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

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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).](image)

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.
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.

![Figure 2. Mouse folliculogenesis](image-url)
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](image)

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 or 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).](image)

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.,
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.](image)
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).
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 result 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 its 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, Gasparini et al., 2007). Bogoglio and colleagues (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 improve 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 Morphological 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 intimately 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, CTNNBD1, 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), NRPI, 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% CO2 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% CO₂ 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% CO₂ 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% CO₂. 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% CO₂ 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% CO₂ 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% CO₂ 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 ºC and 5% CO₂, 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 (10⁶) per ml. After 45 min incubation at 37 ºC, 5% CO₂, capacitated spermatozoa were added to the fertilization drop diluted in Wt medium in order to reach a final concentration of 1.8 x 10⁶ sperm/ml. Before insemination, oocytes were transferred into 100 µl fertilization drop and incubated for 2 hr at 37 ºC, 5% CO₂. 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 ºC) 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% CO₂ 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\textsuperscript{NSN} or MII\textsuperscript{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 instructions: 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.**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer 10X (GeneAmp®)</td>
<td>1 X</td>
</tr>
<tr>
<td>MgCl2 (GeneAmp®, 5 mM)</td>
<td>1 mM</td>
</tr>
<tr>
<td>dNTPmix (GeneAmp®, 10 mM each)</td>
<td>2 mM</td>
</tr>
<tr>
<td>Oligo d(T)16 (GeneAmp®, 50 µM)</td>
<td>2.5 µM</td>
</tr>
<tr>
<td>MuLV reverse transcriptase (GeneAmp®, 50 U/µl)</td>
<td>50 U</td>
</tr>
<tr>
<td>RNase inhibitor (GeneAmp®, 20 U/µl)</td>
<td>20 U</td>
</tr>
</tbody>
</table>
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.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequences</th>
<th>Conc (nM)</th>
<th>Amplicon length (bp)</th>
<th>Tm</th>
<th>Ext. time (sec)</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dnmt3lF</td>
<td>5'-GTGGCGGTACTGAGCTTTTAGA-3'</td>
<td>200</td>
<td>258</td>
<td>60°C</td>
<td>20</td>
<td>40</td>
</tr>
<tr>
<td>Dnmt3lR</td>
<td>5'-CGACATTTGTGACATCTCAGTA-3'</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Luciferase F</td>
<td>5'-AGTCGATGTACAGTGTCAG-3'</td>
<td>200</td>
<td>98</td>
<td>60°C</td>
<td>20</td>
<td>40</td>
</tr>
<tr>
<td>Luciferase R</td>
<td>5'-CAGTGCAATTGTTTGTCAG-3'</td>
<td></td>
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</table>
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](image)

**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.

<table>
<thead>
<tr>
<th></th>
<th>Mean ± S.D.</th>
<th>p value</th>
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<tbody>
<tr>
<td></td>
<td>SN-COCs</td>
<td>NSN-COCs</td>
</tr>
<tr>
<td>COCs diameter (µm)</td>
<td>180 ± 36</td>
<td>160 ± 34</td>
</tr>
<tr>
<td>Number of CC layers</td>
<td>6 ± 0.68</td>
<td>4 ± 0.51</td>
</tr>
<tr>
<td>CCs number</td>
<td>2060 ± 727</td>
<td>1322 ± 416</td>
</tr>
</tbody>
</table>

3.2 Antral oocytes maturation and preimplantation development upon a FL-CCs in 96-well
The isolated fully-grown 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.

<table>
<thead>
<tr>
<th>STAGE</th>
<th>NO-FL</th>
<th>FL-CCs</th>
</tr>
</thead>
<tbody>
<tr>
<td>GV</td>
<td>94.0 ± 9.8</td>
<td>92.7 ± 12.7</td>
</tr>
<tr>
<td>MII</td>
<td>54.0 ± 8.3</td>
<td>64.0 ± 9.4</td>
</tr>
<tr>
<td>2-cell *</td>
<td>15.0 ± 7.6</td>
<td>36.0 ± 15.0</td>
</tr>
<tr>
<td>4-cell **</td>
<td>8.4 ± 2.9</td>
<td>29.0 ± 5.7</td>
</tr>
<tr>
<td>Morula **</td>
<td>4.7 ± 3.7</td>
<td>16.2 ± 3.8</td>
</tr>
</tbody>
</table>

*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 cm² 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 cm² 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 ± 12.8%) (NO-FL), upon unclassified FL-CCs (93.2 ± 7.3%) or on FL-SN-CCs (93.9 ± 8.1%). Instead, it decreased when DOs were cultured upon FL-NSN-CCs (74.4 ± 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.

<table>
<thead>
<tr>
<th>STAGE</th>
<th>Mean ± S.D. % (Number)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NO-FL</td>
</tr>
<tr>
<td>GV</td>
<td>(60)</td>
</tr>
<tr>
<td>MII</td>
<td>87.4 ± 12.8 (51)</td>
</tr>
<tr>
<td>2-cell</td>
<td>35.3 ± 22.1 (19)</td>
</tr>
<tr>
<td>4-cell**</td>
<td>11.4 ± 22.7 (4)</td>
</tr>
<tr>
<td>Morula**</td>
<td>None</td>
</tr>
<tr>
<td>Blastocyst**</td>
<td>None</td>
</tr>
</tbody>
</table>

*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 µm²), those matured in the absence of FL (NO-FL) showed an area of 741.4 ± 290.3 µm² (p< 0.001) compared to FL-CCs 441.0 ± 174.8 µm² (p> 0.05), FL-NSN-CCs 660.0 ± 344.3 µm² (p< 0.001) or FL-SN-CCs 448.1 ± 214.1 µm² (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 ± 22.5%) and 4-cell (55.3 ± 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 ± 8.6%) and blastocyst (3.0 ± 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.](image-url)
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 ± 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-positive (ovulated MII: 73.8 ± 8.4%; FL-SN-CCs: 78.2 ±9%; FL-CCs: 74.5 ± 10.9%) and Oct4-positive cells (ovulated MII: 26.2 ± 8.4%; FL-SN-CCs: 21.8 ± 9%; FL-CCs: 22.8 ± 6%) did not differ significantly in the blastocysts of the three experimental groups (Table 6).

![Figure 18](image.png)

**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

<table>
<thead>
<tr>
<th></th>
<th>FL-CCs</th>
<th>FL-SN-CCs</th>
<th>Ovulated MII</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean number ± S.D. of</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DAPI-positive cells</td>
<td>35.7 ± 8.8</td>
<td>27.0 ± 6.0</td>
<td>29.5 ± 7.7</td>
</tr>
<tr>
<td>Mean ± S.D. % of cells positive to</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>the antibody</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cdx2</td>
<td>74.5 ± 10.9</td>
<td>78.2 ± 9.0</td>
<td>73.8 ± 8.4</td>
</tr>
<tr>
<td>Oct4</td>
<td>22.8 ± 6</td>
<td>21.8 ± 9.0</td>
<td>26.2 ± 8.4</td>
</tr>
</tbody>
</table>
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 µm²), 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 µm² (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>
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<th>Reagent</th>
<th>M2 (1L)</th>
</tr>
</thead>
<tbody>
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<td>NaCl</td>
<td>5.534 g</td>
</tr>
<tr>
<td>KCl</td>
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</tr>
<tr>
<td>CaCl$_2$</td>
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</tr>
<tr>
<td>MgSO$_4$•7H$_2$O</td>
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</tr>
<tr>
<td>KH$_2$PO$_4$</td>
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</tr>
<tr>
<td>NaHCO$_3$</td>
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</tr>
<tr>
<td>BSA</td>
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</tr>
<tr>
<td>Phenol Red</td>
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<tr>
<td>Hepes</td>
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</tr>
<tr>
<td>EDTA</td>
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<tr>
<td>Sodium Lactate</td>
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</tr>
<tr>
<td>Penicillin/Streptomycin</td>
<td>5.00 ml</td>
</tr>
<tr>
<td>Reagent</td>
<td>Amount (per litre)</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>-------------------</td>
</tr>
<tr>
<td>NaCl</td>
<td>5.803 g</td>
</tr>
<tr>
<td>KCl</td>
<td>0.201 g</td>
</tr>
<tr>
<td>NaHCO₃</td>
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</tr>
<tr>
<td>Na₂HPO₄•12H₂O</td>
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</tr>
<tr>
<td>MgCl₂•6H₂O</td>
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</tr>
<tr>
<td>CaCl₂•2H₂O</td>
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</tr>
<tr>
<td>Glucose</td>
<td>1.000 g</td>
</tr>
<tr>
<td>Sodium pyruvate</td>
<td>0.055 g</td>
</tr>
<tr>
<td>Phenol red</td>
<td>0.010 g</td>
</tr>
<tr>
<td>EDTA</td>
<td>1.00 ml</td>
</tr>
<tr>
<td>Sodium lactate (60%)</td>
<td>3.50 ml</td>
</tr>
<tr>
<td>Penicillin/Streptomycin</td>
<td>5.00 ml</td>
</tr>
</tbody>
</table>

Table 2S. Whittingham medium recipe
**Table 3S. M16 medium recipe**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount (per litre)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>5.698 g</td>
</tr>
<tr>
<td>KCl</td>
<td>0.356 g</td>
</tr>
<tr>
<td>CaCl(_2)\cdot2\text{H}_2\text{O}</td>
<td>0.251 g</td>
</tr>
<tr>
<td>KH(_2)PO(_4)</td>
<td>0.136 g</td>
</tr>
<tr>
<td>MgSO(_4)</td>
<td>0.143 g</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.036 g</td>
</tr>
<tr>
<td>NaHCO(_3)</td>
<td>2.101 g</td>
</tr>
<tr>
<td>Phenol red</td>
<td>0.010 g</td>
</tr>
<tr>
<td>Sodium lactate (60%)</td>
<td>5.53 ml</td>
</tr>
<tr>
<td>EDTA</td>
<td>1.00 ml</td>
</tr>
<tr>
<td>Penicillin/Streptomycin</td>
<td>5.00 ml</td>
</tr>
</tbody>
</table>
8. REFERENCES


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