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Mitochondrial functionality in reproduction: from gonads and gametes to embryos and embryonic stem cells

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BACKGROUND: Mitochondria are multitasking organelles involved in ATP synthesis, reactive oxygen species (ROS) production, calcium signalling and apoptosis; and mitochondrial defects are known to cause physiological dysfunction, including infertility. The goal of this review was to identify and discuss common themes in mitochondrial function related to mammalian reproduction.

METHODS: The scientific literature was searched for studies reporting on the several aspects of mitochondrial activity in mammalian testis, sperm, oocytes, early embryos and embryonic stem cells.

RESULTS: ATP synthesis and ROS production are the most discussed aspects of mitochondrial function. Metabolic shifts from mitochondria-produced ATP to glycolysis occur at several stages, notably during gametogenesis and early embryo development, either reflecting developmental switches or substrate availability. The exact role of sperm mitochondria is especially controversial. Mitochondria-generated ROS function in signalling but are mostly described when produced under pathological conditions. Mitochondria-based calcium signalling is primarily important in embryo activation and embryonic stem cell differentiation. Besides pathologically triggered apoptosis, mitochondria participate in apoptotic events related to the regulation of spermatogonial cell number, as well as gamete, embryo and embryonic stem cell quality. Interestingly, data from knock-out (KO) mice is not always straightforward in terms of expected phenotypes. Finally, recent data suggests that mitochondrial activity can modulate embryonic stem cell pluripotency as well as differentiation into distinct cellular fates.

CONCLUSIONS: Mitochondria-based events regulate different aspects of reproductive function, but these are not uniform throughout the several systems reviewed. Low mitochondrial activity seems a feature of 'stemness', being described in spermatogonia, early embryo, inner cell mass cells and embryonic stem cells.

Key words: mitochondria / testis / sperm / oocyte / embryonic stem cells

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Introduction

Mitochondria are double-membrane organelles that play a fundamental role in the cell, and mitochondrial dysfunction has been linked with several pathologies, including infertility (reviewed in Wallace, 1999). Although sharing a general pattern, mitochondria can have distinct features, based on inner membrane invaginations (cristae) and matrix structure. Depending on the cell type and functional status, mitochondria present an extensive range of morphologies, are functionally heterogeneous (Collins *et al*., 2002), and vary in number (reviewed in Meinhardt *et al*., 1999). ATP synthesis by oxidative phosphorylation (OXPHOS) is the primary function associated with mitochondria. During this process electrons derived from the oxidation of substrates are led through redox carriers of the inner membrane electron transfer chain (ETC) to the final acceptor, molecular oxygen (O_2). Electron transfer is associated with proton pumping into the intermembrane space at complexes I, III and IV. This establishes an H^+-based electric and chemical gradient ($\Delta\psi$ and ΔpH, respectively), then used to drive ATP synthesis via complex V (ATP synthase) (Fig. 1) (Darley-Usmar *et al*., 1987; Harris, 1995). OXPHOS is the most efficient way to oxidize substrates; however, glycolytic enzymes are evolutionarily older and have reached catalytic perfection, thus suggesting that glycolysis may be the pathway of choice if glycolytic substrates are plentiful and oxygen is low.

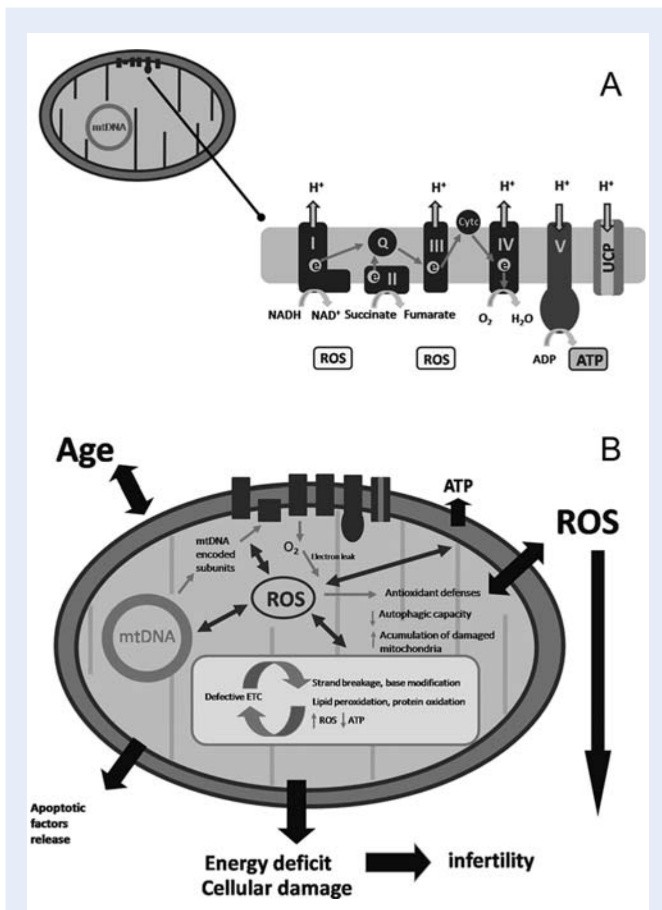

Figure 1 Roles of mitochondria.

(**A**) The electron transfer chain (ETC), of the inner mitochondrial membrane involved in oxidative phosphorylation (OXPHOS) and reactive oxygen special (ROS) production, including ETC complexes (I, II, III, IV and V), electron carriers ubiquinone (Q) and cytochrome c (cyct) and uncoupling proteins (UCP). (**B**) Integration of mitochondrial functions, including ATP and ROS production, activation of apoptosis and effects of oxidative stress.

ATP production can vary to match energy demands, and mitochondria are heterogeneous in different tissues. Long-term adaptations to various rates of ATP utilization can be achieved by modifying the number, morphology and location of mitochondria, as well as the proportions of certain ETC constituents (Meinhardt *et al*., 1999; Nogueira *et al*., 2001). Two morphologic states have been defined in mitochondria: in the orthodox state cristae tend to be tubes or short flat lamellae with few junctions in the peripheral region of the inner membrane, although condensed mitochondria have larger internal compartments with multiple tubular connections (reviewed in Mannella, 2006a; Fig. 2). These states are interchangeable, and seem to be related to mitochondrial status, with mitochondria displaying condensed conformation when ADP is in excess but reverting to the orthodox state when ADP is limiting (Mannella, 2006b). However, changes in mitochondrial architecture may also merely reflect osmotic changes in the local environment (Mannella, 2008).

Mitochondria are also characterized by having their own, maternally inherited genome (Giles *et al*., 1980), mitochondrial DNA (mtDNA). Human mtDNA is a double stranded circular molecule encoding 13 polypeptides that are essential subunits of ETC complexes, 22 tRNAs and 2 rRNAs, that are necessary for their translation (Anderson *et al*., 1981). The maintenance and expression of the mitochondrial genome is controlled by nuclear-encoded factors that are translocated to the mitochondria (St John *et al*., 2007).

All in all, 85–90% of a cell's oxygen is consumed by mitochondria in OXPHOS. However, this comes with an undesirable extra, the production of potentially harmful reactive oxygen species (ROS). Mitochondria are the major ROS generator, with 0.2–2% of the oxygen taken up by the cells converted to ROS by mitochondria (reviewed in Harper *et al*., 2004; Orrenius *et al*., 2007). At several sites along the ETC (namely complexes I and III) electrons can react directly with oxygen or other electron acceptors, and generate free radicals (Muller *et al*., 2004; Grivennikova and Vinogradov, 2006; Fig. 1). As a result, mitochondria need continuous protection, provided by various low-molecular-weight antioxidants, as well as by multiple enzymatic defence systems (for review see Ott *et al*., 2007). However, recent evidence also highlights a specific role of ROS in cell signalling (reviewed in Orrenius *et al*., 2007). Nonetheless, mitochondrial ROS production is very sensitive to the proton-motive force. As their name indicates, UCPs uncouple proton flux through the inner mitochondrial membranes and ATP synthesis, providing a route for proton re-entry (Mattiasson and Sullivan, 2006) (Fig. 1), lowering the proton-motive force and attenuating mitochondrial ROS production (Brand and Esteves, 2005).

Another major role is mitochondrial participation in apoptosis (namely via the 'intrinsic' apoptosis pathway). A wide range of stress stimuli can be transduced to mitochondria, resulting in increased permeability to mitochondrial proteins such as cytochrome c, which plays a prominent role in promoting the caspase cascade of cell execution (reviewed in Khosravi-Far and Esposti, 2004). Once released to the cytoplasm, cytochrome c triggers a series of events leading to the proteolytic activation of executioner caspases 3, 6 and 7 (reviewed in Orrenius *et al*., 2007). Several factors can regulate mitochondria-mediated apoptosis. Notably, Bcl-2 family members may either

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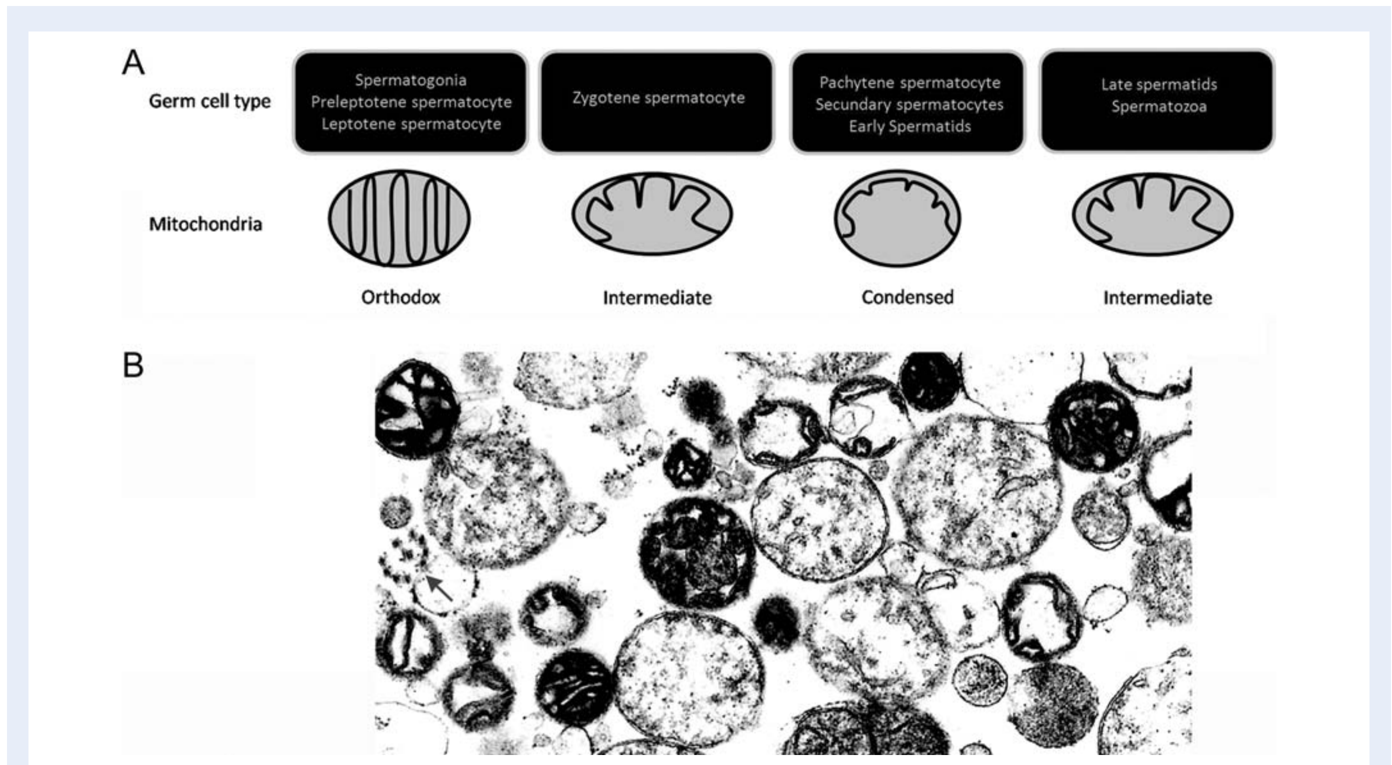

Figure 2 Testis mitochondria.

(**A**) Different types of mitochondria present in male germ cells. See text for discussion. (**B**) Electron microscopy of rat testicular mitochondria highlighting the heterogeneous mix of mitochondrial types shown in A. Arrow highlights a cross-section typical of the sperm axoneme microtubule organization.

promote cell survival (Bcl-2, Bcl-xL, Bcl-w, Mcl-1, A1/Bfl-1) or lead to cell death (Bax, Bak, Bcl-Xs, Bad, Bid, Bik, Hrk, Bok) interacting to form homo- and heterodimers, their relative abundance being the determinant of the threshold of apoptosis (Zamzami *et al*.,1998).

The assessment of mitochondrial functionality can be carried out using oxygen and TPP^+ (tetraphenyl phosphonium) electrodes to analyse respiration and electric membrane potential, respectively. The fluorescent probe Calcium Green can be used to access calcium buffering capacity (Amaral *et al*., 2009), and a range of cationic fluorescent probes, which accumulate in either isolated or cellular mitochondria depending on mitochondrial membrane potential (MMP or $\Delta\Psi$), although controls must always be carried out to ensure proper functional measurements (Ramalho-Santos *et al*., 2007). Loss of mitochondrial function can be mimicked *in vitro* by means of specific drugs that either inhibit the ETC or eliminate the mandatory link between the respiratory chain and the phosphorylation system (OXPHOS uncouplers) (Table I).

Table I Mitochondrial inhibitors and their effects

Effect	Drug	Mode of action
ETC inhibiton	Amytal, Rotenone	Blocks Complex I
	Atpenins, TIFA	Blocks Complex II
	Antimycin A, Myxothiazol	Blocks Complex III
	Azide, Carbon monoxide, Cyanide	Blocks Complex IV
	Oligomycin, DCCD	Blocks Complex V
OXPHOS uncoupling	CCCP, FCCP, Dinitrophenol	Hydrophobic proton carriers
	Valinomycin	K+ ionophore
	Nigericin	K+/H+ mobile carrier
Krebs cycle inhibition	Malonate	Succinate analog; Inhibits succinate dehydrogenase (Complex II)
Transport inhibiton	Atractyloside	Blocks ANT
Ca^{2+} uptake inhibition	Ruthenium red	Inhibits the mitochondrial Ca^{2+} uniporter

CCCP, Carbonyl cyanide m-chloro phenyl hydrazone; DCCD, Diclychlohexylcarbodiimide; FCCP, Carbonyl cyanide-p-trifluoromethoxyphenol hydrozone; TIFA, 2-Thenoyltrifluoroacetate; ANT, Adenine nucleotide translocator.

Search method

A computerized literature search was conducted using Medline and Web of Knowledge for aspects of mitochondrial function in testis, sperm, oocytes, early embryos and embryonic stem cells (ESCs). Mitochondrial morphology, mitochondrial localization, membrane potential, electron transfer complex activity, ATP synthesis, ROS production, antioxidant defences, calcium signalling and apoptosis were the aspects searched for. Given the controversial issue concerning the origin of ATP for different steps of gametogenesis, sperm motility and embryo development, other metabolic pathways were also investigated in these cases. mtDNA was not included as a primary search

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criteria, given that it merits a lengthy separate discussion, beyond the scope of the current review, although some aspects are briefly included where they pertain to other aspects of mitochondrial function. Relevant literature was selected to determine common themes throughout the reproductive system. Given the volume of literature obtained review articles are cited for general or less controversial topics.

Testis mitochondria

Morphology

Morphology, localization and energy metabolism of testicular mitochondria change markedly during spermatogenesis, and three types of mitochondria are recognizable: orthodox-type mitochondria in Sertoli cells, spermatogonia and preleptotene and leptotene spermatocytes; the intermediate form in zygotene spermatocytes; and the condensed form in pachytene and secondary spermatocytes and early spermatids, a conformation that shifts back to the intermediate form in late spermatids and spermatozoa (De Martino *et al.*, 1979) (Fig. 2). An association between germ cell mitochondrial morphology and metabolic status during spermatogenesis was postulated, in which the condensed form presents higher efficiency (De Martino *et al.*, 1979). These morphological changes may be supported/induced by factors released by Sertoli cells. In fact, Activin A was described as an inducer of the condensed form, which may be one of the factors contributing to the regulation of germ cell differentiation by Sertoli cells (Meinhardt *et al.*, 2000).

Leydig cell mitochondria present lamellar cristae in close association, with a gap between apposing lamellae of $\sim$4 nm, a unique feature of steroid-producing cells. Although the functional significance of these structures is unknown, it has been suggested that they are not involved in ATP production since the close apposition of membranes does not allow for the presence of ATP synthase (Prince, 2002).

Testis-specific components

Concomitant with the described structural changes, several mitochondrial proteins, such as heat shock protein (hsp) 60 and 70, Lon protease and sulphidryl oxidase (SOx), are known to be differentially expressed during distinct phases of spermatogenesis (for review see Meinhardt *et al.*, 1999). Specific isoforms are also found in testicular mitochondria, such as cytochrome c and subunit VIb-2 of the cytochrome c oxidase (COX) (Hess *et al.*, 1993; Hüttemann *et al.*, 2003). Finally other proteins are preferentially expressed in the testis (see Table II). Mutations on the mitochondrial 'Drosophila'

Table II **Mitochondrial testicular proteins and their effects on reproduction**

Protein	Unique features	Function	Reference
Mt-Hsp60	Expressed in Sertoli and Leydig cells, spermatogonia and early spermatocytes	Post translational maturation, translocation of polypeptides, binding of unfolded or partially folded proteins	Reviewed in Meinhardt *et al.* (1999)
Mt LON proteases	Only expressed in the mitochondrial matrix of early meiotic cells, such as leptotene and zygotene spermatocytes	Proteolytic ATP-dependent enzyme involved in mtDNA integrity and degradation of misfolded proteins (in bacteria)	Seitz *et al.* (1995)
mt SOx	Only expressed in pachytene and early round spermatids	Oxidation of sulphydryl compounds (ex: glutathione, cystein and thioglycerol)	Seitz *et al.* (1995)
Testicular cytochrome c isoform	Starts to be expressed in zygotene spermatocytes slowly taking the place of somatic cytochrome c	Same functions as somatic cytochrome c but with enhanced anti H_2O_2 capability and higher pro-apoptotic activity	Liu *et al.* (2006), Hess *et al.* (1993)
Subunit Vib-2-of the COX	Testicular isoform of COX subunit Vib-2	Electron transport	Hüttemann *et al.* (2003)
SCaMC-3-Like	Lacks the Ca^{2+}-binding N-extension	ATP-Mg/Pi exchange (catalyze the net accumulation or depletion of mitochondrial adenine nucleotides)	Traba *et al.* (2008)
COXII	High levels of expression in pachytene spermatocytes	Encoded by mitochondrial genome, may reflect higher energy requirements of meiotic cells	Saunders *et al.* (1993)
AACA/slc25a31		ADP/ATP translocases (involved in OXPHOS)	Traba *et al.* (2008)
DSP21	Peripheral membrane protein of the inner mitochondrial membrane	Atypical dual specificity phosphatases	Rardin *et al.* (2008)
GPAT2	Expressed at high levels in the testis and at much lower levels in all other tissues	Synthesis of triacylglycerol and all glycerophospholipids	Wang *et al.*, 2007
PDH2	Compensates for the loss of expression the somatic form (PDH1) associated with the x chromosome	Pyruvate dehydrogenase	Korotchkina *et al.* (2006)
Mt Ferritin	mf Ft expression appears correlate with management of ATP burst in a short time	Highly expressed in human testis, particularly in spermatocytes and Leydig cells	Santambrogio *et al.* (2007)
Mitochondrial fission regulator 1 (Mtfr1)	Highest level of expression in pubertal and adult testis, in particularly by the haploid germ cells and Leydig cells	Associated with the mitochondrial inner membrane that induces mitochondrial fission	Monticone *et al.* (2007)

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protein Merlin, common to somatic cells (ortholog in humans is 'Neurofibromatosis'), produces viable but sterile males, indicating that the induced deregulation on mitochondrial function, although not affecting somatic cells, has profound implications on germ cell differentiation (Dorogova *et al*., 2008). However, this is certainly not a general effect, as mice lacking a testis-specific translocase (Tom 34b) are normal and fertile (Terada *et al*., 2003).

Leydig cell steroidogenesis

Leydig cell steroidogenesis is reliant on mitochondrial functionality as demonstrated using the MA 10 Leydig cell line. Results clearly showed that $\Delta\psi$, ATP synthesis, ΔpH and mitochondrial [Ca^{2+}] are all required for steroid biosynthesis (Hales *et al*., 2005). Myxothiazol (a complex III blocker) inhibits LH-stimulated testosterone production in multiple sites along the steroidogenic pathway (Midzak *et al*., 2007). In addition Leydig cell mitochondria are influenced by ROS, notably during the cholesterol transfer step (Stocco *et al*., 1993). These ROS are also used by macrophages as mediators to modulate Leydig cell activity (Hales, 2002).

Testicular energy metabolism

Testicular mitochondria have different bioenergetic parameters when compared with mitochondria harvested from other tissues. Specifically, testis mitochondria are shown to consume less oxygen to generate approximately the same maximum electric potential as other tissues, and show an age-related modification in phosphorylative efficiency with young animals presenting less efficient phosphorylation (Mota *et al*., 2009; Amaral *et al*., 2009). These observations suggest that, contrary to previous studies, testicular mitochondria should be considered as the primary mitochondrial toxicological model to test the effect of distinct substances on male gametogenesis (Tavares *et al*., 2009).

Testis-specific morphogenetic events suggest that male gonads have a higher energy requirement than ovaries, starting early at the time of *Sry* activation (Matoba *et al*., 2008). Because spermatogonial stem cells (SSCs) are slow-dividing, it is expected that low MMP might be a shared characteristic with other stem cells (see below). The neonate (0–5 days post-partum (dpp)) rat testis cell fraction with the highest concentration of SSCs includes gonocytes, and exhibits low $\Delta\Psi$, although stem cells in rat pup (8–14 dpp) testes appear to have more active mitochondria than their gonocyte precursors, which might reflect increased proliferative activity as this population expands to fill the rapidly increasing number of SSC niches (Ryu *et al*., 2004).

In the adult testis the survival of germ cells is dependent on carbohydrate metabolism, including both anaerobic (glycolysis) and aerobic (OXPHOS) pathways. However, different cell types differ in their preferred substrates (Robinson and Fritz, 1981; Grootegoed *et al*., 1984; Nakamura *et al*., 1984; Bajpai *et al*., 1998; Meinhardt *et al*., 1999). In fact, the formation of the blood-testis barrier and the alteration in the surrounding medium, cause a considerable change in the energy metabolism of germ cells. Spermatogonia in the basal compartment are supplied exclusively by blood components. However, after passage to the luminal compartment germ cells rely on the breakdown of lactate and pyruvate provided by Sertoli cells (reviewed in Boussouar and Benhamed, 2004). Therefore, spermatogonia, mature sperm and the somatic Sertoli cells exhibit high glycolytic activity, whereas spermatocytes and spermatids produce ATP mainly by OXPHOS (Robinson and Fritz, 1981; Grootegoed *et al*., 1984; Nakamura *et al*., 1984; Bajpai *et al*., 1998; Meinhardt *et al*., 1999).

This could also be a matter of opportunity: since seminiferous tubule fluid is rich in lactate and poor in glucose, it is hypothesized that, even though spermatocytes have the machinery to produce energy through glycolysis, they rely mostly on lactate (Bajpai *et al*., 1998). Nonetheless, there are incongruities between availability and usability. Blood vessels, located exclusively between tubules, supply the oxygen needed to perform OXPHOS that only reaches the lumen of the seminiferous tubules by diffusion (Wenger and Katschinski, 2005). The facilitated access of spermatogonia to oxygen would lead us to expect the use of OXPHOS, instead of glycolysis. Similarly, having less access to oxygen, spermatocytes were expected to perform glycolysis. However, the substrate availability imposed by seminiferous tubules compartmentalization, together with ATP demand, may prime the cell to different adaptations. It is also possible that stem cells maintain a low metabolism to avoid ROS-related damage.

Mitochondria-related apoptosis in the testis

In normal spermatogenesis not all germ cells reach maturity, and the normal physiological death of germ cells via apoptosis seems to be a constant event which can be potentiated by various stimuli (reviewed in Sinha-Hikim *et al*., 2003). Caspases are not only effectors of the apoptotic process but can induce its activation through the mitochondrial pathway. Caspase 2 expression is increased in 16 dpp rat testis, when germ cell apoptosis also peaks. The increased amounts of activated caspase 2 in mitochondria was matched by an increased level of cytochrome c release. Specific inhibitors of caspase 2 mitigate the increased cytochrome c release, indicating an important upstream role of mitochondria in germ cell apoptosis during the first wave of spermatogenesis (Zheng *et al*., 2006).

Bcl-2 family members are also crucial during the first spermatogenic wave (Rodriguez *et al*., 1997). Bax is required to induce germ cell death in dividing spermatogonia, at the time point at which their number is regulated in a density-dependent manner (Russell *et al*., 2002), as shown by infertile Bax KO mice that present accumulation of atypical premeiotic germ cells but no mature haploid sperm (Knudson *et al*., 1995). In contrast, male bcl-w-deficient mice display normal testicular development before puberty, although after puberty Sertoli and germ cells of all types are severely reduced in number, and seminiferous tubules contain many apoptotic cells and no mature sperm (Yan *et al*., 2000). In a different study using cynomologus monkeys (*Macaca fascicularis*), hormone deprivation, heat, or both, led to an increase in Bcl-2 levels in testicular lysates and increased cytochrome c and Smac/Diablo release (Jia *et al*., 2007). In mice and humans, hormone deprivation and heat-induced male germ cell death, also induced the mitochondria-dependent apoptotic pathway (Vera *et al*., 2004, 2006). However, recent research described activation of both intrinsic and extrinsic (Fas-mediated) apoptotic pathways in situations of FSH and testosterone depletion in rats and mice (Pareek *et al*., 2007).

Interestingly, inhibition of ATP synthase decreased ATP levels and suppressed cell death, an effect not seen with inhibition of glycolysis,

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indicating that mitochondrial ATP production plays a role in regulating male germ cell apoptosis (Erkkila *et al*., 2006). In a different study, apoptosis was triggered by incubating segments of seminiferous tubules without survival factors (i.e. serum or hormones), and apoptosis in spermatocytes and spermatids was significantly repressed at low levels of oxygen or by inhibitors of mitochondrial transitory permeability, revealing another aspect of mitochondrial function in apoptosis (Erkkila *et al*., 1999). Finally, germ cell mitochondria present a unique feature in apoptosis: testicular cytochrome c isoform shows a 3–5-fold greater apoptotic activity than the somatic isoform (Liu *et al*., 2006). Interestingly, testicular cytochrome c KO mice are fertile but present with highly atrophied testes, with a reduced number of spermatocytes, spermatids and spermatozoa (Narisawa *et al*., 2002).

Testicular ROS and antioxidant defences

Because mitochondria are the major source of intracellular ROS they need constant protection from these species, accomplished through a wide network of mitochondrial non-enzymatic and enzymatic antioxidant defences (reviewed in Ott *et al*., 2007). Thioredoxin glutathione reductase, a redox enzyme, accumulates in testes after puberty, primarily in elongating spermatids at the site of the mitochondrial sheath formation (Su *et al*., 2005). Previously, the same pattern of distribution had been described for glutathione-peroxidase 4 (GPX4) (Roveri *et al*., 1992). Interestingly, prolonged expression of this enzyme during the spermatogenic cycle (observed in transgenic mice bearing the rat GPX4) revealed a number of spermatogenic defects including primary spermatocyte apoptosis, haploid cell loss, seminiferous epithelium disorganization and reduced fertility (Puglisi *et al*., 2007). In agreement with this result, high levels of manganese superoxide dismutase (SOD) were associated with small testis, male infertility, and decreased female fertility (Raineri *et al*., 2001). Results obtained in these studies suggest that any deregulation, or either loss/overexpression of mitochondrial antioxidant enzymes, disrupt normal homeostasis of the seminiferous epithelium resulting in reduced fertility.

Pathological effects of aging on testicular mitochondria

Cellular ageing has been linked to increased ROS production and mitochondrial dysfunction (Harman, 1983; Jonhson *et al*., 1999). Analysis of testis mitochondria has shown differences in ETC complexes and membrane fatty acid composition throughout development and ageing. In fact, the activity of ETC complexes follows a common pattern, with an increase during mitosis and first meiosis of germ cells, and a decrease with ageing. These changes were correlated with a drop in polyunsaturated fatty acid content, increased production of superoxide and reduced SOD activity (Vazquez-Memije *et al*., 2005, 2008). In fact, the balance between pro and antioxidant agents in the ageing testis is shifted towards pro-oxidation (Rebrin *et al*., 2003). Using domestic cat testicular mitochondria it was recently shown that membrane potential reached a maximum in animals with fully active reproductive function, after a pubertal period of increasing values of mitochondrial electric potential (Mota *et al*., 2009). In the rat, there was a peak of functionality in adult animals, a decrease with age, was coupled with an increase in expression and activity of uncoupling protein 2 (UCP-2), suggesting that proton leakage may have a protective role in managing age-dependent mitochondrial dysfunction (Amaral *et al*., 2008). Mitochondria from Leydig cells also present alterations with age, consistent with the proposal that mitochondria-derived ROS may play a role in the decline in testosterone production (reviewed in Zirkin and Chen, 2000).

Sperm mitochondria

During the differentiation of spermatids into sperm (spermiogenesis), some mitochondria, like much of the cytoplasm, are lost in the so-called residual bodies, whilst those remaining rearrange in elongated tubular structures (Ho and Wey, 2007) and are packed helically around the anterior portion of the flagellum. Concomitantly, the number of mtDNA molecules per haploid genome is reduced (Hecht *et al*., 1984), which is probably mediated by the down-regulation of mitochondrial transcription factor A (TFAM) (Larsson *et al*., 1996, 1997). Once the process is concluded mature mammalian sperm possess 22–75 mitochondria arranged end to end in the midpiece (Otani *et al*., 1988). The anchorage of the mitochondrial sheath to the axoneme is supported by the sub-mitochondrial reticulum, a complex of filaments that seems to sustain mitochondrial organization (Olson and Winfrey, 1990). Furthermore, the outer membranes of sperm mitochondria are enclosed in a keratinous structure, the mitochondrial capsule, formed by disulfide bonds between cysteine- and proline-rich selenoproteins, including the sperm-specific phospholipid hydroperoxidase glutathione peroxidase (Ursini *et al*., 1999). This structure appears to confer mechanical stability, and is responsible for some distinctive features of sperm mitochondria, namely the resistance to hypo-osmotic stress, and the unfeasibility of completely isolating these organelles. The fact that some mitochondria are evolutionarily retained in a very specialized sperm region suggests that these organelles fulfil a crucial role in sperm function. However, their physiological significance is still unclear.

Mitochondrial activity and sperm function

Mitochondria may supply sperm with energy for several purposes, including sperm motility. Electron microscopy revealed that sperm from asthenozoospermic samples have disordered mitochondria, significantly shorter midpieces and fewer mitochondrial gyres than normozoospermic counterparts, whereas midpiece widths and tail lengths were similar (Mundy *et al*., 1995). Moreover, enzymatic activities of ETC complexes are positively correlated with human sperm quality, particularly motility (Ruiz-Pesini *et al*., 1998, 2000b). Likewise, the expression of ETC subunits is associated with human sperm quality (Amaral *et al*., 2007).

The relevance of mitochondrial activity in sperm function has been studied at the gene level, namely the significance of sperm mtDNA integrity in male (in)fertility. Although some conflicting results have been published on the effects of specific mitochondrial point mutations and large-scale deletions (for review see St John *et al*., 2007), it seems consensual that the accumulation of multiple mtDNA rearrangements is associated with loss of sperm function (St. John *et al*., 2001). Similarly, both the number and size of mtDNA deletions in either testicular or ejaculated sperm are negatively correlated with ICSI pregnancy outcomes (Lewis *et al*., 2004), clearly showing the deleterious effect of mtDNA

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rearrangements, even when low motility is bypassed. These outcomes have also been supported by transmitochondrial mice models, where the accumulation of pathogenic mtDNA-derived ETC defects was responsible for male infertility (Nakada *et al*., 2006). Furthermore, certain mtDNA haplogroups (groups of specific mtDNA types) have been suggested to predispose for reduced sperm motility (Ruiz-Pesini *et al*., 2000a; Montiel-Sosa *et al*., 2006), although this has been contradicted by others (Pereira *et al*., 2005). On the other hand, a number of reports have suggested that low quality sperm present abnormal mtDNA content (May-Panloup *et al*., 2003; Amaral *et al*., 2007), and the expression of TFAM and of the catalytic subunit of DNA polymerase gamma (POLG), both of which are implicated in the regulation of mtDNA copy number, are both lower in poorer quality sperm (Amaral *et al*., 2007). The relevance of POLG in male infertility has also been confirmed using mouse models: homozygous knock-in mice expressing a deficient POLG presented increased levels of mtDNA mutations and deletions, and showed reduced lifespan and premature onset of aging-related phenotypes, including reduced fertility (Trifunovic *et al*., 2004). In addition, polymorphisms in the CAG-repeat region of POLG are possibly associated with sperm quality, although their true significance in male infertility is questionable (Amaral *et al*., 2007). Overall, and despite the controversies on the exact effect of a particular mtDNA point mutation, deletion or haplogroup in human sperm, there is clear evidence that alterations in the mitochondrial genome can compromise sperm motility and function.

The link between mitochondria-generated ATP and sperm motility/fertilization competence has been illustrated by $\Delta\psi$ evaluation (Fig. 3). Several studies confirmed that motility is related to mitochondrial functional status in humans (Troiano *et al*., 1998), equines (Love*et al*., 2003), rats (Gravance *et al*., 2001), boars (Spinaci *et al*., 2005) and rams (Martinez-Pastor *et al*., 2004). Additionally, at least in humans, sperm fertilization potential (measured as fertilization rates after IVF) is strongly related to $\Delta\psi$ and thus to mitochondrial functionality (Kasai *et al*., 2002; Marchetti *et al*., 2002, 2004b). On the other hand, $\Delta\psi$ seems to negatively correlate with both DNA fragmentation and the generation of ROS (Wang *et al*., 2003). Mirroring the importance of mitochondria to sperm function, subpopulations of mitochondria with high $\Delta\psi$ are enriched in cells with elevated fertilization capabilities (Gallon *et al*., 2006). Interestingly, it has recently been proposed that the ultimate cause for the negative effect of endocannabinoids in male reproduction may be the reduction of sperm mitochondrial activity (Rossato, 2008). These cumulative outcomes strongly suggest $\Delta\psi$ as an indicator of sperm functional status.

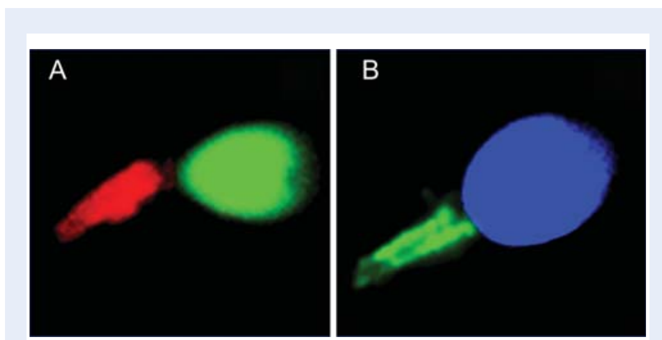

Figure 3 Mitochondria in human sperm.

(**A**) Mitochondrial membrane potential (MMP) detection in live human sperm using Mitotracker Red (red), nuclear DNA is counterstained with SYBR 14 (green). (**B**) Immunodetection of a subunit of COX, part of the electron transfer chain (ETC) (complex IV), in the sperm midpiece (green), nuclear DNA is counterstained with DAPI (blue).

The role of sperm mitochondrial metabolism is controversial

Sperm require ATP mainly for motility, as well as for the cellular events involved in hyperactivation, capacitation and the acrosome reaction. The provenance of the ATP that fuels these events, especially motility, has been discussed for decades: is it derived from OXPHOS, or purely glycolytic? The debate has been confounded by possible species-specific discrepancies, as well as by differences in experimental conditions. The usual approach is to analyse motility and/or ATP levels after incubating sperm in media containing different substrates, in the presence or absence of inhibitors. However, media composition, temperatures and times of incubation, as well as the inhibitors and their concentrations, vary from study to study.

The compartmentalization of mitochondria in the sperm midpiece may limit the availability of OXPHOS-derived ATP for the dynein-ATPases located in the principal piece. This is especially true in species with long sperm tails, such as rodents, as it is doubtful that sufficient ATP can diffuse to the distal end of the flagellum. This problem could be solved via ATP shuttles, but their significance in mammals needs further confirmation (Ford, 2006). Alternatively, ATP can be produced in the principal piece through the glycolytic pathway. Indeed, glycolytic enzymes seem to be compartmentalized in the fibrous sheath, a cytoskeletal element of the principal piece (Krisfalusi *et al*., 2006; Kim *et al*., 2007).

Elegant work was done using hypotonically treated cells, i.e. with disrupted membranes, to solve the difficulty in isolating sperm mitochondria and then analysing oxygen consumption and respiration rates with various substrates (Keyhani and Storey, 1973; Storey, 1978; Carey *et al*., 1981). Results were species-specific, i.e. sperm from distinct species have dissimilar capabilities to metabolize different substrates, suggesting that mitochondrial activity in sperm of a given species is adjusted to the substrate content of the female tract (Storey, 1980).

Human sperm motility and ATP content can be maintained in the presence of a glycolyzable substrate, but decline rapidly in its absence, even in the presence of oxidizable substrates (Peterson and Freund, 1970; Williams and Ford, 2001). On the other hand, the previously stated fact that mutations in the mitochondrial genome are associated with decreased sperm quality suggests that OXPHOS is also relevant. Interesting outcomes were obtained from a patient harbouring a mtDNA point mutation causing reduced activity of the ETC complex I, and whose sperm presented low-motility. Supplementation with succinate, a substrate for complex II, circumventing the effect of the mutation, resulted in an increase in sperm motility (Folgero *et al*., 1993). Furthermore, incubation of human sperm with distinct ETC inhibitors in media containing glucose, resulted in rapid decreases in sperm motility (Ruiz-Pesini *et al*., 2000a; St John *et al*., 2005a, b).

Experiments in rat sperm have shown that 6-chloro-6-deoxysugars prevent glucose metabolism, and have contraceptive action (Ford *et al*., 1981a, b). Sperm became immotile and presented low ATP

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levels when glucose was the only substrate available, but no detrimental effects were observed in the presence of pyruvate and lactate (Ford and Harrison, 1981a, b). Likewise, sperm from control rats presented higher motility and ATP levels in medium with pyruvate and lactate. α-Chlorohydrin has also been used to inhibit glycolysis, in epididymal ram and ejaculated boar sperm, resulting in decreased motility and ATP concentration, but only in the presence of glucose (Ford and Harrison, 1985, 1986). Taken together, these outcomes may indicate a role of OXPHOS in these species, although a contribution of glycolysis is also evident. The results of experiments in mouse epididymal sperm also seem to imply that both glycolysis and OXPHOS are able to sustain sperm motility, although with glycolysis in a predominant role (Mukai and Okuno, 2004).

In testis-specific cytochrome c homozygous knockout mice, males were fertile, although presenting early testicular atrophy, as discussed earlier. Moreover, when compared with wild type, their sperm were less motile, presented lower ATP content and were less successful in *in vitro* fertilization (Narisawa *et al*., 2002). On the other hand, the disruption of the spermatogenic cell-specific glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase-S (GAPDH-S) has also resulted in some remarkable outcomes (Miki *et al*., 2004). Male homozygous KO mice were infertile (but females were fertile) and their sperm showed both decreased motility and ATP levels, although mitochondrial activity was unchanged. These results have been interpreted as proof that glycolysis is essential for sperm motility. However, this view has been challenged (Ford and Harrison, 2006; Ruiz-Pesini *et al*., 2007; Storey, 2008), the reasoning being that the inactivation of GAPDH-S (similarly to 6-chloro-6-deoxysugars, DOG and α-chlorohydrin) block the glycolytic net synthesis of ATP, but the glycolytic ATP-consuming phase still operates. Often neglected is both that glycolysis is usually a prerequisite for OXPHOS and that, unlike OXPHOS, it actually requires ATP to initiate the process, a fact that must always be taken into consideration. Nevertheless, disruption of the glycolytic enzyme lactate dehydrogenase-C_4 (LDH-C_4), normally expressed in spermatogenic cells and also in minor amounts in oocytes, resulted in decreased sperm function and impairment of male fertility in mice (Odet *et al*., 2008). Recently, a new approach has been developed to assess sperm motility, which relies on the use of laser tweezers to measure swimming speeds and force (Nascimento *et al*., 2006). Unexpectedly, no relationship was found between either human or dog sperm motility and $\Delta\psi$, also suggesting a predominant role of glycolysis (Nascimento *et al*., 2008).

The role of OXPHOS versus glycolysis in other events leading to fertilization is believed to be species-specific. To this extent, glucose seems necessary for the acquisition of hyperactivated motility, capacitation and acrosome reaction in both humans and mice (Hoppe, 1976; Fraser and Quinn, 1981; Rogers and Perreault, 1990), and may participate in the protein phosphorylation events occurring in rhesus macaque sperm capacitation (Hung *et al*., 2008). On the other hand, glucose seems to inhibit capacitation in bull and guinea pig (Rogers and Yanagimachi, 1975; Rogers *et al*., 1979; Parrish *et al*., 1989), where oxidizable substrates are required. Also noteworthy are two recent findings using proteomic approaches. The first study concerns sperm epididymal maturation in rodent species (Aitken *et al*., 2007). Apparently, caput epipydimal sperm possess silent mitochondria, although caudal sperm have polarized mitochondria, and therefore epididymal maturation may involve the activation of sperm mitochondria, and mitochondria-generated ATP may facilitate the tyrosine phosphorylation events associated with capacitation. The second study involves the identification of proteomic differences in asthenozoospermic samples (Martinez-Heredia *et al*., 2008). The authors found 17 proteins expressed at different levels in asthenozoospermic samples compared with controls. Interestingly, the list of proteins includes decreased expression of the ETC enzyme COXVIb. In contrast, none of the glycolityc enzymes were affected.

These cumulative reports seem to demonstrate that in the few days it can spend in the female reproductive tract mammalian sperm might be able to utilize both glycolysis and OXPHOS to produce ATP for different purposes. The balance between these two metabolic pathways may vary between species, according to the substrates available during the sperm's route.

Sperm mitochondria and ROS

Although seminal leukocytes were thought to be the only ROS-generators in an ejaculate, it is now well established that sperm are also responsible for some ROS production (Aitken *et al*., 1996). Accordingly, both seminal plasma and sperm possess a number of antioxidant strategies to protect the male gametes against ROS damage. These include enzymes such as SOD, catalase, the glutathione peroxidase/reductase system and non-enzymatic substances, such as ascorbic acid, glutathione and α-tocopherol, among others (reviewed by Tremellen, 2008).

Low and regulated levels of ROS have been implicated in sperm capacitation, acquisition of hyperactivated motility, acrosome reaction and oocyte interaction (for review see de Lamirande *et al*., 1997; Ford, 2004). When the physiological equilibrium between ROS production and scavenging is perturbed, sperm function may be compromised and indeed oxidative stress is implicated in male infertility. Increased ROS levels have been associated with sperm lipoperoxidation damage, decreased motility, DNA fragmentation and increased apoptosis (Agarwal *et al*., 2008). Importantly, it has been recently proposed that mitochondria are a major contributor to the oxidative stress experienced by defective human sperm (Koppers *et al*., 2008).

Mitochondria-related apoptosis in sperm

Although it is well established that a fraction of sperm in any ejaculate presents apoptotic markers (e.g. Varum *et al*., 2007), the mechanisms involved in putative sperm apoptosis are not completely characterized. However, and among other mechanisms, apoptosis can be triggered via the mitochondrial pathway. To this extent, the presence of activated caspase 3 in ejaculated human sperm midpiece was clearly demonstrated (Weng *et al*., 2002), and poorer quality samples exhibited higher levels of active caspase 3 positive-sperm. Furthermore, the activation of caspase 3 is correlated with the externalization of phosphatidylserine (Kotwicka *et al*., 2008). Similar results were obtained for caspase 9 which, when expressed, is also localized in the midpiece (Paasch *et al*., 2004a). Additionally, the treatment of sperm with betulinic acid, an inducer of the intrinsic apoptotic pathway, resulted in loss of $\Delta\psi$ and activation of caspases 3 and 9, with a concomitant decrease in sperm motility (Paasch *et al*., 2004b; Grunewald *et al*., 2005). Indeed, caspase 3 activity seems to be negatively correlated with sperm $\Delta\psi$ (Marchetti *et al*., 2004a). The transcript for the apoptotic marker Bcl-2 is also present in ejaculated sperm, with higher levels

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in infertile men compared with controls (Steger *et al*., 2008). Future studies will be needed to better determine the role of mitochondria in triggering sperm apoptosis, and its relevance in sperm (dys)function.

Other possible roles of sperm mitochondria

The involvement of Ca^{2+} signalling in the regulation of several aspects of mammalian sperm function is very well documented (Felix, 2005; Publicover *et al*., 2008). On the other hand, the ability of mitochondria to store Ca^{2+} has been demonstrated in sperm from diverse species, but its role is unclear (Publicover *et al*., 2007). Thus, although mammalian sperm mitochondria are able to function as intracellular Ca^{2+} stores, a clear role in signalling has not been demonstrated.

Another issue relates to protein synthesis. It is generally accepted that gene expression in mature sperm is restricted to the mitochondria. In fact, mammalian sperm seem to be able to synthesize both mitochondria-encoded RNAs (MacLaughlin and Terner, 1973; Hecht and Williams, 1978; Alcivar *et al*., 1989) and proteins (Ahmed *et al*., 1984; Twaina-Bechor and Bartoov, 1994). More recently, however, and in contrast with previous data (Diez-Sanchez *et al*., 2003), mammalian sperm were suggested to synthesize nuclear-encoded proteins, at least during capacitation (Gur and Breitbart, 2006). The results are particularly odd, and certainly require independent confirmation, not only because they seem to contradict the dogma that sperm are translationally silent cells (at least for nuclear-encoded proteins), but also because they also suggest that translation of nuclear-encoded proteins occurs in mitochondria-type ribosomes localized either inside or outside mitochondria, but with no involvement of the cytoplasmic translation machinery, an event with no equivalent in any other cell type.

Oocyte/embryo mitochondria

Mitochondria are the most abundant and prominent organelle in the early embryo (Motta *et al*., 2000; Sathananthan and Trounson, 2000) and are thought to be exclusively derived from the oocyte (Cummins, 2000). Contradicting a notion still found in a number of textbooks, in mammals the entire sperm enters the oocyte at fertilization (Ankel-Simons and Cummins, 1996), however, sperm mitochondria are diluted beyond detectable levels or destroyed inside the embryo (Sutovsky *et al*., 1999).

Oocyte mitochondrial dysfunction, expressed as declined cell respiration and electron transport, may contribute to diminished fertility, and be the cause of development retardation and arrest in human preimplantation embryos (Fissore *et al*., 2002; Ramalho-Santos *et al*., 2004; Thouas *et al*., 2004). Intracytoplasmatic injection of 'normal' mitochondria can overcome mitochondrial dysfunctions (Nagai *et al*., 2004) and inhibit oocyte fragmentation (Perez *et al*., 2000), stressing the importance of mitochondria in cell death (Fissore *et al*., 2002). In contrast, injection of abnormal mitochondria induces oocyte apoptosis (Perez *et al*., 2007).

Mitochondrial number and structure in the oocyte and early embryo

Oocyte mitochondria must support early embryo development until the resumption of mitochondrial replication, which only occurs post-implantation (reviewed in Dumollard *et al*., 2006). Depending on the species, a mammalian oocyte contains around 10^5-10^8 mitochondria, with 10^5 in human (Chen *et al*., 1995; Jansen and de Boer, 1998). Mitochondria propagate from a restricted founder population present in the primordial germ cell (PGC) (Cummins, 2000; Jansen, 2000), ensuring that mitochondria in the mature oocyte (and therefore in dividing blastomeres) are homogeneous. The mtDNA bottleneck theory (Hauswirth and Laipis, 1982) suggests a restriction in the number of mtDNA molecules to be transmitted from the mother to the offspring, followed by a strong amplification in oocytes (Reviewed in May-Panloup *et al*., 2007). The bottleneck occurs in order to maintain mtDNA homoplasmy and minimize heteroplasmy (Cummins, 2001) Therefore, a selection of a group of mtDNA molecules to repopulate the next generation takes place, and deleterious mutations tend to be eliminated so as not to be transmitted to the offspring. The nature of the mtDNA bottleneck has been recently discussed and different mechanisms have been proposed (Cao *et al*., 2007; Cree *et al*., 2008; Khrapko, 2008). However, further investigations are needed to clarify exactly when and how this phenomenon occurs.

During oogenesis there is an amplification in mitochondrial number in parallel with cytoplasmatic volume increase. Pre-migratory PGC have less than 10 mitochondria, 100 mitochondria are present in ovarian PGCs and 200 in oogonia. Primordial follicle oocytes have 10 000 mitochondria, a number which ultimately increases 10-fold. In the mature oocyte, each of the 10^5 mitochondria possesses a single copy of mtDNA (reviewed in Jansen and de Boer, 1998).

The increase in mitochondrial number during oocyte growth is accompanied by changes in their ultrastructure (Wassarman and Josefowicz, 1978; Motta *et al*., 2000; Au *et al*., 2005). Small oocytes contain mitochondria with numerous transversely-oriented cristae, although growing oocytes present round and oval-shaped mitochondria, with columnar-shaped arched cristae. At ovulation, mitochondria have a spherical immature structure, are highly vacuolated, with a dense matrix and only few cristae. Between the zygote and the 2-cell stage, mitochondria assume a dumb-bell shape and present concentrically located cristae. From the 4-cell to the morula stage, mitochondria have a more elongated structure with transverse cristae.

The total number of mitochondria in a normal human blastocyst is about 14 000, and the average number of mitochondria per cell is about 150 (Van Blerkom, 2008). However, studies in mouse and hamster models show that the average mitochondria per cell is higher in the trophectoderm (TE), which will give rise to the placenta, than in the inner cell mass (ICM), which will give rise to the embryo proper (Barnett *et al*., 1996; Van Blerkom, 2008). There is some controversy regarding the morphological homogeneity of the mitochondria found at the blastocyst stage. Although some authors claim that mitochondria in mouse and human blastocysts are homogenous and elongated elements (Sathananthan and Trounson, 2000), the existence of two types of mitochondria in the mouse blastocyst has been reported: spherical mitochondria in the ICM and elongated mitochondria in the TE. In both types mitochondrial cristae are transversely oriented and their matrix is less dense than the mitochondrial matrix found in earlier developmental stages (Stern *et al*., 1971). Interestingly, although the ICM cells have low $\Delta\psi$ and are almost quiescent, the TE cells are highly polarized and very active producing more ATP

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and consuming more oxygen (Barnett et al., 1996; Houghton, 2006; Van Blerkom et al., 2006).

Localization and distribution of mitochondria in the oocyte and early embryo is highly regulated

During oocyte maturation, and in early embryos, mitochondria are relocated to different regions, probably in response to localized energy demands (reviewed in Bavister and Squirrell, 2000). Throughout maturation mitochondria are mainly found in clusters in close proximity to endoplasmic reticulum membranes (Jansen, 2000), suggesting a possible interaction between the two organelles (Dumollard et al., 2006). In fully-grown germinal vesicle (GV) stage oocytes mitochondria are concentrated in clusters surrounding the nucleus (Jansen, 2000; Sun et al., 2001) and migrate to the periphery of the oocyte after GV breakdown. In metaphase-II (MII) arrested oocytes, mitochondria are mainly present around the meiotic spindle and at the oocyte centre (Dumollard et al., 2004, 2006), accumulating around pronuclei following fertilization, and maintaining close nuclear association through the morula stage. Impaired redistribution of mitochondria may compromise fertilization and embryo development (Au et al., 2005), and blastomeres that receive an insufficient amount of mitochondria remain undivided and undergo fragmentation (Van Blerkom et al., 2000).

In addition, mitochondrial populations present heterogeneity in terms of $\Delta\Psi$. Two populations of mitochondria are present: one with low $\Delta\Psi$, which is more abundant, and a smaller amount with high polarization. Clusters of highly-polarized mitochondria are localized in the subplasmalemmal/pericortical cytoplasm in oocytes and early blastomeres (Van Blerkom et al., 2002). Loss of these mitochondrial domains affects division (Van Blerkom and Davis, 2006), which may be associated with the focal ionic and metabolic regulation (Van Blerkom et al., 2003) involved in oocyte activation and early development (Van Blerkom et al., 2002; Van Blerkom and Davis, 2007).

Energy metabolism in the oocyte and early embryo

During oocyte development, a combination of metabolic pathways is found. Pyruvate and glucose are used by primordial follicles, suggesting that both OXPHOS and glycolysis are involved (Biggers et al., 1967; Boland et al., 1993; Wycherley et al., 2005). Furthermore, glucose used by the cumulus cells may lead to pyruvate production that is utilized by the oocyte (Jansen and Burton, 2004).

Between the primary and pre-ovulatory stages, pyruvate uptake increases 2-fold (Harris et al., 2008), accompanied by an increase in O_2 consumption. However, the average level of ATP seems constant between the GV and MII stages (Van Blerkom et al., 1995). The mature oocyte displays a high ATP turnover, supplied by mitochondrial respiration (Dumollard et al., 2004) and by the uptake of pyruvate (Leese, 1995), which is also the main substrate used by zygotes (Biggers et al., 1967; Leese and Barton, 1984). At fertilization, where higher ATP levels are required to support cortical granules exocytosis, chromosome dysjunction, polar body extrusion and Ca^{2+} homeostasis (Van Blerkom et al., 1995), there is an increase in O_2 use (Magnusson et al., 1977). Therefore, pyruvate is essential for meiotic maturation and to support the first cleavage division (Biggers et al., 1967).

From zygote to morula, the levels of ATP and O_2 used remain basically constant, and it is essentially substrates for OXPHOS that are metabolized (Slotte et al., 1990; Van Blerkom et al., 1995). In later stages the pattern of energy metabolism for the cleaving embryo changes (reviewed in Dumollard et al., 2007). At the morula stage, mitochondrial and metabolic changes occur gradually, and a shift in ATP production to glycolysis is evident (Leese, 1995; Van Blerkom et al., 1995; Thompson et al., 1996). Glucose is the predominant substrate that supports later embryo development (Biggers et al., 1967; Gardner and Leese, 1986; Hardy et al., 1989; Gott et al., 1990), but the increase in glucose uptake at the blastocyst stage is accompanied by a substantial increment in ATP generation and O_2 consumption (Houghton and Leese, 2004), suggesting OXPHOS also takes place (Dumollard et al., 2007). After implantation, levels of O_2 use decrease to those found in pre-blastocyst stages (Houghton and Leese, 2004).

In summary, the uptake of pyruvate is high in the mature oocyte, drops just after fertilization and then peaks before declining again at the morula stage (Gardner and Leese, 1986). Interestingly, if one simultaneously considers independent data for sperm and oocyte, the building consensus is that the male gamete predominantly uses glycolysis to reach the oocyte, although at the same time the female gamete is seemingly more reliant on OXPHOS, despite the same substrate and oxygen availability.

Importantly, intra- and inter-individual variations in oocyte ATP content have been described, and there is a close association between oocyte ATP concentration and developmental competence of the resulting embryo (Van Blerkom et al., 1995). Furthermore, pyruvate and glucose uptake are lower in arrested embryos (Hardy et al., 1989; Gott et al., 1990), which are also unable to switch to a glucose-based metabolism when necessary (Gott et al., 1990). Additionally, blastocysts that implant and develop to term have a significantly higher glucose uptake prior to transfer than those that fail to develop (Gardner and Leese, 1987). As in the case of sperm, it is also possible that these constant changes are simply adjustments to the substrates available in distinct region of the female reproductive tract (Jansen and Burton, 2004). Taking cellular volume into account, Harris and coworkers (2008) found that metabolism is higher in primary follicles, indicating that energy demands are greater. On the other hand, a relatively low metabolism is found in embryos, which seems associated with embryo vitality (Lane and Gardner, 1996; Leese, 2002).

The relevance of mitochondrial activity in terms of mtDNA has been also studied. A correlation between mutations in the catalytic subunit of POLG and premature ovarian failure has been noted and is probably due to an accumulation of mtDNA deletions as has been observed for other tissues (Luoma et al., 2004). Female mice that carry a proofreading-deficient POLG have reduced fertility (Trifunovic et al., 2004). Furthermore, a relationship between oocyte mtDNA copy number and oocyte quality/fertility was observed (Yesodi et al., 2002; Almeida-Santos et al., 2006), and fertilized oocytes present a higher mtDNA copy number than unfertilized oocytes (Almeida-Santos et al., 2006). This may suggest that it is not primarily OXPHOS dysfunction that contributes to diminished fertility, but rather reduced mitochondria/mtDNA copy number that leads to the OXPHOS dysfunction observed and subsequently to poor quality

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oocytes or reduced fertility (Jacobs *et al*., 2006). However, contrary to the situation in males, females that carry mtDNA with pathogenic mutation are fertile. These females produce oocytes with a predominant amount of the mutant mtDNA, oocytes that survive despite severe OXPHOS defects. The mutant mtDNA is maternally transmitted to the F1–F3 progenies, which present mitochondrial dysfunction in various tissues and have a shorter lifespan due to the consequent pathologies that are unrelated to fertility itself (Inoue *et al*., 2000).

Mitochondria in Ca^{2+} signalling

Oocyte mitochondria have an important role in the regulation of sperm-triggered Ca^{2+} waves essential for zygote activation (reviewed in Dumollard *et al*., 2006, 2007), probably acting as a Ca^{2+} store and participating in the generation of the intracellular $[Ca^{2+}]$ oscillations (Tesarik and Sousa, 1996; Liu *et al*., 2001). Sperm-triggered Ca^{2+} oscillations stimulate mitochondrial energy production at fertilization, leading to an increase in O_2 consumption that is maximal during Ca^{2+} release (Dumollard *et al*., 2003), and can be primarily initiated by an influx of Ca^{2+} into the mitochondria (Dumollard *et al*., 2006). Accordingly, the Ca^{2+} signal is directly transmitted to the mitochondrial matrix, leading to the up-regulation of OXPHOS, which is, in turn, necessary for the maintenance of $[Ca^{2+}]_i$ oscillations (Dumollard *et al*., 2004). Thus, Ca^{2+} links ATP supply and demand, allowing for the maintenance of a low-level of OXPHOS, which increases only when ATP is needed to support post-fertilization events, stimulated by Ca^{2+} waves. The production of ROS by the ETC is therefore minimized, ensuring that mitochondria are exposed to low levels of oxidative stress. Furthermore, mitochondria are also important for calcium clearance, i.e. in the maintenance of low levels of cytosolic $[Ca^{2+}]$.

Mitochondrial ROS and apoptosis in oocytes and embryos

Accumulating evidence has shown that ROS play important roles in female reproduction (reviewed in Agarwal *et al*., 2005). However, the mammalian oocyte and embryo are very sensitive to oxidative stress (Liu *et al*., 2000) and if physiological levels of ROS are beneficial, higher levels can disrupt oocyte maturation and embryo development (Harvey *et al*., 2002), and promote embryo fragmentation (Johnson and Nasr-Esfahani, 1994; Yang *et al*., 1998).

Indeed, oxidative stress can induce apoptosis of the oocyte and early embryo (Liu *et al*., 2000). Mitochondria-dependent apoptosis seems to be responsible for the post-natal decline in the female germ cell population (Reynaud and Driancourt, 2000; Tilly, 2001) and for follicular atresia (Kim and Tilly, 2004), as well as in oocytes and early embryos (Liu *et al*., 2000). Mammalian oocytes express several anti- and pro-apoptotic members of the Bcl-2 family and it is the balance between these factors that determines oocyte survival (Liu *et al*., 2000; reviewed in Jurisicova and Acton, 2004). Furthermore, and mirroring the importance of mitochondria in oocyte apoptosis, the apoptotic inducer hydrogen peroxide induces cytochrome c release from oocyte mitochondria, associated with a decrease in $\Delta\Psi$ (Liu *et al*., 2000).

In essence, mature oocytes and early embryos maintain an overall low-level (i.e. 'quiet') metabolism, thus minimizing oxidative stress, but generating the necessary ATP to fulfil cellular functions (Leese, 2002; Leese *et al*., 2007).

Physiological alterations associated with aging

A negative correlation has been described between maternal age and mitochondrial activity (Jansen and de Boer, 1998). Oocytes from older women present declining mitochondrial function, which can contribute to declining fertility, and may be associated with lower embryo development and pregnancy rates (Wilding *et al*., 2001). Ocytes from older women often present aberrant spindle formation, abnormal chromosomal alignment, and consequently, a high occurence of aneuploidy (Battaglia *et al*., 1996; reviewed in Eichenlaub-Ritter *et al*., 2004). It has been suggested that these abnormalities can be due to an inadequate capacity to generate sufficient ATP levels to support these events (Gaulden, 1992). In accordance, oocytes from older women present an accumulation of mtDNA point mutations (Barritt *et al*., 2000) and higher levels of mtDNA deletions (Keefe *et al*., 1995), factors that can ultimately be responsible for aneuploidy and poor implantation rates (Bartmann *et al*., 2004), ensuring that only metabolically intact embryos develop to term (Dumollard *et al*., 2006). These abnormalities can be corrected by injecting cytoplasm from younger oocytes (discussed in Klein and Sauer, 2001). The donor cytoplasm may thus 'rescue' spindle misalignments, due, at least in part, to the mitochondria of the younger oocytes. Moreover, oocytes from older women present a higher volume fraction of mitochondria, indicating compensatory mechanisms (Muller-Hocker *et al*., 1996). In the same vein, mitochondrial dysfunction seems to also play an important role in fragmentation in postovulatory aged oocytes. Indeed, aged oocytes present a lower uptake of pyruvate (Hardy *et al*., 1989). It was also found that, in these oocytes, $[Ca^{2+}]$ oscillations fail to trigger ATP production and instead induce apoptosis (Gordo *et al*., 2002). A decrease in fertility potential, in both maternal aging and postovulatory aged oocytes, seems to be primarily due to damages in mitochondria caused by oxidative stress (Fissore *et al*., 2002), leading to apoptosis (Perez *et al*., 1999).

Mitochondria in embryonic stem cells

ESCs, are derived from the ICM of the blastocyst stage embryo prior to implantation and can be maintained *in vitro* in colonies for prolonged periods without losing the abilities of indefinite self-renewal or differentiation into tissues from all three germ layers (Evans and Kaufman, 1981; Martin, 1981; Thomson *et al*., 1998; Amit *et al*., 2000; Pan and Thomson, 2007). This can be assessed *in vitro* (through embryoid body formation) or *in vivo* (by teratoma formation). Because of these biological properties, human embryonic stem cells (hESCs) have an enormous potential as models to study cell differentiation and for possible replacement cell therapies. Indeed, several groups have shown that under specific culture conditions hESCs can differentiate into various somatic cell types (for review see Gepstein, 2002; Dhara and Stice, 2008; Raikwar and Zavazava, 2009).

Mitochondria number and morphology in ESC

As ESCs are derived from the ICM one should expect that they share metabolic and morphologic features. Indeed, and although there are line-specific differences, it has been shown that undifferentiated hESCs have few mitochondria arranged in small perinuclear clusters,

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and immature morphology, evidenced by the presence of few cristae and low electron lucid matrix (Oh *et al*., 2005; St John *et al*., 2005a, b; Cho *et al*., 2006) (Fig. 4). hESCs colonies are characterized by high nuclear cytoplasmatic ratios and cells tightly packed within colonies.

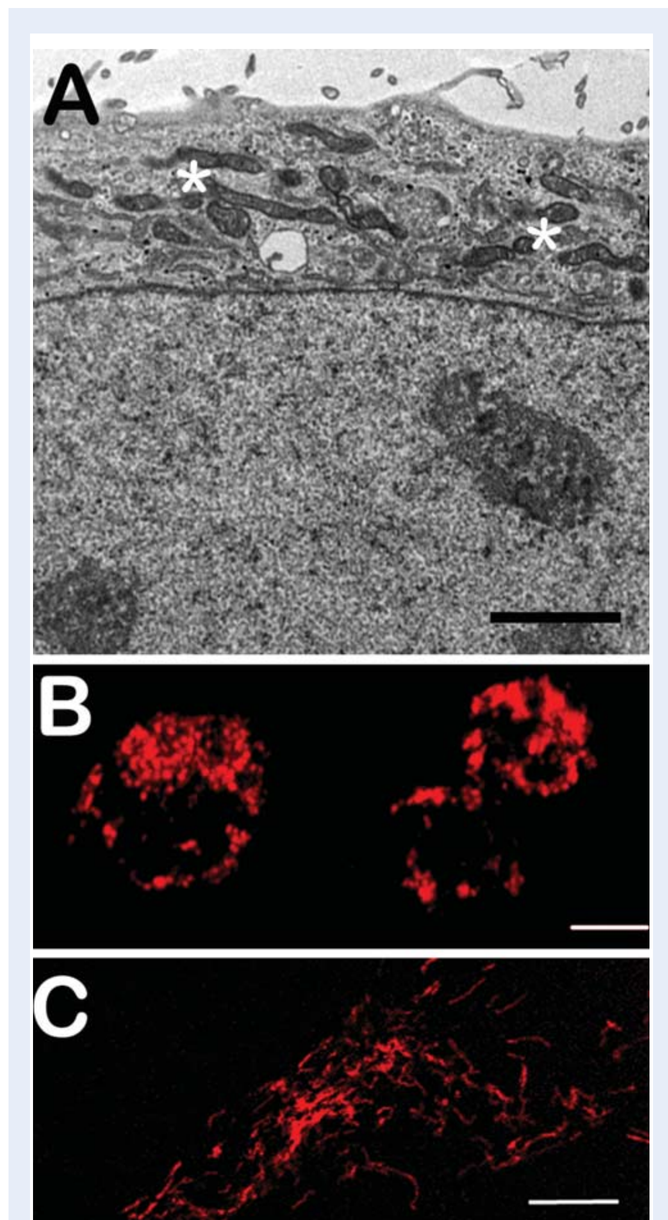

Figure 4 Mitochondria in human embryonic stem cells (hESCs).(**A**) Transmission electron microscopy of hESCs with a large nuclear/cytoplasm ratio and few elliptical mitochondria with small internal matrix space in perinuclear clusters (asterisks). Bar represents 2 μm. (**B**) and (**C**) Both hESCs (B) and mouse embryonic feeder fibroblasts (C) were transfected with the Mito-DsRED vector (red) and imaged by live confocal microscopy. The vector encodes a fusion red fluorescent protein and the mitochondrial targeting sequence from subunit VIII of cytochrome oxidase. (B) Mitochondria distribution in a hESCs undergoing mitosis. Note that the two daughter cells inherit different numbers of mitochondria. Bar represents 20 μm. (C) Mouse fibroblast mitochondria are more elongated that those in hESC. Bar represents 10 μm.

Although there seems to be a paucity of intracellular organization (sometimes discussed as a 'stemness' attribute), this could also be a reflection of reduced cytoplasm. Furthermore, it is well accepted that cells in the periphery of the colony are among the first cells to undergo spontaneous differentiation during *in vitro* culture. Interestingly these cells have higher levels of mitochondria (Cho *et al*., 2006).

MMP and metabolism in ESC

There is some controversy regarding the polarization of mitochondria in undifferentiated versus differentiated ESC. Undifferentiated mouse ESC has been reported to have highly polarized mitochondria, which decreases upon differentiation to cardiomyocytes (Chung *et al*., 2007). On the other hand, no differences in $\Delta\psi$ between undifferentiated hESCs and differentiated hESCs were reported (Saretzki *et al*., 2008). The controversy might be due to the fact that these data come from ESCs of different species, mouse and human, respectively. In addition, mouse ESC were specifically differentiated into cardiomyocytes, whereas with hESCs spontaneous differentiation was studied, and as consequence a mixture of cell lineages would be present. Several studies have differentiated ESCs *in vitro* and observed changes in mitochondrial dynamics during differentiation. As ESCs differentiate the total number of mitochondria increases, as do the number of mitochondria with a more mature morphology (St John *et al*., 2005a, b; Cho *et al*., 2006), similar to that described for SSCs. Concomitantly with an increase of mitochondrial number during ESC differentiation, the rates of O_2 consumption and ATP production in the cell increase, whereas lactate production decreases (Chung *et al*., 2007). These results suggest that during ESC differentiation there is a switch in energy metabolism from glycolysis to OXPHOS. Similar results have been reported in adult stem cells (Piccoli *et al*., 2005; Chen *et al*., 2008).

It seems logical to assume that an increase in the number of mitochondria and OXPHOS in differentiated cells leads to an increase in ROS production, and several authors have shown that that is indeed the case (Cho *et al*., 2006; Saretzki *et al*., 2008). Interestingly, reports regarding antioxidant defences during the process of differentiation differ. Although a decrease in the expression of antioxidants, namely SOD2 and GPX2, has been reported (Saretzki *et al*., 2008), increased expression of GPx1, Cu/Zn SOD, Prx1 and Prx2 was also described (Cho *et al*., 2006). The contradictory results may be due to the fact that these studies looked at different antioxidants, and it is possible that during differentiation there is an increase in certain antioxidants to the detriment of others.

Mitochondrial role in ESC differentiation

Given the distinct mitochondrial properties in undifferentiated versus differentiated ESCs it is logical to assume a role for mitochondria in differentiation. Hypoxic environment prevents spontaneous hESC differentiation (Ezashi *et al*., 2005). In addition, several groups have shown that functional mitochondria are necessary for differentiation. For example, inhibition of mitochondrial respiratory chain complexes I and III, by Rotenone and Antimycin A, respectively, results in reduced cardiomyocyte differentiation, due to an impairment of OXPHOS (Chung *et al*., 2007). Furthermore, glycolytic metabolism is sufficient for maintaining mouse ESC homeostasis; however, in order for cells to differentiate there must be a switch from glycolysis to the

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more efficient OXPHOS (Chung *et al*., 2007). In addition, inhibition of the complex III of mitochondrial respiratory chain by Antimycin A reduced the spontaneous appearance of beating foci formed by differentiating ESCs, probably due to inhibition in calcium signalling (Spitkovsky *et al*., 2004). The same inhibitor has been shown to also boost undifferentiated hESC pluripotency (Varum *et al*., submitted). Again, several authors have reported a similar role for mitochondria in adult stem cell differentiation (Carriere *et al*., 2004; Chen *et al*., 2008). Recently, a correlation between $\Delta\psi$, metabolic rate and the differentiation of mouse ESCs has been described, with cells with lower MMP showing more efficient mesodermal differentiation (but low ability to form teratomas), although a population with higher membrane potential behaved in the opposite fashion, although both populations were indistinguishable in terms of pluripotency markers (Schieke *et al*., 2008).

Mitochondria and ESC apoptosis

Several authors have reported that mitochondrial apoptotic pathways play a role in modulating ESC homeostasis and differentiation. Upon oxidative stress silent mating type information regulation 2 homolog 1 (SIRT1), a deacetylase that catalyzes deacetylation of acetyllysine residues of proteins such as p53, allows mouse ESCs to maintain self-renewal by eliminating the cells that were exposed to endogenous ROS (Vaziri *et al*., 2001; Han *et al*., 2008). Under ROS exposure SIRT1 blocks translocation of the tumor suppressor p53 to the nucleus and induces it's accumulation in the mitochondria of mouse ESCs. p53 can then induce mitochondria-mediated cell death by inducing the release of cytocrome c, SMAC/Diablo and apoptosis inducing factor (Mihara *et al*., 2003; Leu *et al*., 2004; Moll *et al*., 2006). Furthermore, p53 suppresses expression of the key regulator of pluripotency, *Nanog*, in hESCs (Chambers *et al*., 2003; Mitsui *et al*., 2003; Quin *et al*., 2007). These elegant studies suggest that SIRT1 maintains mouse ESC self-renewal under stress by inhibition of p53-mediated suppression of Nanog and by inducing apoptosis of cells that were exposed to endogenous ROS.

Although not necessarily related to mitochondrial function, it has been recently reported that caspase 3 mediates both ESC and hematopoietic stem cell differentiation (Fujita *et al*., 2008; Janzen *et al*., 2008). Another component of the apoptotic machinery that was referred as a mediator of stem cell differentiation is Bcl-2, and mouse ESCs overexpressing this anti-apoptotic protein maintained pluripotency in serum- and feeder-free conditions (Yamane *et al*., 2005). Overall these studies suggest that the mitochondrial apoptotic machinery, besides it's canonical role in apoptosis, is an important mediator of stem cell differentiation.

In summary, although this remains a promising and novel area for research, overall results indicate that modulation of mitochondrial activity may be a useful tool to maintain ESCs in a pluripotent state, or drive differentiation towards a specific lineage. It remains to be established if the same is true of the more recently characterized induced pluripotent (iPS) cells, in which a pluripotent ESC-like state is induced in somatic cells (Takahashi *et al*., 2007; Yu *et al*., 2007; Yamanaka, 2008). Indeed, and although much further research is warranted, both at the basic and applied levels, in all likelihood these iPS cells will essentially replace current hESC lines in much of the research related to pluripotency, differentiation, maintenance of a cell state (i.e. also relevant for putative cell dedifferentiation during cancer), inasmuch as they also represent a technology with the true potential for the generation of embryo and oocyte-free patient-specific cell lines for putative cell replacement therapies.

Conclusions

Mitochondria-based events regulate different aspects of reproductive function, but these are not uniform throughout the several systems reviewed. Reversible switches in mitochondrial activity occur throughout the reproductive system and could reflect changes in substrate availability or signal profound changes that could be used to modulate different processes, such as gamete and embryo quality and cell differentiation. Low(er) mitochondrial activity seems a feature of 'stemness', being described in spermatogonia, early embryo, inner cell mass cells and ESCs. Thus, not only do mitochondria mirror, but also affect cellular state, suggesting the mitochondrial manipulation affects the differentiation of ESCs (and possibly also iPS cells). Furthermore, recent studies showing unexpected and non-canonical relationships between transcription factors and mitochondrial activity (Wegrzyn *et al*., 2009) suggest heretofore unacknowleged levels of complexity in global cell regulation involving the joint coordination of signalling, gene expression and metabolism. Future work should embrace these organelles as targets or indicators of changes invoked by manipulation, toxic injury and other conditioning factors.

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