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Ovarian ageing: the role of mitochondria in oocytes and follicles

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TABLE OF CONTENTS

BACKGROUND: There is a great inter-individual variability of ovarian ageing, and almost 20% of patients consulting for infertility show signs of premature ovarian ageing. This feature, taken together with delayed childbearing in modern society, leads to the emergence of age-related ovarian dysfunction concomitantly with the desire for pregnancy. Assisted reproductive technology is frequently inefficacious in cases of ovarian ageing, thus raising the economic, medical and societal costs of the procedures.

OBJECTIVE AND RATIONAL: Ovarian ageing is characterized by quantitative and qualitative alteration of the ovarian oocyte reserve. Mitochondria play a central role in follicular atresia and could be the main target of the ooplasmic factors determining oocyte quality adversely affected by ageing. Indeed, the oocyte is the richest cell of the body in mitochondria and depends largely on these organelles to acquire competence for fertilization and early embryonic development. Moreover, the oocyte ensures the uniparental transmission and stability of the mitochondrial genome across the generations. This review focuses on the role played by mitochondria in ovarian ageing and on the possible consequences over the generations.

SEARCH METHODS: PubMed was used to search the MEDLINE database for peer-reviewed original articles and reviews concerning mitochondria and ovarian ageing, in animal and human species. Searches were performed using keywords belonging to three groups: 'mitochondria' or 'mitochondrial DNA'; 'ovarian reserve', 'oocyte', 'ovary' or 'cumulus cells'; and 'ageing' or 'ovarian ageing'. These keywords were combined with other search phrases relevant to the topic. References from these articles were used to obtain additional articles.

OUTCOMES: There is a close relationship, in mammalian models and humans, between mitochondria and the decline of oocyte quality with ageing. Qualitatively, ageing-related mitochondrial (mt) DNA instability, which leads to the accumulation of mtDNA mutations in the oocyte, plays a key role in the deterioration of oocyte quality in terms of competence and of the risk of transmitting mitochondrial abnormalities to the offspring. In contrast, some mtDNA haplogroups are protective against the decline of ovarian reserve. Quantitatively, mitochondrial biogenesis is crucial during oogenesis for constituting a mitochondrial pool sufficiently large to allow normal early embryonic development and to avoid the untimely activation of mitochondrial biogenesis. Ovarian ageing also seriously affects the dynamic nature of mitochondrial biogenesis in the surrounding granulosa cells that may provide interesting alternative biomarkers of oocyte quality.

WIDER IMPLICATIONS: A fuller understanding of the involvement of mitochondria in cases of infertility linked to ovarian ageing would contribute to a better management of the disorder in the future.

Key words: granulosa cells / infertility / mitochondria / mitochondrial DNA / mitochondrial haplogroups / ovarian ageing / ovarian reserve

Introduction

Delayed childbearing in modern society leads to the emergence of age-related ovarian dysfunction concomitantly with the desire for pregnancy. This situation is particularly acute today because women often have to delay their first pregnancy for professional reasons. Assisted reproductive technology (ART) is frequently inefficacious in cases of ovarian ageing (Leridon, 2004), thus raising the economic, medical and societal costs of the procedures. Alternatively, oocyte donation raises the problems of the scarcity of donors, and of the potential commodification of oocytes. Societal preservation of oocytes, i.e. preservation of the mothers' own oocytes for use at a later date, also poses various financial and storage problems, and raises ethical issues related to eventual professional pressures and unequal access to medical care. A fuller understanding of the mechanisms underlying the infertility linked to ovarian ageing would contribute to the better management of the disorder in the future.

Reduction of the ovarian reserve in premature and physiological ovarian ageing

The decline in fertility over time results from ovarian ageing, which is characterized by the quantitative and qualitative alteration of the ovarian oocyte reserve (te Velde and Pearson, 2002). In women, as in the vast majority of mammalian females, the oocyte pool, constituted during intrauterine life, is gradually depleted (Faddy *et al.*, 1992), while the number of oocyte abnormalities, such as chromosomal aneuploidies, increases (Hassold and Hunt, 2009). The probability of conception per cycle decreases with age, falling sharply from 25% at age 25 to 12% at age 35, and to as little as 6% at age 42 (Schwartz and Mayaux, 1982; van Noord-Zaadstra *et al.*, 1991). Menopause, the final stage of the ovarian ageing process, occurs at a mean age of 51 years in the Caucasian population, but earlier or later depending on the influence of environmental and genetic factors not yet well defined (te Velde and Pearson, 2002; Broekmans *et al.*, 2009). About 10% of the general population is postmenopausal at age 45. Menopause is preceded by an irregular menstrual cycle that itself is preceded up to 10 years earlier by a period of reduced fertility. The highly variable onset of menopause is related to the great variability of ovarian ageing. Patients experiencing a premature decline of fertility suffer from a condition known as premature ovarian ageing (POA), an entity characterized by subfertility, increased risks of miscarriage and poor response to stimulation during ART procedures (Nikolaou and Templeton, 2003). However, the reality of POA is still under debate. According to some authors, ovarian ageing is a continuous process of physiological ageing up to the pathological premature ovarian insufficiency (POI), characterized by the onset of menopause before age 40. Thus, POA might simply be an intermediate stage reflecting a continuum of phenotypic expressions of various etiologies of ovarian senescence (Gleicher *et al.*, 2009, 2011). The proportion of women with POA, particularly high in the infertile population (Barad *et al.*, 2007), may represent up to 20% of consultations for infertility (Devine *et al.*, 2015). Although chronological age is useful for assessing the chances of pregnancy, it does not necessarily correspond to the biological age, which is critical for predicting the response to ovarian stimulation in the context of ART (Alviggi *et al.*, 2009). Clinically, ovarian ageing is characterized by a diminished ovarian reserve (DOR), which is objectified by markers such as a decrease in anti-Müllerian hormone, an increase in follicle-stimulating hormone (FSH) and a decrease in the antral follicular count (AFC). Although the relevance of these tests used to characterize DOR is still subject to debate, they are commonly used in current practice (Practice Committee of the American Society for Reproductive, 2015). Ovarian ageing also results in poor response to ovarian stimulation and reduced chances of pregnancy with *in vitro* fertilization (IVF) (Bancsi *et al.*, 2002). Independently of the chronological age, DOR is associated with abnormalities usually observed in ovarian ageing, such as increased embryonic aneuploidies (Haadsma *et al.*, 2010; van der Stroom *et al.*, 2011; Katz-Jaffe *et al.*, 2013), fertilization failure (Lekamge *et al.*, 2007) and high rates of pregnancy loss (Levi *et al.*, 2001; Lekamge *et al.*, 2007). The evaluation of the ovarian reserve could predict a woman's total fertility potential (Tremellen and Savulescu, 2014). Thus, when assessing the effects of ageing on fertility, it would be better to consider the patient's ovarian reserve rather than merely her chronological age.

The exhaustion of the follicular pool with ageing depends on two components: its initial size and the importance of the follicular atresia. Studies in the mouse have shown that mitochondria are involved in

these two components and thus are potentially linked to ovarian ageing. On one hand, the depletion of ovarian follicles during reproductive senescence arises from the apoptosis of oocytes and surrounding follicular cells (Hussein, 2005). Mitochondria, via their role in cell survival and apoptosis (Tait and Green, 2010), play a central role in this follicular atresia (Ratts *et al.*, 1995; Hsu *et al.*, 1996; Chipuk *et al.*, 2010; Ene *et al.*, 2013). On the other hand, the initial size of the follicular pool seems to be determined during embryonic life along various pathways, including mitochondrial biogenesis (Aiken *et al.*, 2015).

In addition to this quantitative aspect, the parallel decline of oocyte quality also contributes to the gradual age-related decline in fertility (Bancsi *et al.*, 2002). The decline in oocyte quality is due to increased abnormalities of the nuclear genome and to the deterioration of cytoplasmic quality (Qiao *et al.*, 2014). The ooplasm contains the proteins, RNAs, metabolites and organelles essential for oocyte competence with respect to fertilization and embryo development, which could be adversely affected by ageing. Among these factors, mitochondria are particularly important since they play a major role in the maternal contribution to embryonic development. Indeed, the transfer of isolated mitochondria into an oocyte has in itself been shown to prevent apoptosis in the mouse (Perez *et al.*, 2000) and to promote mouse and pig embryonic development (Van Blerkom *et al.*, 1998; El Shourbagy *et al.*, 2006; Cagnone *et al.*, 2016). Moreover, a recent pangenomic transcriptomic study showed that mitochondrial activity constitutes one of the main pathways differentiating the developmental competence of the bovine oocyte, suggesting that mitochondria are crucial to the process of embryonic development (Nemcova *et al.*, 2016).

Mitochondrial DNA transmission and stability over the generations

The mitochondrion, the site of the terminal catabolism of cellular energetic substrates, is schematized in Fig. 1. Mitochondria constitute the powerhouse of cells, producing the energy required for all cellular functions. Mitochondrial oxidative phosphorylation (OXPHOS) depends on the activity of five multi-enzymatic complexes; complexes I–IV make up the electron transport chain, while complex V, i.e. ATP synthase, produces the ATP required for cellular energetic needs. Mitochondria generate much of the endogenous reactive oxygen species (ROS), a toxic by-product of OXPHOS. These organelles also integrate the mitochondrial permeability transition pores (mtPTPs) that initiate cell death by opening up when the mitochondrial energy function declines. Thus, mitochondrial dysfunctions that inhibit OXPHOS and generate increased ROS lead to apoptosis. Mitochondria are highly dynamic organelles that continuously move, fuse and divide in response to variations of cellular energy demands. Mitochondrial dynamics is mediated by large dynamin GTPases (DRP1, OPA1, MFN1 and MFN2) embedded in mitochondrial membranes. Mitochondrial fission generates new organelles necessary for cellular growth and cell proliferation while facilitating the elimination of damaged mitochondria by mitophagy. Mitochondrial fusion ensures close complementation between organelles to satisfy energy requirements at the cellular level.

Mitochondria possess their own genome in the form of mtDNA, a double-stranded, circular 16 569 bp DNA molecule in humans (Anderson *et al.*, 1981). This molecule is organized in nucleoproteic complexes, called nucleoids, which lack the protective histones associated with the nucleic acids of the nuclear genome (Satoh and Kuroiwa, 1991; Legros *et al.*, 2004). The mtDNA content of each human cell type is highly variable, with the extremes being spermatozoa that contain only a few copies of mtDNA (May-Panloup *et al.*, 2003) and mature oocytes with up to several hundred thousand mtDNA copies (Reynier *et al.*, 2001). Most of the mtDNA sequences are coding sequences involving both DNA strands. Mitochondrial DNA codes for 13 essential subunits of the respiratory chain complexes, 22 tRNAs and two rRNAs, constituting part of the mitochondrial translation machinery. All the other mitochondrial proteins, estimated to number ~1500, are nuclear encoded.

The regulation of mitochondrial biogenesis plays a central role in mitochondrial functions. The factors controlling mtDNA expression are mainly represented by the mitochondrial transcription factor A (TFAM), an ubiquitous, mitochondrial-targeted transcription factor that promotes the replication and transcription of mtDNA, and by the nuclear respiratory factors 1 and 2 (NRF1 and NRF2), which control the expression of the nuclear-encoded respiratory chain components as well as that of TFAM, and regulate mtDNA expression, thus ensuring the necessary coordination between the mitochondrial and nuclear genomes. Mitochondrial function is coordinated with general cell metabolism by the PPAR gamma coactivator 1 (PGC1alpha) and the NAD-dependent deacetylases of the sirtuins family.

The maternal transmission of mtDNA is the rule in most organisms with a genome exclusively transmitted by oocytes (Hutchison *et al.*, 1974; Giles *et al.*, 1980). Several mechanisms contribute to the elimination of paternal mtDNA (Sato and Sato, 2013; Carelli, 2015). One such mechanism involves a process of passive dilution caused by the numerical disproportion of mtDNA molecules between the male and female gametes (Luo *et al.*, 2013). Although spermatozoan mitochondria are found in the oocyte just after fertilization (Ankel-Simons and Cummins, 1996; Cummins, 1998), they are specifically destroyed before the four-cell stage of embryogenesis (Kaneda *et al.*, 1995), suggesting a process of active elimination. Indeed, mitochondria are ubiquinated during spermatogenesis (Sutovsky *et al.*, 1999, 2000), and then recognized and destroyed in the ooplasm through the proteosomal or lysosomal pathways (Sutovsky *et al.*, 1999; Sato and Sato, 2013). The digestion of paternal mtDNA in the ooplasm precedes the complete destruction of mitochondrial structure, ensuring the absence of paternal mtDNA inheritance (Nishimura *et al.*, 2006). The elimination of paternal mtDNA is a highly conserved phenomenon, except in the case of some rare species (Gyllensten *et al.*, 1991; Zhao *et al.*, 2004), implying its physiological and evolutionary importance. Paternal mtDNA inheritance has been described only in some rare pathological cases in humans (Schwartz and Vissing, 2002). The oocyte intervenes in the elimination of paternal mtDNA, and a study showing the accidental persistence of paternal mtDNA in polyploid human embryos suggested that it is linked to poor oocyte quality (St John *et al.*, 2000). Thus, oocyte quality appears to be crucial for ensuring uniparental homoplasmic mtDNA transmission over generations.

During oogenesis, female germ cells accumulate a wide range of components that are essential for early embryonic development. In particular, oogenesis is accompanied by a sharp increase in the

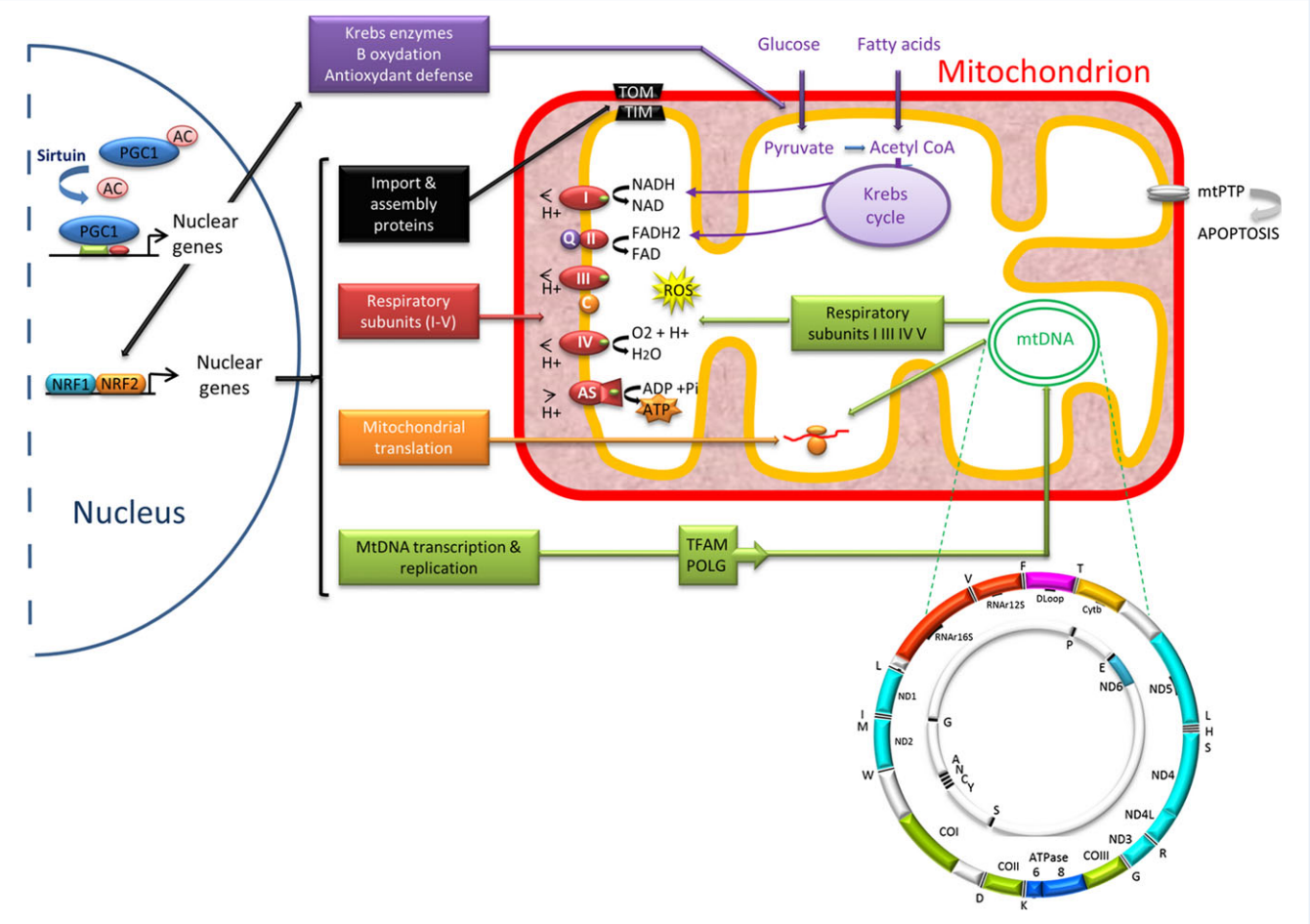

Figure 1 The mitochondrion is the site of the terminal catabolism of energetic molecules. Reduced coenzymes (NADH2 and FADH2) from the Krebs cycle and the oxidation of fatty acids supply electrons to the respiratory chain. OXPHOS is carried out by the five multienzyme complexes I–IV and ATP synthase (AS) embedded in the inner mitochondrial membrane. The production of ATP by the respiratory chain is accompanied by the formation of the toxic ROS. Mitochondrial function is coordinated with the general cell metabolism by PGC1 alpha and the sirtuin family. PGC1 alpha activates nuclear genes involved in fatty acid oxidation, antioxidant defense and the Krebs cycle. In parallel, PGC1 alpha promotes the expression of NRF1 and NRF2, transcription factors of nuclear genes coding for mitochondrial import proteins (TIM and TOM), assembly proteins and respiratory subunits, as well as for factors responsible for mtDNA translation (tRNA and rRNA), transcription and replication (TFAM and POLG). Mitochondria possess their own genome (mtDNA), a double-stranded, circular, 16 569 bp molecule coding for 13 subunits of the respiratory chain complexes (I: ND NADH deshydrogenase (blue), III: Cyt ubiquinone-cytochrome c. reductase (orange), IV CO cytochrome oxydase (green) and ATPase (dark blue), 22 tRNAs and two rRNAs (represented as groups) (red). The organelle also integrates the mitochondrial permeability pore (mtPTP) involved in apoptosis.

mitochondrial mass (Spikings *et al.*, 2007; St John *et al.*, 2010; Mahrous *et al.*, 2012; Cotterill *et al.*, 2013), mainly occurring in the later stages (Cao *et al.*, 2007; Wai *et al.*, 2008; Cree *et al.*, 2009). Thus, whereas primordial germ cells contain only ~100 copies of mtDNA (Jansen and de Boer, 1998; Cao *et al.*, 2007; Wai *et al.*, 2008; Cree *et al.*, 2009), the mature oocytes contain up to several hundred thousand copies, depending on the species examined (Otten and Smeets, 2015).

Studies on mtDNA segregation in pedigrees affected by mtDNA mutations have shown that when point mutations occur, there may be a high-level switching of mutants within a single generation (Hauswirth and Laipis, 1982; Olivo *et al.*, 1983; Marchington *et al.*, 1997; Poulton *et al.*, 1998). This concept has led to the bottleneck theory (Hauswirth and Laipis, 1985), according to which a very small number of mtDNAs may populate the oocyte and consequently the organism. The precise mechanism is not yet well defined but it seems based both on a drastic reduction in the number of mtDNA copies in the primordial germ cells (containing only a small number of founding mitochondria) followed by a great amplification during oogenesis, with a possible preferential replication of a subpopulation of mtDNA molecules (Cao *et al.*, 2007; Wai *et al.*, 2008; Cree *et al.*, 2009; Johnston *et al.*, 2015; reviewed in Mishra and Chan, 2014; Stewart and Chinnery, 2015). The pre-migratory primordial germ cells, containing only a small number of founding mitochondria, could be selected according to their mtDNA integrity or OXPHOS activity (Stewart *et al.*, 2008; Mishra and Chan, 2014; Stewart and Chinnery,

2015), before populating the organism. This mechanism may, to some extent, eliminate abnormal mitochondrial genomes and homogenize the mtDNA, thus preserving mitochondrial integrity over the generations. The bottleneck theory explains how mtDNA may be 'refreshed' and 'purified' from one generation to another as it passes through the 'narrow neck' of the mitochondrial population during early oogenesis (Jansen and de Boer, 1998; Mishra and Chan, 2014; Stewart and Chinnery, 2015). However, this genetic bottleneck is highly complex since its size varies considerably according to the type of mtDNA variants; in fact, various segregation patterns have been observed in human pedigrees transmitting pathogenic mtDNA mutations (Steffann *et al.*, 2015; Wilson *et al.*, 2016).

Usually, mtDNA molecules of all of the cells of an individual are homoplasmic, carrying only one genome with a given nucleotide sequence. But a wide spectrum of deletions or pathogenic and polymorphic mtDNA variants has been described in the heteroplasmic forms. In particular, recent studies using highly sensitive techniques have shown that low-level mtDNA heteroplasmy is common in the general population (Elliott *et al.*, 2008; Payne *et al.*, 2013; Ye *et al.*, 2014). Heteroplasmy of pathogenic variants leads to mitochondrial diseases only when the ratio between mutant mtDNA and wild type mtDNA exceeds a critical threshold. MtDNA mutants also spontaneously accumulate in postmitotic tissues during ageing, particularly in those affected by age-related diseases (Linnane *et al.*, 1989; Arnheim and Cortopassi, 1992).

Mitochondria are major determinants of ovarian ageing

Mitochondria, which are major factors of oocyte quality, may be directly impacted during ovarian ageing (Table I).

Table I mtDNA and oocyte quality.

	Species	Relationship	Oocyte mtDNA integrity	Oocyte mtDNA content	Granulosa cells mtDNA integrity	Granulosa cells mtDNA content	Granulosa cells mitochondrial genes expression or oxidative function	mtDNA haplogroups
Oocyte quality and embryonic development	Human	No			(Au *et al.*, 2005; Chan *et al.*, 2006)			
		Yes	(Brenner *et al.*, 1998; Barritt *et al.*, 1999; Hsieh *et al.*, 2002)	(Reynier *et al.*, 2001; Santos *et al.*, 2006; Wang *et al.*, 2010; Mao *et al.*, 2012; Murakoshi *et al.*, 2013; Xu *et al.*, 2015; Zhao *et al.*, 2016)	(Tsai *et al.*, 2010)	(Pawlak *et al.*, 2015; Ogino *et al.*, 2016)	(Hammond *et al.*, 2015)	
	Pig	No						
		Yes		(El Shourbagy *et al.*, 2006; Spikings *et al.*, 2007; Lee *et al.*, 2014a,b; Cagnone *et al.*, 2016)				
	Mouse	No						
		Yes	(Ross *et al.*, 2013)	(Wai *et al.*, 2010; Ge *et al.*, 2012)				
Oocyte quality and ageing	Human	No	(Chen *et al.*, 1995; Barritt *et al.*, 1999; Brenner *et al.*, 1998)				(Shufaro *et al.*, 2012)	
		Yes	(Keefe *et al.*, 1995; Barritt *et al.*, 2000; Chan *et al.*, 2005)	(Chan *et al.*, 2005; May-Panloup *et al.*, 2005a,b; Duran *et al.*, 2011; Bonomi *et al.*, 2012; Murakoshi *et al.*, 2013; Boucret *et al.*, 2015)	(Tsai *et al.*, 2010)	(Seifer *et al.*, 2002; Boucret *et al.*, 2015)	(Tatone *et al.*, 2006; Pacella-Ince *et al.*, 2014; Boucret *et al.*, 2015)	(May-Panloup *et al.*, 2014)
	Cattle	No		(Cree *et al.*, 2015)				
		Yes		(Yamamoto *et al.*, 2010; Iwata *et al.*, 2011; Takeo *et al.*, 2013a,b; Hammond *et al.*, 2016)			(Cree *et al.*, 2015; Sugiyama *et al.*, 2015)	(Tamassia *et al.*, 2004)
	Mouse	Yes		(Kushnir *et al.*, 2012; Simsek-Duran *et al.*, 2013)				

Mitochondrial dysfunction in ovarian ageing

Mitochondrial involvement in the general ageing process is related to the progressive deterioration of pleiotropic functions with age, both in terms of energy production and in the regulation of the various cellular signaling pathways (Eichenlaub-Ritter, 2012; Bratic and Larsson, 2013; Tilly and Sinclair, 2013). Thus, a study on mouse oocytes has shown an age-associated alteration of gene expression patterns including the genes involved in mitochondrial functions and oxidative stress (Hamatani *et al.*, 2004). Oocyte mitochondria are typically spherical elements with few cristae surrounding a matrix of high-electron density (Motta *et al.*, 2000), and despite this seemingly primitive state, they are an essential source of ATP supply (Van Blerkom *et al.*, 1995; Dumollard *et al.*, 2007a,b). Mitochondria are able to distribute and localize in ooplasmic areas with higher ATP requirements for energy consuming events leading to oocyte cytoplasmic and nuclear maturation including germinal vesicle breakdown, and microtubule assembly and disassembly for meiotic spindle formation. Furthermore, patterns of mitochondrial distribution in the mature oocyte seems to differ among species (Brevini *et al.*, 2005; Dumollard *et al.*, 2007a,b; Yu *et al.*, 2010).

The effects of ageing on oocyte mitochondria have been seen through morphological and functional abnormalities. Indeed, mitochondrial swelling, vacuolization and cristae alteration have been shown to be associated with increasing age, in humans (Muller-Hocker *et al.*, 1996) and in rodents (Kushnir *et al.*, 2012; Simsek-Duran *et al.*, 2013). Mitochondrial membrane potential (Delta ψ), reflecting mitochondrial activity, is also altered with ageing in humans (Wilding *et al.*, 2001) and in mice (Ben-Meir *et al.*, 2015), as is ATP production, at least in mice (Selesniemi *et al.*, 2011; Ben-Meir *et al.*, 2015). In fact, in various species, the failure of ATP production has been shown to have deleterious consequences on chromosome segregation (Eichenlaub-Ritter, 1998, 2012; Schon *et al.*, 2000) and on embryonic development (Van Blerkom *et al.*, 1995; Dumollard *et al.*, 2007a,b; Van Blerkom, 2011; Lee *et al.*, 2014a,b). Moreover, disruption of mitochondrial OXPHOS in mouse oocytes results in meiotic spindle abnormalities and decreased embryonic preimplantation potential (Zhang *et al.*, 2006). A major factor contributing to reduced fertility with reproductive ageing is the aneuploidy associated with meiotic segregation errors, and a recent review proposes that ageing may affect oocyte competence in targeting the cytoplasmic quality and particularly the mitochondria with various consequences including nuclear chromosomal abnormalities (Meldrum *et al.*, 2016).

Mitochondrial DNA is unstable in ovarian ageing

Because of the proximity of mtDNA with the respiratory chain, the lack of protective histones and efficient repair mechanisms, the mtDNA mutation rate is almost 25 times higher than that of nuclear DNA (Lynch *et al.*, 2006). This instability of mtDNA leads to the accumulation of somatic mutations with age. Indeed, a progressive accumulation of deletions and point mutations has been reported in healthy elderly individuals (Cortopassi and Arnheim, 1990; Corral-Debrinski *et al.*, 1992). In mice expressing a proofreading-deficient allele of the nuclear-encoded catalytic subunit of the mtDNA polymerase (POLGA), the resulting increase in mtDNA mutations and deletions is responsible for a premature ageing phenotype (Trifunovic *et al.*, 2004). This mutator phenotype is associated with infertility in males and females mice. Moreover, patients with POLGA mutations can be affected by premature menopause (Luoma *et al.*, 2004) and some single-nucleotide polymorphisms (SNPs) linked to the POLG gene have been associated with age at natural menopause in a Genome-Wide Association study (Day *et al.*, 2015). Recently, using backcrosses between POLGA heterozygote mice, maternally inherited mtDNA mutations *per se* were shown to induce the ageing phenotype and reduce the lifespan of mice possessing the wild-type nuclear genome (Ross *et al.*, 2013, 2014). Interestingly, the ongoing mtDNA mutagenesis in the maternal germ line of mice was found to lead to reduced fertility that could be reversed by the introduction of wild-type mtDNA into females (Ross *et al.*, 2013). Taken together, these findings strongly support the involvement of accumulated mtDNA mutations in ovarian failure.

In the ovary, the follicular pool is definitively constituted during embryonic life and is not subsequently renewed. Thus, the primary oocyte, meiotically blocked at prophase I in the primordial follicle, may, during the long period of quiescence, undergo accumulation of mtDNA rearrangements *t* may be involved in the ageing process. According to the bottleneck concept, the oocyte mtDNA content is formed by the stochastic distribution of mtDNA molecules during oogonial divisions, combined with a turnover of mtDNA molecules during the amplification phase that occurs during oogenesis (Mishra and Chan, 2014; Johnston *et al.*, 2015; Stewart and Chinnery, 2015). The random distribution of mtDNA among the various oocytes may allow discrimination against oocytes containing mtDNA rearrangements leading to functional alteration of mitochondria. It has been proposed that follicular atresia, eliminating primary oocytes carrying deleterious mtDNA mutations, may explain some cases of POI (Krakauer and Mira, 1999). The need for a closely controlled surveillance system ensuring the integrity of maternal mtDNA is supported by observations that single pathogenic mtDNA deletions are not transmitted to the offspring of clinically symptomatic women (Chinnery, 2002).

However, it is difficult to assess how the ageing process produces mtDNA rearrangements in the mature oocyte. Firstly, follicular growth and oocyte maturation are supported by anaerobic glycolysis favored by low oxygen tension (Bermejo-Alvarez *et al.*, 2010; Makanji *et al.*, 2014). Thus, in the ovary, the oocyte evolves in an anoxic environment, which limits OXPHOS and the production of potentially deleterious ROS likely to result in mtDNA rearrangements (Van Blerkom, 2011). Secondly, after the initial period, the mtDNA bottleneck seems to protect the female germ cell line since this phenomenon continues during the growth phase of the oocyte, i.e. after the quiescent phase and up to a few months before ovulation (Wai *et al.*, 2008). Thus, it may at least partially correct the effects of ageing on the integrity of oocyte mtDNA.

In human oocytes, mutated or deleted mtDNA during the IVF process has been estimated at up to 50% (Brenner *et al.*, 1998; Barritt *et al.*, 1999; Chan *et al.*, 2005; Jacobs *et al.*, 2007). However, the link between the mtDNA rearrangements and ageing remains controversial. In studies on oocytes from older women, some authors have reported a greater number of mtDNA abnormalities, such as the common 4977-bp deletion (Keefe *et al.*, 1995; Chan *et al.*, 2005), or some point mutations (Barritt *et al.*, 2000), whereas others have

found no difference in the number of deletions (Brenner *et al.*, 1998; Barritt *et al.*, 1999; Chan *et al.*, 2005). Interestingly, a recent study using a model of cloned cows, 3 and 10 years old, showed that ovarian ageing significantly increased the number of oocytes carrying mtDNA deletions whereas the difference was not significant for mtDNA heteroplasmic point mutations (Hammond *et al.*, 2016). However, in studies in humans, the mutational burden was very low and did not appear to be responsible for direct functional outcomes (Brenner *et al.*, 1998). Indeed, the effects of low levels of heteroplasmy may appear only in the long-term. Recently, a positive correlation has been reported between the number of mtDNA heteroplasmic mutations in the blood and buccal cells of a child and the age of the child's mother at the time of fertilization (Rebolledo-Jaramillo *et al.*, 2014). This association, attributable to oocyte ageing, could have several important consequences. First, through the rapid segregation of heteroplasmic variants, the mitochondrial bottleneck could lead either to homoplasmy, resulting in the complete elimination of particularly deleterious mutations, or to the selection of some variants over the generations (Steffann *et al.*, 2014). Secondly, the purification process might not be efficacious enough to remove some heteroplasmic point mutations (Ye *et al.*, 2014), so that these pathogenic mutations may multiply later in life in a fraction of the cells, leading to age-related mitochondrial dysfunction (Greaves *et al.*, 2014). Finally, inherited and acquired mtDNA mutations may act together to aggravate the phenotype of ageing including infertility (Ross *et al.*, 2013; Stewart and Chinnery, 2015). Somatic mutations, acquired by the oocyte during ageing, may combine with preexisting germline mtDNA rearrangements, leading to decreased oocyte quality. Transmitted by the mother, these germline rearrangements may be directly linked to the maternal age effect. All of these eventualities suggest that the accumulation of mtDNA mutations in oocytes may lead to the decline of fertility with age, and have deleterious consequences for the offspring.

Mitochondrial haplogroups influence ovarian ageing

mtDNA is highly polymorphic with regard to some SNPs used for defining mtDNA haplogroups that indirectly reflect the evolution of human populations (www.mitomap.org) (Torroni *et al.*, 1996). mtDNA haplogroups specifically affect mitochondrial performance related to bioenergetic functions, mitochondrial coupling efficiency and the production of ROS (Gomez-Duran *et al.*, 2010). The role played by the different haplogroups in the promotion or prevention of diseases has been reported in a wide spectrum of pathologies (Ruiz-Pesini *et al.*, 2000; Baudouin *et al.*, 2005; Saxena *et al.*, 2006; Lorente *et al.*, 2013; Nardelli *et al.*, 2013), as well as in complex, age-related traits and longevity (Tanaka *et al.*, 1998; De Benedictis *et al.*, 1999; Carrieri *et al.*, 2001; Ross *et al.*, 2001; Coskun *et al.*, 2003; Niemi *et al.*, 2003; van der Walt *et al.*, 2003; Dominguez-Garrido *et al.*, 2009; Courtenay *et al.*, 2012; Hudson *et al.*, 2013; Rea *et al.*, 2013). We have shown that the JT macro-haplogroup, known to be associated with longevity (De Benedictis *et al.*, 1999; Coskun *et al.*, 2003; Niemi *et al.*, 2003; Rea *et al.*, 2013) and with protection against neurodegenerative disorders (Chagnon *et al.*, 1999; Tranah *et al.*, 2012; Hudson *et al.*, 2013), decreases the risk of a prematurely DOR by two-thirds compared to women carrying the other haplogroups (May-Panloup *et al.*, 2014). Indeed, among the 200 patients we studied, the distribution of the macro-haplogroups was significantly different between the patients with a normal ovarian reserve (NOR) and the DOR patients ($P = 0.02$). The JT macro-haplogroup was significantly under-represented among patients with signs of ovarian ageing, with a frequency of 7.4% compared to that of 20.7% ($P = 0.006$) in the control group and that of 18.8% in the general French population ($P = 0.0012$). Moreover, conventional markers indicated a better ovarian reserve in patients harboring the JT macro-haplogroup, than in women with any other macro-haplogroup. In patients with the JT macro-haplogroup, the mean AFC was significantly higher ($P = 0.004$), the mean value of FSH was significantly lower ($P < 0.001$), and the mean value of estradiol was also significantly lower ($P = 0.04$) (May-Panloup *et al.*, 2014). Thus, certain haplogroups appear be protective against ovarian reserve decline.

Ovarian ageing is associated with reduced oocyte mitochondrial DNA content

The mtDNA content in the oocytes of older women or of women with DOR is significantly lower than that of younger patients or those with NOR (Chan *et al.*, 2005; May-Panloup *et al.*, 2005a,b; Duran *et al.*, 2011; Murakoshi *et al.*, 2013). In our study, we found two distinct ranges of oocyte mtDNA copy numbers in patients with DOR (100 000 ± 99 000 copies) compared to women with NOR (318 000 ± 184 000 copies) ($P < 0.0001$) (May-Panloup *et al.*, 2005a, b). Moreover, polar bodies from oocytes of patients at an advanced reproductive age, i.e. 38 to 45 years old, have been reported to have less mtDNA than those of younger women (Konstantinidis *et al.*, 2014). Similar results have also been found in cows, among which animals over 15 years old have a lower oocyte mitochondrial content than younger animals (Iwata *et al.*, 2011; Takeo *et al.*, 2013a,b).

As shown in various species, the initial mitochondrial mass and mtDNA content of the oocyte are determining factors of fertilizability and embryonic development. In humans, mtDNA copy numbers in oocytes unfertilized because of female-factor infertility, were lower than in those where the treatment was due to male-factor infertility (Reynier *et al.*, 2001; Santos *et al.*, 2006). Comparable results were found in the pig since the addition of 2′3′-dideoxycytidine, which depletes mtDNA copy numbers during oocyte maturation, led to reduced parthenogenetic blastocyst formation (Lee *et al.*, 2014a,b). Finally, in the mouse, the deletion of a single copy of the *TFAM* gene led to the reduction of oocyte mtDNA copy numbers, showing that the mature oocyte has a critical threshold of mtDNA copies, essential for post-implantation development, found to be 40 000 to 50 000 copies in this species (Wai *et al.*, 2010). These authors suggest that the range of mtDNA content described in the mature oocyte of different species (Otten and Smeets, 2015) represents the viable amount determined by mammalian evolution. Indeed, since mtDNA does not gainfully replicate during early embryogenesis, the mitochondrial mass of the fertilizable oocyte must be sufficient for its distribution among the several embryonic blastomeres to ensure the optimal functioning of each cell until the resumption of mtDNA replication. This occurs around implantation at the blastocyst stage, which marks the onset of cellular differentiation within the embryo in larger mammals (May-Panloup *et al.*, 2005a,b; El Shourbagy *et al.*, 2006; Van

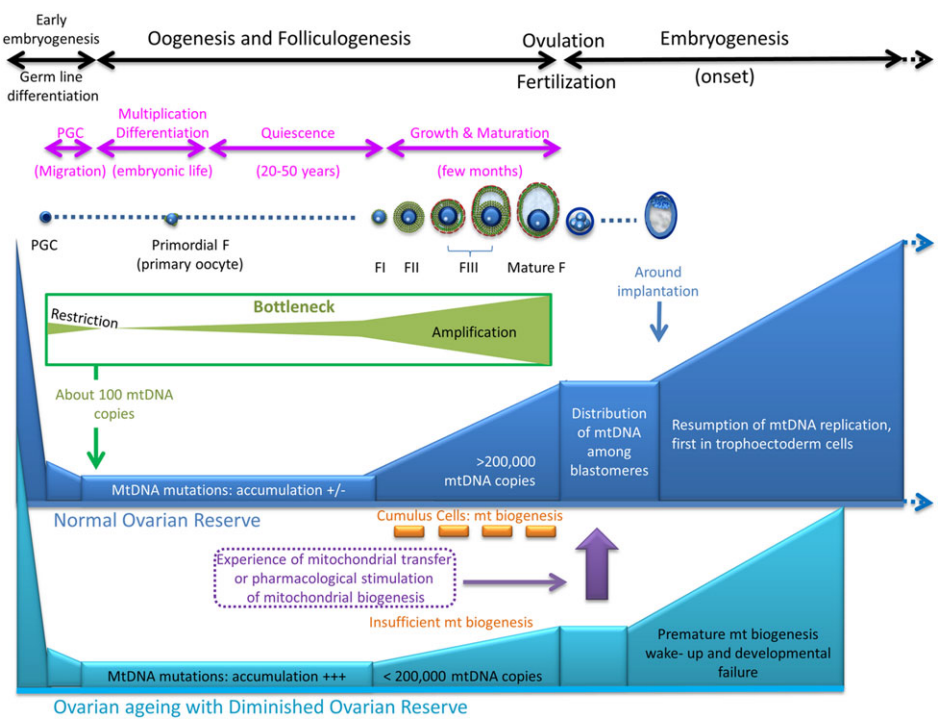

Figure 2 Mitochondrial biogenesis during oogenesis, folliculogenesis (F = Follicule, I primary, II secondary, III tertiary) and early embryonic development. During early maternal embryogenesis, the mtDNA content of each embryonic cell undergoes a drastic reduction. This is followed, during oogenesis, by a restriction and amplification of mtDNA content leading to a refreshed and purified mtDNA pool as schematized in the bottleneck (green), quantitatively sufficient for allocation among the embryonic blastomeres. The close relationship between the oocyte and the surrounding cumulus cells appears to be important for the constitution of the oocyte mitochondrial pool (orange). In DOR patients (light blue), with abnormal mitochondrial biogenesis, the purification and amplification of mtDNA content may be poorer than in NOR patients (dark blue) thus leading to the premature onset of mitochondrial biogenesis during early embryonic development.

Blerkom, 2011; St John, 2013) and later, at the oocyte cylinder stage, in rodents (Piko and Taylor, 1987; Ebert *et al.*, 1988; Thundathil *et al.*, 2005; Kameyama *et al.*, 2007) (Fig. 2).

The close relationship between the oocyte mitochondrial threshold and embryonic developmental competence is demonstrated by the beneficial effect of increasing the oocyte mitochondrial mass either by mitochondrial transfer or pharmacological means.

In humans, the injection of a fraction of the cytoplasm extracted from a young donor oocyte into an aged oocyte was shown to enhance oocyte competence sufficiently to support normal embryonic development (Cohen *et al.*, 1997). This method led to successful human pregnancies with almost 50 births (Cohen *et al.*, 1997; Huang *et al.*, 1999; Lanzendorf *et al.*, 1999; Barritt *et al.*, 2001a,b; Dale *et al.*, 2001). However, these procedures had been conducted without prior animal studies and, given the lack of knowledge of the risks involved and the absence of a strong scientific rationale for the indication of infertility treatment, the US Food and Drug Administration suggested, in 2002, that the technique of donor cytoplasmic transfer should be suspended pending the outcome of further studies. The subject is still under debate (Food and drug administration (FDA) briefing documents – Cellular, Tissue and Gene Therapies Advisory Committee: 'Oocyte Modification in Assisted Reproduction for the Prevention of Transmission of Mitochondrial Disease or Treatment of Infertility').

Whereas several factors present in the ooplasm may be involved in the improvement of oocyte competence, the transfer of isolated mitochondria has in itself proved sufficient in the mouse (Van Blerkom *et al.*, 1998; Perez *et al.*, 2000). Several studies on animal models have shown that the transfer of mitochondria or cytoplasm from developmentally competent donor oocytes into incompetent oocytes improves embryonic quality, reducing fragmentation, increasing the cleavage rate and allowing successful implantation, thus leading to the development of healthy individuals (Table II). The development of these techniques falls within the framework of mitochondrial manipulations (FDA briefing documents – Cellular, Tissue and Gene Therapies Advisory Committee: 'Oocyte Modification in Assisted Reproduction for the Prevention of Transmission of Mitochondrial Disease or Treatment of Infertility'). Mitochondrial manipulations, initially proposed to prevent the transmission of inherited mitochondrial diseases in humans due to pathogenic variants carried by mtDNA (Craven *et al.*, 2010; Paull *et al.*, 2013; Tachibana *et al.*, 2013), have recently been authorized in the UK (3 February 2015). However, such mitochondrial manipulations have come under criticism worldwide (Isasi *et al.*, 2016). These techniques raise the issue of the risks of heteroplasmy linked to the use of mitochondria, or ooplasm, from the third donor (Brenner *et al.*, 2000; Barritt *et al.*, 2001a,b). Indeed, even with non-pathogenic variants, mitochondrial heteroplasmy,

Table II Improvement of oocyte competence by ooplasmic or mitochondrial transfer

Model	Oocyte transfer of :	Bibliography
Mouse	Ooplasm or mitochondria	(Pinkert *et al.*, 1997; Meirelles and Smith, 1998; Van Blerkom *et al.*, 1998; Perez *et al.*, 2000; Nagai *et al.*, 2004; Takeda *et al.*, 2005,2010; Yi *et al.*, 2007; Cheng *et al.*, 2009)
Pig	Ooplasm	(El Shourbagy *et al.*, 2006)
Cattle	Ooplasm or mitochondria	(Hua *et al.*, 2007; Ferreira *et al.*, 2010; Chiaratti *et al.*, 2011)
Human	Ooplasm or mitochondria	(Cohen *et al.*, 1997; Huang *et al.*, 1999; Lanzendorf *et al.*, 1999; Brenner *et al.*, 2000; Barritt *et al.*, 2001a,b; Dale *et al.*, 2001; Harvey *et al.*, 2007; Woods and Tilly, 2015; Oktay *et al.*, 2015)

i.e. the coexistence of two mtDNA genomes with no pathogenic variants, has been reported to alter physiological and cognitive functions in mice (Acton *et al.*, 2007; Sharpley *et al.*, 2012). Beyond a certain percentage of heteroplasmy (Yabuuchi *et al.*, 2012), the coordination between nuclear and mitochondrial genes may break down, particularly in terms of energy production (Reinhardt *et al.*, 2013). To avoid the risk of heteroplasmy, the transfer of autologous mitochondria from somatic cells has been proposed. However, mitochondria of somatic origin possess tissue-specific characteristics and appear to affect embryonic development (Takeda *et al.*, 2005, 2010). The mitochondria of a given individual exhibit tissue-specific differences, particularly in mitochondrial morphology and the proteome (Pagliarini *et al.*, 2008). The best results are obtained with mitochondria from cells of ovarian origin (Schatten *et al.*, 2014). The transfer of mitochondria from follicular cells has given good results in cattle (Hua *et al.*, 2007), and even in humans (Tzeng, 2004). However, the atresia of oocytes is known to be initiated by apoptotic signals emitted by follicular cells (Hussein, 2005), suggesting that mitochondrial transfer from these cells may carry a major risk of oocyte degeneration. The use of mitochondria from somatic cells also raises the issue of the transfer of aged mitochondria, i.e. having the same age as the recipient oocyte. The closest alternative source of mitochondria may be the germ line cells, in particular the so-called germ line stem cells that have not undergone postmitotic stagnation. Indeed, oogonial stem cells have recently been identified in postnatal human ovaries (Johnson *et al.*, 2004) and mouse ovaries (Zou *et al.*, 2009). Using such mitochondria from stem cells, a significant improvement in fertilization rates was shown in women with poor oocyte quality (Oktay *et al.*, 2015).

The importance of mitochondrial threshold in oocyte competence is also shown by the use of pharmacological agents that increase the mitochondrial mass or improve mitochondrial function (Table III). In animals with ovarian ageing, such results have been obtained with various molecules such as: coenzyme Q10, an alternative component of the mitochondrial respiratory chain possessing anti-oxidant properties, which has been shown to decrease ovarian atresia, restore oocyte mitochondrial gene expression and improve mitochondrial

Table III Pharmacological improvement of oocyte competence.

Drug or nutrient	Potential mechanism	Model	Consequence via mitochondrial effect	Auteurs
Rapamycin	Increase the mitochondrial autophagy and renewal	Rat (intraperitoneal injection)	Oocyte pool preservation Increased genital life length	Zhang *et al.*, 2013
		Pig (*in vitro*)	Oocyte developmental capacity	(Lee *et al.*, 2014a,b)
Resveratrol	Inductor of mitochondrial biogenesis and activity via sirtuin pathway	Mouse (oral administration/gastric canula)	Oocyte pool preservation Increased oocyte quantity and quality	(Liu *et al.*, 2013; Cabello *et al.*, 2015)
		Cattle (*in vitro*)	Improved embryonic development Improved embryonic development	(Takeo *et al.*, 2013a,b; Wang *et al.*, 2013; Sato *et al.*, 2014; Sugiyama *et al.*, 2015)
		Pig (*in vitro*)	Improved embryonic development	(Lee *et al.*, 2010; Kwak *et al.*, 2012; Itami *et al.*, 2015)
CoEnz Q10	Alternative component of mitochondrial respiratory chain with anti-oxidant	Mouse (subcutaneous injection)	Improved response to stimulation with increasing number of scalable embryos and births	(Bentov *et al.*, 2010; Bentov and Casper, 2013; Ben-Meir *et al.*, 2015)
		Cattle (*in vitro*)	Improved fecundation, embryonic cleavage, blastocyst formation and implantation	(Stojkovic *et al.*, 1999; Bentov *et al.*, 2010)

activity (Bentov et al., 2010; Bentov and Casper, 2013; Ben-Meir et al., 2015); rapamycin, which may increase mitochondrial autophagy and renewal (Zhang et al., 2013; Lee et al., 2014a,b); or resveratrol, an inductor of mitochondrial biogenesis and activity (Liu et al., 2013; Sugiyama et al., 2015). Whereas rapamycin is reserved for targeted indications in human medicine as an immunosuppressant, resveratrol and coenzyme Q10 have proved their safety (Parkinson Study Group et al., 2014) and coenzyme Q10 has already been used to overcome the effect of age on female fertility (Bentov et al., 2014). The activation of certain key molecules, such as mitochondrial sirtuin 3 (Sirt3), may be one of the best methods of enhancing mitochondrial biogenesis (Kincaid and Bossy-Wetzel, 2013; Pacella-Ince et al., 2014). Indeed, Sirt3, which can be pharmacologically activated by resveratrol, could serve as a key target for the regulation of mitochondrial content in oocytes. It has recently been shown in mice that the inactivation of Sirt3 by SiRNA impairs oocyte developmental competence whereas the injection of Sirt3 mRNA improves mitochondrial biogenesis and oocyte developmental competence (Zhao et al., 2016). This study also human samples and showed that the Sirt3 gene expression was lower in oocytes that failed to mature *in vitro* compared to those with successful *in vitro* maturation, whereas the other members of the sirtuin family remained unaffected.

Ovarian ageing leads to premature mitochondrial biogenesis in the embryo

The role played by mtDNA in early embryonic development has recently been shown to be more complex than initially believed. Indeed, the dynamics of mtDNA replication may be a determining factor of embryonic development. Theoretically, apart from a very short period immediately after fertilization described in rodents (McConnell and Petrie, 2004), mtDNA does not gainfully replicate before the time of implantation (St John et al., 2010), and a down-regulation of mtDNA replication at the end of oocyte growth may be essential to allow successful preimplantation development as shown in the pig (Spikings et al., 2007). But it has also been shown in cattle that under certain conditions, the embryo could prematurely initiate mtDNA replication (Chiaratti et al., 2010). Among older women, the mtDNA content in early-cleavage stage embryos was found to be lower than in those of young women, but paradoxically, at the blastocyst stage, the opposite was observed with an increase of the mtDNA content of blastocysts from older patients (Fragouli et al., 2015). Similarly, aneuploid blastocysts contained a greater number of mtDNA copies than euploid blastocysts (Wells et al., 2014; Fragouli et al., 2015). Moreover, among early-cleavage stage embryos and blastocysts, those which implanted had less mtDNA than those which did not implant (Diez-Juan et al., 2015). Thus, mitochondrial proliferation may be a compensatory mechanism for mitochondrial insufficiency (Wredenberg et al., 2002; Yen et al., 2002; DiMauro and Schon, 2003). The increased mtDNA levels observed in chromosomally abnormal embryos or in embryos with poor implantation potential could be a response to embryonic stress and a sign of developmental abnormality. This type of compensatory increased mtDNA content during the first embryonic cleavages was already observed in embryos carrying the mtDNA pathogenic mutation associated with Mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (Monnot et al., 2013). New generation sequencing did not reveal a greater number of mtDNA mutations in the embryos of older women with high mtDNA levels. This finding argues against the possibility of mitochondrial instability driving the replication of the organelle in such cases (Fragouli et al., 2015).

Fertilization triggers the transition from a relatively quiescent cell into an actively dividing embryo. Despite their low capacity for respiratory activity, embryonic mitochondria provide the energy crucial for the activation of development and for embryo survival. Moreover, mitochondria play a central role through their involvement in various signaling pathways, such as $Ca2^{+}$ signaling and regulation of the intracellular redox potential, particularly important for fertilization and embryonic development (Dumollard et al., 2007a,b). In this context, it may be postulated that an insufficient or faulty mitochondrial pool at the end of oogenesis could trigger an upregulation mechanism leading to an abnormal compensatory increase of mtDNA during early embryogenesis. This could correspond to a premature initiation of mitochondrial biogenesis (Diez-Juan et al., 2015). In humans, such mitochondrial biogenesis is not physiologically activated until the onset of differentiation at the blastocyst stage (Sathananthan and Trounson, 2000; Van Blerkom, 1993, 2011). At this stage, mtDNA replication is resumed and mitochondria differentiate into more active forms with a sharp increase of glycolysis and oxygen consumption (Houghton and Leese, 2004; Dumollard et al., 2007a,b). Prior to this stage, the mitochondria observed are often spherical, containing few cristae and manifesting low OXPHOS activity (Motta et al., 2000). Some authors have suggested that this apparent mitochondrial quiescence (Leese, Sturmey et al., 2007) may be essential to the rapid glycolytic cell proliferation characteristic of this period (Krisher and Prather, 2012). Indeed, during early embryogenesis mitochondria usually have a long lifespan and a quiet metabolism could lower their production of oxidants, i.e. ROS, thus limiting stress damage (Dumollard et al., 2007a,b). The untimely activation of mitochondrial biogenesis could break the fragile balance between energy demand and supply existing in the cleaved stage embryo, leading to oxidative damage as well as dysregulation of the different pathways involving mitochondria. In particular, ROS and intracellular redox-potential are known to regulate some transcription factors critical to early embryonic development (Funato et al., 2006; Liu et al., 2005).

These findings suggest that mitochondrial biogenesis may be crucial during oogenesis for constituting a mitochondrial pool sufficiently large to allow normal early embryonic development. In patients with ovarian ageing, the impaired oocyte quality associated with insufficient mtDNA content could lead to premature mitochondrial biogenesis, leading to the failure of embryonic development (Fig. 2).

Altered metabolic pathways in cumulus cells may reflect impaired mitochondrial biogenesis during oogenesis

In the early stage of oogenesis, primary oocytes are surrounded by a layer of somatic granulosa cells and a basal membrane to constitute primordial follicles. Throughout life, follicles leave the resting pool and enter the growing pool under the influence of intra-ovarian and

endocrine factors (Gilchrist *et al.*, 2004). During the subsequent developmental stages of folliculogenesis, oocyte growth is accompanied by the proliferation and differentiation of granulosa cells. At the antral follicle stage, granulosa cells differentiate into two populations with distinct phenotypes: the cumulus granulosa cells (CGCs) which lie in proximity to the oocyte are intimately involved in oocyte growth and maturation, and the mural granulosa cells, the essential role of which is the steroidogenic activity (Gilchrist *et al.*, 2004). Gap junctions are established between CGCs and the oocyte via cytoplasmic projections through the zona pellucida. These gap junctions persist until the surge of luteinizing hormone that triggers the resumption of oocyte maturation. Within the ovarian follicle, oocyte competence is acquired through bidirectional signaling between the oocyte and the surrounding granulosa cells (Buccione *et al.*, 1990; Eppig *et al.*, 2002; Gilchrist *et al.*, 2008). In particular, oocyte-cumulus complex interactions orchestrate carbohydrate, lipid and protein metabolisms to provide the appropriate balance of energy required for the oocyte to undergo meiosis and fertilization, and to support early embryogenesis (Seli *et al.*, 2014; Dumesic *et al.*, 2015, 2016). Thus, CGCs metabolize glucose from the blood circulation into pyruvate which is then provide to the oocyte to allow ATP production by OXPHOS (Sutton-McDowall *et al.*, 2010). In turn, the oocyte regulates glycolysis in CGCs by inducing the expression of glycolytic key genes (Sugiura *et al.*, 2005, 2007; Su *et al.*, 2009). Similarly, within the oocyte-cumulus complex, fatty acid β-oxidation from lipid produces additional ATP for meiotic resumption (Downs *et al.*, 2009; Dunning *et al.*, 2010; Paczkowski *et al.*, 2013) and amino acid turnover (Colonna and Mangia, 1983; Su *et al.*, 2009) to ensure the metabolic needs of the oocyte. Granulosa cells and oocyte mitochondria, central agents of these metabolic pathways, are thus directly involved in establishing oocyte competence during oogenesis.

The influence of ageing on granulosa cells has been shown in various studies. Thus, the proteome of these cells in women of advanced maternal age (40–45 years old) differs considerably from that in younger women (McReynolds *et al.*, 2012). Similarly, transcriptomic study of granulosa cells shows significant differences between young and older women (Al-Edani *et al.*, 2014), and between women with DOR and those with NOR (Chin *et al.*, 2002; May-Panloup *et al.*, 2012; Skiadas *et al.*, 2012). In this ageing process, mitochondria could be an important factor. Thus, granulosa cells from women over 38 years have been shown to contain higher levels of mtDNA deletions (Seifer *et al.*, 2002) and damaged mitochondria (Tatone *et al.*, 2006).

The impact of ageing on the oocyte within the ovarian follicle may be due to the accumulation of damage in granulosa cells during the long quiescent phase before entering the growing phase (10–50 years) or to the alteration of the ovarian microenvironment perturbing the crosstalk between the granulosa cells and the oocyte during the growth phase (Tatone *et al.*, 2008). An ultrastructural study of the resting follicular pool in human ovaries argues in favor of the first hypothesis revealing age-related morphological changes in mitochondria, particularly with a greater frequency of ruptured mitochondrial membranes, in the granulosa cells of primordial follicles (de Bruin *et al.*, 2004).

However, during folliculogenesis, oocyte and granulosa cells depend closely on the ovarian microenvironment which is altered during ageing, especially under the influence of oxidative stress caused by the mitochondrial respiratory dysfunction that leads to enhanced ROS production (Miquel *et al.*, 1980). Thus, age-related oxidative damage has been reported in granulosa cells and interstitial ovarian tissue in the mouse (Lim and Luderer, 2011). This finding could be linked to the diminished expression of antioxidant enzyme in granulosa cells described in aged mice (Lim and Luderer, 2011) and in women over 38 years (Tatone *et al.*, 2006). Interestingly, in the mouse, suboptimal nutrition *in utero* causes oxidative stress and increases mtDNA copy numbers in somatic ovarian tissue together with accelerated cellular ageing and decline of the ovarian reserve (Aiken *et al.*, 2013). In addition, this study suggests that germ line cells, showing no modification of the mtDNA content, could be protected from such adverse environmental conditions in early life, thus minimizing the consequences on subsequent generations.

Metabolic disorders in the CGCs may reflect mitochondrial dysfunction or faulty mitochondrial biogenesis during folliculogenesis in ovarian ageing. For instance, Sirt3, a regulator of mitochondrial biogenesis, as well as its target, glutamate dehydrogenase, are altered in the CGCs of women with ovarian ageing (Pacella-Ince *et al.*, 2014). In a recent study, we found an under-expression of the *PPARGC1A* (peroxisome proliferator-activated receptor gamma coactivator 1-alpha) gene in the CGCs of DOR patients compared to NOR patients (Boucret *et al.*, 2015). The product of this gene, a master inducer of mitochondrial biogenesis and respiration (Puigserver *et al.*, 1998; Wu *et al.*, 1999), is also involved in protection against oxidative damage related to the ROS produced by the activity of the respiratory chain. At least in the goat, this gene is reported to play a role in follicular development (Zhang *et al.*, 2015). Moreover, CGCs, through the expression of factors involved in mtDNA replication and maintenance, such as TFAM (transcription factor associated with mitochondria), POLGA (a subunit of the mtDNA polymerase gamma) and OPA1 (optic atrophy protein 1, an anti-apoptotic protein ensuring the processing of mitochondrial inner membrane), may play a major role in the constitution of an mtDNA pool sufficiently large to ensure oocyte competence, with this role being impaired in patients with DOR. Indeed, in our study, the combination of clinical biomarkers together with mitochondrial biomarkers, such as the mtDNA content in oocytes and CGCs, and the mRNA of mitochondrial regulators in CGCs, provided a highly predictive indication of mtDNA oocyte content (Boucret *et al.*, 2015).

In a mouse model of fragile X primary ovarian, it has been shown that CGCs and oocytes have decreased mitochondrial content, structurally abnormal mitochondria and reduced expression of critical mitochondrial genes, suggesting the implication of mitochondrial dysfunction in the genesis of this particular case of premature ovarian reserve decline (Conca Dioguardi *et al.*, 2016).

CGCs offer one of the best noninvasive approaches for evaluating the metabolic processes underlying the quality of oocytes (McKenzie *et al.*, 2004; Assou *et al.*, 2006; Cillo *et al.*, 2007; Feuerstein *et al.*, 2007; Gasca *et al.*, 2007; Assou *et al.*, 2008; Hamel *et al.*, 2008; van Montfoort *et al.*, 2008; Adriaenssens *et al.*, 2010; Feuerstein *et al.*, 2012). We have found smaller mtDNA copy numbers not only in oocytes but also in the CGCs of DOR compared to NOR patients. Moreover, for a given oocyte-cumulus complex, the mtDNA content of the CGCs was positively correlated with that of the corresponding oocyte (Boucret *et al.*, 2015). Similarly, in a recent study on the pig,

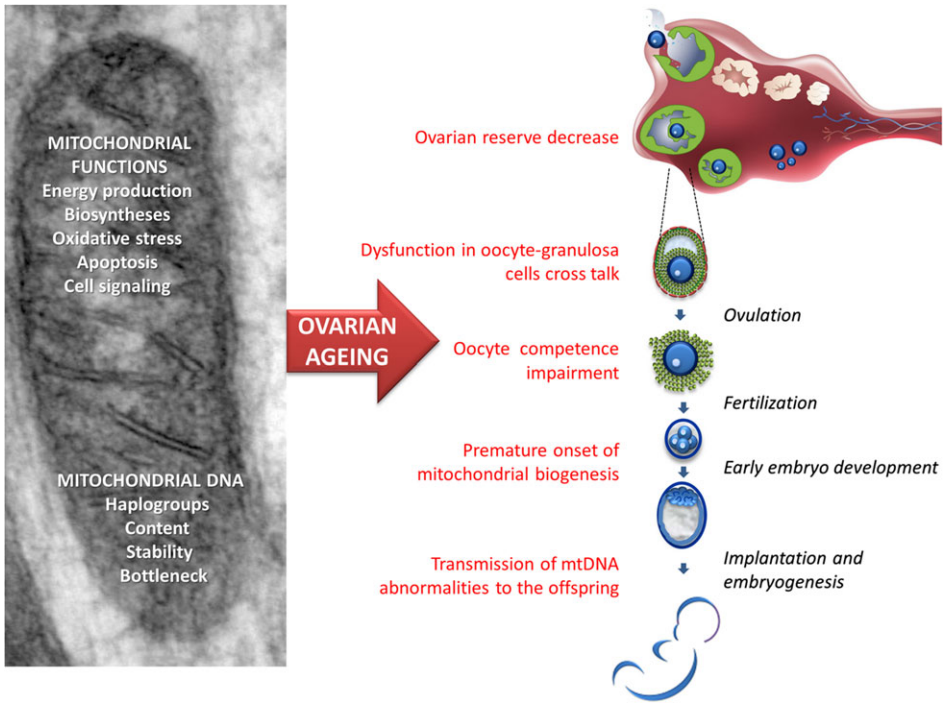

Figure 3 Overview of the multiple implications of mitochondria in ovarian ageing and their deleterious consequences on reproductive function.

the mtDNA content in the oocyte was found to be correlated with that in the CGCs (Pawlak *et al.*, 2015). This implies that, like the mtDNA of the oocyte, the mtDNA of the CGCs surrounding a specific oocyte may assimilate its competence so as to support normal embryonic development. Interestingly, mtDNA copy numbers in CGCs appear to be good predictors of embryo quality in IVF procedures with positive and negative predictive values of 84.4% and 82.1%, respectively (Ogino *et al.*, 2016).

Conclusion

The multiple implications of mitochondria on ovarian ageing and their deleterious consequences on the reproductive function are summed up in Fig. 3. Investigation of the pathophysiological role played by mitochondria in ovarian ageing shows a close relationship between mtDNA and oocyte quality. The mtDNA JT macro-haplogroup constitutes a hereditary factor protecting against DOR. The age-related mtDNA instability, which leads to the accumulation of mtDNA mutations in the oocyte, may play a key role in the deterioration of oocyte quality, in terms of competence and of the risk of transmitting mitochondrial abnormalities to the offspring. Low oocyte mtDNA content, reflecting low mitochondrial content and insufficient biogenesis of the organelle during oocyte maturation, is a significant indicator of poor oocyte quality, which in turn is predictive of poor embryonic development. Abnormal mitochondrial biogenesis, occurring prematurely during early embryogenesis to compensate low mitochondrial content, also attests to the dynamic control exerted by mitochondria on embryonic development. Finally, mitochondrial biogenesis and the mtDNA content in CGCs appear closely correlated with those of oocytes, thus suggesting that CGC mitochondria could usefully serve as biomarkers of oocyte quality.

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Authors' roles

P.M.P. and P.R. reviewed the literature and wrote the paper. They take primary responsibility for the paper. L.B., J.M.C.B., V.D., V.F. and VP participated in the work of the team on which this review is based. C.M. and P.D. are the clinicians responsible for recruiting the patients included in our previous studies. All the authors contributed to the revision of the article, and approved the final version.

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Conflict of interest

None declared.

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