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**Gene expression and human oocyte maturation: transcriptional control and
post-transcriptional mechanisms based on intercellular signalling via exosomal
miRNAs in follicular microenvironment**

Tesi di Dottorato

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1. ABSTRACT

Mammalian reproduction hinges upon the timely ovulation of a fully differentiated oocyte. Growth and development of human oocyte and somatic cell compartments of the ovarian follicle require a series of coordinated events that induce morphological, molecular and functional changes within the follicle, leading to cell differentiation and oocyte maturation. In particular, late oogenesis and early embryogenesis occur in the absence of transcription and rely entirely on maternal mRNAs stored in oocytes during its maturation. Moreover, mechanisms controlling both mRNA stability and their translation (e.g. cytoplasmic polyadenylation, and/or microRNAs) fix the ultimate molecular structure of the mature oocyte. Consequently, mRNA regulation at transcriptional and post-transcriptional level is a crucial step in germ cells and early embryo development. Given their nature of post-transcriptional regulators of gene expression, microRNAs (miRNAs) have recently been highlighted extensively for their possible involvement in translational programming of maternal mRNAs and therefore in the development of mammalian oocytes and embryos.

Studies on oocyte transcriptome are important to understand the biological pathways involved in oogenesis, totipotence and early embryonic development and genes regulating physiological pathways in gametes could represent potential candidate for reproductive disorders. Moreover, given that the bidirectional traffic between the oocyte and its surrounding somatic cells is very important for the acquisition of oocyte competence, the study of the transcriptomic profile of both granulosa/cumulus cells, follicular fluid (FF) and exosomes could improve our knowledge on the complex process of mammalian oocyte maturation as well as offer the opportunity to identify potential non-invasive markers of oocyte quality. These could be used in

Reproductive Medicine, in order to select the best gamete during medically assisted reproduction cycles. Accordingly, it is also useful to cryopreserve these gametes employing protocols that do not affect the biologic quality of oocytes.

This thesis aimed at characterizing mRNA and miRNA expression profiles in the female gamete and its microenvironment, respectively, under physiological and non-physiological conditions in order i) to assess the effect of vitrification on the biomolecular profile of the oocytes, ii) to understand the molecular basis of reproductive ageing and iii) to identify miRNAs in FF to be used as non-invasive biomarkers of oocyte quality.

We demonstrated that vitrification technique might be very helpful for preserving women's fertility, since it keeps unaltered oocyte molecular profile and does not cause degradation of mRNAs essential for oocyte development (i.e. BMP15, FIGLA, GDF9, OCT4, TAF4B).

We determined Apoptotic Machinery (AM) transcriptome in mature MII human oocyte pools from women aged more than 38 years (old) and compared it to those of women aged up to 35 years (young). Subsequently, some AM candidate genes with a key role in apoptotic regulation were selected and analysed in single oocytes. These studies led us to identify AM transcripts never reported in human oocytes so far (BAG3, CD40, CFLAR, TNFRSF21, TRAF2, TRAF3) and to find out other differentially expressed genes in oocytes from older women. In fact, we found a significant upregulation of proapoptotic CD40, TNFRSF10A, TNFRSF21 and downregulation of antiapoptotic BCL2 and CFLAR. Our results demonstrate that during maturation old oocytes selectively accumulate mRNAs potentially able to trigger the extrinsic apoptotic pathway and express at low levels some survival factors: this condition could make old oocytes more inclined to apoptosis. Moreover,

we found TP73 among differentially expressed genes in human MII oocyte pools during reproductive ageing, a process closely related to the production of oocytes with a reduced developmental competence whose main hallmark is aneuploidy. In order to verify the potential involvement of TP73 isoforms in reproductive ageing, we determined their expression in single mature MII oocytes from women younger than 35 and older than 38 years. We found out that TAp73 isoforms are significantly downregulated in oocytes from women of advanced reproductive age. There is evidence that TAp73 interacts with some kinetochore proteins in order to stop the anaphase if chromosomes are not properly attached to the meiotic spindle. The absence of TAp73 removes this cell cycle brake, so causing genomic instability. Consequently, TAp73 downregulation in old oocytes could lead to aneuploidy in the developing embryos, explaining both the reduction of fertility and the increase of newborns with chromosomal abnormalities.

Finally, we profiled the expression of 384 miRNAs in human follicular fluid and its purified exosomal fraction with respect to plasma from the same women, providing the first molecular evidence of these bioactive vesicles inside ovarian follicle. Among the 37 miRNAs that we found upregulated in follicular microenvironment, the majority of them are carried by exosomes (exosome Follicular Fluid miRNAs – eFF-miRNAs) and are involved in signaling pathways critically important for follicle growth, oocyte maturation, and early embryo development (i.e. WNT, MAPK, ErbB and TGF β signaling pathways). Moreover, eFF-miRNAs are able to negatively regulate genes encoding inhibitors of follicle maturation and meiosis resumption as PTEN, MTOR, P21 and RB1. These data could reveal new actors in the molecular communication among cells of ovarian follicle and eFF-miRNAs may represent valuable biomarkers of oocyte quality and reproductive disorders.

Data shown in this thesis were published in 2010 in **Fertility and Sterility** [Di Pietro C, Vento M, Guglielmino MR, Borzi P, Santonocito M et al. *Molecular profiling of human oocytes after vitrification strongly suggests that they are biologically comparable with freshly isolated gametes*. Fertil Steril. 2010 Dec; 94(7):2804-7], in 2011 in **Cell Cycle** [Guglielmino MR, Santonocito M et al. *TAp73 is downregulated in oocytes from women of advanced reproductive age*. Cell Cycle. 2011 Oct 1;10(19):3253-6], in 2013 in **Apoptosis** [Santonocito M, Guglielmino MR et al. *The apoptotic transcriptome of the human MII oocyte: characterization and age-related changes*. Apoptosis. 2013 Feb;18(2):201-11]. The manuscript *Exosomal microRNAs in Human Follicular Fluid: new actors in the communication between oocytes and somatic follicular cells* by Santonocito M, Vento M et al. has been submitted for publication in **Molecular Human Reproduction**.

For brevity, they will be referred in the text by Roman numerals: Paper I, II, III and IV respectively.

2. INTRODUCTION

From oocytes to embryo: a journey full of hurdles

Primordial germ cells (PGCs) are the most primitive undifferentiated sex cells, found initially outside the gonad. During the first month of gestation, these cells migrate to the gonadal ridge from the yolk sac endoderm. In the absence of the testicular determining factor, ovarian differentiation is established at 6–8 weeks of gestation, reflected in a rapid mitosis of the germ cells that peaks around 20 weeks of gestation to a total of about 7 millions of oogonia [Djahanbakhch *et al.*, 2007] (Figure 1).

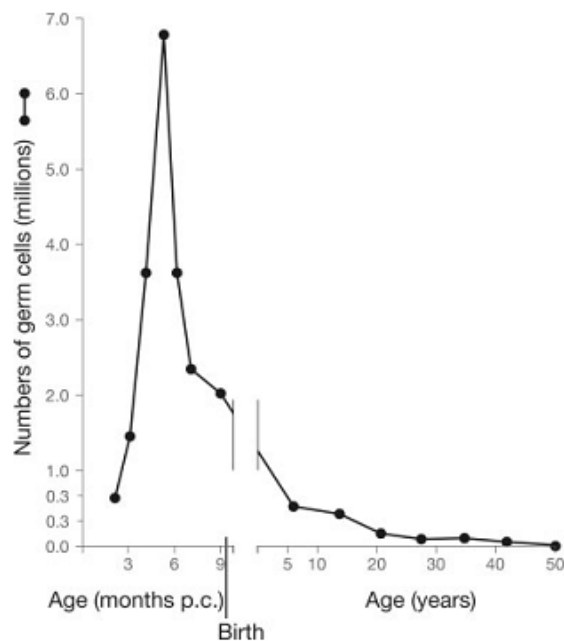


Figure 1. In human females, all germ cells are formed during fetal life. The total number of oocytes is at maximum at 5 months of gestation and drops to about 2 million (in both ovaries) by the time of birth (from Djahanbakhch *et al.*, 2007).

After these multiple divisions oogonia stop proliferating, enter meiosis becoming **primary oocytes** and their development is soon arrested at the diplotene stage of the first meiotic prophase (prophase I). They remain at this stage until puberty, when a surge of luteinizing hormone (LH) induces the resumption of meiosis and ovulation of eggs arrested at metaphase II (MII). Almost all oogonia, which have entered the primary

oocyte stage, are individually surrounded by pregranulosa cells to form a **primordial follicle** or they undergo atresia. Subsequently, the number of follicles decreases dramatically to 1 000 000/ovary at birth and 300 000/ovary by puberty until complete depletion at menopause [Baker, 1963; Baker and Sum, 1976; Gougeon, 1986; Gougeon, 1996; Faddy, 2000] (Figure 1). The fate of each follicle is controlled by endocrine as well as paracrine factors [Richards *et al.*, 1995]. After puberty, during each ovarian cycle, hundreds of resting primordial follicles enter the growing follicle pool developing through primordial, primary, and secondary stages before acquiring an antral cavity. At the antral stage, most follicles undergo atretic degeneration, whereas a few of them, under the cyclic gonadotropin stimulation that occurs after puberty, reach the preovulatory stage (Figure 2). Concomitantly, increases in local growth factors allow a positive selection of the dominant follicle from this cohort, thus ensuring its final growth and eventual ovulation, whereas the remaining cohort is led to its ultimate demise [Hirshfield, 1991; Gougeon 1996; MCGee and Hsueh, 2000] (Figure 2).

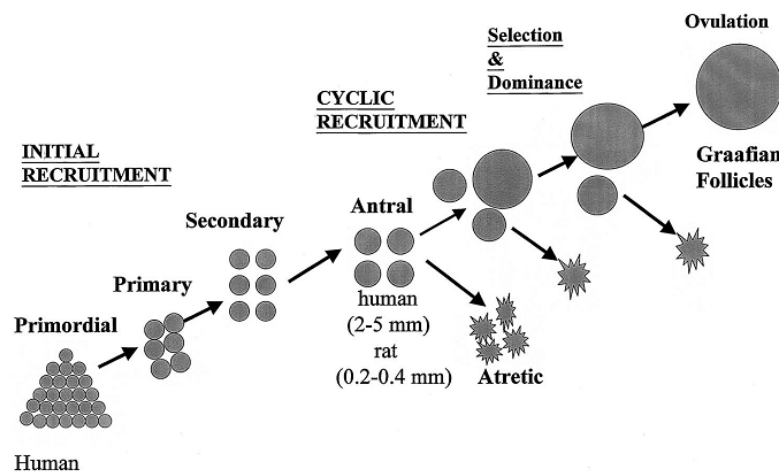


Figure 2. Follicle development and recruitment in human ovaries. Primordial follicles undergo initial recruitment to enter the growing pool of primary follicles. During cyclic recruitment, increases in circulating FSH allow a cohort of antral follicles to escape apoptotic demise. Among this cohort, a leading follicle emerges as dominant by secreting high levels of estrogens and inhibins to suppress pituitary FSH release. The result is a negative selection of the remaining cohort, leading to its ultimate demise. Concomitantly, increases in local growth factors allow a positive selection of the dominant follicle, thus ensuring its final growth and eventual ovulation (from MCGee and Hsueh, 2000).

The decline in oocyte number can be attributed to several mechanisms: germ cells in the cortical area migrating to the surface of the ovary and becoming incorporated within the surface epithelium or being eliminated in the peritoneal cavity, regression during meiosis, and failure to become encapsulated with granulosa cells to become primordial follicles. Once all the primordial follicles have been formed, continuous loss of oocytes occurs through the physiological process of follicular growth and atresia, which continues throughout the woman's life [Djahanbakhch *et al.*, 2007].

Since the pool of oocytes in the mammalian ovary is already fixed after birth, ovarian senescence is linked to the decreasing supply and eventual exhaustion of the pool of primordial follicles. It has been generally accepted that, in most mammalian species, oocytes cannot renew themselves in postnatal or adult life. However, a few recent studies challenge the idea that mammalian species have lost the capacity for oocyte production after birth, showing that female germline stem cells still exist in mammalian ovaries after birth, but this concept is still controversial [Johnson *et al.*, 2004]. These hypotheses, which need further confirmations, suggest that the pool of primordial follicles in adult mammalian ovary may represent a dynamic rather than a static population, which is characterized by structures continuously subjected to degeneration and differentiation processes.

Oogenesis and Folliculogenesis: coordinated processes under hormonal regulation

Oocyte development is the final outcome of a sophisticated biological process that is hormonally regulated and tightly connected to the differentiation of other highly specialized cells within the follicle, which provides for and influences the quality of the oocyte (Figure 3).

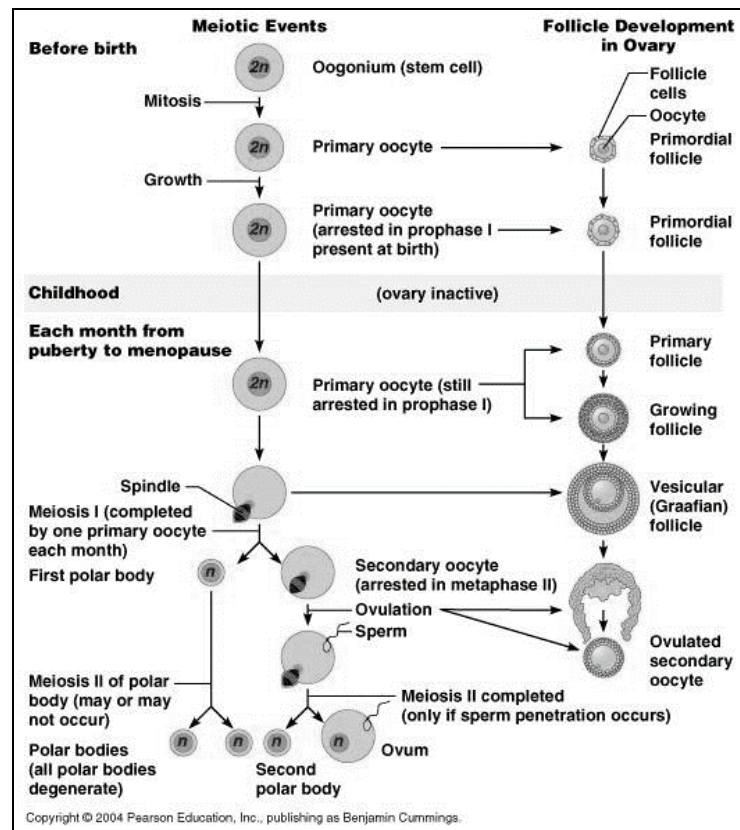


Figura 3. Synchronization between oogenesis and folliculogenesis.

The oogenesis begin when PGCs migrate to the gonad and start to proliferate by mitosis giving rise to oogonia. Following this event and before follicle formation, mitotic divisions stop and germ cells initiate meiosis, becoming primary oocytes [Ginsburg *et al.*, 1990] (Figure 3). Meiosis initiate with a prophase stage, a complex phase that is subdivided into five stages: leptotene, zygotene, pachytene, diplotene and diakinesis. Within the first period of the prophase, a series of crucial events occur, involving the pairing of homologous chromosomes, synapsis (close association between these chromosomes), and recombination or “crossing over” (exchange of genetic material). Subsequently, oocytes progress to the diplotene stage where they enter into a prolonged resting phase called “dictyate” [Hunt and Hassold, 2008]. Prophase events are vital for germ cell survival and meiotic progression, and errors occurring along this stage, as well as throughout the consecutive phases of meiosis, may originate and/or contribute to

female meiotic aneuploidies. Oocytes remain arrested at the dictyate stage of meiosis I throughout oogenesis until childhood when, each month from puberty to menopause, one primary oocyte resumes and completes meiosis I, giving rise to a secondary oocyte arrested at metaphase II stage. These oocytes will complete meiosis II only after ovulation if sperm penetration occurs thus fertilizing the egg (Figure 3).

However, the oocyte cannot reach this point of development without the support of its follicle. Folliculogenesis is an intricate process by which one follicle per cycle is selected to develop fully and thereby create a mature oocyte. Soon after oocytes enter the prolonged diplotene stage of meiosis, the precursors to the follicular somatic cells encompass the oocyte in a single squamous layer to form primordial follicles. The large population of non-growing primordial follicles serves as the source of developing follicles and oocytes until the end of a female's reproductive life. Until that time, there is a continuous recruitment of follicles from this pool, beginning with the formation of primary follicles. The oocyte in a primary follicle begins its extensive growth phase, and the surrounding follicular cells, now called granulosa cells (GC), become cuboidal and proliferative. When the growing oocytes are surrounded by more than one layer of granulosa cells, the follicle is called a secondary follicle. Early preantral follicles are independent of gonadotrophins for their initial growth, as evidenced by the fact that development to the primary and secondary stage can take place in the absence of hormones [Fortune and Eppig, 1979], although follicle stimulating hormone receptors (FSHR) are present on the GCs of these early follicles [O'Shaughnessy *et al.*, 1996; Oktay *et al.*, 1997]. Primary to secondary follicle transition is rather driven by local intraovarian paracrine factors produced by oocytes and their companion granulosa cells [Kol and Adashi, 1995]. The latter phase of folliculogenesis is termed antral

folliculogenesis, and its progress is entirely dependent on gonadotropic hormones, follicle stimulating hormone (FSH) and LH (Figure 4).

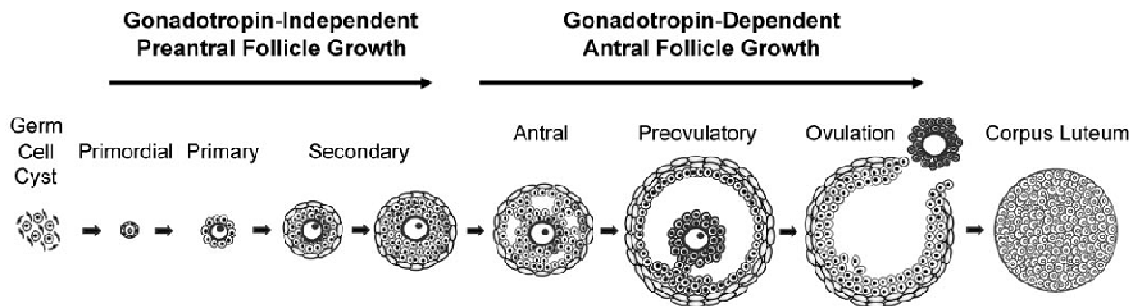


Figure 4. Classification of the major stages of mammalian folliculogenesis. In humans, preantral follicular development does not require stimulation by the pituitary gonadotropins whereas antral follicle growth is entirely dependent on them (Edson *et al.*, 2009).

FSH induces luteinizing hormone receptor (LHR) expression in GCs, which will be required for follicles to respond to LH, the latter being crucial for triggering the ovulatory process [Sánchez and Smitz, 2012]. The antral stage is characterized by the appearance of a fluid-filled cavity, the antrum, which begins to form when follicles reach a critical size and a critical number of granulosa cells [Boland *et al.*, 1994]. The follicular fluid (FF) consists of a complex mixture of proteins, metabolites, and ionic compounds, which are secreted from the granulosa and thecal cells and combined with plasma components that cross the blood–follicular barrier via the thecal capillaries [Angelucci *et al.*, 2006; Hanrieder *et al.*, 2008; Hanrieder *et al.*, 2009; Rodgers and Irving-Rodgers, 2010]. This fluid provides a very important microenvironment and contains regulatory molecules that are important for the maturation and quality of the oocytes [Revelli *et al.*, 2009]. Certain lipids, proteins, vitamins, and metabolites in the FF have been found to be associated with reproductive diseases [Kim YS *et al.*, 2006], oocyte quality [Berker *et al.*, 2009], embryo quality, and the outcome of *in vitro* fertilization (IVF) attempts [Wu *et al.*, 2007; Wallace *et al.*, 2012]. Additionally, the

appearance of the antral cavity establishes the morphological and functional separation of granulosa cells into mural granulosa cells (MGC), which line the follicle wall, and the cumulus cells (CC), which surround the oocyte. Each follicle is enveloped by a basal lamina, a specialized sheet of extracellular matrix that separates the internal follicle from the third somatic follicular cell type, the theca cells. Theca cells are a vascularized cell layer that defines the outer boundary of the antral follicle (Figure 5).

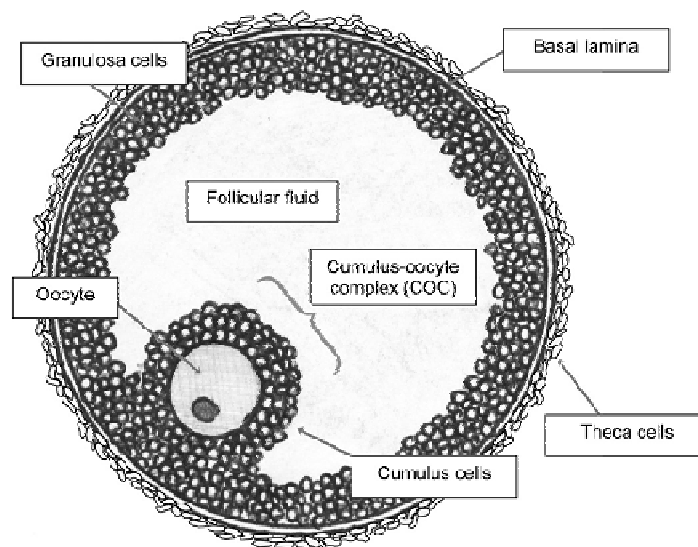


Figure 5. Schematic representation of an antral follicle (from Hennet and Combelles, 2012).

As mentioned earlier, the antral phase of follicular development is characterized by dependency on gonadotrophins, FSH and LH, which are cyclically secreted by the pituitary gland. Circulating levels of FSH support the growth of a group of antral follicles until the largest follicle begins producing estradiol (E₂), an FSH suppressor, and switches to dependence on LH. This follicle is termed the dominant follicle, and this event is called follicle selection. As FSH levels decline, the rest of the antral follicles will regress in a process called atresia and the dominant follicle will be the only follicle capable of reaching ovulation. Atresia is the process by which all follicles regress if they do not reach ovulation, and as it progresses into its latest stages, the granulosa cell and cumulus cell populations die, followed by resorption of FF and

oocyte death. The underlying mechanism of this condition is apoptosis, or programmed cell death. Finally, preovulatory follicles containing fully-grown oocytes are ready to undergo ovulation, which is triggered by the preovulatory surge of LH (Figure 6). Ovulation is characterized by the rupture of the follicle wall and the release of the cumulus–oocyte complex; at this time the oocyte has resumed meiosis and has progressed to the metaphase II stage of meiosis (Figure 7).

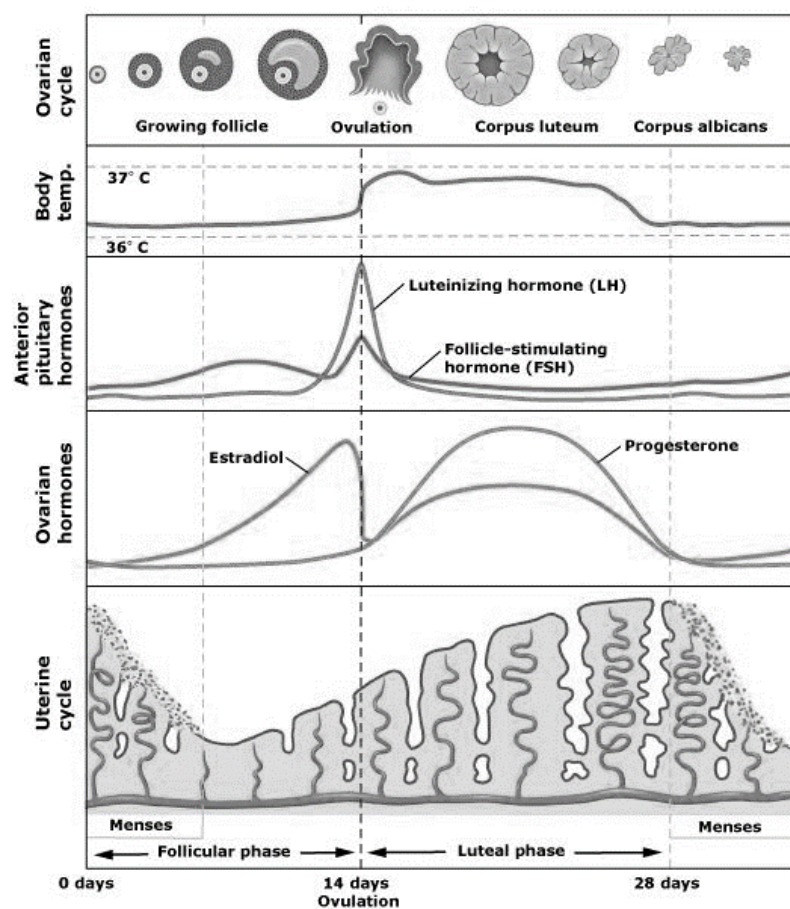


Figure 6. The ovarian and uterine cycles regulated by gonadotropin hormones.

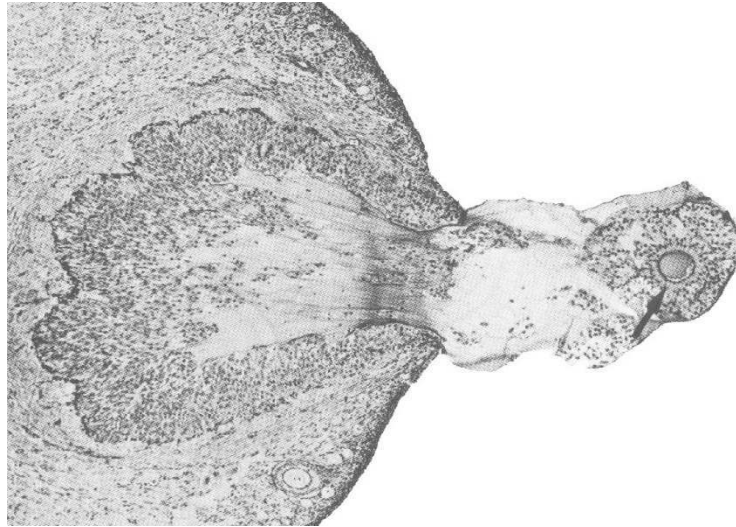


Figure 7. Mature MII oocyte surrounded by cumulus cells ovulated from a mature follicle.

After ovulation, granulosa and theca cells become luteal cells and are responsible for the production of E2 and progesterone (P), the latter is a female hormone predominantly expressed in the *corpus luteum* (Figure 8), which plays a central role in the reproductive events associated with pregnancy establishment and maintenance [Eppig, 2001; Kwintkiewicz and Giudice, 2009]. In particular, high levels of progesterone causes a subsequent decrease in FSH levels due to its negative feedback effects on the hypothalamic-pituitary axis, thus inhibiting the development of other follicles. If egg fertilization does not occur, the *corpus luteum* breaks down causing a decrease in estrogen production with a subsequent increment of FSH secretion due to a positive feedback of these hormones on hypothalamus and pituitary gland, so the reproductive cycle begins again.

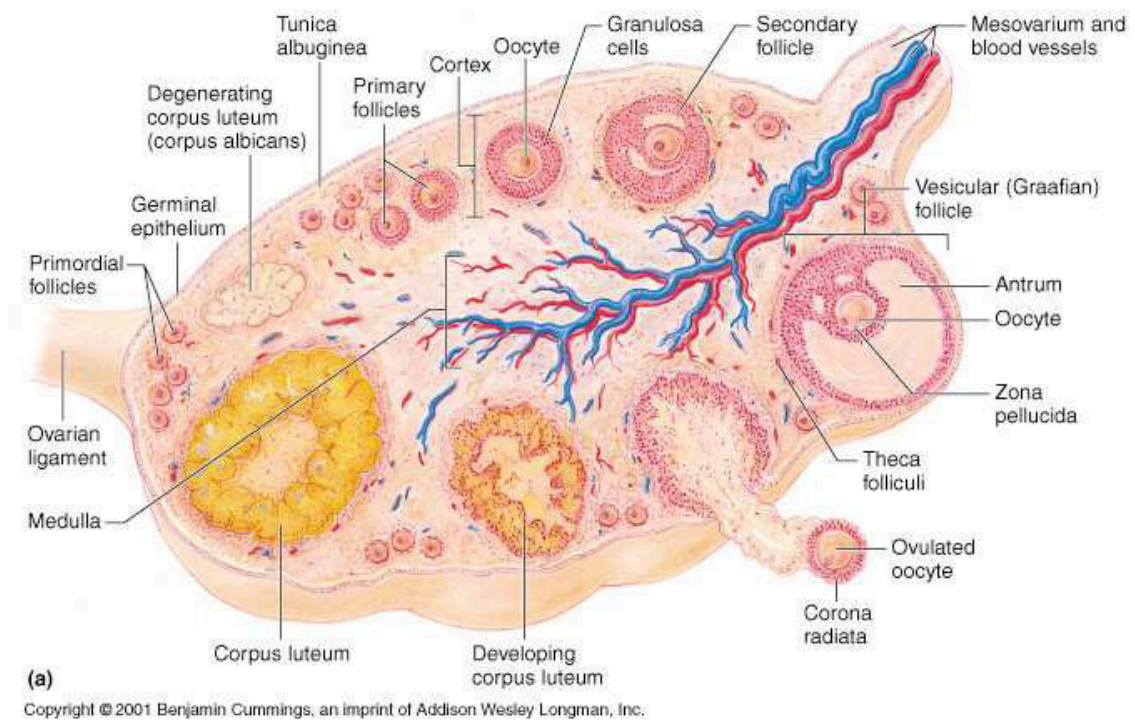


Figure 8. Folliculogenesis and ovulation.

Therefore, the mechanisms underlying oogenesis, folliculogenesis and pregnancy maintenance take place on different scales and rely on a subtle interplay between regulatory signals derived from multiple organs, including the hypothalamus, the pituitary gland and the ovaries that form a functional endocrine axis, known as the hypothalamic-pituitary-ovary (HPO) axis, with hormonal regulations and feedback loops [Bacchus, 1975] (Figure 9). Neurons in the hypothalamus secrete Gonadotropin-releasing Hormone (GnRH) to regulate anterior pituitary gland gonadotrope cells. Gonadotrope cells produce FSH and LH, which control ovarian follicle development and ovulation by stimulating the granulosa cells to synthesize and secrete steroid hormones as well as peptidic hormones, such as estradiol and inhibin (INH) respectively, whose cumulated ovarian release impacts FSH secretion (Figure 9). During their development, antral follicles become more and more sensitive to FSH, then to LH, and they secrete increasing amounts of E2 and INH. The large estradiol amounts

secreted by the preovulatory follicle affect the secretion of GnRH from the hypothalamus and end up by triggering the GnRH ovulatory surge. As a result, the pituitary LH surge occurs and brings about the ovulation event [Clément and Monniaux, 2012], the end-point of follicular phase of the menstrual cycle during which the oocyte is released (Figure 8, 9).

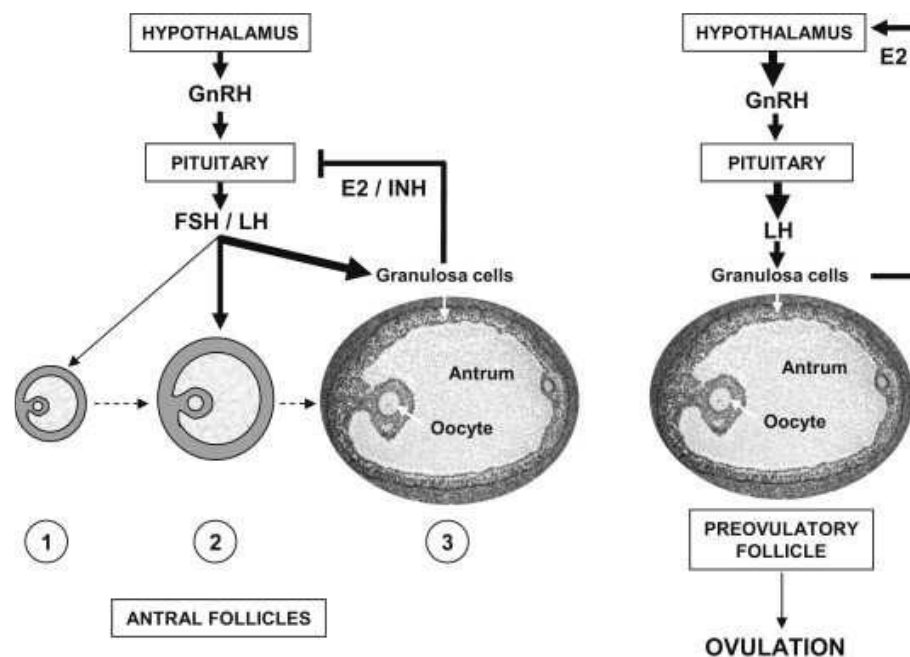


Figure 9. Endocrine feedback loops between the ovary and the hypothalamic-pituitary axis. Left panel: The terminal development of follicles from the small antral growing stage (stage 1) is under the control of FSH secreted by the pituitary gland. In turn, the granulosa cells secrete steroid hormones, such as E2, as well as peptidic hormones, such as INH, whose cumulated ovarian release impacts FSH secretion. During their development, antral follicles become more and more sensitive to FSH, then to LH, and they secrete increasing amounts of E2 and INH (stages 2 and 3). Right panel: The large estradiol amounts secreted by the preovulatory follicle(s) (stage 3) impact the secretion of GnRH from the hypothalamus and end up by triggering the GnRH ovulatory surge. As a result, the pituitary LH surge occurs and results in the ovulation event during which the oocyte is released (from Clément and Monniaux, 2012).

Following ovulation, somatic cells of the ruptured follicle form the *corpus luteum* giving rise to the luteal phase of the menstrual cycle (Figure 8). During this phase, it secretes high levels of progesterone and moderate levels of estradiol to inhibit further release of GnRH and thus secretion of LH and FSH through negative feedback mechanisms, and transforms proliferating endometrium into secretory endometrium. If

pregnancy does not occur, *corpus luteum* degenerates in *corpus albicans* and stops producing progesterone and estrogen, while the secretory endometrium breaks down and sheds during the ensuing menstrual period, thus the cycle starts again.

The role of intracellular communication in ovarian follicle development

Growth and development of the somatic and germ cell compartments of the ovarian follicle occur in a highly coordinated and mutually dependent manner, which means that all these processes rely on continuing cross talk between the oocyte and its somatic cells. Historically, the oocyte was relegated to a passive role in the regulation of folliculogenesis; the key drivers being endocrine and ovarian somatic cell derived hormones and growth factors. However, over the past 20 years, it has become increasingly clear that the oocyte is a pivotal regulator of folliculogenesis, and there exists an important bi-directional communication axis between the oocyte and somatic cells [Albertini and Barret, 2003]. This kind of relationship is mainly based on two means of intercellular communication mediated by gap junctions, also known as connexins, and paracrine factors (Figure 10).

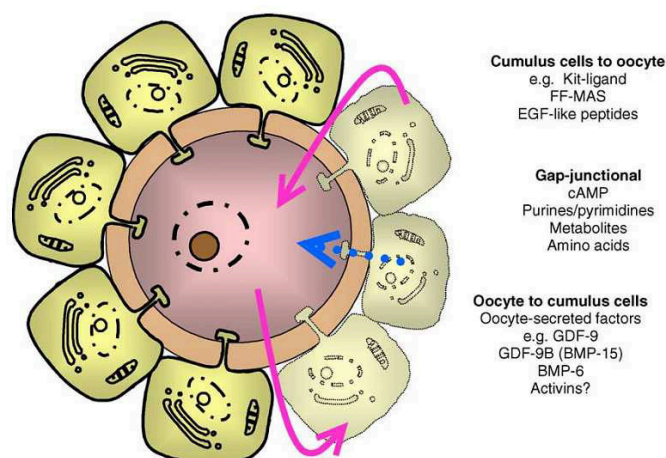


Figure 10. Oocyte-granulosa cell communication is essential for normal growth and development of both the oocyte and the follicle. Communication occurs via paracrine signalling (curved arrows) and gap-

junctional exchange of small regulatory molecules (straight arrow). This is a bi-directional communication axis (from Gilchrist *et al.*, 2004).

The highly specialized cumulus cells have trans-zonal cytoplasmic processes, which penetrate through the zona pellucida (a glycoprotein membrane surrounding the plasma membrane of the oocyte that is secreted by both the oocyte and the follicular cells) and abut the oocyte membrane, forming the cumulus-oocyte complex (COC) [Albertini *et al.*, 2001]. Gap junctions at the ends of these processes allow the transfer of small molecular weight molecules between oocyte and cumulus cell, and also between cumulus cells, whereas larger molecules are transported by receptor-mediated endocytosis [Gilchrist *et al.*, 2004]. Molecules that pass via gap junctions include ions, metabolites, and amino acids that are necessary for oocyte growth, as well as small regulatory molecules that control oocyte development. In particular, connexins 43 (Cx43) and 37 (Cx37), play an important role in the maintenance of this communication: Cx43 is expressed by granulosa cells and required to form gap junctions between granulosa cells whereas Cx37, expressed in oocytes at all stages of follicle development, is crucial for an oocyte–granulosa cell gap junctional communication. In the absence of Cx43, gap junctions between somatic cells do not form and folliculogenesis arrests at the unilaminar stage [Juneja *et al.*, 1999; Gittens *et al.*, 2003; Gittens and Kidder, 2005], whereas mutation of Cx37 abolishes the production of mature Graafian follicles and fully grown oocytes and its meiotic competence is compromised [Simon *et al.*, 1997; Carabatsos *et al.*, 2000]. These experimental evidences demonstrate that this mode of communication in the ovary is essential for development and fertility, and is thought to play a key role in disseminating local and endocrine signals to the oocyte via the cumulus cells [Simon *et al.*, 1997; Buccione *et al.*, 1990].

A second means of communication between oocytes and granulosa cells is via paracrine signalling (Figure 10). Now there is widespread interest in paracrine factors secreted by oocytes and their role in the regulation of key granulosa cell processes and *vice versa*. For example, before the LH surge, oocytes do not only influence granulosa cell proliferation [Vanderhyden *et al.*, 1992; Gilchrist, 2006] and differentiation [Diaz *et al.*, 2007; Diaz *et al.*, 2007], but very importantly, oocytes regulate metabolic activity of cumulus cells within the COC (aminoacid uptake, glycolysis and cholesterol biosynthesis) [Eppig, 2005; Sugiura *et al.*, 2005; Su *et al.*, 2008]. After the LH surge, oocytes regulate the expression of cumulus genes responsible for the mucification/expansion process [Sánchez and Smitz, 2012] (Figure 11).

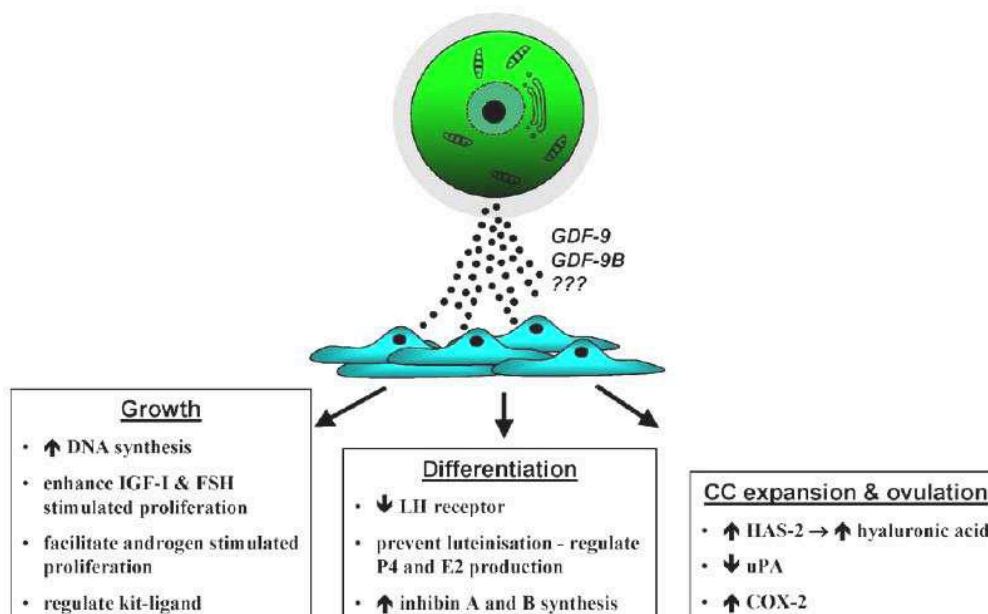


Figure 11. Oocytes secrete soluble growth factors that regulate fundamental aspects of granulosa cell functions, including growth, differentiation, cumulus cell expansion and ovulation. The direct actions and secondary consequences of oocyte-secreted factors impact on fertility (from Hawkins and Matzuk, 2010).

Much of scientific interest has focused on oocyte-secreted TGF- β superfamily members, in particular growth differentiation factor-9 (GDF-9), GDF-9B (also called bone morphogenetic protein-15; BMP-15) (Figure 10, 11). Interest in these oocyte-secreted

factors has been fostered not only by the need to improve our understanding of fundamental mechanisms regulating folliculogenesis, but also because altered expression of some of these oocyte paracrine factors has profound effects on ovarian function and fertility, and perhaps ovarian disease [Buccione *et al.*, 1990; Simon *et al.*, 1997; Gilchrist *et al.*, 2004; Yeo *et al.*, 2009]. Targeted deletion to produce *gdf9*^{-/-} mice causes an infertile phenotype in which follicles halt at the one-layer stage with enlarged oocytes thus demonstrating that GDF-9 is needed for granulosa cells to proliferate [Dong *et al.*, 1996; Gosden, 2002]. This feedback communication between oocytes and somatic cells is also necessary to coordinate meiotic resumption and ovulation. Moreover, it has been demonstrated that immature or incompetent oocytes which fail to interact appropriately in this communication loop are not released [Albertini, 1992; Albertini *et al.*, 2003; Plancha *et al.*, 2005]. The ovulation process may therefore be considered a checkpoint event whereby only healthy and meiotically competent oocytes engineer their own release, thus ensuring a sort of natural selection mechanism of the best gamete among all those belonging to the reproductive pool [Russell and Robker, 2007].

Although many studies have contributed in pointing out to the significance of the interaction between oocyte and its surrounding somatic cells, there is still much to be learned about molecular pathways controlling early stages of oogenesis, folliculogenesis and oocyte maturation, which are determined by complex activation and interplay of many factors acting in a stage-specific manner. Since the follicle-oocyte dialogue seems to be a prerequisite to ensure a proper development and to preserve fertility, it is extremely important to elucidate the molecular basis of these events.

Storage and regulation of maternal RNAs

During oogenesis, oocytes increase in size (approximately 35-120µm in human) and in volume (~100-fold) [Eppig and O'Brien, 1996; Picton *et al.*, 1998]. Fully-grown and meiotically competent murine oocytes have been estimated to contain ~6 ng of total RNA which is almost ~200 times the amount of RNA found in a typical somatic cell [Sánchez and Smitz, 2012]. Throughout this period, oocytes synthesize and accumulate RNAs (mRNAs, rRNAs, tRNAs, small RNAs) and proteins that are vital for their appropriate growth and maturation, and indispensable for the development into a viable embryo [Moore and Lintern-Moore, 1978; Bachvarova, 1985; Eichenlaub-Ritter and Peschke, 2002]. Synthesis of transcripts is highest in the earliest phases of development, which coincides with active proliferation of follicular cells; however, by the end of growth (antral stages) and at the time of oocyte maturation, silencing of transcriptional activity and a selective degradation of some mRNAs will be the predominant processes [Bachvarova *et al.*, 1985; De La Fuente *et al.*, 2004; Su *et al.*, 2007]. In mouse, a decrease of ~30% in total RNA has been described to occur during maturation [Paynton *et al.*, 1988]. Additionally, during the maternal-zygotic transition, embryonic transcription is initiated and other maternal mRNAs are inactivated or degraded through co-ordinated post-transcriptional regulation. Accumulating evidence indicates that a class of small silencing RNAs, particularly microRNAs (miRNAs), is implicated in the elimination of maternal transcript by their binding to the 3' untranslated region (UTR) of target RNAs. So far, very few studies have analysed the expression of and the role played by miRNAs during oocyte growth and preimplantation development [Tang *et al.*, 2007]. Thus, it is imperative to examine transcription not only in the oocyte but also in somatic cells as well as FF when investigating what is needed to make a developmentally competent egg. The identification of oocyte-quality molecular markers that could be used to predict the developmental competence of oocytes more precisely

could be of help in establishing more objective criteria for the selection of the best gamete during medically assisted reproduction cycles.

MicroRNAs

miRNAs are short 20–22 nucleotides (nts) RNA molecules that function as negative regulators of gene expression in eukaryotic organisms. These highly conserved RNAs regulating gene expression constitute about 1-5% of predicted genes in animals genomes, and 10-30% of protein-coding genes are probably regulated by miRNAs. Recently, many small non-coding RNAs have been also identified in prokaryotic organisms and viruses. Since they were recognized in 2001, the biological significance of these newly identified small RNAs was elucidated, because more and more evidences suggest that miRNAs play an essential role in multiple biological processes through negative regulation of gene expression at a post-transcriptional level. They perform their functions by partial pairing with one or more 3'UTR of mRNA targets to promote their degradation or transcriptional repression [Bartel, 2009]. The majority of known miRNAs are evolutionarily conserved among species, demonstrating that miRNA mediated gene silencing pathways have essential roles in development, cell differentiation, cell proliferation, cell death, chromosome structure and virus resistance, signalling transduction, disease and cancer [Lee and Ambros, 2001; Zhang *et al.*, 2007]. From a functional point of view, it has been demonstrated that most of miRNAs are able to recognize several mRNA targets; on the other hand, one specific mRNA can be regulated by more than one miRNA [Kim and Nam, 2006]. Moreover, studies from the last years have demonstrated that there is altered expression of miRNA genes in many human malignancies.

Genomic location of miRNA genes

It was estimated that the human genome contains 1600 miRNA loci, encoding for 2042 mature miRNAs, distributed in all chromosomes except for Y chromosome, according to the latest release of miRBase database, the primary online repository for all miRNA sequences and annotation, where all known and newly identified miRNAs are deposited (www.mirbase.org). The identification of miRNA genes is the result of a combination of directional cloning together with computational approaches, based on the examination of genomic sequences to identify phylogenetic conservation of known miRNA genes, taking in account the high level of 5' region conservation in all miRNA sequences. An integrative approach leading to the identification of a significantly higher number of miRNAs than previously expected in humans combined bioinformatic predictions with microarray analysis and sequence-directed cloning, allowing the identification of a huge amount of non-conserved miRNAs [Bentwich *et al.*, 2005]. Around 50% of the known miRNAs are organized in genomic clusters and are transcribed as polycistronic primary transcripts. Generally, miRNAs belonging to the same cluster are structurally and functionally related to each other, demonstrating their origin as a result of duplication events during evolution; on the other hand, some miRNAs lying in close genomic regions can be unrelated to each other. One possible explanation for the existence of clustered miRNAs with related functions or structure is the possibility that they may play a synergic role in regulating the same genes, or different genes involved in the same pathway [Kim and Nam, 2006]. Despite it was originally inferred that most of miRNAs are located in intergenic regions, more recent analysis have shown that a vast majority of mammalian miRNA genes is located in well-defined transcription units, many of them within introns of coding genes in the sense orientation. Based on these findings, miRNAs can be categorized as follows:

a) intronic miRNAs in coding regions;

- b) intronic miRNAs in non-coding regions;
- c) exonic miRNAs in coding regions;
- d) exonic miRNAs in non-coding regions (Figure 12).

Based on the splicing pattern, some miRNAs have mixed features; moreover, for miRNAs comprised within coding regions, as expected for genes sharing the same promoters, miRNAs usually have similar expression profiles to their host transcript [Kim and Nam, 2006].

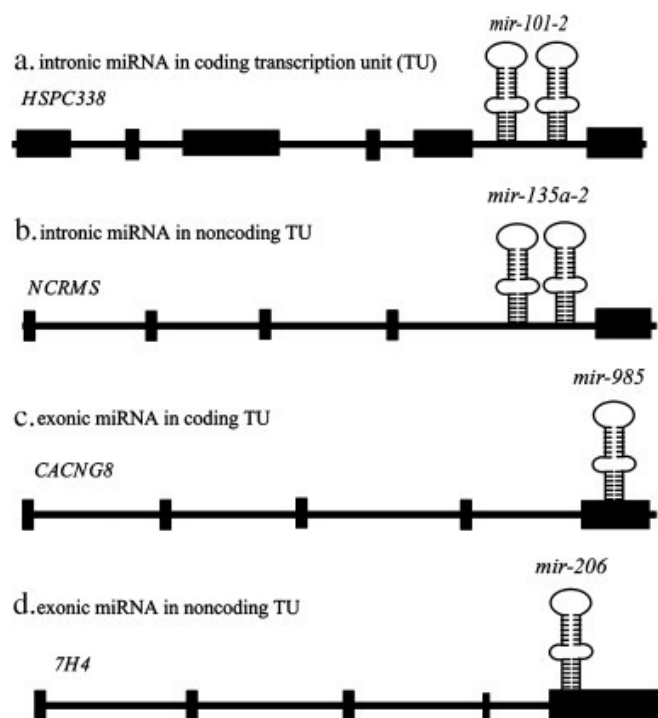


Figure 12. Genomic organization of miRNA genes (from Wahid *et al.*, 2010).

miRNA biogenesis

miRNA biogenesis is a multistep process where a mature miRNA is generated from a miRNA gene, with several enzymes playing critical roles in the process (Figure 13). As it was already discussed, intronic miRNAs share their regulatory elements and primary transcript with their pre-mRNA host genes, and are undoubtedly transcribed by RNA

polymerase II (pol II). This gives a possible mechanism for coordinated miRNA and protein coding gene expression [Rodriguez *et al.*, 2004].

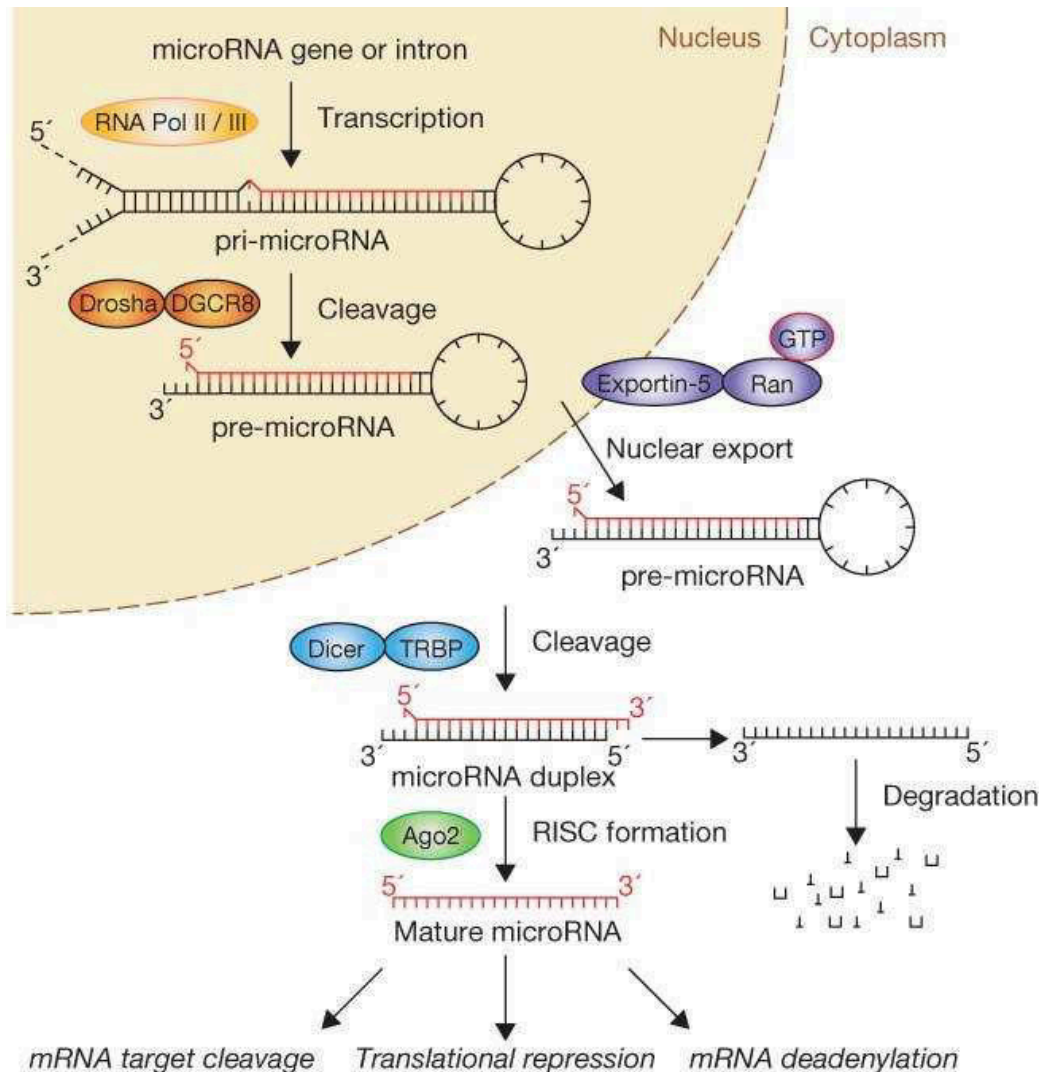


Figure 13. miRNA biogenesis pathways and their regulation (from Winter *et al.*, 2009).

All the characterized promoters for the other miRNA genes contain general RNA polymerase II transcriptional regulatory elements previously found in protein-coding genes: this, together with other experimental evidences, strongly suggests that all miRNAs are pol II products [Lee *et al.*, 2004]. miRNA gene transcription by pol II produces a pri-miRNA of several hundreds of nts in length, with a 5' cap and a polyadenylated 3', which forms a hairpin stem-loop secondary structure within the

nucleus and enters a large complex called microprocessor complex (500–650 kDa). The main components of this complex are Drosha (an RNase III endonuclease) and the essential cofactor DGCR8/Pasha (containing two doublestranded RNA binding domains). Drosha asymmetrically cleaves both strands of the hairpin stem at sites near the base of the primary stem loop thus releasing a 60- to 70-nucleotides pre-miRNA with a 5' phosphate and a 2-nucleotide 3' overhang (Figure 13). This process is highly specific and predetermines the sequence of mature miRNA [Lee *et al.*, 2004]. The pre-miRNAs are then transported to the cytoplasm by Exportin-5 (Exp5) (a member of the Ran transport receptor family): this transport process requires energy, a specific hairpin secondary structure, and is Ran-GTP dependent. When the pre-miRNA reaches the cytoplasm, one of its ends has already been predetermined by Drosha cleavage site selection. Once in the cytoplasm, the pre-miRNAs enter the RISC loading complex (RLC), made by Dicer (a second RNase endonuclease), TRBP (able to bind double stranded RNAs), PACT (protein activator of PKR) and Ago-2 [Gregory *et al.*, 2005; Haase *et al.*, 2005; Lee *et al.*, 2006]. Dicer recognizes the 3' overhangs of the pre miRNA through the PAZ domain, and its cleavage 22-nt from the end of the substrate releases a mature double-stranded miRNA (miRNA:miRNA* duplex) with 5' phosphates and a 2-nt 3' overhang [Gregory and Shiekhattar, 2005]. Finally, miRNA:miRNA* duplex is unwound by helicase into two single strands: in the majority of cases miRNA*, which is the less thermodynamically stable strand at 5' end, is degraded, while the leading strand is incorporated into a ribonucleoprotein effector complex known as RNA-induced silencing complex (RISC) which induces gene silence at a post-transcriptional level [Zhang *et al.*, 2007].

miRNA mediated post-transcriptional repression

After being processed, miRNAs are assembled into ribonucleoprotein complexes called

RISC (RNA-induced silencing complexes), which identify miRNA targets by the perfect or nearly perfect complementarity between the miRNA and the mRNA. The better characterized components of RISC are Argonaute (AGO) proteins, with PAZ and PIWI domains: in mammals, four AGO proteins (AGO1 to AGO4) function in the miRNA repression but only AGO2 functions in RNA interference (RNAi), which is the process guiding the endonucleolytic cleavage of the target mRNA. Beyond Ago proteins, the complex contains other proteins playing critical roles as regulators of the main effectors of RISC inhibitory functions [Tolia and Joshua-Tor, 2007]. The complementary sites for miRNAs associated with RISC reside in the 3'UTRs of target mRNAs and are generally present in multiple copies as a necessary condition to reach an efficient translational inhibition [Easow *et al.*, 2007]. The specific site of mRNA recognition in miRNA molecules is called seed region, and is comprised between nucleotides 2-8; generally, in metazoan miRNAs bind the target mRNA with mismatches and bulges, in contrast, most plant miRNAs bind with near-perfect complementarity to sites within the coding sequence of their targets [Carthew and Sontheimer, 2009] (Figure 14).

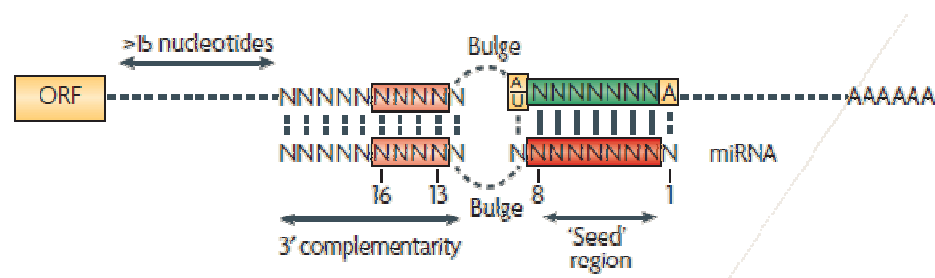


Figure 14. Principles of microRNA–mRNA interactions in metazoan. The base pairing is perfect at the seed region and at miRNA 3' half, while bulges and mismatches can be present in the central region (from Filipowicz *et al.*, 2008).

Once associated to the target mRNA, RISC inhibitory functions can take place through several possible mechanisms:

- translational repression, in presence of mismatches in the core part of the seed region;
- mRNA deadenylation and decay in case of perfect complementarity with the target mRNA (Figure 15).

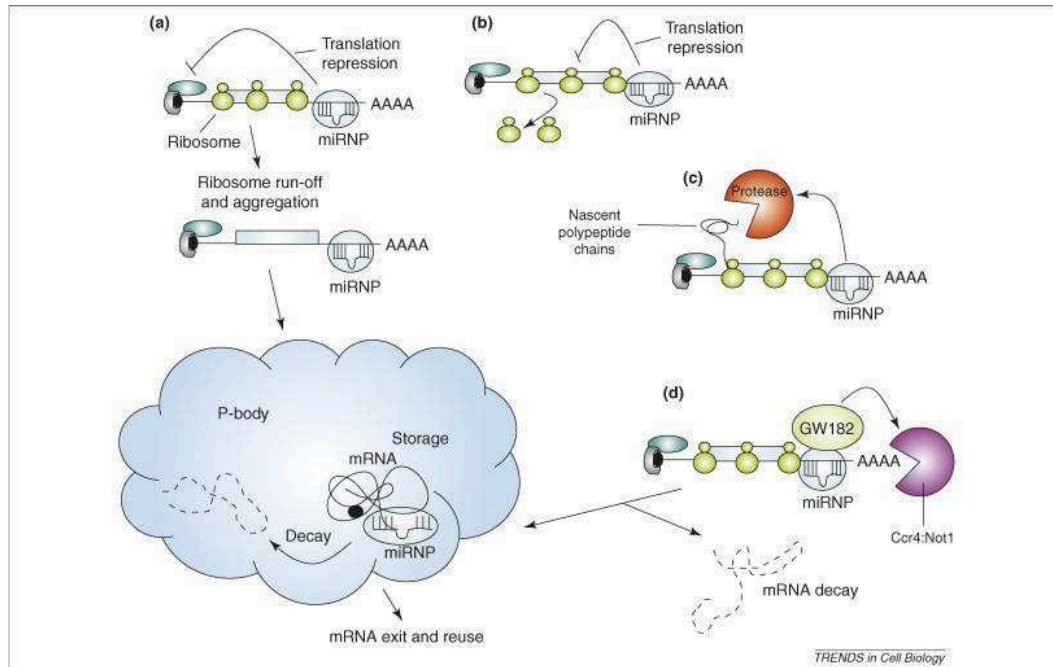


Figure 15. Mechanisms of miRNA mediated post-transcriptional repression: a) repression of transcription initiation; b) inhibition of protein chain elongation; c) lysis of nascent peptide; d) mRNA deadenylation (from Pillai *et al.*, 2007).

Translational repression can occur at the initiation or at post-initiation steps. Initiation of translation of most cellular mRNAs starts with the recognition of the mRNA 5'-terminal-7-methylguanosine (m^7G) cap by the eukaryotic translation initiation factors (eIF) 4E, 4F and 4G, which together with PAPB1 are responsible for the association between the mRNA ends, triggering the translation process [Filipowicz *et al.*, 2008]. Ago2 and related proteins can compete with eIF4E for m^7G binding, preventing translation. An alternative mechanism of miRNA action was recently proposed at a post-initiation step, since studies with reporter mRNAs targeted by either synthetic or endogenous miRNAs have shown that repressed mRNAs were associated with active polysomes; nevertheless, how miRNAs could modulate the elongation or termination

process remains unclear [Petersen *et al.*, 2006]. In eukaryotes, mRNA degradation can follow two pathways, both of them initiated by a gradual shortening of the mRNA poly(A) tail. The mRNA body can then be degraded by progressive 3'→5' decay, which is catalysed by the exosome, or by the removal of the cap followed by 5'→3' degradation, catalysed by the exonuclease XRN1. The final steps of mRNA degradation occur in P-bodies, cytoplasmic structures enriched in proteins participating in miRNA repression functions such as AGO proteins and GW182, and miRNAs themselves [Filipowicz *et al.*, 2008]. Recent findings demonstrated that lowered mRNA levels account for most ($\geq 84\%$) of the decreased target's protein production: by using ribosome profiling to measure the overall effects on protein production and compare these to simultaneously measured effects on mRNA levels, Guo *et al.* showed that changes in mRNA levels closely reflect the impact of miRNAs on gene expression and indicate that destabilization of target mRNAs is the predominant reason for reduced protein output [Guo *et al.*, 2010].

miRNAs: new candidates for the regulation of the human COC

As previously described, in the ovarian follicle, the maturing oocyte is nurtured and supported by CCs, the surrounding somatic cells. Disruption or deregulation of the CC interactions with the oocyte can affect oocyte quality and consequently embryo development and pregnancy outcome. Much knowledge on human oocytes and CCs has been generated over recent years mainly owing to technological advances in gene expression analysis [Assou *et al.*, 2011]. Although such techniques have allowed entire profiling of the transcriptional activity in single human MII oocytes and the surrounding CCs [Assou *et al.*, 2006; Kocabas *et al.*, 2006; Grondahl *et al.*, 2010], leading to the identification of several transcripts that are crucial for oogenesis and folliculogenesis,

however, the post-transcriptional regulation of these transcripts needs to be elucidated. This is particularly important also because the stability and translation of the maternal mRNAs, that are accumulated during oocyte maturation [Niakan *et al.*, 2012] and that drive human preimplantation development, are controlled by post-transcriptional regulatory mechanisms [Bettegowda and Smith, 2007]. The miRNA repertoires are cell type specific and change markedly during development [Carthew and Sontheimer, 2009]. Changes in miRNA expression profiles have been linked to pathologies, such as cancer [Ventura and Jacks, 2009]. Moreover, miRNAs have been associated with infertility as shown in female mice in which Dicer, an essential factor in miRNA biogenesis, was genetically ablated [Murchison *et al.*, 2007; Nagaraja *et al.*, 2008]. Furthermore, the expression of several miRNAs, such as miR132 and miR212, has been found to be regulated by gonadotropins (FSH and LH) [Fiedler *et al.*, 2008; Yao *et al.*, 2009], even if this mechanism has not been fully investigated so far. Finally, analysis of mRNA expression during mouse and bovine oogenesis shows that a large proportion of maternal genes are regulated by miRNAs [Tang *et al.*, 2007]. Therefore, regulation of gene expression in the oocyte throughout oogenesis at the transcriptional and post-transcriptional level is a crucial process that is tightly controlled in a stage-dependent manner, and this process ultimately ensures that the oocyte will mature and acquire full developmental competence. Thus, miRNA profiling might help us to better understand the regulation of transcripts involved in human reproduction.

miRNAs in body fluids: new powerful biomarkers

MiRNAs are involved in virtually all biologic processes and, because a single miRNA can target hundreds of mRNAs, aberrant miRNA expression is involved in the initiation of many diseases, including cancer. Distinguishable abnormalities in miRNA genes and

expression patterns were identified in almost all types of cancer, thus providing a strong rationale for the application of miRNAs as diagnostic/prognostic biomarkers. Expression profiles of miRNAs have been found significantly altered in numerous types of human cancers when compared with their corresponding normal tissues, among different subtypes within the same type of cancers, or among individual patients suffering from a same type of cancer but having different prognoses [Yan *et al.*, 2008; Lebanony *et al.*, 2009]. The analyses of miRNA signatures are in general limited to tissue biopsies; however, in the last few years several studies have shown the diagnostic and prognostic usefulness of circulating miRNAs, released by some cell types both under normal and pathological conditions [Chen *et al.*, 2008] (Table 1). One of the first studies measuring miRNA levels in serum demonstrated that levels of miR-21 were associated with relapse-free survival in patients with diffuse large B-cell lymphoma [Lawrie *et al.*, 2008]. Currently, one of the most complete studies highlighting the role of circulating miRNAs was carried out on a cohort of 303 non-small-cell lung cancer (NSCLC) patients through Solexa sequencing, and led to the identification of eleven serum miRNAs significantly altered between longer-survival and shorter-survival groups, while some of them were associated to overall survive [Hu *et al.*, 2010].

Table 1. Serum miRNAs as biomarkers for cancer (from Kosaka *et al.*, 2010).

Type of cancer	Biomarker candidate
Diffuse large B-cell lymphoma (DLBCL)	Expression levels of miR-155, miR-210 and miR-21 were higher in DLBCL patient than control sera
Prostate cancer	High miR-21 expression was associated with relapse-free survival Serum levels of miR-141 can distinguish patients with prostate cancer from healthy controls
Ovarian cancer	The levels of the 8 specific miRNAs were similar between cellular and exosomal miRNAs. Exosomal miRNA from ovarian cancer patients exhibited similar profiles, which were significantly distinct from profiles observed in benign disease miR-21, -92, -93, -126 and -29a were significantly overexpressed in the serum from cancer patients compared to controls
Non small cell lung cancer	Eleven serum miRNAs were found to be altered more than 5-fold between longer-survival and shorter-survival groups, and levels of four miRNAs were significantly associated with overall survival miR-92a decreased in the plasmas of acute leukemia patients
Acute myeloid leukemia (AML) Acute lymphoblastic leukemia (ALL) Breast cancer	Increased miR-195 levels in patients were reflected in tumors, and circulating levels of miR-195 and let-7a decreased in cancer patients postoperatively, to levels comparable with control subjects miR-155 was differentially expressed in the serum of women with hormone-sensitive compared to women with hormone-insensitive breast cancer
Gastric cancer	The plasma concentrations of miR-17-5p, miR-21, miR-106a, and miR-106b were significantly higher in patients than controls, whereas let-7a was lower in patients
Pancreatic cancer	Circulating miR-210 levels are elevated in pancreatic cancer patients
Pancreatic ductal adenocarcinoma	The combined analyses of four miRNAs (miR-21, miR-210, miR-155, and miR-195a) in plasma can discriminate patients from normal healthy individuals
Squamous cell carcinoma (SCC) of tongue	Plasma miR-184 levels were significantly higher in tongue SCC patients in comparison with normal individuals, and the levels were significantly reduced after surgical removal of the primary tumors
Colorectal cancer	Both miR-17-3p and miR-92 were significantly elevated in the patients, and the plasma levels of these miRNAs were reduced after surgery
Hepatocellular carcinoma (HCC)	An increased amount of miR-500 was found in the sera of the HCC patients, and its levels in sera returned to normal after the surgical treatment

So far, miRNAs have been found in human serum, plasma, and other body fluids [Takamizawa *et al.*, 2004; Peter, 2009; Wurz *et al.*, 2010] and specific compositions and concentrations were found for each body fluid analysed. The miRNA profiles of these fluids have been found to be associated not only with cancer and other diseases [Calin and Croce, 2006; Chun-Zhi *et al.*, 2010; Pineau *et al.*, 2010; Zhang *et al.*, 2010] but also with healthy conditions. In fact, in healthy individuals the levels of cell-free miRNAs present in sera are stable [Chen *et al.*, 2008; Gilad *et al.*, 2008]. Mitchell and colleagues showed that under healthy conditions, the serum miRNA profile is similar to that of circulating blood cells [Mitchell *et al.*, 2008]. Thus, alterations of serum miRNA levels may be indicative of physiological or pathological changes and may possibly be

used as surrogate biomarkers [Cortez and Calin, 2009]. For example, circulating miRNAs were found in the sera of pregnant women [Gilad *et al.*, 2008]: miR-526a, miR-527 and miR-520d-5p showed a considerably high fold-change and could be used to distinguish pregnant from non-pregnant women with high accuracy. Moreover, placenta derived miRNAs (e.g. miR-141, miR-149, miR-299-5p and miR-517a) are detectable in the maternal plasma, and their concentrations decrease directly after childbirth [Chim *et al.*, 2008; Luo *et al.*, 2009]. Therefore, miRNAs have been discussed as novel non-invasive markers for prenatal diagnosis [Chim *et al.*, 2008]. miRNAs have also been identified in the ovarian tissues of other species, such as mouse [Ahn *et al.*, 2010], goat [Zhang *et al.*, 2013], ruminant [McBride *et al.*, 2012], etc. Moreover, miRNAs has been found in equine and human FF [Da *et al.*, 2012; Sang *et al.*, 2013] whose composition is tightly correlated with the developmental competence of the human oocyte [Wallace *et al.*, 2012]. It contains essential substances involved in follicle growth, oocyte fertilization, glucose and lipoprotein metabolism which have been found to be associated with reproductive disorders such as premature ovarian failure (POF) and polycystic ovary syndrome (PCOS) [Dai and Lu, 2012]. Accordingly, the discovery of miRNAs in body fluids, particularly in FF, opens up the possibility of using them as non-invasive biomarkers in human reproductive diseases; moreover, given that FF is easily available during oocyte pick up, these miRNAs can be used as non-invasive predictors of oocyte quality in order to select the best gamete during medical assisted reproductive cycles.

Circulating miRNA stability and possible release mechanisms

To be able to use circulating miRNAs as a diagnostic marker, we need to gain a better understanding of the mechanisms by which miRNAs are released in the bloodstream.

Cell-free miRNAs in body fluids are stable under harsh conditions that would normally degrade most RNAs, including boiling, low/high pH, extended storage and multiple freeze-thaw cycles [Chen *et al.*, 2008]. Moreover, there is evidence that serum miRNAs are particularly resistant to RNases digestion, since they can be detected in this body fluid containing high levels of these enzymes; this implies that they should be released in the bloodstream in a form that protects them from degradation. Some hypotheses have proposed that they are secreted in protected protein–miRNA complexes (AGO2, nucleophosmin 1-NPM1, and other RNA-binding proteins); another hypothesis is that they are produced as by-products of dead cells [Vickers *et al.*, 2011; Xu *et al.*, 2012]. Recent studies have demonstrated a novel mechanism of cell communication using miRNAs released in microvesicles (up to 1 μm), or in small membrane vesicles called exosomes (10-100 nm) [Kosaka *et al.*, 2010] (Figure 16).

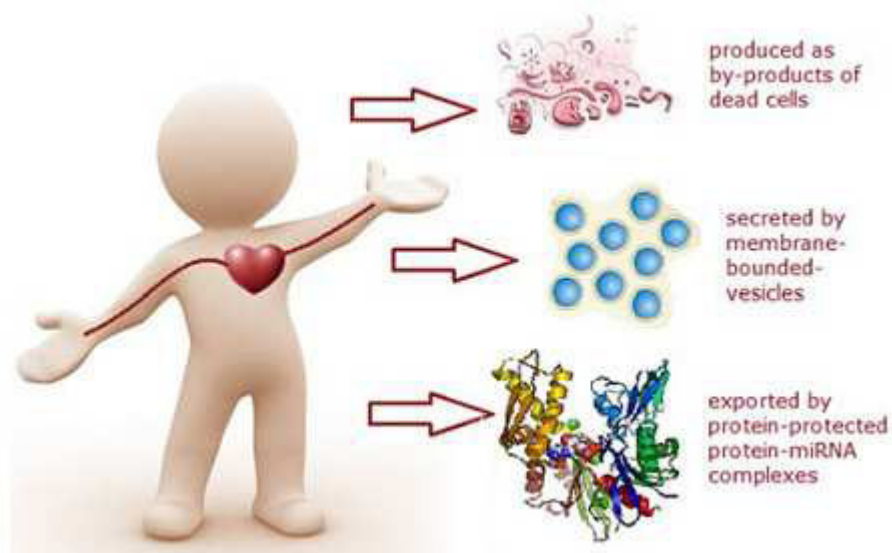


Figure 16. Origin of circulating miRNAs (from Xu *et al.*, 2012).

These small vesicles of endocytic origin, which can also contain mRNAs and proteins in addition to miRNAs, can be formed through inward budding of endosomal

membranes, giving rise to intracellular multivesicular bodies (MVB) that later fuse with the plasma membrane, releasing the exosomes into the extracellular environment. In any case, circulating miRNAs enter the bloodstream and are taken up by the recipient cells by endocytosis or, hypothetically, by binding to receptors present at the recipient cellular membrane capable of recognizing RNA-binding proteins (Figure 17). However, more studies are necessary to elucidate how miRNAs are loaded into exosomes and how they can be internalized by recipient cells.

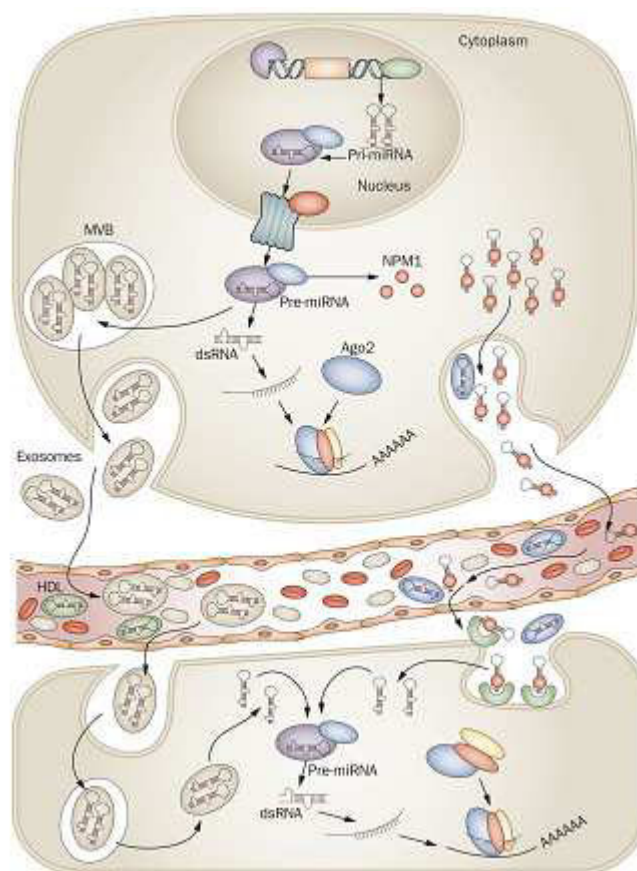


Figure 17. Biogenesis and mechanism of action of circulating miRNAs (from Cortez *et al.*, 2011).

Defining exosomes

Although potentially any vesicle released by a cell carries cell type specific membrane and cytosolic components, there are a number of features that should be taken into consideration for the characterization of exosomes. Exosomes are between 40 and 100

nm in diameter, appear with a characteristic cup-shaped morphology (after negative staining) or as round well delimited vesicles as observed by transmission and cryo-electron microscopy, respectively [Conde-Vancells *et al.*, 2008]. Exosomes float on sucrose gradient to a density that ranges from 1.13 to 1.19 g/ml [Thery *et al.*, 2006]. Apart from their morphology, their unique protein and lipid composition enable their identification. Notably, these include cytoplasmic proteins such as tubulin, actin, actin-binding proteins, integrins, annexins and Rab proteins as well as molecules responsible for signal transduction (protein kinases, heterotrimeric G-proteins) [Thery *et al.*, 2001; Skokos *et al.*, 2001; Skokos *et al.*, 2001]. Most exosomes also contain MHC class I molecules [Wolfers *et al.*, 2001; Blanchard *et al.*, 2002] and heat-shock proteins such as Hsp70 and Hsp90 [Thery *et al.*, 2001; Thery *et al.*, 2002]. The protein family most commonly associated with exosomes is the tetraspanins including CD9, CD63, CD81 and CD82 [Escola *et al.*, 1998; Bard *et al.*, 2004; Chaput *et al.*, 2005] (Figure 18). While some of the proteins that are found in the proteome of many exosomal membrane preparations may merely reflect the cellular abundance of the protein, others are specifically enriched in exosomes and can therefore be defined as exosomal marker proteins (e.g. Alix, flotillin, TSG101, CD63). Another feature of exosomes is their enrichment in raft-lipids such as cholesterol, sphingolipids, ceramide and glycerophospholipids with long and saturated fatty-acyl chains [Wubbolts *et al.*, 2003; Subra *et al.*, 2007; Trajkovic *et al.*, 2008]. Conversely, other exosomal proteins directly represent the proteome of the source cells. For example, analysis of urinary vesicles showed a link between exosomes containing aquaporin-2 and their origin from the urogenital tract [Pisitkun *et al.*, 2004].

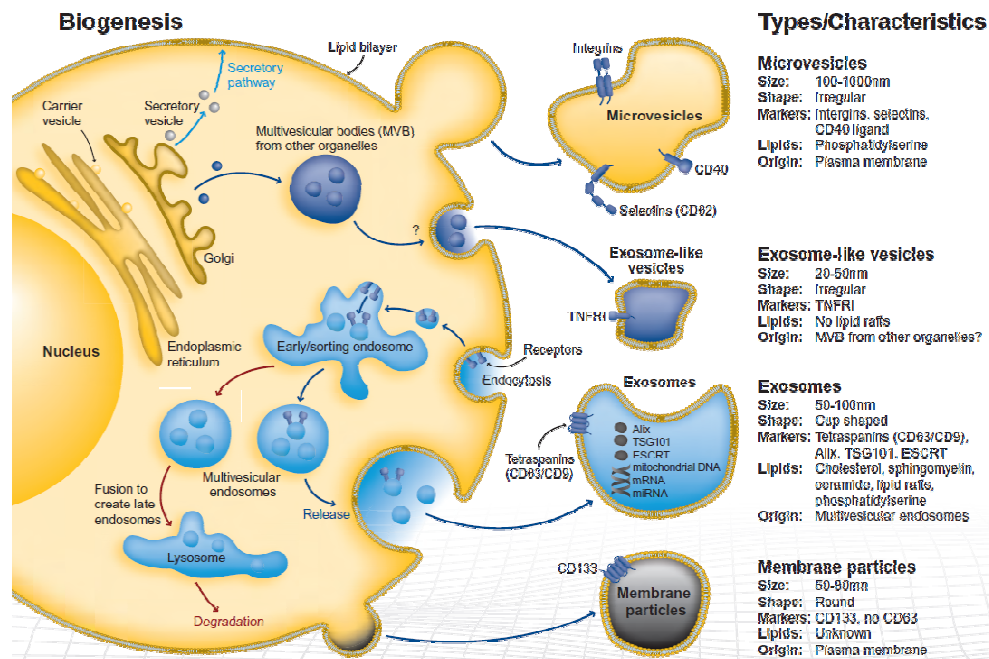


Figure 18. Biogenesis, characteristics and functions of secreted extracellular vesicles.

Exosomes in biological fluids

Exosomes have been detected in many biological fluids, where they are secreted both under physiological and pathological conditions (e.g. blood, plasma, urine and cancerous pleural effusions); besides they are actively produced by many cultured cell types [Mathivanan *et al.*, 2010]. Among the possible biological functions exerted by exosomes, they have been demonstrated to play a significant role in signalling, immune response, and tumor development [Denzer *et al.*, 2000; Anand, 2010; Zhang and Grizzle, 2011]. The increased levels of tumor-derived exosomes in plasma and malignant effusions of patients with cancer suggest that exosomes can be a rich source for the discovery of blood-based diagnostic biomarkers of disease. One of the first reports showing the existence of miRNAs in exosomes was published by Valadi *et al.*, who reported that exosomes released from human and murine mast cell lines contain mRNAs and miRNAs [Valadi *et al.*, 2007]. Since then, many studies started to focus on the expression profiling of miRNAs from exosomes isolated from plasma of diseased patients, in order to identify new miRNA markers that are easily measurable in blood

samples. Two pivotal studies led to the identification of eight putative miRNA markers for the diagnosis of ovarian cancer (miR-21, miR-141, miR-200a, miR-200c, miR-200b, miR-203, miR-205, and miR-214) and twelve specific miRNAs (miR-17-3p, miR-21, miR-106a, miR-146, miR-155, miR-191, miR-192, miR-203, miR-205, miR-210, miR-212, and miR-214) for lung cancer, respectively: in both cases it was demonstrated that miRNA profiling could be performed in the absence of tissue and accurately reflects the tumor's profile [Taylor and Gercel-Taylor, 2008; Rabinowits *et al.*, 2009]. However, up to date very little is known about the presence of exosomes in FF since the only paper published on this subject is limited to equines. da Silveira and colleagues reported the presence of cell-secreted vesicles containing miRNAs and proteins within equine ovarian FF and demonstrated that several exosomal miRNAs, being expressed exclusively or at higher levels in FF from old mares with respect to the young counterpart, could represent novel biomarkers of the age-related decline in oocyte quality and competence [da Silveira *et al.*, 2012].

It is well known that a proper cell communication within the ovarian follicle is critical for the growth and maturation of a healthy oocyte that can be fertilized and develop into an embryo. Thus, the detection of exosomes even in human FF could reveal a new mechanism of communication between oocyte and follicular cells, as well as elucidate the critical role played by their miRNA cargo in the signalling pathways required for follicle development.

Women's infertility: reasons and remedies

On average, optimal female fertility is maintained until 30 years of age and then decreases sharply as women towards the menopause, a process referred to as female reproductive ageing. It is dictated by a gradual decrease in both the quantity and the

quality of the oocytes and, therefore, tightly correlated with the depletion of the pool of ovarian follicles and aneuploidy increase. Accordingly, the modern trend to delay childbearing in the last 30 years has notably increased the risk of infertility. Nowadays infertility affects approximately 15% of reproductive-age couples [Uyar *et al.*, 2013] and in addition to medical consequences, has significant social and financial implications. Other than reproductive ageing, the main causes responsible for altering fertility are environmental factors (i.e. smoke, diet, environmental pollution), life-style related risks (sports, jobs), diseases (i.e. endometriosis, POF, PCOS) and medical treatments (i.e. chemotherapy, radiotherapy, surgery). Among the treatment modalities available to infertile couples, IVF offers the highest success rates of pregnancy and live-birth outcomes. However, as experienced by many couples with infertility problems, an ovulated MII oocyte is not always a good egg, as it may resist fertilization or, when fertilized, may not be competent to sustain development. Since the quality of the female gamete has an impact on rates of preimplantation, implantation and pregnancy, a critical step in IVF treatment is assessment of oocyte competence to determine the most viable gamete to be transferred. To this aim, eggs have been studied so far to identify non-invasive markers that would help the selection of the gametes to fertilize.

Another challenge in IVF is the choice of the best option to preserve the oocytes of those women at risk of losing their fertility (i.e. after disease, surgery or chemotherapy), thus safeguarding their potential to become pregnant in the future. To date oocyte cryopreservation is considered the best helpful technique for fertility preservation and, particularly, vitrification is the most suitable protocol to be used for this purpose. Unlike the slow-cooling method, ultrarapid freezing rates applied for vitrification allow the formation of a glass-like egg, free of ice crystals that normally destroy the integrity of the cell.

Reproductive ageing

Female reproductive ageing is the process dictated by a gradual decrease in both the quantity and the quality of the oocytes. The pool of ovarian follicles is progressively reduced during female reproductive lifespan, which ends with menopause at a mean age of 50–51 years. With the decay in follicle numbers, oocyte quality also diminishes: in fact, fertilization rates decrease while the frequency of miscarriages increases [Broekmans *et al.*, 2007]. Moreover, during in vitro fertilization cycles older women tend to produce less oocytes after hormonal stimulation and derived embryos have a lower implantation potential [Jones, 2008]. The two mechanisms mainly involved in the decrease of oocyte number and miscarriage increase with ageing are apoptosis and aneuploidies.

Apoptosis: overview of cell death signalling pathways

Programmed cell death (apoptosis) is a common type of cell death associated with morphological features that had been repeatedly observed in various tissues and cell types. Characteristic apoptotic features include cell membrane blebbing, cell shrinkage, chromatin condensation and DNA fragmentation, finally ending with the packaging of dead cell content into apoptotic bodies, which are recognized by neighboring cells or macrophages and cleared by phagocytosis, thereby avoiding an inflammatory response in surrounding tissues. Phagocytosis reaction is triggered by phosphatidylserine signal: it is normally present on the cytoplasmic side of the cell membrane, but exposure to the external surface of the cell membrane occurs when a cell undergoes apoptosis [Savill and Fadok, 2000]. Apoptosis is distinct from necrosis in which the cells suffer a major insult, leading to a loss of membrane integrity, swelling and disruption of the cells. During necrosis, the cellular contents are released uncontrolled into the environment which results in damage of surrounding cells and a strong inflammatory response in the

corresponding tissue [Leist and Jaattela, 2001] (Figure 19).

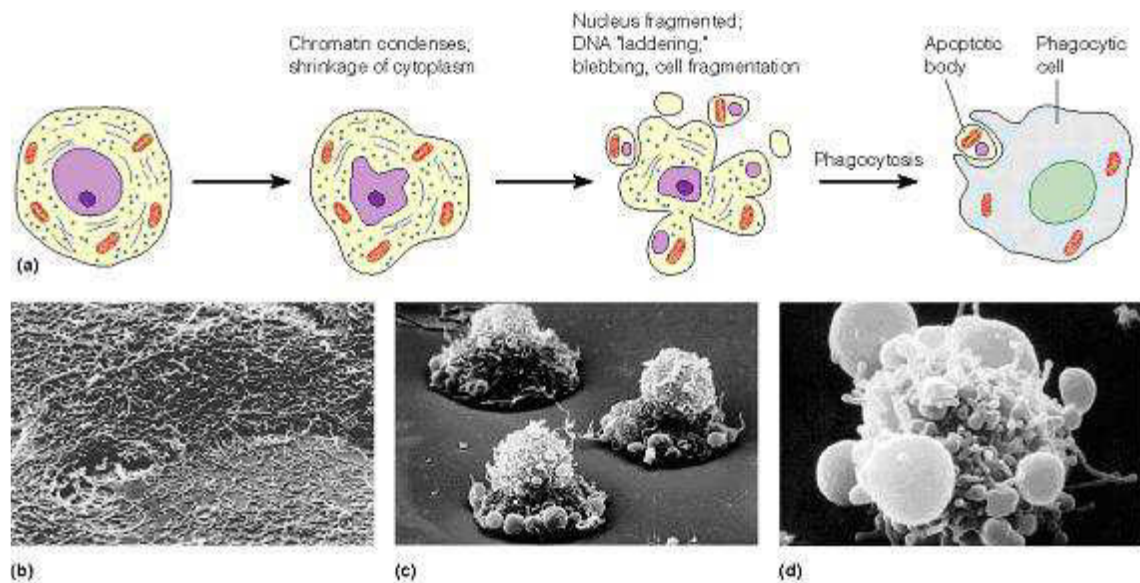


Figure 19. Morphological features of apoptotic cells.

Apoptosis is an essential part of life for multicellular organisms that plays an important role in development and maintenance of tissue homeostasis [Prindull, 1995]. During development many cells are produced in excess which eventually undergo programmed cell death and thereby contribute to sculpturing organs and tissues [Meier *et al.*, 2000]. Cellular apoptosis is tightly controlled by complex regulatory networks and balanced in a physiological context. Pro-survival signals enhance the expression and/or activity of anti-apoptotic regulatory molecules thereby keeping in check the activation of pro-apoptotic factors. A set of various anti-apoptotic molecules and mechanisms has been identified such as NF- κ B, AKT, BCL2, CFLAR and the IAP family of proteins. Every step in the apoptotic cascade is monitored and controlled by certain pro-survival signals [Vogelstein and Kinzler, 2004]. Pro-apoptotic factors such as BAX, BID and caspases can counteract those inhibitory molecules when apoptotic demise of a cell is timely and imperative. Caspases activation is a hallmark of apoptosis since this family of proteins are the main effectors of programmed cell death [Jin and El-Deiry, 2005]. Caspases are

synthesized as inactive zymogens that can be cleaved to form active enzymes following the induction of apoptosis. To date, about 14 mammalian caspases have been identified and classified into three groups based on their function, even if all of them share a number of common features (Table 2).

Table 2. Classification of mammalian caspases family.

Name of caspase	Other names	Tetrapeptide preference	Function	Size of pro-domain	Pro-domain molecule	Adaptor protein
Caspases important for execution and signalling events of apoptosis						
Caspase-2	ICH-1/mNedd2	DEHD/ VDVAD	Initiator	Long	CARD	RAIDD
Caspase-8	MACH/FLICE/ Mch5	LETD/IETD	Initiator	Long	DED	FADD
Caspase-9	ICE-LAP6/ Mch6	LEHD	Initiator	Long	CARD	Apaf-1
Caspase-10	Mch4, FLICE2	IEAD	Initiator	Long	DED	FADD
Caspase-3	CPP32/ Apopain/Yama	DMQD/ DEVD	Effector	Short		None
Caspase-6	Mch2	VEID/VEHD	Effector	Short		None
Caspase-7	Mch3/ICE- LAP3/CMH-1	DEVD	Effector	Short		None
Caspases involved in control of inflammation						
Caspase-1	ICE	WEHD/ YEVD		Long	CARD	
Caspase-4	TX/ICH-2/ ICE _{REL-II}	LEVD/(W/L) EHD		Long	CARD	
Caspase-5	TY/ICE _{REL-III}	(W/L)EHD		Long		
mCaspase-11	ICH-3			Long		
mCaspase-12				Long		
Caspase-13	ERICE			Long		
mCaspase-14	MICE			Short		None

Failure of this complex regulation system results in pathological conditions such as developmental defects, autoimmune diseases, neurodegeneration or cancer [Thompson, 1995]. Intensive efforts have been made to explore the molecular mechanisms of the apoptotic signalling pathways including the initiation, mediation, execution and regulation of apoptosis. Cells undergo apoptosis through two major pathways, the extrinsic pathway (death receptor pathway) or the intrinsic pathway (the mitochondrial pathway) (Figure 20).

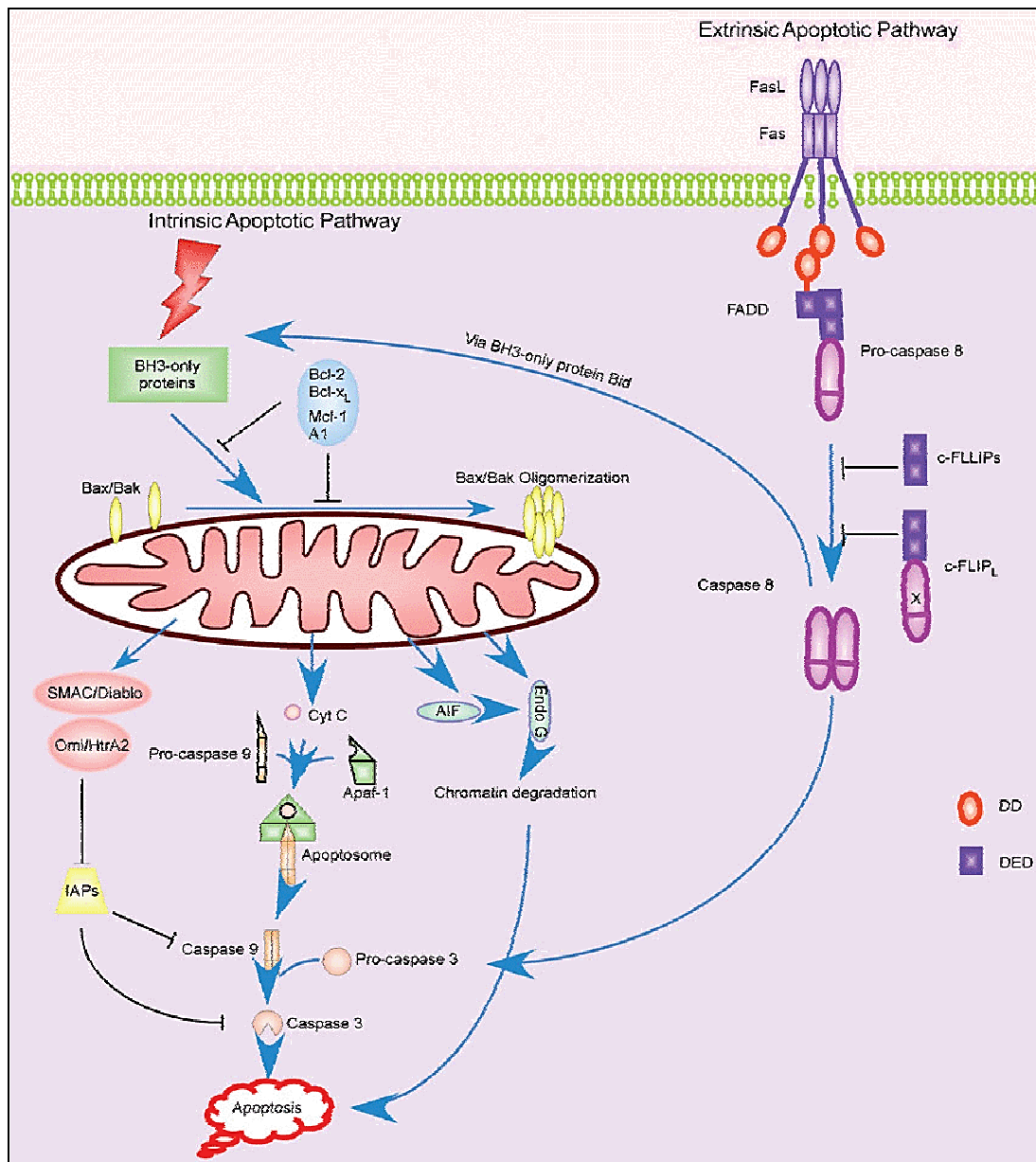


Figure 20. Cell death signalling pathways (from Zhang *et al.*, 2005).

The extrinsic pathway is mediated by cell surface death receptors, such as Fas, tumor necrosis factor receptor (TNFR), or TRAIL receptors. Death ligand stimulation results in oligomerization of the receptors and recruitment of the adaptor protein Fas-associated death domain (FADD) and caspase-8, forming a death-inducing signaling complex (DISC). Autoactivation of caspase-8 at the DISC is followed by activation of effector caspases, including caspase-3, -6 and -7, which function as downstream effectors of the cell death program [Ashkenazi and Dixit, 1998]. The intrinsic pathway is mediated by

diverse apoptotic stimuli, which converge at the mitochondria. Release of cytochrome c from the mitochondria to the cytoplasm initiates a caspase cascade. Cytosolic cytochrome c binds to apoptosis protease-activating factor 1 (Apaf-1) and procaspase-9, generating an intracellular DISC-like complex known as “apoptosome”. Within the apoptosome, caspase-9 is activated, leading to processing of caspase-3 [Shi, 2002]. The two pathways of apoptosis, extrinsic/death receptor and intrinsic/mitochondrial, converge on caspase-3 and subsequently on other proteases and nucleases that drive the terminal events of programmed cell death.

Because deregulated apoptosis lies at the heart of development of many diseases including cancer, understanding cell death signalling pathways represents an attractive target for therapeutic intervention.

Apoptosis in the ovary: molecular mechanisms

One of the earliest descriptions of programmed cell death was by Walther Flemming who, in 1885, published detailed observations on cellular degeneration in the rabbit ovary [Flemming, 1885]. This cell death, he noted, was characterized by chromatin condensation along the inner side of the nuclear envelope, followed by nuclear fragmentation and the ultimate budding of the cell into small remnants. This study, probably the first to illustrate the degradation of DNA during apoptosis, was published almost a century before Kerr et al. would contribute what many consider to be a landmark paper in which the term “apoptosis” was first used [Kerr *et al.*, 1972].

Apoptosis occurs almost incessantly in the ovaries throughout fetal and postnatal life and it claims up to 99.9% of the cells in the mammalian female germ line. During fetal life, apoptosis is localized to the oocytes, whereas in adult life, it is detected in granulosa cells of secondary and antral follicles (Figure 21).

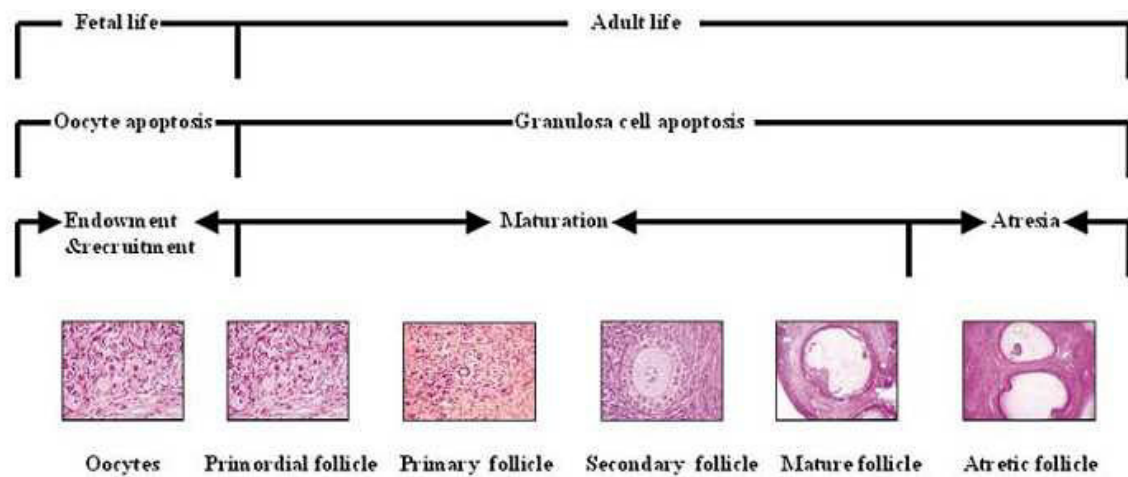


Figure 21. The development of the ovarian follicle during fetal and adult life. During fetal life, apoptosis is found in the oocytes, in adult life in the granulosa cells. (from Hussein, 2005).

In many respects, female germ cells can be considered analogous to grains of sand within an hourglass in that the continuous trickle of oocytes out of the initial endowment provided at birth in most, if not all, vertebrate species functions as the “female biological clock” (Figure 22).

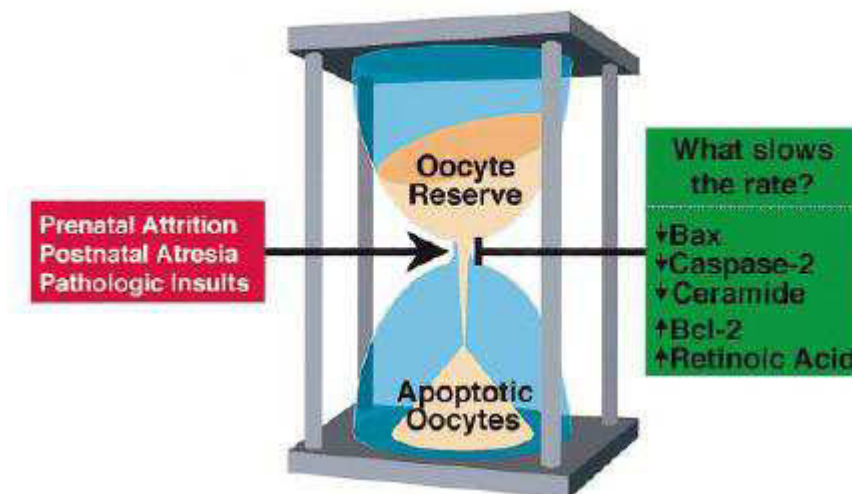


Figure 22. Analogy drawn between the loss of oocytes via apoptosis and the trickling of grains of sand through an hourglass to define the female biological clock. In the upper reservoir, each grain of sand can be considered a healthy oocyte. The rate at which healthy oocytes pass irrevocably into the apoptotic pool due to several causes (red box) defines how long the female biological clock will tick. The rate at which the healthy oocyte reserve is decimated by apoptosis is precisely controlled by a number of molecules that comprise an evolutionarily conserved cell death program (green box) (from Morita and Tilly, 1999).

Oocyte depletion from the ovarian reserve eventually drives irreversible infertility and ovarian failure, which is defined menopause in women. Accordingly, understanding how and why the female body creates, only to delete, so many germ cells is imperative. New insights into the mechanisms that underlie germ-cell apoptosis have been provided by the study of oocyte death in lower organisms and in genetically manipulated mice that lack apoptosis-regulatory proteins. The main objectives of each reproductive cycle in female mammals are to ovulate an egg(s) that is fully competent for fertilization and embryonic development, and to prepare the accessory reproductive organs (the oviducts and uterus) for the gestation and birth of healthy offspring. The egg that is selected for ovulation and potential fertilization must beat hundreds of competitors along the way, through, as yet, poorly described selection mechanisms [Morita and Tilly, 1999]. Therefore, each oocyte exists under a continuous threat of death and this feature, along with the fact that female germ cells outlive almost all other cells in the body, makes oocytes a unique model system for studying programmed cell death. Hypothetically, mechanisms underlying the exhaustion of the ovarian reserve of follicles include: (i) a death by neglect due to a deficit in survival factors produced by somatic neighbouring cells; (ii) death by defect or 'quality control' to eliminate meiotic anomalies; and (iii) a death by self-sacrifice or "altruistic death".

In other examples of developmental cell death, such as the high loss of neurons during the formation of the central nervous system [Giehl, 2001], competition between cells for a limiting amount of growth ('survival') factors has been proposed as a key component of organogenesis [Jacobson *et al.*, 1997]. Cells that receive insufficient support from their environment simply wither and die (Figure 23A).

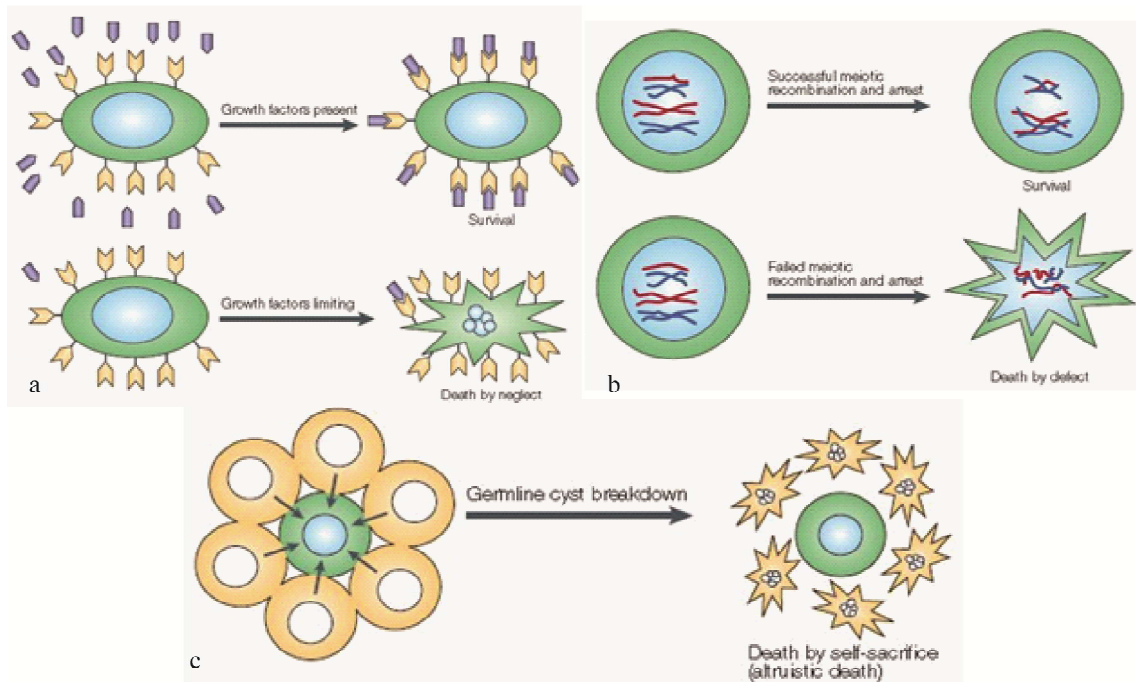


Figure 23. Hypotheses to explain mammalian fetal ovarian germline death. **a)** Apoptosis is actively suppressed by growth factors which act through receptors that are expressed on the germ-cell surface; however, if growth-factor availability is low, intracellular survival signals no longer keep apoptosis repressed and death by neglect ensues. **b)** Oocytes that successfully complete meiotic recombination become arrested in prophase I and are used to form primordial follicles; however, failure of meiotic recombination results in germ-cell death by defect. **c)** In germ-cell cysts that form during development, ‘nurse’ germ cells (yellow) transport macromolecules and organelles (arrows) into one germ cell, which is destined to become the oocyte (green), to perhaps coordinate meiotic events or mitochondrial inheritance (from Tilly, 2001).

Evidence for ‘death by neglect’ in the developing female germ line comes mainly from studies of gametogenic failure in mutant female mice that lack germ-cell survival factors, such as stem-cell factor (SCF) [Mintz and Russell, 1957]. Furthermore, *in vitro* culture of fetal mouse ovaries in the absence of serum or cytokines leads to a rapid induction of germ-cell apoptosis, and germline death in this artificial situation of acute neglect can be prevented by adding exogenous survival factors [Morita *et al.*, 1999]. However, despite mounting evidence for death by neglect, there is no proof that it is a normal component of oogenesis.

A second theory for why so many germ cells are lost during fetal ovarian development is that apoptosis eliminates oocytes with meiotic pairing or recombination anomalies

(Figure 23B). The idea is that a surveillance (or quality-control) mechanism detects and removes defective oocytes and retains meiotically competent oocytes for the formation of primordial follicles.

The third hypothesis rely on the fact that the massive apoptosis occurring in the ovary during oogenesis could be due to an altruistic death by oocyte-surrounding cells (Figure 23C). In *Drosophila*, *Xenopus laevis* and mice it has been shown that during female gametogenesis the oocyte is interconnected to its surrounding “nurse cells” by specialized intercellular bridges, thus arranging structures termed germline cysts [Pepling *et al.*, 1999; Pepling and Spradling, 2001]. In germ-cell cysts, nurse cells transport macromolecules and organelles into one germ cell, which is destined to become the oocyte, to perhaps coordinate meiotic events or mitochondrial inheritance. Coincident with primordial follicle formation, the cyst breaks down through a mechanism which involves nurse germ-cell death by self-sacrifice for the benefit of their sister germ cell, the oocyte (Figure 23C). Although there is no direct evidence of the contribution of these cells as in invertebrates, similar clusters of cells that undergo apoptosis have been identified in lower vertebrates but the fate and the role of these germline cysts remain unclear [Pepling and Spradling, 1998; Pepling and Spradling, 2001]. In light of these observations, we need to uncover the function of germline cysts in mammals. For example, perhaps a unidirectional transfer of ‘nutrients’ from the nurse cells might be needed to ensure that the oocyte survives from when it becomes physically dissociated from its supporting sister cells until it becomes fully re-enveloped by the supportive pre-granulosa cells during primordial follicle formation [Tilly, 2001]. With these advances in understanding the occurrence and regulation of apoptosis in the germ line, we still do not know why the human female produces so many germ cells during prenatal gametogenesis, when two thirds or more of the oocytes that are

generated will be lost before birth through apoptosis. And why do more than 95% of the oocytes that survive the perinatal waves of programmed cell death subsequently die in postnatal life? The answer probably lies, at least in part, in the supposition that not all oocytes are created equal. So oocyte apoptosis can be viewed in a context analogous to Darwin's "survival-of-the-fittest" theory of evolution [Darwin, 1860]. Therefore, especially in those species such as humans that ovulate only a single egg per cycle for fertilization, the continual deletion of presumably inferior oocytes by apoptosis might maximize the chances of reproductive success, thus ensuring that only the real competent egg will be available for fertilization.

Aneuploidy

For what concerns the decrease of oocyte competence to produce a vital embryos during ageing, chromosomal abnormalities are a prominent example of defects causing implantation failure. Several hypothetical mechanisms have been proposed to explain the increase of aneuploidy with ageing, as defects in chromosome recombination or of chromosome cohesion at meiosis. An additional cause may be the long time interval between protracted meiotic arrest stage and MI restart, which could make oocytes unable to detect and correct recombination errors arisen at prophase during fetal life [Hunt and Hassold, 2008]. Because of their well known involvement in the control of cell cycle, apoptosis and genome stability including ploidy, the members of the p53 family (p53, p63, p73) could be etiologically involved in reproductive disorders [Collavin *et al.*, 2010; Levine *et al.*, 2011]. The role of the p53 family as guardian of maternal reproduction has been recently investigated. This family consists of three members (p53, p63 and p73), conserved from invertebrates to mammals [Dötsch *et al.*, 2010]. The roles of the p53 gene and its encoded protein as tumor suppressor and guardian of the genome have been firmly established [Levine *et al.*, 2006]. Maintenance

of germ-line genomic integrity is a major identified function of these proteins in *Caenorhabditis elegans* and *Drosophila melanogaster* [Dötsch *et al.*, 2010]. In vertebrates, including *Homo sapiens*, these proteins have been suggested to also regulate maternal reproduction [Levine *et al.*, 2011]. Recently, p53 role in regulating embryo implantation by transcriptionally controlling the expression of LIF (Leukemia Inhibitory Factor) was demonstrated [Hu *et al.*, 2009]. Moreover, loss of the p53, p63 and p73 genes in female mice leads to a significant decrease of fertility [Levine *et al.*, 2011]. Finally, single nucleotide polymorphisms (SNPs) in both human p53 and p73 genes have been associated to women reproductive disorders [Feng *et al.*, 2011]. Despite the role of the majority of p53 family members has been well established, much more remains to be discovered, even because the fact that different isoforms can display opposing functions has to be considered. For example, the p73 gene encodes two major groups of protein isoforms, endowed with opposite biological functions, which are synthesized through alternative promoters. Proapoptotic TAp73 activates a set of p53-responsive genes and induces cell cycle arrest or apoptosis in response to DNA damage. $\Delta Np73$ isoforms lack the N-terminal transactivation domain (TA) and perform a dominant negative role on proapoptotic isoforms [Dötsch *et al.*, 2010]. They are frequently upregulated in various human cancers, including myeloid leukemia, neuroblastoma, ovarian cancer and, moreover, the truncated isoforms perform an important role in brain development [Moll and Slade, 2004].

Given all these considerations and the fact that they are key regulators of many signalling pathways involved in cancer, development and ageing, the study of p53 family members could lead us to understand how the ovarian microenvironment can influence oocyte transcriptome and proteome during ageing, and to identify the molecular bases of female ageing.

3. MATERIALS AND METHODS

Women enrolled in the studies

Study subjects included in all the studies were healthy women selected by an IVF Center (Servizio di PMA, Azienda Ospedaliera Cannizzaro, Catania, Italy) whose primary infertility was due to a male factor. Accordingly, we excluded pathologies that could influence oocyte quality such as endometriosis, ovarian insufficiency and polycystic ovaries. Therefore, patients had undergone intracytoplasmic sperm injection (ICSI), which included gonadotropin administration to induce multiple follicular development and transvaginal oocyte aspiration (Figure 24). Approval by the Hospital Ethical Committee and patients' informed consent were obtained for the use of supernumerary oocyte, FF and plasma samples employed in all our studies.

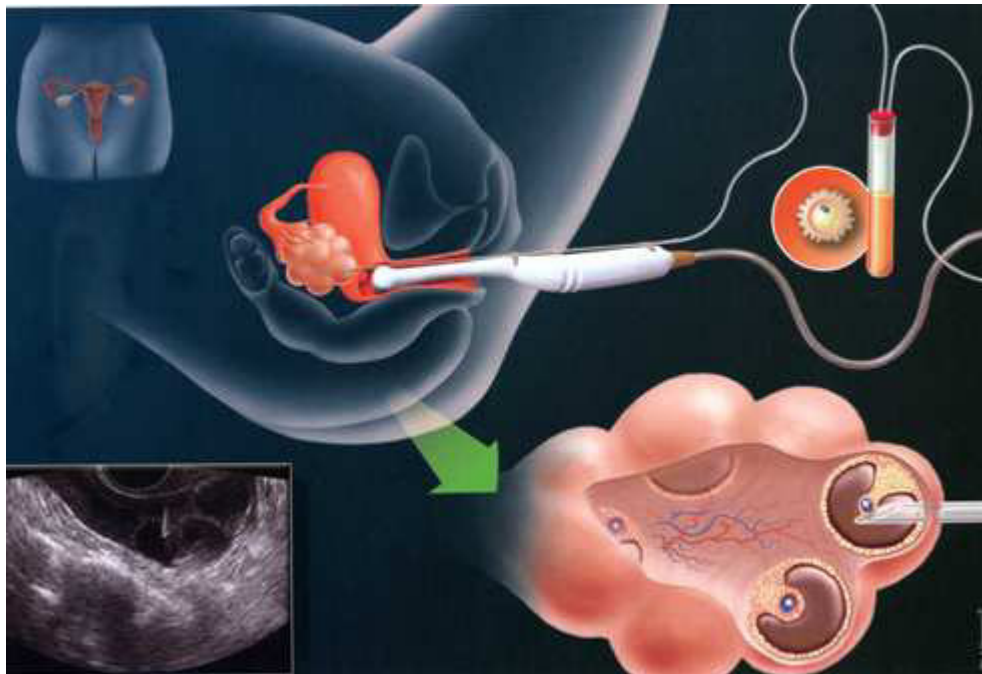


Figure 24. Oocyte pick-up through vaginal access under ultrasound guidance.

Sample collection

Human metaphase II oocytes

To avoid factors that could alter the oocyte transcriptome, we analysed only mature MII oocytes with good morphology taken from women who were within the selection criteria described above. Hormonal stimulation was performed by using classical protocols involving treatment with GnRH agonists (triptorelin or buserelin), followed by ovarian stimulation with recombinant follicle stimulating hormone (rFSH) and human menopausal gonadotropin (HMG). These were administered in individual doses to each patient starting when the estradiol concentration was <40 ng/l. Stimulation was monitored by using both serum estradiol concentrations and ultrasound measurements of follicle numbers and diameters. Ovulation was induced with 10,000 IU of human chorionic gonadotropin (HCG; Gonasi), when the leading follicles reached 18–20 mm in diameter and the serum estradiol concentration per follicle was 150–200 ng/l. Ovarian follicles containing mature MII oocytes were retrieved through vaginal access under ultrasound guidance, 34–36 h after HCG administration (Figure 24). Cumulus-enclosed oocytes were separated from follicular fluid and placed in medium for a short incubation (37°C in a humidified 5% CO₂ atmosphere). 2 h after retrieval, the oocytes were removed from the surrounding cumulus cells with hyaluronidase 80 IU/ml (Origio, Denmark, EU). After incubation for a few seconds, the oocytes were rinsed three times in culture medium (IVF; VitroLife AB, Kungsbacka, Sweden). Finally, oocytes were rinsed several times in RNase-free water to remove proteins from the culture medium, placed in the same-quality water in PCR tubes and frozen in liquid nitrogen.

Follicular fluid

After ovarian follicles were retrieved as previously described, follicular fluid was separated from cumulus-oocyte complex through manual aspiration with a 10 ml syringe and centrifuged at 2800 rpm for 20 min in order to remove cell debris. The supernatant was immediately transferred into a clean polypropylene tube and stored at -20°C for further analysis. Samples with massive blood contamination were excluded from further analysis.

Plasma

Blood samples from each patient were collected into commercially available anticoagulant-treated (EDTA-treated) tubes. Cells were removed from plasma by centrifugation at 1800 rpm for 10 min. The resulting supernatant was immediately transferred into a clean polypropylene tube and stored at -20°C .

Sample preparation

Vitrification protocol

For the vitrification and warming procedure, we used the Irvine Vitrification Freeze Kit (Vit Kit-Freeze; Vit Kit-Thaw, Santa Ana, CA) including two vitrification and three warming solutions. The two vitrification solutions have to be used in sequence and comprise the equilibration solution, a HEPES-buffered solution of Medium-199 containing gentamicin sulfate (35 mg/mL), 7.5% (vol/vol) each of dimethyl sulfoxide and ethylene glycol, and 20% (vol/vol) dextran serum supplement; and the vitrification solution, a HEPES-buffered solution of Medium-199 containing gentamicin sulfate (35 $\mu\text{g/mL}$), 15% (vol/vol) each of dimethyl sulfoxide and ethylene glycol, 20% (vol/vol) dextran serum supplement, and 0.5 mol/L sucrose. The three warming solutions include the thawing solution, a HEPES-buffered

solution of Medium-199 containing gentamicin sulfate (35 µg/mL), 1.0 mol/L sucrose, and 20% (vol/vol) dextran serum supplement; the dilution solution, a HEPES-buffered solution of Medium-199 containing gentamicin sulfate (35 µg/mL), 0.5 mol/L sucrose, and 20% (vol/vol) dextran serum supplement; and the washing solution, a HEPES-buffered solution of Medium-199 containing gentamicin sulfate (35 µg/mL) and 20% (vol/vol) dextran serum supplement. Vitrification procedure was performed at 23°C to 25°C (room temperature). After three oocytes were transferred with a minimal volume of medium from the culture dish (37°C–5% CO₂) into the 20 µl drop of culture medium (Gamete; Vitrolife, Goteborg, Sweden) for 1 min, the drop was merged first to 20 µl equilibration solution drop with the tip of the transfer pipette; spontaneous mixing of the two solutions was allowed to occur for 2 min; subsequently, a second 20 µl drop of equilibration solution was merged to the previously merged drops and left for 2 min; then, the oocytes were transferred with minimal volume of solution to the bottom of a third 20 µl equilibration solution drop for 3 min. After a quick incubation in three subsequent 20 µl drops of vitrification solution for 5 seconds, 5 seconds, and 10 seconds each, the oocytes were aspirated in a small volume of vitrification solution and immediately loaded onto a high-security vitrification kit (HSV; CryoBioSystem, L'Aigle, France) on the distal part of the capillary gutter. The capillary was inserted immediately into the HSV straw and sealed by using a thermal sealer (CryoBioSystem). In less than 1 min from the beginning of exposure to the vitrification solution, the sample was vitrified by plunging the HSV in the liquid nitrogen. Warming was performed at room temperature. The device was cut keeping the area containing the oocytes immersed in the liquid nitrogen. The capillary was extracted and immediately plunged into the first thawing solution, in which the oocytes were incubated for 1 min and then

transferred into a second 20 μ l thawing solution drop for another minute. Then the oocytes were incubated in two subsequent 20 μ l drops of dilution solution for 2 min each and in three subsequent 20 μ l drops of washing solution for 2 min each. Single oocytes were rinsed several times in ribonuclease-free water to remove any trace of proteins from the culture medium; then they were placed in Eppendorf tubes in 2 μ l ribonuclease-free water and frozen in liquid nitrogen to avoid RNA degradation.

Exosome purification

A volume of 120 ml of follicular fluid was collected in 50 ml tubes and centrifuged at 3000 rpm for 15 min at 4°C to pellet cells and debris. The supernatant was transferred to ultracentrifuge tubes and ultracentrifuged at 19400 rpm (16500 x g) for 30 min at 4°C followed by filtration through a 0.2 μ m syringe filter. Finally exosomes were pelleted by ultracentrifugation at 38800 rpm (120000 x g) for 70 min at 4°C in a Beckman Optima L-100 XP ultracentrifuge using a Ti70 rotor (Beckman Coulter, Germany). Exosome pellets were resuspended in PBS for Nanosight and FACS analysis or Trizol reagent (Invitrogen, Sweden) for RNA isolation.

Size determination of exosomes

Following the manufacturer's instructions, we diluted the sample with sterile PBS to reach a particle concentration suitable for analysis with Nanoparticle Tracking Analysis (NTA). The sample was injected into the LM10 unit (approximately 300 μ l) with a 1 ml sterile syringe. The capturing and analyzing settings were manually set according to the protocol. Using the NanoSight LM10 instrument, vesicles were visualized by laser light scattering and analyzing Brownian motion of these vesicles captured by videos. Number of tracks always exceeded 200 and five size distribution measurements were taken for the same sample. Recorded videos were then analysed

with the NTA software which provides high-resolution particle size distribution profiles and concentration measurements of the vesicles in solution.

Flow cytometry of exosomes

Aldehyde/sulfate latex beads (4 μm , Invitrogen, Sweden) were serially diluted with PBS to reach a final concentration of 2900 beads/ μl . Vesicles from FF sample were incubated with 25 μl diluted latex beads at 37°C for 30 minutes and then overnight at 4°C with gentle agitation. After one wash in PBS, exosome coated beads were incubated with 20 μl 1M glycine (Sigma-Aldrich, Sweden) for 30 min at room temperature to block remaining binding sites. After two washes in PBS with 1% FBS, exosome coated beads were stained with phycoerythrin (PE) conjugated CD63, CD81, CD9 antibody or isotype control (BD Biosciences Pharmingen, Belgium) and incubated for 1h at 4°C with gentle agitation. After the third wash in PBS/FBS 1% solution, samples were resuspended in PBS and analysed on FACSCantoII (Becton Dickinson, San Diego CA) with FlowJo software (TreeStar, Inc, USA).

Samples used in our studies: classification

Experimental plan and samples used in Paper I, II, III and IV are reported in the tables below. Oocytes collected from women younger than 35 years of age are named ‘young oocytes’, while those collected from women older than 38 years are called ‘old oocytes’. In addition, FVB mice (from the Animal Facility of IST – Genoa) used in Paper II were also split in two age groups: younger than 5–8 weeks (young) and older than 6–7 months (old). Housing and treatment of animals were in accordance with the Italian and European Community guidelines and approved by the internal Ethic Committee.

Table 3. Molecular profiling of human oocytes after vitrification strongly suggests that they are biologically comparable with freshly isolated gametes (Fertility and Sterility 2010).

Women	Oocyte from each patient	Total number of oocytes	Analysed genes
5	2 fresh	10 fresh	HPRT-BMP15-GDF9-FIGLA-OCT4-TAF4B
	3 vitrified	15 vitrified	

Note: we used 25 MII oocytes collected from five different women. For each patient, we collected two fresh and three vitrified oocytes, choosing those with optimal morphology, for a total of 10 fresh and 15 vitrified oocytes.

Table 4. TAp73 is downregulated in oocytes from women of advanced reproductive age (Cell Cycle 2011).

Human Oocytes	Group	Analysed Genes
1 - 6	Old	HPRT-TAp73-ΔNp73
7 - 16	Young	
Mouse Oocytes	Group	
Pool (1-3)	Old	
Pool (4-6)	Young	

Note: we used 16 MII single human oocytes (from 1 to 16), 6 from older and 10 from younger women, and oocyte pools from old and young mice in order to analyse the expression profile of p73 transcript variants.

Table 5. The apoptotic transcriptome of the human MII oocyte: characterization and age-related changes (Apoptosis 2013).

Oocytes	Group	Analysed Genes
Pool (1-5)	Old	RT ² Profiler™ PCR Array System
Pool (6-10)	Young	
11 - 15	Old	HPRT-NAIP-BCL2L1 short-BCL2L1 long-BCL2L1
16 - 21	Young	
22 - 26	Old	HPRT-MCL1 short-MCL1 long-TNFRSF10A-TNFRSF21
27 - 32	Young	
33 - 37	Old	HPRT-BCL2-CFLAR-CD40-BAD
38 - 43	Young	

Note: In this study we included 20 women, 12 of which were younger than 35 and 8 older than 38 years of age. Five MII oocytes were collected from each woman from both groups and separately pooled to obtain sufficient mRNA to analyse the 84 Apoptotic Machinery genes. For single cell analysis, we collected a total of 33 MII oocytes (from 11 to 43), 15 from older and 18 from younger women.

Table 6. Exosomal microRNAs in Human Follicular Fluid: new actors in the communication between oocytes and somatic follicular cells (Mol Hum Reprod submitted).

Pool	Sample	Analysed miRNAs
1	Total Follicular Fluid	TaqMan Human MicroRNA Array A Cards
2		
3		
4	Exosomes from Follicular Fluid	
5		
6		
7	Plasma	
8		
9		

Note: follicular fluid and plasma were collected from the same 15 women, mixed and divided into aliquots in order to obtain pools of FF and plasma for exosome purification and miRNA expression profile analysis.

Primer design

Primers used in our studies were designed with Primer Express version 3.0 software (Applied Biosystems, Foster City, CA) or Primer-BLAST (<http://www.ncbi.nlm.nih.gov/tools/primer-blast>) and are reported in details in the original publications.

mRNA profiling in oocytes

RNA isolation, reverse transcription and profiling by Real-Time PCR

Pooled or single human (or mouse) oocytes in RNase-free water were incubated at 100°C for 1 min and 30 sec and 1 min, respectively, in order to release nucleic acids. Total RNA was incubated with DNase I Amplification Grade (Invitrogen, Carlsbad, CA, USA) and reverse transcribed in the same tube by using Superscript II (Invitrogen S.R.L, Milan, Italy) for human samples and WT-Ovation kit (Nugen Technologies) for mouse samples according to the manufacturer's instructions. cDNA from human oocyte pools was mixed up with SYBR Green PCR master mix

and added to each well of the Human Apoptosis RT² Profiler PCR Array (SuperArray Bioscience Corporation, MD, USA) in order to profile the expression of 84 key genes involved in programmed cell death plus five housekeeping genes (ACTB, B2M, GAPDH, HPRT1, RPL13A) through quantitative PCR (qPCR). Each 96-well plate also comprises controls for genomic DNA contamination, RNA quality, and general PCR performance (Figure 25).

	1	2	3	4	5	6	7	8	9	10	11	12
A	ABL1	AKT1	APAF1	BAD	BAG1	BAG3	BAG4	BAK1	BAX	BCL10	BCL2	BCL2A1
B	BCL2L1	BCL2L10	BCL2L11	BCL2L2	BCLAF1	BFAR	BID	BIK	NAIP	BIRC2	BIRC3	BIRC4
C	BIRC6	BIRC8	BNIP1	BNIP2	BNIP3	BNIP3L	BRAF	NOD1	CARD6	CARD8	CASP1	CASP10
D	CASP14	CASP2	CASP3	CASP4	CASP5	CASP6	CASP7	CASP8	CASP9	CD40	CD40LG	CFLAR
E	CIDEA	CIDEB	CRADD	DAPK1	DFFA	FADD	FAS	FASLG	GADD45A	HRK	IGF1R	LTA
F	LTBR	MCL1	NOL3	PYCARD	RIPK2	TNF	TNFRSF10A	TNFRSF10B	TNFRSF11B	TNFRSF1A	TNFRSF21	TNFRSF25
G	CD27	TNFRSF9	TNFSF10	CD70	TNFSF8	TP53	TP53BP2	TP73	TRADD	TRAF2	TRAF3	TRAF4
H	B2M	HPRT1	RPL13A	GAPDH	ACTB	HGDC	RTC	RTC	RTC	PPC	PPC	PPC

Figure 25. Schematic view of 96-well Real Time PCR SuperArray plate.

cDNA from single human oocytes has been used in our studies to perform qPCRs in order i) to validate the data from oocyte pools, by performing five different single reactions per oocyte and repeating them with five old oocytes and six young oocytes (Paper III, Table 5); ii) to analyse genes encoding proteins essential for oocyte-specific functions and development with the aim to assess the effects of vitrification on oocyte biologic quality (Paper I, Table 3); iii) to determine the expression of TAp73 and Δ Np73 transcript variants with the purpose of verifying the potential involvement of p73 in women reproductive ageing (Paper II, Table 4).

microRNA profiling in follicular fluid, plasma and exosomes

miRNA isolation, reverse transcription and profiling by Microfluidic Cards

Total RNA from FF and plasma samples was extracted using the miRNeasy kit (Qiagen) according to the manufacturer's directions. In brief, 400 µl of each FF and plasma pool were mixed thoroughly with 2 ml of QIAzol reagent. Following a brief incubation at room temperature, 400 µl of chloroform were added and the solution was mixed vigorously. The samples were then centrifuged at 10000 rpm for 15 min at 4°C. The upper aqueous phase was carefully transferred to a new tube and 1.5 volumes of ethanol were added. The samples were applied directly to RNA binding columns and washed. Total RNA was eluted in 30 µl of nuclease-free H₂O.

Total RNA from follicular fluid exosomes was isolated using TriZol reagent (Invitrogen, Sweden) according to the manufacturer's instructions. Prior to precipitating the RNA with isopropyl alcohol, we added 20 µg RNase-free glycogen (Invitrogen) as carrier to the aqueous phase and stored the samples overnight at -80°C. The RNA pellets were dissolved in RNase-free water. Detection and quality control of RNA was performed using an Agilent 2100 Bioanalyzer (Agilent Technologies Sweden AB, Kista, Sweden) and 2100 Expert software.

Three microliters of RNA isolated from FF, plasma and exosome samples were reverse transcribed using the TaqMan MicroRNA Reverse Transcription Kit and Megaplex Primer Human Pool A (Applied Biosystems, Foster City, CA, USA), according to the Megaplex Protocol. Subsequently, 4 µl of these RT products were pre-amplified with TaqMan PreAmp Master Mix-2X and Megaplex PreAmp Primers Human Pool A (Applied Biosystem), and then diluted by adding 75 µl of RNase free water up to a final volume of 100 µl.

As a platform for the profiling of miRNA expression in FF, plasma and exosome samples we chose the TaqMan Low Density Array (TLDA) technology (Applied Biosystems), that is microfluidic cards enabling the simultaneous amplification and accurate quantitation of up to 384 miRNAs present in the Sanger miRBase v18.0 (Figure 26).

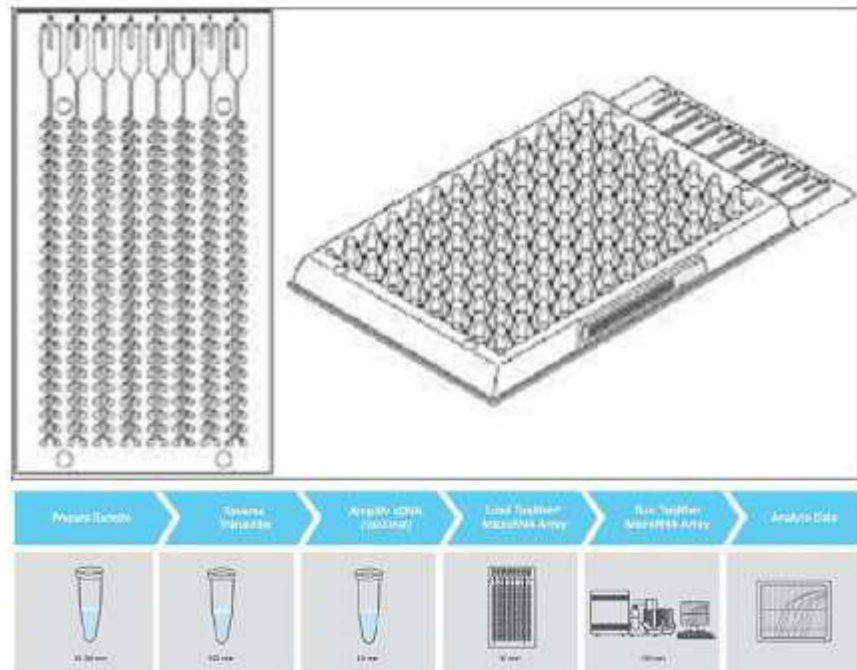


Figure 26. Applied Biosystems 7900HT TaqMan low density array and workflow for the analysis of miRNA transcriptome.

Nine microliters of pre-amp products were mixed up with TaqMan Universal PCR Master Mix II, no AmpErase UNG (Applied Biosystems) and 100 μ l of this PCR mix were dispensed into each port of the TaqMan Human MicroRNA Array A Cards v2.0 (Applied Biosystems); finally, the microfluidic card was centrifuged and mechanically sealed.

All qPCR reactions performed in our studies were carried out in a 7900HT Fast Real Time PCR system (Applied Biosystems). All the experiments were performed in triplicates.

Expression data analysis

Paper I

As a first step for data analysis, we performed the relative quantification of our results through the $2^{-\Delta\Delta Ct}$ method by using HPRT as reference gene [Livak and Schmittgen, 2001; Mamo *et al.*, 2007] and a single fresh oocyte as calibrator. To statistically validate our results, we compared the ΔCt values in fresh and vitrified oocytes by using the independent Student's t-test ($p < 0.01$).

Paper II

We calculated the relative levels of mRNA expression in each sample according to the $2^{-\Delta\Delta Ct}$ method [Livak and Schmittgen, 2001], using HPRT as reference gene for normalization and young oocytes as calibrators. To statistically evaluate our results, young and old oocyte ΔCt values were analysed by unpaired (two sample) Student's t-test ($p < 0.01$). Fold change in Results section are shown as mean of the natural logarithm (ln) of relative quantity (RQ) of mRNAs.

Paper III

The relative levels of mRNA expression for each gene in each sample were calculated according to the $2^{-\Delta\Delta Ct}$ method [Livak and Schmittgen, 2001]. For pool data normalization, we used the mean of the five housekeeping genes included in the 96-well plate while for single assay we used HPRT as reference gene. In both analysis young oocytes were used as calibrator samples. The corresponding fold changes are shown as ln of mRNA RQ values. We considered as up- or downregulated those genes having a ln of expression fold change ≥ 1 and ≤ -1 , respectively. To statistically evaluate the results, young and old oocyte ΔCt values were analysed by unpaired (two sample) Student's t test ($p < 0.05$ and $p < 0.01$).

Network analysis

Global Apoptotic Machinery (AM) network was generated by retrieving through cytoscape plug-in APID2NET (www.cytoscape.org) all the available experimental interactions among 84 AM genes analysed. To create the AM network specific for the comparison of old versus young oocyte pools, we applied on APID2NET- interaction maps the expression profile dataset concerning this comparison: both colour and shape gradients of nodes indicate expression fold changes and gene functions. Analysis of network centrality (NC) was performed by using the plug-in Network Analyzer, which allows to retrieve all centrality parameters of a node from an established network. The degree of a node inside a biological network does not mirror its real functional importance, but rather the network general topological features. For this reason, we focused our analysis also on other centrality metrics: node degree (ND), betweenness (B), closeness (C), stress (S). These parameters are biologically important, because they demonstrate the ability of a protein to functionally connect to and be relevant for others within complex signalling networks [Joy *et al.*, 2005]. Mathematical details of these centrality metrics are reported by Brandes and Erlebach [Brandes and Erlebach, 2005]. We inferred the possible correlation between altered regulation of AM genes and their NC by comparing the different NC in the two different gene expression classes [differentially expressed (DE) genes and unchanged genes] by the Wilcoxon Rank Sum test ($p\text{-value} < 0.05$).

Paper IV

No data are available on reliable reference genes for validation of miRNA expression in follicular fluid. Therefore, in order to identify the most suitable miRNA to be used as reference gene for normalization, we used DataAssist v.3 software (Applied

Biosystems) and geNorm Algorithm [Vandesompele *et al.*, 2002]. This analysis allowed us to select miR-25, miR-28-3p, miR-145 and miR-126, miR-28-3p, miR-145 as endogenous controls to normalize RNA amount in FF vs plasma and exosomes vs plasma comparisons, respectively. ΔCt values were calculated for each sample by using all endogenous controls. To identify DE miRNAs we used three different SAM (Significance Analysis of Microarrays, <http://www.tm4.org>) tests (Tusher, 5th percentile and Minimum S Value) applying a two-class unpaired test among ΔCt and using a p-value based on all possible permutations; imputation engine: K-nearest neighbors (10 neighbors); False Discovery rate <0.05. We selected only DE miRNAs in common to all endogenous controls and at least to two SAM tests and we calculated their RQ values through the $2^{-\Delta\Delta\text{Ct}}$ method, by using the above mentioned miRNAs as endogenous controls and the average of ΔCt of each plasma sample as calibrator in both analysis. We considered as upregulated those miRNAs having a ln of RQ values > 2.5. RQ values obtained with the three different endogenous controls for the same miRNA were reciprocally comparable, therefore in Results section we exclusively referred to miR-145. Accordingly, we used only miR-145 to calculate the mean RQ and standard deviation relative to triplicate, according to Livak and colleagues [Livak and Schmittgen, 2001]. The mean fold change is shown as natural logarithm of RQ values and the error is estimated by evaluating the $2^{-\Delta\Delta\text{Ct}}$ equation using $\Delta\Delta\text{Ct}$ plus standard deviation and $\Delta\Delta\text{Ct}$ minus the standard deviation [Livak and Schmittgen, 2001].

miRNA target prediction, gene ontology and pathway analysis

The analysis on DE miRNA targets was carried out by using a combination of several approaches to obtain a short list of reliable targets. A first list of predicted and experimentally validated targets of DE miRNAs was extracted from miRecords,

by interpolation between the highest number of the 11 prediction tools (<http://mirecords.biolead.org>). To improve our prediction, we also used TarBase (<http://www.diana.pcbi.upenn.edu/tarbase>) and miRTarBase (<http://mirtarbase.mbc.nctu.edu.tw>), two databases that have accumulated more than thirty thousand experimentally supported targets. An additional filtering was performed by using starBase (sRNA target Base), a database for predicted miRNA-target interactions, overlapped with data from Argonaute cross-linked immunoprecipitation-sequencing (CLIP-Seq) (<http://starbase.sysu.edu.cn>); CLIP-Seq experiments are based on cross-linking between RNA and proteins, followed by immunoprecipitation coupled with high-throughput sequencing: the application of this technique is useful for the identification of miRNA binding sites. Finally, we enriched our list of targets by retrieving specific information about validated miRNA targets from literature.

The Gene Ontology (GO) functional classification of miRNA targets was performed by using the g:Profiler tool (<http://biit.cs.ut.ee/gprofiler/>): this approach led us to focus on mRNAs specifically involved in biological process category (e.g. developmental process, regulation of cellular process, growth and cellular process). For this analysis we used the g:GOST (Gene Ontology Statistics) tool of g:Profiler with a p-value of 0.05 and the significance was determined by the adjusted Bonferroni correction.

Pathway analysis was performed by using the prediction software DIANA-microT-4.0 (beta version) (<http://diana.cslab.ece.ntua.gr/pathways/>) which allowed us to retrieve some signalling pathways predicted to be regulated by FF and exosome miRNAs. The probability value is reported as negative \log_{10} (p-value).

4. RESULTS

MOLECULAR PROFILING OF HUMAN OOCYTES AFTER VITRIFICATION STRONGLY SUGGESTS THAT THEY ARE BIOLOGICALLY COMPARABLE WITH FRESHLY ISOLATED GAMETES

To assess the effects of vitrification on the biomolecular profile of oocytes, we analysed 8 genes encoding critically important proteins for embryo development through RT-PCR Real-Time and compared this partial transcriptome with that of freshly collected gametes isolated from the same women. Our data clearly show that the expression profile of the genes chosen as biomarkers (BMP15, FIGLA, GDF9, OCT4 and TAF4B) did not change between fresh and vitrified oocytes ($0.3 < 2^{\Delta\Delta Ct} < 1.3$) (Figure 27A). To statistically validate our results, we compared the ΔCt values in fresh and vitrified oocytes. Even though some were collected from the same woman, we consider each oocyte as an independent sample: this is due to the specific differentiation history even of gametes from a single individual. In fact, each gamete may undergo specific genomic mutations or specific epigenetic DNA modifications, and show an evident level of molecular diversity, leading to differences in oocyte competence, fertilization grade, and pregnancy rate. Accordingly, we used the independent Student's t-test and demonstrated that there are no significant variations between fresh and vitrified-thawed oocytes ($p < 0.01$) (Figure 27B). This was further confirmed by comparing five different oocytes (two fresh and three vitrified), collected from a woman during the same ovarian stimulation protocol (Figure 27C).

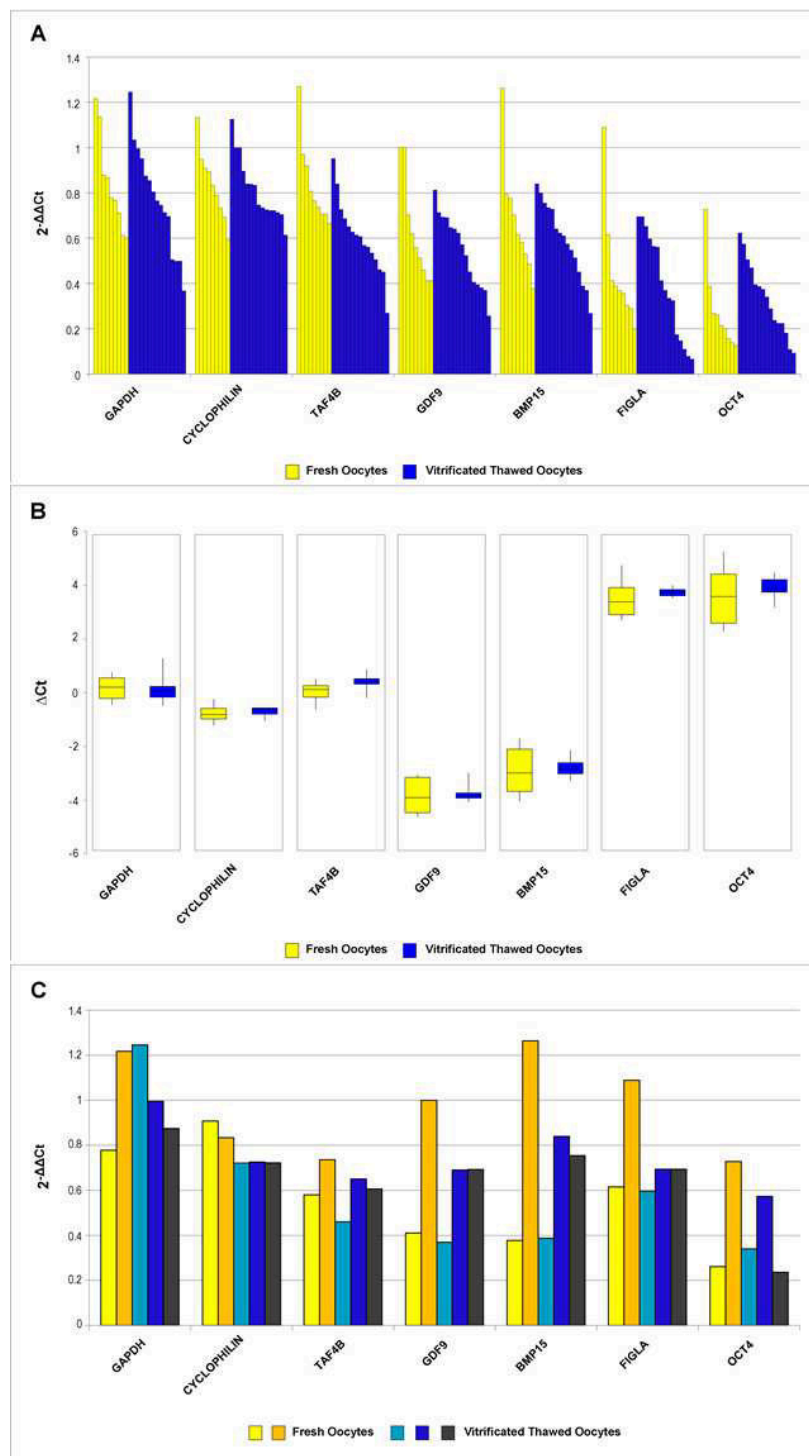


Figure 27. Gene expression profile in fresh and vitrified oocytes. (A) The histogram shows the expression profiles of analysed genes in 9 fresh (yellow) and 15 vitrified oocytes (blue). Expression values are shown as $2^{-\Delta\Delta Ct}$ normalized using HPRT as reference gene and the same fresh oocyte as calibrator. (B) Box and whisker plot the analysed genes in fresh and thawed oocytes after vitrification. It provides a simple description of a distribution of values by depicting the 25th and 75th percentile values as the bottom and top of a box, respectively. The Y axis represents the average ΔCt values. The median expression values of fresh and vitrified thawed oocytes are marked by horizontal lines in the boxes. (C) Expression profile of analysed genes in five oocytes collected from a woman during the same ovarian stimulation protocol. Data are shown as $2^{-\Delta\Delta Ct}$ using HPRT as reference gene and the same fresh oocyte as calibrator. Statistical analysis of data demonstrates that the expression profiles of the analysed genes are not significantly different ($p < 0.01$).

This article proves for the first time that the vitrification protocol keeps unaltered the oocyte molecular profile and does not cause messenger RNA degradation, as normally found in poor-quality oocytes. Our molecular data, together with published results on oocyte survival, oocyte fertilization, and pregnancy rates, confirm that vitrification might be very helpful for preserving women's fertility [Kuwayama, *et al.*, 2005; Kuwayama, 2007; Chang *et al.*, 2008; Cao *et al.*, 2009; Wennerholm *et al.*, 2009; Cobo *et al.*, 2009].

TAp73 IS DOWNREGULATED IN OOCYTES FROM WOMEN OF ADVANCED REPRODUCTIVE AGE

In order to verify the potential involvement of p73 in women reproductive ageing, we searched quantitative expression differences of both TAp73 and Δ Np73 transcript variants. In this study, we analysed by RT-PCR Real Time 16 MII oocytes, 10 from women younger than 35 and 6 from women older than 38 years of age. We found out that TAp73 isoforms are significantly downregulated in oocytes from women of advanced reproductive age whereas Δ N isoforms are expressed in human oocytes, but their expression is age independent (Figure 28A). To confirm these data, we performed a similar analysis in pools of mouse oocytes split in two age groups: younger than 5–8 weeks (young) and older than 6–7 months (old). We found a significant TAp73 downregulation in the older cohort (Figure 28B). To statistically evaluate the results, young and old oocyte Δ Ct values were analysed by unpaired (two sample) Student's t-test ($p < 0.01$). To verify if this downregulation was dependent on epigenetic modifications, methylation analysis by bisulfite conversion and pyrosequencing was performed in order to detect the methylation status of 6 CpGs in the CpG island located at the promoter region of mouse TAp73 gene. Our results show that methylated CpG sites were less than 5% in both groups:

accordingly, promoter methylation does not explain the low levels of TAp73 expression in old mice (Figure 28).

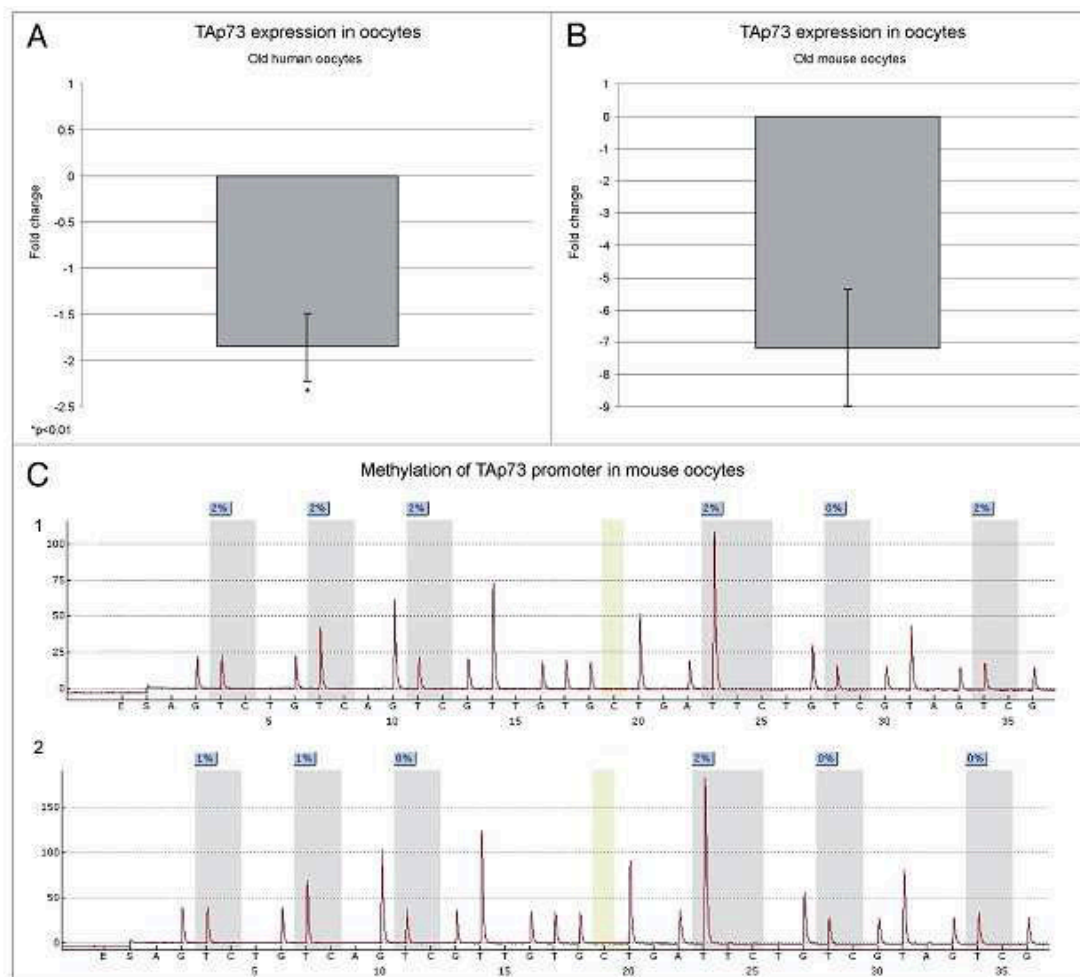


Figure 28. (A) Downregulation of TAp73 in old human oocytes. Data are shown as average natural logarithm of RQ values calculated by the $2^{-\Delta\Delta C_t}$ method using HPRT for normalization and young oocytes as calibrators. Y error bars represent variability among different samples, showing the maximum and minimum FC values. (B) The histogram shows the downregulation of TAp73 in old mouse oocytes. Data are presented as average natural logarithm of RQ values \pm standard deviation in pools of mouse oocytes. The experiment was performed in triplicate. (C) Methylation analysis in both young (1) and old (2) mouse oocytes was performed by bisulfite conversion and pyrosequencing to detect the methylation status of 6 CpGs in the CpG island, located at the promoter region of mouse TAp73 gene. Each sample was analysed in duplicate, including an internal control, to measure the extent of bisulfite modification.

THE APOPTOTIC TRANSCRIPTOME OF THE HUMAN MII OOCYTE: CHARACTERIZATION AND AGE-RELATED CHANGES

HT analysis of AM in human oocytes

Several AM mRNAs are expressed in human oocytes. For 58 transcripts, we obtained Ct values <35 in old and/or young oocytes (Table 7, Groups 1–2). On the contrary, 26 mRNAs had Ct values >35 or not determined, so they can be considered absent or expressed at very low levels in human eggs (Table 7, Group 3). Some mRNAs present in groups 1 or 2 such as BAG3, CD40, CFLAR, TNFRSF21, TRAF2 and TRAF3 have never been described in human oocytes. Comparing old versus young oocytes we found that the expression profile of AM genes was strongly dependent on maternal age: 36 genes showed an age-dependent expression. Among significantly DE genes, we found 12 proapoptotic genes upregulated and 9 antiapoptotic genes downregulated in old oocytes (Figure 29). In general, our results show that the number of upregulated proapoptotic genes in old oocytes is higher than that of downregulated ones. Similarly, the number of downregulated antiapoptotic genes in old oocytes is higher than that of upregulated ones (Figure 29).

NC analysis shows that CFLAR, FADD, TRADD, CASP9, BID, TRAF2, CASP10, TNFRSF1A, CASP3, BCL2 and CASP8 are the nodes having the highest centrality score among the analysed AM genes (Figure 30). When we compared centrality parameters with expression data, we found that among DE genes BCL2 and CFLAR were associated to high centrality values within the AM network (Wilcoxon Rank Sum test): B (p-value=0.043), C (p-value=0.04), ND (p-value=0.043), S (p-value=0.054) (Figure 30B). On the contrary, most DE genes were associated to low centrality values (Figure 30B). We identify 12 potentially interesting mRNAs to be validated on single oocytes. BCL2 and CFLAR perform a central role in AM

network and are downregulated in old oocytes (Figure 29, 30). BAD, BCL2L11, CD40, NAIP, TNFRSF10A and TNFRSF21 are markedly overexpressed or downregulated in old compared to young oocytes ($RQ > 3$ or $RQ < -3$) (Figure 29). Finally, we analysed BCL2L1 long and BCL2L1 short, MCL1 long and MCL1 short, to detect transcript variants whose isoforms perform pro- or antiapoptotic functions (Table 5).

Table 7. Expression groups of Apoptotic Machinery genes in human oocytes.

Group 1 *(Ct < 30)	ABL1, BAG3, BAX, BCL2L1, BCL2L10, BNIP3L, CASP6, CASP9, CD40, IGF1R, MCL1, NOD1, RIPK2, TNFRSF21, TP73, TRAF2, TRAF3
Group 2 *(30 < Ct ≤ 35)	AKT1, APAF1, BAD, BAG1, BAG4, BAK1, BCL10, BCL2, BCL2L11, BCLAF1, BFAR, BIRC2, BIRC4, BIRC6, BNIP1, BNIP2, BNIP3, BRAF, CARD6, CASP1, CASP2, CASP3, CASP4, CASP7, CD27, CFLAR, CIDEB, CRADD, DFFA, FADD, HRK, NAIP, PYCARD, TNFRSF10A, TNFRSF10B, TNFRSF25, TNFRSF9, TP53, TP53BP2, TRADD, TRAF4
Group 3 *(Ct > 35 o ND**)	BCL2A1, BCL2L2, BID, BIK, BIRC3, BIRC8, CARD8, CASP10, CASP14, CASP5, CASP8, CD40LG, CD70, CIDEA, DAPK1, FAS, FASLG, GADD45A, LTA, LTBR, NOL3, TNF, TNFRSF11B, TNFRSF1A, TNFSF10, TNFSF8

*Ct value referred at least to one group (old or young oocytes); **Not Determined.

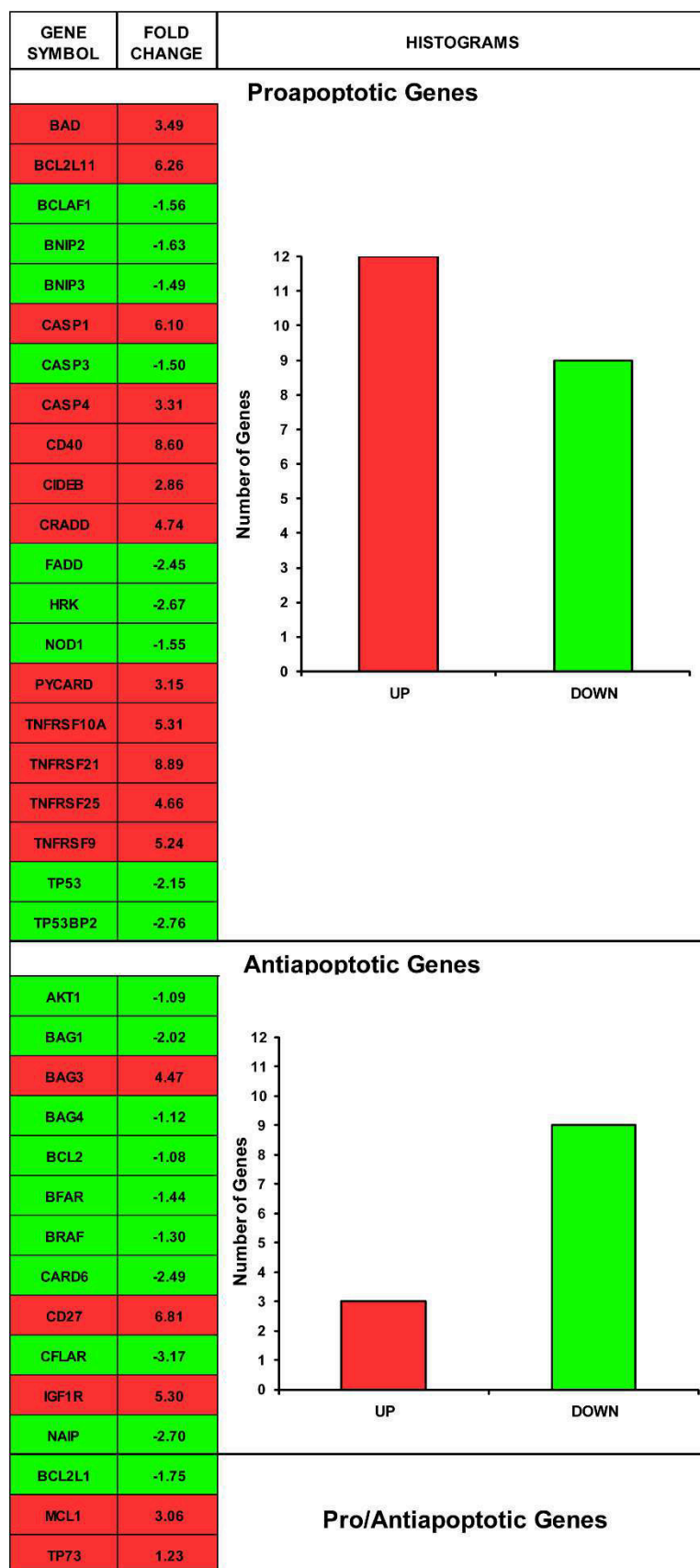


Figure 29. Differentially expressed AM genes in oocytes from older women. Data are shown as \ln of RQ values of DE genes in old with respect to young oocyte pools. Upregulated genes are shown in red, downregulated genes in green.

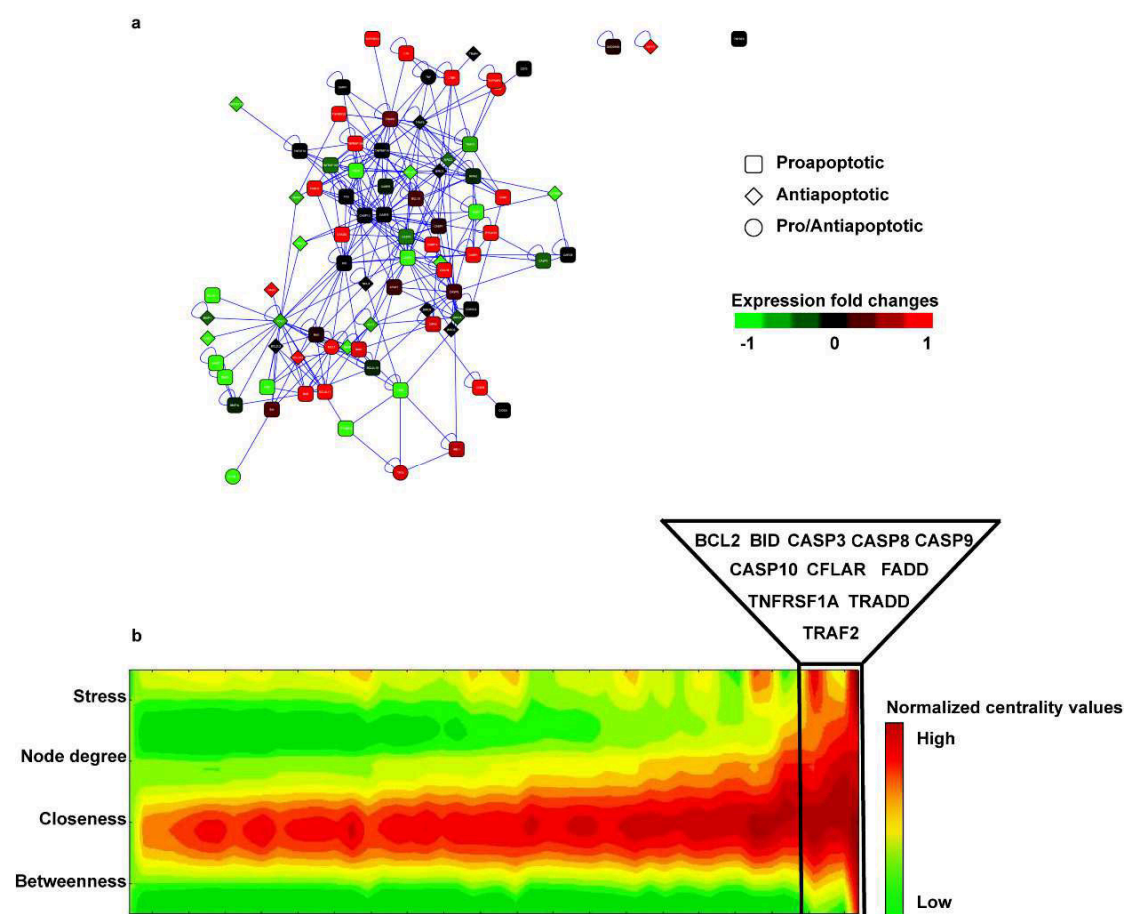


Figure 30. AM network structure based on different expression profile in oocyte pools from older with respect to younger women. (A) Network of interaction among 84 AM genes: squares=proapoptotic proteins; diamonds=antiapoptotic proteins, circles=pro/antiapoptotic proteins. Data are shown as \ln of RQ values of DE genes in old with respect to young oocyte pools. The colours indicate the expression fold change of AM genes according to the bar shown on the right of the network (B) Heat map of centrality values of AM proteins: X-axis represents the AM proteins with the highest centrality, Y-axis represents different centrality parameters. The colours indicate the centrality levels, according to the colour bar shown on the right of the matrix. The emphasized genes have the higher values of all centrality parameters with respect to all genes considered. B= betweenness; C= closeness; ND= node degree; S= stress.

Single cell analysis

We found a high variability of expression profiles among oocytes; maternal age is only one of the factors that determine the transcriptome of mature oocytes, even though a very important one. In fact, molecular diversity can be determined by intraspecies genomic variability and also by external stimuli. Experimental studies suggest that environmental influences might affect follicle development and oocyte

maturation influencing oocyte competence and reproductive success [Hunt and Hassold, 2008]. Moreover, even oocytes from the same woman can show variability (e.g., genome structure and epigenetic modifications) leading to differences in oocyte competence, fertilization grade and pregnancy rates. Nevertheless, statistical analysis by unpaired (two sample) Student's t-test confirmed that the expression differences between old and young oocytes are significant for BCL2, CFLAR, CD40, TNFRSF10A and TNFRSF21 (dependently on the specific mRNA $p < 0.05$ or $p < 0.01$, see Figure 31). We found that BCL2 and CFLAR are downregulated while CD40, TNFRSF10A and TNFRSF21 are upregulated in old oocytes (Figure 31). Analysis of BCL2L1 and MCL1 transcript variants showed that the antiapoptotic isoforms prevail on the proapoptotic in both groups of age and no difference was present in relation to maternal age.

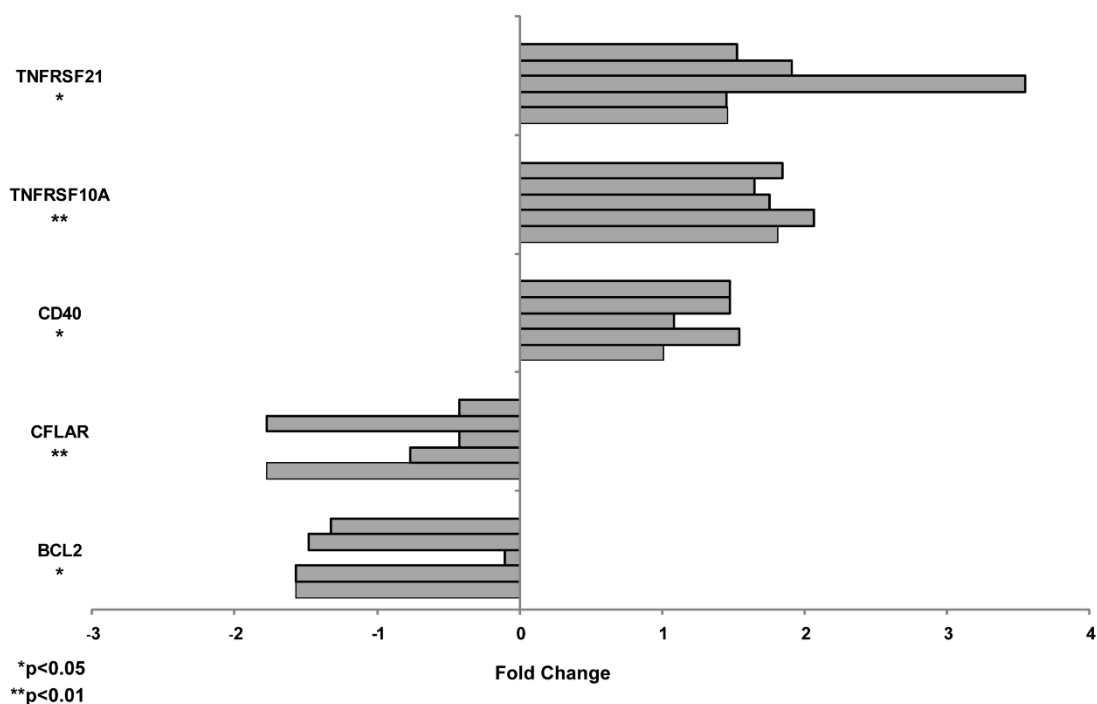


Figure 31. DE genes in single oocytes from older women. Downregulation of BCL2, CFLAR and upregulation of CD40, TNFRSF10A, TNFRSF21 in single old oocytes. Data are shown as natural logarithm of RQ values of DE genes. Unpaired Student's t-test among ΔC_t values between old and young oocytes (** $p < 0.01$, * $p < 0.05$).

EXOSOMAL microRNAs IN HUMAN FOLLICULAR FLUID: NEW ACTORS IN THE COMMUNICATION BETWEEN OOCYTE AND SOMATIC FOLLICULAR CELLS

Exosome characterization

To confirm that the analysed structures are exosomes, we performed size determination of the particles present in the ultracentrifuge pellet of follicular fluids; this was followed by flow cytometry analysis for the detection of exosomal markers. The results revealed the presence of vesicles with an average size of 40 nm in diameter in the follicular fluid samples, which is consistent with the characteristic size range of exosomes [Théry *et al.*, 2002] (Figure 32A). Moreover, our data demonstrate that our samples were positive for tetraspanin proteins CD63 and CD81, known to be enriched in exosomes, but not for CD9 [Théry *et al.*, 2002] (Figure 32B). This could be due to the fact that exosomes may express different surface markers, based on their source of origin and their specific physiological state. Analysis of exosome RNA content revealed no contamination with cellular RNA. In fact, vesicle preparation from our sample contained a relatively large amount of small-sized RNA, with no 18S and 28S ribosomal RNA (Figure 32C).

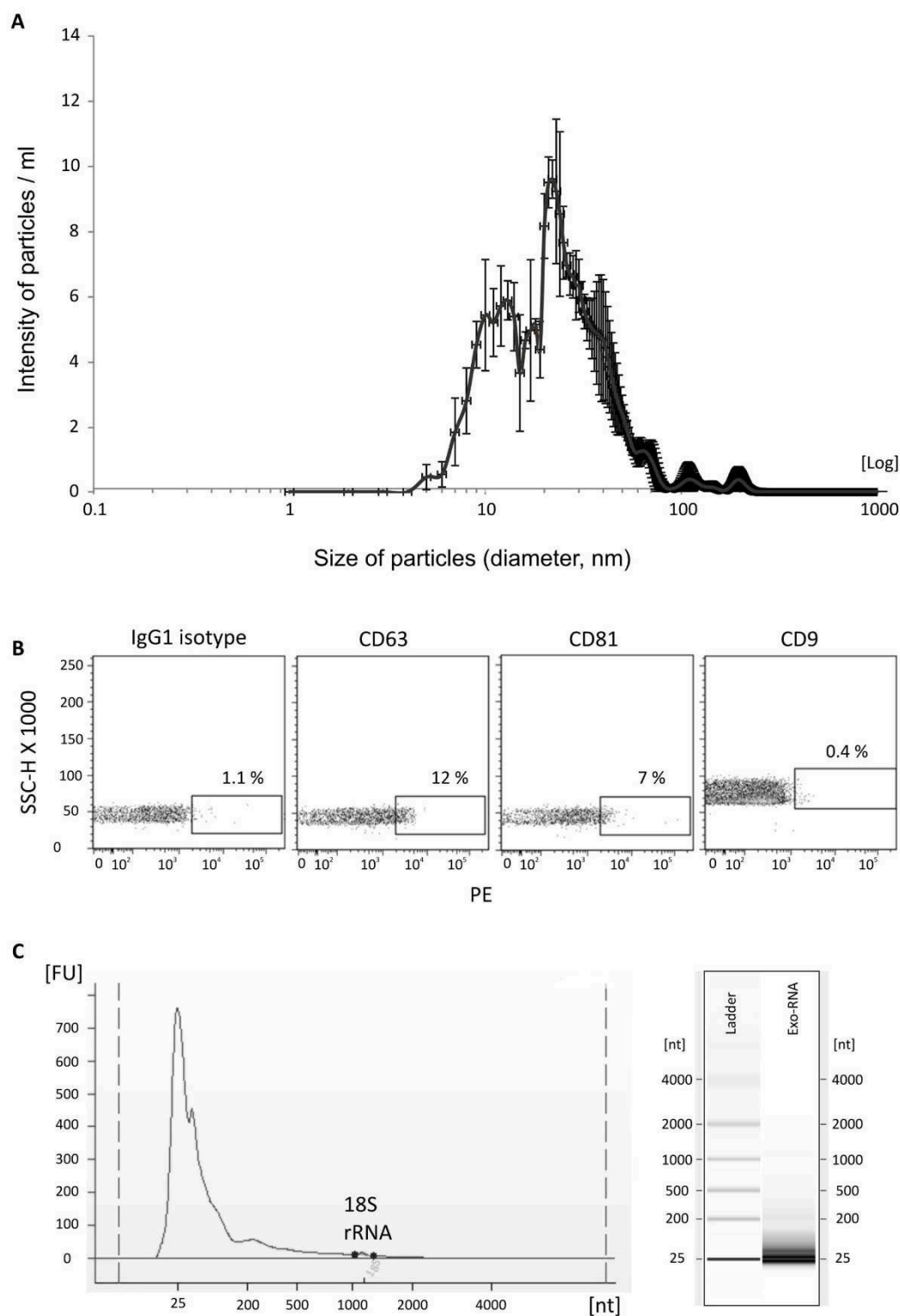


Figure 32. Characterization of exosomes. (A) Particle sizes in ultracentrifuge pellet consistent with size range of exosomes. Pelleted structures are vesicles whose diameter size range from 10 nm to 100 nm with a peak size between 30 and 50 nm. Average vesicle size was 40 nm. (B) The presence of the typical exosomal surface markers CD63, CD81 and CD9 was determined using FACS and compared to the corresponding isotype control. Follicular fluid exosomes were positive for the first two tetraspanins. Vesicles were bound to latex beads and stained with antibodies against exosome associated proteins (CD63, CD81 and CD9). (C) Bioanalyzer results showing RNA content in exosomes. Exosomes are enriched in small RNAs, the two peaks for rRNA are absent.

Expression profile of microRNAs

We compared miRNAs purified from total FF and from FF exosomes with those from plasma from the same women. We discovered that 37 miRNAs are upregulated: specifically, we found 15 upregulated miRNAs in total FF (Figure 33A) and 32 upregulated eFF-miRNAs in exosomes (Figure 33B). In particular, we found 10 miRNAs (27%) shared between total FF and exosomes, and 22 (60%) detectable only in exosomes. Interestingly, we found 5 (13%) FF specific miRNAs (Figure 33). Among the identified eFF-miRNAs, we found 11 miRNAs (miR-204, miR-212, miR-218, miR-337-5p, miR-410, miR-455-5p, miR-489, miR-493, miR-508-3p, miR-654-3p, miR-886-5p) that have not been previously described in exosomes (www.exocarta.org).

Additionally, we found miR-218, miR-508, miR-654-3p that have never been described in ovarian cells, while miR-10b, miR-29a, miR-99a, miR-125b, miR-132, miR-202, miR-212 and miR-874 have been reported to be highly expressed in granulosa or in cumulus cells [Fiedler *et al.*, 2008; Assou *et al.*, 2013; Velthut-Meikas *et al.*, 2013].

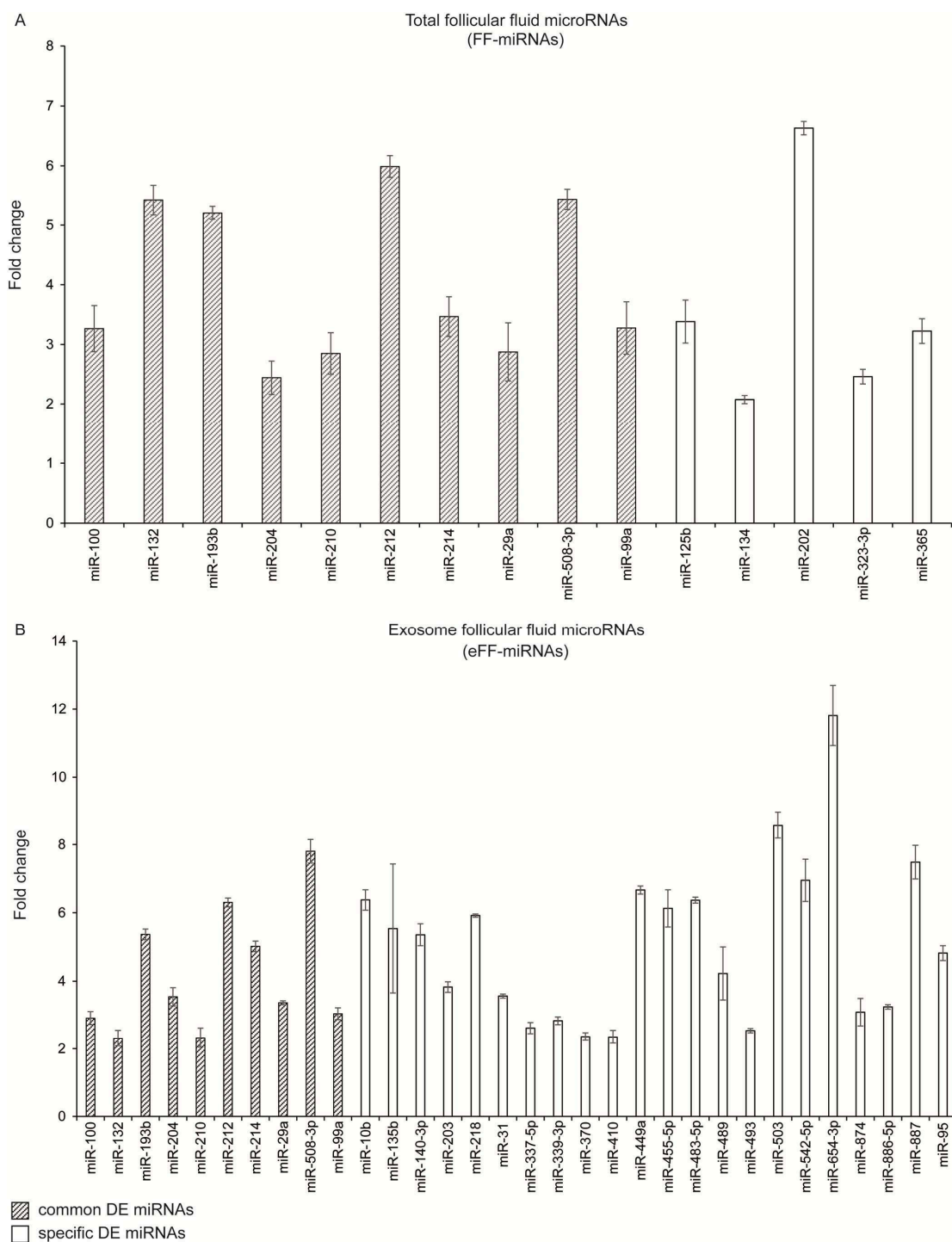


Figure 33. Upregulated miRNAs in total follicular fluid (A) and exosomes (B). Lined bars show the 10 miRNAs shared between FF and exosomes, whereas white bars represent 5 and 22 miRNAs which are specific of FF and exosomes, respectively. Fold change is shown as natural logarithm of relative quantity (RQ) values, which were calculated through the $2^{-\Delta\Delta Ct}$ method by using miR-145 as endogenous control and plasma as calibrator sample. Error bars represent Standard Deviation.

Genomic Analysis

Genomic analysis showed that 23 miRNAs are localized in 13 different clusters; a number of the upregulated miRNAs are part of the same cluster (Table 8), which could suggest sharing of a common promoter. miR-132 and miR-212, located in a cluster at 17p13.3, have been found highly upregulated in mouse mural granulosa cells following LH/hCG induction [Fiedler *et al.*, 2008]. Their expression is regulated by GnRH in mouse L β T2 gonadotrope cell line [Yuen *et al.*, 2009]. Interestingly, miR-134, miR-323-3p, miR-410 and miR-654-3p are localized within the large miR-379/miR-656 cluster, exclusively present in placental mammals and involved in embryonic development [Glazov *et al.*, 2008]. We found that the coding sequence of 13 miRNAs is located inside genes encoding for proteins and 24 in extragenic regions or inside non-coding genes. Interestingly, miR-218 is located within slit homolog 2 (*Drosophila*) (SLIT2) gene, which encodes the secreted SLIT glycoprotein Roundabout (ROBO) receptors ligand. SLIT and ROBO have been proposed to be regulated by steroid hormones and to modulate physiological cell functions in the ovary [Dickinson and Duncan, 2010].

Table 8. Genomics of Follicular Fluid microRNAs.

Extragenic microRNAs *			miRNAs located in protein encoding genes			
microRNA	Chromosomal position	Clustered miRNAs	microRNA	Chromosomal position	Clustered miRNAs	Host Gene
miR-886-5p	5q31.1		miR-214	1q24.3	miR-199a-2 miR-3120	DNM3
miR-339-3p	7p22.3		miR-135b	1q32.1		LEMD1
miR-29a	7q32.3	miR-29b-1	miR-10b	2q31.1		HOXD3
miR-31	9p21.3		miR-95	4		ABLIM2
miR-202	10q26.3		miR-218	4p15.31		SLIT2
miR-210	11p15.5		miR-449a	5q11.2	miR-449b miR-449c	CDC20B
miR-100	11q24.1	let-7a-2	miR-887	5p15.1		FBXL7
miR-125b	21q21.1	miR-99a miR-125b let-7c	miR-874	5q31.2		KLHL3
miR-99a			miR-489	7q21.3	miR-653	CALCR
miR-134	14q32.31	Cluster miR- 379/656	miR-204	9q21.12		TRPM3
miR-323-3p			miR-455-5p	9q32		COL27A1
miR-410			miR-483-5p	11p15.5		IGF2
miR-654-3p			miR-140-3p	16q22.1		WWP2
miR-203	14q32.33	miR-203b miR-203a	(*) Or located in non-coding genes.			
miR-337-5p	14q32.2	miR-493 miR-665 miR-431 miR-433 miR-127 miR-432 miR-337				
miR-370						
miR-493						
miR-193b	16p13.12	miR-365a miR-193b				
miR-365						
miR-212	17p13.3	miR-132 miR-212				
miR-132						
miR-503	Xq26.3	miR-542 miR-503 miR-424 miR-450a-2 miR-450a-1 miR-450b				
miR-542-5p						
miR-508-3p	Xq27.3	miR-507 miR-506				

Gene Ontology and Pathway Analysis

Gene-Ontology analysis of validated and predicted targets, independently performed on the 15 FF-miRNAs and 32 eFF-miRNAs, revealed that the most significant Biological Processes shared are: *developmental process, regulation of cellular process, cell differentiation, cell communication* (Figure 34). Moreover, we found that *cell-cell signaling* (157 targets) and *developmental process involved in reproduction* (68 targets) were significant only for eFF-miRNAs (Figure 34).

Comparative pathway analysis showed that *WNT, MAPK, ErbB* and *TGF β signaling pathways* are the most significant, with negative \log_{10} (p-value) included between 11.04 and 4.12. *Cell communication, cell cycle, PPAR Notch, p53* and *Hedgehog signaling pathways* are significant for eFF-miRNAs (Figure 35). Our results revealed that miR-29a, miR-132, miR-135b, miR-203, miR-212 and miR-214 regulate most of the pathways, whereas miR-99a, miR-100, miR-483-5p and miR-887 are involved in regulation of only few of them (Figure 35).

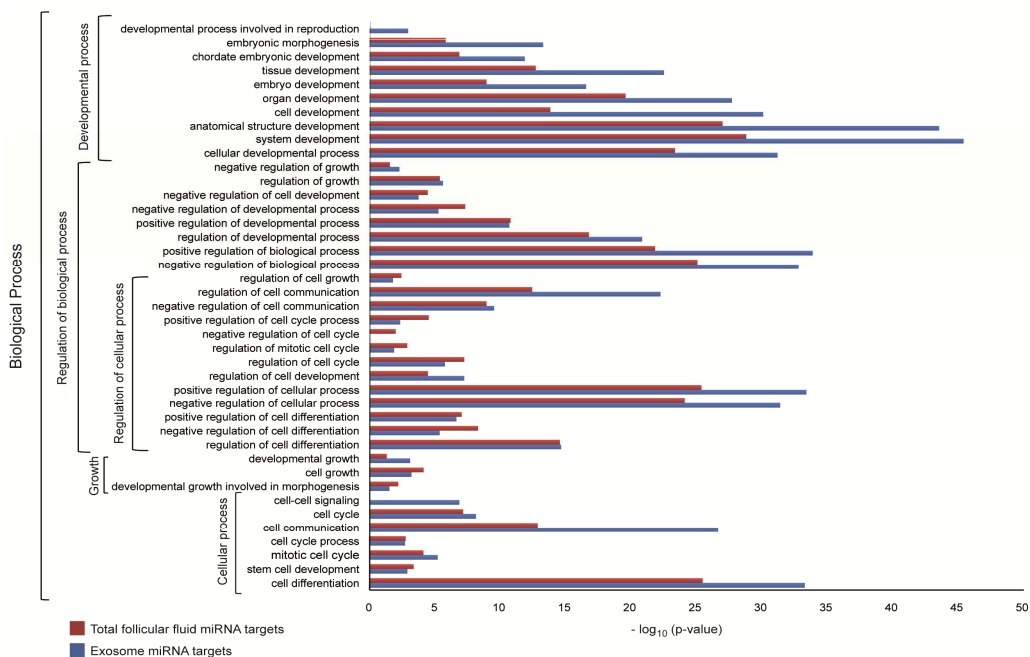


Figure 34. Gene Ontology analysis based on differentially expressed miRNA targets. Gene ontology (GO) terms within Biological Process category for the upregulated miRNA targets in total follicular fluid (red bars) and exosomes (blue bars) are shown. X-axis represent the $-\log_{10}$ (p-value), the significance was determined by the adjusted Bonferroni correction.

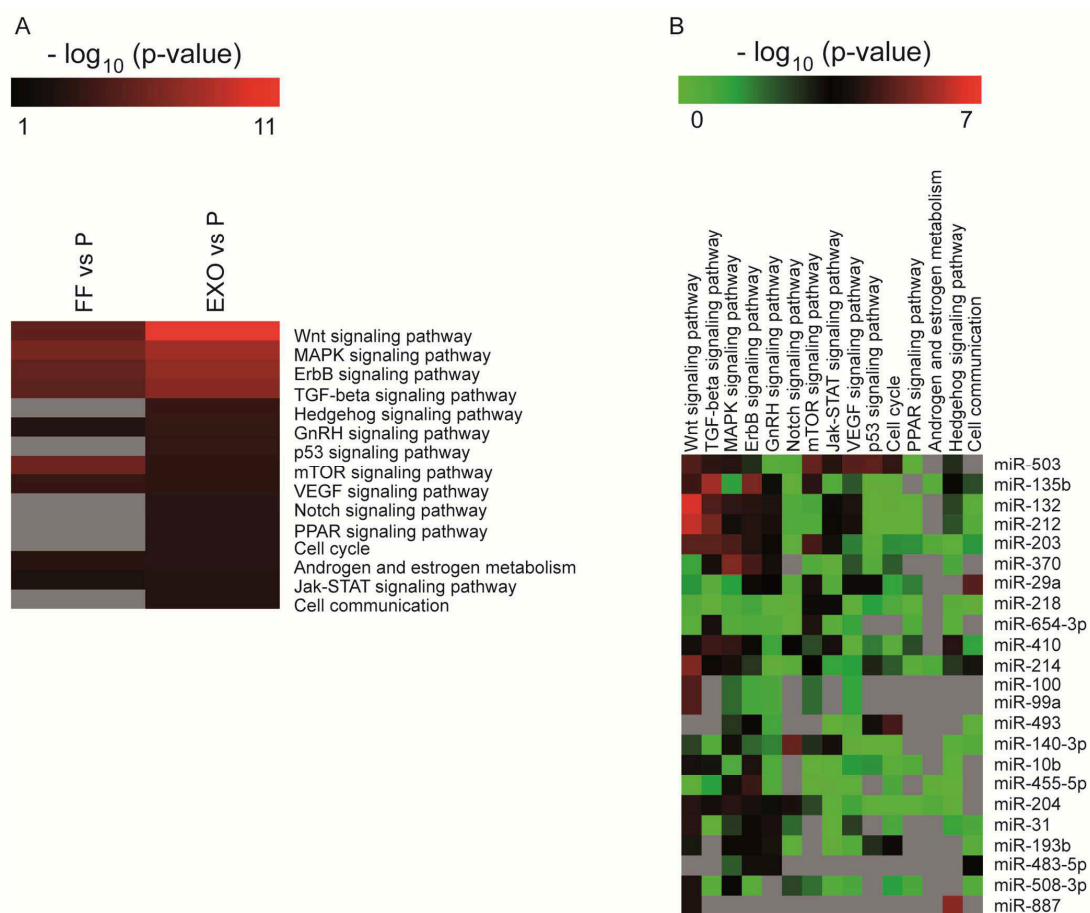


Figure 35. Heat-map representations of signaling pathways. (A) Significant signaling pathways regulated by follicular fluid and exosomal miRNAs. (B) FF-miRNAs and eFF-miRNAs involved in the regulation of signaling pathways. Grey boxes indicate that a miRNA does not control the matched pathway reported in the x-axis. The probability values are reported as $-\log_{10}(\text{p-value})$ for both panels.

Validated Targets

miRNA biological functions depend on those of their target genes: based on bioinformatic tools (miRecords, TarBase, miRTarBase, starBase) and supported by literature analysis, we found that expression levels of proteins involved in follicular development and oocyte maturation can be regulated by several eFF-miRNAs. Specifically, we found that SIRT1 is regulated by miR-132, miR-140-3p and miR-449a, PTEN by miR-29a, miR-132 and miR-214, MTOR by miR-99a and miR-100, DNMT3B and DNMT3A by miR-29a and miR-132, RB1 by miR-132 and miR-212.

In Table 9 we have listed nine miRNAs and their peculiar features, which make them the most interesting miRNAs involved in follicle maturation.

Table 9. Most interesting eFF-miRNAs and their peculiar features.

miRNA	Notes	Target Genes*	References
miR-29a	It is highly expressed in human cumulus cells; It plays an important role in female gonadal development modulating methylation of genomic DNA.	DNMT3A DNMT3B	Takada <i>et al.</i> 2009, Denis <i>et al.</i> 2011, Assou <i>et al.</i> 2013.
miR-99a	In cluster with let-7c which is highly expressed in cumulus cells; Its deregulation is involved in ovarian cancer.	MTOR	Nam <i>et al.</i> 2008, Doghman <i>et al.</i> 2010, Velthut-Meikas <i>et al.</i> 2013.
miR-100	Its deregulation is involved in ovarian cancer by MTOR pathway	MTOR	Nagaraja <i>et al.</i> 2010
miR-132	Involved in steroidogenesis; Involved in polycystic ovary syndrome; Regulated by LH/hCG; Highly expressed in granulosa and cumulus cells.	SIRT1 PTEN RB1 DNMT3A	Fiedler <i>et al.</i> 2008, Strum <i>et al.</i> 2009, Park <i>et al.</i> 2011, Jin <i>et al.</i> 2012, Miller <i>et al.</i> 2012, Wong <i>et al.</i> 2013, Sang <i>et al.</i> 2013.
miR-212	Regulated by LH/hCG; Highly expressed in granulosa and cumulus cells.	PTEN RB1	Fiedler <i>et al.</i> 2008, Park <i>et al.</i> 2011, Wong <i>et al.</i> 2013.
miR-214	It is involved in ovarian cancer targeting PTEN	PTEN	Yang <i>et al.</i> 2008, Yin <i>et al.</i> 2010.
miR-218	It is absent in plasma; Negative regulator of three Wnt signaling inhibitors; It is located in SLIT2 gene encoding the secreted SLIT glycoprotein Roundabout (ROBO) receptors ligand. The SLITs and ROBOs seem to be regulated by steroid hormones and regulate physiological cell functions in ovary.	LHR	Dickinson <i>et al.</i> 2010, Hassan <i>et al.</i> 2012.
miR-508-3p	It is absent in plasma; Present in seminal plasma but absent in other biological fluid.	TSC1	Weber <i>et al.</i> 2010
miR-654-3p	Located in miR-379/miR-656 miRNA cluster, exclusively present in placental mammals; p21 inhibition induced oocyte maturation.	P21	Kovac <i>et al.</i> 2001, Glazov <i>et al.</i> 2008, Wu <i>et al.</i> 2010.

(*) All targets are experimental validated except for LHR and TSC1

Our molecular analysis of eFF-miRNAs demonstrates that many of them are involved in the regulation of biological processes and signaling pathways important for follicle maturation (Figure 34, 35). We represent a schematic view of different pathways inside follicular cells and oocyte showing that eFF-miRNAs are able to negatively regulate genes encoding for the inhibitors of follicle maturation and meiosis resumption [Sun *et al.*, 2009; Zhang *et al.*, 2009; Conti *et al.*, 2012] (Figure 36).

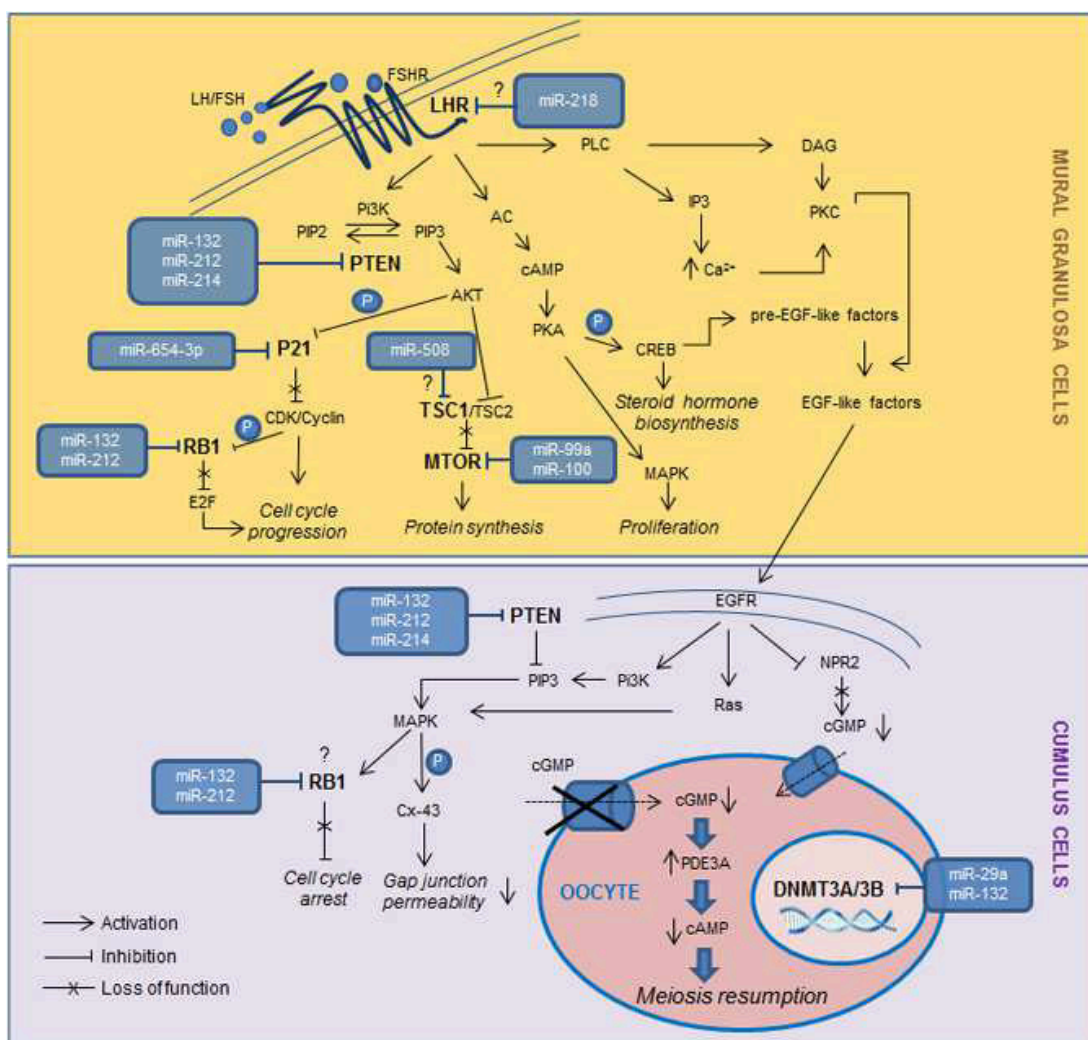


Figure 36. Ovarian follicle, signaling pathways and eFF-miRNAs. Model summarizing follicle signalling pathways. eFF-miRNAs could act along these ways and possibly regulate the processes of follicular development and meiotic resumption.

5. DISCUSSION

The quality of the female gamete has an impact on rates of preimplantation, implantation and clinical pregnancy. During the whole differentiation process of the oocyte, several intra- and external ovarian factors may adversely affect the female reproductive system and contribute to reduced fertility. In fact, an ovulated MII oocyte is not always a good egg, as it may resist fertilization or, when fertilized, may not be competent to sustain development. Many studies in animal models and in humans revealed the association between decrease of oocyte competence and altered gene expression. For example, several papers demonstrated significant expression profile differences during reproductive ageing, comparing oocytes coming from young and old women. [Hamatani *et al.*, 2004; Steuerwald *et al.*, 2007; Grøndahl *et al.*, 2010]. Thus, eggs have been intensively studied with the aim of understanding the main causes of oocyte competence decrease, and identifying non-invasive markers that would help the selection of the gametes to fertilize. Accordingly, studies on oocyte transcriptome are important to understand the biological pathways involved in oogenesis, totipotency and early embryonic development and genes regulating physiological pathways in gametes could represent potential candidate for reproductive disorders. Moreover, given that the bidirectional traffic between the oocyte and its surrounding somatic cells is very important for the acquisition of oocyte competence, the analysis of each component of the ovarian follicle is needed. The study of granulosa cells, cumulus cells and follicular fluid, whose composition depend on the secretory activity of somatic follicular cells and oocyte, could improve our knowledge on the complex process of mammalian oocyte maturation.

For these reasons, we decided to analyse the transcriptome of human MII oocytes, focusing our attention on different issues that could influence oocyte quality such as cryopreservation protocols employed in Assisted Reproductive Technologies, and reproductive ageing. Subsequently, we aimed at characterizing miRNA role in follicular fluid by isolating exosomes and by analysing the expression profiles of their miRNA cargo.

Firstly, we demonstrated that vitrification protocol keeps unaltered oocyte molecular profile and does not cause degradation of mRNAs essential for oocyte development (i.e. BMP15, GDF9, FIGLA, OCT4, TAF4B), as normally found in poor-quality oocytes. Our molecular data, together with published results on oocyte survival, oocyte fertilization, and pregnancy rates, confirm that vitrification might be very helpful for preserving women's fertility. In particular, this technique could be advantageous especially for those women at risk of losing their ovarian functions after disease, surgery, or chemotherapy [Grifo and Noyes, 2010]. The evidence of the advantages of this technique is the recent birth of the first baby conceived from a woman, previously affected by cancer, using a vitrified oocyte (26th October 2013, Milan, Italy).

Subsequently, we focused our attention on gene expression profile changes during reproductive ageing, in order to identify mRNAs that play a critical role in oocyte maturation. Apoptosis involvement in this process is well established even if their molecular bases were still poorly characterized. In fact, apoptosis is of critical importance for oocyte differentiation and early embryonic development: it plays a crucial role in establishing oocyte competence after intrinsic genetic, epigenetic and cytoplasmic changes essential for producing a normal offspring [Miao *et al.*, 2009; Boumela *et al.*, 2009]. We analysed the expression profile of 84 AM genes, during

reproductive ageing, in human MII oocyte pools and further confirmed our data in single oocytes. These studies led us to identify AM transcripts never reported in human oocytes so far (BAG3, CD40, CFLAR, TNFRSF21, TRAF2, TRAF3) and others which are well known and characterized (BAX, BCL2L10, MCL1, TAp73). We demonstrated that the expression profile was strongly dependent on maternal age: 36 out of the 84 AM genes showed an age-dependent expression (Figure 29). In particular, we found that death receptors such as CD40 and TNFRSF21, and death receptor-associated factors such as TRAF2 and TRAF3 are overexpressed, while survival factors such as BCL2 and CFLAR are downregulated in oocytes from older women. Accordingly, the upregulation of most proapoptotic genes and downregulation of most antiapoptotic genes represent strong evidence that the molecular profile of old oocytes is more inclined to apoptosis in comparison to their young counterpart (Figure 29). Network analysis confirms the relevance of apoptosis to female gametogenesis: it shows a significant correlation between AM genes, whose expression is constant during ageing, and centrality (Figure 30). Given their leading role in the apoptotic process, perturbation of high centrality genes could impair the network in a non-reversible way: accordingly, alteration of their expression could have been negatively selected during evolution. On the other hand, peripheral network modifications could be tolerated without invalidating oocyte development [He and Zhang, 2006]. Nevertheless, antiapoptotic CFLAR and BCL2, which are downregulated in old oocytes, are associated to high centrality values (Figure 30). Based on our analysis of oocyte AM transcriptome, mature MII oocytes from older women could be more inclined to apoptosis and sensitive to external death signals. As a result, unfit or poorly fit gametes and embryos could be eliminated by activating intrinsic or extrinsic apoptotic pathways. In nematodes and

in mammals, apoptosis is able to counteract the decline of oocyte quality [Andux and Ellis, 2008; Kujjo *et al.*, 2010]. The theory of *death by defect*, which aims to explain why so many germ cells are lost during fetal ovarian development, could also be applied to postnatal life: apoptosis could represent a general mechanism of natural selection [Tilly *et al.*, 1997; Tilly, 2001].

Among differentially expressed genes in human MII oocyte pools during reproductive ageing, we also found TAp73. This process is closely related to the production of oocytes with a reduced developmental competence whose main hallmark is aneuploidy. It has been demonstrated that aneuploidies are associated with altered transcript levels affecting genes encoding proteins involved in spindle assembly and chromosome alignment [Fragouli *et al.*, 2010]. Moreover, several genes involved in cell cycle regulation, spindle function, meiosis, oxidative stress and protective mechanisms have been found differentially expressed during ageing [Eichenlaub-Ritter *et al.*, 2004; Grøndahl *et al.*, 2010]. Given their well known involvement in the control of cell cycle, apoptosis and genome stability including ploidy, the members of the p53 family (p53, p63, p73) could be etiologically associated with this process. In order to verify the potential involvement of p73 isoforms in women reproductive ageing, we determined their expression in single mature MII oocytes from old and young women. In particular, we found out that TAp73 isoforms are significantly downregulated in oocytes from women of advanced reproductive age. Our data are the first on TAp73 downregulation in human old oocytes: they integrate other existing literature results and suggest an important role for this member of the p53 family as guardian of maternal reproduction in mammals. In fact, TAp73^{-/-} mice show defects in spindle organization during mitosis and meiosis, develop tumors and produce oocytes that even if

fertilized generate non-vital embryos [Tomasini *et al.*, 2008]. Tomasini and colleagues reported TAp73 interactions with the kinetochore proteins Bub1, Bub3, BubR1 components of the spindle assembly checkpoint (SAC) complex [Tomasini *et al.*, 2009]. If the chromosomes are not properly attached to the spindle, this complex stops the anaphase promoting complex (APC) by negatively regulating CDC20. Absence of TAp73 removes this cell cycle brake, so causing genomic instability. Consequently, TAp73 downregulation in oocytes from women of advanced reproductive age could lead to aneuploidy in the developing embryos, explaining both the reduction of fertility and the increase of newborns with chromosomal abnormalities. Methylation analysis we also performed on TAp73 promoter showed that methylated CpG sites were less than 5%, thus revealing that its downregulation was not dependent on epigenetic modifications. Accordingly, it could involve other signalling molecules such as miRNAs: recent data on RNA silencing during oogenesis and early oocyte development have revealed the critical role of miRNAs in post-transcriptional gene regulation in this system [Ohnishi *et al.*, 2010; Svoboda and Flemr, 2010].

The deregulation of follicular fluid miRNAs could affect oocyte transcriptome and competence. The presence of miRNAs in FF has been recently demonstrated, therefore these small RNAs could be involved in a proper cell communication between the oocyte and its surrounding somatic cells [da Silveira *et al.*, 2012; Sang *et al.*, 2013]. It is common opinion that FF represent a very important microenvironment for the development of the oocyte: its biochemical composition, related to the metabolic activity of ovarian cells, reflects the physiological state of the follicle. Accordingly, the analysis of FF components may provide useful information on oocyte quality and pathways involved in follicle differentiation and

development [Revelli *et al.*, 2009]. Finally, given that FF is easily available during oocyte pick-up, it could represent an optimal source of non-invasive molecular markers of oocyte quality. Nevertheless, it has not been investigated if FF miRNAs are released from blood cells or from cells of the follicular microenvironment.

The last subject of this thesis is therefore the isolation and characterization of miRNAs in both human follicular fluid and its purified exosomal fraction. We profiled the expression of 384 miRNAs in both samples with respect to plasma collected from the same women. This analysis revealed that 37 miRNAs are upregulated in follicular microenvironment and the majority of them are carried by exosomes, providing the first molecular evidence of bioactive vesicles involved in intercellular signaling inside ovarian follicle (Figure 32, 33). In fact, exosomes are able to mediate cellular communication carrying miRNAs and also mRNAs and proteins [Valadi *et al.*, 2007]. Given that the miRNAs identified in our paper are upregulated in FF respect to plasma, we propose that they are synthesized in follicular microenvironment by granulosa cells, cumulus cells or oocyte. Interestingly, miR-218, miR-455-5p and miR-508-3p seem to be FF specific, since we could not detect them in plasma samples. Moreover, we identified some miRNAs previously never described in ovary.

Our molecular analysis of eFF-miRNAs demonstrates that many of them are involved in the regulation of biological processes and signaling pathways important for follicle maturation (Figure 34, 35). In fact they are able to negatively regulate genes encoding for the inhibitors of follicle maturation and meiosis resumption such as PTEN, MTOR, P21 and RB1 [Sun *et al.*, 2009; Zhang *et al.*, 2009; Conti *et al.*, 2012] (Figure 36). eFF-miRNAs could also be involved in epigenetic modifications, which are important during gamete maturation and early embryo development. We

found that DNMT3A and DNMT3B are regulated by miR-29a and miR-132. DNMT3 silencing could explain hypomethylation in preimplantation embryo and in inner cell mass [Swales and Spears, 2005]. Recent studies have suggested a link between the use of assisted reproductive techniques and the increased incidence of normally rare imprinting disorders [Swales and Spears, 2005]. A greater understanding of the mechanisms regulating epigenetics is important in assessing the safety of these techniques.

Our results strongly suggest that exosomes are originated from follicle and that they may be exploited to exchange molecular signals (especially miRNAs) among follicular cells [Valadi *et al.*, 2007]. Currently, it is difficult to precisely pinpoint which cell type synthesizes and secretes exosomes and their molecular cargo: eFF-miRNAs could be released by somatic follicular cells or by the oocyte and in addition, they could act in autocrine or paracrine manner. Our results allow us to propose their involvement in the communication between oocytes and somatic follicular cells. Moreover, eFF-miRNA deregulation could cause alterations in the pathways involved in follicle growth and oocyte maturation, as it has been demonstrated for intracellular ovarian miRNAs [Baley and Li, 2012]. Therefore, they could represent potential biomarkers of oocyte quality and reproductive disorders.

6. CONCLUSIONS AND FUTURE PERSPECTIVES

A deeper knowledge of oocyte transcriptome and of the mechanisms regulating gene expression at transcriptional and post-transcriptional level would be of great utility to our understanding of the mechanisms involved in oogenesis and early embryonic development. It is important to remember that miRNAs are able to mediate post-transcriptional regulation also in paracrine manner, thus they could represent a way of communication among follicular cells.

This thesis aimed at characterizing mRNA and miRNA expression profiles in the female gamete and its microenvironment, respectively, under physiological and non-physiological conditions in order i) to assess the effect of vitrification on the biomolecular profile of the oocytes, ii) to understand the molecular basis of reproductive ageing and iii) to identify miRNAs in FF to be used as non-invasive biomarkers of oocyte quality.

The data reported in this thesis show for the first time that the vitrification protocol keeps unaltered the molecular profile of the oocyte and does not cause RNA degradation, confirming that this technique might be very helpful for preserving women's fertility. The characterization of AM gene expression profile in old and young oocytes contribute to clarify the molecular mechanisms of AM involvement in the natural selection strategy of removing low quality oocytes and preventing unfit or poorly fit embryos. Old oocytes produce low levels of survival factors as BCL2 and CFLAR and this condition make them more inclined to apoptosis. We demonstrated for the first time that TAp73 isoforms are significantly downregulated in oocytes from women of advanced reproductive age. TAp73 downregulation in old oocytes

could cause meiotic defects leading to aneuploidy in the developing embryos. In fact this protein perform a pivotal role in stopping the anaphase if chromosomes are not properly attached to the meiotic spindle. This could explain both the reduction of fertility and the increase of newborns with chromosomal abnormalities in women of advanced reproductive age.

Finally, we demonstrated the presence of exosomes in human follicular fluid, providing the first molecular evidence of these bioactive vesicles inside ovarian follicle. Moreover, we characterized their miRNA cargo, identifying new actors in the molecular communication among cells of ovarian follicle.

Further analysis will aim to compare FF samples collected from young and old women in order to investigate possible quantitative and qualitative differences in content and number of cell-secreted vesicles between these two groups. Furthermore, we will characterize miRNAs expression profile in old and young oocytes. These results could lead us to identify differentially expressed miRNAs between young and old women.

At any rate and in spite of several literature data, the events determining specific mRNA phenotypes in human oocytes and in early embryo are not yet clarified. Major efforts will be required to analyse the complex interactions among the different molecular players and to identify functional links among the different factors. This should allow to understand how the ovarian microenvironment can influence the oocyte transcriptome and proteome during eggs maturation and to characterize the molecular bases of reproductive potential decline caused by ageing or by reproductive disorders as POF or polycystic ovary.

7. REFERENCES

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