REPRODUCTION
REVIEW

Mitochondrial regulation in spermatogenesis

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Abstract

The classic roles of mitochondria in energy production, metabolism, and apoptosis have been well defined. However, a growing body of evidence suggests that mitochondria are also active players in regulating stem cell fate decision and lineage commitment via signaling transduction, protein modification, and epigenetic modulations. This is particularly interesting for spermatogenesis, during which germ cells demonstrate changing metabolic requirements across various stages of development. It is increasingly recognized that proper male fertility depends on exquisitely controlled plasticity of mitochondrial features, activities, and functional states. The unique role of mitochondria in germ cell ncRNA processing further adds another layer of complexity to mitochondrial regulation during spermatogenesis. In this review, we will discuss potential regulatory mechanisms of how mitochondria swiftly reshape their features, activities, and functions to support critical germ cell fate transitions during spermatogenesis. In addition, we will also review recent findings of how mitochondrial regulators coordinate with germline proteins to participate in germ cell-specific activities.

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Introduction

Each cell contains hundreds to thousands of mitochondria, and each mitochondrion contains multiple copies of mitochondrial genomic DNA (mtDNA) (Cole 2016). As the 'powerhouse' of the cell, mitochondria need to rapidly adapt their functions in response to changing cellular metabolic needs, substrate availabilities, as well as physiological and pathological cues. Notably, it is not just enzymatic activities in the tricarboxylic acid cycle (TCA cycle, also called citric acid cycle or Krebs cycle) and mitochondrial respiration that control mitochondrial performance. Mitochondrial features, including their morphology, architecture, numbers, subcellular localization, and interaction with other organelles, also vary in a cell type and developmental stage-dependent manner to critically affect the mitochondrial functional state. The plasticity of mitochondrial features is largely regulated by mitochondrial dynamics (i.e. fusion and fission), which not only enable coordinated response of individual mitochondria to extracellular stimuli but also reshape mitochondrial features to indirectly control their metabolic profiles and functions (Mishra & Chan 2016).

Further, mounting evidence suggests that mitochondria are not just a 'powerhouse' of the cell, they are increasingly recognized as active players in stem cell differentiation and development. For example, metabolites from the TCA cycle may function as epigenetic cofactors to initiate transcriptional reprogramming (Wellen *et al.* 2009, Cai *et al.* 2011, Hwang *et al.* 2016, van der Knaap & Verrijzer 2016), while reactive oxygen species (ROS) from mitochondrial respiration act as signaling molecules or protein oxidation modifiers to regulate transcription and protein activity (Zhang *et al.* 2016*b*). In spermatogenesis, the process of postnatal male germ cell development, millions of sperm are generated per day from mouse adult testes, which require highly coordinated mitochondrial and metabolic activities. Interestingly, germline mitochondria differ remarkably in features, activities, and functions across varying stages of spermatogenesis (Varuzhanyan & Chan 2020), suggesting that developmental stimuli and metabolic needs are intertwined at mitochondria to regulate germ cell fate decision and male fertility. Hereby, we reviewed recent key findings of mitochondrial regulation during spermatogenesis, including a growing understanding of metabolic transitions at critical stages in spermatogenesis, germ cell-specific mitochondrial functions, as well as coordination between germline factors and common mitochondrial modulators that are shared with somatic cells.

Mitochondria at a glance

Mitochondria are double-membrane organelles that are found in almost all eukaryotic cells. The double-membrane divides mitochondria into five distinct regions: the outer mitochondrial membrane (OMM), intermembrane space, the inner mitochondrial membrane (IMM), cristae, and the matrix (Fig. 1. Mitochondrial structure). The OMM acts as a barrier

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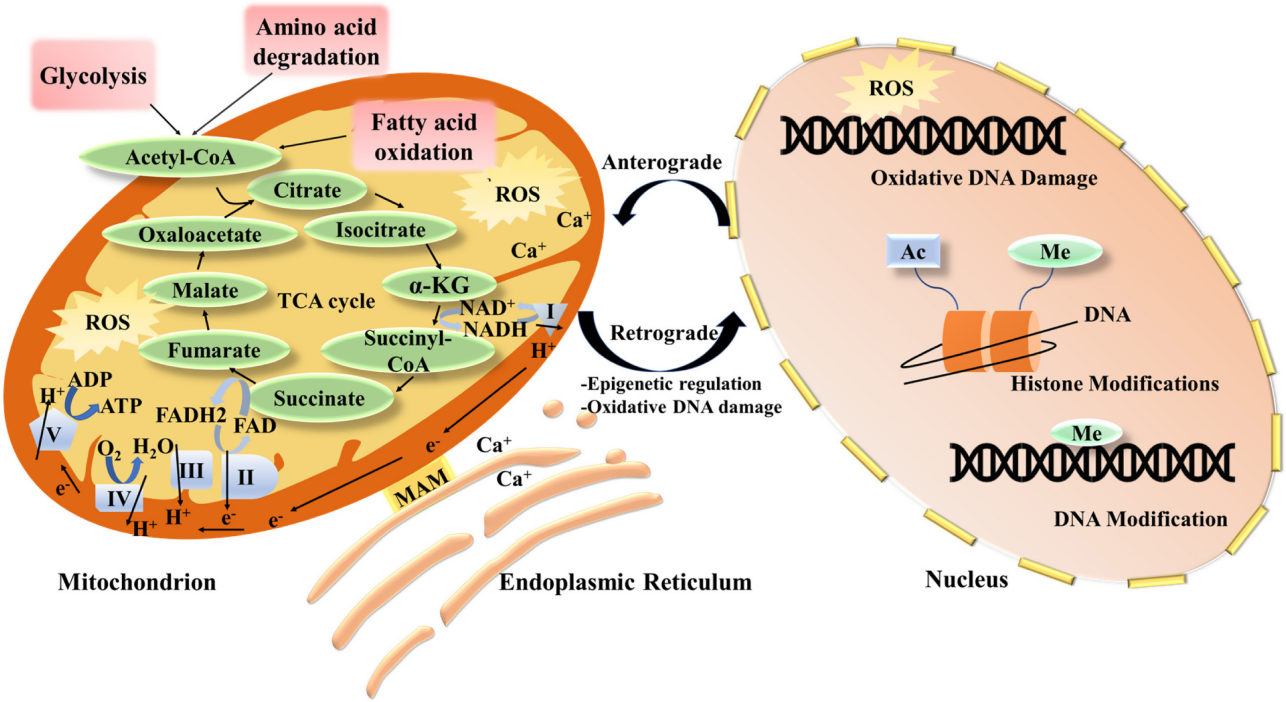

Figure 1 Mitochondrial metabolism and communication network with nucleus and ER. The TCA cycle begins with the reaction that combines acetyl-CoA with oxaloacetate to form citrate. NADH and FADH2 that are generated from the TCA cycle provide electrons passing through ETC-I to IV to O_2, releasing energy to establish a proton gradient across IMM for final ATP production via ATP synthase in ETC-V. Metabolites that are produced in the TCA cycle such as acetyl-CoA and α-KG serve as cofactors for epigenetic regulators that modulate chromatin modifications and DNA methylation. ROS that are generated during OXPHOS may induce protein oxidative modifications or serve as a secondary messenger to trigger signaling cascades. Excess ROS may cause DNA damage in the mitochondria or in the nucleus. The communication between mitochondria and ER is facilitated by mitochondrial associate membrane (MAM), which regulates lipid metabolism, calcium homeostasis, and mitochondria dynamics. I-V, ETC I-V; V, ATP synthase; Ac, acetyl group; Me, methyl group.

to macromolecule diffusion and regulates the release of signals (e.g. ROS and pro-apoptotic molecules like cytochrome c) from mitochondria to the cytoplasm (Li *et al.* 1997, Rostovtseva & Colombini 1997, Adrain *et al.* 2001). The IMM is linked to the OMM via several contact sites that are necessary for metabolites exchange, coordination of mitochondrial dynamics, and protein transport (Reichert & Neupert 2002). Cristae are pouch-like structures that contain the machinery for oxidative phosphorylation (OXPHOS) (Mannella 2006, Quintana-Cabrera *et al.* 2018), while the mitochondrial matrix is the site of the TCA cycle, as well as mtDNA replication, transcription, and protein translation (Kuhlbrandt 2015).

The mtDNA is a circular double-stranded molecule with ~16 kb that encodes 13 polypeptides, 2 ribosomal RNAs, and 22 tRNAs (Anderson *et al.* 1981, Bibb *et al.* 1981). The mtDNA replication does not coincide with the cell cycle and occurs independently from nuclear DNA (Bogenhagen & Clayton 1977). DNA polymerase gamma (POLG) with 3′–5′ exonuclease proofreading activity is the primary polymerase that mediates mtDNA replication (Ropp & Copeland 1996, Copeland & Longley 2003). The mtDNA encodes polypeptides in enzyme complexes of electron transport chain (ETC), including NADH CoQ reductase (complex I), cytochrome b (complex III), subunits I, II, and III of cytochrome c oxidase (complex IV), and subunits 6 and 8 of the H+ ATPase (Anderson *et al.* 1981, Bibb *et al.* 1981). Other mitochondrial proteins are encoded by the nuclear DNA and imported into mitochondria by translocase complexes at OMM and IMM (Reichert & Neupert 2002, Chacinska *et al.* 2009). For example, mitochondrial transcription factor A (TFAM) is a nuclear DNA coded protein responsible for recruiting POLG to activate mtDNA transcription and subsequent mtDNA packaging into nucleoids (Fisher & Clayton 1988, Kanki *et al.* 2004). Disruption of *Tfam* causes embryonic lethality and decreases mtDNA copy number in mice (Larsson *et al.* 1998), whereas upregulated TFAM protein level in mtDNA mutant mice partially rescues mitochondrial functions (Jiang *et al.* 2017).

Mitochondrial features and functions vary by cell type. Cells that require a lot of energy, such as muscle and liver cells, contain hundreds or thousands of mitochondria (Anversa *et al.* 1980). In contrast, some other cells, such as mature red blood cells, have few mitochondria (Tablin & Weiss 1985). Mature mammalian spermatozoon contains ~75 mitochondria in its midpiece (Otani *et al.* 1988). The mitochondrial morphology also varies from sacks and ovals to tubular

shapes, depending on cell type (Nixon *et al.* 1994, Das *et al.* 2012). To precisely coordinate the responses of individual mitochondria to various developmental cues, physiological changes, and pathological stress, mitochondria need to share their membrane and exchange their contents via continuous mitochondrial fusion (mitofusion) and fission, collectively known as mitochondrial dynamics (Chan 2012, Mishra & Chan 2014). Disrupting fusion or fission increases the heterogeneity of mitochondria (Chen *et al.* 2005, 2007).

In many mammalian cells, mitochondria present as a branched reticular network that radiates from the nucleus (Frazier *et al.* 2006). This unique spatial distribution allows mitochondria to readily connect and exchange materials with other subcellular organelles (Vance 1990, Prachar 2003, Desai *et al.* 2020). For example, mitochondria and endoplasmic reticulum (ER) are often closely associated via tethering at specific sites, so-called mitochondria-associated membrane (Vance 1990). The interaction between mitochondria and ER is crucial for mitochondrial fission (Friedman *et al.* 2011), lipid synthesis (Vance 1990), calcium signaling (Rizzuto *et al.* 1998, de Brito & Scorrano 2008), and mtDNA maintenance (Lewis *et al.* 2016). Mitochondria also communicate with the nucleus via anterograde (from the nucleus to mitochondria) and retrograde (from mitochondria to the nucleus) signaling pathways, which are facilitated by the contacts between mitochondria and the nucleus (Prachar 2003, Desai *et al.* 2020). These close contacts enable the transport of mitochondria-derived molecules (such as cholesterol and ROS) to the nucleus, regulating gene expression (Desai *et al.* 2020).

The mitochondrial features (i.e. morphology, subcellular distribution, and interaction with other organelles) are largely regulated by mitochondrial dynamics (Bereiter-Hahn & Voth 1994). For example, elevated mitofusion leads to elongated tubular mitochondria that favor OXPHOS, while fission increases small mitochondria (Mishra & Chan 2016). Further, mitochondrial dynamics contribute to mitochondrial health by coupling with the autophagic machinery to remove dysfunctional mitochondria for degradation (Twig *et al.* 2008, Hailey *et al.* 2010, Youle & van der Bliek 2012, Abeliovich *et al.* 2013, Pickles *et al.* 2018). Dysregulated mitofusion and fission may cause mtDNA instability and reduced respiratory capacity (Chan 2012).

Functions of Mitochondria as more than just the powerhouse of the cell

Mitochondria play a central role in bioenergetics and metabolism through the TCA cycle, β-oxidation, and OXPHOS (Nsiah-Sefaa & McKenzie 2016). The TCA cycle starts from acetyl coenzyme A (CoA), which is generated from oxidative decarboxylation of pyruvate, β-oxidation of fatty acids, and amino acid degradation (Krebs 1970) (Fig. 1). NADH and FADH2 from the TCA cycle provide electrons passing through ETC to O_2, releasing energy to mitochondrial respiratory complexes I–IV to establish a proton gradient across IMM (Mitchell 1961). The proton gradient further powers ATP synthase in complex V to drive ATP synthesis (Mitchell 1961). Compared to 2 ATP generated from glycolysis, the TCA cycle and OXPHOS produce ~32 ATP per glucose (Rich 2003). Mitochondria are therefore called the 'powerhouse' of the cell (Siekevitz 1957, Dunn & Grider 2021).

Importantly, accumulating evidence suggests that mitochondria contribute to multiple cellular and biological processes, such as cell signaling, proliferation, and lineage commitment, well beyond their canonical roles in metabolism and OXPHOS (Osellame *et al.* 2012, Chandel 2014, Chandel 2015, Chakrabarty & Chandel 2021). Metabolites generated through the TCA cycle not only provide precursors for anabolism of nucleotides, lipids, and amino acids but also serve as cofactors for enzymes in epigenetic regulation (Wellen *et al.* 2009, Cai *et al.* 2011, Hwang *et al.* 2016). For instance, acetyl-CoA provides the acetyl group to histone acetyltransferases for histone acetylation and thus plays an essential role in transcription activation (Wellen *et al.* 2009). Pluripotent stem cells (PSCs) need high levels of acetyl-CoA and histone acetylation for the maintenance of pluripotency (Moussaieff *et al.* 2015). Another key product of the TCA cycle, α-ketoglutarate (α-KG), serves as cofactors for ten-eleven translocation enzymes and Jumonji family histone demethylases, which are responsible for demethylation of DNA and histones (Klose *et al.* 2006, Yang *et al.* 2014). In general, α-KG promotes demethylation, while succinate and fumarate act as antagonists to α-KG-dependent functions (van der Knaap & Verrijzer 2016). An elevated ratio of α-KG to succinate has been shown to promote DNA and histone demethylation, which is key to maintain the pluripotency of naive mouse PSCs (Carey *et al.* 2015). By contrast, α-KG accelerates early endoderm and neuroectoderm differentiation in primed human and mouse PSCs (TeSlaa *et al.* 2016, Zhu *et al.* 2020). Overall, these studies highlight that these metabolites from mitochondrial metabolism are important regulators for transcription and histone modification that ultimately dictate stem cell fate decisions.

Further, ROS from OXPHOS may alter protein functions via oxidative modification or act as a secondary messenger to trigger signaling cascades, but excessive ROS production will cause oxidative damages (Chandel *et al.* 1998, Lo Conte & Carroll 2013, Holmstrom & Finkel 2014). Intracellular ROS exist primarily in three forms: superoxide anions (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH^-). H_2O_2 is thought to be the main ROS form in signaling transduction, likely due to its long half-life and the ability to quickly diffuse across membranes (Holmstrom & Finkel 2014).

Oxidation of cysteine thiol groups is the most common oxidative protein modification (Bigarella *et al.* 2014). Such a modification may cause functional changes of proteins in their stability, subcellular localization, and communication with other proteins, which, in some cases, affect cell fate decision (Dansen *et al.* 2009, Velu *et al.* 2017). Cellular redox homeostasis is maintained by the balance between ROS-producing and ROS-scavenging systems. A moderate increase of ROS may promote cell proliferation and survival (Le Belle *et al.* 2011, Morimoto *et al.* 2013). However, excessive ROS will overwhelm the cellular antioxidant capacity and trigger apoptosis (Guo *et al.* 2010, Ito *et al.* 2016). Thus, cellular ROS is a double-edged sword, and its levels must be tightly regulated to maintain normal cell functions.

Mitochondria in male germ cells

Dynamic changes of mitochondrial features during spermatogenesis

Mammalian spermatogenesis is a complex multi-step process supported by a rare population of spermatogonial stem cells (SSCs) (Oakberg 1971, Roosen-Runge & Holstein 1978, Hara *et al.* 2014, de Rooij 2017). In mice, SSCs undergo mitotic divisions to produce more SSCs and support a pool of undifferentiated spermatogonia (Mecklenburg & Hermann 2016). In response to developmental signals, such as retinoic acid, undifferentiated spermatogonia transition into type A1 differentiating spermatogonia, which will further develop into type B spermatogonia to form spermatocytes and then haploid spermatids via meiosis. Spermatids subsequently enter spermiogenesis to mature into spermatozoa, by going through nuclear condensation, acrosome formation, and tail development. Finally, mature but non-motile spermatozoa will be released from the seminiferous epithelium to the epididymis via a process known as spermiation. The entire process takes approximately 35 days from type A1 spermatogonia to spermiation in mice (Clermont 1972, Griswold 2016).

During spermatogenesis, mitochondrial morphology, size, and localization change markedly (Fig. 2), as reported by early studies using transmission electron microscopy (TEM). Three different morphological types of mitochondria have been described in germ cells – 'orthodox', 'intermediate', and 'condensed' (De Martino *et al.* 1979, Hess *et al.* 1993, Meinhardt *et al.* 1999, Vertika *et al.* 2020). Spermatogonia and early spermatocytes usually carry the conventional orthodox type of mitochondria that are small and spherical organelles with greater matrix and thin cristae (De Martino *et al.* 1979, Hess *et al.* 1993, Meinhardt *et al.* 1999). While mitochondria are randomly dispersed in the cytoplasm of rodent germ cells (De Martino *et al.* 1979, Meinhardt *et al.* 1999), in prepubertal spermatogonia from humans and pigs, mitochondria appear to be distributed at one side of the nucleus close to the basement membrane of testicular tubules (Voigt *et al.* 2021). In zygotene and pachytene spermatocytes at the early stages of meiosis, the mitochondria with 'intermediate' configuration are found around the nucleus (De Martino *et al.* 1979, Meinhardt *et al.* 1999). Late spermatocytes and early spermatids contain condensed forms of mitochondria with enlarged matrix and vesicular cristae, randomly

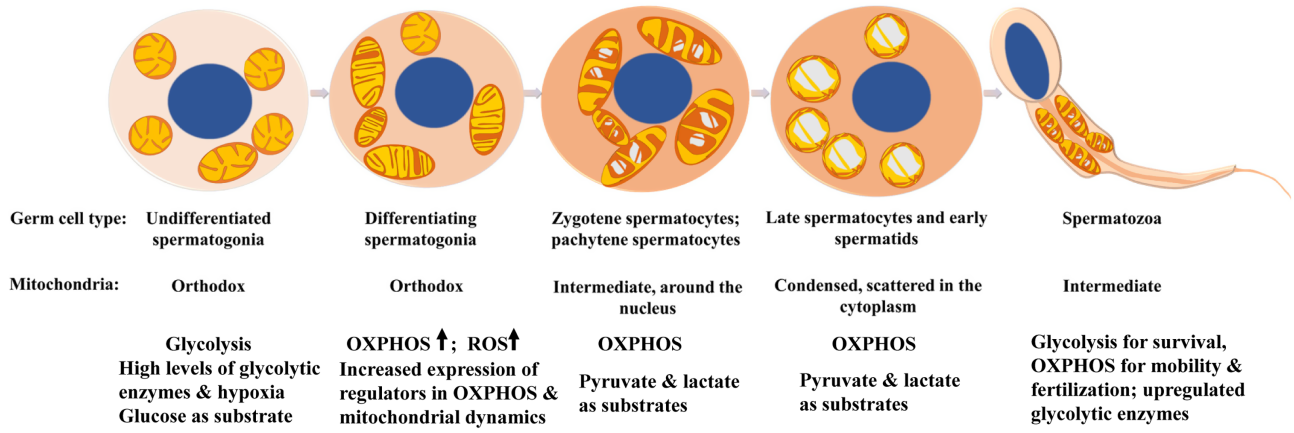

Figure 2 Alteration of mitochondrial morphology and metabolism during spermatogenesis. Spermatogonia carry orthodox mitochondria, which are small, spherical organelles containing large matrix and thin cristae. Compared to undifferentiated spermatogonia, differentiating spermatogonia have higher mitochondrial respiration and ROS levels, as well as increased expressions of mitochondrial regulators. Zygotene and pachytene spermatocytes tend to have elongated mitochondria (intermediate) that are localized around the nucleus. In late spermatocytes and spermatids, mitochondria are small and condensed with an enlarged matrix and vesiculate cristae. In spermatozoa, mitochondria of 'intermediate' type are helically arranged end to end in the midpiece of flagella. Lactate and pyruvate are required for energy production via OXPHOS in both spermatocytes and spermatids. Spermatozoa utilize glycolysis for survival but require both glycolysis and OXPHOS for motility and fertilization.

distributed throughout the cytoplasm (De Martino *et al.* 1979). In elongated spermatids, mitochondria with 'intermediate' configuration are aligned along the outer dense fibers in the forming tail (Hess *et al.* 1993). At the end of spermiogenesis, mitochondria are helically arranged end-to-end in the midpiece of flagella in spermatozoa (De Martino *et al.* 1979). These dramatic changes in mitochondrial features are critical to spermatogenesis and thus need to be precisely regulated to support the cascades of key events in germ cell development.

Mitochondria and piRNA biogenesis in male germ cells

In postnatal germ cells, nuage, the amorphous electron-dense granule with no limiting membrane has been identified in the cytoplasm using TEM (Fawcett *et al.* 1970, Eddy 1974). These germline granules consist of ribonucleoproteins and participate in the biogenesis of P-element-induced wimpy testis (PIWI)-interacting RNA (piRNA) (Paniagua *et al.* 1985, Aravin *et al.* 2006, Girard *et al.* 2006, Watanabe *et al.* 2006). Based on its size, subcellular location, and components, nuage is classified as intermitochondrial cement (IMC, also named pi-body), piP-body, and chromatoid body in male germ cells (Eddy 1975, Paniagua *et al.* 1985, Chuma *et al.* 2009). Both IMC and piP-body are in close proximity to mitochondria and contain mitochondrion-localized proteins (Eddy 1974, Aravin *et al.* 2009, Chuma *et al.* 2009). Clustered mitochondria with 'cementing materials' are uniquely present at IMC in pro-spermatogonia, postnatal spermatogonia, and early stage spermatocytes (Eddy 1974, Chuma *et al.* 2009), though it remains unclear how mitochondria or IMC structurally and functionally communicate with other types of nuage.

The piRNAs are small ncRNAs with ~24–34 nucleotides in length, which have been identified in diverse species (Aravin *et al.* 2006, Girard *et al.* 2006, Grivna *et al.* 2006, Lau *et al.* 2006, Watanabe *et al.* 2006). Two classes of piRNAs have been described during mouse spermatogenesis: pre-pachytene and pachytene piRNAs. Pre-pachytene piRNAs maintain germline DNA integrity by repressing the expression of transposable elements, a function essential for germ cell development and male fertility. Pre-pachytene piRNAs are mainly processed by PIWI proteins MILI and MIWI2 in pro-spermatogonia, postnatal spermatogonia, and spermatocytes before the pachytene stage (Aravin *et al.* 2008, Kuramochi-Miyagawa *et al.* 2008). By contrast, pachytene piRNAs are expressed abundantly in spermatocytes and round spermatids, and their biogenesis is mediated by MILI and MIWI (Deng & Lin 2002, Aravin *et al.* 2006, 2007, 2008, Reuter *et al.* 2011). Pachytene piRNAs display enormous diversity in their sequences and their functions remain to be a highly debated topic in the field (Vourekas *et al.* 2012, Homolka *et al.* 2015, Dai *et al.* 2019, Wu *et al.* 2020).

Bioenergetic preference is precisely regulated to support critical events in spermatogenesis

Mammalian SSCs and spermatogonia are located at the base of seminiferous tubules, while spermatocytes and spermatids sequentially migrate from the basal compartment toward the adluminal region (Roosen-Runge & Holstein 1978). It has been implicated that progenitors and differentiating spermatogonia preferentially resided in regions adjacent to the interstitial space with easy access to O_2 provided by testicular vasculature, whereas the SSCs located in an avascular hypoxic environment (Chan *et al.* 2014, DeFalco *et al.* 2015, Lord & Nixon 2020). The differences in oxygen availability may contribute to different metabolic preferences in SSCs and their differentiating counterparts. In alignment with this observation, several studies support that high glycolysis level favors long-term self-renewal of SSCs. For example, rodent SSCs show increased regenerative capacity when cultured under a glycolysis-optimized condition (Helsel *et al.* 2017). In a study by Kanatsu-Shinohara *et al.*, *Myc*/*Mycn* deficiency reduced glycolytic activity, which led to compromised self-renewal of SSCs (Kanatsu-Shinohara *et al.* 2016). By contrast, upregulation of glycolysis by small molecule PS48 or by enforced expression of PDPK1 or AKT1 promoted the establishment of SSC primary culture from the C57BL/6 mouse strain (Kanatsu-Shinohara *et al.* 2016), which is otherwise proven difficult under conventional conditions.

Although a low oxygen tension environment appears to be beneficial for the long-term maintenance of undifferentiated spermatogonia *in vitro* (Oatley *et al.* 2016, Helsel *et al.* 2017), several studies suggest that ROS is required for rodent SSC maintenance (Morimoto *et al.* 2013, 2019, 2021). For example, the addition of ROS inhibitors suppressed SSC self-renewal, whereas hydrogen peroxide increased cell proliferation (Morimoto *et al.* 2013). ROS produced by NADPH1 oxidase 1 (NOX1) drives SSC self-renewal through a ROS-BCL6B-NOX1 feed-forward loop (Morimoto *et al.* 2019). Notably, although ROS may be generated from OXPHOS, the self-renewal promoting function is likely mediated by ROS from a process other than mitochondrial respiration in SSCs. Chemical suppression of mitochondria-derived ROS or deficiency of mitochondrion-specific topoisomerase does not affect SSC self-renewal (Morimoto *et al.* 2021). Therefore, the requirement of ROS in SSC maintenance does not necessarily contradict the high glycolysis level in SSCs. We found that compared to differentiating spermatogonia, mitochondrial OXPHOS activity was much lower in SSCs (Chen *et al.* 2020*b*). In this case,

suppressing OXPHOS or ROS from mitochondrial respiration will only have a modest effect on the total ROS pool of SSCs and thus have little impact on SSC proliferation.

Unlike SSC self-renewal or spermatogonial proliferation, spermatogonial differentiation relies more on mitochondrial respiration. We show that during spermatogenesis, energy production is shifted from glycolysis to OXPHOS to meet the energy demand in spermatogonial differentiation (Chen *et al.* 2020*b*). Inhibiting enzymatic activities in ETC reduces the formation of differentiating spermatogonia but has lesser effects on spermatogonial proliferation (Chen *et al.* 2020*b*). Our data (Chen *et al.* 2020*b*), coupled with other evidence summarized in two recent reviews (Lord & Nixon 2020, Park & Pang 2021), further revealed that glycolytic enzymes and hypoxia-responsive factors are highly expressed in human and mouse SSCs, while regulators reflecting mitochondrial biogenesis, activities, and OXPHOS are upregulated in differentiating progenitors. These findings support the notion that a metabolic shift from glycolysis to mitochondrial respiration is required for spermatogonial differentiation (Fig. 2).

As the process of spermatogenesis advances, spermatocytes and spermatids at the adluminal compartment require lactate and pyruvate for survival (Grootegoed *et al.* 1984, Bajpai *et al.* 1998, Courtens & Ploen 1999). Lactate appears to suppress apoptosis of spermatocytes and spermatids through activation of FAS receptor signaling pathways (Erkkila *et al.* 2002). Recently, Varuzhanyan *et al.* reported higher expression of the mitochondrial pyruvate carrier MPC1 in spermatocytes than in spermatogonia, suggesting that meiotic and post-meiotic spermatocytes may rely heavily on mitochondrial OXPHOS activity (Varuzhanyan *et al.* 2019). In spermatozoa, possibly because ATP-dependent homologous recombination and meiosis complete, energy production goes back to glycolysis, and the expression levels of glycolytic enzymes including hexokinase, glyceraldehyde-3-phosphate dehydrogenase, and phosphofructokinase increase (Westhoff & Kamp 1997, Bunch *et al.* 1998, Mori *et al.* 1998). Despite a long-standing debate on whether glycolysis is the primary energy production source for sperm, it is generally agreed that sperm exhibit great versatility in their metabolic preference and functional demand. Sperm can survive purely on glycolytic energy (Peterson & Freund 1970, Storey & Kayne 1978, Nascimento *et al.* 2008), but they do require OXPHOS for mobility and fertilization (Auger *et al.* 1989, Ruiz-Pesini *et al.* 1998, Ford 2006, Tourmente *et al.* 2015).

Germ cell-specific mitochondrial regulators

Multiple germ cell-specific variants of glycolytic enzymes have been reported, such as HK1S (Mori *et al.* 1993), PGK2 (Robinson *et al.* 1989), and GPAT2 (Wang *et al.* 2007). These enzymes appear to have unique structural or functional properties to support specific metabolic requirements during spermatogenesis (Ramalho-Santos *et al.* 2009). Similarly, mitochondria also have germ cell-specific regulators. For example, the expression of testicular cytochrome c isoform (C_T) increases during the zygotene-to-pachytene transition and becomes the predominant form in sperm (Hess *et al.* 1993). Compared to somatic cytochrome c, C_T has a higher catalytic potential for ROS destruction and stronger pro-apoptotic activity (Liu *et al.* 2006), suggesting a unique functional mechanism that protects sperm from ROS-induced oxidation and eliminates those with damaged DNA due to oxidative stress. An isoform of COX subunit VI has also been reported in sperm, which implicates germ cell-specific regulation in energy production (Huttemann *et al.* 2003).

Several mitochondrial heat shock proteins are known to be specifically expressed in germ cells and play critical roles in spermatogenesis. For example, HSP60 is highly expressed in spermatogonia and primary spermatocyte and subsequently diminished as spermatogenesis advances (Meinhardt *et al.* 1995, Paranko *et al.* 1996). HSP60 reappears in mature and ejaculated spermatozoa (Asquith *et al.* 2004, Lachance *et al.* 2010), supporting its role in fertilization. Another heat shock protein, HSPA2 (or HSP70-2), facilitates the assembly of mitochondrially encoded subunits of the ATP synthase complex (Herrmann *et al.* 1994). In addition, HSPA2 is expressed at high levels in pachytene spermatocytes (Allen *et al.* 1988, Zakeri *et al.* 1988). HSPA2 deficiency causes infertility, probably due to failure in desynapsis (Dix *et al.* 1997), arrest of G2/M transition (Zhu *et al.* 1997), and defects in HSPA2-dependent chromatin condensation and histone replacement/modification (Scieglinska & Krawczyk 2015).

Interaction between mitochondria and piRNA regulators

The main regulators in piRNA biogenesis are PIWI proteins (Lehtiniemi & Kotaja 2018). They belong to PIWI/Argonaute (PIWI/AGO) family and are evolutionarily conserved with the presence of PAZ (Piwi-Argonaute-Zwille) and PIWI domains (Hutvagner & Simard 2008). PIWI proteins are predominantly expressed in germ cells and indispensable for spermatogenesis. Four PIWI members have been identified in humans, Hiwi, Hili, Hiwi2, Hili2, and three in mice, Miwi, Mili, Miwi2 (Cox *et al.* 1998, Kuramochi-Miyagawa *et al.* 2001, Deng & Lin 2002, Sasaki *et al.* 2003, Carmell *et al.* 2007). In addition to PIWI family members, several RNA-binding proteins also contribute to piRNA biogenesis on the surface of mitochondria at IMC, including TDRD1 (Huang *et al.* 2011*b*), DDX4 (Kuramochi-Miyagawa *et al.* 2010), and MOV10L1 (Frost *et al.* 2010, Zheng *et al.*

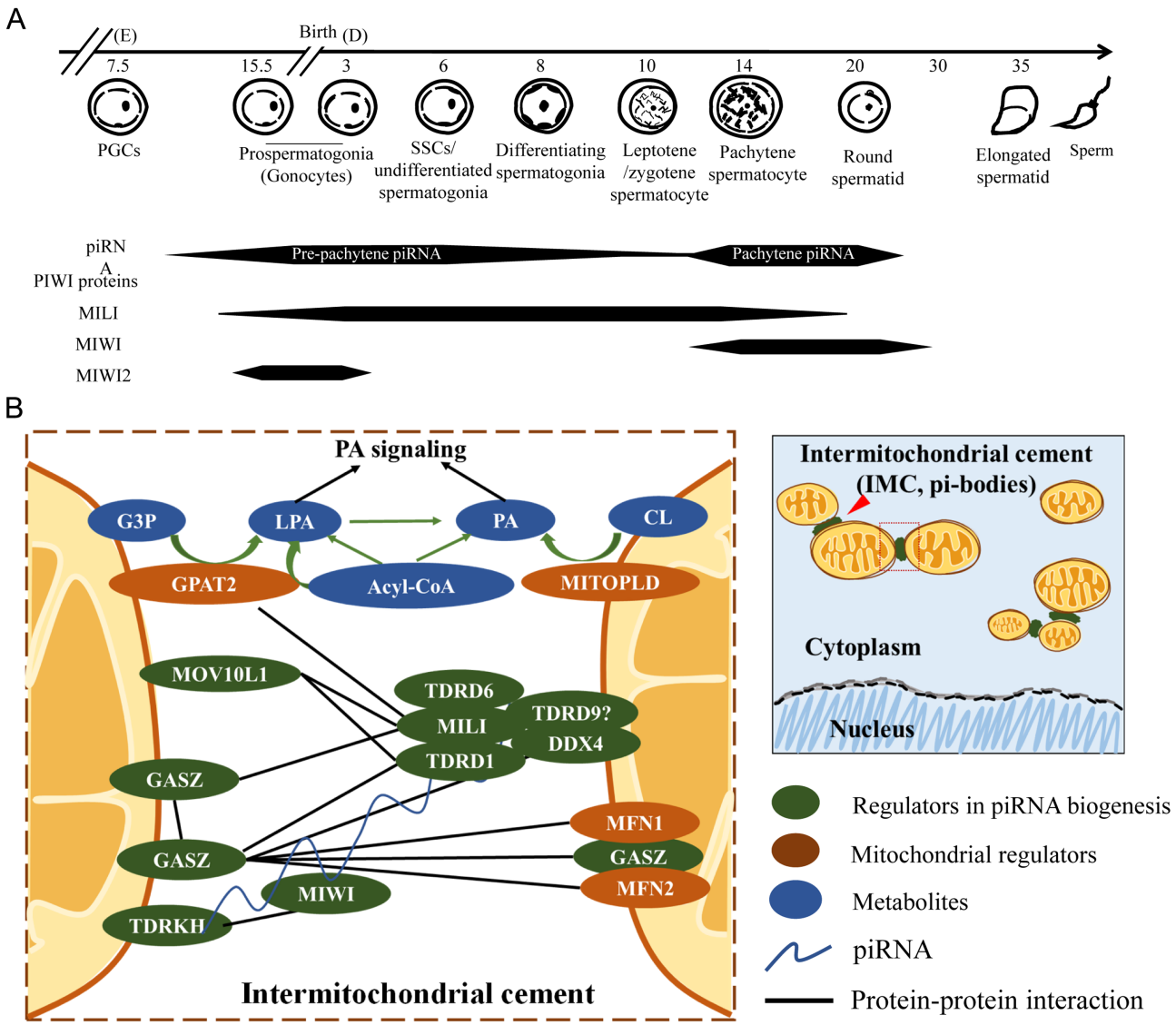

Figure 3 Interaction of germ cell-specific piRNA regulators and mitochondrial modifiers. (A) Expression of PIWI proteins and piRNAs during mouse spermatogenesis. (B) Protein interactions in piRNA biogenesis at the OMM of IMC (left panel) during mouse spermatogenesis. The structure of IMC is shown in the right panel. G3P, glycerol-3-phosphate; LPA, lysophosphatidic acid; PA, phosphatidic acid; CL, cardiolipin.

2010). None of these proteins possess mitochondrial localization signal (MLS), and thus they rely on other mitochondrion-localized proteins to be recruited or to functionally communicate with IMC (Fig. 3).

So far, only a few germ cell-specific proteins are known to be localized directly at mitochondria. Among those, GASZ (also called ASZ1) protein contains four Ankyrin repeats, one sterile alpha motif, and one basic zipper domain (Yan *et al.* 2002, Ma *et al.* 2009). GASZ is localized at the OMM via a c-terminal MLS (Altshuller *et al.* 2013, Zhang *et al.* 2016*a*). Although *Gasz* knockout leads to reduced piRNA biogenesis, to date, there is no evidence that GASZ proteins interact directly with RNAs. Instead, GASZ contributes to piRNA production as an anchorage by recruiting multiple RNA-binding proteins to IMC, including MILI, MIWI, TDRD1, and MVH (Ma *et al.* 2009, Altshuller *et al.* 2013, Zhang *et al.* 2016*a*). We show that deletion of MLS in GASZ dislocates it with MILI and MVH from mitochondria to cytoplasm in germ cells (Zhang *et al.* 2016*a*). Consequently, no IMC is formed, and piRNA expression is dramatically reduced, with spermatogenesis arrested at pachytene spermatocytes (Zhang *et al.* 2016*a*). These data suggest that the mitochondrial localization of GASZ is critical for its role in piRNA biogenesis.

A TUDOR family protein, TDRKH/TDRD2 (TUDOR and KH domain-containing protein 2) is also localized at the OMM via an MLS (Saxe *et al.* 2013). TDRKH plays a crucial role in primary piRNA biogenesis (Saxe *et al.* 2013) and recruits MIWI but not MILI to mitochondria (Chen *et al.* 2009, Zhang *et al.* 2017, Ding *et al.* 2019). Upon deletion, *Tdrkh* mutant mice have drastically

reduced mature piRNAs, leading to germ cell DNA instability, spermiogenic arrest, and male infertility (Saxe *et al.* 2013, Ding *et al.* 2019).

Unlike the germ cell-specific genes described above, *MitoPLD* (*PLD6*), a member of the mammalian phospholipase D superfamily, was originally identified in mammalian somatic cells as a profusogenic factor at the OMM (Choi *et al.* 2006). MITOPLD facilitates mitochondrial fusion by hydrolyzing cardiolipin to generate phosphatidic acid (PA) (Choi *et al.* 2006, Huang *et al.* 2011*a*). PA subsequently recruits phosphatase LIPIN 1, which converts PA to diacylglycerol and promotes mitochondrial fission (Gao & Frohman 2012, Baba *et al.* 2014, Adachi *et al.* 2016). MITOPLD is partially colocalized with perinuclear IMC components and participates in primary piRNA biogenesis (Huang *et al.* 2011*a*, Watanabe *et al.* 2011, Gao & Frohman 2012, Hong *et al.* 2021). In *Mitopld*-deleted pro-spermatogonia, components from IMC/pi-body (e.g. MILI and TDRD1) and piP-body (e.g. MIWI2) show polar aggregation with mitochondria and gamma-tubulin, a marker for centrosome (Huang *et al.* 2011*a*, Watanabe *et al.* 2011). In turn, the spermatogenesis in *MitoPLD* knockout mice is arrested at the meiotic stage with diminished IMC formation and decreased piRNA expression (Huang *et al.* 2011*a*, Watanabe *et al.* 2011). Similar polar aggregation of piRNA pathway proteins has been observed in other germ cell-specific gene mutants, including *Mov10l1* (Zheng & Wang 2012) and *Tdrkh* (Saxe *et al.* 2013, Ding *et al.* 2019) knockout mice. However, no evidence shows that MITOPLD directly interacts with these regulators in piRNA biogenesis (Huang *et al.* 2011*a*, Watanabe *et al.* 2011). Interestingly, in Drosophila, piRNA biogenesis is dependent on a *Drosophila* MITOPLD homolog *Zucchini* (*Zuc*) (Pane *et al.* 2007). Crystal structural analyses also show that mouse MITOPLD may act in primary piRNA processing directly as a nuclease (Ipsaro *et al.* 2012). It remains elusive how MITOPLD affects piRNA biogenesis in mammals. Neither is clear on whether mitochondrial organization at IMC or subcellular localization regulated by MITOPLD directly contributes to piRNA biogenesis.

Glycerol-3-phosphate acyltransferase 2 (GPAT2) is another example of mitochondrial proteins that participate in piRNA biogenesis (Nakagawa *et al.* 2012, Shiromoto *et al.* 2013). GPAT2 catalyzes the conversion of glycerol-3-phosphate and long-chain acyl-CoA to lysophosphatidic acid in *de novo* glycerolipid synthesis(Goossens 2008). The motif IV of GPAT2 anchors this protein to the OMM (Nakagawa *et al.* 2012, Shiromoto *et al.* 2013). Although GPAT2 is detected in some human cancer lines (Wang *et al.* 2007, Garcia-Fabiani *et al.* 2015), in normal tissues, GPAT2 is mainly expressed in germ cells starting from embryonic day 13.5 (Shiromoto *et al.* 2019). GPAT2 has been identified to be an essential interacting partner of MILI in primary piRNA biogenesis (Shiromoto *et al.* 2013). *Gpat2* knockout leads to impaired IMC formation and defective piRNA production in mice (Shiromoto *et al.* 2019). Although both MITOPLD and GPAT2 participate in mitochondrial phospholipid biosynthesis, it is unknown whether PA signaling plays a role in IMC formation and piRNA biogenesis.

Mitochondrial dynamics and spermatogenesis

Mitochondria are highly dynamic organelles, undergoing coordinated cycles of fission and fusion (Bereiter-Hahn & Voth 1994, Chan 2012). Mitofusion is regulated primarily by two GTPases, mitofusin (MFN)1 and MFN2 at the OMM (Eura *et al.* 2003, Chen *et al.* 2007), and OPA1 GTPase at the IMM (Alexander *et al.* 2000, Delettre *et al.* 2000), while fission is mediated by DRP1 GTPase and several adaptors (e.g. FIS1, MFF, and MID49/51), which bring DRP1 to mitochondria (Frank *et al.* 2001, Loson *et al.* 2013). Emerging evidence suggests that mitochondrial dynamics alter mitochondrial features and functions in a developmental stage- and cell type-specific manner, which in turn impacts cell fate decision. For example, disrupting mitofusion specifically impairs the self-renewal of neural stem cells but not the survival of their committed progenitors (Khacho *et al.* 2016). By contrast, inhibition of mitofusion during somatic cell reprogramming upregulates the formation of induced PSCs (Zhong *et al.* 2019).

Mitochondrial dynamics in spermatogenesis also demonstrate a developmental stage-specific regulation (Zhang *et al.* 2016*a*, Varuzhanyan *et al.* 2019, Chen *et al.* 2020*a*). MFN1 and MFN2 are upregulated during spermatogonial differentiation – loss of either destroys male fertility but leaves undifferentiated spermatogonia unaffected (Zhang *et al.* 2016*a*, Varuzhanyan *et al.* 2019, Chen *et al.* 2020*a*). Using a Ddx4-CRE driver, we show that conditional deletion of *Mfn1* or *Mfn2* from pro-spermatogonia in mice causes mitochondrial dysfunction, increased ROS levels, DNA oxidation, and apoptosis mostly in differentiating spermatogonia and spermatocytes, which in turn lead to male infertility (Chen *et al.* 2020*a*, Zhang *et al.* 2016*a*). Similarly, other studies demonstrate that MFN1 and/or MFN2 disruption by Stra8-CRE also cause male infertility, albeit with a relatively delayed meiotic defect (Varuzhanyan *et al.* 2019, Wang *et al.* 2021), possibly due to Stra8-CRE expression only in a fraction of spermatogonia but not in every SSC. Notably, at the early stage of *Mfn1* or *Mfn2* deficiency before puberty, spermatogonia exhibit swollen and enlarged mitochondria, indicating that reduced mitofusion leads to decreased fission to compensate for imbalanced mitochondrial activities (Chen *et al.* 2020*a*, Zhang *et al.* 2016*a*). At this stage, impaired mitofusion in MFN1 or MFN2 mutant mice by Ddx4-CRE does not reduce ATP production (Chen *et al.* 2020*a*, Zhang *et al.* 2016*a*). Instead, it pathologically elevates ROS level that causes DNA oxidation and apoptosis in differentiating

spermatogonia (Chen *et al.* 2020*b*). In this case, blocked spermatogonial differentiation in MFN1 or MFN2 mutant mice may not directly result from reduced ATP production but rather from oxidative stress due to failure in rebalancing mitofusion and fission. When deficiency of mitofusion persists, MFN1/2 mutant germ cells display reduced expression of mitochondrial respiratory complexes and OXPHOS activity (Varuzhanyan *et al.* 2019), which in turn compromises mitochondrial functions and blocks spermatogonial differentiation and meiosis in adult spermatogenesis.

Data from *Drosophila* studies support an indispensable role of mitochondrial fission in spermatogonial maintenance (Senos Demarco *et al.* 2019). Loss of function of *drp1* upregulates ROS production, which increases cell death in germline stem cells and spermatogonia (Senos Demarco *et al.* 2019). In mammals, several studies suggest that defects of mitochondrial fission severely jeopardize late spermatogenesis. For example, conditional knockout of *Fis1,* a *DRP1* adaptor, by Stra8-CRE results in early spermatid arrest with giant multinucleated cells (Varuzhanyan *et al.* 2021*b*). In addition, *Fis1* mutant spermatids exhibit an accumulation of dysfunctional mitochondria with altered ultrastructure and defects in mitophagy (Varuzhanyan *et al.* 2021*b*). Mice with deletion of *Mff,* another *DRP1* adaptor*,* also have reduced fertility and sperm count (Varuzhanyan *et al.* 2021*a*). The remaining sperm in *Mff* mutants display aberrant morphology and motility with decreased respiratory chain complex IV activity (Varuzhanyan *et al.* 2021*a*).

Although sperm need mitochondrial metabolism for energy production to support their mobility and fertilization, the activities of mitochondrial dynamics in sperm are likely kept minimal to ensure proper mitochondrial organization at the base of flagellum. Indeed, low levels of mitofusion and fission in sperm have been reported (Pham *et al.* 2012). Therefore, haploid spermatids likely have high tolerance for perturbations in mitochondrial dynamics. Indeed, our recent study revealed that upon conditional deletion of both *Mfn1* and *Mfn2* in post-meiotic germ cells by Prm1-CRE, mice displayed normal male fertility with functional sperm (Miao *et al.* 2021).

The common machinery for mitochondrial dynamics has been largely elucidated, but the mechanisms of its cell-specific regulation remain elusive. In addition, although MFN1 and MFN2 share more than 80% homology in their protein sequences, the cellular reliance on MFN1 vs MFN2 and their exact functional mechanisms vary by cell type. In germ cells, MFN2 regulates mitochondrial functions through both mitofusion and ER homeostasis (Chen *et al.* 2020*a*), whereas MFN1 predominantly contributes to mitofusion (Chen *et al.* 2020*a*). In our study, both MFN and MFN2 interact with GASZ, a germ cell-specific IMC protein at the OMM (Zhang *et al.* 2016*a*). Interestingly, enhanced GASZ expression in germ cells promotes MFN1/2 dependent mitochondrial aggregation and fusion (Zhang *et al.* 2016*a*), indicating a germ cell-specific regulation of mitochondrial functions. New evidence also shows that MFN2 interacts with several germ cell-specific proteins including a translation regulator MSY2 (YBX2) to regulate piRNA biogenesis and control mRNA fate indirectly during spermatogenesis (Wang *et al.* 2021).

Conclusion and perspectives

In this review, we discussed three key aspects of mitochondrial regulation in postnatal germ cell development. First, as the cellular machinery for the TCA cycle, OXPHOS, and ATP production, mitochondria features, activities, and functions need to be precisely regulated in a germ cell developmental stage-specific manner to support critical transitions during spermatogenesis. To this end, current research focuses on how oxygen accessibility, energy demands, and substrate availability affect energy preference across different germ cell developmental stages. It however remains unclear how altered mitochondrial metabolism drives germ cell fate decisions via epigenetic regulation, signaling pathways, or other post-transcriptional modulation.

Secondly, mitochondria play a critical role in regulating germ cell-specific functions such as piRNA biogenesis. The unique subcellular mitochondrial organization and mitochondrial localization of germ cell-specific proteins are likely vital to this function. Although contributions of classic mitochondrial regulators (e.g. MITOPLD and GPAT2) to piRNA biogenesis have been reported, it remains elusive how these regulators modulate mitochondrial localization or organization to affect piRNA biogenesis. Neither is clear if other mechanisms, for example PA signaling or microtubule-dependent organelle trafficking, are involved.

Thirdly, during spermatogenesis, the dynamic changes in mitochondrial features, activities, and functions are exquisitely regulated by both germ cell-specific factors and known mitochondrial regulators that are shared with somatic cells. Recent findings show that mitochondrial features and activities are tailored by germline factors to adapt for germ cell development. In addition, emerging evidence reveals critical interactions between germ cell-specific factors and classic mitochondrial regulators (e.g. MFNs). This represents a promising research topic to dissect the underlying mechanisms of how such interactions enable mitochondria to participate in germ cell-specific biological processes and prompt mitochondrial responses to various developmental cues, metabolic needs, and physiological or pathological stress.

This line of basic research also bears clinical significance. Infertility affects ~48.5 million couples worldwide, half of which is due to male factors (Agarwal

et al. 2015). Strong evidence links mitochondrial dysfunction (e.g. low mtDNA copy number, high mtDNA mutation load, and swollen mitochondria) to reduced male fertility in humans (Folgero *et al.* 1993, Kao *et al.* 1995, Mundy *et al.* 1995, Carra *et al.* 2004, Sousa *et al.* 2011, Amaral *et al.* 2013, Hamada *et al.* 2013, Demain *et al.* 2017, Wu *et al.* 2019, Vertika *et al.* 2020). Low MFN2 expression was also reported in human asthenozoospermia (Fang *et al.* 2018). However, the mechanisms of these phenomena and whether correcting mitochondrial defects may remedy certain cases of male infertility remain unknown. Studies on mitochondrial regulations of spermatogenesis will fill in these critical knowledge gaps, by revealing novel mechanistic controls of mammalian spermatogenesis and mitochondrial determinants of male reproduction, and may eventually lead to new strategies to improve male reproductive health.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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Author contribution statement

Z Zhang and J Miao drafted the manuscript. Y Wang revised the manuscript.

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