al.,* 2008a, b; Combelles *et al.,* 2005; Vanhoutte *et al.,* 2009; Zhou *et al.,* 2010; Tahaei *et al.,* 2011).

In brief, this culture system is made of three principal steps: 1) CCs, isolated mainly from ovulated MII-COCs (MII-CCs) or from GV-COCs (GV-CCs) are cultured for 48-72 hr until they reach at least 80% confluence; 2) before in vitro maturation (IVM) the spent medium is replaced with maturation medium 3) DOs are placed on top of the CCs feeder layer and matured for 15 hr.
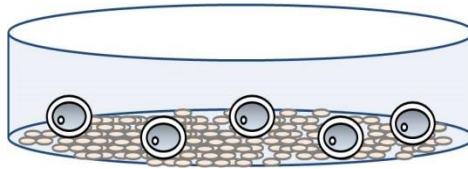
Figure 9. Schematic representation of *in vitro* oocyte maturation upon a feeder layer of CCs.

Since it was demonstrated that the CCs feeder layer co-culture gives better rate of blastulation when compared with the use of conditioned medium and dispersed cumulus cells (Ge *et al.,* 2008b), the attention moves to obtain a robust protocol for CCs feeder culture which allow the oocyte to be in contact with a high number of CCs. Mouse DOs matured upon FL-CCs show a better meiotic spindle morphology, mitochondria and cortical granules redistribution and an improved developmental capacity (Ge *et al.,* 2008a, b; Zhou *et al.,* 2010) compared to those oocytes cultured in the absence of FL (NO-FL). A further enhancement to FL-CCs co-culture could arise from the addition of both cysteamine and cysteine (Zhou *et al.,* 2010), inactivated human follicular fluid (Malekshah *et al.,* 2005) or trans-retinoid acid (t-RA) (Tahaei *et al.,* 2011) into the culture medium. As for the bovine, FL-CCs enhances DOs development and prevents the pellucid zone hardening (Zhao *et al.,* 2014). Also in human, a FL-CCs in a 3D culture system toughly improves maturation (Combelles *et al.,* 2005) and development (Vanhoutte *et al.,* 2009) of DOs.

Although the key role played by CCs-oocyte communication in promoting the correct development of the oocyte is well known (Luciano *et al.,* 2011; Lodde *et al.,* 2013; Li *et al.,* 2013), we still need to clarify the mechanisms by which FL-CCs could influence the maturation and developmental potential of DOs cultured upon. To this end, co-culture experiments could contribute to shed light on the events occurring among the somatic and germ cell components during the GV-to-MII transition.

Nowadays, two are the main hypothesis that we can speculate in order to explain the positive effect of such system: a bidirectional communication between oocyte and FL-CCs via GJ channels and/or via paracrine molecules.

Only one study demonstrated a re-formation of gap junctions between DOs and cumulus clumps cultured together (Feng *et al.,* 2013), whereas the

majority validates the hypothesis of a paracrine cross-talk among DOs and CCs in *in vitro* co-culture system (for a review see Russel *et al.,* 2016).

Among the CCs molecules involved in this communication of particular interest are endogenous small noncoding RNAs (miRNA), key regulators of the ovarian follicular development (Murchison *et al.,* 2007, Tang *et al.,* 2007; Hennebold *et al.,* 2010). Enclosed within macrovesicles, miRNA are non-coding RNA of ~22 nucleotides identified in the follicular fluid of several species (for a review see Di Pietro, 2016).

Namely, *Let-7 miR21* (Assou *et al.,* 2013b), *miR-720* (Moreno *et al.,* 2015), *miR-202-5p*, *miR-206*, *miR-16-1-3p*, *miR-1244* (Machtinger *et al.,* 2017), *miR-132* and *miR-320* (Sang *et al.,* 2013*), mir-23a* (Yang *et al.,* 2012) have been related to the oocyte capacity to be fertilized and further develop into a good-quality embryo. The releasing of microRNAs by cumulus cells could represent one of the mechanisms of paracrine communication among CCs and the oocyte grown up to MII-stage inside a co-culture system.

1.10 Markers of the oocyte developmental competence

Assisted reproductive treatments require the selection of the oocyte which could better sustain the embryo development. The capacity to identify those oocytes with the highest developmental potential has a crucial role for both fertilization and embryo development. In this direction, many efforts have been made to find morphological and molecular markers predictive of the oocyte quality.

1.10.1 Morphologycal markers

The easiest method of oocytes selection, adopted by numerous embryologists, is based on the evaluation of a number of morphological features (i.e., ZP, perivitelline space, cytoplasm, PB-I) by means of simple observation through light microscopy.

Among the features analyzed, the semblance of the *zona pellucida* (ZP) has been correlated with the success of fertilization, implantation and clinical pregnancy (Rama Raju *et al.,* 2007; Zhou *et al.,* 2014; Shi *et al.,* 2014; Omidi

et al., 2013; Sauerbrun-Cutler *et al.,* 2015; Sousa *et al.,* 2015; Andolfi *et al.,* 2016; González-Ortega *et al.,* 2016; Ashourzadeh *et al.,* 2015; Safian *et al.,* 2017; Canosa *et al.,* 2017). A dark (Rama Raju *et al.,* 2007; Shen *et al.,* 2005; Shi *et al.,* 2014; Sauerbrun-Cutler *et al.,* 2015) or disproportionate (Rama Raju *et al.,* 2007; Zhou *et al.,* 2014) ZP is associated with bad-quality oocytes and impaired embryo formation. Moreover, an abnormal size (Xia *et al.,* 1997; Mikkelssen *et al.,* 2001; Rienzi *et al.,* 2008) and granularity (Hassan-Ali *et al.,* 1998) of the perivitelline space (PS) (Chamayou *et al.,* 2006; Ten *et al.,* 2007; Inoue *et al.,* 2007; de Cássia S Figueira *et al.,* 2010; Setti *et al.,* 2011; Hassa *et al.,* 2014) as well as the presence of vacuoles, inclusions, central granulations or smooth endoplasmic reticulum clusters in the ooplasm (Otsuki *et al*., 2004; 2007; Wilding *et al.,* 2007; Balaban *et al.,* 2008; Rienzi *et al.,* 2008; Setti *et al.,* 2011) have been recognized as signs of poor embryo quality. Such defective oocytes fertilize worse and have low chances of successful pregnancy (Otsuki *et al.,* 2004; Balaban *et al.,* 2008).

Regarding the first polar body, a fragmentation or a disproportionate size leads to a reduction of fertilization and cleavage rates (Xia *et al.,* 1997; Mikkelssen *et al.,* 2001; Rienzi *et al.,* 2008; Navarro *et al.,* 2009); whereas an ovoid or round shape is an indicator of good quality oocytes (Ebner *et al.,* 2000).

However, a weakness of this methodology is the high heterogeneity of approaches and the variability of the operator's evaluation which compromise the possibility to standardize the method. In this direction, numerous groups have been tried to find more objective criteria for the oocytes selection.

Recently, a new methodology, named Time-Lapse Imaging (TLI), starts to be adopted for morphological assessment of the female gamete quality. This method allows the gamete observation, without any perturbation of the culture condition, inside an incubator chamber equipped with a camera.

The latter is connected to a software which could record oocyte/embryo progression. Since during the recording period a great number of images could be capture and analyzed by a mathematical tools, the variability of operators action has been eliminated. Through time lapse imaging, the timing of first cytokinesis, the passage among first and second mitosis (Wong *et al.,* 2010; Cruz *et al.,* 2012; Wong *et al.,* 2013) and the alteration of the egg chromatin

organization (Belli *et al.,* 2014) have been linked to the oocyte developmental capability. Moreover, a very recent study (Bui *et al.,* 2017) demonstrated a correlation among the oocyte developmental potential and its cytoplasmic movements, observed by TLI, during its GV-to-MII transition *in vitro*.

1.10.2 Molecular markers

As discussed before, many transcripts and proteins expressed by the oocyte have already been identified as markers of developmental competence, nevertheless their detection implies the sacrifice of the gamete. In the need to find an objective, but still non-invasive method of selection for choosing the ideal oocyte, some groups point out different sources of markers i.e., cumulus cells which are intimated connected to the oocyte over the whole folliculogenesis, and thus, could reflect the health of the gamete enclosed.

Various studies focused on cumulus cell transcriptome analysis allow the identifications of specific genes and protein prognostic of oocyte developmental potential.

In human CCs, the up-regulation of specific genes [*PTX3* (Zhang *et al.,* 2005; Gebhardt *et al.,* 2011), *PTGS2* (McKenzie *et al.,* 2004; Gebhardt *et al.,* 2011; Wathlet *et al.,* 2011), *HAS2* (McKenzie *et al.,* 2004; Cillo *et al.,* 2007; Ekart *et al.,* 2013), *GREM1* (McKenzie *et al.,* 2004; Cillo *et al.,* 2007), *BCL2L1*, *PCK1* (Assou *et al.,* 2008), *PR* (Ekart *et al.,* 2013), *SDC4* (Wathlet *et al.,* 2011), *VCAN* (Gebhardt *et al.,* 2011; Ekart *et al.,* 2013), *FDX1*, *CYP19A1*, *SERPINE2*, *3bHSD1* (Hamel *et al.,* 2008), *PGK1* and *RGS2* (Hamel *et al.,* 2010)] was linked to successful embryo formation and implantation. On the contrary, a higher expression of *BDNF* (Anderson *et al.,* 2009), *CCND2*, *CTNND1*, *CXCR4*, *GPX3* (van Montfoort *et al.,* 2008), *NFIB* (Assou *et al.,* 2008, 2010), *TLR4* (Gu *et al.,* 2016) or *RUNX2*, *GPX3* (Huang *et al.,* 2013), *LHR* (Maman *et al.,* 2012), *AMHR2*, *LIF* (Devjak *et al.,* 2016) or proteins [*CITED2* (Fang *et al.,* 2016)] found in human CCs are related to poor oocytes and embryo quality.

As for mouse CCs, up-regulation of *Has2*, *Ptx3*, *Ptgs2*, *Tnfaip6* (Diaz *et al.,* 2006; Vigone *et al.,* 2013) and *Metap2* (O'Shea *et al.,* 2012) transcripts, as well as *FSHr*, *LHr* (Calder *et al.,* 2003), *PTGS2*, *HAS2*, *GREM1* (Assidi *et al.,* 2008), *THBS1*, *EREG* (Assidi *et al.,* 2010), *FGF11*, *IGFBP4*, *SPRY1*, *GPC4*

(Kussano *et al.,* 2016; Melo *et al.,* 2017), *GATM* (Bunel *et al.,* 2015), *NRP1*, *CALM1* (Assidi *et al.,* 2015) in bovine CCs was positively correlated to good preimplantation development and pregnancy outcome.

In recent times, new approaches as microarrays and next-generation sequencing opened up new perspectives in this field.

By means of these new methods an higher number of cumulus cells genes could be analyzed and associated with the quality of the enclosed oocyte. *LHCGR, ANGPTL1, TNIK* (Huang *et al.,* 2013), *BAI2, POU5F1, HDAC1* (Xu et al., 2015), *STC2, NPY1R, GM2A, GREM1 and OSBPL6* (Assou *et al.,* 2013a) in human and *Has2*, *Ptx3*, *Ptgs2* in mouse (Vigone *et al.,* 2013) were recognize as predictive of the oocyte quality. Therefore, Borup *et al.,* 2016 identified a pool of 30 genes expressed in CCs as predictive of gamete quality and embryo development with accuracy of 80%.

As for cumulus cell gene expression, also follicular fluid could be considered a rich source of new non-invasive markers for predicting oocyte developmental potential since it is easily available. In the human follicular fluid, high level of *ALBU_Human* was correlated to the inability of the oocytes to be fertilized (Chen *et al.,* 2016), whereas *ITIH1_Human* and *CO3_Human* as well as *TNF-a* and *G-CSF* (Niu *et al.,* 2017, Raucci *et al.,* 2011) were reported as predictive of successful oocyte development.

2. MATERIALS AND METHODS

2.1 Animals

CD1 mice (4-6 week-old females and 3-4 month-old males) were purchased from Charles River (Como, Italy). Animals were maintained under controlled conditions of 22 °C, 60% air humidity and a light/dark cycle of 12:12 hours. Research on mice was done after the approval of the Animal Ethics Committee of the University of Pavia.

2.2 Media for follicle isolation, manipulation and culture

M2 isolation medium (Supplementary material: Table 1S) was used for COCs collection. DOs were matured in αMEM-Glutamax medium (Life Technologies) supplemented with 5% FBS, 1 mg/ml fetuin, 26 µg/ml pyruvate, Pen/Strep, 50 mU/ml rFSH, 5 ng/ml EGF.

FL-SN-CCs, FL-NSN-CCs and FL-CCs were cultured in αMEM-Glutamax medium supplemented with 15% FBS, 25.3 mg/ml Sodium Pyruvate, 100 IU/ml penicillin, 75 µg/ml streptomycin and 1 mg/ml fetuin until confluence. All the media were prepared and equilibrated at 37 °C, 5% CO_2 overnight the day before the experiment.

2.3 Hormonal treatment

Female were injected with 10 IU Folligon (Intervet Srl, Italy) 48 hr before the sacrifice. For the superovulation of *in vivo* matured oocytes, females were injected with 10 IU Folligon and 48 hr after with 10 IU hCG (Corulon, Intervet Srl, Italy).

2.4 Morphological characterization of Cumulus Oocyte Complexes

The diameter in size, the cell layers of CCs and the number of CCs surrounding SN- and NSN-COCs was determined as described below.

Using a micrometer ocular of an inverted microscope (Olympus IX71), the size in diameter of 83 and 17 COCs enclosing SN (SN-COCs) or NSN (NSN-COCs) oocytes, respectively, was measured.

Instead, to determine the number of CC layers surrounding SN-COCs or NSN-COCs, COCs were fixed in 4% PFA in 1X PBS supplemented with 2 U/µL RNase, 0.5% Tween 20 and 1 µg/ml propidium iodide (PI) for 45 minutes at 37° C. Then, they were placed overnight in 1X PBS supplemented with 1 µg/ml PI, 2 U/µL RNase and 0.5% Tween 20. Slides were mounted in 1,4-

diazabicyclo[2.2.2]octane (DABCO, Sigma-Aldrich) and observed under a confocal microscope (Zeiss LSM 510 objective APO 63× NA 1.4).

The total number of CCs surrounding SN-COCs or NSN-COCs was determined after COCs fixation in 4% PFA in 1X PBS supplemented with DAPI (0.2 µg/ml in 1X PBS).

By using a hand-pulled sterile Pasteur micropipette with an internal size in diameter of 150 µm, fixed COCs were pipetted in and out several times until the somatic and germ cell components were separated. Whilst oocytes were stained with the fluorochrome Hoechst 33342 and classified as SN or NSN based on their chromatin conformation, the CCs were spread over a slide, mounted in DABCO and counted under a fluorescence microscope (Olympus BX60) using the Cell Sens software (Olympus).

2.5 Isolation of fully-grown antral follicles

The ovarian surface was punctured using a sterile 21G needle in M2 medium (Supplementary material: Table 1S) and only intact fully grown antral follicles with more than three layers of CCs were collected.

2.6 Cumulus cell feeder layer preparation in 96-well plate

Following their isolation, a group of 18-25 COCs were washed twice in drops of fresh α-MEM-Glutamax medium (Life Technologies) supplemented with 15% FBS, 25.3 mg/ml Sodium Pyruvate, 100 IU/ml penicillin, 75 µg/ml streptomycin and 1 mg/ml Fetuin. The CCs were first separated from the oocyte by gently pipetting the COCs in and out through mouth-controlled sterile glass hand-pulled Pasteur micropipette and then cultured in 150 µl α-MEM-Glutamax medium (supplemented with 15% FBS, 25.3 mg/ml Sodium Pyruvate, 100 IU/ml penicillin, 75 µg/ml streptomycin and 1 mg/ml Fetuin) in 96-well plate (PerkinElmer, USA) covered with oil at 37.5 °C, 5% CO_2 for 72 hr.

2.7 In vitro maturation of fully-grown antral oocytes in 96-well plates

After COCs isolation and denudation, groups of 20 DOs were transferred into single wells containing 40 µl (2 µl/oocyte) of α-MEM-Glutamax medium (supplemented with 5% FBS, 25.3 mg/ml Sodium Pyruvate, 100 IU/ml penicillin, 75 µg/ml streptomycin, 1 mg/ml Fetuin, 50 mIU/ml FSH and 10

ng/ml EGF) for *in vitro* maturation. Oocytes were cultured at 37°C and 5% CO_2 either in the absence of FL (NO-FL) or upon a FL-CCs.

2.8 Cumulus cell feeder layer preparation in micro-Insert 4-well plates

The isolated fully-grown antral follicles were washed twice in drops of fresh α-MEM-Glutamax medium (Life Technologies) (supplemented with 15% FBS, 25.3 mg/ml Sodium Pyruvate, 100 IU/ml penicillin, 75 µg/ml streptomycin and 1 mg/ml Fetuin). CCs were separated from the oocyte by gently pipetting the COCs in and out through a mouth-controlled sterile glass hand-pulled Pasteur micropipette and cultured in 18 µl α-MEM-Glutamax medium (supplemented with 15% FBS, 25.3 mg/ml Sodium Pyruvate, 100 IU/ml penicillin, 75 µg/ml streptomycin and 1 mg/ml Fetuin) in micro-Insert 4-well plates (Ibidi, Germany) at 37.5 °C, 5% CO_2.

The medium (18µL) was replaced every 24 hr. To keep humidity and to avoid evaporation, 1.5 ml of the same medium was placed around the micro-Insert 4-well covered with lid.

2.9 Cumulus cell feeder layer preparation using SN-CCs or NSN-CCs in micro-Insert 4-well plates

Isolated fully-grown antral follicles were transferred in drops of fresh α-MEM-Glutamax medium (supplemented with 15% FBS, 25.3 mg/ml Sodium Pyruvate, 100 IU/ml penicillin, 75 µg/ml streptomycin and 1 mg/ml Fetuin) where CCs were mechanically separated from the oocyte as described above. Following the separation of CCs from their enclosed oocytes, DOs were singularly transferred into 5 µl droplets of M2 medium containing Hoechst 33342 (0.05 µg/ml, Sigma-Aldrich) and incubated for 15 min at room temperature in the dark. Stained oocytes were observed with a AX70 microscope (Olympus, Japan) under UV fluorescent light and classified as SN or NSN depending on the presence or absence of a ring of Hoechst-positive chromatin surrounding their nucleolus (Zuccotti *et al.,* 1995). Cumulus cells surrounding either SN- or NSN-COCs were separately transferred into micro-Insert 4-well plates containing 18µl of α-MEM- Glutamax medium (supplemented with 15% FBS, 25.3 mg/ml Sodium Pyruvate, 100 IU/ml penicillin, 75 µg/ml streptomycin and 1 mg/ml Fetuin) and cultured at 37.5 °C and 5% CO_2 for 72 hr. The medium (18µl) was replaced every 24 hr. To keep

humidity and to avoid medium evaporation, 1.5 ml of the medium was placed around the micro-Insert 4-well covered with a lid.

2.10 *In vitro* maturation of fully-grown antral oocytes in micro-insert 4-well plates

Four to five fully-grown antral oocytes were transferred into single micro-insert 4-well containing 18 μl of α-MEM- Glutamax medium (supplemented with 5% FBS, 25.3 mg/ml Sodium Pyruvate, 100 IU/ml penicillin, 75 μg/ml streptomycin, 1 mg/ml Fetuin, 50 mIU/ml FSH and 10 ng/ml EGF) and cultured at 37 °C, 5% CO_2 in the absence of FL-CCs (NO-FL), upon a FL-CCs, FL-SN-CCs or FL-NSN-CCs for 15 hr.

2.11 Variable timing oocytes *in vitro* maturation upon FL-SN-CCs

In a further set of experiments, DOs were transferred into single micro-insert 4-well containing 18 μl of α-MEM- Glutamax medium (supplemented with 5% FBS, 25.3 mg/ml Sodium Pyruvate, 100 IU/ml penicillin, 75 μg/ml streptomycin, 1 mg/ml Fetuin, 50 mIU/ml FSH and 10 ng/ml EGF) and cultured at 37 °C, 5% CO_2 upon FL-SN-CCs following three different experimental conditions:

1. upon SN-FL-CCs for 3 hours and then without FL (NO-FL) for further 12 hr;
2. without FL (NO-FL) for 6 hours and then upon SN-FL-CCs for further 9 hr;
3. upon SN-FL-CCs for 6 hours and without FL (NO-FL) for further 9 hr.

2.12 Isolation of ovulated metaphase II oocytes

Ovulated MII oocytes were isolated by puncturing the oviducts of superovulated female mice in M2 medium supplemented with 500 IU/ml hyaluronidase type II (Sigma-Aldrich).

2.13 *In vitro* fertilization

Those oocytes that extruded the first polar body were fertilized after 15-16 hr of *in vitro* culture. Sperm were isolated from the epididymis using the swim-up method consisting of best motile sperm swimming from the bottom to the top fraction of the medium. In brief, cauda epididymides, isolated from 3-4 month-old males, were transferred in 3 ml tube and punctured with a 21G

needle in 1500 µl of Whittingham medium (Wt) (Supplementary material: Table 2S). After 20 min incubation at 37 °C and 5% CO_2, 300 µl of top fraction of the medium was used to prepare the capacitation drop covered with mineral oil. To evaluate the sperm concentration/ml, a drop of sperm was placed in the counting Burker chamber and the number of sperm in 10 squares was counted. This count was performed on two diagonals and the average value was calculated. The number of sperm counted in 10 squares represents the concentration in millions (10^6) per ml. After 45 min incubation at 37 °C, 5% CO_2, capacitated spermatozoa were added to the fertilization drop diluted in Wt medium in order to reach a final concentration of 1.8 x 10^6 sperm/ml. Before insemination, oocytes were transferred into 100 µl fertilization drop and incubated for 2 hr at 37 °C, 5% CO_2. The latter were then moved into a fresh drop of Wt medium (2 µl/oocyte) covered with mineral oil and incubated for another hour.

At the end of this period the oocytes were transferred into pre-warmed (37 °C) M16 medium (Supplementary material: Table 2S), through a mouth-controlled sterile glass hand-pulled Pasteur micropipette, to remove excess sperm and incubated in drops (2 µl/oocyte) to allow embryonic development. The fertilization rate was calculated based on the number of MII oocytes that reached the 2-cell stage.

2.14 First polar body area

At the end of 15 hr maturation period, DOs that reached MII were photographed under an inverted microscope (Olympus IX71). The PB-I area was measured using Cell Sens software (Olympus).

2.15 Immunofluorescence

Oct4 and Cdx2 protein markers of the blastocyst inner cell mass (ICM) and trophectoderm (TE), respectively, were analyzed by immunofluorescence. Blastocysts obtained from ovulated MII oocytes and from *in vitro* matured MII oocytes were fixed with 4% paraformaldehyde (PFA) in 1X PBS for 20 min. After fixation, cells were washed three times with PBT (0.1 % Tween 20 in 1X PBS) to eliminate any fixative residue and permeabilized with 0.5 % Triton X100 in 1X PBS for 20 min at room temperature. To suppress non-specific binding of antibodies, blastocysts were incubated in the block solution

[0.5% Blocking reagent (Roche), 0.1 M Tris-HCl, 0.15 M NaCl, 0.05% Tween 20] for 20 min at room temperature. Then, embryos were incubated with anti-OCT4 primary antibody (1:500, Abcam) for 1 hr at 37°C, washed three times with PBT for 25 min each, and incubated with the secondary Alexa Fluor 488 conjugated goat anti-rabbit IgG antibody (1:400, Molecular Probes). Following, blastocysts were incubated with anti-CDX2 primary antibody (1:50, Cell Signalling) for 1 hr at 37°C, washed three times with PBT for 25 min each, and incubated with anti-rabbit-TRITC conjugated antibody (1:750, Sigma-Aldrich).

After immunostaining, embryos were rinsed 3 times with PBT for 15 min and counterstained with 4',6-diamidino-2-phenylindole (DAPI, 0.2 µg/ml in 1X PBS) for 5 min. Slides were mounted in Vectashield (Vector Lab, Italy) and observed under a fluorescence microscope (Olympus BX60) equipped with z-stack acquisition.

2.16 Statistical analysis

Statistical analysis was carried out using Sigma Stat 3.0 software. The data were expressed as mean ± standard deviation (SD).

Data were evaluated using the Student's t-test or Mann-Whitney U test (depending on the distribution of the data set). Differences were considered significant when $p \leq 0.05$.

2.17 Further experiments

2.17.1 Antral oocyte-cumulus cells clumps co-culture

Fully grown antral follicles (COCs) were collected in M2 isolation medium (Supplementary material: Table 1S).

Cumuli were gently pipetted in and out through a mouth-controlled sterile glass hand-pulled Pasteur micropipette to remove CCs and the respective oocytes were stained with 0.5 µg/ml Hoechst 33342. After classification in SN or NSN oocytes based on their chromatin conformation, DOs were cultured together with CCs for 15 hr at 37 °C, 5% CO_2 following four experimental conditions:

1. SN-DOs cultured with SN-CCs;
2. SN-DOs cultured with NSNCCs;

3. NSN-DOs cultured with NSN-CCs;
4. NSN-DOs cultured with SN-CCs.

Those oocytes that reach metaphase II stage (MII^{NSN} or MII^{SN}) were collected in groups of 10. Cells were stored at -80°C until RNA extraction.

2.17.2 *Dnmt3L* gene expression in oocytes cultured together with CCs clumps

For the analysis of pools of MII oocytes RNA was extracted from cell pellets with the RNeasy Mini Kit (Quiagen).

Pellet was loosened by gently flicking the tube, RLT buffer was added and samples were vortexed and homogenized to disrupt the cells. 1 volume of 70% ethanol was added to the whole lysate which was transferred to an appropriate spin column and centrifuged at ≥ 10.000 rpm for 15 seconds.

To prevent contamination, it is recommended to make sure of doing DNase digestion directly on-column by adding DNase to the spin column membrane for 15 min. Then, washing with RW1 buffer the enzyme was eliminated and the spin column membrane was cleaned by adding RPE buffer. Next, the RNA was diluted with RNase-free water.

The total RNA was then retro-transcribed into cDNA using the following istructions: for each sample 17.0 µl reaction mix (Table 1) were added to 3 µl RNA to reach a total volume of 20 µl.

Table 1. PCR mix components for the retro-transcription.

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
MuLV reverse trascriptase (GeneAmp®, 50 U/µl)	50 U
RNase inhibitor (GeneAmp®, 20 U/µl)	20 U

The reaction was conducted using an Applied Biosystems GeneAmp 9700® thermocycler at the following setting condition:

- 25°C, 10 min;
- 42°C, 15 min;
- 99°C, 5 min.

After the retrotranscription process samples were maintained at 4°C until the PCR-analysis.

cDNA of 10 single oocytes groups were analyzed through q-RT-PCR.

15.0 µl reaction mix were added to 5 µl cDNA of each sample to reach the final volume of 20 µl and the reaction was performed using a Rotorgene 6000 thermocycler with the following amplification program:

- 95°C, 5 min;
- 95°C, 10 sec;
- 62.5°C, 20 sec;
- 72°C, 20 sec; 40 cycles.

Data obtained by q-RT-PCR were normalized on *Luciferase* gene expression. *Dnmt3L* and *Luciferase* primers were shown in Table 2.

Table 2. Sequences and conditions of *Dnmt3l* and *Luciferase* primers (forward and reverse) used for qRT-PCR analyses.

Primer	Sequences	Conc (nM)	Amplicon length (bp)	T_m	Ext. time (sec)	Cycles
Dnmt3lF	5'-GTGCGGGTACTGAGCCTTTTTAGA-3'	200	258	60°C	20	40
Dnmt3lR	5'-CGACATTTGTGACATCTTCCACGTA-3'					
Luciferase F	5'-AGTCGATGTACACGTTCGTCA-3'	200	98	60°C	20	40
Luciferase R	5'-CAGTGCAATTGTTTTGTCACG-3'					

3. RESULTS

3.1 Morphological characterization

3.1.1 Number of cumulus cells and cumulus cells layers surrounding SN or NSN cumulus-oocyte-complexes.

In the need to prepare a FL starting from a similar number of cells, the diameter, the number of CC layers and of CCs surrounding each of the two type of antral oocytes was determined. SN-(180 ± 36) or NSN-(160 ± 34) Cumulus Oocyte Complexes (COCs) differed significantly in their size in diameter ($p < 0.05$) (Table 3), although the corresponding oocytes maintained the same size. Therefore, the number of CCs layer was counted though a confocal imaging analysis (Figure 10, 11) of both SN- and NSN-COCs.
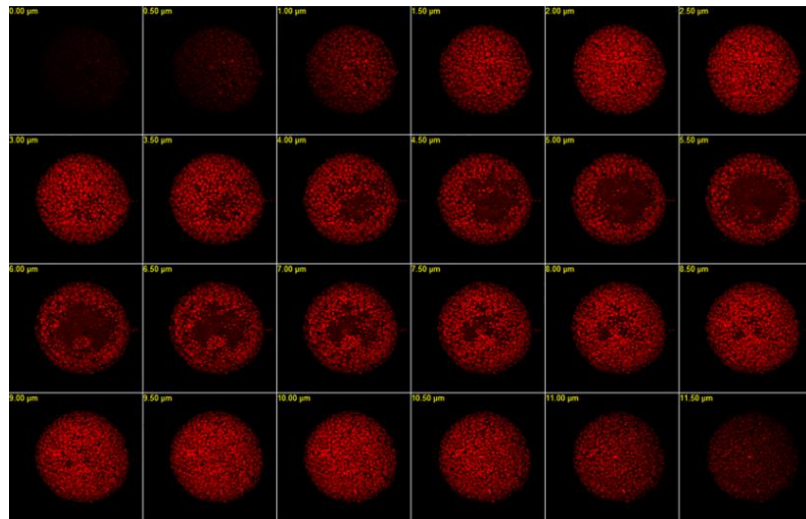
Figure 10. Representative confocal microscope image of follicle 50 μm sections stained with the fluorochrome PI.

Figure 11 showed a significantly ($p < 0.01$) higher number of layers surrounding SN (6 ± 0.68) compared to NSN (4 ± 0.51) oocytes. According with this result, the mean number of CCs, counted on slide spreads after DAPI staining, resulted significantly ($p < 0.02$) higher in SN (2060 ± 727) compared to NSN (1322 ± 416) COCs (Table 3).
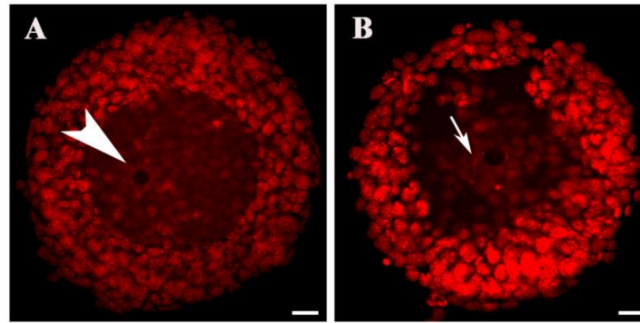
Figure 11. Representative confocal microscope image of an NSN-COC (A) and SN-COC (B) stained with the fluorochrome PI. Arrowhead indicates a ring of chromatin surrounding the nucleolus of an SN-COC, whereas the arrow points indicates diffused chromatin spots non-surrounded nucleolus of an NSN-COC. Bar, 20 μm.

Table 3. Diameter, number of CC layers and total CCs number of antral fully-grown SN- and NSN-COCs.

	Mean ± S.D.		
	SN-COCs	**NSN-COCs**	*p* value
COCs diameter (μm)	180 ± 36	160 ± 34	0.05
Number of CC layers	6 ± 0.68	4 ± 0.51	0.01
CCs number	2060 ± 727	1322 ± 416	0.02

3.2 Antral oocytes maturation and preimplantation development upon a FL-CCs in 96-well

The isolated fully-gown antral follicles were used as a source of CCs to prepare each single FL-CCs in a 96-well plate (Figure 12).
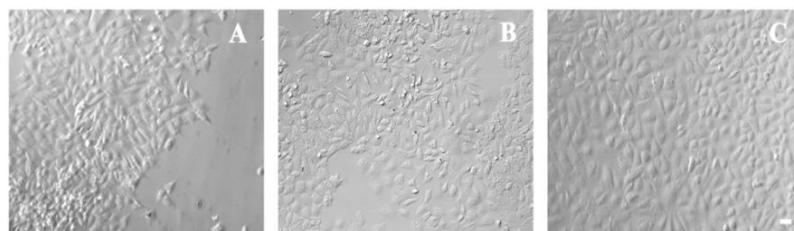
Figure 12. Establishment of a FL-CCs in 96-well plate after (A) 24 hr (B) 48 hr and (C) 72 hr. Bar, 10 μm.

Group of ~ 10 DOs were matured in the absence of FL (NO-FL) or upon a FL-CCs for 15 hr on 96-well plate.

DOs maturation rate to MII did not vary significantly when they were cultured in the absence of a FL (NO-FL) (94.0 ± 9.8%) or upon a FL-CCs (92.7% ± 12.7) ($p > 0.05$). Then, those GV oocytes that extruded the first polar bodies were *in vitro* fertilized. Although up to the 4-cell stage the developmental frequency did not differ significantly in the presence (FL-CCs) or absence (NO-FL) of a FL, in the further development 8.4% ± 2.9 of DOs cultured in absence of FL (NO-FL) reached the morula stage compared to 29.0% ± 5.7 of FL-CCs group ($p < 0.01$). Oocytes that were cultured upon a FL-CCs successfully progress to the blastocyst stage with a rate attested at 16.2% ± 3.8, whereas most of those DOs cultured in absence of FL (NO-FL) stops and only 4.7% ± 3.7 of them complete the preimplantation development ($p < 0.05$). Table 4 summarizes the rates of 2-cell, 4-cell, morula and blastocyst embryos obtained after 24, 48, 72 and 96 hr of culture.

Table 4. GV-to-MII transition of denuded oocytes matured in the presence or absence of a FL-CCs on 96-well plate.

STAGE	Mean ± S.D. % (Number)	
	NO-FL	**FL-CCs**
GV	(61)	(60)
MII	94.0 ± 9.8 (57)	92.7 ± 12.7 (55)
2-cell[*]	54.0 ± 8.3 (31)	64.0 ± 9.4 (35)
4-cell[**]	15.0 ± 7.6 (7)	36.0 ± 15.0 (15)
Morula[**]	8.4 ± 2.9 (3)	**29.0 ± 5.7 (12)**
Blastocyst[**]	4.7 ± 3.7 (2)	**16.2 ± 3.8 (8)**

[*]The maturation rate was calculated based on the number of MII oocytes.
[**]The developmental rate was calculated based on the number of 2-cell embryos.

In a further experiment, I tried to prepare a FL of selected CCs derived from either SN-COCs or NSN-COCs. However, there was an obstacle encountered towards the realization of this experimental design on 96-well plates. Since the antral compartment of the mouse ovary contains about 65% of SN-COCs and 35% of NSN-COCs (Zuccotti *et al.,* 1995), the lower number of the latter jeopardized our earlier tentative of producing a confluent FL-NSN-CCs on 96-well plates. In fact, the 96-well growing area of 0.3 cm^2 requires an high number of NSN-COCs (~ 25) and therefore animals (~ 15) for each FL preparation.

This critical point was overcome by using, for the first time, a miniaturized 4-well Petri dish (Ibidi, Germany) with a growing area of 0.03 cm^2 and 15-18 µl volume *per* well (Figure 13A), which allowed to reach complete confluence of both FL-SN-CCs [Figure 13B (i, iii)] and FL-NSN-CCs [Figure 13B (ii, iv)] after 72 hr of culture.
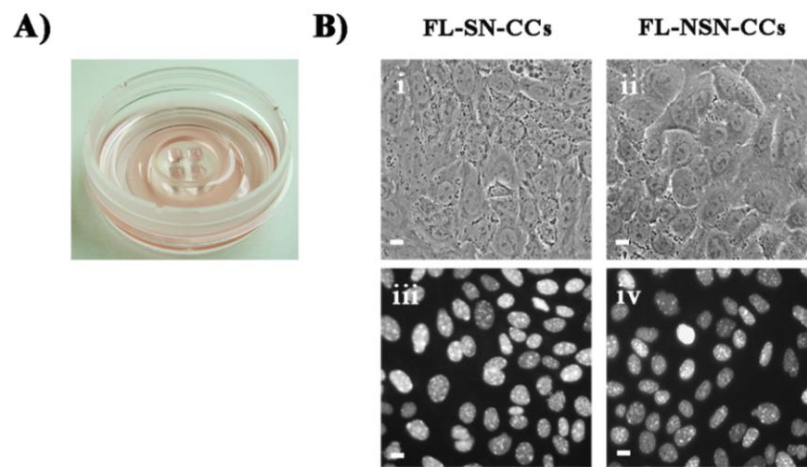
Figure 13. Cumulus cells feeder layer. (A) Micro-Insert 4-well (Ibidi); (B) Representative images of FL-SN-CCs (i, iii) and FL-NSN-CCs (ii, iv) taken under phase contrast and after Hoechst 33342 staining. Bar, 5 µm.

3.3 Antral oocyte maturation upon a FL-NSN-CCs, FL-SN-CCs or FL-CCs in micro-Insert 4-well plates

DOs were matured in absence of FL (NO-FL), upon a FL-CCs, FL-NSN-CCs or FL-SN-CCs in micro-Insert 4-well.

Below a representative images of CCs proliferation (Figure 14) and of MII-DOs cultured upon a confluent FL-CCs (Figure 15a, b) in micro-Insert 4 well.
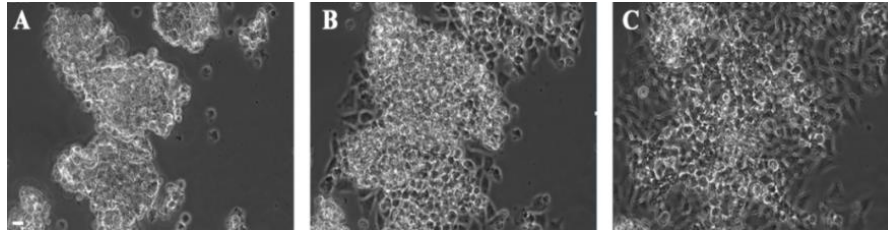
Figure 14. Representative bright field images of cumulus cell proliferation in micro-Insert 4-well (Ibidi, Germany) after 0 (A), 8 (B) and 10 (C) hr of culture observed in BioStation (IM, Nikon). Bar, 5 μm.
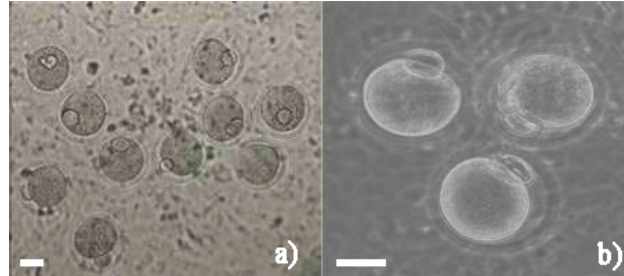
Figure 15. Mouse oocytes after 15 hr of culture upon FL-CCs. a) oocytes observed under inverted microscope (Olympus IX71), objective 20X; b) bright field image of MII oocytes upon a FL-CCs in micro-insert 4 well observed in BioStation (IM, Nikon). Bar, 40 μm.

A similar trend of DOs maturation rate to MII it was observed when they were cultured in the absence of a FL ($87.4 \pm 12.8\%$) (NO-FL), upon unclassified FL-CCs ($93.2 \pm 7.3\%$) or on FL-SN-CCs ($93.9 \pm 8.1\%$). Instead, it decreased when DOs were cultured upon FL-NSN-CCs ($74.4 \pm 10.7\%$), in the comparison with FL-CCs ($p= 0.009$) or FL-SN-CCs ($p= 0.033$) (Table 5).

Table 5. GV-to-MII transition and developmental competence of DOs matured in the absence of a FL, upon a FL-CCs, FL-NSN-CCs or FL-SN-CCs.

STAGE	Mean ± S.D. % (Number)			
	NO-FL	FL- CCs	FL-NSN-CCs	FL-SN-CCs
GV	(60)	(140)	(51)	(160)
MII	87.4 ± 12.8 (51)	93.2 ± 7.3 (130)	74.4 ± 10.7 (39)	93.9 ± 8.1 (147)
2-cell[*]	35.3 ± 22.1 (19)	69.8 ± 21.6 (90)	68.9 ± 37.2 (25)	58.1 ± 26.1 (66)
4-cell[**]	11.4 ± 22.7 (4)	46.2 ± 30.7 (57)	36.9 ± 23.0 (12)	65.8 ± 23.1 (40)
Morula[**]	None	34.2 ± 23.6 (39)	4.7 ± 7.5 (2)	42.6 ± 16.5 (26)
Blastocyst[**]	None	19.0 ± 15.0 (21)	none	26.9 ± 13.2 (16)

[*]The maturation rate was calculated based on the number of MII oocytes.

[**]The developmental rate was calculated based on the number of 2-cell embryos.

Following fertilization, the frequency of MII oocytes that developed to the 2-cell stage varied significantly only when comparing, among the four experimental groups, NO-FL (35.3 ± 22.1%) *vs.* FL-CCs (69.8 ± 21.5) (p= 0.023) (Table 5). However, when the FL was prepared with classified CCs, FL-SN-CCs gave the highest developmental frequency of 4-cell (66.0 ± 23.1%) and morula (42.6 ± 16.5%) stage, significantly different (*p*< 0.05) to those obtained with FL-NSN-CCs (4-cell: 36.9 ± 23%, *p*= 0.045; morula: 4.7 ± 7.5%, *p*< 0.001). None of the embryos of the NO-FL and FL-NSN-CCs experimental group developed further, whereas those oocytes that were cultured upon FL-CCs (19.0 ± 15.0%) or FL-SN-CCs (26.9 ± 13.2%) reached the blastocyst stage (*p*= 0.355).

3.4 First polar body size analysis

The PB-I size is a predictive factor of the MII oocyte quality (Xia *et al.,* 1997; Mikkelssen *et al.,* 2001; Rienzi *et al.,* 2008; Navarro *et al.,* 2009).

To evaluate the influence of FL-CCs on the oocyte meiotic asymmetric division, I measured the first polar body (PB-I) area of the oocytes cultured following four experimental conditions:

1. DOs cultured in the absence of FL (NO-FL);

2. DOs cultured upon FL-CCs;

3. DOs cultured upon FL-SN-CCs;

4. DOs cultured upon FL-NSN-CCs.

When the PB-I area of oocytes of the four experimental conditions was compared to that of control ovulated MII oocytes (403.0 ± 190.6 μm^2), those matured in the absence of FL (NO-FL) showed an area of 741.4 ± 290.3 μm^2 ($p < 0.001$) compared to FL-CCs 441.0 ± 174.8 μm^2 ($p > 0.05$), FL-NSN-CCs 660.0 ± 344.3 μm^2 ($p < 0.001$) or FL-SN-CCs 448.1 ± 214.1 μm^2 ($p > 0.05$). (Figure 16).
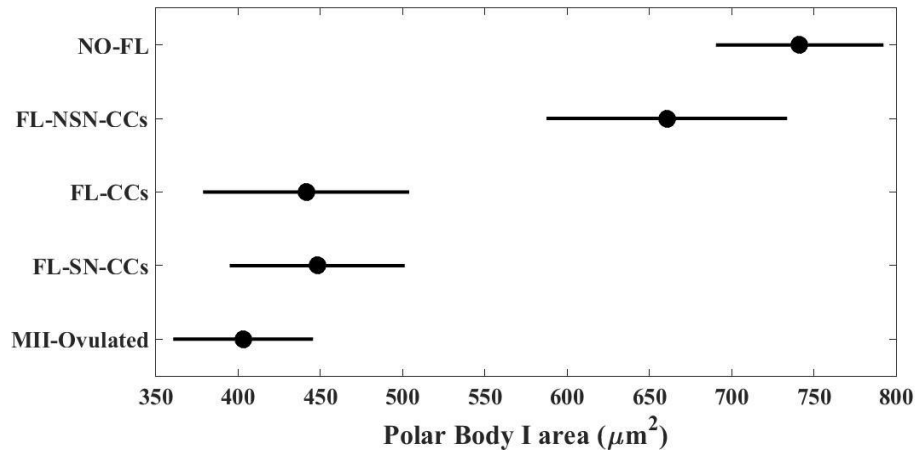
Figure 16. Polar body I area analysis of DOs cultured in absence of FL-CCs (NO-FL), upon a FL-NSN-CCs, FL-CCs, FL-SN-CCs and *in vivo* matured oocytes.

3.5 Variable timing of oocyte *in vitro* maturation upon FL-SN-CCs

Since it was demonstrated the beneficial effect of FL-SN-CCs on the oocyte developmental competence acquisition, I addressed the question of whether the oocytes require the whole 15 hr culture upon FL-SN-CCs to became competent or a shorter amount of time. To this end, DOs were matured upon a FL of SN-CCs following three different experimental conditions:

1. DOs cultured in the absence of FL for 3 hr and then upon FL-SN-CCs for further 12 hr;

2. DOs cultured in the absence of FL-CCs (NO-FL) for 6 hr and then upon FL-SN-CCs for further 9 hr;

3. DOs cultured upon FL-SN-CCs for 6 hr and then in the absence of FL-CCs (NO-FL) for further 9 hr.

DOs cultured in the absence of CCs (NO-FL) for the first three hours and then upon FL-SN-CCs for the remaining 12 hr display although the developmental progression to the 2- ($61.0 \pm 22.5\%$) and 4-cell ($55.3 \pm 10.5\%$) stages was similar ($p > 0.05$) to that obtained when DOs were cultured for the whole 15 hr, the frequency of development to morula ($25.3 \pm 8.6\%$) and blastocyst ($3.0 \pm 5.2\%$) decreased significantly ($p = 0.017$).
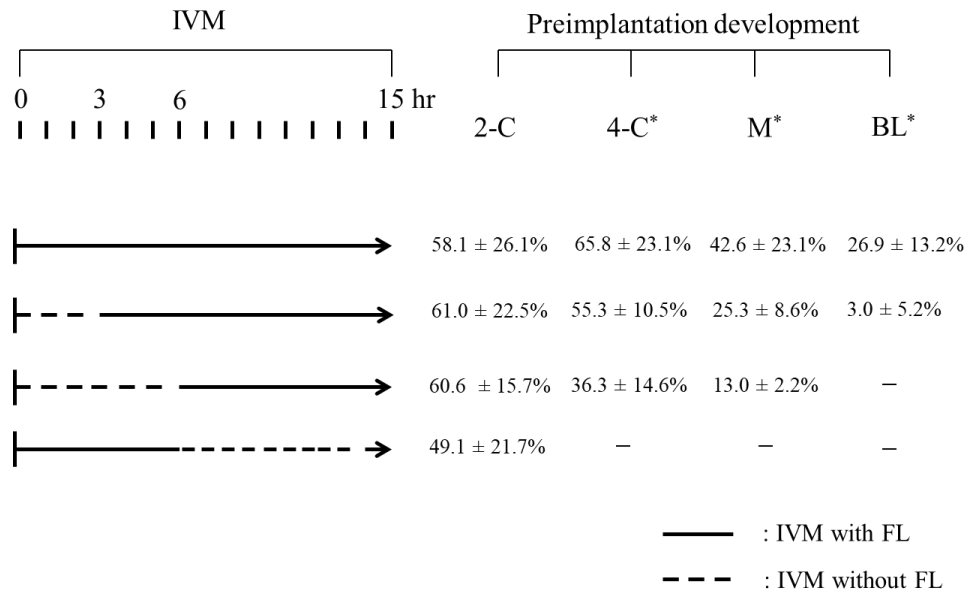
Figure 17. GV oocytes in vitro maturation in the presence (continuous line) or absence (dashed line) of a FL-SN-CCs and their preimplantation developmental competence. *, the rate of development was calculated based on 2-cell embryos.

If the initial culture in the absence of CCs was prolonged to 6 hr, the developmental progression is negatively affected. Indeed, the rate of embryo that achieve the 4-cell and morula stages decreased significantly ($p< 0.05$) with none of the embryos reaching blastocyst.

Instead, when GV oocytes were cultured upon FL-SN-CCs for 6 hr and then in the absence of FL for another 9 hr, although $49.1 \pm 21.7\%$ of embryos reached the 2-cell stage, the developmental potential was totally compromised since none of them was able to overcome the 2-cell block.

Altogether, these results demonstrated the necessity of a continuous interaction among the FL-CCs and the oocytes cultured upon during the whole period of GV-to-MII transition *in vitro*.

3.6 Analysis of blastocyst quality

The presence of the Cdx2 and Oct4 proteins, markers of the trophectoderm and inner cell mass cell lines was investigated in blastocysts obtained in the experimental groups of DOs matured upon FL-CCs or FL-SN-CCs, together with those obtained following fertilization of ovulated MII oocytes. All blastocysts from each group were fixed on slide and analyzed by immunocytochemistry (Figure 18).

The total number of blastomeres, attested at 29.5 ± 8.6 (ovulated MII), 27 ± 5.6 (FL-SN-CCs) and 35.7 ± 8.8 (FL-CCs), as well as the frequency of Cdx2- (ovulated MII: $73.8 \pm 8.4\%$; FL-SN-CCs: $78.2 \pm 9\%$; FL-CCs: $74.5 \pm 10.9\%$) and Oct4-positive cells (ovulated MII: $26.2 \pm 8.4\%$; FL-SN-CCs: $21.8 \pm 9\%$; FL-CCs: $22.8 \pm 6\%$) did not differ significantly in the blastocysts of the three experimental groups (Table 6).
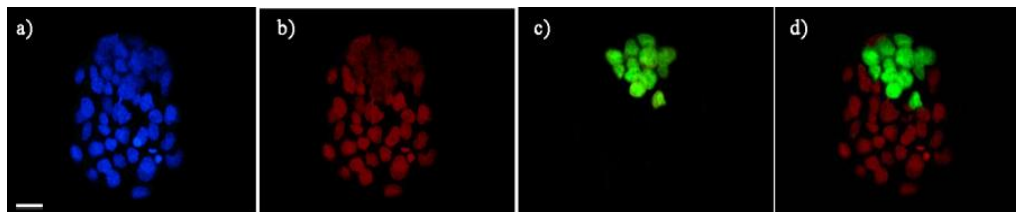
Figure 18. Analysis of proteins markers of blastocyst quality. a) blastomeres DAPI-positive; b) blastomeres Cdx2-positive, c) blastomeres Oct4-positive, d) combined image Oct4-Cdx2. Bar, 5 μm.

Table 6. Percentage of Cdx2- and Oct4-positive blastomeres in blastocysts developed from GV oocytes matured upon FL-CCs, FL-SN-CCs or from ovulated MII oocytes

		FL-CCs	FL-SN-CCs	Ovulated MII
Mean number ± S.D. of DAPI-positive cells		35.7 ± 8.8	27.0 ± 6.0	29.5 ± 7.7
Mean ± S.D. % of cells positive to the antibody	**Cdx2**	74.5 ± 10.9	78.2 ± 9.0	73.8 ± 8.4
	Oct4	22.8 ± 6	21.8 ± 9.0	26.2 ± 8.4

4. DISCUSSION

A third of women undergoing oncological treatment is at risk of premature ovarian failure. *In vitro* maturation of DOs to MII and their cryopreservation is a strategy for preserving women fertility.

However, protocols for cryopreserving intact COCs are still inadequate, as with struggle they preserve the CCs-to-oocyte bidirectional communication (Fuku *et al.,* 1995; Ruppert-Lingham *et al.,* 2003), reflecting the inability of cryoprotectants to penetrate the COC. On the contrary, the cryopreservation protocols of DOs are more robust.

To this regard, many attempts have been made to culture DOs of human or model animals in the presence either of dispersed or of a FL-CCs. The results obtained so far indicate a developmental competence of DOs matured upon FL-CCs, similar to that of intact COCs (Zhou *et al.,* 2010), but better than that acquired when DOs are cultured either with dispersed CCs (Ge *et al.,* 2008b), in conditioned medium (Ge *et al.,* 2008b) or in the absence of CCs (NO-FL) (Ge *et al.,* 2008a, b; Zhou *et al.,* 2010).

With this in mind, I first set up a co-culture system, on 96-well plate, based on the culture of DOs upon a FL of unclassified CCs and compared the results with those obtained in the absence of FL (NO-FL). In this experimental setting, the rate of oocyte meiotic resumption did not differ significantly between NO-FL and FL-CCs groups. But, following fertilization, the rate of embryos that developed to morula and blastocyst stage was significantly higher in FL-CCs group compared to NO-FL, highlighting the beneficial role of CCs on the gamete developmental potential.

However, the major limit of the latter culture system was the lack of a selection-step among the COCs used as a source of CCs. Indeed, the antral compartment of the ovary contains fully-grown follicles that are potentially developmentally competent and others that are incompetent, an aspect that might be critical to the preparation of the DOs/CCs co-culture system, since the isolated CCs might derive from competent or incompetent oocytes.

The purpose of our study was to test the efficiency of maturation to MII and acquisition of developmental competence of DOs when cultured upon a feeder layer of selected CCs derived either from SN (FL-SN-CCs) or from NSN (FL-NSN-CCs) oocytes, in the comparison with DOs cultured in the absence of FL or with a FL made of a mixture of unclassified CCs.

Following their isolation, SN-CCs or NSN-CCs were first used to prepare a FL on 96-well plates (growing area of 0.3 μm^2), but the high number of COCs required for the preparation of the FL in each single well, jeopardized our first tentative. In fact, on a 96-well plate, although a confluent FL was obtained with ~ 16 SN-COCs, corresponding to ~ 33,000 CCs per well, the same number of NSN-CCs would require ~ 25 NSN-COCs, isolated from almost ~ 15 females.

Therefore, we changed strategy. The next experiments were carried out using plates with a much smaller growing area, i.e., 0.03 μm^2 (micro-Insert 4-well plate), a choice that allowed SN- or NSN-CCs to reach complete confluence in 72 hr, reducing the number of COCs to 5 or 8, respectively, and the number of mice down to 1/3.

Thus, I was able to establish a co-culture system of DOs upon a FL of selected CCs.

DOs cultured for 15 hr upon FL-CCs or FL-SN-CCs reached with an higher frequency and with a regular PB-I size (marker of oocyte quality) the MII-stage, in the comparison with NO-FL and FL-NSN-CCs. FL-NSN-CCs significantly reduced the quality (determined by measuring PB-I area) and the maturation capacity of the oocytes.

Following fertilization, in the presence of FL-SN-CCs, preimplantation development proceeded better. Whilst DOs cultured upon FL-NSN-CCs or with NO-FL displayed the most consistent 2-cell block, oocytes matured upon FL-CCs or FL-SN-CCs successfully progressed to the 4-cell stage with a rate of 46.2 % and 65.8 %, respectively. None or only a meagre number of DOs, matured with NO-FL or FL-NSN-CCs, achieved the morula stage. Completion of preimplantation development was obtained only by oocytes matured upon FL-CCs or FL-SN-CCs with a mean frequency (19% and 26.9%, respectively) and blastocyst quality not significantly different.

Most importantly, only DOs matured upon FL-SN-CCs developed to blastocyst with the same rate of COCs routinely cultured in our laboratory (26.9 ± 13.2 vs. 28.9 ± 7.8%, respectively; p = 0.614); whereas the developmental rate achieved with oocytes cultured upon FL-CCs is significantly lower (p= 0.01).

In summary, for the first time, here we show that the presence of FL-SN-CCs leads to the acquisition of the oocyte meiotic and developmental competence; whereas the absence of CCs (NO-FL) or the presence of NSN-CCs ends up with meiotic and developmental failure.

Furthermore, with the aim of identifying the role of FL-SN-CCs on the acquisition of the female gamete developmental competence, I addressed the question whether the beneficial influence of FL-SN-CCs requires the whole GV-to-MII transition period or a shorter amount of time to achieve its beneficial effect.

When DOs were first cultured in the absence of CCs (NO-FL) for the first three hours (when dramatic events such as GVBD occurs) and then upon FL-SN-CCs for the remaining 12 hr, the progression to the 2- and 4-cell did not vary among the two groups suggesting correct oocyte-to-embryo transition during phase I of preimplantation development (Zernicka-Goetz *et al.,* 2009). Instead, the frequency of development to blastocyst decreased significantly, indicating an alteration to key developmental processes, such as the second main epigenetic reorganization occurring during phase II (Zernicka-Goetz *et al.,* 2009). However, if the initial culture in the absence of CCs was prolonged to 6 hr, although the developmental rate up to the 2-cell stage remained unaltered, in the further development the oocytes arrested their progression, with none of the embryos reaching morula and blastocyst stages. On the contrary, in the last experimental setting, when DOs were matured on FL-SN-CCs during the initial 6 hr and then in NO-FL, the acquisition of developmental competence was totally compromised, with a complete block at the 2-cell stage.

The developmental scenario, described above, suggests the presence of a key developmental CC-factor/s released in the presence of FL-SN-CCs during the whole 15 hr culture period.

The key questions that will need to be addressed in the future is concerned with the nature of this CC-factor/s and whether, the latter, interacts with another oocyte-factor/s to trigger a cascade of events leading to the acquisition of the gamete developmental competence.

Different are the candidate molecules that could be released by FL-SN-CCs. The lack of TZPs in our experimental system makes a direct transmission of

proteins, RNA transcripts or other large molecular weight molecules unlikely, and suggests a possible communication role for soluble factors and/or extracellular vesicles, including exosomes and microvesicles. Exosomes, containing proteins, mRNA or miRNAs have been abundantly identified in the follicular fluid of matured antral follicles of several species (Sang et al., 2013; Machtinger et al., 2015; Moreno et al., 2015). In particular, the expression of specific miRNAs, as part of the cargo carried by exosomes [*miR21* (Assou *et al.,* 2013b), *miR-720* (Moreno *et al.,* 2015), *miR-202-5p*, *miR-206*, *miR-16-1-3p*, *miR-1244* (Machtinger *et al.,* 2017), *miR-132*, *miR-320* (Sang *et al., 2013), mir-23a* (Yang *et al.,* 2012)], has been related to the oocyte capacity to be fertilized and further develop into a good-quality embryo.

Thus, the release of exosomes by cumulus cells could represent a possible mechanism of paracrine communication between FL-SN-CCs and the oocytes in our co-culture system.

5. CONCLUSION

In conclusion, our results confirm the beneficial effect of CCs on the oocyte quality during its GV-to-MII transition also in a miniaturize *in vitro* culture system and, furthermore, show the positive contribution of FL-SN-CCs to the acquisition of meiotic and developmental competence. Instead, the influence of FL-NSN-CCs appears absent, since the oocyte meiotic resumption and embryo progression in their presence is similar to that obtained with NO-FL. The development of a new culture system, now ready to be tested and used also for other mammalian species (i.e., bovine, monkey and humans) sets the basis to further unravel the molecules involved in the cross-talk between oocyte and its companion cumulus cells during the GV-to-MII transition.

6. FURTHER PERSPECTIVES

The identification of the molecule/s involved in oocyte-to-FL-SN-CCs communication is the main objective I am aiming at in the future. To this regard, I set up the following experiments:

1. *in vitro* maturation of DOs together with SN- or NSN-CCs and q-RT-PCR analysis of genes marker of the oocyte developmental competence (i.e., *Dnmt3L*, *Nobox*, *Oct4*);

2. *in vitro* maturation of DOs upon FL-SN-CCs preventing the physical contact among the somatic and germ cell components;

3. identification and analysis of putative exosomal components in FL-SN-CCs conditioned medium;

4. blastocyst-transfer in the uterus of pseudo-pregnant females.

In vitro maturation of DOs together with SN- or NSN-CCs and q-RT-PCR analysis of genes marker of the oocyte developmental competence (i.e., Dnmt3L, Nobox, Oct4)

DOs were matured in four experimental conditions:

1. SN-DOs with SN-CCs,
2. SN-DOs with NSNCCs,
3. NSN-DOs with NSN-CCs,
4. NSN-DOs with SN-CCs.

Oocytes that extruded the PB-I after 15 hr were pooled in groups of 10 and frozen at -80°C. The expression of *Dnmt3L* was evaluated by q-RT-PCR analysis.

DnmtL is involved in the maintenance of the methylation pattern (Branco et al., 2008; Kurihara et al., 2008) and maternal imprinting (Bourc'his and Bestor, 2004).

The up-regulation of *Dnmt3L* has been correlated with high quality oocyte and it is predictive of successful embryo development (Bourc'his and Bestor, 2004; Branco *et al.,* 2008; Kurihara *et al.,* 2008). Figure 19 shows an increased level of *Dnmt3L* when SN-DOs were cultured together with SN-CCs compared to NSN-CCs ($p < 0.001$). Instead, the expression of Dnmt3L was down-regulate in NSN oocyte either when cultured with SN-CCs or NSN-CCs ($p = 0.110$).
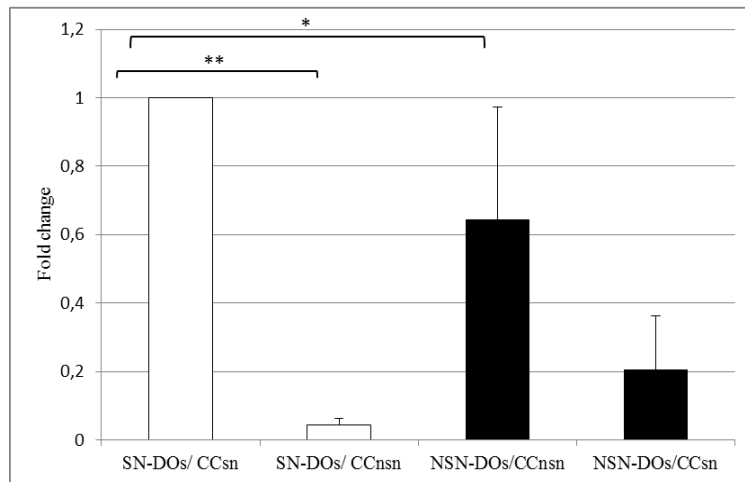
Figure 19. Dnmt3L gene expression in MII oocyte matured in four different culture conditions

The expression of *Nobox* and *Oct4* will be further analyzed on the same samples.

In vitro maturation of DOs upon FL-SN-CCs preventing the physical contact among the somatic and germ cell components

A further experiment that I would like to carry on is the establishment of a culture system which allow to investigate whether the direct contact between FL-SN-CCs and oocytes is required for the acquisition of the oocyte developmental competence. This can be done by growing a FL-SN-CCs in micro droplets on the bottom of a petri dish that will be twist (Figure 20). Meanwhile the FL-SN-CCs would remain attached to the bottom of the petri, the oocytes, by means of gravity force, will fall down preventing a physical interaction among the somatic and germ cell components.
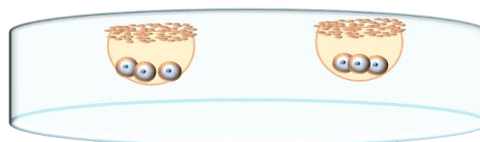
Figure 20. Co-culture system preventing a physical contact among FL-CCs and DOs.

Identification and analysis of putative exosomal components (miRNAs) in FL-SN-CCs conditioned medium

The question that will need to be addressed in the future is concerned with the nature of this CC-factor/s and whether it is able alone to trigger a cascade of events leading to the acquisition of the gamete developmental competence. Different are the candidate molecules that could be released by SN-CCs and among them, the more plausibles are extracellular vesicles, including exosomes and microvesicles. Exosomes, widely identified in the follicular fluid of matured antral follicles of several species (Sohel *et al.,* 2013; da Silveira *et al.,* 2012; Diez-Fraile *et al.,* 2014) and also, in culture media (Kropp and Khatib, 2015; Capalbo *et al.,* 2016; Borges *et al.,* 2016; Andrade *et al.,* 2017) could contain small (~22 nucleotides) non-coding RNAs, named microRNAs (miRNAs) (for a review see Di Pietro, 2016; Russell *et al.,* 2016). The level of expression of a number of specific miRNAs has been correlated to oocytes fertilization (*miR-202-5p*, *miR-206*, *miR-16-1-3p* and *miR-1244*) and developmental competence (*miR-766-3p*, *miR663b*, *miR-132-3p* and *miR-16-5p*) (Sang *et al.,* 2013; Machtinger *et al.,* 2015; Moreno *et al.,* 2015).

In the future I would analyze the FL-SN-CCs conditioned culture medium through flow cytometry or electron microscopy to investigate the presence of exosomes. Furthermore, it would be interesting to identify specific miRNA released by FL-SN-CCs in the comparison with NSN-CCs, and to pinpoint their role in the acquisition of oocyte developmental competence, performing a specific gene silencing during oocyte IVM.

Blastocyst-transfer in the uterus of pseudo-pregnant females

Finally, to evaluate the capability of blastocyst, obtained by *in vitro* fertilization of those oocytes cultured upon FL-CCs or FL-SN-CCs, to complete their development to term, I would transfer the embryos into the uterus of pseudo-pregnant females.

7. SUPPLEMENTARY MATERIALS

Table 1S. M2 medium recipe

Reagent	M2 (1L)
NaCl	5.534 g
KCl	0.356 g
$CaCl_2$	0.251 g
$MgSO4 \cdot 7H_2O$	0.293 g
KH_2PO_4	0.162 g
$NaHCO_3$	0.350 g
BSA	4.000 g
Phenol Red	0.010 g
Hepes	4.969 g
EDTA	1.00 ml
Sodium Lactate	5.65 ml
Penicillin/Streptomycin	5.00 ml

Table 2S. Whittingham medium recipe

Reagent	Amount (per litre)
NaCl	5.803 g
KCl	0.201 g
$NaHCO_3$	2.106 g
$Na_2HPO_4 \cdot 12H_2O$	0.056 g
$MgCl_2 \cdot 6H_2O$	0.102 g
$CaCl_2 \cdot 2H_2O$	0.264 g
Glucose	1.000 g
Sodium pyruvate	0.055 g
Phenol red	0.010 g
EDTA	1.00 ml
Sodium lactate (60%)	3.50 ml
Penicillin/Streptomycin	5.00 ml

Table 3S. M16 medium recipe

Reagent	Amount (per litre)
NaCl	5.698 g
KCl	0.356 g
$CaCl_2 \cdot 2H_2O$	0.251 g
KH_2PO_4	0.136 g
$MgSO_4$	0.143 g
Glucose	0.036 g
$NaHCO_3$	2.101 g
Phenol red	0.010 g
Sodium lactate (60%)	5.53 ml
EDTA	1.00 ml
Penicillin/Streptomycin	5.00 ml

8. REFERENCES

Amhle A., Dean J. Mouse genetics provides insight into folliculogenesis, fertilization and early embryonic development. *Hum. Reprod. Update*. **8**(5): 395-403 (2002).

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. *Reprod.* **138**(4): 629-637 (2009).

Andolfi L., Masiero E., Giolo E., Martinelli M., Luppi S., Dal Zilio S., Delfino I., Bortul R., Zweyer M., Ricci G., Lazzarino M. Investigating the mechanical properties of zona pellucida of whole human oocytes by atomic force spectroscopy. *Integr. Biol.* **8**: 886-893 (2016).

Armon L., Ben-Ami I., Ron-El R., Eisenbach M. Human oocyte-derived sperm chemoattractant is a hydrophobic molecule associated with a carrier protein. *Fertil. Steril.* **102**(3): 885-90 (2014).

Ashourzadeh S., Khalili M.A., Omidi M., Mahani S.N., Kalantar S.M., Aflatoonian A., Habibzadeh V. Non invasive assays of in vitro matured human oocytes showed insignificant correlation with fertilization and embryo development. *Arch. Gynecol. Obstet.* **292**(2): 459-63 (2015).

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.* **79**(2): 209-222 (2008).

Assidi M., Dieleman S.J., Sirard M.A. Cumulus cell gene expression following the LH surge in bovine preovulatory follicles: potential early markers of oocyte competence. *Reprod.* **140**(6): 835-52 (2010).

Assidi M., Montag M., Sirard M.A. Use of both cumulus cells' transcriptomic markers and zona pellucida birefringence to select developmentally competent oocytes in human assisted reproductive technologies. *BMC Genomics.* **1**: S9 (2015).

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.* **14**(12): 711-71 (2008).

Assou S., Al-edani T., Haouzi D., Philippe N., Lecellier C.H., Piquemal D., Commes T., Aït-Ahmed O., Dechaud H., Hamamah S. MicroRNAs: new candidates for the regulation of the human cumulus-oocyte complex. *Hum. Reprod.* **28**(11): 3038-49 (2013b).

Assou S., Haouzi D., De Vos J., Hamamah S. Human cumulus cells as biomarkers for embryo and pregnancy outcomes. *Mol. Hum. Reprod.* **16**(8): 531-8 (2010).

Assou S., Haouzi D., Dechaud H., Gala A., Ferrières A., Hamamah S. Comparative gene expression profiling in human cumulus cells according to ovarian gonadotropin treatments. *Biomed. Res. Int.* **2013**: 354582 (2013a).

Backonja U, Hediger M.L., Chen Z., Lauver D. R., Sun L., Peterson C.M., Buck Louis G.M. . Beyond Body Mass Index: using antropometric measures and body composition indicators to assess odds of an endometriosis diagnosis. *J Women Health.* doi: 10.1089/jwh.2016.6128 (2017).

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.* **23**(8): 1778-85 (2008).

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

Biggers J.D., Whittingham D.G. and Donahue R.P. The pattern of energy metabolism in the mouse oocyte and zygote. *Proc. Natl. Acad. Sci. USA* **58**(2): 560–567 (1967).

Blondin P., Sirard M.A. Oocyte and follicular morphology as determining characteristics for developmental competence in bovine oocytes. *Mol. Reprod. Dev.* **41**(1): 54-62 (1995).

Bogliolo L., Ariu F., Fois S., Rosati I., Zedda M.T., Leoni G., Succu S., Pau S., Ledda S. Morphological and biochemical analysis of immature ovine oocytes vitrified with or without cumulus cells. *Theriogenology.* **68**(8): 1138-49 (2007).

Borges E. Jr, Setti A.S., Braga D.P., Geraldo M.V., Figueira R.C., Iaconelli A. Jr. miR-142-3p as a biomarker of blastocyst implantation failure - A pilot study. *JBRA Assist. Reprod.* **20**(4): 200-205 (2016).

Borup R., Thuesen L.L., Andersen C.Y., Nyboe-Andersen A., Ziebe S., Winther O., Grøndahl M.L. Competence Classification of Cumulus and Granulosa Cell Transcriptome in Embryos Matched by Morphology and Female Age. *Plos One.* **11**(4): 0153562 (2016).

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

Brambillasca F., Guglielmo M.C., Coticchio G., Mignini Renzini M., Dal Canto M., Fadini R. The current challenges to efficient immature oocyte cryopreservation. *J. Assist. Reprod. Genet.* **30**(12): 1531-9 (2013).

Bui T.T., Belli, M., Fassina L., Vigone G., Merico V., Garagna S., Zuccotti, M. Cytoplasmic movement profiles of mouse surrounding nucleolus and not-surrounding nucleolus antral oocytes during meiotic resumption. *Mol. Reprod. Dev.* **9999**: 1-7 (2017).

Bunel A., Jorssen E.P., Merckx E., Leroy J.L., Bols P.E., Sirard M.A. Individual bovine in vitro embryo production and cumulus cell transcriptomic analysis to distinguish cumulus-oocyte complexes with high or low developmental potential. *Theriogenology.* **83**(2): 228-37 (2015).

Calder M.D., Caveney A.N., Smith L.C., Watson A.J. 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.* **1**: 14 (2003).

Canosa S., Adriaenssens T., Coucke W., Dalmasso P., Revelli A., Benedetto C., Smitz J. Zona pellucida gene mRNA expression in human oocytes is related to oocyte maturity, zona inner layer retardance and fertilization competence. *Mol. Hum. Reprod.* **23**(5): 292-303 (2017).

Capalbo A., Ubaldi F.M., Cimadomo D., Noli L., Khalaf Y., Farcomeni A., Ilic D., Rienzi L. MicroRNAs in spent blastocyst culture medium are derived from trophectoderm cells and can be explored for human embryo reproductive competence assessment. *Fertil. Steril.* **105**(1): 225-35 (2016).

Carabatsos M.J., Sellitto C., Goodenough D.A., Albertini D.F. Oocyte-granulosa cell heterologous gap junctions are required for the coordination of nuclear and cytoplasmic meiotic competence. *Dev. Biol.* **226**(2): 167-79 (2000).

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.* pp. 27–44 (2005).

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*. **13**(5): 661-7 (2006).

Check J.H. Diagnosis and treatment of cervical mucus abnormalities. *Clin. Exp. Obstet. Gynecol.* **33**(3): 140-2 (2006).

Chen F., Spiessens C., D'Hooghe T., Peeraer K., Carpentier S. Follicular fluid biomarkers for human in vitro fertilization outcome: Proof of principle. *Proteome Sci.* **14**:17 (2016).

Chow K.M., Cheung M.C., Cheung I.K. Psychosocial interventions for infertile couples: a critical review. *J. Clin. Nurs.* **25**(15-16): 2101-13 (2016).

Cillo F., Brevini T.A., Antonini S., Paffoni A., Ragni G., Gandolfi F. Association between human oocyte developmental competence and expression levels of some cumulus genes. *Reprod.* **134**(5): 645-50 (2007).

Cohen-Dayag A., Tur-Kaspa I., Dor J., Mashiach S., Eisenbach M. Sperm capacitation in humans is transient and correlates with chemotactic responsiveness to follicular factors. Proc. *Natl. Acad. Sci. U S A.* **92**: 11039-43 (1995).

Combelles C.M., Chateau G. The use of immature oocytes in the fertility preservation of cancer patients: current promises and challenges. *Int. J. Dev. Biol.* **56**(10-12): 919-29 (2012).

Combelles C.M., Fissore R.A., Albertini D.F., Racowsky C. In vitro maturation of human oocytes and cumulus cells using a co-culture three-dimensional collagen gel system. *Hum. Reprod.* **20**(5): 1349-58 (2005).

Coticchio G., Dal-Canto M., Guglielmo M.C., Mignini-Renzini M., Fadini R. Human oocyte maturation *in vitro*. *Int. J. Dev. Biol.* **56**(10-12): 909-18 (2012).

Crozet N, Motlik J, Szollosi D. Nucleolar fine structure and RNA synthesis in porcine oocytes during early stages of antrum formation. *Biol. Cell.* **72**: 323-328 (1984).

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.* **25**(4): 371-81 (2012).

da Silveira J.C., Veeramachaneni D.N., Winger Q.A., Carnevale E.M., Bouma G.J. Cell-secreted vesicles in equine ovarian follicular fluid contain miRNAs and proteins: a possible new form of cell communication within the ovarian follicle. *Biol. Reprod.* **86**: 71 (2012).

de Cássia S Figueira R., de Almeida Ferreira Braga D.P., Semião-Francisco L., Madaschi C., Iaconelli A. Jr, Borges E. Jr. Metaphase II human oocyte morphology: contributing factors and effects on fertilization potential and embryo developmental ability in ICSI cycles. *Fertil. Steril.* **94**(3): 1115-7 (2010).

Debey P., Szöllösi M.S., 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.* **36**(1): 59-74 (1993).

Devjak R., Burnik Papler T., Verdenik I., Fon Tacer K., Vrtačnik Bokal E. Embryo quality predictive models based on cumulus cells gene expression. *Balkan J. Med. Genet.* **19**(1): 5-12 (2016).

Di Pietro C. Exosome-mediated communication in the ovarian follicle. *J. Assist. Reprod. Genet.* **33**: 303-311 (2016).

Diamond M.P., Legro R.S., Coutifaris C., Alvero R., Robinson R.D., Casson P.A., Christman G.M., Huang H., Hansen K.R., Baker V., Usadi R., Seungdamrong A., Bates G.W., Rosen R.M., Schlaff W., Haisenleder D., Krawetz S.A., Barnhart K., Trussell J.C., Santoro N., Eisenberg E., Zhang H. Sexual function in infertile women with polycystic ovary syndrome and unexplained infertility. *Am. J. Obstet. Gynecol.* doi: 10.1016/j.ajog.2017.04.034 (2017).

Diaz F.J., O'Brien M.J., Wigglesworth K., Eppig J.J. The preantral granulosa cell to cumulus cell transition in the mouse ovary: development of competence to undergo expansion. *Dev. Biol.* **299**(1): 91-104 (2006).

Diez-Fraile A., Lammens T., Tilleman K., Witkowski W., Verhasselt B., De Sutter P, Benoit Y, Espeel M, D'Herde K. Age-associated differential microRNA levels in human follicular fluid reveal pathways potentially determining fertility and success of in vitro fertilization. *Hum Fertil (Camb).* **17**(2): 90-8 (2014).

Dong J., Albertini D.F., Nishimori K., Kumar T.R., Lu N., Matzuk M.M. Growth differentiation factor-9 is required during early ovarian folliculogenesis. *Nature.* **383**(6600): 531-5 (1996).

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

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

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

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

Driancourt M.A., Reynaud K., Cortvrindt R., Smitz J. Roles of KIT and KIT LIGAND in ovarian function. *Rev. Reprod.* **5**:143-152 (2000).

Dumollard R, Duchen M, Sardet C. Calcium signals and mitochondria at fertilisation. Semin. Cell Dev. Biol. 2006;17:314–323.

Ebner T., Moser M., Sommergruber M., Puchner M., Wiesinger R., Tews G. Developmental competence of oocytes showing increased cytoplasmic viscosity. *Hum. Reprod.* **18**: 1294-8 (2003).

Ebner T., Yaman C., Moser M., Sommergruber M., Feichtinger O., Tews G. Prognostic value of first polar body morphology on fertilization rate and embryo quality in intracytoplasmic sperm injection. *Hum. Reprod.* **15**(2): 427-30 (2000).

Eisenbach M., Giojalas L.C. Sperm guidance in mammals – an unpaved road to the egg. *Nat. Rev. Mol. Cell. Biol.* **7**: 276–85 (2006).

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.* **28**(11): 2930-2942 (2013).

Eppig J.J., Wigglesworth K., Pendola F.L. The mammalian oocyte orchestrates the rate of ovarian follicular development. Proc. Natl. Acad. Sci. USA. **99**(5): 2890-4 (2002).

Fang Y., Shang W., Wei D.L., Zeng S.M. Cited2 protein level in cumulus cells is a biomarker for human embryo quality and pregnancy outcome in one in vitro fertilization cycle. *Fertil. Steril.* **105**(5): 1351-1359 (2016).

Fasano G., Demeestere I., Englert Y. In-vitro maturation of human oocytes: before or after vitrification? *J. Assist. Reprod. Genet.* **29**: 507-512 (2012).

Feng G., Shi D., Yang S., Wang X. Co-culture embedded in cumulus clumps promotes maturation of denuded oocytes and reconstructs gap junctions between oocytes and cumulus cells. *Zygote.* **21**(3): 231-7 (2013).

Fuku E., Xia L., Downey B.R. Ultrastructural changes in bovine oocytes cryopreserved by vitrification. *Cryobiology.* **32**(2): 139-56 (1995).

Gakamsky A, Schechtman E, Caplan SR, Eisenbach M. Analysis of chemotaxis when the fraction of responsive cells is small-application to mammalian sperm guidance. *Int. J. Dev. Biol.* **52**: 481-7 (2008).

Galloway S.M., McNatty K.P., Cambridge L.M., Laitinen M.P., Juengel J.L., Jokiranta T.S., McLaren R.J., Luiro K., Dodds K.G., Montgomery G.W. Mutations in an oocyte-derived growth factor gene (BMP15) cause increased ovulation rate and infertility in a dosage-sensitive manner. *Nat. Genet.* **25**: 279–283 (2000).

Gao L.L., Zhou C.X. , Zhang X.L., Liu P., Jin Z., Zhu G.Y., Ma Y. , Li J., Yang Z.X., Zhang D. ZP3 is Required for Germinal Vesicle Breakdown in Mouse Oocyte Meiosis. Sci. Rep. 7: 41272 (2017).

Gasparrini B., Attanasio L., De Rosa A., Monaco E., Di Palo R., Campanile G. Cryopreservation of in vitro matured buffalo (Bubalus bubalis) oocytes by minimum volumes vitrification methods. *Anim. Reprod. Sci.* **98**: 335–342 (2007).

Ge L., Han D., Lan G.C., Zhou P., Liu Y., Zhang X., Sui H.S. and Tan J.H. Factors affecting the in vitro action of cumulus cells on the maturing mouse oocytes. *Mol. Reprod. Devel.* **75**:136-142 (2008b).

Ge L., Sui H.S., Lan G.C., Liu N., Wang J.Z. and Tan J.H. Coculture with cumulus cells improves maturation of mouse oocytes denuded of the cumulus oophorus: observations of nuclear and cytoplasmic events. *Fertil. Steril.* **90**(6): 2376-88 (2008a).

Gebhardt K.M., Feil D.K., Dunning K.R., Lane M., Russell D.L. Human cumulus cell gene expression as a biomarker of pregnancy outcome after single embryo transfer. *Fertil. Steril.* **96**(1): 47-52 (2011).

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

Gilchrist R.B. Recent insights into oocyte-follicle cell interactions provide opportunities for the development of new approaches to in vitro maturation. *Reprod. Fertil. Dev.* **23**: 23-31. (2011).

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

Gilchrist R.B., Ritter L.J., Armstrong D.T. Oocyte-somatic cell interactions during follicle development in mammals. *Anim. Reprod. Sci.* **82**–**83**: 431–446 (2004).

González-Ortega C., Cancino-Villarreal P., Alonzo-Torres V.E., Martínez-Robles I., Pérez-Peña E., Gutiérrez-Gutiérrez A.M. Polarized light microscopy for evaluation of oocytes as a prognostic factor in the evolution of a cycle in assisted reproduction. *Ginecol. Obstet. Mex.* **84**(4): 217-27 (2016).

Gu B.X., Wang X., Yin B.L., Guo H.B., Zhang H.L., Zhang S.D., Zhang C.L. Abnormal expression of TLRs may play a role in lower embryo quality of women with polycystic ovary syndrome. *Syst. Biol. Reprod. Med.* **62**(5): 353-8 (2016).

Guimarães A.L., Pereira S.A., Kussano N.R., Dode M.A. The effect of pre-maturation culture using phosphodiesterase type 3 inhibitor and insulin, transferrin and selenium on nuclear and cytoplasmic maturation of bovine oocytes. *Zygote.* **24**(2): 219-29 (2016).

Hassa H., Aydın Y., Taplamacıoğlu F. The role of perivitelline space abnormalities of oocytes in the developmental potential of embryos. J. *Turk. Ger. Gynecol. Assoc.* **15**(3): 161-3 (2014).

Hassan-Ali H., Hisham-Saleh A., El-Gezeiry D., Baghdady I., Ismaeil I., Mandelbaum J. Perivitelline space granularity: a sign of human menopausal gonadotrophin overdose in intracytoplasmic sperm injection. *Hum. Reprod.* **13**(12): 3425-30 (1998).

Hennebold J.D. Preventing granulosa cell apoptosis through the action of a single microRNA. *Biol. Reprod.* **83**(2): 165-7 (2010).

Hennet M.L., Combelles C.M. The antral follicle: a microenvironment for oocyte differentiation. *Int. J. Dev. Biol.* **56**: 819-831 (2012).

Hervè J.C., Bourmeysterb N., Sarrouilhec D., Duffyd H.S. Gap junctional complexes: From partners to functions. *Prog Biophys. Mol. Biol.* **94**(1-2): 29-65 (2007).

Higaki S., Kishi M., Koyama K., Nagano M., Katagiri S., Takada T., Takahashi Y. Early germinal vesicle breakdown is a predictor of high preimplantation developmental competent oocytes in mice. *Zygote.* **25**(1): 41-48 (2017).

Hillier S.G. Current concepts of the roles of follicle stimulating hormone and luteinizing hormone in folliculogenesis. *Hum. Reprod.* **9**(2): 188-91 (1994).

Huang X., Hao C., Shen X., Zhang Y., Liu X. RUNX2, GPX3 and PTX3 gene expression profiling in cumulus cells are reflective oocyte/embryo competence and potentially reliable predictors of embryo developmental competence in PCOS patients. *Reprod. Biol. Endocrinol.* **11**: 109 (2013).

Huang Z., Wells D. The human oocyte and cumulus cells relationship: new insights from the cumulus cell transcriptome. *Mol. Hum. Reprod.* **16**(10): 715-25 (2010).

Inoue A., Akiyama T., Nagata M., Aoki F. The perivitelline space-forming capacity of mouse oocytes is associated with meiotic competence. *J. Reprod. Dev.* **53**(5): 1043-52 (2007).

Inoue A., Nakajima R., Nagata M., Aoki F. Contribution of the oocyte nucleus and cytoplasm to the determination of meiotic and developmental competence in mice. *Hum. Reprod.* **23**: 1377-84 (2008).

Jedrusik A., Parfitt D.E., Guo G., Skamagki M., Grabarek J.B., Johnson M.H., Robson P., Zernicka-Goetz M. Role of Cdx2 and cell polarity in cell allocation and specification of trophectoderm and inner cell mass in the mouse embryo. *Genes Dev.* **22**(19): 2692-706 (2008).

Jin H.X., Song W.Y., Xin Z.M., Dai S.J., Chen Z.J., Sun Y.P. Effects of cumulus cells on vitreous cryopreservation of human mature oocytes and clinical pregnancy outcomes. *Reprod. Sci.* **19**(2): 216-20 (2012).

Juengel J.L., McNatty K.P. The role of proteins of the transforming growth factor-beta superfamily in the intraovarian regulation of follicular development. *Hum. Reprod. Update* **11**(2): 143-60 (2005).

Juneau C., Kraus E., Werner M., Franasiak J., Morin S., Patounakis G., Molinaro T., de Ziegler D., Scott R.T. Patients with endometriosis have aneuploidy rates equivalent to their age-matched peers in the in vitro fertilization population. *Fertil. Steril.* doi: 10.1016/j.fertnstert.2017.05.038 (2017).

Kageyama S., Liu H., Kaneko N., Ooga M., Nagata M., Aoki F. Alterations in epigenetic modifications during oocyte growth in mice. *Reprod.* **133**(1): 85-94 (2007).

Knight P.G., Glister C. Local roles of TGF - superfamily members in the control of ovarian follicle development. *Anim. Reprod. Sci.* **78**: 165-183 (2003).

Koopman P., Gubbay J., Vivian N., Goodfellow P., Lovell-Badge R. Male development of chromosomally female mice transgenic for Sry. *Nature.* **351**(6322): 117-21 (1991).

Kropp J. and Khatib H. mRNA fragments in in vitro culture media are associated with bovine preimplantation embryonic development. *Frontiers in Genetics.* **6**: 273 (2015).

Kussano N.R., Leme L.O., Guimarães A.L., Franco M.M., Dode M.A. Molecular markers for oocyte competence in bovine cumulus cells. *Theriogenology.* **85**(6): 1167-76 (2016).

Kuwayama M., Vajta G., Kato O., Leibo S.P. Highly efficient vitrification method for cryopreservation of human oocytes. *Reprod. Biomed. Online.* **11**(3): 300-8 (2005).

Lefèvre B., Gougeon A., Nome 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.* **29**: 523–531 (1989).

Levasseur D.N., Wang J., Dorschner M.O., Stamatoyannopoulos J.A., Orkin S.H. Oct4 dependence of chromatin structure within the extended Nanog locus in ES cells. *Genes. Dev.* **22**(5): 575-580 (2008).

Levine J.M., Kelvin J.F., Quinn G.P., Gracia C.R. Infertility in reproductive-age female cancer survivors. *Cancer* **121**(10): 1532-9 (2015).

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

Li M., Yu Y., Yan J., Yan L.Y., Zhao Y., Li R., Liu P., Hsueh A.J., Qiao J. The role of cilostazol, a phosphodiesterase 3 inhibitor, on oocyte maturation and subsequent pregnancy in mice. *Plos one.* **7**(1): e30649 (2012).

Li R., Albertini D.F. The road to maturation: somatic cell interaction and self-organization of the mammalian oocyte. *Nat. Rev. Mol. Cell. Biol.* **14**: 141-152 (2013).

Liu C.F., Liu C., Yao H.H. Building pathways for ovary organogenesis in the mouse embryo. *Curr. Top. Dev. Biol.* **90**: 263-90 (2010).

Lodde V., Franciosi F., Tessaro I., Modina S.C., Luciano A.M. Role of gap junction-mediated communications in regulating large-scale chromatin configuration remodeling and embryonic developmental competence acquisition in fully grown bovine oocyte. *J. Assist. Reprod. Genet.* **30**(9): 1219-26 (2013).

Luciano A.M., Franciosi F., Lodde V., Perazzoli F., Slezáková M., Modina S. Cryopreservation of immature bovine oocytes to reconstruct artificial gametes by germinal vesicle transplantation. *Reprod. Domest. Anim.* **44**(3): 480-8 (2009).

Luciano A.M., Franciosi F., Modina S.C., Lodde V. Gap Junction-Mediated Communications Regulate Chromatin Remodeling During Bovine Oocyte Growth and Differentiation Through cAMP-Dependent Mechanism(s). Biology of Reproduction. **85**: 1252–1259 (2011).

Luciano A.M., Lodde V., Beretta M.S., 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.* **71**(3): 389-97 (2005).

Machtinger R., Rodosthenous R.S., Adir M., Mansour A., Racowsky C., Baccarelli A.A., Hauser R. Extracellular microRNAs in follicular fluid and their potential association with oocyte fertilization and embryo quality: an exploratory study. *J. Assist. Reprod. Genet.* **34**(4): 525-533 (2017).

Machtinger R., Rodosthenous R.S., Adir M., Mansour A., Racowsky C., Baccarelli A.A., Hauser R. Extracellular microRNAs in follicular fluid and their potential association with oocyte fertilization and embryo quality: an exploratory study. *J. Assist. Reprod. Genet.* **34**(4): 525-533 (2017).

Makekshah A.K., Moghaddam A.E., Daraka S.M. Comparison of conditioned medium and direct co-culture of human granulosa cells on mouse embryo development. *Indian J. Expe. Biol.* **44**: 189-192 (2006).

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. 97(3): 592-598 (2012).

Mandelbaum J., Anastasiou O., Lévy R., Guérin J.F., de Larouzière V., Antoine J.M. Effects of cryopreservation on the meiotic spindle of human oocytes. *Eur. J. Obstet. Gynecol. Reprod. Biol.* **113** Suppl 1: S17-23 (2004).

Mandl A.M., Zuckerman S. The growth of the oocyte and follicle in the adult rat. *J. Endocrinol.* **8**(2): 126-32 (1952).

Mao L, Lou H, Lou Y, Wang N, Jin F. Behaviour of cytoplasmic organelles and cytoskeleton during oocyte maturation. *Reprod. BioMed. Online.* **28**: 284-299 (2014).

Mårdh P.A. Tubal factor infertility, with special regard to chlamydial salpingitis. *Curr. Opin. Infect. Dis.* **17**(1): 49-52 (2004).

McKenzie L.J., Pangas S.A., Carson S.A., Kovanci E., Cisneros P., Buster J.E., Amato P., Matzuk M.M. Human cumulus granulosa cell gene expression: a predictor of fertilization and embryo selection in women undergoing IVF. *Hum. Reprod.* **19**(12): 2869-74 (2004).

McLaren A., Southee D. Entry of mouse embryonic germ cells into meiosis. *Dev. Biol.* **187**(1): 107-13 (1997).

McNatty K.P., Hudson N.L., Whiting L., Reader K.L., Lun S., Western A., Heath D.A., Smith P., Moore L.G., Juengel J.L. The effects of immunizing sheep with different BMP15 or GDF9 peptide sequences on ovarian follicular activity and ovulation rate. *Biol. Reprod.* **76**: 552–560 (2007).

Melo E.O., Cordeiro D.M., Pellegrino R., Wei Z., Daye Z.J., Nishimura R.C., Dode M.A. Identification of molecular markers for oocyte competence in bovine cumulus cells. *Anim. Genet.* **48**(1): 19-29 (2017).

Mikkelsen A.L., Lindenberg S. Morphology of *in-vitro* matured oocytes: impact on fertility potential and embryo quality. *Hum. Reprod.* **16**(8): 1714-8 (2001).

Modina S., Luciano A.M., 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.* **106**(2): 241-248 (2001).

Molyneaux K., Wylie C. Primordial germ cell migration. *Int. J. Dev. Biol.* **48**: 537-44 (2004).

Moore R.K., Erickson G.F., Shimasaki S. 2004. Are BMP15 and GDF-9 primary determinants of ovulation quota in mammals? *Trends Endocrinol. Metab.* **15**(8): 356-361 (2004).

Moreno J.M., Nunez M.J., Quinonero A., Martinez S., de la Orden M., Simón C., Pellicer A., Díaz-García C., Domínguez F. Follicular fluid and mural granulosa cells microRNA profiles vary in in vitro fertilization patients depending on their age and oocyte maturation stage. *Fertil. Steril.* **104**(4): 1037-46 (2015).

Moreno J.M., Nunez M.J., Quinonero A., Martinez S., de la Orden M., Simón C., Pellicer A., Díaz-García C., Domínguez F. Follicular fluid and mural granulosa cells microRNA profiles vary in in vitro fertilization patients depending on their age and oocyte maturation stage. *Fertil. Steril.* **104**(4): 1037-46 (2015).

Navarro P.A., de Araújo M.M, de Araújo C.M., 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.* **104**(3): 226-9 (2009).

Nikseresht M, Tori MA, Rasti T, Kashani IR, Mahmoudi R. The nuclear maturation and embryo development of mice germinal vesicle oocytes with and without cumulus cell after vitrification. *J. Clin. Diagn. Res.* **9**: 1-4 (2015).

Niu Z., Ye Y., Xia L., Feng Y., Zhang A. Follicular fluid cytokine composition and oocyte quality of polycystic ovary syndrome patients with metabolic syndrome undergoing in vitro fertilization. *Cytokine*. **91**: 180-186 (2017).

Norris R.P., Ratzan W.J., Freudzon M., Mehlmann L.M., Krall J., Movsesian M.A., Wang H., Ke H., Nikolaev V.O., Jaffe L.A. Cyclic GMP from the surrounding somatic cells regulates cyclic AMP and meiosis in the mouse oocyte. *Development.* **136**(11): 1869-78 (2009).

Omidi M., Khalili M.A., Nahangi H., Ashourzadeh S., Rahimipour M. Does women's age influence zona pellucida birefringence of metaphase II oocytes in in-vitro maturation program? *Iran J. Reprod. Med.* **11**(10): 823-8 (2013).

O'Shea L.C., Mehta J., Lonergan P., Hensey C., Fair T. Developmental competence in oocytes and cumulus cells: candidate genes and networks. *Syst. Biol. Reprod. Med.* **58**(2): 88-101 (2012).

Otsuki J., Nagai Y., Chiba K. Lipofuscin bodies in human oocytes as an indicator of oocyte quality. *J. Assist. Reprod. Genet.* **24**(7): 263-70 (2007).

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.* **19**(7): 1591-7 (2004).

Pangas, S.A., Matzuk M.M. The art and artifact of GDF9 activity: Cumulus expansion and the cumulus expansion-enabling factor. *Biol. Reprod.* **73**(4): 582-5 (2005).

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.* **22**(2): 219-231 (1989).

Pesce M., Schöler H.R. Oct-4, control of totipotency and germline determination. *Mol. Reprod. Dev.* 2000, **55**(4): 452-457.

Rama Raju G.A., Prakash G., Krishna K.M., Madan K. Meiotic spindle and zona pellucida characteristics as predictors of embryonic development: a preliminary study using PolScope imaging. *Reprod. Biomed. Online.* **14**(2): 166-74 (2007).

Rankin T, Soyal S, Dean J: "The mouse zona pellucida: folliculogenesis, fertility and pre-implantation development". *Mol. Cell. Endocrinol.* **163**: 21-5 (2000).

Raucci F., Di Fiore M.M. Localization of c-kit and stem cell factor (SCF) in ovarian follicular epithelium of a lizard, Podarcis s. sicula. *Acta Histochem.* **113**(6): 647-55 (2011)

Rienzi L., Ubaldi F.M., Iacobelli M., Minasi M.G., Romano S., Ferrero S., Sapienza F., Baroni E., Litwicka K., Greco E. Significance of metaphase II human oocyte morphology on ICSI outcome. *Fertil. Steril.* **90**(5): 1692-700 (2008).

Ruppert-Lingham C.J., Paynter S.J., Godfrey J., Fuller B.J., Shaw R.W. Developmental potential of murine germinal vesicle stage cumulus-oocyte complexes following exposure to dimethylsulphoxide or cryopreservation: loss of membrane integrity of cumulus cells after thawing. *Hum. Reprod.* **18**(2): 392-8 (2003).

Russell D.L., Gilchrist R.B., Brown H.M., Thompson J.G. Bidirectional communication between cumulus cells and the oocyte: Old hands and new players? *Theriogenology.* **86**(1): 62-8 (2016).

Russell D.L., Gilchrist R.B., Brown H.M., Thompson J.G. Bidirectional communication between cumulus cells and the oocyte: Old hands and new players? *Theriogenology.* **86**(1): 62-8 (2016).

Safian F., Khalili M.A., Ashourzadeh S., Omidi M. Analysis of meiotic spindle and zona pellucida birefringence of IVM oocytes in PCOS patients. *Turk. J. Med. Sci.* **47**(1): 368-373 (2017).

Saitou M., Yamaji M. Primordial germ cells in mice. *Cold Spring Harb Perspect Biol.* **4**(11) (2012).

Sang Q., Yao Z., Wang H., Feng R., Wang H., Zhao X., Xing Q., Jin L., He L., Wu L., Wang L. Identification of MicroRNAs in human follicular fluid: characterization of MicroRNAs that govern steroidogenesis in vitro and are associated with polycystic ovary syndrome in vivo. *J. Clin. Endocrinol. Metab.* **98**(7): 3068-79 (2013).

Sang Q., Yao Z., Wang H., Feng R., Wang H., Zhao X., Xing Q., Jin L., He L., Wu L., Wang L. Identification of MicroRNAs in human follicular fluid: characterization of MicroRNAs that govern steroidogenesis in vitro and are

associated with polycystic ovary syndrome in vivo. *J. Clin. Endocrinol. Metab.* **98**(7): 3068-79 (2013).

Sasaki H., Matsui Y. Epigenetic events in mammalian germ-cell development: reprogramming and beyond. *Nat. Rev. Gen.* **9**: 129-140 (2008).

Sauerbrun-Cutler M.T., Vega M., Breborowicz A., Gonzales E., Stein D., Lederman M., Keltz M. Oocyte zona pellucida dysmorphology is associated with diminished in-vitro fertilization success. *J. Ovarian Res.* **8**: 5 (2015).

Scaramuzzi R.J., Campbell B.K. Physiological regulation of ovulation rate in the ewe: A new look at an old problem. *In book: Reproductive Physiology of Merino Sheep - Concepts and Consequences.* The University of Western Australia, Perth, Australia. pp. 71-84 (1990).

Setti A.S., Figueira R.C., Braga D.P., Colturato S.S., Iaconelli A. Jr, Borges E Jr. Relationship between oocyte abnormal morphology and intracytoplasmic sperm injection outcomes: a meta-analysis. *Eur. J. Obstet. Gynecol. Reprod. Biol.* **159**(2): 364-70 (2011).

Shen Y., Stalf T., Mehnert C., Eichenlaub-Ritter U., Tinneberg H.R. High magnitude of light retardation by the zona pellucida is associated with conception cycles. *Hum. Reprod.* **20**(6): 1596-606 (2005).

Shi W., Xu B., Wu L.M., Jin R.T., Luan H.B., Luo L.H., Zhu Q., Johansson L., Liu Y.S., Tong X.H. Oocytes with a dark zona pellucida demonstrate lower fertilization, implantation and clinical pregnancy rates in IVF/ICSI cycles. *Plos One* **9**(2): 89409 (2014).

Shimasaki S., Moore R.K., Otsuka F., Erickson G.F. The bone morphogenetic protein system in mammalian reproduction. *Endocr. Rev.* **25**(1): 72–101 (2004).

Sohel M.M., Hoelker M., Noferesti S.S., Salilew-Wondim D., Tholen E., Looft C., Rings F., Uddin M.J., Spencer T.E., Schellander K., Tesfaye D. Exosomal and non-exosomal transport of extracellular microRNAs in follicular fluid: implications for bovine oocyte developmental competence. *Plos One*. **8**: e78505 (2013).

Sousa M., Teixeira da Silva J., Silva J., Cunha M., Viana P., Oliveira E., Sá R., Soares C., Oliveira C., Barros A. Embryological, clinical and ultrastructural

study of human oocytes presenting indented zona pellucida. *Zygote*. **23**: 145-157 (2015).

Stewart C.L. Oct-4, scene 1: the drama of mouse development. *Nat. Genet.* **24**(4): 328-30 (2000).

Stival C., Puga Molina Ldel C., Paudel B., Buffone M.G., Visconti P.E., Krapf D. Sperm Capacitation and Acrosome Reaction in Mammalian Sperm. *Adv. Anat. Embryol. Cell. Biol.* **220**: 93-106 (2016).

Strumpf D., Mao C.A., Yamanaka Y., Ralston A., Chawengsaksophak K., Beck F., Rossant J. Cdx2 is required for correct cell fate specification and differentiation of trophectoderm in the mouse blastocyst. *Development*. **132**(9): 2093-102 (2005).

Su Y.Q., Sugiura K., Eppig J.J. Mouse Oocyte Control of granulosa cell development and function: paracrine regulation of cumulus cell metabolism. *Semin. Reprod. Med*. **27**(1): 32-42 (2009).

Su Y.Q., Sugiura K., Wigglesworth K., O'Brien M.J., Affourtit J.P., Pangas S.,. Matzuk M.M., and Eppig J.J. Oocyte regulation of metabolic cooperativity between mouse cumulus cells and oocytes: BMP15 and GDF9 control cholesterol biosynthesis in cumulus cells. *Development*. **135**(1): 111-21 (2008).

Sun F., Bahat A., Gakamsky A., Girsh E., Katz N., Giojalas L.C. Human sperm chemotaxis: both the oocyte and its surrounding cumulus cells secrete sperm chemoattractants. *Hum. Reprod.* **20**: 761-7 (2005).

Sutton-McDowall M.L., Gilchrist R.B., Thompson J.G. The pivotal role of glucose metabolism in determining oocyte developmental competence. *Reproduction.* **139**(4): 685-95 (2010).

Tahaei L.S., Eimani H., Yazdi P.E., Ebrahimi B., Fathi R. Effects of retinoic acid on maturation of immature mouse oocytes in the presence and absence of a granulosa cell co-culture system. *J. Assis. Reprod. Genet.* **28**(6): 553-8 (2011).

Tang F., Kaneda M., O'Carroll D., Hajkova P., Barton S.C., Sun Y.A., Lee C., Tarakhovsky A., Lao K., Surani M.A. Maternal microRNAs are essential for mouse zygotic development. *Genes Dev.* **21**(6): 644-8 (2007).

Ten J., Mendiola J., Vioque J., de Juan J., Bernabeu R. Donor oocyte dysmorphisms and their influence on fertilization and embryo quality. *Reprod. Biomed. Online.* **14**(1): 40-8 (2007).

Tharasanit T., Colleoni S., Galli C., Colenbrander B., Stout T.A. Protective effects of the cumulus-corona radiata complex during vitrification of horse oocytes. *Reprod.* **137**(3): 391-401 (2009).

Trivers K.F., Fink A.K., Partridge A.H., Oktay K., Ginsburg E.S., Li C., Pollack L.A. Estimates of young breast cancer survivors at risk for infertility in the U.S. *Oncologist.* **19**(8): 814-22 (2014).

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.* **14**(3): 157-168 (2008).

Vanegas J.C., Chavarro J.E., Williams P.L., Ford J.B., Toth T.L., Hauser R., Gaskins A.J. Discrete survival model analysis of a couple's smoking pattern and outcomes of assisted reproduction. *Fertil. Res. Pract.* doi: 10.1186/s40738-017-0032-2 (2017).

Vanhoutte L., Nogueira D., De Sutter P. Prematuration of human denuded oocytes in a three-dimensional co-culture system: effects on meiosis progression and developmental competence. *Hum. Reprod.* **24**(3): 658-69 (2009).

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

Wassarman P.M., Litscher E.S.: "Mammalian fertilization: the egg's multifunctional zona pellucida". *Int. J. Dev. Biol***52**: 66576 (2008).

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.* **26**(5): 1035-1051 (2011).

Wear H.M., McPike M.J., Watanabe K.H. From primordial germ cells to primordial follicles: a review and visual representation of early ovarian development in mice. *J. Ovarian Res.* **9**(1): 36 (2016).

Wilding M., Di Matteo L., D'Andretti S., Montanaro N., Capobianco C., Dale B. An oocyte score for use in assisted reproduction. *J. Assist. Reprod. Genet.* **24**: 350–358 (2007).

Wong C., Chen A.A., Behr B., Shen S. Time-lapse microscopy and image analysis in basic and clinical embryo development research. *Reprod. Biomed. Online* **26**: 120-9 (2013).

Wong C.C., Loewke K.E., Bossert N.L., Behr B., De Jonge C.J., Baer T.M., Reijo Pera R.A. Non-invasive imaging of human embryos before embryonic genome activation predicts development to the blastocyst stage. *Nat. Biotechnol.* **28**: 1115–1121 (2010).

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.* **12**(8): 1750-5 (1997).

Xu X., Chen D., Zhang Z., Wei Z., Cao Y. Molecular signature in human cumulus cells related to embryonic developmental potential. *Reprod. Sci.* **22**(2): 173-80 (2015).

Yanagimachi R. Fertility of mammalian spermatozoa: its development and relativity. *Zygote*. **2**: 371-2 (1994).

Yang X., Zhou Y., Peng S., Wu L., Lin H.Y., Wang S., Wang H. Differentially expressed plasma microRNAs in premature ovarian failure patients and the potential regulatory function of mir-23a in granulosa cell apoptosis. *Reprod.* **144**(2): 235-44 (2012).

Ying Y., Liu X.M., Marble A., Lawson K.A., Zhao G.Q. Requirement of Bmp8b for the generation of primordial germ cells in the mouse. *Mol. Endocrinol.* **14**(7): 1053-63 (2000).

Zernicka-Goetz M., Morris .SA., Bruce A.W. Making a firm decision: multifaceted regulation of cell fate in the early mouse embryo. Nat. Rev. Genet. **10**(7): 467-77 (2009).

Zhang X., Jafari N., Barnes R.B., Confino E., Milad M., Kazer R.R. Studies of gene expression in human cumulus cells indicate pentraxin 3 as a possible marker for oocyte quality. *Fertil. Steril.* **83**(1): 1169-79 (2005).

Zhao X.M., Ren J.J., Du W.H., Hao H.S., Liu Y., Qin T., Wang D., Zhu H.B. Effect of mouse cumulus cells on the in vitro maturation and developmental potential of bovine denuded germinal vesicle oocytes. *Zygote.* **22**(3): 348-55 (2014).

Zhou C.J., Wang D.H., Niu X.X., Kong X.W., Li Y.J., Ren J., Zhou H.X., Lu A., Zhao Y.F., Liang C.G. High survival of mouse oocytes using an optimized vitrification protocol. *Sci. Rep.* **6**: 19465 (2016)

Zhou H.X., Ma Y.Z., Liu Y.L., Chen Y., Zhou C.J., Wu S.N., Shen J.P., Liang C.G. Assessment of mouse germinal vesicle stage oocyte quality by evaluating the cumulus layer, zona pellucida, and perivitelline space. *Plos One.* **9**(8): 105812 (2014).

Zhou P., Wu Y.G., Wei D.L., Li Q., Wang G., Zhang J., Luo M.J. and Tan J.H. Mouse Cumulus-Denuded Oocytes Restore Developmental Capacity Completely When Matured with Optimal Supplementation of Cysteamine, Cystine, and Cumulus Cells. *Biol. Reprod.* **82**: 759-768 (2010)

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

Zuccotti M., Merico V., Belli M., Mulas F., Sacchi L., Zupan B., Redi C.A., Prigione A., Adjaye J., Bellazzi R., Garagna S. OCT4 and the acquisition of oocyte developmental competence during folliculogenesis. *Int. J. Dev. Biol.* **56**: 853-8 (2012).

Zuccotti M., Merico V., Bellone M., Mulas F., Sacchi L., Rebuzzini P., Prigione A., Redi C.A., Bellazzi R., Adjaye J., Garagna S. Gatekeeper of pluripotency: a common Oct4 transcriptional network operates in mouse eggs and embryonic stem cells. *BMC Genomics* **12**: 1-13 (2011).

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

Zuccotti M., Merico V., Sacchi L., Bellone M., Brink T.C., Bellazzi R., Stefanelli M., Redi C.A., Garagna S., Adjaye J. Maternal Oct-4 is a potential key

regulator of the developmental competence of mouse oocytes. BMC Dev. Biol. 8: 97 (2008).

Zuccotti M., Merico V., Sacchi L., Bellone M., Brink T.C., Stefanelli M., Redi C.A., Bellazzi R., Adjaye J., Garagna S. Oct-4 regulates the expression of Stella and Foxj2 at the Nanog locus: implications for the developmental competence of mouse oocytes. *Hum. Reprod.* **24**: 2225-2237 (2009b).

Zuccotti M., Piccinelli A., Giorgi Rossi P., Garagna S., Redi C.A. Chromatin organization during mouse oocyte growth. *Mol. Reprod. Dev.* **41**(4): 479-85 (1995).

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