UNIVERSITÀ DEGLI STUDI DI CATANIA

DOTTORATO DI RICERCA IN BIOLOGIA, GENETICA UMANA E BIOINFORMATICA: BASI CELLULARI E MOLECOLARI DEL FENOTIPO-XXVIII CICLO

DIPARTIMENTO DI SCIENZE BIOMEDICHE E BIOTECNOLOGICHE
SEZIONE DI BIOLOGIA E GENETICA "GIOVANNI SICHEL"

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MicroRNA signature changes in human ovarian follicle in relation to reproductive aging

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Anno Accademico 2014-2015

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1. ABSTRACT

Small non-coding class of RNAs includes different types of molecules involved in different steps of RNA synthesis, processing, translation, as well as RNA modulation of transcription initiation, RNA degradation or protein synthesis block. Among these, microRNAs (miRNAs) represent the most studied and better characterized class of ncRNAs in terms of function and impact in human health and disease. miRNAs are short non-coding RNAs involved in the control of gene expression in different species. Specifically, miRNAs play a major role as master regulators of protein-coding genes. They target specific mRNAs for cleavage or translational repression. miRNA role in ovarian follicles has been characterized and the existence of miRNAs in human follicular fluid has been recently demonstrated. It was also reported that altered regulation of miRNA expression affects crucial pathways for follicle growth and oocyte maturation in several reproductive diseases. In addition, global miRNA analysis, on different tissues and organs and animal models has also shown that aging can influence mirna expression. Decreased female fertility with advanced maternal age has been widely documented. Although it is widely recognized that a key aspect that explains infertility in reproductive aging is the decline in oocyte quality, which also associates with higher risk of birth defects, genetic disorders and miscarriage, the molecular mechanisms underlying reproductive aging in female mammals are poorly understood. This thesis has aimed at evaluating impact of maternal age on miRNA expression profiles in human ovarian follicle and characterizing the pathways significantly affected by female ageing, identifying their regulator miRNAs. The manuscript includes three studies that for concision, they will be referred in the text, by Roman numerals, as Study I, II and III respectively. The Study I has been focused on the characterization of

microRNAs in human follicular fluid (FF), the Study II on the miRNome changes in Follicular fluid exosomes from women of two different age groups; while miRNA identification in human MII oocyte and their expression profile changes in relation to reproductive aging have been shown in the Study III. Firstly, it was ascertained whether miRNAs are cargo of FF exosomes and whether they are involved in the regulation of follicle maturation. At a later stage, TaqMan Human microRNAs cards were performed to verify differently expressed miRNAs in FF respect with plasma samples, collected from 15 healthy women who underwent to intracytoplasmic sperm injections (ICSI). 37 miRNAs had significantly higher expression levels in human FF. 32 are carried by exosomes and involved in critically important pathways for follicle growth and oocyte maturation. Specifically, nine of them target and negatively regulate mRNAs expressed in the follicular microenvironment encoding inhibitors of follicle maturation and meiosis resumption. In order to reveal the contribution of miRNAs to female reproduction aging, we examined their expression changes during aging in human follicular fluid, using TLDA technology. Different miRNA distribution was found in FF exosomes from old and younger women. We detected about 50 miRNAs in FF exosomes which showed highly significant differences related to aging and were predicted to regulate ECM-receptor interaction, PI3K-Akt, p53, TGF-beta, HIF-1 and mTOR signaling pathways. Finally, we identified 57 miRNAs constantly expressed in 12 MII oocytes and 12 miRNAs displaying altered regulation in women of advanced reproductive age. By computational approach we explored the possible functions of differentially expressed miRNAs inside human germ cells, and confirmed experimentally miRNA differential expression in murine MII oocyte. Finally, a significant negative correlation miRNA-targets was found for miR-29a, miR-203 and specific mRNAs, that have been ascribed responsible for mechanisms modulating

epigenetic changes underlying compromised oocyte quality caused by advanced maternal age. Data shown in this thesis were published in 2014 in Fertility and Sterility [Santonocito M, Vento M, Guglielmino MR, Battaglia R, Wahlgren J, Ragusa M, Barbagallo D, Borzì P, Rizzari S, Maugeri M, Scollo P, Tatone C, Valadi H, Purrello M, Di Pietro C. 2014. *Molecular characterization of exosomes and their microRNA cargo in human follicular fluid: bioinformatic analysis reveals that exosomal microRNAs control pathways involved in follicular maturation*. Fertil Steril;102(6):1751-61.e1] and also presented at the 30th Annual Meeting of the European Society of Human Reproduction and Embriology, held in Munich, Germany [R. Battaglia, M. Santonocito, M. Vento, M.R. Guglielmino, J. Wahlgren, M. Ragusa, D. Barbagallo, P. Borzì, S. Rizzari, M. Maugeri, P. Scollo, C. Tatone, H. Valadi, M. Purrello, C. Di Pietro. "*MicroRNAs upregulated in follicular fluid are carried by exosomes: new actors in the communication between oocyte and somatic follicular cells?*" 30th Annual Meeting of the European Society of Human Reproduction and Embriology, Munich, Germany from 29 June to 2 July 2014. Oral communication] and 16th World Congress on Human Reproduction held in Berlin, Germany [Rosalia Battaglia, Marilena Vento, Placido Borzì, Marco Ragusa, Davide Barbagallo, Paolo Giovanni Artini, Paolo Scollo, Carla Tatone, Michele Purrello, Cinzia Di Pietro. *The miRNome landscape changes in human ovarian follicles in relation to aging.* 16th World Congress on Human Reproduction held in Berlin, Germany on March 18-21 2015. Oral communication].

2. INTRODUCTION

From Germline Stem Cells toward fertilization-competent Oocyte

Human Primordial Germ Cells

In most multicellular organisms, including mammals, germ cells are the origin of new organisms and ensure the perpetuation of the genetic and epigenetic information across the generations. They prepare for totipotency during their ontogeny through genetic and epigenetic regulations of their genome function. Specification of the germ cell lineage is fundamental in development and the correct transmission of the genome to the next generation [Saitou and Yamaji, 2012]. There appear to exist at least two pathways for the specification of germ cell fate. In one pathway, which is called 'preformation', embryonic cells that inherit maternal determinants from the egg proceed to form the germ cell lineage, whereas in the other, which is called 'epigenesis', pluripotent cells formed early in development are induced by signals from adjacent tissues to form the germ cell lineage. In mice, and presumably in all mammals, germ cell fate is induced by 'epigenesis' [Saitou and Yamaji, 2010], thus it is not an inherited trait from the egg, but is induced in the in pluripotent epiblast cells by signaling molecules [Saitou, 2009]. In many animals, primordial germ cells (PGCs) are the first germline cell population which colonize the developing gonads by active migration [Richardson and Lehmann, 2010]. The precursors of PGCs are early committed and specified in the epiblast before gastrulation and rapidly moved into an extraembryonic region where PGCs are determined. PGCs reenter into the embryo proper during early gastrulation to reach the developing gonads, the gonadal ridges (GRs). During this journey, while undergoing proliferation, PGCs begin extensive nuclear reprogramming (activation of genes for pluripotency and epigenetic changes of the genome involving DNA demethylation and

histone code) to regain differentiation totipotency and reset the genomic imprinting. These processes are completed after their arrival into the GRs [Coticchio *et al*., 2013]. After some cycles of proliferation, PGCs initiate to differentiate either toward the spermatogenic or the oogenic pathway, into gonocytes or oogonia within testes and ovaries, respectively (Figure 1).

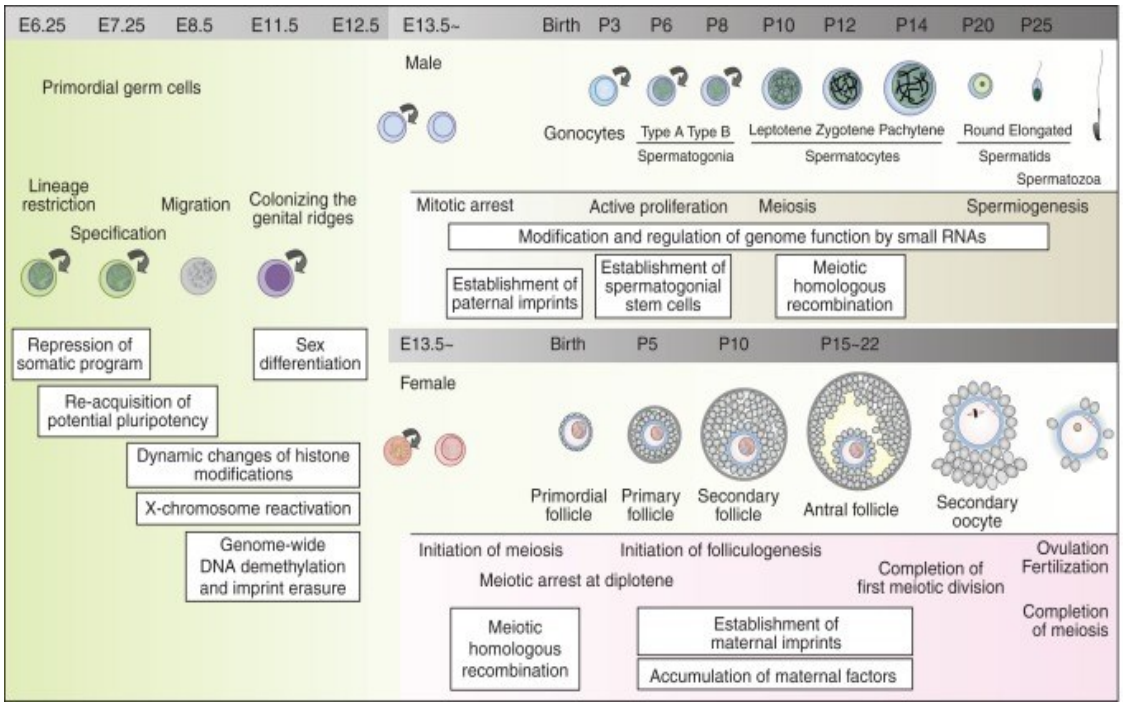


Figure 1: Schematic representation of germ cell development in mice. (Left) PGC development (from E6.25 to ~E12.5); (upper right) male germ cell development (from ~E13.5); (lower right) female germ cell development (from ~E13.5). [from Saitou and Yamaji, 2012].

The spermatogenic pathway most typically involves the establishment of spermatogonial stem cells (or male germline stem cells [GSCs]) with an enormous mitotic potential, whose homeostasis is likely accomplished by a stochastic balance of self-renewal and differentiation and not by regulated asymmetric cell division, whereas

it appears that female GSCs in adults are rare across the phylogenetic spectrum [Spradling *et al*., 2011]. However, it is still generally accepted that the oogenesis is dependent on PGSs set established in human ovaries during fetal period of life and does not increase following birth [Virant-Klun, 2015], although several recent research studies have identified functional oogonial stem cells in the postnatal ovary of several different species including humans [Oatley and Hunt, 2012; White *et al*., 2012]. In the absence of the testicular determining factor, the differentiation of PGCs into oogonia occur 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 2).

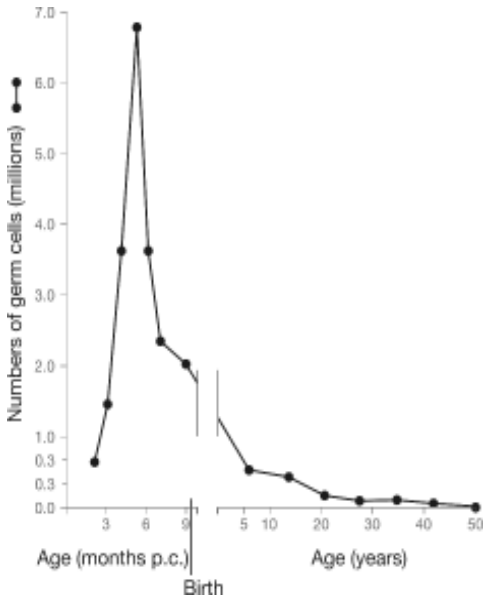


Figure 2: In human females, all germ cells are formed during fetal life. The total number of oocytes is at maximum at 5 months of gestation and drops to about 2 million (in both ovaries) by the time of birth. [from Djahanbakhch *et al*., 2007]

The mitotic proliferation of oogonia lasts several weeks and overlaps the period of their entry into meiosis (10–11 weeks) [Kurilo, 1981]. By entering the prophase of meiosis,

oogonia become primary oocytes and their development is soon arrested at the diplotene stage of the first meiotic prophase. 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). When oocytes enter the diplotene stage of the meiotic prophase I, they must be furnished with several flattened somatic cells, termed pregranulosa cells, to form the so-called primordial follicle or they undergo atresia [Nilsson *et al*., 2013]. At this stage, primordial follicles have one of two fates: (1) recruitment into the growth phase, with the possibility of ovulation or (2) death. The fate of each follicle is controlled by endocrine as well as paracrine factors [Richards *et al*., 1995]. At later stages, growth and differentiation and the selection of the cohort are largely dependent on FSH activity [Fauser and van Heusden, 1997]. Two important regulation steps can be identified during "the life history of ovarian follicles": the initiation of growth of follicles from the primordial pool (initial recruitment) and rescue of the antral follicles from atresia (cyclic recruitment), which is the result of the increase in circulating FSH during each reproductive cycle (Figure 3).

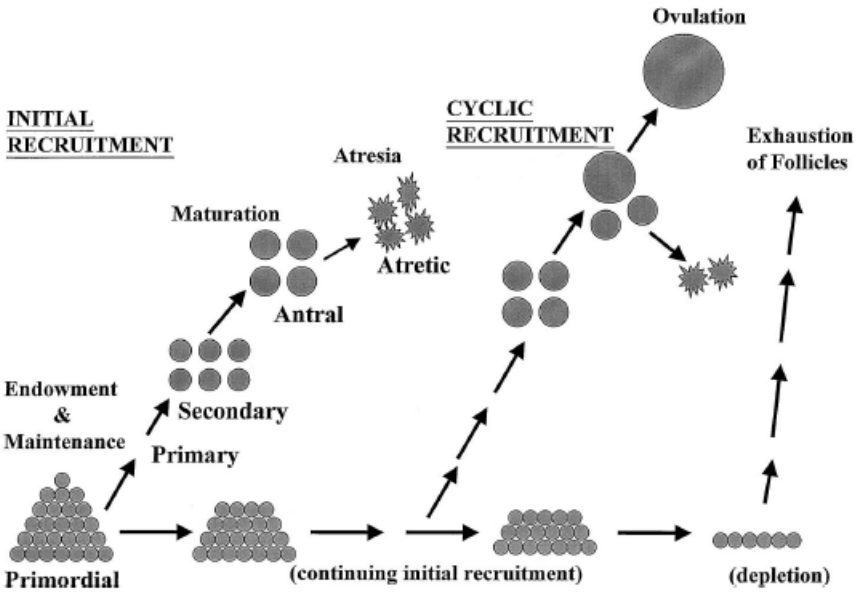


Figure 3: Life history of ovarian follicles: endowment and maintenance, initial recruitment, maturation, atresia or cyclic recruitment, ovulation, and exhaustion [from McGee and Hsueh, 2000].

In this way, the follicles develop 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. Among this cohort, a single leading follicle, the Graafian follicle, eventually emerges as dominant by secreting high levels of estrogens and inhibins to suppress pituitary FSH release. This event results in a negative selection of the remaining cohort, leading to its ultimate demise and concomitantly in an increase of local growth factors and vasculature allowing a positive selection of the dominant follicle, thus ensuring its final growth and eventual ovulation. Eventually, depletion of the pool of resting follicles leads to ovarian follicle exhaustion and senescence [McGee and Hsueh, 2000; Woodruff and Shea, 2011]. Throughout life, the number of primordial follicles drastically declines, so the supply of follicles decreases to 1 000 000/ovary at birth and 300 000/ovary by puberty until complete depletion at the menopause or reproductive senescence begins [Faddy, 2000] (Figure 2). The decline in oocyte number from the peak of 7 000 000 oogonia at 20 weeks of gestation to 300 000/ovary at puberty 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. This process continues throughout the woman's life, during fetal life, infancy, childhood, puberty, pregnancy and periods of anovulation [Djahanbakhch *et al*., 2007].

Female Gametogenesis

Oogenesis is defined as the formation, development, and maturation of an oocyte accompanied by re-initiation and completion of the first meiotic division and the subsequent progression to MII that entails the completion of nuclear and cytoplasmic processes that are essential for fertilization and the subsequent early embryo development [Küpker *et al*., 1998]. In mammals, it is a process that is initiated during fetal development but completed many years later at the time of fertilization. The precursors to oocytes are the primordial germ cells (PGCs), which are specified early in embryogenesis outside the embryo proper. Subsequently, PGCs migrate and colonize the urogenital ridge to establish the undifferentiated gonad [Coticchio *et al*.,2013]. Human oocytes are amongst the most long-lived cells in the body. The meiotic division of oocytes begins prenatally in the in the foetal ovary when primary oocytes from oogonial mitotic divisions enter meiotic S-phase and initiate prophase of meiosis I (Fig. 4). For this, the replicated homologous chromosomes each containing two chromatids connected physically by cohesion complexes condense chromatin, pair and recombine with the aid of a synaptonemal complex, a unique meiotic pairing structure during leptonene, zygotene and pachytene until diplotene stage of prophase I of meiosis. Unlike in male meiosis in which there is a continuous progression from prophase I to completion of first and second meiotic divisions in the testis of the sexually mature male, oocytes become meiotically arrested for long periods of time at prophase/G2 phase of meiosis I (termed dictyate stage), in the human for up to five decades, until they complete meiosis. At this stage the oocyte nucleus is called the germinal vesicle (GV), as this stage refers to the GV stage of maturity [Virant-Klun *et al*., 2013]. Primordial follicles containing the dictyate stage blocked, prophase I meiotically arrested primary oocytes are formed prior to or shortly after birth from nests of oocytes

(nest breakdown) and recruit granulosa cells. These primordial follicles first have to be recruited into the growing stage, many become atretic and die, and a fraction undergoes an extensive growth phase from the primary to the large antral stage of folliculogenesis before the oocytes reach full growth and acquire the competence to resume maturation to metaphase II and emit a first polar body containing one set of dyads/metaphase II chromosomes with two sister chromatids [Eichenlaub-Ritter, 2012] (Figure 4).

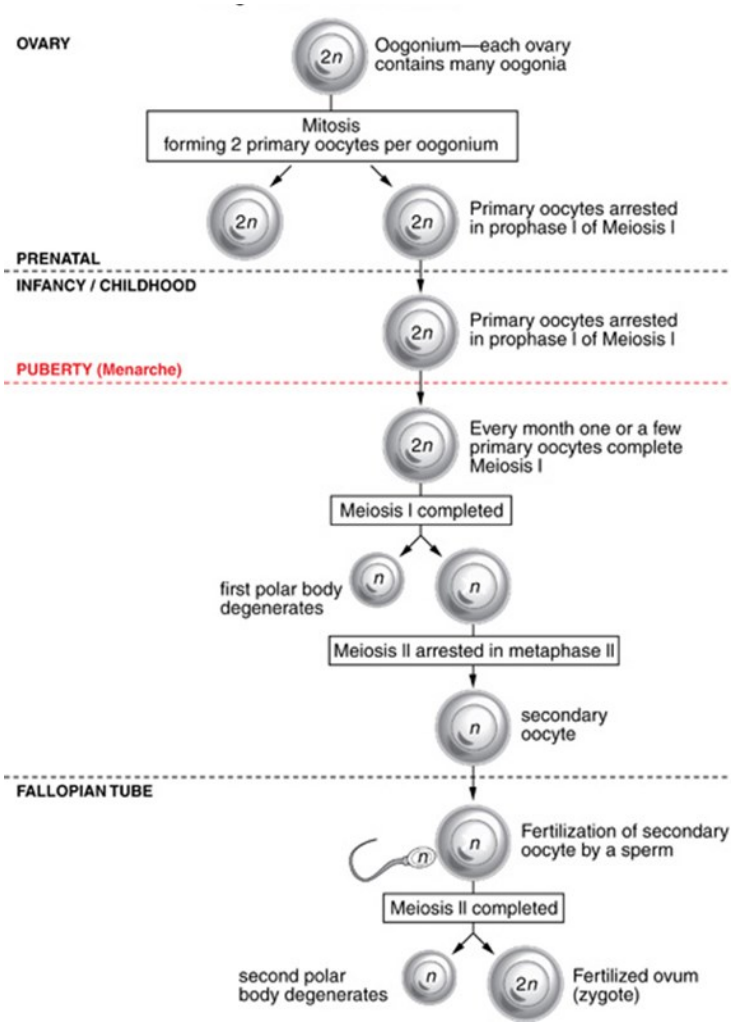


Figure 4: Gamete creation in the female gonads and meiotic divisions.

Since the follicle pool is restricted in size, the recruitment of primordial follicles and follicular atresia eventually lead to ovarian depletion, when cycles eventually become irregular and finally pool size reaches a critical size, ovulation seizes and menopause occurs. Apart from dictyate stage, there is a second meiotic arrest in oocytes, in most mammals at the metaphase of the second meiotic division (MII) [Ikeda and Yamada, 2014]. Although chromosomes are aligned and attached to spindle fibres, a condition that leads to anaphase progression in mitosis, metaphase II arrest occurs. The MII-stage oocyte has the potential to be fertilized. The fusion between a sperm and the oocyte plasma membrane at fertilization triggers the resumption of meiosis II whereby sister chromatids segregate to either the oocyte or the second haploid polar body (Figure 4). Once the second meiotic division is completed, a haploid male and female pronucleus are subsequently formed in the zygote [Nicholas *et al*., 2009; Eichenlaub-Ritter, 2012; Virant-Klun *et al*., 2013].

Folliculogenesis

In our species folliculogenesis begins when the oocyte is surrounded by somatic cells to form primordial follicles, which are generally believed to represent the stock of oocytes available to the female during her reproductive years. Folliculogenesis, or development of ovarian follicle, accompanies the oocyte development, preparing a single oocyte from a primordial follicle for ovulation [Hawkins and Matzuk, 2008]. At the beginning a primordial follicle emerges from the surrounding cohort to become a primary follicle (figure 5). This transition is known as primordial follicle activation. It is characterized by dramatic growth of the oocyte itself, accompanied by proliferation and differentiation of the surrounding pregranulosa cells [McGee and Hsueh, 2000]. Furthermore, the oocyte begins deposition of the zona pellucida, and stromal cells become organized into theca cell layers outside the basement membrane following a

highly co-ordinated process that involves a number of autocrine and paracrine factors that have been demonstrated to arrest or induce the recruitment of primordial ovarian follicle. Among these, Nobox (newborn ovary homeobox-encoding gene), Sohlh I and Lhx8, are oocyte-specific genes found correlated with the transition from primordial to primary follicles in mice [Rajkovic *et al*., 2004] [Pangas *et al*., 2006].

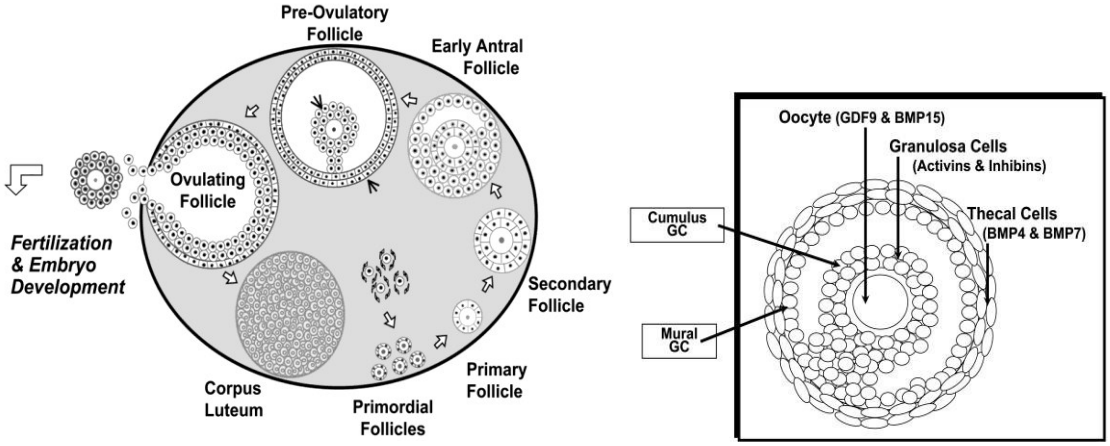


Figure 5: Stages of ovarian follicle development and anatomy of antral ovarian follicle [from Hawkins and Matzuk, 2008].

Primary follicles develop into secondary follicles with continued growth of the oocyte and the somatic granulosa and thecal cells. From multilayer secondary follicle, GCs become FSH responsive in most mammal species. In the presence of FSH, these follicles begin to grow even more and are competent to develop into antral follicles. Without FSH, the follicles become atretic [Hawkins and Matzuk, 2008]. The transition from the pre-antral to the antral stage is under the control of both FSH and paracrine factors secreted by the oocyte. In 2008, Diaz et al. suggested that this transition is controlled by TGF-β ligands, which might be processed differently depending on the

presence of the convertase protein PCSK6 in granulosa cells [Diaz *et al*., 2008]. Reached the antral stage, the appearance of islets filled with follicular fluid (FF), within the GC intercellular space, begins. The fusion of FF pockets leads to the formation of the antrum cavity, synonym of tertiary follicle. The appearance of the antral cavity establishes the morphological and functional separation of granulosa cells into mural granulosa cells (MGCs), localized close to the basal lamina of the ovarian follicle, and the cumulus cells (CCs), which surround the oocyte [Coticchio *et al*.,2013] (Figure 5). Although formation of the antrum is not fundamental for the acquisition of full developmental potential, the follicular fluid represents an essential microenvironment for the oocyte, enriched in nutritional and regulatory molecules as well apoptotic factors. It is well known that high concentrations of estradiol and low concentrations of insulin-like growth-factor binding proteins (IGFBP-2, -4, and -5) in the follicular fluid are the hallmark of dominant and pre-ovulatory follicles [Fortune *et al*., 2004]. As mentioned earlier, the antral phase of follicular development is characterized by dependency on FSH and LH, which are cyclically secreted by the pituitary gland. FSH, binding to its receptor, activates the cAMP/protein kinase A pathway, thus promoting cell proliferation, the differentiation of follicle cells into cumulus and mural granulosa cells, and the acquisition of meiotic competence. The layer of theca cells with exposure to low levels of LH, produces androgens in humans that are converted to estrogen via a member of the cytochrome P450 superfamily, CYP19 (aromatase) in the granulosa cells. FSH induces granulosa cell proliferation, induction of aromatase, and increased FSH receptors on the granulosa cells, thus leading to a very high estrogen microenvironment [Hawkins and Matzuk, 2008]. This high estrogen down regulates FSH from the anterior pituitary and begins the process of selecting for a single dominant follicle. Follicles that are not at the appropriate stage and are not able to maintain a high

estrogen microenvironment without stimulation from FSH degenerate and become atretic follicles. The very high estrogen levels feed back to the anterior pituitary to induce the LH surge, which ultimately leads to ovulation [Hawkins and Matzuk, 2008] During ovulation, the oocyte is expulsed from the follicle with cumulus granulosa cells surrounding it. The physical expulsion of the mature oocyte from the follicle appears to be the result of an LH-induced increase in collagenase and prostaglandins. The prostaglandins may cause localized contractions in the smooth muscles of the ovary and may also increase the flow from the ovarian capillaries, increasing fluid pressure in the antrum [Diaz-Infante *et al*., 1974; Koos *et al*., 1983]. The final phase of folliculogenesis that leads to meiotic resumption and germinal vesicle break down is triggered by a surge of LH and results from the release from the inhibitory action exerted by the follicle cells surrounding the oocyte and the interruption of the action of cAMP or other inhibitory molecules on the oocyte [Mehlmann, 2005]. Following the LH surge, phosphodiesterase type 3A (PDE3A) is activated, the level of cAMP falls and protein kinase A is inactivated. As a consequence, Cdc25 phosphatase is activated and removes inhibitory phosphatases from the Cdk1 subunit of the MPF, chromosomes start to condense and germinal vesicle break down occurs [Zuccotti *et al*., 2011]. At this stage, the remaining follicular cells in the ovary become luteinized as part of the corpus luteum which secretes progesterone. Corpus luteum play a central role in the reproductive events associated with pregnancy establishment and maintenance [Eppig, 2001]. During the luteal phase, the granulosa cells within the corpus luteum also produce inhibin A, an α:βA heterodimeric member of the transforming growth factor β (TGFβ) superfamily, which acts as an endocrine hormone to suppresses pituitary FSH, inhibiting growth of other ovarian follicles. With no fertilization or implantation of the embryo, the corpus luteum degenerates in corpus albican, possibly in response to activin, homodimers

(βA:βA or βB:βB) or heterodimers (βA:βB) that share the β subunits with inhibin A and inhibin B (α:βB). When inhibin and progesterone levels fall with regression of the corpus luteum, suppression of FSH is released. At the luteal-follicular transition, FSH levels increase, and the next cycle begins [Hawkins and Matzuk, 2008] (Figure 6).

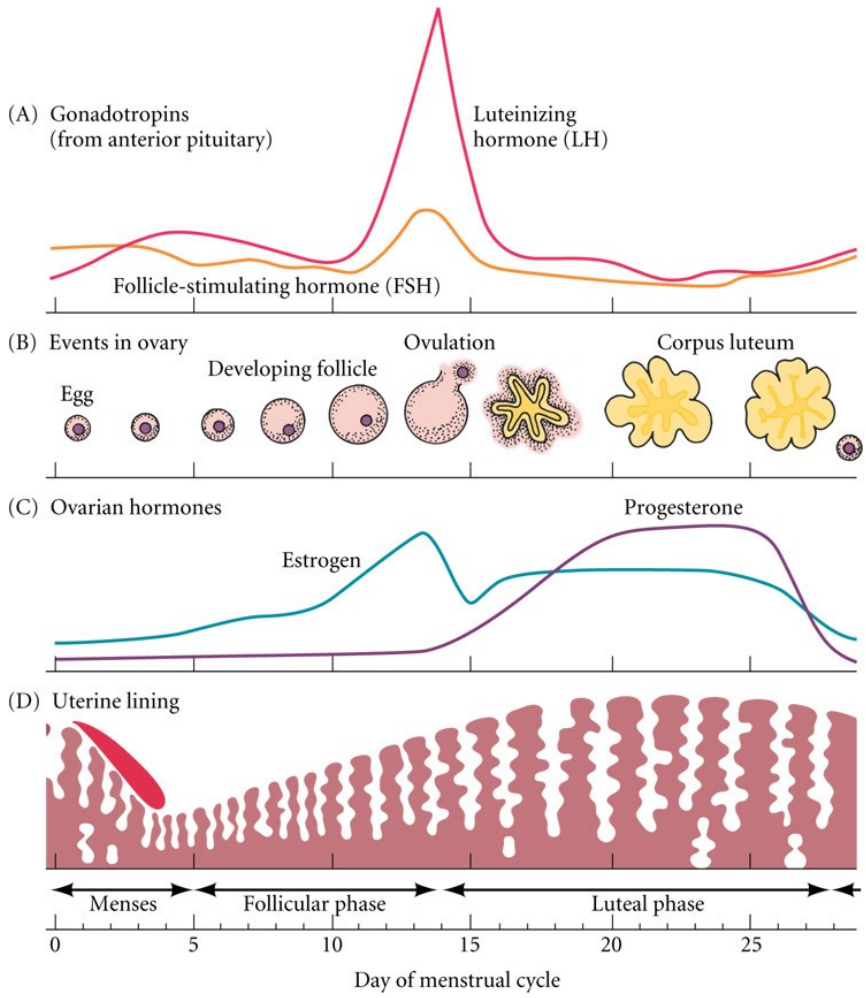


Figure 6: The coordination of ovarian and uterine cycles controlled by the pituitary and the ovarian hormones [Gilbert, 2013]

The role of the oocyte–cumulus cell dialogue in oocyte developmental competence

Ovarian folliculogenesis requires complex regulatory mechanisms involving both extrinsic (endocrine) and intrinsic signalling pathways. In the case of intrinsic signalling pathways, several intra- ovarian peptides, which are members of several growth factor families appear to influence follicle growth and maturation through a paracrine signalling [Kidder and Mhawi, 2002]. Albertini *et al*. reviewed evidence that such signalling is facilitated by transzonal projections or follicle cell extensions that traverse the zona pellucida and terminate on the oocyte cell [Albertini *et al*., 2001]. The cumulus cells and oocyte form a closely connected complex called the cumulus oocyte complex (COC); the COC is connected by gap junctions between cumulus cells, as well as between cumulus cells and oocytes [Kidder and Mhawi, 2002]. Moreover, the establishment of this bidirectional communication allows the production of developmentally competent oocytes [Cecconi *et al*., 2004; Gilchrist *et al*., 2008]. Oocyte-follicular cell contacts are not permanent structures, but rather function as specific 'devices' continuously adapting their morphology in response to the activity of both oocyte and cumulus cells [Zuccotti *et al*., 2011]. In ovarian follicle, the physical contact between somatic cells and between somatic cells and oocyte is mediated by the presence of connexins (Cx), which are expressed from the early stage of development [Gittens and Kidder, 2005; Gittens *et al*., 2005]. In particular, Cx43 and Cx45 have been identified between granulosa cells. The number of Cx43 gap junctions per granulosa cell increases concomitantly with follicle development and, in particular, during the transition from the preantral to the antral stage. In the absence of Cx43, gap junctions between somatic cells do not form and folliculogenesis arrests at the unilaminar stage [Gittens and Kidder, 2005]. On the contrary, Cx37 is found in gap junction between the oocyte and granulosa cells at the primary follicle stage and is required for fertility

[Simon *et al*., 1997]. Mice lacking Cx37 do not ovulate and oocytes are meiotically incompetent [Carabatsos *et al*., 2000]. Bevens and Harris first reported differential permeabilities of connexins to biological signaling molecules [Bevans et al., 1998]. Cx43 and Cx37 form channels that have different permeability properties and Cx43-positive and Cx37-positive plaques do not overlap suggesting that each Cx could play a specific physiological role, e.g. the transfer of different signals between the different compartments of the developing follicles. Molecules that pass via gap junctions include ions, metabolites and amino acids that are necessary for oocyte growth, as well as small regulatory molecules such as cAMP that control oocyte nuclear maturation, and gap-junctional signaling is a key means of disseminating local and endocrine signals to the oocyte via CCs [Albertini *et al*., 2001]. Gap junctions had long been thought to control meiotic arrest delivering meiosis-arresting substances like cAMP to the oocyte and just recently gap junctional closure has been uncovered as a key regulatory component of this pathway [Norris *et al*., 2008]. Moreover in regulating of meiotic events central is the role of cGMP that appears to decrease in the oocyte in response to LH and therefore releases the oocyte from meiotic arrest, as a consequence of EGF-mediated closure of connexin 43 gap junctions that may be manifest at both the level of communication between the oocyte and granulosa as well as the lateral integration of metabolism between the cumulus cells themselves [Norris *et al*., 2009; Norris *et al*., 2010]. It has recently become evident that the oocyte in fact is a central regulator of follicular cell function and thereby plays a critical role in the regulation of oogenesis, ovulation rate and fecundity [Eppig, 2001; McNatty *et al*., 2004; Gilchrist and Thompson, 2007]. The oocyte achieves this by secreting soluble growth factors, oocyte-secreted factors (OSFs), which act on neighboring follicular cells to regulate a broad range of GC and CC functions [Nekola and Nalbandov, 1971; Buccione *et al*., 1990; Vanderhyden *et al*.,

1990]. More recently, there was a great deal of scientific interest in GDF9 and BMP15 biology. These molecules are members of the transforming growth factor β (TGFβ) superfamily, and apart from being required for early folliculogenesis, are central regulators of GC/CC differentiation and may be associated with the pathogenesis of ovarian dysfunction [Shimasaki *et al*., 2004; Juengel and McNatty, 2005; McNatty *et al*., 2007]. More recently, Sugiura *et al*. demonstrated that GDF-9 and BMP-15, together with 17β-estradiol, co-ordinate cumulus cell development and expansion [Sugiura et al., 2010]. It was proposed that Oocytes secrete soluble growth factors, notably GDF9 and BMP15 and probably others, that lead to the activation of SMAD2/3 [Dragovic *et al*., 2007] and MAPK signaling in CCs, which in turn regulate a multitude of CC gene expression and key CC functions (Figure 7). Appropriate CC function and maintenance of the COC microenvironment is dependent on OSFs. In conjunction with maternal signals such as FSH and EGF, CCs pass regulatory growth factors and small metabolites back to the oocyte via paracrine and gap-junctional signaling. This bidirectional CC–oocyte communication loop appears to regulate unknown processes in the oocyte that improves its quality, embryo development and fetal viability [Gilchrist *et al*., 2008] (Figure 7). FSH and EGF signalling contribute to the acquisition of oocyte developmental competence by prolonging gap-junctional communication, which mediates the exchange of factors necessary for optimal oocyte developmental competence and subsequent fetal development [Yeo *et al*., 2009]. In this specific context, it is not surprising that also addition of recombinant GDF-9 during mouse oocyte IVM significantly increases blastocyst quality and fetal survival [Yeo *et al*., 2008].

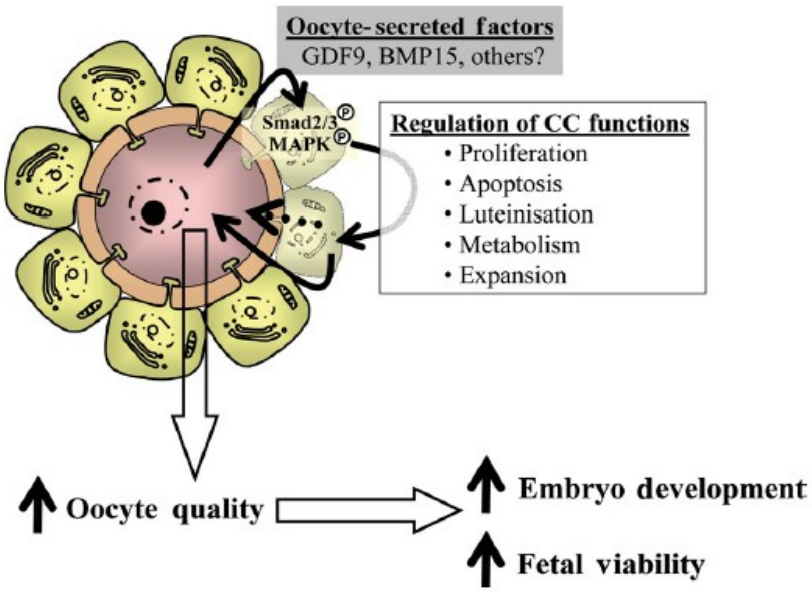


Figure 7: OSF regulation of CC function and oocyte quality [from Gilchrist *et al*., 2008].

Among the different mechanisms of autocrine and paracrine communication inside the ovarian follicle, over the last few years, an alternative mechanism has come to light. Follicular Fluid (FF), accumulated inside the antral follicle, represents an additional means of communication between oocyte and somatic cells. FF is the product of both the transfer of blood plasma constituents, that cross the blood-follicular barrier, and of the secretory activity of granulosa and thecal cells [Fortune JE, 1994]. FF consists of a complex mixture of nucleic acids, proteins, metabolites, and ions, which are secreted by the oocyte, granulose and thecal cells, combined with plasma components that cross the blood–follicular barrier via thecal capillaries [Revelli *et al*., 2009; Rodgers and Irving-Rodgers, 2010]. It represents a very important microenvironment for the development of the oocyte and its biochemical composition reflects the physiological status of the follicle. It is reasonable to think that the analysis of FF components may provide useful information on oocyte quality, and the subsequent potential to achieve fertilization and

embryo development [Leroy *et al*., 2004; Revelli *et al*., 2009]. In fact, hormones, growth factors, cytokines, and chemokines secreted by follicular cells in FF are able to promote oocyte maturation and an alteration of biochemical composition of FF is related to low quality oocytes. By the FF the oocyte and somatic follicular cells could interchange nutrients, as well as molecular signals. However the main molecular actors of this alternative communication tool will be discussed in detail as follows.

Transcriptional activity in oocytes

During oocyte maturation, fertilization and early embryo development until zygotic genome activation, which occurs at the 2-cell stage in mice and 4- to 8-cell stage in humans, transcription is suppressed and gene expression is dependent upon the timely activation of stored mRNAs [Braude *et al*., 1988; Schultz, 1993]. 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]. Synthesis of transcripts is highest in the earliest phases of development,which coincides with active proliferation of follicular cells. However, maternal mRNAs are degraded in a well-defined temporal, transcript-specific pattern [Evsikov *et al*., 2006; Su *et al*., 2007., Chen *et al*., 2011] in synchrony with maturation; as a result, mRNA content is decreased by 30% in MII oocytes. Infact, almost half of the 85 pg mRNA stored during oocyte growth is degraded during meiosis resumption and by the MII stage the oocyte carries about 35 pg mRNA; of this, about half undergoes stabilization through selective deadenylation of the poly(A) tail at the 3′ region [Paynton *et al*., 1988]. In this way, a vast number of transcript important for germinal vesicle block and for the following stages of meiosis resumption are eliminated while transcripts associated with the maintenance of MII oocyte features or

playing important roles during the early stages of development are kept stabilized through deadenylation [Su *et al*., 2007] [Thélie *et al*., 2007]. Soon after fertilization and by the time the embryonic genome is first expressed, translation of maternal mRNAs, necessary for the early phases of development, began through the polyadenylation of the 3′ untranslated region operated by embryonic cis regulatory cytoplasmic polyadenylation elements [Mendez and Richter, 2001; Racki and Richter, 2006]. Moreover it is important to pinpoint that during this quiescent period, fundamental changes in nuclear function also take place as the differentiated cells, oocyte and sperm, unite to give rise to an embryonic genome. These changes, known as "genome reprogramming," transform the genome to the state of totipotency, a critical event for the successful development of an embryo [Dean *et al*., 2003]. However, transcriptional quiescence of the genome requires post-transcriptional and post-translational mechanisms to orchestrate the multitude of processes participating in meiotic maturation, fertilization, and reprogramming of the nascent embryonic genome [Solter *et al*., 2004]. Recent advances include identification of RNA-binding proteins that function as specificity factors to direct the maternal degradation machinery to its target mRNAs; the action of small silencing RNAs (ssRNAs) and signaling pathways that trigger production and/or activation of the clearance mechanism in early embryos; and mechanisms for spatial control of transcript clearance [Walser and Lipshitz, 2011]. Little is known about the role played by ssRNAs during oocyte growth and preimplantation development. The few studies that have analyzed the presence of ssRNAs in oocytes have showed that their average amount does not vary during maturation, although single miRNAs may vary considerably [Tang *et al*., 2007]. Moreover, these maternal ssRNAs have been presumed to represent the only contribution to the zygote, since the paternal miRNAs, brought into the zygote by the

sperm, do not seem to contribute significantly to the total miRNAs in the zygote [Amanai *et al*., 2006]. At 1-cell and 2-cell stage of development, the amount of these decreases by 60 %, suggesting an active process of degradation that coincides with a global RNA degradation occurring at this time of development [Zuccotti *et al*., 2011].. Moreover emerging evidences indicate important the role of miRNAs, a class of ssRNAs, during early development as supported by recent studies that have demonstrated their crucial involvement in regulating ES cell pluripotency and differentiation [Gangaraju and Lin, 2009]. Clearly, further understanding and deciphering of changes that the oocyte transcriptome and epigenome undergoes may expand our knowledge on their potential role in the context of oocyte maturation.

The new world of RNAs

Although Jacob and Monod had suggested in 1961 the centrality of RNA in the flow of genetic information, establishing the concept of messenger RNA in their paper "Genetic Regulatory Mechanisms", for many decades proteins represented not only the primary structural and functional components of cells, but also the main regulatory components of the genome in both simple and complex organisms [Jacob and Monod, 1961]. This protein-centred view was, in fact, considered as rather simplistic and misleading when applied to higher organisms [Ragusa *et al*., 2015]. In this way, recent advances in the fields of RNA biology and genome research have reassessed the old assumption and provided significant evidence of the importance of RNAs as "riboregulators" outside of their more conventional role as accessory molecules [Prasanth and Spector, 2007]. The number of protein-coding genes that structure the human genome has long been a source of discussion. With the publication of the final draft of the Human Genome Project, the number of protein-coding genes was revised to between 20 000 and 25 000. Most recently, Clamp and co-workers used evolutionary comparisons to suggest that the most likely figure for the protein-coding genes would be at the lower end of this continuum, just 20 500 genes. This number is lower than expected and very close to the number of protein-coding genes in *C. elegans*, suggesting that maybe these genes alone are not sufficient to appropriately and completely explain the complexity of higher eukaryotes [International Human Genome Sequencing Consortium, 2004; Szymanski and Barciszewski, 2006; Ezkurdia *et al*., 2014]. Recent high-throughput studies of the human transcriptome have revealed that about 85%-90% of our genome is dynamically and pervasively transcribed and that ~98% of the transcriptional output of the human genome represents non-protein-coding RNAs (ncRNAs) [Mattick, 2005; Prasanth and Spector, 2007]. Many observations have, only recently, suggested convincingly that

ncRNAs significantly contribute to the complex molecular signalling needed to regulate structures and functions in different cells and developmental programming [Amaral *et al*., 2008]. Hence, dysregulation of these ncRNAs is being found to have relevance not only to tumorigenesis, but also to neurological, cardiovascular, developmental and other many pathological conditions [Taft *et al*., 2010; Esteller, 2011]. It is not precisely known how many ncRNA genes are present in the human genome. The number of known functional ncRNA genes has risen dramatically in recent years and over 800 ncRNAs (excluding tRNAs, rRNAs and snRNAs) have been catalogued in mammals, at least some of which are alternatively spliced [Pang *et al*., 2005]. ncRNA genes are difficult to identify because of their structural heterogeneity: extreme length variation from 20 nucleotides to > 100 kb; absence of Open Reading Frames (ORFs); no or low evolutionary conservation in many cases; no preferential localization within the genome; and relative tolerance to point mutations [Ragusa *et al*., 2015]. ncRNAs are divided into several different categories according to size and function. ncRNAs are commonly classified, according to their length, in two different classes: (1) long non-coding RNAs (lncRNAs), which are longer than 200 nucleotides (nt); and (2) small non-coding RNAs, whose length is equal to or less than 200 nt [i.e., microRNAs (miRNAs), small interfering RNAs (siRNAs), PIWI-interacting RNAs (piRNAs) (Table I). Other classifications have been proposed to categorize ncRNAs according to functional features into two main classes: (1) housekeeping ncRNAs; and (2) regulatory ncRNAs. Housekeeping ncRNAs are constitutively expressed and required for normal function and viability of cell. They include transfer RNAs (tRNAs), ribosomal RNAs (rRNAs), small nuclear RNAs (snRNAs), small nucleolar RNAs (snoRNAs), and telomerase RNAs [Zhu *et al*., 2013]. Conversely, regulatory ncRNAs can be expressed in a cell-specific way, or during defined stages of development and cell differentiation, and

finally in response to external stimuli [Brosnan and Voinnet, 2009]. Regulatory ncRNAs can affect the expression of other genes at the level of transcription or translation. This category comprises miRNAs, siRNAs, lncRNAs (i.e., the RNA molecules more closely involved in cancer biology), and piRNAs [Adams *et al*., 2014].

Table I. Class of non-coding RNA in mammals [from Taft *et al*., 2010].

NcRNA class	Characteristics	References
Established ncRNA classes		
Long (regulatory) non-coding RNAs (lncRNAs)	The broadest class, lncRNAs, encompass all non-protein-coding RNA species >~200 nt, including mRNA-like ncRNAs. Their functions include epigenetic regulation, acting as sequence-specific tethers for protein complexes and specifying subcellular compartments or localization	Reviewed in [97,98]
Small interfering RNAs (siRNAs)	Small RNAs ~21–22 nt long, produced by Dicer cleavage of complementary dsRNA duplexes. siRNAs form complexes with Argonaute proteins and are involved in gene regulation, transposon control and viral defence	Reviewed in [15–17,227]
microRNAs (miRNAs)	Small RNAs ~22 nt long, produced by Dicer cleavage of imperfect RNA hairpins encoded in long primary transcripts or short introns. They associate with Argonaute proteins and are primarily involved in post-transcriptional gene regulation	Reviewed in [16,17,70]
PIWI-interacting RNAs (piRNAs)	Dicer-independent small RNAs ~26–30 nt long, principally restricted to the germline and somatic cells bordering the germline. They associate with PIWI-clade Argonaute proteins and regulate transposon activity and chromatin state	Reviewed in [15,17]
Promoter-associated RNAs (PARs)	A general term encompassing a suite of long and short RNAs, including promoter-associated RNAs (PASRs) and transcription initiation RNAs (tiRNAs) that overlap promoters and TSSs. These transcripts may regulate gene expression	Reviewed in [38,39]
Small nucleolar RNAs (snoRNAs)	Traditionally viewed as guides of rRNA methylation and pseudouridylation. However, there is emerging evidence that they also have gene-regulatory roles	Reviewed in [228]
Other recently described classes		
X-inactivation RNAs (xiRNAs)	Dicer-dependent small RNAs processed from duplexes of two lncRNAs, Xist and Tsix, which are responsible for X-chromosome inactivation in placental mammals	131
Sno-derived RNAs (sdRNAs)	Small RNAs, some of which are Dicer-dependent, which are processed from small nucleolar RNAs (snoRNAs). Some sdRNAs have been shown to function as miRNA-like regulators of translation	229–231
microRNA-offset RNAs (moRNAs)	Small RNAs ~20 nt long, derived from the regions adjacent to pre-miRNAs. Their function is unknown	232,233
tRNA-derived RNAs	tRNAs can be processed into small RNA species by a conserved RNase (angiogenin). They are able to induce translational repression	Reviewed in [44]
MSY2-associated RNAs (MSY-RNAs)	MSY-RNAs are associated with the germ cell-specific DNA/RNA binding protein MSY2. Like piRNAs, they are largely restricted to the germline and are ~26–30 nt long. Their function is unknown	234
Telomere small RNAs (tel-sRNAs)	Dicer-independent ~24 nt RNAs principally derived from the G-rich strand of telomeric repeats. May have a role in telomere maintenance	43
Centrosome-associated RNAs (crasiRNAs)	A class of ~34–42 nt small RNAs, derived from centrosomes that show evidence of guiding local chromatin modifications	42

microRNAs: genomics, biogenesis and function

Small non-coding class of RNAs includes different types of molecules involved in different steps of RNA synthesis, processing, translation, as well as modulation of transcription initiation [i.e., piRNAs, promoter associated small RNAs (PASRs)], RNA

degradation or protein synthesis block (e.g., miRNAs, siRNAs), RNA maturation (e.g., snoRNAs) [Dogini *et al*., 2014]. Among these, microRNAs (miRNAs) represent the most studied and better characterized class of ncRNAs in terms of function and impact in human health and disease. Specifically, miRNAs are small endogenous 18-25 nucleotides (nt) long, that regulate gene expression, in animals and plants, post-transcriptionally in a sequence-specific manner [Bartel, 2004]. miRNAs had been discovered for the first time in 1993 by Ambros and colleagues when two independent studies identified the real nature of the C. elegans heterochronic gene lin-4 as small non-coding RNA (ncRNA) [Lee *et al*., 1993]. At that time, these findings were not adequately appreciated by the scientific community, because this small ncRNA was considered a specific tool, employed by the worms, to control somehow the expression of the heterochronic coding genes. After seven years, Reinhart *et al*. identified another *C. elegans* 22 nt non-coding RNA, the let-7 [Reinhart *et al*., 2000]. Surprisingly, it was also found to be complementary to the 3'UTR of another gene, lin-41, promoting its translational knockdown. This discovery triggered scientist curiosity to look for other small ncRNAs and, later, independent groups have identified these small ncRNAs also in other different organisms and cellular systems providing evidence for the existence of a large class of small ncRNAs with potential regulatory roles that was named microRNAs [Pasquinelli *et al*., 2000; Lagos-Quintana *et al*., 2001; Lau *et al*., 2001; Lee and Ambros, 2001]. Currently, 2603 human and 1920 murine mature miR entries are reported in the miRbase sequence repository (mirbase.org; November 2015). Animal miRNAs are phylogenetically conserved (~55% of *C. elegans* miRNAs have homologues in humans) and mammalian miRNA genes have also multiple isoforms (paralogues), which are probably generated by duplication (human let-7 gene accounts for 8 different isoforms distributed in 11 genomic loci) [Di Leva *et al*., 2014]. MiR-

encoding genes are present in the genome as intergenic clusters or individual transcriptional units. In both cases, their expression is under the control of promoters and enhancers with similar characteristics and regulation to those for protein-coding genes [Liu *et al*., 2013]. Several miRNAs, however, are embedded in the intronic sequences of other genes (miRtrons) and are then co-transcribed accordingly with the encompassing genes [Curtis *et al*., 2012]. miRNAs are canonically produced through a multistep process that, starting in the nucleus and ending into the cytoplasm, is composed by three main events: cropping, export and dicing [Kim *et al*., 2009] (Figure 8). Following the canonical pathway, miRNA genes are generally transcribed by RNA polymerase II into primary transcripts of several hundred nucleotides (pri-miRNAs), bearing secondary hairpin structures and undergoing 5′ capping and 3′ polyadenylation [Ha and Kim, 2014]. In few cases, miRNA genes are transcribed by RNA polymerase III [Diebel *et al*., 2014]. While still inside the nucleus, the microprocessor complex, formed by the RNase-III *Drosha* and its co-factor Dgcr-8, cleaves the pri-miRNAs into ~ 70 nt-long precursor molecules (pre-miRNAs) [Liz *et al*., 2014]. Dgcr-8 is a dsRNA binding protein that recognizes the proximal ~10 bp of stem of the pri-miRNA hairpin, positioning the catalytic sites of the RNase III enzyme Drosha [Yeom *et al*., 2006]. Pre-miRNAs are, then, transported to the cytoplasm by exportin-5 and the cofactor Ran-GTP [Yi *et al*., 2003; Lund *et al*., 2004]. Once in the cytoplasm, pre-miRNAs are further cleaved, near the terminal loop, releasing the ~ 22 nt-long double-stranded molecules by a complex that includes another RNase-III, *Dicer*, in combination with its RNA-binding cofactor, Tbrp [Winter *et al*., 2009]. Whereas mammals typically encode a single DICER that can generate several classes of small RNAs, *Drosophila* and *C. elegans* have two types of Dicer [Lee *et al*., 2004]. Human Dicer interacts with two related proteins TRBP (TAR RNA-binding protein) and PACT (also known as PRKRA)

that, as miRNA duplexes are produced, associate with Argonaute (Ago) proteins, forming the RNA-induced silencing complex (RISC) [Schwarz *et al*., 2003; Chendrimada *et al*., 2005; Lee *et al*., 2006]. After Dicer-mediated maturation, miRNAs are loaded on the RISC as single strands. Mature miRNA duplexes are in fact separated in a guide strand (mature miRNA) and a passenger strands (miRNA*). The guide strand usually presents the lower free energy and weakest base pair at 5′ and is preferentially loaded on the RISC complex, whereas the complementary passenger strand is preferentially degraded [Khvorova *et al*., 2003]. The RISC complex includes Argonaute proteins, such as Ago-2, and targets the miRNA to its messenger RNA (mRNA) targets. Also, Ago-2-binding protein, Gw-182, shuttles the miR-loaded, active RISC complex to discrete foci in the cytoplasm, called processing bodies (P-bodies), where several enzymes are available for mRNA decapping, deadenylation and degradation [Leung and Sharp, 2013]. The seed sequences of the miRNA (7 nt sequences mapping to positions 2–8 at the molecule's 5′ end) are considered essential for the selection of the target mRNAs. According to the binding complementarity of the seed sequence, miRNAs repress gene expression by targeting the 3′ untranslated region (3′-UTR) of mRNA. If the pairing is complete (100% sequence complementarity between the miRNA and mRNA), the mRNA will be degraded; however, if the sequence is only partially complementary, this will result in translational inhibition of the mRNA [Cannell *et al*., 2008]. The latter is achieved through various mechanisms, including RISC-mediated destabilization of ribosomal assembly or translation continuation [Kim *et al.,*2014]. Non-canonical processing of miRNA transcripts, typical for miRtrons, relies on direct processing of the pre-miRNAs during intron splicing of the harboring gene mRNA. Other microprocessor-independent sources of pre-miRNAs include small nucleolar

RNAs (snoRNAs), transfer RNA precursors (tRNAs) and short hairpin RNAs (shRNAs) [Yang and Lai, 2011].

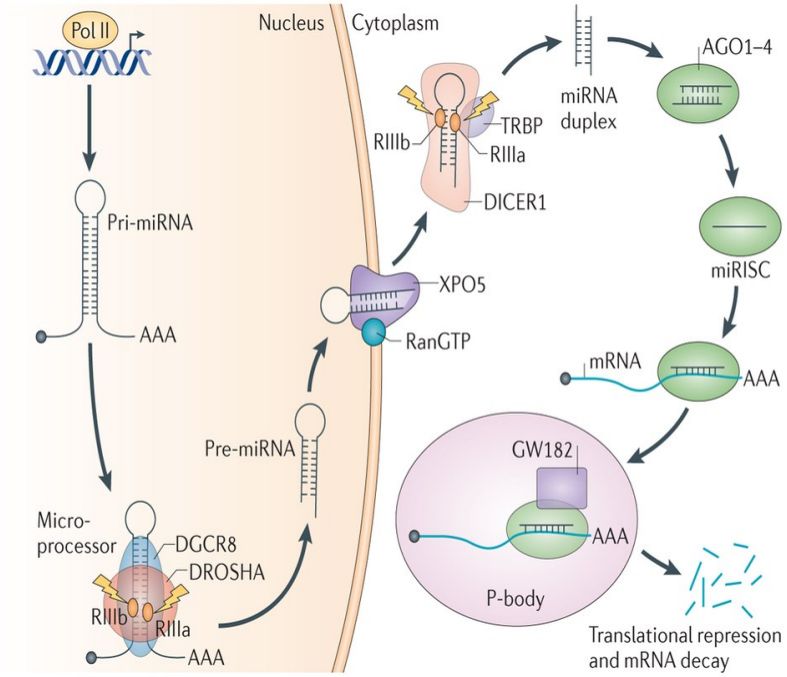


Figure 8: Mirna Biogenesis. miRNA genes are transcribed as primary miRNAs (pri-miRNAs) by RNA polymerase II (Pol II) in the nucleus. The long pri-miRNAs are cleaved by Microprocessor, which includes DROSHA and DiGeorge syndrome critical region 8 (DGCR8), to produce the 60–70-nucleotide precursor miRNAs (pre-miRNAs). The pre-miRNAs are then exported from the nucleus to the cytoplasm by exportin 5 (XPO5) and further processed by DICER1, a ribonuclease III (RIII) enzyme that produces the mature miRNAs. One strand of the mature miRNA (the guide strand) is loaded into the miRNA-induced silencing complex (miRISC), which contains DICER1 and Argonaute (AGO) proteins, directs the miRISC to target mRNAs by sequence complementary binding and mediates gene suppression by targeted mRNA degradation and translational repression in processing bodies (P-bodies). TRBP, transactivation-responsive RNA-binding protein [from Lin and Gregory, 2015].

Modulation of miRNAs

Many factors regulate miRNA biogenesis, hence affect the downstream miRNA-mediated gene repression. Both miRNA precursors and mature miRNAs undergo A-to-I RNA editing by deaminases, affecting the miRNA maturation process and activity [Tomaselli *et al*., 2013], or 3′ uridylation that can influence Drosha- or Dicer-dependent

processing steps. Also, abundance of Ago-2, which has intrinsic endonuclease activity in mammals, or agonist proteins directly affects RISC activity levels [Slezak-Prochazka *et al*., 2010]. Such regulation of miRNA processing fine-tunes their intracellular levels, and modulates their biological activity. Indeed, miRNA intracellular concentration directly relates to their ability to affect mRNA translation. Another important aspect of intrinsic miRNA regulation, consists of turnover and degradation. After mRNA targeting. and in the presence of still unidentified signals, the miRNA guide strand is released and degraded. Processes underlying average half-life of miRNAs have been supposed involve exoribonucleases, although still remain largely unknown [Gantier *et al*., 2011; Großhans and Chatterjee, 2011].

Vesicular and non-vesicular trafficking of miRNAs

Besides operating intracellularly, miRNAs can be released from the producing cells in the surrounding areas or in the circulation. Intriguingly, circulating miRNAs are traceable in plasma or serum and other biological fluids and appear resistant to harsh conditions such as RNase activity, pH changes, boiling, freeze-thawing and storage at room temperature [Chen *et al*., 2008; Weber *et al*., 2010; Cortez *et al*., 2011]. In these fluids, miRNAs display remarkable stability and resistance to degradation, possibly because of their association with carriers. Non-vesicle carriers encompass protein complexes, including Ago-2 and Nucleophosmine-1, and lipoprotein complexes, such as high-density lipoproteins (HDLs). Among the vesicle-based carriers, exosomes are emerging as important regulators of long-range miRNA shuttling [Valadi *et al*., 2007; Atay and Godwin, 2014]. Exosomes are small vesicles (30-100 nm diameter) that enclose their cargo with a lipidic bi-layer and are generated through inward budding of

endosomal membranes, which gives rise to intracellular multivesicular bodies (MVB). In addition to exosomes, cells can also shed other types of extracellular membrane vesicles, namely, microvesicles, microparticles and apoptotic bodies, after various biological stimuli, including induction of programmed cell death [Majno and Joris, 1995; Aupeix *et al*., 1997]. Exosomes are different from the other extracellular vesicles because the latter are the result of a direct budding process of the plasma membrane and seem to have properties distinct from those of exosomes. Exosomes mature though a still unknown process, most likely at the interface with the Golgi reticulum and later fuse with the plasma membrane releasing the intraluminal vesicles into the extracellular environment. This process completes the exocytosis event [Waldenström and Ronquist, 2014] (Figure 9).

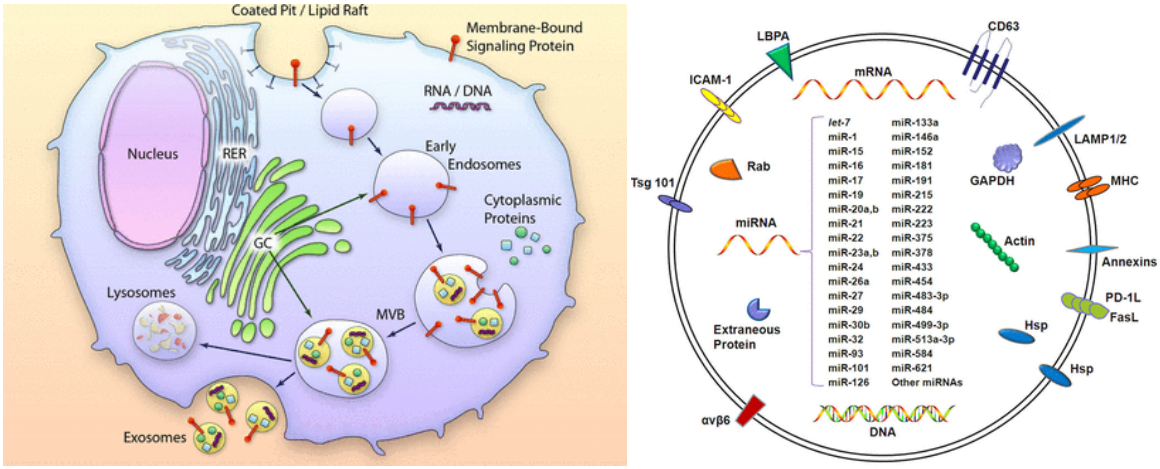


Figure 9: Biogenesis and composition of Exosomes [from Waldenström and Ronquist, 2014].

However, exosome biogenesis and secretion are multifaceted mechanisms and they are different depending on the cell type and cargo sequestered. Exosomes can be formed at

least via two distinct pathways: the first utilizes the Endosomal Sorting Complexes Required for Transport (ESCRT) machinery, while the second, independent from ESCRT complexes, involves lipids or tetraspanins [Van Niel *et al*., 2011; Mayers and Audhya, 2012]. In the extracellular space, exosome interaction with recipient cells can take place in different ways: by ligand-receptor interactions, by fusion, or by internalization via receptor-mediated endocytosis [Urbanelli *et al*., 2013]. Therefore, exosomes can act as paracrine or autocrine mediators and also as endocrine mediators that through the blood stream are able to target cells located at distant sites in the body [Cortez *et al*., 2011]. The first evidence of exosome secretion was demonstrated in reticulocytes undergoing maturation into red blood cells [Johnstone *et al*., 1987]. In 1996, Raposo et al. demonstrated in B-lymphocytes that both the limiting membrane and the internal vesicles contain major histocompatibility complex class II, and the released exosomes were perceived to have a role in antigen presentation in vivo. Subsequently, it was realized that many cell types release exosomes [Raposo *et al*., 1996]. Exosomes are released by a variety of "normal" cells including mast cells (MC), dendritic cells, reticulocytes, epithelial cells, B-cells, trophoblastic cells, and neural cells, as well as a variety of tumor cells. In addition, exosomes are found in various biological fluids including bronchoalveolar lavage, blood, ascites, urine, pregnancy associated sera, breast milk, saliva, follicular fluid and malignant effusions [Sang *et al*., 2013; Atay and Godwin, 2014; Santonocito *et al*, 2014]. Several studies have demonstrated that exosomes from different cellular origins share some common characteristics. Such characteristics are the lipid bilayer with exceptionally high cholesterol/phospholipid ratio, size, density, and a basic collection of lipid and protein composition. Over the last few years, proteomic analysis of exosomes has identified common characteristic marker proteins on their surface and in their lumen [Mathivanan

et al., 2010] (Figure 9). Because of their endosomal origin, exosomes contain several proteins involved in the ESCRT complex (e.g., TSG101, Alix) and in transport and fusion (e.g., Rab11, Rab7, Rab2 and various annexins) [Atay and Godwin, 2014]. Further markers expressed in or on exosomes include tetraspanins (CD9, CD63, CD81, CD82) and heat shock proteins (HSC70 and HSP90) [Mathivanan *et al*., 2010]. Moreover, in 2007, Valadi et al. demonstrated that exosomes contain both mRNAs and miRNAs, which can be delivered to another cell, and can be functional in this new location. They proposed to call this RNA "exosomal shuttle RNA" [Valadi *et al*., 2007]. Therefore, exosomes are able to transfer macromolecules to target cells, but the mechanisms that allow the selection of the correct have not yet been characterized, however, it has been demonstrated that the cells are able to modify the number and the cargo of exosomes depending on specific conditions [Lo Cicero *et al*., 2015]. Moreover, it has been shown that there is a specific repertoire of miRNAs selectively exported to exosomes, whereas others are usually excluded, indicating that an active sorting mechanism occurs at the RNA level [Van Niel *et al*., 2011]. However, at present, the relative abundance of vesicular or carrier-based complexes for circulating miRNAs in the bloodstream is still debated. Some reports suggest that the majority of circulating miRNAs are exosome-borne whereas others suggest that miRNAs in body fluids are predominantly vesicle-free [Turchinovich *et al*., 2011; Gallo *et al*., 2012]. These discrepancies are probably due to still different and rather incomparable methodologies of investigation. While our understanding of intercellular communication has been mainly based on the secretion of soluble factors, such as growth factors and neurotransmitters, cytokines, and chemokines, and their specific recognition by cell-surface receptors, evidence indicating that cells also communicate via the direct exchange of DNA and RNA has been growing over the past several years [Deregibus *et*

al., 2007; Ehnfors *et al*., 2009]. Additionally, following the notion of exosomes as miRNA carriers, notwithstanding the lack of details concerning putative receptors and intracellular processing, pre- or mature miRNAs are delivered to other cell where they exert their specific regulation [Salido-Guadarrama *et al*., 2014]. In this way, miR-based cross-communication is emerging as unconventional mechanism regulating intercellular communication.

miRNAs and ovarian function

Currently, over 2000 human miRNAs have been identified by cloning and sequencing approaches. It is predicted that miRNA genes account for 1%-2% of the human genome and control the expression of at least 30-50% of all protein coding genes [Krol *et al*., 2010]. By modulating the expression of target transcripts, miRNAs may, therefore, affect many different signaling pathways and cellular processes, such as cell proliferation, differentiation, migration, angiogenesis and apoptosis: accordingly, they are considered potential oncogenes or tumor suppressors in cancer development and progression [Bueno and Malumbres, 2011]. The miRNA repertoires are cell type specific and change markedly during development [Carthew and Sontheimer, 2009]. Crucial and time-dependent events such as degradation of the LH receptor transcripts or translational repression of Connexin 43, after LH surge, in ovarian granulosa cells have suggested that gene expression may, also, be under control of miRNAs in reproductive tissues [Carletti and Christenson, 2009]. miRNAs constitute the most abundant class of small RNAs in the ovary. By using microarrays, high-throughput quantitative polymerase chain reaction, and next-generation sequencing techniques miRNA population in the ovaries was identified in human and other species. Regardless of

species, let-7 family, miR-21, miR-99a, miR-125b, miR-126, miR-143, miR-145, and miR-199b were found to be the most commonly abundant miRNA populations [Li *et al*., 2015]. Many studies demonstrate also that miRNAs are essential for follicle development in different species [Abramov *et al*., 2013; Yang *et al*., 2013; Zhang *et al*., 2014]. miRNAs are involved in the entire process of ovarian follicle development, including follicle growth, atresia and ovulation in which different growth factors contribute to stage-specific functions in different cell types [Li *et al*., 2015]. Studies of the role of miRNAs in ovarian function have been highlighted primarily through Dicer, a ribonuclease that is required for the synthesis and processing of mature functional miRNAs. As the function of Dicer and its products (miRNAs and siRNAs) were studied in the female reproductive tract, the essential role for these post-transcriptional gene regulators in female fertility was revealed [Carletti and Christenson, 2009]. Dicer is expressed in both oocytes and granulosa cells of the mouse ovarian follicle. Oocytes contain 10–15-fold higher levels of Dicer transcripts than any other cells and/or tissues [Imbar and Eisenberg, 2014]. In mice, knockout (KO) of Dicer results in postimplantation embryonic lethality [Bernstein *et al*., 2003]. In the germ cell-specific Dicer1 knockout mouse model, in which Cre recombinase is driven by the zona pellucida 3 (Zp3) promoter, Dicer1-deficient oocytes are unable to complete meiosis, and arrest with defects in meiotic spindle organization and chromosome congression [Murchison *et al*., 2007]. Moreover mouse mutant with a hypomorphic Dicer allele (Dicer d/d) resulted in Dicer1 deficiency with subsequent female infertility due in part to the insufficiency of corpus luteum (CL) function [Otsuka *et al*., 2008]. Likewise, in ovarian granulose cells, Dicer1 cKO in mouse led to an increased primordial follicle pool endowment, accelerated early follicle recruitment, and more follicle degeneration in the cKO ovaries [Lei *et al*., 2010]. Dicer1 regulates follicle development by

downregulating miR-503, an ovary-specific miRNA, as well as miR-503 target genes, such as anti-Müllerian hormone (AMH); inhibin beta A subunit (INHBA); cytochrome P450, family 17, subfamily a, polypeptide 1 (Cyp17a1); cytochrome P450, family 19, subfamily a, polypeptide 1 (Cyp19a1); zona pellucida glycoproteins (ZPs); growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15) [Li *et al*., 2015]. All these studies demonstrate that Dicer plays important roles in follicle growth and oocyte maturation. Moreover, a study of miRNA expression in mouse mural granulosa cells identified miR-132 and miR-212 upregulation following LH/hCG induction. Interestingly, knockdown of both miR-212 and miR-132 resulted in decreased protein levels, but not of mRNA levels, of C-terminal binding protein 1 (CTBP1) which is a known target of miR-132, and whose gene product acts as a corepressor of nuclear receptor genes [Fiedler *et al*., 2008]. Further studies to determine how these miRNAs cause these changes in CTBP1 expression would be useful to establish the precise relationship. Sirotkin et al. investigating the role of miRNAs in granulosa cells, by using 80 individual artificial miRNA precursors, that mimic endogenous miRNAs, to transfect cultured primary ovarian granulosa cells, demonstrated the direct involvement of miRNAs in controlling both proliferation and apoptosis in these cells [Sirotkin *et al*., 2010]. Another study, in rodents, revealed a biphase regulation of miRNAs by FSH. The expression of 31 miRNAs was altered during FSH-mediated progesterone secretion of cultured granulosa cells. Specifically, 12 h after FSH treatment, miRNAs mir-29a and mir-30d were significantly down-regulated. However, their expression increased two and threefold respectively, 48 h after FSH exposure [Yao *et al*., 2010]. These two miRNAs could be, hence, involved in the fine-tuning of FSH-mediated granulose cell function. Moreover using a deep-sequencing approach specific miRNAs that are abundant in MII oocyte (MIR184,

MIR100 and MIR10A) or CCs (MIR29a, MIR30d, MIR21, MIR93, MIR320a, MIR125a and the LET7 family) were identified in women who underwent IVF procedures[Assou *et al*., 2013]. In the same study, predicted target genes of the oocyte miRNAs were associated with the regulation of transcription and cell cycle, whereas genes targeted by CC miRNAs were involved in extracellular matrix and apoptosis. Comparison of the predicted miRNA target genes and mRNA microarray data resulted in a list of 224 target genes that were differentially expressed in MII oocytes and CCs, including PTGS2, CTGF and BMPR1B, important for cumulus–oocyte communication. Functional analysis using primary CC cultures revealed that BCL2 and CYP19A1 mRNA levels were decreased upon MIR23a overexpression, suggesting an important role of MIR23a in controlling transcripts that are involved in the ovulatory follicle apoptosis [Assou *et al*., 2013]. Given their important roles in normal physiology, it is not surprising that miRNAs have been shown to play contributory roles in ovarian diseases such as ovarian cancer, polycystic ovarian syndrome (PCOS) and premature ovarian failure (POF) [Yang *et al*., 2012; Zheng *et al*., 2013]. Sang et al. assessed miRNA expression in human follicular fluid of PCOS patients and identified numerous miRNAs that play important roles in steroidogenesis. miR-132 and miR-320 are expressed at a significantly reduced level in the follicular fluid of polycystic ovary patients compared with healthy controls. In addition, miR-132, miR-320, miR-520c-3p, miR-24 and miR-222 regulate estradiol concentrations, and miR-24, miR-193b, and miR-483-5p regulate progesterone concentrations in PCOS patients [Sang et al., 2013].

Recent studies indicate also that miRNA single-nucleotide polymorphisms (SNPs) are associated with disease susceptibility. A study related to miRNA polymorphism analysis identified the association between combined genotypes and haplotypes of miR-146aC>G, miR-196a2T>C, and miR-499A>G and POF risk in Korean women; the

research study indicate that the transcriptional aberration of miR-146a and miR-196a2 induced by miRNA SNPs is potentially involved in POF development [Rah et al., 2013]. In this context, identifying miRNAs, that are specific in different reproductive compartments, and their target genes is fundamental to better understand the underlying mechanisms in normal ovarian physiology and in reproductive disorders.

miRNAs and Oocyte aging

Aging is a complex time-dependent biological process that takes place in every cell and organ, predisposing organism to degenerative changes that affect normal biological functions [Qian *et al*., 2015]. Decreased female fertility with advanced maternal age is by now well documented [Kenny *et al*., 2013]. The fertility decline is slow and steady in 30 to 35 years old women; however it accelerates past 35 years, due to the decrease in oocyte quality and ovarian reserve [Faddy *et al*., 1992; Alviggi *et al*., 2009]. Therefore, female age is crucial and oocyte aging is a common cause of assisted reproduction technology failures [Tarín *et al*., 2014]. Although it is widely recognized that a key aspect that explains infertility in reproductive aging is the decline in oocyte quality, which also associates with higher risk of birth defects, genetic disorders and miscarriage, the molecular mechanisms underlying reproductive aging in female mammals are poorly understood [Kenny *et al*., 2013]. Ovarian physiology is unique in that follicles might remain in a 'resting' phase for a long time and start growing even in the fifth decade of life. It is reasonable, therefore, to hypothesize that during the reproductive lifespan, the ovarian microenvironment is gradually perturbed. Reproductive aging is associated with the impairment of specific functions of oocytes and granulosa cells, along with general dysfunctions typical of the cellular ageing process, such as altered mitochondrial activity, energetic failure and changes in genes

and protein expression profiles [Tatone *et al*., 2011]. A relevant consequence of this condition is an increased susceptibility to meiotic errors, enhancing the risk of chromosomal abnormalities, in the mature oocyte [Vogt *et al*., 2008] (Figure 10). In addition to these, recent studies suggest that epigenetic mechanisms may be altered in oocytes with aging, affecting the expression of DNA methyltransferases, which catalyze the methylation of DNA [Lopes *et al*., 2009]. Quantitative decrease of follicles is associated with production of oocytes with a reduced developmental competence, whose one of the main feature is aneuploidy [Broekmans *et al*., 2007; Jones, 2008]. The human oocyte is, in fact, extraordinary susceptible to meiotic errors leading to aneuploidy and autosomal or gonosomal numerical chromosomal aberrations in the zygote and embryo. Aneuploidy is the leading cause of pre- and postimplantation developmental arrest, implantation failure, spontaneous abortion or birth of a chromosomally unbalanced child [Nagaoka *et al*., 2012]. Thus in assisted reproduction more than 75% of blastocysts from women over the age of forty are aneuploidy [Eichenlaub-Ritter, 2012]. Correlations between children with Down syndrome and maternal age have been recognized already for many years. Cohesins loss, that are the key proteins regulating chromosome separation, may be responsible for age-related meiotic segregation errors in mammalian oocytes [Revenkova *et al*., 2004]. In humans, the meiosis-specific cohesin subunits, REC8 and SMC1B, were found to be decreased in oocytes of women aged 40 and over, compared with those of women aged around 20 years [Tsutsumi *et al*., 2014].

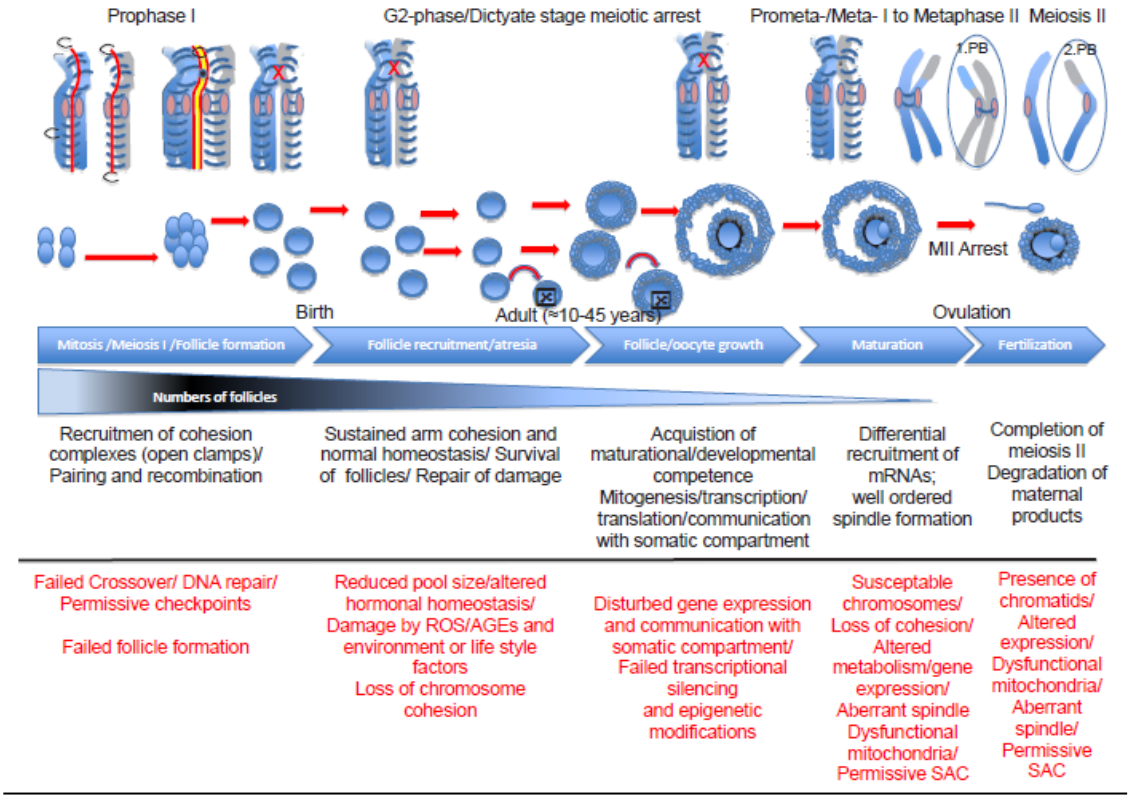


Figure 10: Oocyte and follicle development, meiotic stages, pool size, key events in development and suggested disturbances contributing to age-related meiotic errors. Black lettering: normal development; red lettering: disturbances related to oocyte ageing and suscepibility to meiotic errors [from Eichenlaub-Ritter, 2012].

Moreover, literature data report also TAp73 downregulation in human old oocytes, suggesting an important role for this member of the p53 family as guardian of maternal reproduction in mammals. TAp73 was described interact with the kinetochore protein components of the spindle assembly checkpoint (SAC) complex. 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 [Guglielmino *et al*., 2011]. Furthermore, it

is known that, the age-related decline in fertility is modulated by oxidative stress with consequences on mitochondrial DNA (mtDNA) integrity, resulting in mithochondrial dysfunction [Agarwal *et al*., 2005]. Dysfunctional mitochondria are currently discussed as major factor in predisposition to chromosomal nondisjunction during first and second meiotic division and mitotic errors in embryos, and in reduced quality and developmental potential of aged oocytes and embryos [Eichenlaub-Ritter *et al*., 2011]. As the amount of mitochondria decreases, the mutation of mitochondrial DNA may become increased and mitochondrial function is also affected in the oocytes of advanced-age female bovine, hamsters and mice [Eichenlaub-Ritter *et al*., 2011; Simsek-Duran *et al*., 2013]. Interestingly, an alteration in transcriptome profile of human oocyte with female aging was also reported [Grøndahl *et al*., 2010]. It is well known that the transcriptome of the MII oocytes is established during maturation through a process mainly based on degradation of specific transcripts. Profiling of global gene expression patterns in human and mouse oocytes revealed that ageing alters expression of many genes, and that many maternal mRNAs in old oocytes are not properly degraded during maturation [Hamatani *et al*., 2004; Leoni *et al*., 2007; Pan *et al*., 2008; Grøndahl *et al*., 2010; Santonocito *et al*., 2013]. Thus, oocytes produced during reproductive aging were seen exhibit a defective mRNA storage which could reduce the oocyte quality and, hence, affect oocyte susceptibility to external apoptotic stimuli. Recently, a growing body of evidence supports the hypothesis that the aging process is regulated by a continuous crosstalk between reactive oxygen species (ROS) and SIRT1 [Di Emidio *et al*., 2014]. Although SIRT1 levels were found increased in aged oocyte , the SIRT1 protein was undetectable in aged oocytes. Taken together, all these data could justify the decrease of antioxidant response that normally occurs with aging and suggest a possible involvement of oxidative stress responsive miRNAs in

germ cells. In fact, a negative correlation between Sirt1 mRNA and miR-132 levels was observed when young oocytes exposed to oxidative stress were compared with young control oocytes, and when aged oocytes were compared with young control oocytes [Di Emidio *et al*., 2014]. Moreover, it has been found that female aging alters the expression of variety of genes in human cumulus cells that are essential for oocyte quality and potential targets of specific miRNAs previously identified, by the same group, in cumulus cells, such as miR-425, miR-744, miR-146b, and Let-7d for younger (<30 years) and middle-aged (31–34 years) women and miR-202 and Let-7e for elder (>37 years) women [Al-Edani *et al*., 2014]. Additionally, 79 and 41 miRNAs were detected in microvesicles and exosomes, isolated from equine follicular fluid respectively, and three miRNAs are significantly higher in exosomes isolated from follicular fluid of old mares compared with young ones [da Silveira *et al*., 2012]. miRNA expression profiling of the follicular fluid of younger (<31 years) and older (>38 years) individuals was also investigated and the expression of four miRNAs was found to be different. These miRNAs was found involved in carbohydrate digestion and absorption, p53 signalling and other biological processes that may be related with fertility[Diez-Fraile *et al*. 2014]. However, the correlation between miRNA expression and oocyte quality during reproductive ageing is still unknown.

Circulating miRNAs as biomarkers of reproductive disorders

A promising research field on miRNAs has been opened as the identification of miRNAs circulating in the blood and other biological fluids (e.g., urine, saliva, amniotic fluid, pleural fluid) was executed [Mitchell *et al*., 2008] [Gilad *et al*., 2008]. MiRNAs have been shown to be protected by RNase digestion and esistant to severe chemical-physical conditions [Chen *et al*., 2008]. Accordingly, miRNAs are stable in plasma and

serum and their expression pattern are emerging as highly tissue-specific, and unique also, for each cancer type [Cortez *et al*., 2011] (Table II).

Table II. A compendium of circulating miRNAs with potential as biomarkers for cancer [from Cortez *et al*., 2011].

miRNAs	Cancer type	Body fluid source	Healthy subjects (*n*)	Patients (*n*)	Clinical correlations
miRs-21, 155 and *210*	Diffuse large B-cell lymphoma	Serum	43	60	High *miR-21* expression was associated with relapse-free survival[68]
miR-141	Prostate	Serum	25	25	Serum levels of *miR-141* distinguished patients from healthy subjects[69]
miRs-141, 16, 92a, 92b, 103, 107, 197, 34b, 328, 485-3p, 486-5p, 574-3p, 636, 640, 766, and *885-5p*	Prostate	Serum	15	6	Serum levels were significantly higher in patients compared to controls[70]
miRs-486, 30d, 1 and *499*	Lung	Serum	–	243	Serum levels were differentially expressed between patients with longer and shorter survival. The four-miRNA signature was an independent predictor of overall survival[72]
miRs-21, 92, 93, 126 and *29a*	Ovarian	Serum	11	19	*miRs-21, 92* and *93* were overexpressed in patients with normal preoperative cancer antigen 125[73]
miRs-17-3p and *92*	Colorectal	Plasma	50	90	Plasma levels decrease after surgery; differentiated colorectal from gastric cancer and normal individuals[74]
miRs-92a and *29a*	Colorectal	Plasma	59	157	Plasma levels significantly higher in patients with advanced-stage cancer than healthy controls[75]
miRs-17-5p, 21, 106a and *106b*	Gastric	Plasma	69	30	Plasma miRNA levels reflected the tumor miRNAs in most cases; miRNAs were significantly reduced in post-operative samples[76]
miR-195 and *let7-a*	Breast	Serum	44	83	Serum levels were decreased after tumor resection and correlated with nodal and estrogen-receptor status[77]
miRs-21, 210, 155, and *196a*	Pancreas	Plasma	36	49	Plasma levels discriminate patients from healthy controls[78]
miR-210	Pancreas	Plasma	25	22	Plasma levels were significantly elevated in two independent patient cohorts[79]
miR-500	Liver	Serum	40	40	Increased levels found in patients with hepatocellular carcinoma; *miR-500* serum levels returned to normal after surgical treatment[80]
miR-206	Rhabdomyo-sarcoma	Serum	17	8	Serum levels of the muscle-specific miRNA *miR-206* were significantly higher in patients with rhabdomyosarcoma tumors than in patients with other types of tumors or in the control group[81]
miR-184	Tongue	Serum	20	20	Serum levels were significantly reduced after surgical removal of the primary tumors[82]
miR-92a	Acute leukemia	Plasma	20	20	Decreased levels in plasma samples of acute leukemia patients[84]
miRs-125a and *200a*	Oral squamous-cell	Saliva	50	50	Lower levels in the saliva of patients than control subjects[86]
miR-31	Oral squamous-cell	Plasma Saliva	21 8	43 9	Increased levels in patients compared with controls; level in most patients declined after surgery[87]
miRs-126, 152 and *182*	Bladder	Urine	9	47	Increased levels in patients compared with controls[88]

One of the first studies measuring miRNA levels in serum of patients with diffuse large B-cell lymphoma, discovered that high levels of miR-21 correlated with improved relapse-free survival [Lawrie *et al*., 2008]. Since then, additional studies have assessed the potential use of serum or plasma miRNAs as biomarkers in different types of cancer and diseases. In the ovary, a recent study is showing the use of plasma/serum miRNAs as a noninvasive marker for early diagnosis and treatment of ovarian cancer [Zheng *et al*., 2013]. Plasma/serum miRNA expression profiling has been found to vary significantly with changed physiologic or pathologic condition, such as pregnancy, heart failure, and sepsis [Chim *et al*., 2008; Wang *et al*., 2010; Marfella *et al*., 2013].

Moreover, placenta derived miRNAs (e.g. miR-141, miR-149, miR-299-5p, and miR-135b) are detectable in the maternal plasma, and their concentrations decrease directly after childbirth [Chim *et al*., 2008]. Therefore, miRNAs have been discussed as novel non-invasive markers for pregnancy monitoring [Chim *et al*., 2008]. It is of great advantage that miRNAs can be identified also in follicular fluid retrieved in the in vitro fertilization program [Sang *et al*., 2013; Santonocito *et al*., 2014]. When the oocytes are removed for in vitro fertilization, the follicular fluid is discarded in daily medical practice, but can represent a suitable material to study the ovarian physiology and pathologies and the oocyte quality. It has been reported that miRNAs can readily be detected within membrane-enclosed vesicles of human follicular fluid [Sang *et al*., 2013; Santonocito *et al*., 2014]. It has also been proposed that miRNA containing subcellular vesicles can be taken up by cells and cause changes in gene expression of the recipient cell. It is, also, discussed the concept that miRNAs can function as hormones through their secretion in plasma and acting on distant sites in the body [Cortez *et al*., 2011]. Surprisingly, it has been demonstrated also that human embryos secrete miRNAs into culture media, that could represent new biomarkers for embryo development and implantation [Kropp *et al*., 2014]. miRNAs, hence, constitute a new class of biomarkers easily detectable in a non-invasive manner (i.e., blood collection), that can complement existing conventional markers, including metabolites, antigens, and mRNA transcripts to assess and monitor the physiopathologic state of the body. miRNAs have, therefore, a great potential to be used for diagnosis, prognosis and theraphy of different diseases. This new approach has the potential to revolutionize present clinical management, including determining ovarian reserve, ovarian endocrine function, evaluating the efficacy of ovulation-inducing drugs, and evaluating the process of embryo implantation.

3. MATERIALS AND METHODS

Experimental design and sample classification

The first part of the study was focused on the characterization of miRNA expression profile in human follicular fluid (FF). FF and plasma samples were collected from 15 healthy women who underwent to intracytoplasmic sperm injections (ICSI) at the IVF Center in Catania. From samples, exosomes were purified for the miRNA content to be analysed. FF exosomes were characterized by nanosight, flow cytometry, and exosome-specific surface markers. Expression miRNA profiles from total and exosomal FF were compared with those from plasma of the same women. Next, by using TaqMan Low Density Array technology, we analysed the expression profile of 384 miRNAs from exosomes of 30 FF samples and 12 MII oocytes. Oocytes collected from women younger than 35 years of age are termed 'young oocytes', while those collected from women older than 38 years are called 'old oocytes'. Furthermore, CD-1 mice from the University of L'Aquila were also split in two age groups: young (4-5 weeks) and reproductively old (36 weeks) mice. All the experiments were carried out in accordance with the guidelines for the care and use of laboratory animals approved by the Animal Care Committee of the University of L'Aquila. Experimental design and samples used in our studies are reported in the tables below.

Table III. I Study: Molecular characterization of exosomes and their microRNA cargo in human follicular fluid: bioinformatic analysis reveals that exosomal microRNAs control pathways involved in follicular maturation (Fertil Steril 2014).

Pool	Sample type	miRNA detection system
I-III	Total Follicular fluid	TaqMan Human MicroRNA Array A Cards
IV-VI	Exosomes from follicular fluid	
VII-IX	Plasma	

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.

Table IV. II Study: The miRNome changes in Exosomes from human follicular fluid in relation to aging.

Pool	Sample type	miRNA detection system
I-III	Exosomes from follicular fluid of old women	TaqMan Human MicroRNA Array A Cards
IV-VI	Exosomes from follicular fluid of young women	

Note: Follicular fluid samples were collected from 30 healthy women mixed and divided into aliquots in order to obtain 3 pools from FF of old women and 3 pools from FF of young women for exosome purification and miRNA expression profile analysis.

Table V. III Study: MiRNAs in human MII oocyte and their expression profile changes in relation to aging

Pool	Sample type	Molecular detection system
I-III	Oocytes (MII) from old women	TaqMan Human MicroRNA Array A Cards
IV-VI	Oocytes (MII) from young women	
I-III	Oocytes (MII) from CD-1 old mice	Single TaqMan expression assays for miRNAs TaqMan RT-PCR assays analysis of gene expression
IV-VI	Oocytes (MII) from CD-1 young mice	

Note : A total of 12 mature MII (metaphase II) oocytes, two oocytes per woman, showing normal morphology, were collected from individual follicles of 6 healthy women, three of which were younger than 35 and three older than 38 years of age, and used to profile the transcriptome with TaqMan Human MicroRNA Array A Cards. Moreover, mature oocytes arrested at metaphase II stage (MII) were isolated from CD-1 mice and pooled in experimental groups of 30 oocytes. Three pools of oocytes were obtained from young mice (4-5 weeks) and three from old mice (36 weeks) and used for miRNA and Gene expression analysis.

Women enrolled in the studies

All patients included in these studies were healthy women who underwent to intracytoplasmic sperm injections (ICSI) at the IVF CENTER in Catania, Servizio di PMA –Azienda Ospedaliera Cannizzaro. Such women were known to have male-dependent primary infertility. Patients had not endometriosis, polycystic ovaries, ovarian insufficiency or metabolic syndromes. Moreover, heavy smokers and overweight women were excluded from the study. Our selection criteria were designed to avoid limiting factors in female fertility which could affect sample quality. Once approved by the Hospital Ethical Committee the study started with sample collection. Patients undergoing ICSI treatment, were subjected to gonadotropin administration to induce multiple follicular development and transvaginal oocyte aspiration (Figure 11). Our research followed the tenets of the Declaration of Helsinki; informed consent was obtained from the subjects for the use of supernumerary oocytes, FF and plasma samples employed in our studies.

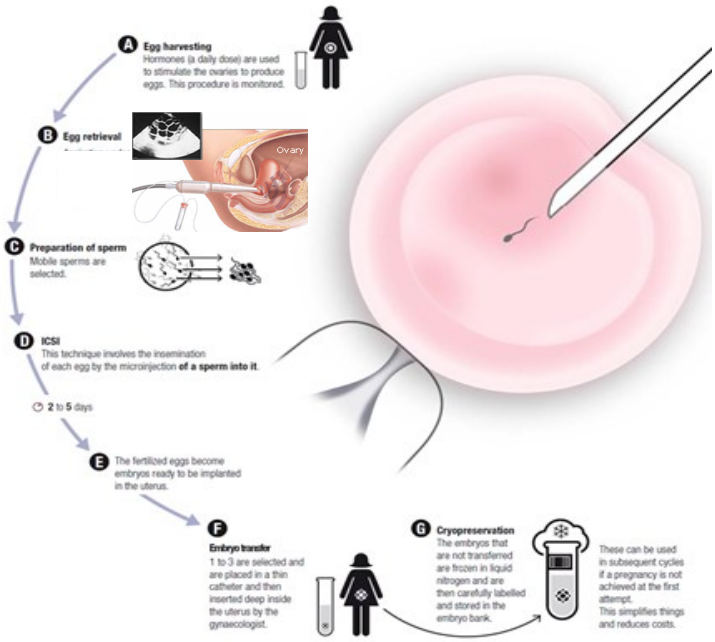


Figure 11: intracytoplasmic sperm injections (ICSI) procedure with oocyte retrieval under ultrasound guidance.

Sample collection

Human metaphase II oocytes

At the IVF CENTER in Catania, the oocyte were collected from patients that had been treated with GnRH agonists (triptorelin or buserelin), to induce multiple follicular development, followed by ovarian stimulation with recombinant follicle stimulating hormone (rFSH) and human menopausal gonadotrophin (HMG). Stimulation was monitored using serum E2 concentrations as well as ultrasound measurements of follicle numbers and diameters. Ovulation was induced with 10,000 IU of human chorionic gonadotrophin (HCG; Gonasi), when the leading follicles reached 18–20 mm diameter and the serum oestradiol concentration per follicle was 150−200 ng/l. Transvaginal ultrasound–guided pick-up of ovarian follicles was performed 34–36 hours after hCG injection. Cumulus-enclosed oocytes were separated from follicular fluid, placed in medium and incubated for two hours at 37°C in a humidified 5% CO2 atmosphere. After incubation, they were treated with 80 IU/ml of hyaluronidase (SynvitroHyadase; Medicult, Jyllinge-Denmark) and the oocytes were at last rinsed three times in culture medium (IVF; VitroLife AB, Kungsbacka, Sweden). Special care was taken to ensure the complete lack of corona cells from oocytes in order to select for each patient the three best MII oocyte to be used for microinjection to perform ICSI procedures. Oocyte were evaluated by an inverted microscope at magnification ×200 for structural parameter assessment such as zona pellucida, polar body and cytoplasm. At the end of the injection, supernumerary eggs were prepared for further molecular studies, choosing those with optimal morphology. 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. Once collected, the oocytes were separately placed in six independent Eppendorf tubes and rinsed in

RNase-free water several times to remove any trace of cell culture medium. The oocytes were moved in PCR tubes in 2 µl water and stored at -80°C before RNA extraction. Ethical considerations have restricted us in using MII-stage oocytes, therefore only those that had failed to fertilize were employed.

Follicular fluid

Follicular fluid was sampled by transvaginal ultrasound–guided puncture and aspiration of follicles 18 to 20 mm in diameter. Follicular fluid samples were centrifuged for 20 minutes at 2,800 rpm at 4 °C to remove follicular cell residues and any blood traces; 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. We recovered about 20 mL of FF from each woman. FF of individual follicles was kept separated until decumulation of the oocytes to collect only the FF in which nuclear mature oocytes (metaphase II) had been identified. To obtain a homogenous pool of samples, 18 mL of FF from each woman was mixed and 270 mL of pooled FF was obtained. Three aliquots of 400 mL for miRNAs were purified from total FF, while two aliquots of 120 mL were used for exosome characterization and exosomal miRNA purification.

Plasma

Blood samples were collected from study patecipants in commercially available EDTA-containing tubes. Cells were removed from plasma by centrifugation for 10 minutes at 1,800 rpm at 4 °C. The resulting supernatant was immediately transferred into a clean polypropylene tube using a Pasteur pipette and stored at 20 °C. 5 mL of blood from each woman was collected, 2 mL of plasma was obtained, and 1 mL was used to make a pool of 15 mL. We used three aliquots of 400 mL for miRNA purification.

Mouse oocyte collection

For oocyte isolation CD-1 young (4-5 weeks) and reproductively old (36 weeks) mice, that is near the end of their reproductive lifespan, were stimulated by 7.5 IU pregnant mare's serum gonadotropin (PMSG) (Folligon; Intervet-International, Boxmeer, Holland) to induce follicular development and, 48 h later, by 7.5 IU human chorionic gonadotropin (hCG) (Profasi HP 2000; Serono, Roma, Italy) to induce ovulation. All the experiments were carried out in accordance with the guidelines for the care and use of laboratory animals approved by the Animal Care Committee of the University of L'Aquila. 15 h after hCG somministration, oviducts were dissected from mice and cumulus oocyte complexes (COCs) collected by puncture of the oviduct. Afterward dissociation of cumulus-oocyte complexes in hyaluronidase 0.1% (Sigma-Aldrich), mature oocytes arrested at metaphase II stage (MII) were isolated and pooled in experimental groups of 30 oocytes. Three pools of oocytes obtained from young mice and three from old mice were washed in RNase-free water, transferred in 10 μL of Trizol and sunk in liquid nitrogen. Samples were stored at −80°C until use.

Sample preparation

Exosome purification

Exosomes were isolated from FF and subsequently characterized according to a previously published protocol with minor modifications (Lässer *et al*., 2012). A volume of 120 mL of FF was collected in 50-mL tubes and centrifuged at 3,000 rpm for 15 minutes at 4 °C to pellet debris. The supernatant was transferred to ultracentrifuge tubes and ultracentrifuged at 16,500 x *g* for 30 minutes at 4 °C, followed by filtration through a 0.2-mm syringe filter. Finally, exosomes were pelleted by ultracentrifugation at 120,000 x *g* for 70 minutes at 4 °C in a Beckman Optima L-100 XP ultracentrifuge

using a Ti70 rotor (Beckman Coulter). Exosome pellets were resuspended in Trizol (Invitrogen) for RNA isolation or in phosphate-buffered saline (PBS) for nanosight and fluorescence-activated cell sorter analysis. For the nanoparticle tracking analysis (NTA), samples were diluted with sterile PBS following the manufacturer's instructions. Samples (approximately 300 mL) were injected into the LM10 unit with a 1-mL sterile syringe. Capturing and analyzing settings were manually set according to the protocol. Using the NanoSight LM10 instrument, vesicles were visualized by laser light scattering, and Brownian motion of these vesicles was captured on video. The number of tracks always exceeded 200, and five size distribution measurements were taken for each sample. Recorded videos were then analyzed with NTA software, which provided high resolution particle size distribution profiles and concentration measurements of the vesicles in solution.

Exosome characterization

Flow cytometry analysis was performed according to a previously published protocol (Wahlgren *et al*., 2012.). Aldehyde/sulfate latex beads of 4 mm (Invitrogen) were serially diluted with PBS to reach a final concentration of 2,900 beads/mL. Vesicles from FF samples were incubated with 25 mL of 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 mL 1 M glycine (Sigma-Aldrich) for 30 minutes at 20 °C to block any remaining available binding sites. After two washes in PBS with 1% fetal bovine serum (FBS), exosome-coated beads were stained with phycoerythrin-(PE-) conjugated CD63, CD81, or CD9 antibodies or isotype control (BD Biosciences Pharmingen) and incubated for 1 hour at 4 °C with gentle agitation. After

the third wash in PBS/FBS 1% solution, samples were resuspended in PBS and analyzed on FACSCantoII (Becton Dickinson) with FlowJo software (TreeStar, Inc.).

miRNA profiling in follicular fluid, plasma and exosomes

miRNA Isolation, Reverse Transcription, Preamplification, and miRNA expression profile

All experiments were performed in triplicate. Three aliquots of 400 µl of FF and plasma samples were thawed completely on ice. For fluids, miRNA isolation was performed by using Qiagen miRNeasy Mini Kit (Qiagen GmbH), according to the Qiagen Supplementary Protocol for the purification of small RNAs from serum and plasma. Briefly, 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 (Figure 12). Total RNA was eluted in 30 µl of RNAse-free water.

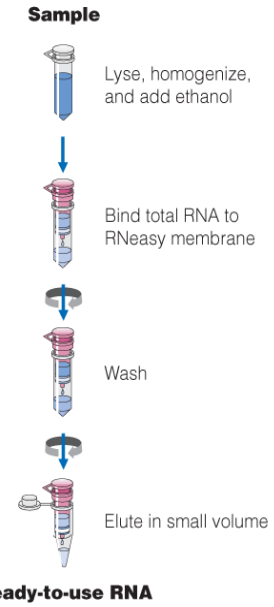


Figure 12.: Copurification of miRNA and total RNA through spin columns (Qiagen GmbH).

Total RNA was isolated from three aliquots of FF exosomes using Trizol reagent (Invitrogen), according to the manufacturer's instructions. Before precipitating the RNA with isopropyl alcohol, 20 µg RNase-free glycogen (Invitrogen) as carrier was added to the aqueous phase and the samples were stored for 16 hours at -80 °C. RNA pellets were dissolved in RNase-free water. Detection and quality control of RNA were performed using an Agilent 2100 Bioanalyzer (Agilent Technologies Sweden AB) and 2100 Expert software. Expression profiles of 384 miRNAs were investigated by using TaqMan low-density array technology (Applied Biosystems) in a 7900HT fast real time PCR system (Applied Biosystems). Each card contains dried Primers and Probes for up to 380 miRNAs and controls. cDNA product (with or without pre-amplification) specific to card A or card B is loaded onto the respective array for real time PCR. This technology is highly specific and enables highly parallel profiling of up to 384 miRNAs present in the Sanger miRBase v18.0, allowing the amplification of only mature miRNAs [Chen *et al*., 2005] (Figure 13).

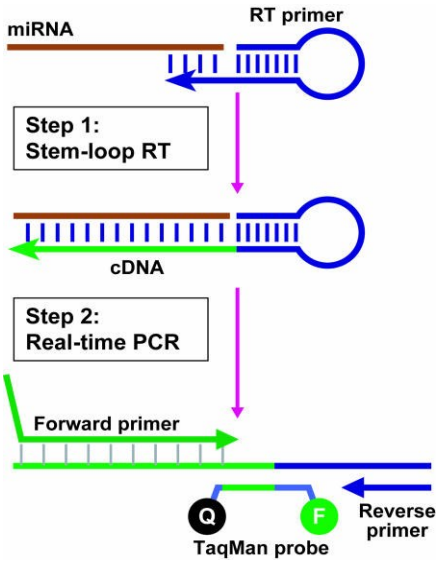


Figure 13: Schematic description of TaqMan miRNA assays. TaqMan-based real-time quantification of miRNAs includes two steps, stem–loop RT and real-time PCR. Stem–loop RT primers bind to at the 3′ portion of miRNA molecules and are reverse transcribed with reverse transcriptase. Then, the RT product is quantified using conventional TaqMan PCR that includes miRNA-specific forward primer, reverse primer and a dye-labeled TaqMan probes. The purpose of tailed forward primer at 5′ is to increase its melting temperature (Tm) depending on the sequence composition of miRNA molecules [Chen *et al*., 2005].

RNA (3 µl of each sample) was reverse-transcribed (RT) by using the TaqMan MicroRNA Reverse Transcription Kit and Megaplex RT Primer Pool A (Applied Biosystems). Four microliters of RT products were preamplified with TaqMan PreAmp Master Mix 2x and Megaplex PreAmp Primers Pool A (Applied Biosystems). Pre-amplification products were subsequently diluted in 75 µl of RNase free purified water. Nine microliters of each amplified product was mixed with Universal Master Mix II, no UNG (Applied Biosystems), and 100 µl of this PCR mix were loaded into each port of the TaqMan Human MicroRNA Array A Cards v2.0 (Applied Biosystems); according to the manufacturer's instructions. 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) (Figure 14). All the experiments were performed in triplicates.

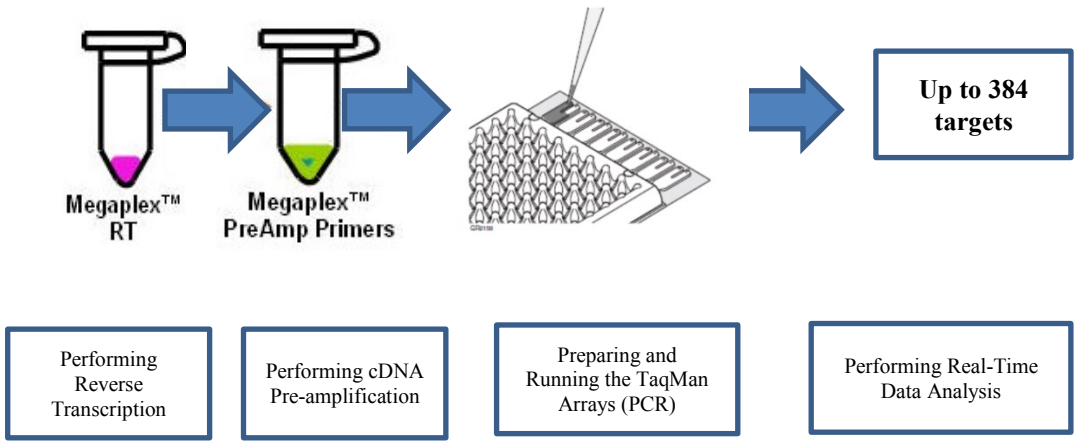


Figure 14: workflow on miRNA quantification through Applied Biosystems 7900HT TaqMan low density array.

miRNA profiling in human MII oocytes

Total RNA isolation, miRNA Reverse Transcription and miRNA expression profile.

The six couples of MII oocytes retrieved, three couples of patients with a mean age of 35 years and three of patients with a mean age of 38, were separately placed in six independent Eppendorf tubes and rinsed in RNase-free water several times to remove any trace of cell culture medium. The oocytes were moved in PCR tubes in 2 µl water and stored at -80°C before RNA extraction. All samples were incubated, after adding water (2 µl), for 1′ at 100°C according to a previously published protocols with minor modifications [El Mouatassim *et al*., 1999; Di Pietro *et al*., 2008] in order to release nucleic acids. Although the lysis reactions, as expected, can release DNA and other cellular components, a specific reverse transcription system followed by a Probe-based quantitative PCR allows the detection of mature miRNA molecules. To profile the transcriptome of 384 miRNAs, 3.2 µl of RNAs were reverse-transcribed (RT) in 7.5 µl reaction using TaqMan MicroRNA Reverse Transcription Kit and Megaplex RT Primer Human Pool A (Applied Biosystems). One cDNA Preamplification step from 2.5 µL of RT reaction product, using Megaplex PreAmp Primers Pool A and TaqMan PreAmp Master Mix (2x)(Applied Biosystems), was run in a final volume of 25 µl. Pre-amplification products, diluted in 75 µl of RNase free purified water, were loaded on TaqMan Low Density Arrays (TLDAs) TaqMan Human MicroRNA Array A (Applied Biosystems) according to the manufacturer's protocol. qRT-PCR reactions were performed in a 7900 HT Fast Real Time PCR System as follows: 10′ at 94.5°C, 30″ at 97°C and 1′ at 59.7°C for 40 cycles.

Expression Data Analysis

Study I

No data are available on reliable reference genes to be used for miRNA normalization in FF. Accordingly, we used DataAssist version 3 software (Applied Biosystems) and the geNorm algorithm (http://medgen.ugent.be/jvdesomp/genorm/) to identify appropriate endogenous controls. GeNorm is based on the geometric average of multiple internal control genes, and it is especially useful for normalization of data from a large and unbiased set of genes (e.g., miRNA expression profiling) [Vandesompele *et al.*, 2002; Mestdagh *et al*., 2009]. DataAssist is a data analysis tool that is useful to compare samples when using the comparative CT (DDCT) method for calculating relative quantitation of gene expression. In a first step, three miRNAs common to two programs and having a low score in both (the lower the score, the better the normalization) were selected as housekeeping genes. The miRNAs miR-25, miR-28-3p, and miR-145 were identified for comparison between FF and plasma, and miR-126, miR-28-3p, and miR-145 were identified for exosomes versus plasma. The DCt values were independently calculated by using the three selected housekeeping genes, for each sample. To identify differentially expressed (DE) miRNAs, three different significance analysis of microarrays (SAM; http://www.tm4.org) tests (Tusher, 5th percentile and minimum S value) were used, applying a two-class unpaired test among DCt and using a P value based on all possible permutations: imputation engine, K-nearest neighbors (10 neighbors); false discovery rate, <0.05. We considered as DE miRNAs only those with three housekeeping genes in common after at least two SAM tests. The DCt values of DE miRNAs were used to calculate relative quantity (RQ) values as a natural logarithm of 2^{-DDCt} and the average of DCt of each plasma sample as calibrator.

miRNAs with a natural logarithm of RQ values R2.5 were considered as upregulated (highly expressed) in FF or exosomes compared with plasma. For each DE miRNA, nine RQ values (three for each housekeeping gene) were obtained that were reciprocally comparable, and RQ values obtained with the housekeeping miR145 were used to perform additional statistical analysis according to Livak and Schmittgen [Livak and Schmittgen, 2001]. The mean fold change was calculated as a natural logarithm of RQ values, and the error was estimated by evaluating the 2^{-DDCt} equation using DDCt plus SD and DDCt minus SD [Livak and Schmittgen, 2001].

Study II

An accurate selection of candidate reference miRNAs for a suitable data normalization was executed. After calculating mean and median of miRNA Ct values for each plate and the pairwise Pearson correlation for all miRNAs, the two more stable miRNAs, miR-320 and miR-342-3p showing constant expression levels between individual samples and positive correlation were identified (Figure 15). The canonical comparative cycle threshold (ΔΔCt) method to perform relative quantification was applied and Ct values were normalized to the internal controls, miR-320, miR-342-3p, mean and median Ct of each sample. Mean Ct of FF exosomes from young patients were employed as calibrator sample. In this analysis, we applied Significance Analysis of Microarrays (SAM) method to extract the statistical significant DE miRNAs between exosomes from FF of women younger than 35 and women older than 38 analyzed with TaqMan Human MicroRNA Array A cards. SAM allowed us to identify statistically significant miRNAs by carrying out miRNA specific tests (Tusher, minimum S value and the 5th , 50th and 90th percentiles) applying a two-class unpaired test among ΔCt and using a p value based on 100 permutations; imputation engine: K-nearest neighbors (10 neighbors); False Discovery rate <0.05. Relative quantities (RQ) were calculated by

Livak and Schmittgen method. For each DE miRNA, twelve RQ values (three for each housekeeping gene) were obtained that were reciprocally comparable, and RQ values obtained with the housekeeping miR-320 were used to perform additional statistical analysis. The mean fold change was calculated as a natural logarithm of RQ values, and the error was estimated by evaluating the $2^{-\Delta\Delta Ct}$ equation using ΔΔCt plus SD and ΔΔCt minus SD.

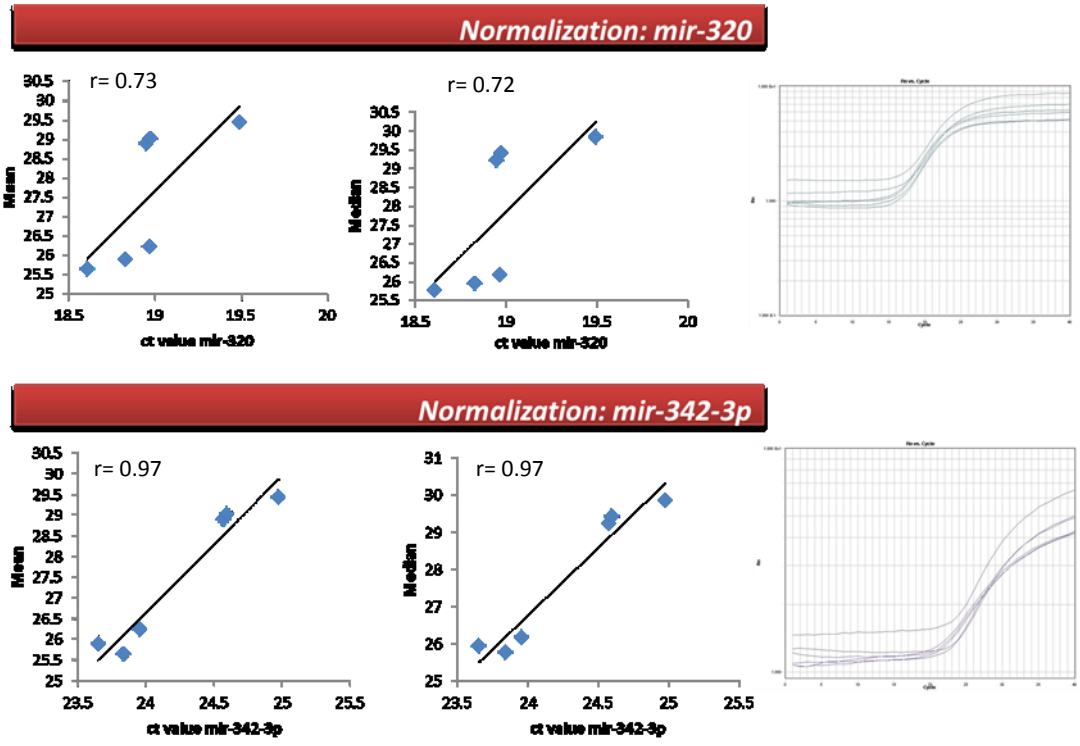


Figure 15: Scatter diagrams showing on X axis the Ct values of miR-320 and miR-342-3p and on Y axis the mean Ct or Median Ct value**.** Pearson's correlation coefficient is denoted by r.

Study III

The 384 qRT-PCR reaction efficiency in all samples was primarily evaluated. The average threshold cycle (Ct) and the standard error mean as a measure of variability in microRNA content for two groups of patients were compared. miRNAs with a Ct less than 37 and a Standard Deviation (SD) <5 were considered as detected, while undetermined Ct values were managed as 40. To normalize miRNA expression profiling data, median and the average expression of the plate and the pairwise Pearson correlation for all miRNAs were calculated to identify the more stable miRNAs that showed constant expression levels between individual samples. The miRNAs miR-320 (p=0.9), miR-342-3p (pearson correlation=0.9), miR-372 (p= 0.9), RNU48 (0.9) and U6 snRNA (0.9) were identified as candidate housekeeping miRNAs for comparison between young and older MII oocytes. As a result, geNorm and Normfinder statistical algorithms were used to confirm their stability and suitability as endogenous controls. Validation using geNorm suggested miR-342-3p (score 0.11) and miR-372 (score 0.11), having a low score in both (the lower the score, the better the normalization), as the most stable ones. NormFinder indicated that the combination of miR-342-3p and miR-372 had the highest stability value (0.005). Differential expression of miRNAs, between young and old oocytes, measured with qRT-PCR on TLDAs was identified by different Significance Analysis of Microarrays (SAM) tests such as Tusher, minimum S value and the 5th , 50th and 90th percentiles, applying a two-class unpaired test among ΔCt and using a p value based on 100 permutations; imputation engine: K-nearest neighbors (10 neighbors); False Discovery rate <0.30. We considered as DE miRNAs only those with two housekeeping miRNAs (and media e mediana) in common after at least two SAM tests. The relative quantification was performed with miR-342-3p, miR-372, the mean and median Ct of each sample value as endogenous controls.

The relative expression was obtained applying the 2−ΔΔCt method, where ΔCt values were independently calculated by using mir-342-3p, mir-372 and ct mean as housekeeping miRNAs and the average DCt of qRT-PCR reactions from oocytes of young patients were the calibrator sample. The mean fold change was calculated as a natural logarithm of RQ values, and the error was estimated by evaluating the $2^{-\Delta\Delta Ct}$ equation using ΔΔCt plus SD and ΔΔCt minus SD according to Livak and Schmittgen method. Since relative quantification with different reference miRNAs did not differ significantly and produced similar relative expression data, only RQ values obtained with the housekeeping miR-342 were shown.

miRNA Target Prediction, Gene Ontology (GO) and Pathway Analysis

Study I

Targets of upregulated miRNAs were explored by using a combination of different approaches. By interpolation among the highest numbers of 11 prediction tools, the first series of experimentally validated and predicted targets were extracted from miRecords (http://mirecords.biolead.org) [Xiao *et al*., 2009]. To improve prediction, TarBase, version 5.0 (http://www.diana.pcbi.upenn.edu/tarbase), and miRTarBase, version 3.5 (http://mirtarbase.mbc.nctu.edu.tw), were also used. These are two databases that have accumulated more than 50,000 experimentally supported targets. An additional filtering was performed by using starBase, version 2.0, a database for predicted miRNA-target interactions that are 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, our list was

enriched by retrieving from the literature specific information on validated miRNA targets. The Gene Ontology (GO) and Pathway analysis was independently performed on miRNAs upregulated in total FF and miRNAs upregulated in exosomes. The GO functional classification of miRNA targets was focused on the biological processes, in which target mRNAs are involved. For this analysis, the g:GOSt (GO statistics) tool of g:Profiler (http://biit.cs.ut.ee/gprofiler/) was used and Bonferroni correction for a P value of 0.05 was applied. Pathway analysis was performed by using the prediction software DIANA-microT-4.0 (beta version; http://diana.cslab.ece.ntua.gr/pathways/). DIANA-miRPath uses miRNA targets that have been predicted with high accuracy based on DIANA-microT-CDS. In our analysis, we input upregulated miRNAs, and the software performs an enrichment analysis of multiple miRNA targets genes comparing the set of miRNA targets to all known KEGG pathways. It retrieved signalling pathways enriched with gene targets of the miRNAs sorting them by P values (P-value threshold .05 and microthreshold (0.8) [Vlachos *et al*., 2012].

Study II

All miRNAs significantly different between the two groups were loaded into Diana-miRPath v2.0 (http://www.microrna.gr/miRPathv2) selecting experimentally validated target genes for DE miRNAs. Experimentally validated miRNA targets were found through Tarbase v6.0 database, hosting more than 65 000 miRNA–gene interactions; 16.5- to 175-fold more than any other available manually curated database [Vergoulis *et al*., 2012]. The human pathway collection from KEGG for DE miRNAs was used to perform an over-representation analysis selecting the option *Union of Genes* from server and applying the false discovery rate (FDR) method (Benjamini and Hochberg), a P value <0.05 and a microT threshold of 0.8.

Study III

The potential and validated target of DE miRNAs were explored by a computational approach based on a combination of three different tools: starBase v2.0, miRTarBase 4.5 and TarBase v7.0. An enrichment analysis for biological process and pathway of target genes was, then, performed using protein-coding genes associated with maturation of oocyte in human (http://okdb.appliedbioinfo.net/) [Piras *et al*., 2014; Kocabas *et al*., 2006] and expressed in aging together. List of genes involved in normal aging process is obtained from GenAge, a curated database of genes related to aging in humans and in model organisms (http://genomics.senescence.info/genes/models.html). Final target list was then uploaded to The PANTHER (Protein ANalysis THrough Evolutionary Relationships) Classification System, version 10.0; a comprehensive, curated database of protein families, trees, subfamilies and functions, available at http://pantherdb.org, designed to classify proteins (and their genes) in order to facilitate high-throughput analysis. The Gene Ontology (GO) analysis was applied to analyze the main functions of target genes of de miRNAs. The functional classification of miRNA targets was focused on the GO experimentally observed biological process. Statistical overrepresentation test was executed and the Bonferroni correction for multiple testing was used to correct the P value. GOs with a P value <0.05 were chosen. DE miRNA target genes were subsequently employed in a signalling pathways enrichment analysis in Diana-miRPath v2.0 (http://www.microrna.gr/miRPathv2). The predefined list with genes expressed in MII human oocyte and aging-related was uploaded to carry out pathway analysis of in silico predicted and experimentally validated miRNA gene targets, according to the Kyoto Encyclopedia of Genes and Genomes (KEGG). The false discovery rate (FDR) method (Benjamini and Hochberg), as a correction for

multiple hypothesis testing, was implemented to select the biological pathways with a threshold of significance defined by a P value <0.05 and a microT threshold of 0.8.

Network analysis

In sorting candidate miRNAs to be validated, the miRNAs were required to have high homology with the murine miRNA sequence because validation assays were based on human sequence. Based on a homology search of mature microRNA sequences between human and mouse, deposited in miRBase 21 (http://www.mirbase.org) and fold change filtering, a set of seven conserved miRNAs were selected. A query list of 125 target genes was entered in Cytoscape version 3.2.1 (www.cytoscape.org) including predicted and validated miRNA targets from our pathway analysis. The biological network of seven conserved miRNA target genes was built by retrieving the corresponding interactome data through Cytoscape plug-in GeneMANIA v.3.4.0, selecting for physical, genetic and pathway interactions. Subnetwork was derived by taking only first neighbors between miRNAs and target genes in the network. Network key genes, common between human and mouse, were also screened using Diana-miRPath v2.0. The resulting network was analyzed using the Cytoscape plug-in CentiScaPe v.2.1, to calculate the centrality parameters of individual nodes. Nodes in networks can be characterized by several parameters, which evaluate their importance in the network's structure, from different viewpoints. In our study, were analysed only few of the relevant network biologically parameters used in describing functional associations as well as physical interactions between proteins [Raman *et al*., 2014; Batool *et al*., 2014]. Based upon Degree, Betweenness, Closeness and Eccentricity, important were selected for molecular examination: DNA (cytosine-5-)-methyltransferase 3 alpha (DNMT3A),

DNA (cytosine-5-)-methyltransferase 3 beta (DNMT3B), phosphatase and tensin homolog (PTEN) and transcription factor A, mitochondrial (TFAM).

miRNA and Gene expression analysis

Total RNA for miRNA and gene expression analysis was prepared using TRIzol (Life Technologies) according to the protocol provided by the manufacturer. 3 µl of Total RNA were used for miRNA-specific reverse transcription (RT) to obtain miRNA-specific cDNAs. Four-fifths of the cDNA total volumes were analyzed with quantitative real-time polymerase chain reaction (qRT–PCR) using TaqMan MicroRNA Assays (Applied Biosystems). All real-time PCR reactions were performed in 20 µl volume containing 10 µl of TaqMan Universal PCR Master Mix (Applied Biosystems),1 µl of miRNA-specific TaqMan MicroRNA Assay (Applied Biosystems), 6 µl of RT product, and 3µl of nuclease free water. We assayed the presence of seven miRNAs previously identified at different expression levels through TLDAs: let-7b (Assay ID 002619), miR-126 (Assay ID 002228), mir-19a (Assay ID 000395), mir-192-5p (Assay ID 000491), miR-203 (000507), miR-29a (Assay ID 002112) and miR-494 (Assay ID 002365). These assays specifically detect mature miRNAs. For each reaction, the following amplification profile was performed: 95°C for 10 min for the first cycle; 95°C for 15 s and 60°C for 1 min for 40 cycles. The results were normalized to the expression level of U6 and analyzed by the ΔΔCt method. For the expression of target mRNAs for miR-192-5p, miR-203, mir-29a and miR-494 murine-specific primers were designed using Primer-Blast software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). To assess Pten, Tfam, Dnmt3a and Dnmt3b gene expression in mouse oocytes, cDNA was synthesized from 100 ng of total RNA using SuperScript® II Reverse Transcriptase (Invitrogen) and random hexamer primers (Roche). Polymerase chain reaction (PCR) analyses were carried out by Power SYBR Green PCR Master Mix kit (Applied

Biosystem) in a final volume of 20 µl containing 2 µl of cDNA, 10 µl of SYBR Green, 0.4 µl of Primers and 7.6 of RNAse free water. Primer sequences are shown in table below.

GENE NAME	Forward	Reverse
Dnmt3a	5′-ACCGCAAAGCCATCTACGA-3′	5′-GCCACTGTCACTTTCATCACTG-3′
Dnmt3b	5′-CGTCGAGCATCATCTTCAGCAA-3′	5′-GCTCTTAGGTGTCACTTCTTCCA-3′
Pten	5′-AATTCCCAGTCAGAGGCGCTATGT-3′	5′-GATTGCAAGTTCCGCCACTGAACA-3′
tFam	5′-TCTCATCCGTCGAAGTGTGAAAC -3′	5′-GCCTAATCCCAATGACAACTCCG-3′
Hprt	5′-TCCATTCCTATGACTGTAGATTTTATCAG -3′	5′-AACTTTTATGTCCCCCGTTGACT -3′

For each reaction, 40 cycles of amplification with the following profile were performed: 95°C for 10 min for the first cycle; 95°C for 15 sec and 60°C for 1 min for 40 cycles; then 95°C for 15 s and 60°C for 15 s. Relative expression levels for each gene were calculated by ΔΔCt method, obtained by normalization of the level of each transcript to expression of Hprt and to the normalized level of transcript of the young oocytes as control group.

Statistical analysis

The value for the mean DCT and the standard error of the mean were calculated using the statistical function of Microsoft Excel 2010. Statistical significance was determined by an unpaired t-test (two-tailed) from delta Ct values of the older oocytes and the controls. The miRNA-target correlation was performed by Pearson correlation test. A P-value of <0.05 was considered statistically significant.

4. RESULTS

Study I: Molecular characterization of exosomes and their microRNA cargo in human follicular fluid: bioinformatic analysis reveals that exosomal microRNAs control pathways involved in follicular maturation.

Exosome Characterization and miRNA Isolation

The nanovesicles isolated from human FF had an average size of 40 nm in diameter, which is consistent with the characteristic size range of exosomes [Théry *et al*., 2002] (Figure 16A). Moreover, our samples were positive for the tetraspanin proteins CD63 and CD81, which are known to be enriched in exosomes [Théry *et al*., 2002] (Figure 16B). RNAs isolated from exosomes were small-sized RNAs and lacked bands corresponding to cellular 18S and 28S ribosomal RNAs (Figure 16C).

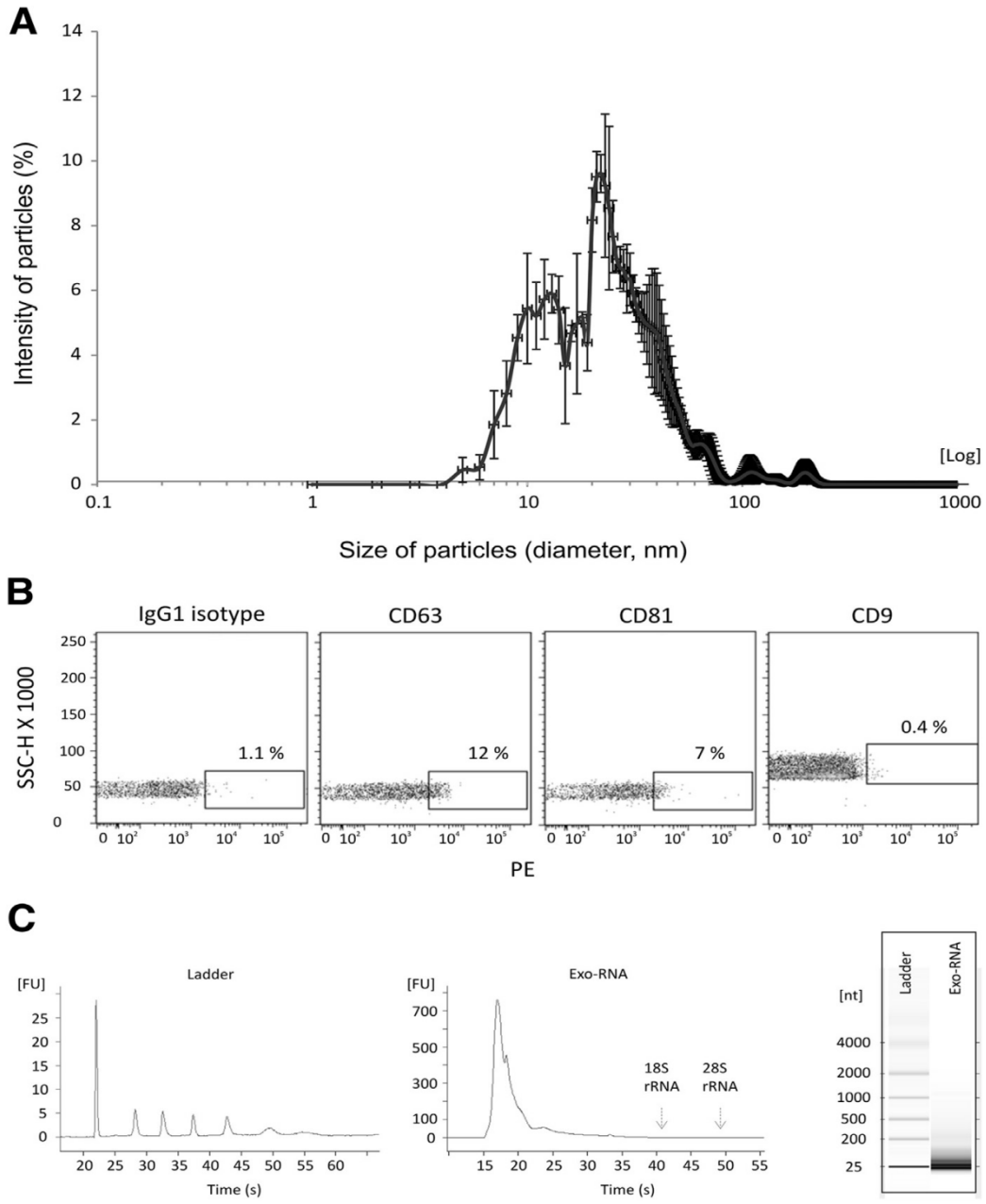


Figure 16: Characterization of exosomes. (A) Nanoparticle tracking measurement of particle size and concentration. Pelleted fractions from FF are vesicles whose diameter size ranged from 10 to 100 nm, with a peak size between 30 and 50 nm. Average vesicle size was 40 nm. Particle size is consistent with exosome size range. Error bars represent SDs obtained from five measurements of the same sample. (B) Flow cytometry detection of surface molecules on exosomes from FF. Exosomes, bound to latex beads, were immunostained by using monoclonal antibodies against the tetraspanins CD9, CD63, and CD81 and analyzed by flow cytometry. The antibodies were compared with their appropriate isotype control IgG1. FF exosomes were positive for CD63 and CD81. (C) Exosomal RNA analyzed using a Bioanalyzer. RNA pattern, isolated from FF exosomes, is visualized in Bioanalyzer as electrophoretic data and a gel-like image. The results show that exosomes are enriched in small RNAs and contain no 18S and 28S ribosomal RNAs. Abbreviations: SSC-H= side scatter height; FU = fluorescence; nt = nucleotides.

Expression Profile of miRNAs

We identified 37 miRNAs upregulated in human FF compared with in plasma (Figure 17). Specifically, 15 miRNAs were found to be upregulated in total FF compared with in plasma; 10 miRNAs were carried by exosomes, while five were not (Figure 17). Twenty-two miRNAs were present exclusively in exosomes. They were undetectable in total FF because their relative concentration was higher in purified exosomes compared with in total FF (Figure 17). The five miRNAs detected only in total FF and absent in exosomes could be free circulating miRNAs associated with protein complexes (Figure 17). Interestingly, miR-508-3p, whose fold change value was among the highest, seems to be follicle specific, since we could not detect it in plasma (Figure 17). Among the 37 miRNAs previously cited, we found miR-10b, miR-29a, miR-99a, miR-125b, miR-132, miR-202, miR-212, and miR-874, which had been reported to be highly expressed in granulose or in cumulus cells in humans and in mice [Fiedler *et al*., 2008; Assou *et al*., 2013; Velthut-Meikas *et al*., 2013].

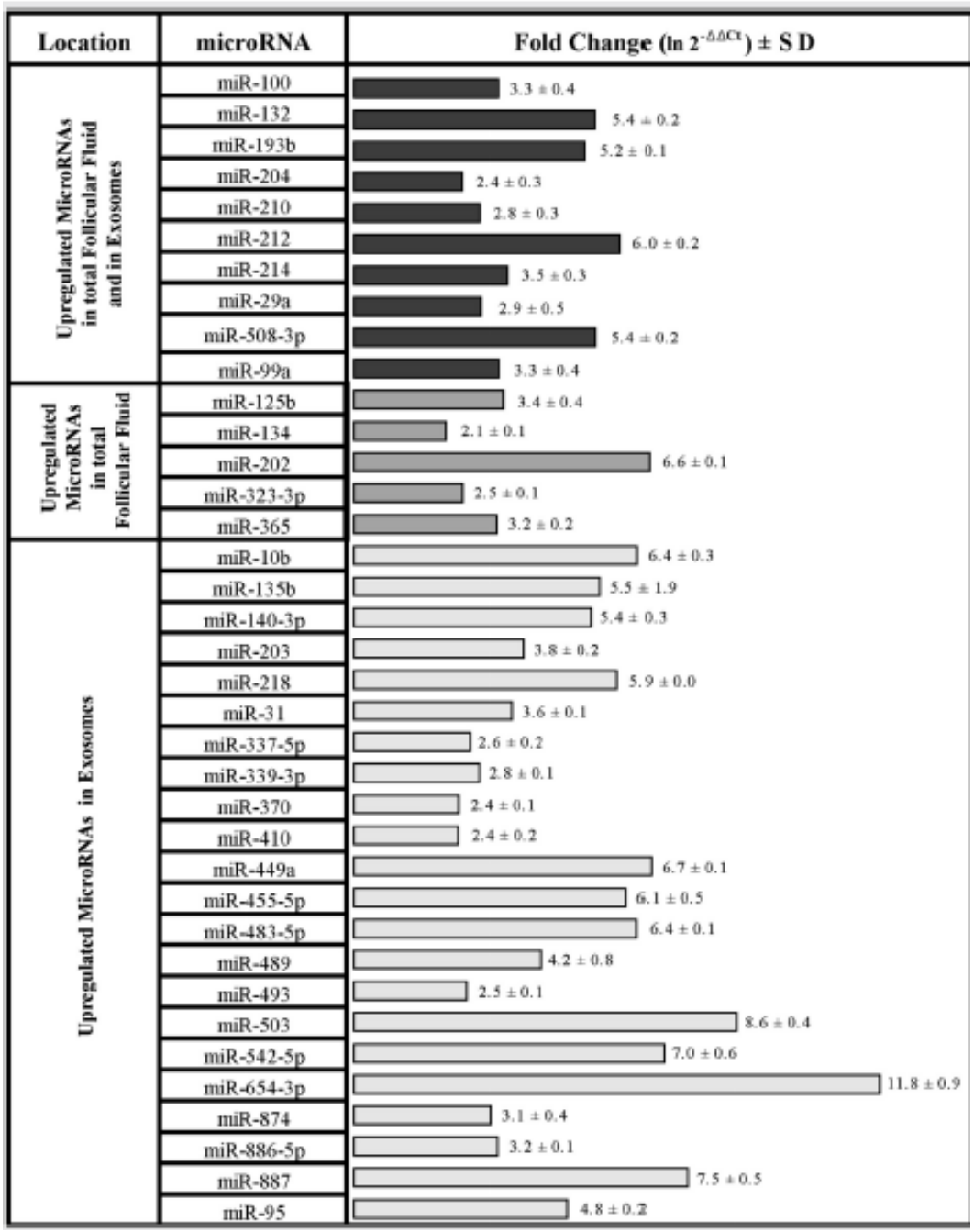


Figure 17: MicroRNA expression in total FF and exosomes compared with plasma. Black bars show the 10 upregulated miRNAs in total FF and in exosomes. Gray bars represent five upregulated miRNAs exclusively in the total FF, and light gray bars represent 22 upregulated miRNAs in exosomes only. Fold change is shown as the natural logarithm of RQ values, which were calculated through the 2^{-DDCt} method (by using miR-145 as an endogenous control and plasma as the calibrator sample) and respective SD values.

Genomic Analysis

Genomic analysis showed that 22miRNAs (miR-29a, miR-99a, miR-100, miR-125b, miR-132, miR-134, miR-193b,miR-203, miR-212, miR-214, miR-323-3p, miR-337-5p, miR-365, miR-370, miR-410, miR-449a, miR-489, miR-493, miR-503, miR-508-3p, miR-542-5p, and miR-654-3p) are localized in 13 different clusters; a number of the upregulated miRNAs were part of the same cluster (Table VI). Interestingly, miR-134, miR-323-3p, miR-410, and miR-654-3p were localized within the large miR-379/miR-656 cluster, which is exclusively present in placental mammals and involved in embryonic development [Glazov *et al*., 2008]. We found that genes encoding 13 miRNAs were located inside genes encoding proteins, while those for the other 24 miRNAs were located in extragenic regions or inside noncoding genes.

Table VI. Upregulated FF miRNAs and their genomic localization

Extragenic miRNAs[a]			miRNAs located in protein encoding genes			
miRNA	**Chromosomal position**	**Clustered miRNAs**	**miRNA**	**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	CDC20 B
miR-100	11q24.1	let-7a-2	miR-887	5p15.1		FBXL7
miR-125b	21q21.1	miR-99a	miR-874	5q31.2		KLHL3
miR-99a		miR-125b let-7c	miR-489	7q21.3	miR-653	CALCR
miR-134	14q32.31	Cluster	miR-204	9q21.12		TRPM3
miR-323-3p		miR-379/miR-656	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				
miR-337-5p	14q32.2	miR-493				
miR-370		miR-665				
miR-493		miR-431 miR-433 miR-127 miR-432 miR-337				
miR-193b	16p13.12	miR-365a				
miR-365		miR-193b				
miR-212	17p13.3	miR-132				
miR-132		miR-212				
miR-503	Xq26.3	miR-450a-2				
miR-542-5p		miR-542 miR-450a-1 miR-450b miR-503 miR-424				
miR-508-3p	Xq27.3	miR-507 miR-506				

[a] Or located in noncoding genes.

GO and Pathway Analysis

GO analysis of validated and predicted targets was independently performed on miRNAs upregulated in total FF and miRNAs upregulated in exosomes. GOs were used to functionally categorize miRNA target genes that were identified in a range of biological processes. The GO terms ranged from one to three levels of detail, and only significant GO terms are reported. The maximum P value was .048. Figure 3A shows the significant GOs, and in the x-axis the negative log10 (P value) is reported. The most significant biological processes involving the targets of our upregulated miRNAs are developmental, regulation of cellular process, cell differentiation, and cell communication (Figure 18A). Pathway analysis showed that WNT, MAPK, ErbB, and TGFb signaling pathways, which are shared by total FF and exosome miRNAs, are the most significant, with a negative log10 (P value) between 11.04 and 4.12 (Figure 18B). Figure 18C specifically shows the miRNAs involved in the regulation of the significant pathways presented in Figure 3B. It can be observed that WNT and MAPK signaling pathways are clearly targeted by most miRNAs with a very small P value. On the other hand, it can be seen that some miRNAs (e.g., miR-132, miR-212, and miR-214) could regulate at least eight different pathways (Figure 18C). To perform a more accurate analysis, our computational approach was validated using literature data. Its application confirmed that miR-29a, miR-99a, miR-100, miR-132, miR-212, miR-214, miR-218, miR-508-3p, and miR-654-3p are particularly interesting. In fact, their expression inside granulose or cumulus cells and their regulatory role were demonstrated by their targeting mRNAs involved in ovarian physiology and pathology [Kovac *et al*., 2001; Fiedler *et al*., 2008; Sang *et al*., 2013]. In Figure 19 we present a schematic view of the pathways inside follicular cells and oocytes, and we pinpoint where our miRNAs could perform their action.

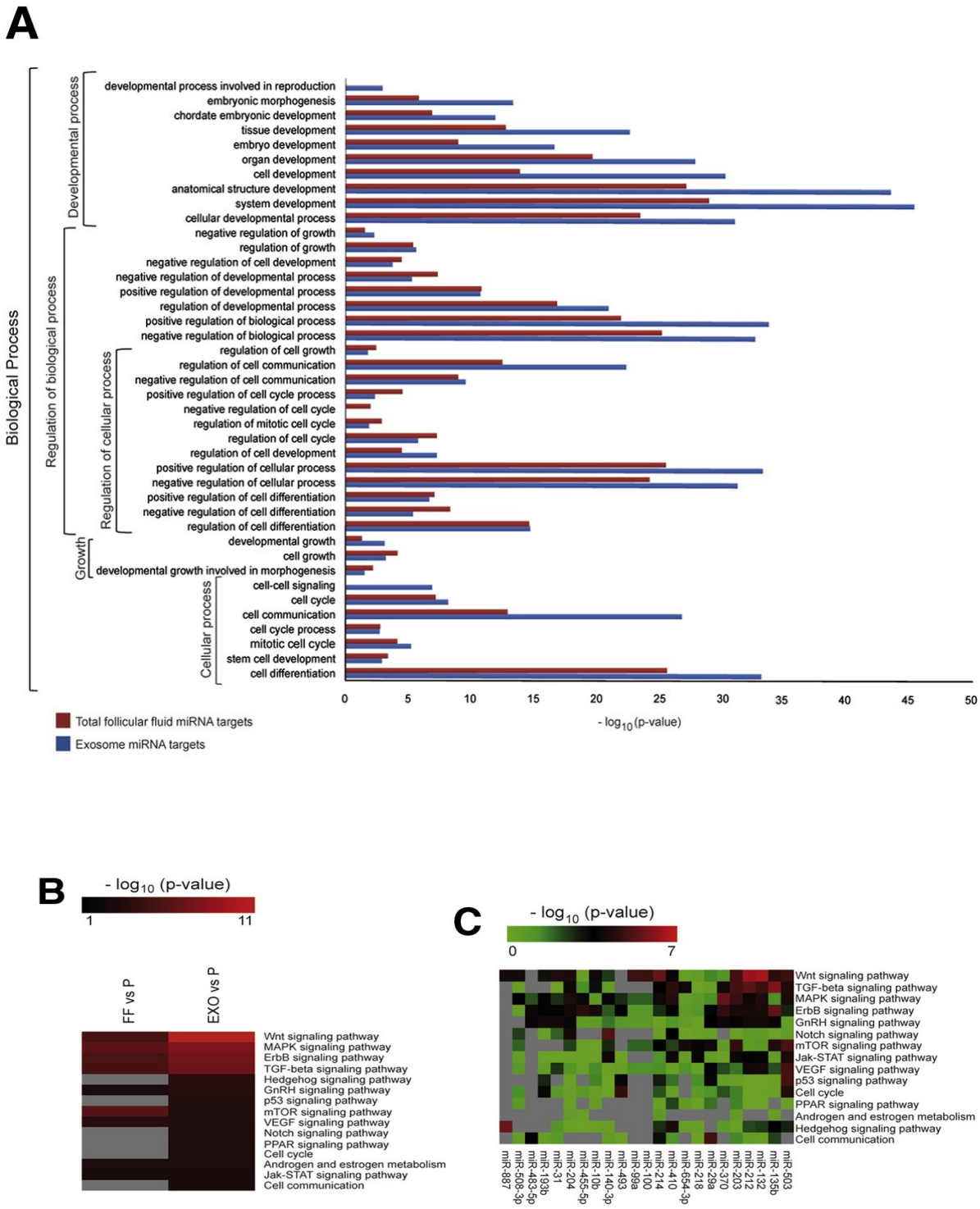


Figure 18: GO analysis based on highly expressed miRNA targets and heat-map representations of signaling pathways. (A) GO terms within the biological process category for highly expressed miRNA targets in total FF (red bars) and in exosomes (blue bars) are shown. The x-axis represents the -log10 (P value); the significance was determined by the adjusted Bonferroni correction. (B) Signaling pathway heat map regulated by total FF and exosomal miRNAs. Gray boxes indicate that the pathway is not significant. (C) miRNAs versus pathways. The probability values are reported as -log10 (P value) for both panels. Abbreviations: EXO ¼ exosomes; P ¼ plasma.

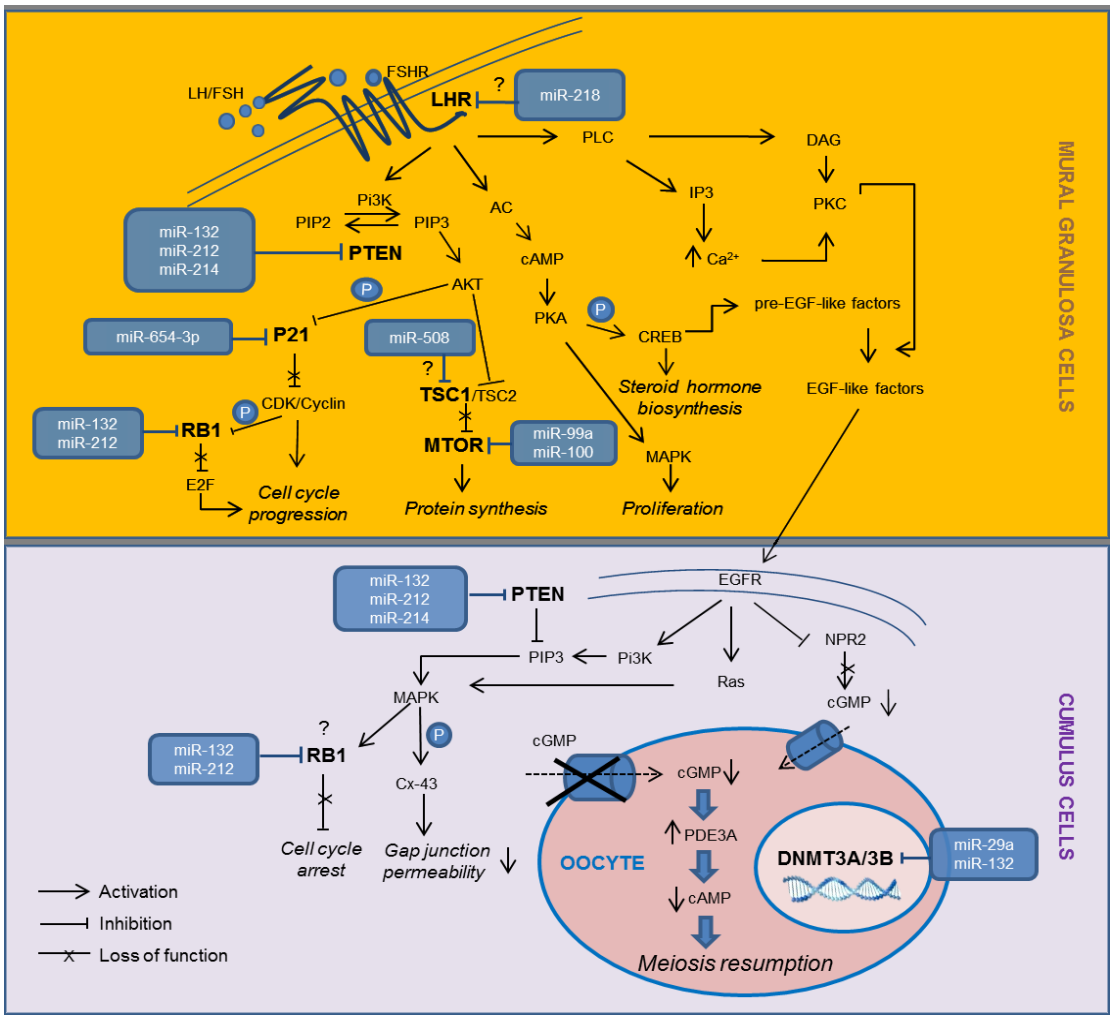


Figure 19: Ovarian follicle, signaling pathways, and FF miRNAs. Model summarizing crucial LH- and FSH-induced signaling pathways resulting in oocyte meiosis resumption. Hormone-receptor binding activates the AKT, PKA, and MAPK pathways and then induces the EGF-like factors' expression in granulosa cells. EGF-like factors act on EGFR to activate the RAS-MAPK pathway, contributing to phosphorylation of Cx-43 and the resulting decrease in gap junction permeability. These events would reduce NPR2 activity, induce PDE1 activation, and decrease cGMP levels in oocytes. Within the oocyte, activated PDE3A degrades cAMP and meiosis resumes. Identified miRNAs could act along these pathways and regulate the processes of follicular development and meiotic resumption. Abbreviations: FSHR = FSH receptor; LHR = LH receptor; AC = adenylate cyclase; cAMP =cyclic adenosine 30,50-monophosphate; PKA = cAMP-dependent protein kinase; CREB = cAMP responsive element binding protein; MAPK = mitogen-activated protein kinase; EGF = epidermal growth factor; EGFR = EGF receptor; PI3K = phosphatidylinositol 3 kinase; PIP2 = phosphatidylinositol 4, 5-bisphosphate; PIP3 = phosphatidylinositol-3,4,5-trisphosphate; AKT = v-akt murine thymoma viral oncogene homolog (also known as PKB); CDK = cyclin-dependent kinase; E2F = E2F transcription factor; PLC = phospholipase C; IP3 = inositol 1,4,5-trisphosphate; DAG = 1,2-diacylglycerol; PKC = protein kinase C; Cx-43 = connexin 43; NPR2 = natriuretic peptide receptor B/guanylate cyclase B; cGMP = cyclic guanosine 30,50-monophosphate; PDE3A =phosphodiesterase 3A, cGMP-inhibited.

Study II: The miRNome changes in Exosomes from human follicular fluid in relation to aging.

Expression Profile of miRNAs

We identified the presence of more than 100 miRNAs in FF exosomes. About 50 miRNAs in FF exosomes showed highly significant differences related to aging (Figure 20A), some of which (miR- 618, miR-155, miR-433, miR-340, miR-296, miR-138) are particularly abundant in exosomes from FF of women older than 38 (Figure 20B). In detail, miR- 618, miR-155, miR-433, miR-340, and miR-296 were overexpressed more than seven-fold in older women compared to control group of younger women. However, some miRNAs were found strongly downregulated in older women's FF exosomes compared to those from younger women: miR-199a, miR-324-3p and miR-519a (Figure 20B).

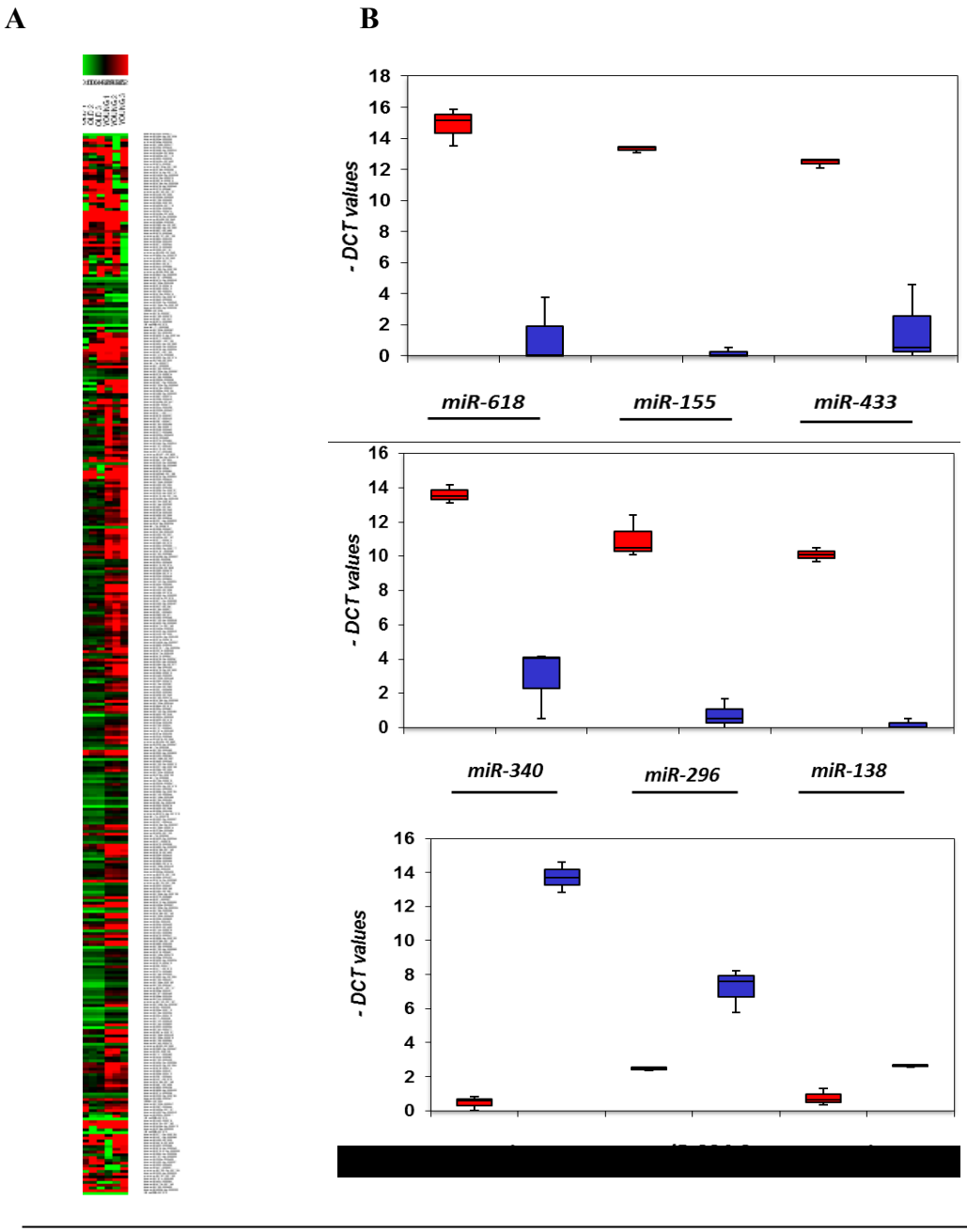


Figure 20: miRNA expression profile in exosomes from human follicular fluid. (A) Heat map diagram of miRNAs expression profiles in old and young women' s follicular fluid exosomes. Each row represents a microRNA and each column, a sample. The colour scale illustrates the relative level of microRNA expression: red, higher the reference channel; green, below than the reference. (B) Box and Whisker plot of DE miRNAs in old women (red boxes) respect with younger women (blue boxes). DCT values were reported as DCT *(-1).

Pathway Analysis

A comprehensive bioinformatics analysis using DIANA-miRPath allowed us to predict putative targets for differentially expressed miRNAs identified. Validated targets for up-regulated miRNAs are involved in 27 KEGG pathways (Table VII). According to DIANA-miRPath predictions, the most significant are: *ECM-receptor interaction*, *PI3K-Akt*, *p53*, *TGF-beta*, *HIF-1* and *mTOR* signaling pathway. On the contrary, validated target for down-regulated miRNAs are predicted to regulate 14 different biological pathways (Table VIII).

Table VII. KEGG pathways for targets of over-expressed miRNAs in reproductively old women's FF exosomes.

Pathways	p value	Target Genes	miRNAs
ECM-receptor interaction	<1e-16	15	9
Colorectal cancer	<1e-16	33	24
Small cell lung cancer	<1e-16	44	25
Transcriptional misregulation in cancer	<1e-16	58	27
Glioma	<1e-16	23	27
Melanoma	<1e-16	26	30
Chronic myeloid leukemia	<1e-16	34	31
PI3K-Akt signaling pathway	<1e-16	121	32
Pancreatic cancer	<1e-16	38	33
Prostate cancer	<1e-16	38	34
Hepatitis B	<1e-16	63	36
Bladder cancer	<1e-16	29	39
Pathways in cancer	<1e-16	154	49
HTLV-I infection	7.66E-15	52	23
Viral carcinogenesis	9.77E-15	59	23
Cell cycle	6.92E-14	37	24
p53 signaling pathway	3.78E-13	34	25
Non-small cell lung cancer	1.42E-12	24	23
Focal adhesion	6.11E-12	51	20
TGF-beta signaling pathway	2.68E-11	29	16
HIF-1 signaling pathway	5.85E-10	25	23
ErbB signaling pathway	7.80E-10	26	20
Prion diseases	7.46E-09	2	2
Endometrial cancer	7.46E-09	21	19
mTOR signaling pathway	1.36E-06	27	21
Renal cell carcinoma	3.65E-06	19	20
Acute myeloid leukemia	0.007285663	20	14

Table VIII. KEGG pathways for targets of under-expressed miRNAs in reproductively old women's FF exosomes

Pathways	p value	Target Genes	miRNAs
Melanogenesis	8.463723e-06	4	2
HTLV-I infection	8.273219e-05	5	2
Wnt signaling pathway	0.0009806106	3	1
Notch signaling pathway	0.001548697	2	1
Basal cell carcinoma	0.003006559	2	1
Pathways in cancer	0.003645282	4	2
Prostate cancer	0.01148955	2	2
HIF-1 signaling pathway	0.01808764	2	2
Adherens junction	0.01808764	2	2
Hepatitis B	0.01808764	2	2
Osteoclast differentiation	0.03049907	2	1
Dorso-ventral axis formation	0.0313866	1	1
Epstein-Barr virus infection	0.03161971	2	2
Jak-STAT signaling pathway	0.03664671	2	2

Study III: MiRNAs in human MII oocyte and their expression profile changes in relation to aging

miRNA identification

In order to study the *miRNome* of human oocytes and to identify possible age-related alterations we analyzed by TLDAs-based method the expression of 384 miRNAs from 12 human MII oocytes, six from three of women older than 38 years and six from three women younger than 35 years. To perform data normalization, we selected miR-342 and miR-372 as housekeeping miRNAs, by using median and average expression of the plate to find more stable ones. Combining geNorm and Normfinder algorithms suggested miR-342 and miR-372 as stable endogenous controls in RT-qPCR analysis. We found 69 miRNAs expressed in human oocyte, whose cycle threshold (Ct) values were displayed in Table IX. 57 miRNAs, that show a mean Ct value ≤ 36 and SD $\pm$ 3.5, are normally present and expressed at the same level in the 12 oocytes from two groups (Table IX). Although the Ct values were very low, we taken into consideration miR-328, miR-518f, miR-523 and miR-618 because the progress of the reaction and amplification curves suggested that these miRNAs are present and highly expressed in human oocytes. SAM analysis revealed some statistically significant changes in miRNA expression from oocytes of older women respect to those from younger. Particularly, 12 miRNAs displaying significant expression changes with aging were identified. Mean Ct and SD values (Table IX) and expression differences for the two groups of women were reported (Figure 21A). Fold Change values of the 12 Differentially Expressed (DE) miRNAs as ln of Relative Quantity (RQ) were shown in Figure 21B. Interestingly, miR-203 and miR-192 are highly expressed in aged MII oocytes, with RQ values about 100-fold (ln RQ=4.6) and 78-fold (ln RQ=4.3) respectively, compared to younger ones. Likewise, let-7e, miR-126, miR-136, miR-29a, miR-371-3p, miR-484 and miR-494 are

importantly up-regulated. Nevertheless, three miRNAs were strongly downregulated in oocytes from older women compared to those from younger: miR-19a (ln RQ= -3.5), let-7b (ln RQ= -4.6) and miR-519d (ln RQ= -5.4) (Figure 21B).

Table IX. Summary of miRNAs detected by TaqMan Low-Density Arrays (TLDAs) Quantitative PCR analysis of young and older oocyte.

Common miRNAs						DE miRNAs				
							Young		Older	
miRNA	Ct	SD	miRNA	Ct	SD	miRNA	Ct	SD	Ct	SD
miR-328	14.0	± 0.6	miR-197	31.3	± 1.4	miR-136	28.5	± 1	27.1	± 0.1
miR-518f	14.1	±0.8	miR-454	31.5	± 1.7	miR-484	30.5	± 1.1	29.6	± 0.4
miR-523	16.0	± 0.7	miR-193b	31.6	± 2.4	miR-371-3p	31.5	± 1.6	30.8	± 0.3
miR-618	16.5	± 1.0	miR-891a	31.8	± 2.6	miR-192	37.8	± 2.8	31.9	± 1.6
miR-363	19.1	± 0.7	miR-125a-5p	32.2	± 1.5	miR-494	34.0	± 2	31.6	± 0.6
miR-372	25.8	± 1.0	miR-24	32.5	± 1.1	let-7e	33.2	± 1.9	32.7	± 1.5
miR-19b	27.1	± 1.6	miR-28-3p	32.5	± 1.3	miR-19a	29.9	± 2.6	36.1	± 0.9
miR-518d	27.1	± 0.2	miR-508	32.5	± 2.5	miR-29a	34.1	± 2.7	33.4	± 0.9
miR-628-5p	27.1	± 1.2	miR-888	32.6	± 2.8	miR-126	35.3	± 4.6	34.5	± 0.1
miR-17	27.7	± 1.6	miR-574-3p	32.7	± 1.8	miR-519d	31.1	± 2	40	± 0
miR-155	27.8	± 2.3	miR-125b	32.8	± 1.1	let-7b	32.2	± 1.9	40	± 0
miR-20a	28.0	± 1.4	miR-223	32.8	± 1.6	miR-203	40	± 0	34.4	± 1.5
miR-320	28.2	± 0.6	miR-885-5p	32.8	± 2.1					
miR-218	28.4	± 2.1	miR-886-5p	32.8	± 2.3					
miR-191	28.7	± 1.1	miR-517a	33.0	± 2.3					
miR-342-3p	29.1	± 1.0	miR-374	33.1	± 2.6					
miR-132	30.1	± 1.8	miR-483-5p	33.2	± 0.4					
miR-16	30.1	± 1.4	miR-138	33.4	± 2.8					
miR-519a	30.1	± 2.6	miR-195	33.4	± 1.2					
miR-31	30.2	± 2.0	miR-202	33.5	± 3.3					
miR-425-5p	30.2	± 2.0	miR-489	33.8	± 3.4					
miR-30b	30.4	± 1.6	miR-518e	34.2	± 3.1					
miR-362	30.4	± 2.8	miR-20b	34.4	± 1.2					
miR-487a	30.4	± 1.9	miR-323-3p	34.5	± 3.5					
miR-146a	30.9	± 1.3	miR-26a	34.9	± 3.3					
miR-886-3p	30.9	± 1.4	miR-106b	35.0	± 2.8					
miR-184	31.0	± 0.6	miR-146b	35.6	± 2.7					
miR-30c	31.1	± 1.8	miR-212	35.9	± 2.9					
miR-106a	31.2	± 1.5								

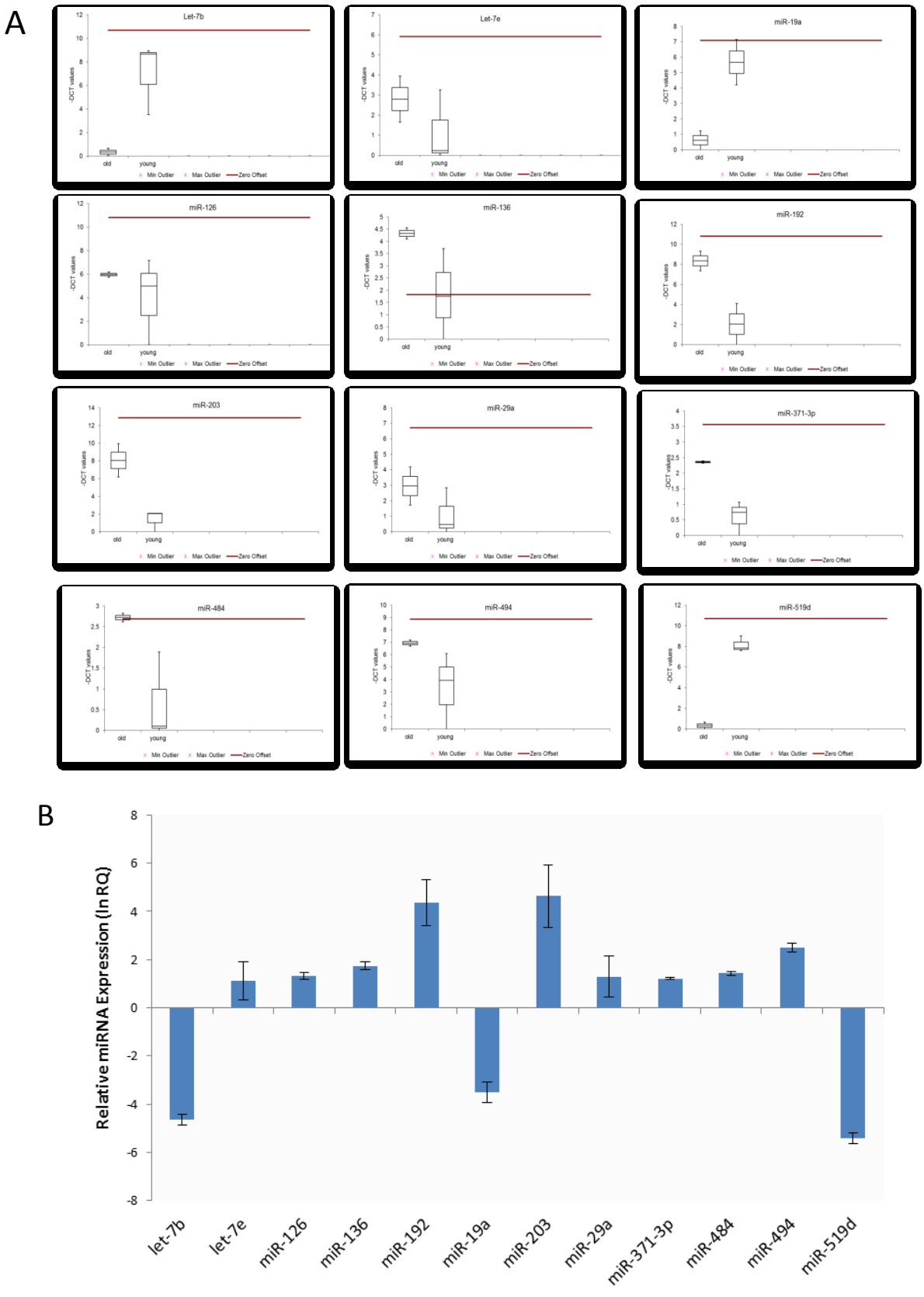


Figure 21: Significant expression changes of age-associated miRNAs. **A**. Expression differences of let-7b, let-7e, miR-126, miR-136, miR-192, miR-19a, miR-203, miR-29a, miR-371-3p, miR-484, miR-494, miR-519d in MII oocyte of old and young women. Values on the y-axis are reported as (–)DCT. **B.** Expression fold change of 12 DE miRNAs in old oocyte with respect young oocyte. Relative miRNA expression levels on *y- axis* are reported as the natural logarithm of RQ values.

mRNA targets, Gene Ontology and pathway analysis

1637 protein-coding genes were predicted as targets for 12 DE miRNAs by starBase v2.0, miRTarBase v4.5 and TarBase v7.0 prediction tools. The interpolation of potential and, then ,of the already experimentally-validated miRNA targets, with specific oocyte genes and human genes aging-related allowed us to define, reducing false positives, 153 relevant candidate targets for regulation by DE miRNAs. Of these 153, 126 are expressed in human oocyte and 27 were also described to regulate aging processes in human. The GO analysis, on candidate target genes of oocyte-aging, performed by using PANTHER public database showed that regulation of *gene expression*, *cell cycle process*, *chromosome organization*, *RNA metabolic process*, *response to stress*, *apoptotic process*, *chromatin modification* and *remodelling*, *response to oxygen-containing compound*, *cell aging, cellular senescence* and *signal transduction in response to DNA damage* were significantly enriched GOs in DE mirna targets in terms of biological process (Figure 22). Candidate target genes were found to be significantly enriched in 13 KEGG pathways (p value< 0.05) (Figure 22). Notably these include *mTOR, VEGF*, *PI3K-Akt*, *ErbB*, *Insulin*, *p53,HIF1* signalling pathways, *Progesterone-mediated oocyte maturation*, *Endometrial cancer, Cell cycle*, *Oocyte meiosis* and *Dorso-ventral axis formation* (Figure 22).

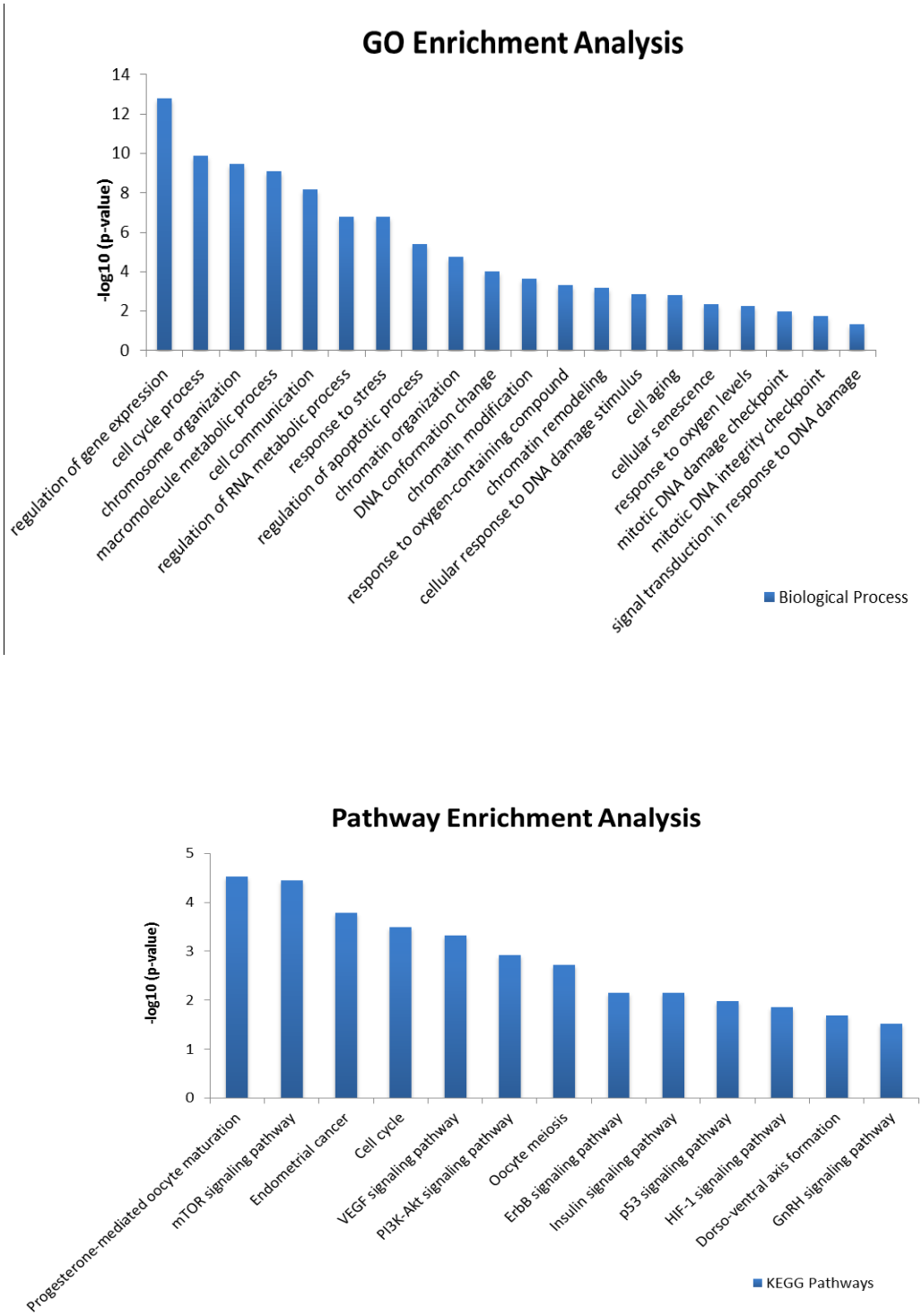


Figure 22: GO and pathway enrichment analysis for DE miRNAs. Significant GOs in terms of Biological Process and crucial biological Pathways for DE miRNAs identified in human MII oocyte are shown. The y-axis represents the -log10 (P value); the significance was determined by the adjusted Bonferroni correction.

Identification of miRNAs for validation in aging mouse model

Although the systems used to control gene expression have many similarity in mice and humans, some biological processes have not been fully conserved through evolutionary time. Searching for murine conserved homologs of DE miRNAs identified in human oocyte showed that let-7b-5p, let-7e-5p, miR-126-3p, miR-136-5p, miR-192-5p, miR-19a-3p, miR-203a-3p, miR-29a-3p, miR-484, miR-494-3p have 100% identity matching between human and mouse. On the contrary, interestingly two candidate miRNAs, miR-371a-3p and miR-519d-3p, identified in human, are miRNAs primate specific. To validate TLDA experiment results we decided to investigate the presence in murine MII oocytes of seven miRNAs: let-7b, mir-19a, miR-126, miR-29a, miR-494, mir-192-5p and miR-203.

Network analysis and mRNA selection

Biological networks of some target mRNAs for conserved DE miRNAs (yellow nodes) were shown in Figure 23. The network contains 101 nodes and 532 edges (gene relationship, pathway involvement and physical interactions). 51 common genes between human and mouse were indicated as blue nodes. The centrality features, Degree, Betweenness, Closeness and Eccentricity were chosen to identify topological important nodes. After calculating the value of the 4 features for each node, the average values of Degree, Betweenness, Closeness and Eccentricity were 6.4, 0.0042, 40.5, 0.25 respectively. As a result,20 conserved important nodes with Degree >6.4, Betweenness>0.0042, Closeness>40.5 and Eccentricity>0.25 were selected: ADSS, ALCAM, BRWD1,DNMT3A, DNMT3B, EDN1, EFNB2, FOXJ2, PTEN, PURA, RAP1A, RAP1B, RBFOX2, RHOB, RICTOR, RRM2, SOCS3, STK38, TFAM,

UBE2A. Among them, ALCAM, DNMT3A, DNMT3B, PTEN, RBFOX2,RRM2, SOCS3 and TFAM are validated targets for DE miRNAs age-associated. According to miRNA target prediction, miR-29a was identified as candidate miRNA targeting DNMT3A and DNMT3B. Fabbri et al. reported the regulatory role of miR-29s in epigenetic normalization of lung cancer [Fabbri *et al*., 2007]. Starbase also predicts DNMT3B as a potential target of miR-203. Moreover, studies from literature document the loss of expression miR-203 associated with expression of the hypermethylation defect in breast cancer cell lines [Sandhu *et al*., 2012]. Moreover, the interaction between mir-19a-3p, mir-29a-3p, mir-494-3p and PTEN has been also validated [Pezzolesi *et al*., 2008; Liu *et al*., 2010; Kong *et al*., 2011; Liu *et al*., 2012; Wang *et al*., 2013]. In addition, Yamamoto et al. reported miR-494 as mitochondrial biogenesis regulator by downregulating mtTFA [Yamamoto *et al*., 2012]

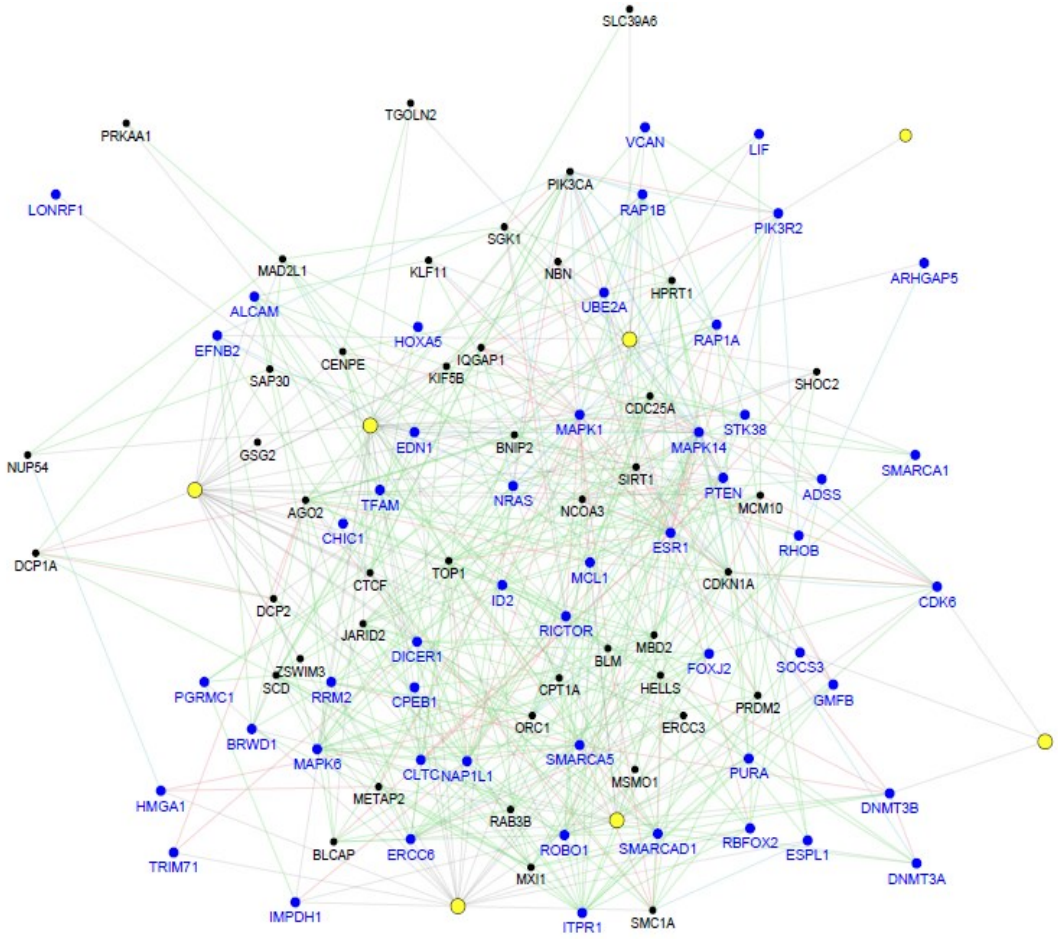


Figure 23. Network of evolutionarily conserved DE miRNAs and their target genes.

Validation with Single assays

We confirmed the up-regulation of 4 on 7 miRNAs. Particularly, mir-203 (ln RQ 1.8), mir-29a (ln RQ 1.5), miR-494 (ln RQ 1.5) and miR-192 (ln RQ 1.3) (Figure 24). Interestingly, miR-203 and miR-29a showed differences with statistical significance less than 0.05. To further confirm biological role of identified miRNAs, we investigate the expression of Dnmt3a target of miR-29a, Dnmt3b target of miR-29a and miR-203, Pten target of miR-29a and miR-494 and Tfam target of miR-494 in the same murine samples. We found that the relative expression amounts of targets genes decreased significantly in MII oocytes from reproductively old mice (Figure 24). Changes in both miRNAs and target genes analysed in the same samples, showed a significant negative correlation among miR-29a and its target (Dnmt3a, Dnmt3b, Pten) and between miR-203 and Dnmt3b, once older oocytes were compared with young control oocytes (Figure 25).

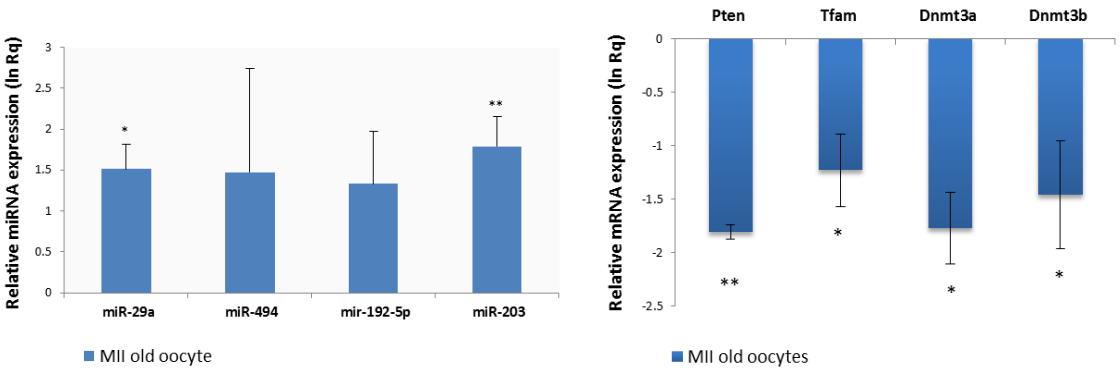
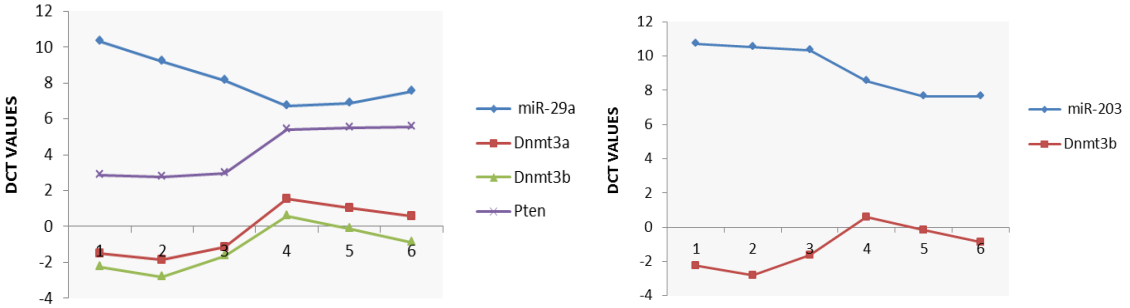


Figure 24: miRNA validation and Target detection by qRT–PCR in mouse MII oocyte. Relative expression, as the natural logarithm of RQ values, of miRNA and Targets, in older oocyte compared with young controls, were reported on y-axis. * p≤0.05; ** p≤0.01.



	Pearson	P value
miR-29a/Dnmt3a	-0.89528727	0.015959
miR-29a/Dnmt3b	-0.89089241	0.017484
miR-29a/Pten	-0.85080714	0.032062

	Pearson	P value
miR-203/Dnmt3b	-0.80522	0.05

Figure 25: miRNA-Target expression correlations. The graphs show the expression trend as DCT values for miR-29a and Dnmt3a,Dnmt3b, Pten and for miR-203 and Dnmt3b. Statistical comparisons by Pearson correlation reveals a significant miRNA-Target anticorrelation in older oocytes when compared with youngcontrols.

5. Discussion

After miRNA discovery in *Caenorhabditis elegans* in 1993 by Victor Ambros and colleagues [Lee *et al*., 1993], hundreds of scientific descriptions of the importance of these small noncoding RNAs, as key factors in the control of gene expression and in maintenance of genome stability started to push them to the limelight, over the last two decade. The function of miRNAs began, in this way, to be revealed and it was soon reported that miRNAs can influence many biological processes and are highly related to human diseases including cancer [Sassen *et al*., 2008]. Technological advances, based on microarray and subsequently next generation sequencing, allowed miRNA profiling establishing a foundation for research studies regarding use of miRNA as biomarker for the classification and diagnosis of disease. Although the analysis of the miRNA profile in human tissues showed the great potential of miRNAs as a disease markers, a less invasive method related to identification of miRNAs in body fluids has made this analysis more practical [Kim, 2015]. The existence of extracellular miRNAs in body fluids of humans was soon reported in several studies [Chen *et al*., 2008; Chim *et al*., 2008; Mitchell *et al*., 2008]. While the exact mechanism which make stable the circulating miRNAs in body fluids was not fully elucidated, the observation that many miRNAs were copurified with exosomes [Valadi *et al*., 2007] elicited the hypothesis that extracellular miRNAs in plasma are protected by the small membranous vesicles. Moreover, studies reporting the exchange of miRNAs (and also mRNAs) between cells through exosome-mediated transfer, led to a revolutionary paradigm: the existence of inter-cellular and inter-organ communication by means of vesicle-encapsulated miRNAs [Turchinovich *et al*., 2015]. Although miRNAs have been extensively investigated in serum and plasma [Mitchell *et al*., 2008; Arroyo *et al*., 2011], research on miRNAs in follicular fluid is in its infancy. Since the presence of miRNAs in FF has

also been demonstrated, these small RNAs have been predicted to be involved in a proper cell communication between the oocyte and its surrounding somatic cells [da Silveira *et al*., 2012]. Such intercellular communications allow the oocyte to determine its own fate by influencing the intrafollicular microenvironment, which in turn provides the necessary cellular functions for oocyte developmental competence, which is defined as the ability of the oocyte to complete meiosis and undergo fertilization, embryogenesis, and term development [Dumesic *et al*., 2015]. The follicular fluid provides the microenvironment in which oocytes develop, mature, and ovulate. Because the follicular fluid affects oocyte development, its composition has been investigated as a possible predictor of oocyte and embryo quality [Feng *et al*., 2015]. The first topic of the present thesis was to characterize miRNAs in human follicular fluid (FF). We used TaqMan low-density array to investigate the expression profile of 384 microRNAs from FF compared with plasma collected from the same women. 37 miRNAs upregulated in human FF compared with plasma were identified and 32 of them are carried by exosomes (Figure 17). We studied miRNAs upregulated in FF compared with plasma; this allowed us to hypothesize that they were not released by blood cells but specifically by ovarian follicle cells. Exosomes are able to mediate cellular communication carrying miRNAs and also mRNAs and proteins [Valadi *et al*., 2007]. The presence of exosome miRNAs in human follicular fluid means that an unexplored communication tool could exist inside ovarian cells. By applying many different computational tools, we performed multiple searches in public databases and literature data to confirm that identified miRNAs could carry out their biological role inside ovarian follicles. GO and pathway analysis demonstrated that the targets of upregulated miRNAs could perform their role inside ovarian follicles during follicular maturation (Figure 18) [Richards *et al*., 2002; Russell and Robker, 2007; Zhang *et al*., 2009; Conti *et al*., 2012]. Moreover

the outcome of the complex interaction among the different molecules involved in these pathways is the elimination of meiosis-inhibiting factors and/or the accumulation or activation of oocyte maturation signals [Zhang *et al*., 2009]. Interestingly, we found that miR-29a, miR-99a, miR-100, miR-132, miR-212, miR-214, miR-218, miR-508-3p, and miR-654-3p could trigger meiosis resumption by negatively regulating genes encoding for follicle maturation inhibiting factors [Sun *et al*., 2009; Reddy *et al*., 2010] (Figure 19). We propose that identified miRNAs are synthesized by ovarian follicle cells and that they could be transferred to recipient cells through FF exosomes. It is known that exocytosis and endocytosis mechanisms have been described in granulose cells and oocytes [Grant and Hirsh, 1999; Krishnamurthy *et al*., 2003; Shimada *et al*., 2007; Liu M, 2011], but further studies are necessary to identify specific exosome surface markers able to discriminate the vesicles released by somatic follicular cells or by the oocyte and to ascertain whether their cargo acts in an autocrine or paracrine manner. Although circulating miRNAs have been examined in several diseases, the role of miRNAs, in particular circulating miRNAs, in human aging has only begun to be explored [Noren Hooten *et al*., 2013]. In order to reveal the contribution of sncRNAs, especially miRNAs, to female reproduction aging, we examined their expression changes during aging in human follicular fluid, using TLDA technology. MiRNA expression profiling of the follicular fluid of younger and older women revealed a set of strongly differentially expressed miRNAs (Figure 20). Moreover, linking DE miRNAs to their target genes allowed us to identify the pathways more affected by the aging process (Table VII and VIII). Bioinfomatic analysis revealed, in fact, that identified miRNA targets are enriched in genes involved in signaling pathways which are essential for maintenance of the dormant pool of primordial follicles, follicle maturation, oocyte growth, fertilization and regulation of maternal reproduction [Adriaenssens *et al*., 2009;

Levine *et al*., 2011; Tsui *et al*., 2014; Wang *et al*., 2014; Cheng *et al*., 2015]. Recently, Diez-fraile et al. demonstrated that age-associated differential miRNA levels in human follicular fluids may reveal pathways potentially determining fertility and success of in vitro fertilization [Diez-Fraile *et al*., 2014]. We speculate that specific set of miRNAs, synthesized by somatic and germinal cells of ovarian follicles, could be released in FF contributing to the aging process in an autocrine, paracrine and even endocrine manner. To the best of our knowledge, very limited data are available about gene expression post-transcriptional regulators in MII human oocytes, such as miRNAs. In recent years, maternal gene expression was described as an important biological process naturally occurring in oocyte maturation and early cleavage stages [Cui *et al*., 2007]. A progressive transcriptional activity decrease during oocyte nuclear maturation was observed in MII oocytes compared with GV or MI oocytes [Assou *et al*., 2006]. In the absence of transcription, the regulation of gene expression in oocytes is controlled almost exclusively at the level of mRNA translation and stabilization and posttranslational modifications of proteins [Susor *et al*., 2015]. The last subject of this thesis was, hence, related to characterization of human MII oocytes transcriptome and identification of possible age-related changes. By TLDAs, 69 microRNAs were identified in mature MII-stage oocytes. These miRNAs were previously described in cumulus, granulosa cells and follicular fluid [Assou *et al*., 2013; Velthut-Meikas *et al* 2013; Santonocito e*t al.*, 2014; McGinnis *et al*., 2015]. Interestingly, few of them have been previously found also in blastocysts [Rosenbluth *et al*., 2013]. We suppose that oocyte contains a number of miRNAs that are manufactured during oocyte maturation, and then utilized during early stages of development, before activation of the embryonic genome. Moreover, data from literature shown that the identified miRNAs are within three eminent clusters, the chromosome 19 miRNA cluster (C19MC), the chromosome

14 microRNA cluster C14MC and miR-371-3 cluster [Morales-Prieto *et al*., 2013] which members have been detected in the placenta, but also in other compartments and are highly expressed in human undifferentiated cells possibly marking a "naïve" state of ESCs [Morales-Prieto *et al*., 2013]. In this study 12 miRNAs were reported as DE with aging (Figure 21). A comprehensive analysis of DE miRNAs role in oocyte aging revealed that several target genes are associated with own features of the oocytes produced in advanced reproductive age [Eichenlaub-Ritter U, 2012]. Moreover, we demonstrated that 4 of them, whose sequences and functions are evolutionarily conserved (Figure 23) in human and mouse, showed the same differential expression in murine oocytes (Figure 24). Although miRNAs had been thought to preferentially affect the level of proteins rather than mRNAs, it has been shown that miRNAs also reduce the level of mRNAs [Bagga *et al*., 2005; Guo *et al*., 2010]. A significant under-expression of PTEN, DNMT3A, DNMT3B and TFAM was found in reproductively old mice. Described as essential for the appropriate embryo development [Di Cristofano *et al*., 1998], the maintenance of mitochondrial DNA [Kang *et al*., 2007] and responsible for mechanisms underlying compromised oocyte quality, including epigenetic changes and mitochondrial dysfunction, caused by advanced maternal age [Yue *et al*., 2012], thus the changed expression of deregulated miRNA target might be the reason of the lower reproductive potential with aging. Although the false-positive predictions always exist, we suggest that these targets have high possibility of being regulated by miRNAs which are involved in reproductive aging.

6. CONCLUSIONS AND FUTURE PERSPECTIVES

This study identified exosomal miRNAs highly represented in human FF and involved in the regulation of ovarian follicular pathways. We believe that, as it has been demonstrated for intracellular ovarian miRNAs, their altered expression could be associated with reproductive disorders. We propose that these miRNAs could represent non-invasive molecular markers of oocyte quality in ART. To our knowledge this is also the first report about the determination of age associated miRNAs in human oocyte in a high-throughput manner. However, enriched GO and pathway analyses shown in this study, suggest that, the identified miRNAs function in known aging processes. Further, identified miRNA expression confirmation in mouse model revealed additional evidence for miRNA role in modulating epigenetic mechanism and oocyte quality. Although a better understanding of epigenetic changes is desired, we suggest that age-associated variation of miRNA expression levels might cause a reciprocal trend in the expression change of specific target during reproductive aging. As regards the events determining specific miRNA expression in human oocyte, in early embryo and in reproductive disorders our knowledge is still not complete and more efforts are needed to shed light on the still numerous open questions. Moreover, functional studies, possibly by using Follicular In Vitro Maturation systems, will be further necessary for the development of miRNAs in the follicular fluid as novel biomarkers for embryo quality and fertility related phenotypes.

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