Cell-free nucleic acids as non-invasive biomarkers of gynecological cancers, ovarian, endometrial and obstetric disorders and fetal aneuploidy

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Submitted on January 10, 2014; resubmitted on May 12, 2014; accepted on May 23, 2014

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BACKGROUND: Proper folliculogenesis is fundamental to obtain a competent oocyte that, once fertilized, can support the acquisition of embryo developmental competence and pregnancy. MicroRNAs (miRNAs) are crucial regulators of folliculogenesis, which are expressed in the cumulus–oocyte complex and in granulosa cells and some can also be found in the bloodstream. These circulating miRNAs are intensively studied and used as diagnostic/prognostic markers of many diseases, including gynecological and pregnancy disorders. In addition, serum contains small amounts of cell-free DNA (cfDNA), presumably resulting from the release of genetic material from apoptotic/necrotic cells. The quantification of nucleic acids in serum samples could be used as a diagnostic tool for female infertility.

METHODS: An overview of the published literature on miRNAs, and particularly on the use of circulating miRNAs and cfDNA as non-invasive biomarkers of gynecological diseases, was performed (up to January 2014).

RESULTS: In the past decade, cell-free nucleic acids have been studied for potential use as biomarkers in many diseases, particularly in gynecological cancers, ovarian and endometrial disorders, as well as in pregnancy-related pathologies and fetal aneuploidy. The data strongly suggest that the concentration of cell-free nucleic acids in serum from IVF patients or in embryo culture medium could be related to the ovarian hormone status and embryo quality, respectively, and be used as a non-invasive biomarker of IVF outcome.

CONCLUSIONS: The profiling of circulating nucleic acids, such as miRNAs and cfDNA, opens new perspectives for the diagnosis/prognosis of ovarian disorders and for the prediction of IVF outcomes, namely (embryo quality and pregnancy).

Key words: MicroRNAs / cell-free DNA / infertility / non-invasive biomarkers
Introduction

MicroRNAs (miRNAs) are small (19–25 nucleotides), single-stranded, non-coding RNA molecules that bind specifically to, and post-transcriptionally regulate, several messenger RNAs (mRNAs) (Thomas et al., 2010). miRNAs play important physiological roles and miRNA dys-regulation can lead to pathologies. In fertility, miRNAs are associated with the functional regulation of gonadal somatic cells [Leydig and Sertoli cells in testis, and granulosa and cumulus cells (CCs) in the ovary] involved in steroid synthesis. For example, in male mice, deletion of Dicer (a protein essential for miRNA maturation) in Sertoli cells leads to infertility due to the complete absence of spermatozoa and progressive testicular degeneration (Hossain et al., 2012). In female mice, Dicer inactivation leads to infertility due to multiple defects in ovarian functions, including abnormal cycles and an abnormal response to gonadotrophin (Follicle-stimulating hormone), leading to ovulation problem (Nagaraja et al., 2008).

During follicular development, oocytes are in close contact with the surrounding CCs to form the cumulus–oocyte complex (COC). The crosstalk between oocytes and CCs occurs through gap junctions (Albertini et al., 2001). This paracrine signaling is crucial for the acquisition of developmental competence in oocytes and CCs (Gilchrist et al., 2008). These reciprocal regulations are carefully modulated by some key genes that are themselves regulated by miRNAs (Assou et al., 2013a). Some miRNAs are found in body fluids and as they are contained in exosomes, they are highly stable because they are protected from RNases. The potential use of these circulating miRNAs as novel, non-invasive diagnostic/prognostic biomarkers is the focus of many investigations (Mitchell et al., 2008) and they are already used as biomarkers for the diagnosis and prognosis of several gynecological and pregnancy disorders (Carletti and Christenson, 2009).

Similarly, cell-free DNA (cfDNA) molecules, which are released mostly by apoptotic or necrotic cells, are also found in body fluids and can be used as biomarkers of pathological conditions (Schwarzenbach et al., 2011). Indeed, cfDNA has been detected in human semen (Chou et al., 2004). This cell-free seminal DNA contains DNA epigenetic information that is essential for proper spermatogenesis (Wu et al., 2013a). Circulating cfDNA in the bloodstream is also being used to detect gynecological abnormalities, whereas fetal cfDNA in maternal blood constitutes a non-invasive biomarker for fetal aneuploidy (Lo et al., 1999; Bischoff et al., 2002, 2005; Bauer et al., 2006; Lo and Chiu, 2008; Wright and Burton, 2009; Lambert-Messerlian et al., 2014).

In this review, we describe first the biogenesis of circulating cell-free miRNAs and DNA. Then, we present an analysis of the available data on circulating nucleic acids in gynecological diseases and in pregnancy and discuss their potential role in the ‘oocyte–nich’ crosstalk and in the hormonal regulation of folliculogenesis. Finally, we discuss the evidence suggesting that cell-free nucleic acids could be used as non-invasive biomarkers of IVF outcomes.

Methods

A summary of the general knowledge on cellular and circulating miRNAs was compiled based on seminal articles in this research field. A systematic review of the current literature in the English language on cellular and circulating nucleic acids (miRNAs and DNA) in relation to mammalian, including human, reproduction was performed. All the selected articles were searched in journal databases, such as PubMed (http://www.ncbi.nlm.nih.gov/sites/entrez), using key words, including ‘miRNA’, ‘circulating miRNA’, ‘cell-free DNA’, ‘oocyte’, ‘cumulus cells’, ‘embryo’, ‘pregnancy’ and ‘biomarkers’. The search retrieved c.10,000 articles; of which, 284 were included in this review.

Results

MicroRNAs

General considerations on cellular miRNAs

miRNAs belong to the ‘small RNA’ family and are evolutionarily conserved from invertebrates to vertebrates (Lagos-Quintana et al., 2001). miRNAs were first identified in Caenorhabditis elegans at the beginning of the 1990s (Lee et al., 1993). They are non-coding single-stranded RNA molecules of 19–25 nucleotides in length that arise from inter- or intragenic genomic regions. In mammals, miRNAs are usually complementary to a small region in the 3′ untranslated region (UTR) of mRNAs.

miRNAs are derived from primary transcripts (called pri-miRNAs) that are folded into hairpins and are synthesized via the classical transcription process using polymerase II (Lee et al., 2004; Rodriguez et al., 2004). Pri-miRNAs are then cleaved by a protein complex formed by Drosha (an enzyme of the RNase III complex) and its partner, the nuclear protein DiGeorge critical region 8 (DGCR8). This cleavage leads to ~70 nucleotide-long pre-miRNAs that are exported by Exportin 5 to the cytoplasm (Yu et al., 2003). Pre-miRNAs are then cleaved by Dicer, another protein of the RNase III complex, to eliminate the terminal loop and release double-stranded miRNAs of ~22 nucleotides (Hutvagner et al., 2001). Only one strand of each mature miRNA is then incorporated in the miRNA-induced silencing complex (miRISC), which includes either AGO1 or AGO2 proteins from the Argonaute family. In the miRISC complex, single-stranded miRNAs can interact with and silence their target mRNAs in two different ways. If the miRISC complex contains AGO2, the targeted mRNA is degraded. On the other hand, the presence of AGO1 in the RISC complex promotes translation repression (Hutvagner and Simard, 2008). Thus, miRNAs can regulate protein levels by promoting mRNA degradation and also by attenuating protein translation.

miRNAs are predicted to be involved in the silencing of more than half of mammalian genes (Friedman et al., 2009). Based on sequence homology, one single miRNA could regulate at least 200 mRNAs and consequently the expression of the corresponding proteins (Esquela-Kerscher and Slack, 2006). Some miRNAs are tissue-specific, while others are expressed in more than one tissue (Reedy et al., 2009). It is now acknowledged that miRNAs play a crucial role in the physiological regulation of many cellular processes. Moreover, miRNA expression must be very tightly and dynamically regulated to allow the specific modulation of different mRNAs, for instance during embryo development, cell transitions or cell environmental changes. In 2002, it was reported, for the first time, that deletion and down-regulation of specific miRNAs could be implicated in cancer (Calin et al., 2002). Further studies have demonstrated that miRNA mutations, biogenesis defects or deregulation can affect miRNA-mediated gene silencing, and this may result in serious diseases, such as cancers (Calin and Croce, 2006; Esquela-Kerscher and Slack, 2006), cardiovascular diseases (Latronico et al., 2007; van Rooij and Olson, 2007), neurological disorders (Esau and Monia, 2007; Fiore et al., 2008; Kathiresan et al., 2008; Li et al., 2008; Liu et al., 2008; Madan et al., 2008; Nunez et al., 2008; Pal et al., 2008; Rich et al., 2008; Safra et al., 2008; Skowronski et al., 2008; Watkinson et al., 2008; Wright et al., 2008; Yu et al., 2008).
Schratt, 2007; Hansen et al., 2007; Perkins et al., 2007), ischemia (Silvestri et al., 2009), heart failure (Adachi et al., 2010; Ai et al., 2010; Tijssen et al., 2010; Wang et al., 2010b), hepatitis (Wang et al., 2012c), Crohn’s disease, sepsis (Wang et al., 2010c), tuberculosis (Singh et al., 2013), diabetes (Farr et al., 2013; Roberts and Porter, 2013) and obesity (Weiler et al., 2006).

Circulating miRNAs in gynecological disorders and pregnancy

Most miRNAs are localized inside the cell; however, a significant number of miRNAs have been detected also in extracellular body fluids, such as serum, plasma, urine, spinal fluid, saliva and follicular fluid (Wang et al., 2010b; Weber et al., 2010; Zubakov et al., 2010; Zen and Zhang, 2012; Sang et al., 2013). These circulating miRNAs could be used as biomarkers of specific conditions, because they are relatively abundant (especially in blood) and quite stable due to their confinement within vesicles where they are protected from RNases. Both serum and plasma are suitable for the analysis of miRNAs (Mitchell et al., 2008). miRNAs are selectively and actively secreted from cells and packaged into appropriate carriers. They are then transported to targeted or receptor-specific recipient cells where they recognize and repress mRNA targets within recipient cells (Boon and Vickers, 2013). miRNA intercellular transport is performed by different subclasses of miRNAs carriers, such as membrane-derived vesicles (exosomes and microparticles), lipoproteins and ribonucleoprotein complexes (Valadi et al., 2007; Zhang et al., 2010; Vickers et al., 2011). Exosomes (small vesicles of 40–100 nm in diameter) and microparticles (100–4000 nm in diameter) have different biogenesis and secretory mechanisms (Thery, 2011).

During apoptosis, cells can release even larger microparticles or apoptotic bodies to transport specific miRNAs (Zernecke et al., 2009). Extracellular miRNAs can be transported also by high-density lipoproteins (HDLs) and low-density lipoproteins (LDLs), abundant in plasma (Vickers et al., 2011). Biophysical studies have also shown that miRNAs can associate also with protein complexes, including AGO2, the main functional component of the cytoplasmic miRNA ribonucleoprotein complex (Arroyo et al., 2011; Turchinovich et al., 2011). Many observations suggest that miRNA export mechanisms are selective and regulated (Wang et al., 2010d). For example, the miRNA profiles of extracellular vesicles are not representative of their parent cell type, but of specific sets of miRNAs. Indeed, the exosomal-, HDL- and LDL-miRNA signatures are distinct, although some miRNAs can be found in all carrier subclasses (Vickers et al., 2011). Furthermore, specific miRNA profiles are consistent among individuals and each biological fluid has its own physiological miRNA signature (Valadi et al., 2007; Vickers et al., 2011). This suggests that a specific miRNA profile in serum or plasma could be associated with some pathological conditions. Since Lawrie et al. (2008) showed that the serum level of specific miRNAs was higher in patients with lymphoma than in healthy controls, cell-free miRNAs have been assessed in many different pathological conditions, including gynecological and pregnancy disorders, in order to identify tissue-specific miRNAs that may constitute non-invasive diagnostic tools. Moreover, as the amount of specific circulating miRNAs has been associated with tumor development and malignant progression (Schwarzenbach et al., 2011), circulating cell-free nucleic acids are now used not only as diagnostic biomarkers, but also as prognostic tools.

Table I lists the circulating miRNAs used as biomarkers in gynecological disorders. For example, miR-205 expression is significantly up-regulated and let-7f significantly lower in plasma samples from patients with epithelial ovarian cancer (EOC), especially in patients with Stage I EOC, compared with healthy controls (Zheng et al., 2013). Likewise, miR-483-5p plasma level is higher in patients with Stage III and IV EOC than in those with Stage I and II EOC, consistent with its expression pattern in tumor tissues (Zheng et al., 2013). miR-200a, miR-200b, miR-200c and miR-103 are significantly overexpressed in serum samples from patients with serous EOC compared with controls (Kan et al., 2012). Moreover, circulating miR-92 is overexpressed in serum samples from patients with EOC compared with healthy controls (Guo et al., 2013).

In breast tumors, many miRNAs are differentially expressed in patients versus healthy women (Table I; Wang et al., 2010a; Wu et al., 2011; van Schooneveld et al., 2012; Cuk et al., 2013a,b). However, only seven are concomitantly overexpressed in the tumor and in serum. Among them, miR-1, miR-92a, miR-133a and miR-133b have been validated as the most important diagnostic markers for breast cancer (Chan et al., 2013). Another study showed that the serum level of miR-182 is significantly higher in patients with breast cancer compared with controls (Wang et al., 2013a). In addition, miR-182 serum levels were considerably lower in patients with estrogen receptor- or progesterone receptor-positive breast tumors than in those with estrogen receptor- or progesterone receptor-negative cancers (Wang et al., 2013a). All these data suggest that circulating miRNAs might also be used as biomarkers to diagnose and identify breast cancer type.

Recently, abnormal miRNA expression in the bloodstream has been associated also with several metabolic disorders, including obesity, diabetes and gynecological pathologies, such as polycystic ovary syndrome (PCOS), premature ovarian failure (POF) and endometriosis (Fernandez-Valverde et al., 2011; Hulsmans et al., 2011; Gilabert-Estelles et al., 2012; Rottiers and Naar, 2012; Chen et al., 2013). Indeed, miRNAs play a crucial role in metabolism regulation (Rottiers and Naar, 2012). For example, miR-33a and miR-33b, which are located within the sterol regulatory element-binding protein 1 and 2 (SREBP 1 and 2) genes, regulate cholesterol and lipid metabolism in concert with their host genes (Gerin et al., 2010; Horie et al., 2010; Marquart et al., 2010; Najafi-Shoushtari et al., 2010; Davalos et al., 2011). Moreover, miR-103 and miR-107 are involved in controlling insulin and glucose homeostasis, and miR-34a is a key regulator of hepatic lipid homeostasis (Wilfred et al., 2007; Takanabe et al., 2008; Xie et al., 2009; Trajkovski et al., 2011). Therefore, circulating miRNAs may act as endocrine signaling molecules and could be used as potential biomarkers of metabolic diseases. For instance, it has been shown recently that miR-138, miR-15b and miR-376a might constitute reliable predictive biomarkers in obesity (Pescador et al., 2013). Specifically, miR-138 and miR-376a could be used as a powerful predictive tool to differentiate obese patients from diabetic patients, obese diabetic patients and healthy controls. Moreover, miR-138 and miR-503 can differentiate between diabetic and obese diabetic patients (Pescador et al., 2013).

PCOS is one of the most common endocrine disorders among women of reproductive age and is considered one of the leading causes of female infertility (Azziz et al., 2004). The main features of PCOS are dysovulation (resulting in irregular menstrual cycles or amenorrhea and thus ovulation-related infertility), excessive levels of androgenic hormones (resulting in hirsutism) and insulin resistance, often associated with obesity, Type 2 diabetes and high cholesterol levels.
A recent study showed that miR-21, miR-27b, miR-103 and miR-155 levels are decreased in the bloodstream of obese men and women, whereas they are increased in women with PCOS compared with healthy controls (Murri et al., 2013). These data suggest that the concomitant evaluation of different miRNAs might be used as biomarker to differentiate real obesity from obesity associated with PCOS.

Some miRNAs are differentially expressed in the plasma of women with POF compared with normal responder women (Table I). Some of these miRNAs may regulate granulosa cell proliferation and apoptosis by affecting different signaling pathways. For example, miR-23a may regulate apoptosis by decreasing XIAP expression in human granulosa cells (Yang et al., 2012).

Moreover, miR-30b and miR-30d are significantly up-regulated in receptive endometrium, whereas miR-494 and miR-923 are down-regulated (Altmae et al., 2013). In some endometrial disorders, endometrium receptivity can be altered and several studies have focused on the identification of miRNAs that may be deregulated in these diseases. Twenty-seven miRNAs were shown to be differentially expressed in women suffering from endometriosis in comparison with healthy controls (Jia et al., 2013). miR-17-5p, miR-20a and miR-22 in particular were dramatically decreased in the plasma from patients with endometriosis compared with controls (Jia et al., 2013). Moreover, the serum levels of miR-199a and miR-122 were higher in patients with endometriosis compared with controls, whereas miR-145, miR-141, miR-542-3p and miR-9 were lower (Wang et al., 2013b). Finally, the relative expression of miR-199a and miR-122 has been used to discriminate between severe and mild endometriosis and thus constitutes a reliable biomarker to follow endometriosis progression (Wang et al., 2013b).

The discovery of fetal miRNAs in the maternal bloodstream has paved the way to their possible use for non-invasive prenatal diagnosis. Specifically, placental miR-141, miR-149, miR-299-5p and miR-135b can be easily detected in maternal plasma during pregnancy and after delivery their plasma concentration significantly decreases (Chim et al., 2005). In particular, miR-141 plasma level increases as pregnancy progresses into the third trimester. This promising new fetal biomarker appears to be more reliable for pregnancy monitoring than the currently used chorionic somatomammotropin hormone 1 mRNA level, because it is more stable in maternal plasma (Chim et al., 2008).

Pre-eclampsia is one of the leading causes of maternal and fetal/neonatal mortality (Sibai et al., 2005). One study showed that miR-210 level is up-regulated, whereas miR-152 is down-regulated in serum samples from patients with pre-eclampsia (Gune et al., 2011). Thus, miR-210 quantification in maternal serum could be used to improve pre-eclampsia diagnosis using non-invasive methods. Another study showed that miR-24, miR-26a, miR-103, miR-130b, miR-181a, miR-342-3p and miR-574-5p are significantly increased in plasma from pregnant women with severe pre-eclampsia (Wu et al., 2012). The study of their target genes suggests that these miRNAs could be involved in many different functions, such as the regulation of metabolic processes, control of cell cycle and signaling pathways, including the mitogen-activated protein kinase and other signaling pathways.
kinase and the transforming growth factor-β (TGF-β signaling pathways), or pathways involved in cancer metastasis. In addition, they could also play important roles in pre-ecplasma development and its severity and might constitute potential biomarkers for this disease (Wu et al., 2012). Moreover, some miRNAs, particularly miR-323-3p, could improve the accuracy of ectopic pregnancy detection in association with plasma hCG and progesterone levels (Zhao et al., 2012).

In conclusion, due to their accessibility and stability (miRNAs circulate confined within exosomes), different circulating miRNAs could be used, alone or in combination, as non-invasive biomarkers of gynecological cancers and gynecological disorders.

**Role of miRNAs in the oocyte–niche relationship and in the hormonal regulation of folliculogenesis**

During the early stages of follicular development, a specific crosstalk between the oocyte and follicular cells is established. At the pre-antral secondary stage, follicular cells differentiate into two types: granulosa cells that cover the follicle and CCs that are directly in contact with the oocyte. Then, the COC is formed and the oocyte–CC dialog is organized via tight junctions. Oocyte secreting factors participate in CC differentiation and proliferation. Reciprocally, CCs provide nutrients for the oocyte development and maturation (Mori et al., 2000; Eppig, 2005; Sugiera et al., 2005; Gilchrist et al., 2008). These mutual regulations are mainly controlled by the growth differentiation factor-9 (GDF9) and the bone morphogenetic protein 15 (BMP15) genes that belong to the TGF-β family and are expressed in the oocyte. GDF9 induces the expression of many genes in CCs, including genes implicated in oocyte maturation and quality, such as Hyaluronic Acid Synthase 2 (HAS2), and genes involved in fertilization and embryo development, such as Cyclo-Oxygenase 2 (COX-2) and Gremlin 1 reviewed in Gilchrist et al. (2008) and Assou et al. (2010). Therefore, oocyte quality and its ability to contribute to the formation of a ‘competent’ embryo with a strong potential to implant in the endometrium and lead to a successful pregnancy can be predicted by using indirect CC markers. miRNAs have been identified in human oocytes (Xu et al., 2011b; Assou et al., 2013a). In mouse, oocyte miRNAs are not essential for meiosis (Suh et al., 2010). Indeed, Dgc8 is required only for miRNA processing, whereas Dicer is also implicated in small interfering RNA (siRNA) processing. Dicer loss in mouse oocytes results in meiotic arrest and severe spindle and chromosomal segregation defects, whereas Dgc8 loss showed no phenotype (Murchison et al., 2007; Tang et al., 2007; Suh et al., 2010). This suggests that siRNAs rather than miRNAs are involved in oocyte meiosis. On the other hand, miRNAs also could have a major role in the regulation of follicular cell functions, such as steroidogenesis, apoptosis, luteinization as well as in ovulation process (Hawkins and Matzuk, 2010). For example, treatment of mouse mural granulosa cells with LH leads to the deregulation of a set of miRNAs (particularly miR-132 and miR-212 overexpression) that are possibly important for the control of ovarian functions (Fiedler et al., 2008). Overexpression of miR-93 could disturb ovary development. Indeed, miR-93 targets the mRNA encoding LHXB8, a protein that contains a Lim homeodomain required for the transition from primordial to primary follicle (Pangas et al., 2006).

Table II lists the miRNAs found in the COC, granulosa cells, the follicular fluid and the corpus luteum. Table III summarizes the miRNAs involved in folliculogenesis and Table IV the miRNAs involved in hormonal regulations.

Many studies have shown that hormones from the hypothalamic–pituitary–gonadal axis, which are essential for sexual maturation and reproductive function in mammals, are also involved in the regulation of some miRNAs. Gonadotropin-releasing hormone (GnRH) stimulates the synthesis and the secretion of the pituitary gonadotrophins Luteinizing hormone (LH) and Follicle-stimulating hormone (FSH) that then regulate the production of gonadal steroids and gametogenesis (Conn and Crowley, 1994; Kaiser et al., 1997). GnRH also induces the expression of multiple miRNAs, particularly miR-132 and miR-212, which are encoded by the same gene that is induced by GnRH (AK006051) (Godoy et al., 2011). LH acts on ovarian granulosa cells to induce ovulation and luteinization, resumption of oocyte meiosis and CC expansion that are crucial steps for ovulation. Moreover, LH acts as a survival factor by preventing apoptosis of granulosa cells (Robker and Richards, 1998; Chaffin et al., 2001). Interestingly, LH also up-regulates miR-132, miR-212 and miR-21 in mural granulosa cells (Fiedler et al., 2008). miR-21 is overexpressed in many tumors, including breast, pancreatic, colorectal and esophageal cancer, and thus is considered as an oncogenic miRNA (Cho, 2007; Dillhoff et al., 2008; Vergheze et al., 2008). miR-21 depletion induces caspase-dependent apoptosis of mouse granulosa cells in vitro and in vivo (Carlett et al., 2010), highlighting the physiological anti-apoptotic role of miR-21 in normal tissues. miR-200b and miR-429 depletion inhibits LH synthesis by repressing transcription of the gene encoding the β subunit of LH. This results in a lower serum LH concentration and the absence of the LH surge, leading to ovulation failure (Hasuwa et al., 2013). Thus, the hypothalamic–pituitary–gonadal axis requires miR-200b and miR-429 to ensure ovulation. Finally, miR-122 is involved in the down-regulation of LH receptor expression by increasing the expression of LH receptor mRNA-binding protein via activation of SREBPs (Azhar, 2013; Menon et al., 2013).

FSH has a crucial role in both follicle development and granulosa cell proliferation and differentiation. Several miRNAs, including miR-143, miR-125b, miR-21 and the let-7 family, are involved in follicular development in the mouse (Yao et al., 2009). The expression of these RNAs is very low in primordial follicles, but they become readily detectable in granulosa cells of primary, secondary and antral follicles. miR-143, let-7a and miR-15b are negatively regulated by FSH (Yao et al., 2009). Moreover, miR-133b is involved in FSH-induced estrogen production, by binding to the 3’ UTR of FoxL2 and thus reducing FOXL2 protein level in granulosa cells (Dai et al., 2013). FOXL2 is expressed in the ovaries and is necessary for granulosa cell function (Schmidt et al., 2004), particularly through regulation of steroidogenesis genes, including StAR and CYP19A1 that are essential for promoting estradiol production (Psarska et al., 2011; Caburet et al., 2012).

The involvement of miRNAs in the hormonal regulation during folliculogenesis and in the oocyte–niche crosstalk could be exploited for identifying new non-invasive biomarkers of fertility. Moreover, the development of therapies that block the expression or mimic the functions of specific miRNAs may represent a new therapeutic strategy for many gynecological disorders.

**Cirulating cell-free DNA**

**Biology of circulating cfDNA**

DNA fragments are found in the blood circulation. Circulating cfDNA are double-stranded molecules with low molecular weight than genomic DNA, in the form of short fragments (between 70 and 200
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<td>GC</td>
<td>Rat</td>
<td>Regulation by FSH</td>
<td>Targets of miR-29a: COL4AI and BMF Targets of miR-30d: RNF2 and EED</td>
<td>Pro-apoptotic role Follicular atresia</td>
<td>Lin et al. (2012)</td>
</tr>
<tr>
<td>miR-181a</td>
<td>GC</td>
<td>Mouse</td>
<td>–</td>
<td>ACVRIIA</td>
<td></td>
<td>Zhang et al. (2013c)</td>
</tr>
<tr>
<td>miR-145</td>
<td>GC</td>
<td>Mouse</td>
<td>–</td>
<td>ACVRIB, CCND2</td>
<td></td>
<td>Yan et al. (2012)</td>
</tr>
<tr>
<td>miR-224</td>
<td>Pre-antral GC</td>
<td>Mouse</td>
<td>Up-regulation by TGF-β1/SMAD pathway</td>
<td>SMAD4</td>
<td>GC proliferation Ovarian estrogen release (CYP19A1)</td>
<td>Carletti et al. (2010)</td>
</tr>
<tr>
<td>miR-21</td>
<td>Mural GC</td>
<td>Mouse</td>
<td>Up-regulation by hCG</td>
<td>PDCD4, PTEN, tropomyosin-1 and sprouty homolog 2</td>
<td>Anti-apoptotic role</td>
<td>Fiedler et al. (2008)</td>
</tr>
</tbody>
</table>

Continued
<table>
<thead>
<tr>
<th>miRNAs</th>
<th>Expression</th>
<th>Species</th>
<th>Regulation</th>
<th>Target genes</th>
<th>Functions</th>
<th>References</th>
</tr>
</thead>
</table>
| miR-503         | GC         | Mouse (Amhr2-Dicer1) | Stimulation by gonadotrophins | Down-regulation of ACVR2a (ActRlla), ACVR2b (ActRlb), FSHR, BCL2 and CCND2 | Ovarian development  
|                 |            |                |                       |                                                  | Proliferation during folliculogenesis  
|                 |            |                |                       |                                                  | (down-regulation during early follicular development, increase during later stage before ovulation and decline during luteinization) | Lei et al. (2010)  
|                 |            |                |                       |                                                  | Nagaraja et al. (2008) |
| miR-132 and     | Mural GC   | Mouse          | Up-regulation by hCG  | 77 mRNA, CTBP1 protein synthesis                  | Ovarian function of CTBP1  
| miR-212         |            |                |                       |                                                  | unknown                  | Fiedler et al. (2008) |
| miR-23a         | GC         | Human          | –                     | XIAP Caspase-3                                    | Pro-apoptotic role       | Yang et al. (2012) |
| miR-21          | GC lines (KGN) | Human       | –                     | COL4A1 mRNA                                       | Basement membrane  
|                 |            |                |                       |                                                  | surrounding the GC layer and granulosa-embedded extracellular structure | Mase et al. (2012) |
| Pre-miR-10a,    | GC         | Human          | –                     | CyclinB1 TdT, caspase-3 PCNA                      | Apoptosis and cell proliferation | Sirotkin et al. (2010) |
| miR-105 and     |            |                |                       |                                                  |                                                  |                         |
| miR-182         |            |                |                       |                                                  |                                                  |                         |
| miR-15a         |            |                |                       |                                                  |                                                  |                         |
| FF              |            |                |                       |                                                  |                                                  |                         |
| miR-654-5p      | GC transfected with exosomes | Bovine | –                     | ITGA3 SOCS4 MAP3K1 BRMS1L ZNFX1 CD44 VEGFA       | Tumor progression (melanoma)  
| miR-640         |            |                |                       |                                                  | Apoptosis and cell proliferation (retinal development)  
| miR-526b        |            |                |                       |                                                  | Ovarian primordial follicle activation  
| miR-373         |            |                |                       |                                                  | Neonatal development  
|                 |            |                |                       |                                                  | Early endometrial response to pregnancy  
|                 |            |                |                       |                                                  | Network of matrices in COC extracellular space  
|                 |            |                |                       |                                                  | Neovascularization and vascular permeability during pre-antral follicle development |
| miR-181A,       | FF         | Equine         | –                     | TGF-β signaling (24 genes)                        | Follicle development and growth  
| miR-375 and     |            |                |                       |                                                  | Oocyte maturation           | Da Silveira et al. (2012) |
| miR-513a-3p     |            |                |                       |                                                  |                                                  |                         |
| miR-222, miR-193b and miR-520c3p | FF | Human | –                     | PTEN, ESR1 IL-1A, IL-10, IL-12B, IL-37, IL-8 TGF-β1 PDK3 HMGA2, RAB5B TGF-β1 | Tumor suppressor, negative regulation of insulin signaling and glucose metabolism in adipose tissue  
| miR-191,        |            |                |                       |                                                  | Steroidogenesis process  
| miR-483-5p,     |            |                |                       |                                                  | Immune system  
| miR-146a,       |            |                |                       |                                                  | Reproductive aging, cell proliferation, metabolic diseases  
| miR-320, miR-24, |            |                |                       |                                                  | Regulation of glucose metabolism  
| miR-574-3p,     |            |                |                       |                                                  | PCOS (Shi et al. (2012)  
| miR-1290 and    |            |                |                       |                                                  | Steroidogenesis |
| miR-518a        |            |                |                       |                                                  |                                                  |                         |
| miR-132, miR-24 and miR-320 | FF | Human | –                     |                                                  |                                                  |                         |
| miR-24          |            |                |                       |                                                  |                                                  |                         |
| miR-132, miR-320 and miR-520-3p | FF | Human | –                     |                                                  |                                                  |                         |
| miR-222, miR-24, |            |                |                       |                                                  |                                                  |                         |
| miR-193b and miR-483-5p | FF | Human | –                     |                                                  |                                                  |                         |

Continued
base pairs in length) or long fragments up to 21 kb. Two different mechanisms (not mutually exclusive) could explain the presence of cfDNA in the blood circulation. The first one is a passive mechanism due to the release of nuclear and mitochondrial DNA during the destruction of apoptotic and necrotic cells (Schwarzenbach et al., 2011). In normal conditions, cell debris is phagocytosed by macrophages and thus the cfDNA level in blood remains low in healthy individuals (Pisetsky and Fairhurst, 2007). However, after phagocytosis of necrotic cells, DNA might be partially released into the bloodstream inside nucleosomes where it is protected from enzymatic degradation (Holdenrieder et al., 2001a,b). This mechanism occurs in both healthy individuals and patients with benign diseases. The second mechanism is an active one probably through cell secretion (Gahan et al., 2008). Many studies have reported high concentrations of cfDNA in plasma or serum of patients with cancer or other severe diseases (Laktionov et al., 2004). Moreover, recent studies using genome-wide sequencing of plasma DNA have revealed that circulating tumor DNA represents the tumor genome and reflects the clonal genomic evolution of cancers (Murtaza et al., 2013). Circulating cfDNA should be rapidly degraded by nucleases, and it has been shown that mutated cfDNA is degraded more rapidly than non-mutated cfDNA (Diehl et al., 2005).

Circulating cfDNA for the non-invasive diagnosis of gynecological and pregnancy disorders

Changes in the levels of circulating DNA have been associated with several diseases, including gynecological and fetal disorders (Table V). cfDNA could be used for the early detection and monitoring of gynecological malignancies. For example, circulating cfDNA can be measured to detect EOC at early stages (Zhang et al., 2013b). The total cfDNA concentration in blood samples from patients with ovarian cancer is higher, particularly at advanced stages of the disease, than in healthy controls (Kamat et al., 2006b). Very high pre-operative plasma levels of cfDNA are significantly associated with decreased patients’ survival and constitute an independent predictor of death from ovarian cancer (Kamat et al., 2010; No et al., 2012). EOC is rarely detected early and it is not easy to determine whether an adnexal mass is malignant or benign. Interestingly, patients with EOC or endometriosis have significantly different levels of circulating cell-free mitochondrial DNA, but not of circulating cell-free nuclear DNA (Zachariah et al., 2008). cfDNA originating from promoters can be methylated. The methylation profile of this cfDNA could also be used to differentiate between some benign and malignant tumors (Liggett et al., 2011a,b). The level of tumor-specific DNA in plasma increases progressively with the tumor burden. On the other hand, it can decrease following chemotherapy. Indeed, tumor-specific plasma DNA levels were significantly higher in mice without treatment compared with animals treated with a combination of cytotoxic chemotherapy and anti-angiogenic agents against ovarian carcinoma (Kamat et al., 2006a). Thus, tumor-specific cfDNA may be a useful biomarker of therapeutic response as well. This was confirmed by a recent paper showing that exome-wide analysis of circulating tumor DNA could complement the current invasive biopsy approaches to identify mutations associated with acquired drug resistance in advanced cancer (Murtaza et al., 2013).

In the case of endometrial cancer, measurement of cfDNA is not useful for the detection of this malignancy. However, changes in cfDNA levels in a given patient after surgery/drug treatment may be a prognostic biomarker (Tanaka et al., 2012).

DNA isolated from maternal blood is a mixture of fetal and maternal DNA in proportions that change during pregnancy progression. Although it is called fetal DNA, it derives from apoptotic placental cells (Hupertz and Kingdom, 2004; Hahn et al., 2005).

Maternal obesity is associated with increased circulating total cfDNA, but not with fetal cfDNA. This could be due to less efficient clearance of cfDNA in obese women (Vora et al., 2012). However, it is more likely to be the result of increased production of total cfDNA because decreased clearance would also lead to an increase in fetal cfDNA. In obese pregnant women, active remodeling of adipose tissue via adipocyte necrosis and/or apoptosis of the stromal vascular fraction results in higher release of cfDNA of maternal origin in the circulation (Haghiac et al., 2012). In addition, the total cfDNA level is correlated with the maternal BMI and the gestational weight gain (Lapaire et al., 2009; Haghiac et al., 2012).

Circulating cfDNA levels are higher in pregnant women with pre-eclampsia or abnormal placental invasion, as reported by different studies using real-time quantitative PCR for the male-specific SRY (sex-
Table III miRNAs implicated in folliculogenesis.

<table>
<thead>
<tr>
<th>miRNAs</th>
<th>Expression</th>
<th>Species</th>
<th>Regulation</th>
<th>Target genes</th>
<th>Functions</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-143</td>
<td>Ovary</td>
<td>Mouse</td>
<td>–</td>
<td>Genes related to the cell cycle</td>
<td>Primordial follicle formation, Suppression of pre-granulosa cell proliferation</td>
<td>Zhang et al. (2013a)</td>
</tr>
<tr>
<td>miR-145</td>
<td>Neonatal ovary</td>
<td>Mouse</td>
<td>–</td>
<td>Tgfbr2</td>
<td>Initiation of primordial follicle development and maintenance of primordial follicle quiescence</td>
<td>Yang et al. (2013)</td>
</tr>
<tr>
<td>Dicer-1</td>
<td>Oviductal, uterine mesenchyme, granulosa cells from pre-antral and small antral follicles</td>
<td>Mouse (Amhr2-Dicer1)</td>
<td>–</td>
<td>Follicle development-related genes such as Amh, Inhba, Cyp17a1, Cyp 19a1, Zps, Gdf9, Bmp15</td>
<td>Reproductive tract abnormalities (primary oviductal defect leading to infertility), Follicle cell proliferation, differentiation and apoptosis, Follicle development and atresia (accelerated early follicle recruitment and reduction in the number of pre-ovulatory follicles), Oocyte maturation, Estrous cycle: reduction in the number of natural or induced ovulations</td>
<td>Leib et al. (2010), Gonzalez and Behringer (2009), Pastorelli et al. (2009), Hong et al. (2008), Nagaraja et al. (2008)</td>
</tr>
<tr>
<td>miR-125b</td>
<td>Granulosa cells</td>
<td>Mouse</td>
<td>–</td>
<td>–</td>
<td>Follicular development: low expression in primordial follicles and increased expression in primary, secondary and antral follicles</td>
<td>Yao et al. (2009)</td>
</tr>
<tr>
<td>miR-21</td>
<td>–</td>
<td>Mouse</td>
<td>Negative control by FSH</td>
<td>–</td>
<td></td>
<td></td>
</tr>
<tr>
<td>miR-7a</td>
<td>–</td>
<td>Mouse</td>
<td>–</td>
<td>–</td>
<td></td>
<td></td>
</tr>
<tr>
<td>miR-143</td>
<td>–</td>
<td>Mouse</td>
<td>–</td>
<td>–</td>
<td></td>
<td></td>
</tr>
<tr>
<td>miR-15b</td>
<td>–</td>
<td>Mouse</td>
<td>–</td>
<td>–</td>
<td></td>
<td></td>
</tr>
<tr>
<td>miR-709</td>
<td>Ovary</td>
<td>Newborn mouse</td>
<td>–</td>
<td>Nobox</td>
<td>Folliculogenesis (transition from primordial to primary follicle stage), Oogenesis</td>
<td>Choi et al. (2007b)</td>
</tr>
<tr>
<td>miR-93</td>
<td>Ovary</td>
<td>Mammalian</td>
<td>–</td>
<td>Lhx8</td>
<td>Folliculogenesis (transition from primordial to primary follicle stage), Oogenesis</td>
<td>Zhao and Rajkovic (2008), Pangas et al. (2006)</td>
</tr>
</tbody>
</table>

Fetal cell-free DNA (cfDNA) is detectable in the plasma of pregnant women up to a few hours after birth and could thus be used for non-invasive prenatal testing to detect chromosomal abnormalities (Hui and Bianchi, 2013). Indeed, fetal cfDNA is considered a reliable non-invasive biomarker of fetal aneuploidy (Bischoff et al., 2002, 2005; Azziz et al., 2004; Bauer et al., 2006; Wright and Burton, 2009; Abd El Naby et al., 2013; Canick et al., 2013). First, the presence of fetal cfDNA in the amniotic fluid was explored as a non-invasive method for the early detection of fetal chromosomal abnormalities. Then, it was demonstrated that specific fetal aneuploidies, such as trisomy 13, 18 or 21, can be detected in fetal cfDNA from maternal serum samples (Dan et al., 2012; Norton et al., 2012; Palomaki et al., 2012; Sparks et al., 2012; Zimmermann et al., 2012; Fairbrother et al., 2013; Nicolaides et al., 2014). Moreover, fetal cfDNA in maternal plasma is also used to detect pathogenic copy number variations using target region capture sequencing, for instance in the case of family history of thalassemia (Ge et al., 2013). Fetal cfDNA is used also for fetal sex determination in pregnant women who are carriers of X-linked genetic disorders in order to avoid invasive chorionic villus sampling, generally performed at 11–13 weeks of gestation (Miura et al., 2011; Abd El Naby et al., 2013). Fetal cfDNA enrichment in amniotic fluid and in the maternal bloodstream, detected by using the improved new technologies, will contribute...
to more sensitive and accurate prenatal diagnosis in the near future and might greatly extend the scope of non-invasive prenatal diagnosis.

**Nucleic acids as emerging non-invasive diagnostic biomarkers of female infertility**

**Circulating miRNAs and ovarian function**

Several studies have shown that miRNAs are involved in intercellular signaling (Valadi et al., 2007). In order to identify the miRNAs that are implicated in the CC–oocyte crosstalk and that regulate key genes implicated in folliculogenesis and ovarian function, we analyzed by deep sequencing the miRNAs present in mature metaphase II oocytes and in the associated CCs (Assou et al., 2013a). Only 3 miRNAs were found to be expressed in oocytes (miR-184, miR-100 and miR-10a) and 32 in CCs.

Among the miRNAs expressed in the COC (Assou et al., 2013a), some of them were found also in the plasma (Rekker et al., 2013) (Fig. 1). It could be interesting to select some of these miRNAs, based on their possible involvement in folliculogenesis, and investigate whether their blood level could be used as a marker of IVF outcome. Moreover, the possible

<table>
<thead>
<tr>
<th>miRNAs</th>
<th>Expression</th>
<th>Species</th>
<th>Regulation</th>
<th>Target genes</th>
<th>Functions</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-378</td>
<td>GC (in antral follicle growth)</td>
<td>Pig</td>
<td>Up-regulation by hCG (activation of CAMP/PKA/ERK)</td>
<td>Fox2</td>
<td>Inhibition of Fox2-mediated transcriptional repression of StAR and CYP19A1</td>
<td>Menon et al. (2013)</td>
</tr>
<tr>
<td>miR-122</td>
<td>Ovary</td>
<td>Rat</td>
<td>Up-regulation by LHR mRNA-binding protein</td>
<td>Fox2</td>
<td>Inhibition of Fox2-mediated transcriptional repression of StAR and CYP19A1</td>
<td>Azhar (2013)</td>
</tr>
<tr>
<td>miR-133b</td>
<td>GC</td>
<td>Mouse</td>
<td>Down-regulation by TGF-β I in pre-antral follicles</td>
<td>Rbms1 (DNA-binding protein that activates MYC)</td>
<td>Stimulation of CYP19A1 and estradiol levels</td>
<td>Dai et al. (2013)</td>
</tr>
<tr>
<td>miR-200b and miR-429</td>
<td>Pituitary gland</td>
<td>Mouse</td>
<td>Up-regulation by TGF-β I in pre-antral follicles</td>
<td>Smad4</td>
<td>GC proliferation CYP19A1 stimulation Ovarian estrogen release</td>
<td>Hasuwa et al. (2013)</td>
</tr>
<tr>
<td>miR-383</td>
<td>GC (culture in vitro) and oocyte</td>
<td>Mouse</td>
<td>Up-regulation by TGF-β I in pre-antral follicles</td>
<td>Smad4</td>
<td>Decrease in estradiol secretion Stimulation of estradiol secretion Decrease in progesterone secretion</td>
<td>Yin et al. (2012)</td>
</tr>
<tr>
<td>miR-224</td>
<td>Pre-antral GC</td>
<td>Mouse</td>
<td>Up-regulation by TGF-β I / SMAD pathway</td>
<td>TGF-β I</td>
<td>Decrease in estradiol secretion Stimulation of estradiol secretion Decrease in progesterone secretion</td>
<td>Parker and Schimmer (1997)</td>
</tr>
<tr>
<td>miR-24, miR-132, miR-320 and miR-520-3p</td>
<td>FF</td>
<td>Human</td>
<td>--</td>
<td>--</td>
<td>Suppress estradiol production Inhibition of progesterone release stimulation of progesterone release Increase of progesterone output</td>
<td>Sirotkin et al. (2009)</td>
</tr>
<tr>
<td>miR-193b and miR-483-5p</td>
<td>GC (culture in vitro)</td>
<td>Human</td>
<td>--</td>
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<td></td>
</tr>
<tr>
<td>51 miRs 36 or 57 miRs 10 miRs miR-15a and miR-188 miR-107</td>
<td>GC (culture in vitro)</td>
<td>Human</td>
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the abundance of cfDNA can change in abnormal situations, we hypothesized that variations, particularly an increase, in circulating cfDNA might reflect ovarian reserve disorders. cfDNA is easily quantifiable in serum, and in women undergoing IVF it would be interesting to compare cfDNA concentration with FSH, LH, AMH and estradiol levels at Day 3 of the cycle, patient characteristics and IVF outcome. Recently, it was reported that increased plasma cfDNA levels are associated with low pregnancy rates in IVF programmes (Czamanski-Cohen et al., 2013). However, the only correlation was between cfDNA and pregnancy outcome, once the patient was pregnant. The same group also reported a reduction in the higher than normal plasma cfDNA levels in a group of women undergoing IVF procedures following the practice of stress-reduction techniques, suggesting that these techniques may facilitate physiological changes leading to a reduction in plasma cfDNA levels and ultimately an improved IVF outcome (Czamanski-Cohen et al., 2014).

**Table V** cfDNA in gynecological pathologies and fetal cfDNA in the maternal circulation.

<table>
<thead>
<tr>
<th>Pathologies</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>cfDNA</td>
<td>Vietnamese patients (Zhang et al., 2013b); Maternal cancer (Kamat et al., 2010); Liggett et al. (2011a); Dobrzycka et al. (2011); Kamat et al. (2006a, b)</td>
</tr>
<tr>
<td>Endometrial cancer</td>
<td>Tanaka et al. (2012); Dobrzycka et al. (2010)</td>
</tr>
<tr>
<td>Maternal obesity</td>
<td>Vora et al. (2012); Haghic et al. (2012); Lapaire et al. (2009)</td>
</tr>
<tr>
<td>Pre-eclampsia/HELLP syndrome</td>
<td>Miranda et al. (2013); Lazar et al. (2010); Lazar et al. (2009); Swinkels et al. (2002)</td>
</tr>
<tr>
<td>Fetal cfDNA</td>
<td>Hahn et al. (2011); Lazar et al. (2010); Lazar et al. (2009); Levine et al. (2004); Bianchi et al. (2004); Cotter et al. (2004); Zheng et al. (2002); Hahn and Holzgreve (2002); Swinkels et al. (2002); Zhong et al. (2001); Leung et al. (2001); Lo et al. (1999)</td>
</tr>
<tr>
<td>Abnormal placental invasion</td>
<td>Sekizawa et al. (2002)</td>
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<tr>
<td>Pre-term delivery</td>
<td>Leung et al. (1998)</td>
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<tr>
<td>Aneuploidy</td>
<td>Horsting et al. (2014); Nicolaidas et al. (2014); Russo and Blakemore (2014); Robinson et al. (2014); Bianchi and Wilkins-Haug (2014); Grenzink et al. (2013); Verweij et al. (2013); Benn et al. (2013); Walsh and Goldberg (2013); Canick et al. (2013); Langlois et al. (2013); She et al. (2013); Webb and Murphy (2012); Hou et al. (2012); Wang et al. (2012a); Dan et al. (2012); Lazar et al. (2012); Wright and Burton (2009); Lo and Chiu (2008); Zimmermann et al. (2005); Montagnana et al. (2007); Deng and Li (2007); Bischoff et al. (2005); Bianchi (2004); Wataganara and Bianchi (2004); Spencer et al. (2003); Farina et al. (2003); Wataganara et al. (2003); Lee et al. (2002); Bischoff et al. (2002); Ohashi et al. (2001); Zhong et al. (2000); Lo et al. (1999)</td>
</tr>
<tr>
<td>Fetal sex determination</td>
<td>X-linked genetic disorders Khorram et al. (2013); Wight et al. (2012); Hou et al. (2012); Hill et al. (2012); Miura et al. (2011); Zimmermann et al. (2008); Deng and Li (2007); Montagnana et al. (2007); Lo (2005); Chen et al. (2004); Honda et al. (2002); Costa et al. (2002); Sekizawa and Saito (2001); Costa et al. (2001)</td>
</tr>
<tr>
<td>α-Thalassemia</td>
<td>Ge et al. (2013); Sinchotiyakul et al. (2012)</td>
</tr>
<tr>
<td>β-Thalassemia</td>
<td>Li et al. (2011); Chen et al. (2008); Lo (2005); Chiu et al. (2002b)</td>
</tr>
<tr>
<td>Achondroplasia</td>
<td>Lim et al. (2011); Saito et al. (2000)</td>
</tr>
<tr>
<td>Myotonic dystrophy</td>
<td>Amiricci et al. (2000)</td>
</tr>
<tr>
<td>Cystic fibrosis</td>
<td>Gonzalez-Gonzalez et al. (2005); Gonzalez-Gonzalez et al. (2002)</td>
</tr>
<tr>
<td>Huntington’s disease</td>
<td>Gonzalez-Gonzalez et al. (2003)</td>
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<tr>
<td>Congenital adrenal hyperplasia</td>
<td>Rijnders et al. (2001); Chiu et al. (2002a)</td>
</tr>
<tr>
<td>Hemolytic disease of fetus and newborn</td>
<td>Illanes and Soothill (2009)</td>
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</table>

cfDNA, cell-free DNA; HELLP: hemolysis, elevated liver enzymes, low platelets.

**cfDNA as a biomarker of ovarian function**

cfDNA is released into the circulation following physiological and pathological cell necrosis and apoptosis. Based on the finding that the abundance of cfDNA can change in abnormal situations, we hypothesized that variations, particularly an increase, in circulating cfDNA might reflect ovarian reserve disorders. cfDNA is easily quantifiable in serum, and in women undergoing IVF it would be interesting to compare cfDNA concentration with FSH, LH, AMH and estradiol levels at Day 3 of the cycle, patient characteristics and IVF outcome. Recently, it was reported that increased plasma cfDNA levels are associated with low pregnancy rates in IVF programmes (Czamanski-Cohen et al., 2013). However, the only correlation was between cfDNA and pregnancy outcome, once the patient was pregnant. The same group also reported a reduction in the higher than normal plasma cfDNA levels in a group of women undergoing IVF procedures following the practice of stress-reduction techniques, suggesting that these techniques may facilitate physiological changes leading to a reduction in plasma cfDNA levels and ultimately an improved IVF outcome (Czamanski-Cohen et al., 2014).

**miRNA and cfDNA in embryo culture medium**

miRNAs are involved in the regulation of mammalian embryo development (Foshay and Gallicano, 2009; Medeiros et al., 2011). Global miRNA expression profiling suggests that miRNA synthesis and degradation dynamically coexist during preimplantation embryo development (Yang et al., 2008). In addition, intracellular miRNAs might modulate the transition of human embryonic stem cells (hESCs) to the differentiated cells that form the early germ layers (Wong et al., 2012). Many miRNAs are expressed in developing mammalian embryos and...
hESCs, including miR-320, miR-92a, let-7a and miR-146b (Yang et al., 2008; McCallie et al., 2010; Merkerova et al., 2010). Recent reports indicate that deregulated miRNA expression in the embryo is associated with human infertility (McCallie et al., 2010) and the embryo miRNA expression profile varies according to its chromosomal make-up and sex (Tzur et al., 2008; Rosenbluth et al., 2013).

As miRNAs have been detected in the culture medium following release by cells grown in culture (Hergenreider et al., 2012), it would be possible to quantify the embryonic miRNAs released in the medium in order to monitor embryo health during preimplantation in vitro culture. Currently, human embryo selection for transfer into the uterus is based mainly on morphology (Sakkas et al., 2001; Fenwick et al., 2002). The culture medium could routinely be discarded at each step of the in vitro embryo development. Thus, the CC gene expression profiling, together with miRNA quantification in culture medium, could improve the monitoring of preimplantation embryo health, and provide a non-invasive approach to predict oocyte competence and pregnancy outcome (Assou et al., 2008, 2011, 2013b). Changes in metabolite (pyruvate, glucose or amino acids) levels in the embryo culture medium might reflect embryo viability, and these metabolites have been assessed as potential biomarkers of embryo quality (Seli et al., 2007, 2010, 2011). Different methods (visual inspection, CC gene profiling, metabolite and miRNA quantification in the embryo culture medium) could thus be applied to improve embryo selection and ultimately IVF outcome.

Recently, it has been reported that the presence of cfDNA released into embryo culture medium from mitochondria is associated with poor embryo quality during cleavage (Stigliani et al., 2013). Thus, as for miRNAs, the analysis of cfDNA released into the culture medium by embryos might offer the possibility to develop a non-invasive test for the selection of the embryos with the highest implantation potential.

**Conclusions and further perspectives**

In recent years, our understanding of the biology of circulating nucleic acids has greatly progressed and powerful technologies for their analysis

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**Figure 1** miRNAs expressed in human CCs and oocytes that are also present in human plasma. The Venn diagram has been generated crossing the miRNAs identified in the COC by Assou et al. (2013a), and the miRNAs present in plasma as described in Reeker et al. (2013). CC, cumulus cell.
have been developed. Consequently, cell-free nucleic acids, such as cfDNA and miRNAs, will play an increasing role as non-invasive tools for the detection/prognosis of ovarian disorders and the monitoring of human preimplantation embryo health during in vitro culture. Indeed, blood and spent embryo culture medium are easily accessible and are therefore ideal materials for assessing ovarian and embryo health (Fig. 2). For example, the evaluation of cfDNA and miRNAs in the serum of infertile women might allow an assessment of ovarian reserve. Similarly, the quantification of cfDNA and miRNAs in microdrops of embryo culture medium at Day 3 and Day 5/6 post-fertilization could help in the monitoring of embryo development and pregnancy outcome. Ultimately, specific embryonic miRNAs secreted into the culture medium might be attractive candidate biomarkers to predict embryo quality and pregnancy outcomes.

Authors’ roles
S.T., S.A., E.S., S.B. and S.H. were involved in the study design and manuscript preparation. S.T. conducted literature review, manuscript drafting on circulating cell-free nucleic acids, and S.A. on nucleic acids in embryo culture medium and figure drafting. T.A.-E. and D.H. provided technical help and advices. S.H. provided final manuscript approbation.

Funding
We thank the direction of the University-Hospital of Montpellier and Ferring Pharmaceutical Companies for their support.

Conflict of interest
The authors declare that there is no conflict of interest as defined by the guidelines of the International Committee of Medical journal Editors (ICMJE; www.icmje.org).

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