Mitochondria in early development: linking the microenvironment, metabolism and the epigenome

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Abstract

Mitochondria, originally of bacterial origin, are highly dynamic organelles that have evolved a symbiotic relationship within eukaryotic cells. Mitochondria undergo dynamic, stage-specific restructuring and redistribution during oocyte maturation and preimplantation embryo development, necessary to support key developmental events. Mitochondria also fulfil a wide range of functions beyond ATP synthesis, including the production of intracellular reactive oxygen species and calcium regulation, and are active participants in the regulation of signal transduction pathways. Communication between not only mitochondria and the nucleus, but also with other organelles, is emerging as a critical function which regulates preimplantation development. Significantly, perturbations and deficits in mitochondrial function manifest not only as reduced quality and/or poor oocyte and embryo development but contribute to post-implantation failure, long-term cell function and adult disease. A growing body of evidence indicates that altered availability of metabolic co-factors modulate the activity of epigenetic modifiers, such that oocyte and embryo mitochondrial activity and dynamics have the capacity to establish long-lasting alterations to the epigenetic landscape. It is proposed that preimplantation embryo development may represent a sensitive window during which epigenetic regulation by mitochondria is likely to have significant short- and long-term effects on embryo, and offspring, health. Hence, mitochondrial integrity, communication and metabolism are critical links between the environment, the epigenome and the regulation of embryo development.

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Introduction

Mitochondria are double membrane-bound organelles that generate ATP through oxidative phosphorylation (OXPHOS), the components of which are partially encoded by their own genome. They represent the most abundant organelle in the oocyte and undergo significant structural and positional changes during preimplantation development (Motta *et al.* 2000, Sathananthan & Trounson 2000). This dynamic nature is essential for modulating key cellular events, coincident with the changing energy requirements of the embryo (Gardner 1998), yet the significance of mitochondria during preimplantation embryo development extends beyond the generation of ATP. This review integrates recent advances in our understanding of the dynamics of mitochondria, and the interactions between mitochondria and the cytoskeleton, and other organelles, that ensure mitochondrial, and oocyte and embryo, integrity and facilitate signalling. The implications of deficits in mitochondrial dynamics and signalling for embryo viability and offspring health are also explored, with an emphasis on the emerging understanding that modulation of metabolic flux contributes to the regulation of the nuclear epigenetic landscape (Donohoe & Bultman 2012, Harvey *et al.*

2016), proposing a novel role for mitochondria as mediators of metaboloepigenetic processes that programme the oocyte and preimplantation embryo. Consequently, the utility of mitochondria as a biomarker is limited by deficiencies in our understanding of the physiological and cellular consequences of *in vivo* and *in vitro* manipulation of mitochondrial function, and the link between mitochondrial movement and metabolite signalling during preimplantation embryo development that mediate alterations in the epigenome.

Mitochondrial function and cellular homeostasis

Mitochondria are primarily recognised as the powerhouse of cells, synthesising ATP through OXPHOS. The electron transport chain (ETC) comprises five complexes (I–V) that regulate the transfer of nicotinamide adenine dinucleotide (NADH) and succinate through the ETC, and the generation of an electrochemical gradient across the inner mitochondrial membrane to drive ATP synthase (complex V). Respiratory activity is determined by the assembly of respiratory chain complexes into functional supercomplexes within the inner mitochondrial membrane (Cogliati *et al.* 2013), although single complexes are also believed to exist

to enable flexibility and adaptation to environmental changes (Porras & Bai 2015). The environment created within the inner mitochondrial membrane is distinct from the cytosol (Tzagoloff 1982, Herrmann & Riemer 2010), hence the mitochondrion possesses shuttles which regulate specific metabolite levels between the cytoplasm and the mitochondria and contribute to metabolic homeostasis (Herrmann & Riemer 2010, Wang *et al.* 2014). Mitochondrial metabolism also plays a role in the synthesis of macromolecules, such as fatty acids, amino acids and nucleotides, to support proliferation (reviewed by Ahn & Metallo 2015). These diverse functions underscore the significance of mitochondria as regulators of cellular metabolism, however mitochondrial function extends beyond the traditional confines of metabolic regulation.

Historically, mitochondria have been perceived as signalling effectors, that is, mere bystanders in the response to various stimuli. However, mitochondria are increasingly viewed as active participants in numerous pathways, acting as initiators and transducers of signals through the modulation of metabolite availability and changes in redox state (Chandel 2014). Beyond the production of ATP via OXPHOS, mitochondria participate in numerous cellular functions, including Ca^{2+} homeostasis (Duchen 2000, Rizzuto *et al.* 2012), stress responses (Zhao *et al.* 2002, Jovaisaite *et al.* 2014), apoptosis (reviewed by Tait & Green 2013) and have been proposed to be involved in the regulation of chromosome segregation (Schon *et al.* 2000, Qian *et al.* 2012). A consequence of OXPHOS is the production of reactive oxygen species (ROS), yet in stark contrast to the damaging effects of ROS at high concentrations, physiological levels are necessary to act as signalling molecules to directly modulate numerous processes through the modification of kinases, transcription factor activity, growth factors and metabolic enzymes (reviewed Harvey *et al.* 2002, by Hamanaka & Chandel 2010, Lees *et al.* 2017). Consequently, mitochondria are central to cellular and metabolic homeostasis and are key players in diverse physiological (and pathological) processes.

Importantly, mitochondrial function has also been connected to cell specification (Chung *et al.* 2007), where lineage commitment necessitates functional mitochondrial adaptation to support differentiation. Cellular energy demand varies significantly depending on cell function and activity, therefore modulation of energy production to accommodate changing physiological demands is essential and likely underpins cell identity. Indeed, once considered mere by-products of cellular metabolism, recent evidence supports a role for mitochondrial metabolites as signalling molecules that regulate gene expression, through the regulation of epigenetics, and consequently drive lineage decisions (Shyh-Chang *et al.* 2013a, Folmes & Terzic 2014, Harvey *et al.* 2016). Mitochondrial activity is an

absolute requirement for stem cell differentiation, as inhibiting mitochondrial activity with antimycin A or CCCP (carbonyl cyanide *m*-chlorophenyl hydrazine) prevents embryonic stem cell (ESC) differentiation (Mandal *et al.* 2011, Pereira *et al.* 2013). Mitochondrial metabolism in particular controls the levels of the key co-factors including acetyl-CoA, α -ketoglutarate (α KG) and NADH/NAD⁺, as well as other tricarboxylic acid (TCA) intermediates including citrate and succinate, which act as key substrates for epigenetic modifiers (Wallace & Fan 2010). Studies in ESC have begun to highlight the significance of nutrient regulation in establishing the epigenome (reviewed by Harvey *et al.* 2016), however, our understanding of the consequences of modulating nutrient and metabolic intermediate levels on the epigenetic landscape in the oocyte, and during preimplantation embryo development, remains rudimentary.

Dynamic regulation of the preimplantation epigenome

Preimplantation embryo development is characterised by extensive reprogramming of the epigenetic landscape to support and regulate stage-specific events including embryonic genome activation and lineage specification. Following fertilisation, the paternal genome undergoes a wave of active DNA demethylation (Mayer *et al.* 2000, Oswald *et al.* 2000). Subsequently, passive demethylation proceeds with successive cleavage divisions, persisting through to the morula stage (Monk *et al.* 1987, Santos *et al.* 2002, Zaitseva *et al.* 2007), with the exception of imprinted genes and some repetitive elements. Formation of the blastocyst results in the establishment of the first cell lineages, in which the pluripotent inner cell mass (ICM) exhibits higher levels of *de novo* methylation compared with the trophectoderm (Reik *et al.* 2003). In addition to DNA methylation, chromatin remodelling and histone modifications also play a critical role in programming the embryonic epigenetic landscape. However, in contrast to DNA methylation, the dynamics of acetylation (ac) and histone methylation (me) during early development is largely limited to studies on the expression of histone (de)acetylases, methyltransferases and demethylases (reviewed Shi & Wu 2009, by Mason *et al.* 2012). Immunofluorescent analyses have however documented asymmetry in a number of pronuclear modifications (Adenot *et al.* 1997, Santos *et al.* 2005, van der Heijden *et al.* 2005, Boskovic *et al.* 2012), along with the presence of other marks in both pronuclei (Zhou *et al.* 2014, Rollo *et al.* 2017) and stage-specific changes (Ma & Schultz 2008, Boskovic *et al.* 2012, Liu *et al.* 2016b). Consequently, normal development relies on coordinated changes in DNA methylation, and histone acetylation and methylation, which underpin

the activation and silencing of specific genes (reviewed by Santos & Dean 2004, Lucas 2013, Messerschmidt *et al.* 2014, Marcho *et al.* 2015).

Aberrant epigenetic patterns have been correlated with alterations in the microenvironment surrounding the developing embryo, as well as with subsequent developmental failure (Lucas 2013, Vickaryous & Whitelaw 2005, Ma & Schultz 2008). *In vitro* embryo culture has been shown to accelerate DNA demethylation of rabbit embryos (Reis e Silva *et al.* 2012) and significantly lower histone 3 lysine 4 trimethylation (H3K4me3; Wu *et al.* 2012), while exposure of *in vivo* derived bovine embryos to *in vitro* culture conditions induces significant hypermethylation or hypomethylation depending on the timing of exposure, and include alterations in metabolic pathways (Salilew-Wondim *et al.* 2018). Further, increased acetylation of histone 3 lysine 9 (H3K9ac) has recently been proposed as a biomarker of culture-induced perturbations (Rollo *et al.* 2017). Importantly, inhibition of histone deacetylase activity with trichostatin A reduces (Adenot *et al.* 1997) or inhibits (Ito *et al.* 2000) blastocyst development, and perturbations in the programming of the preimplantation epigenome have been shown to reduce implantation (Ding *et al.* 2012) and disturb post-implantation decidualisation (Gao *et al.* 2012). While it remains to be determined whether epigenetic perturbations persist into foetal tissues, combined, these studies reveal that the preimplantation embryonic epigenome is sensitive to alterations in the surrounding nutrient environment, with alterations negatively impacting on subsequent development. Significantly, the activity of epigenetic modifiers that regulate the deposition and removal of DNA and histone marks depends on the availability of specific metabolites. Conceivably, the changing energy requirements, and therefore metabolic pathway activity, of the preimplantation embryo may drive epigenetic programming, such that perturbed metabolic activity transmits alterations in the cellular microenvironment which modify the epigenetic landscape (Gardner & Harvey 2015).

Metabolic regulators of acetylation and methylation

Levels of histone acetylation are modulated by histone acetyltransferases (HATs) and histone deacetylases (HDACs). Acetylation by histone acetyltransferases requires acetyl-CoA, a metabolic intermediate whose availability dictates histone acetylation levels, and consequently gene transcription. High glycolytic rates drive acetyl-CoA synthesis in mitochondria, although acetyl-CoA can also be produced in the cytosol from citrate, fatty acid and ketone body oxidation and the catabolism of amino acids, or within the nucleus (Wellen *et al.* 2009). Recently, glucose flux through glycolysis has been shown to regulate ESC acetyl-CoA levels, and therefore histone acetylation (Moussaieff *et al.* 2015).

Supplementing human ESC with acetate, a precursor of acetyl-CoA, increases acetylation levels, blocks histone deacetylation and reduces differentiation, while inhibition of acetyl-CoA production from glucose results in the loss of pluripotency (Moussaieff *et al.* 2015). Alterations in glucose use can therefore significantly modify not only downstream metabolite levels, but also alter histone acetylation, through altered acetyl-CoA levels, and subsequently regulate differentiation. The significance of this is such that incorrect timing of substrate availability in the early embryo may accelerate or delay key events through gene activation and is particularly relevant given the changing energy requirements of the preimplantation embryo (reviewed by Gardner & Harvey 2015) and the significance of glucose use as a marker of human embryo viability (Gardner *et al.* 2011). As alterations in glucose uptake can significantly alter acetyl-CoA availability, it will be important to understand the impact of altering acetyl-CoA levels during preimplantation development. A role for appropriate modulation of acetyl-CoA levels to support embryo development, potentially via the epigenome, is however plausible given the identification of the embryotrophic factor promoting blastocyst development within bovine serum albumin as the acetyl-CoA precursor citrate (Gray *et al.* 1992).

In contrast, repression of gene transcription is in part mediated through histone deacetylation. In addition to Class I and II HDACs, which are dependent on zinc as a co-factor for their activity, nicotinamide adenine dinucleotide (NAD⁺) availability regulates a family of conserved NAD⁺-dependent histone deacetylases known as sirtuins (SIRT6), which deacetylate targets involved in the modulation of cellular metabolism and mitochondrial biogenesis, cell survival, DNA repair, differentiation and ROS production (Houtkooper *et al.* 2012). Of note, alterations in sirtuin activity have been shown to alter embryo development. Inhibition of sirtuin activity with nicotinamide or sirtinol significantly reduces porcine (Kwak *et al.* 2012) and mouse (Kawamura *et al.* 2010) blastocyst development, conceivably through alterations in acetylation. It will however be important to examine the regulation of sirtuins, and sirtuin regulated changes in the epigenome, during preimplantation development in response to nutrient availability, particularly given the expanded use of monophasic culture systems in preference to biphasic media. Equally, investigation of the significance of NAD⁺ in regulating sirtuin activity during preimplantation development is warranted. NAD⁺ is synthesised *de novo* from the amino acid tryptophan (Bender 1983) or recycled via the NAD⁺ salvage pathway from nicotinamide and derivatives of vitamin B3 (Canto *et al.* 2015). In order to ensure the continuation of glycolysis, NAD⁺ is also recycled via the conversion of pyruvate to lactate, the production of which increases with blastocyst development (Gardner & Harvey 2015). NADH reducing equivalents are also shuttled across the

impermeable inner mitochondrial membrane via the malate aspartate shuttle (MAS) to maintain $\text{NAD}^+:\text{NADH}$ (Lu *et al.* 2008). Significantly, the activity of the MAS is responsible for oxidative metabolism during embryo development (Lane & Gardner 2005) and inhibition of MAS activity with amino-oxyacetate impairs embryo metabolism and ATP production, accompanied by reduced blastocyst development and placental and foetal growth (Mitchell *et al.* 2009, Wakefield *et al.* 2011). Whether these changes are accompanied by alterations in the foetal epigenome remains to be determined. These studies however highlight a role for NAD^+ regulation in programming development, and a requirement for coordinated compartmentalisation of metabolites between the cytoplasm and mitochondria to maintain mitochondrial function and ensure normal development.

In a similar manner, the activity of DNA methyltransferases (DNMTs), which catalyse the addition of methyl groups to the 5' position of cytosine residues within cytosine-guanine dinucleotides (CpG) and result in the formation of 5-methylcytosine (5mC), as well as histone methyltransferases which catalyse the transfer of 1 (me), 2 (me₂) or 3 (me₃) methyl groups to lysine and arginine residues on histones, rely on metabolic intermediate availability. The amino acid methionine is the precursor of the universal methyl donor S-adenosylmethionine (SAM), catalysed by methionine adenosyltransferases (MATs), that acts as a co-factor in methylation reactions (Finkelstein 1990). Methionine has been found to be essential for mouse and bovine blastocyst formation, as omission of methionine from culture reduces mouse blastocyst formation, and morula and blastocyst H3K4me₃ (Sun *et al.* 2018), while addition of the methionine antagonist ethionine following embryonic genome activation (EGA) impairs blastocyst development and cell number (Menezo *et al.* 1989, Ikeda *et al.* 2012, Kudo *et al.* 2015) accompanied by a reduction in H3K9me₃ modification of Nanog and Tead4 (Kudo *et al.* 2015). Importantly, this loss in developmental potential coincides with the increase in SAM synthesis that occurs following compaction (Menezo *et al.* 1989), suggesting a requirement for appropriate metabolite availability at the time of EGA. A requirement for SAM is also evidenced by studies that demonstrate that inhibition of MAT2A, which catalyses the formation of SAM, significantly reduces bovine and mouse blastocyst development and cell number and increases apoptosis (Ikeda *et al.* 2017, Sun *et al.* 2018). Conversely, SAM supplementation during bovine embryo culture enhances hatching, accompanied by significant alterations in the global DNA methylation landscape (Shojaei Saadi *et al.* 2016). Likewise, methionine deprivation reduces SAM levels in human ESC, accompanied by a rapid decrease in H3K4me₃ and reduction in global DNA methylation, which can be abrogated through SAM supplementation, with changes

in methylation potentiating subsequent differentiation (Shiraki *et al.* 2014). These data reveal that methionine is important not only for maintaining the pluripotent epigenome but its availability has the potential to alter the timing of lineage specification.

Maternal methionine supplementation has likewise been associated with alterations in bovine blastocyst gene expression (Penagaricano *et al.* 2013), demonstrating that even subtle changes in methionine availability can impact development. Indeed, methionine utilisation has been proposed as a biomarker of human embryo developmental potential (Houghton *et al.* 2002). Considerably, deficiencies in metabolites that intersect the folate and methionine cycles, including folate (O'Neill 1998, Kwong *et al.* 2010) and vitamin B12 (Sinclair *et al.* 2007), are also known to impact embryo development, and more significantly, alter post-natal growth and health and DNA methylation (Sinclair *et al.* 2007). Threonine, metabolised via the folate cycle, likewise regulates SAM levels in mouse ESC (Wang *et al.* 2009a, Shyh-Chang *et al.* 2013b). The cytoplasmic folate cycle intersects with mitochondrial formate availability (Bao *et al.* 2016), while mitochondrial NADH oxidation, and therefore TCA activity, has recently been linked to methionine metabolism (Lozoya *et al.* 2018), highlighting a link between mitochondrial activity and methylation. Mitochondrial one-carbon metabolism therefore integrates nutrient, amino acid metabolism and mitochondrial activity, such that differences in pathway flux can profoundly alter the epigenome.

Conversely, α -KG, a product of the oxidation of glucose and glutamine via the mitochondrial TCA not only modulates the activity of Ten-Eleven translocation (TET) dioxygenases (Xiao *et al.* 2012) but also regulates the activity of Jumanji (JM) demethylases which remove methyl groups from lysine residues (Tsukada *et al.* 2006). The TET family of enzymes (TET1, 2 and 3) use oxygen to catalyse the oxidative decarboxylation of α -KG, generating a reactive enzyme-bound intermediate which converts 5mC to 5-hydroxymethylcytosine (5hmC). At the pronuclear stage, Tet3 mediates active demethylation of the paternal genome (Gu *et al.* 2011, Iqbal *et al.* 2011, Wossidlo *et al.* 2011) with 5hmC progressively lost through to the 8-cell stage (Inoue & Zhang 2011). In contrast, H3K9me₂ of maternal chromatin enables the recruitment of Dppa3 (also known as Stella) to exclude Tet3 from binding and enabling its passive demethylation (Wossidlo *et al.* 2011, Nakamura *et al.* 2012). Tet3 expression in porcine embryos is regulated by EGA and modulates Nanog (Gu *et al.* 2011, Lee *et al.* 2014b), suggesting an involvement in lineage specification. Tet1 is detectable throughout preimplantation development in the mouse (Ito *et al.* 2010) and from the 4-cell stage in porcine embryos (Lee *et al.* 2014b) and becomes enriched in the ICM compared with the trophectoderm at the blastocyst stage, being absolutely required for specification toward

the ICM compartment (Ito *et al.* 2010). The differential expression pattern plausibly reflects the differences in metabolism between these two lineages. Indeed, glucose- and glutamine-derived α -KG modulate mouse ESC histone and TET-dependent DNA demethylation (Carey *et al.* 2015), suggesting that glycolytic activity supports DNA demethylation and plausibly that appropriate uptake of glucose is likely necessary to establish correct (de)methylation dynamics. This may in part explain the significance of the relationship between glucose uptake and embryo viability (Gardner & Leese 1987, Gardner *et al.* 2011). Indeed, *in vitro* culture has been associated with increased DNA methylation relative to *in vivo* derived embryos in both the mouse and rat (Zaitseva *et al.* 2007), suggesting dysregulation of demethylating enzymes. It will therefore be important to determine whether modulation of α -KG impacts embryo development, accompanied by changes in methylation.

Appropriate regulation of histone demethylation is also required for normal preimplantation embryo development, mediated by JMJ demethylases which remove the methyl groups from lysine residues in histones in a Fe(2+) and α -KG-dependent manner. Knockdown of maternal JMJD3 (also known as KDM6B), required for the demethylation of H3K27me3 from the 2-cell stage, significantly reduces mouse (Canovas *et al.* 2012) and bovine blastocyst development accompanied by a reduction in both ICM and trophectoderm cell numbers (Chung *et al.* 2017), with JMJD3 activity correlated with α -KG availability in mouse ESC (Carey *et al.* 2015). Moreover, knockdown of JMJD2C (Wang *et al.* 2010) and the JmJc demethylase KDM5B (Huang *et al.* 2015) impairs mouse and porcine blastocyst development respectively, accompanied by perturbations in histone methylation, while inhibition of H3K4 demethylation impairs mouse embryo cleavage at the time of EGA (Shao *et al.* 2008), further indicating that appropriate, stage-specific regulation of the epigenetic landscape is required for development.

Combined, these studies identify that metabolism, and in particular mitochondrial activity, acts as a primary interface between the environment and the epigenome (Fig. 1). As a consequence, perturbations in metabolic pathway activity alter the production and availability of co-factors required for epigenetic modifier activity, resulting in an altered, and heritable, nuclear epigenetic landscape (reviewed by Donohoe & Bultman 2012, Brown *et al.* 2015, Harvey *et al.* 2016) at the time of lineage specification when the epigenetic landscape is being programmed. Therefore, perturbations in the nutrient environment surrounding the oocyte and preimplantation embryo may modulate long-term offspring health and viability through alterations in metabolism that impact the programming epigenome. However, it is also important to consider the dynamic nature of mitochondria within the oocyte and early embryo, as a means to deliver not only ATP but also

facilitate and regulate the deposition and removal of epigenetic modifications in a stage-specific manner.

Mitochondrial dysfunction and fertility

In order to support the dynamic changes that occur during preimplantation embryo development, mitochondria within the oocyte and early embryo have primarily been regarded as necessary to generate adequate ATP to fuel the first few days of embryo development (Dumollard *et al.* 2004). In line with this, the mammalian preimplantation embryo undergoes significant changes in its metabolism during development, where metabolic pathway activity and ATP content vary significantly between human oocytes and embryos (Leese *et al.* 1986, Chi *et al.* 1988, Hardy *et al.* 1989, Gott *et al.* 1990, Slotte *et al.* 1990, Conaghan *et al.* 1993, Gardner & Harvey 2015). Growing oocytes preferentially metabolise pyruvate via OXPHOS, while their somatic compartment, the cumulus cells, are more glycolytic, metabolising glucose to supply the pyruvate required by the oocyte (reviewed by Dumesic *et al.* 2015). Similarly, the early embryo is characterised by low levels of oxidative metabolism and oxygen consumption, utilising pyruvate, lactate and amino acids to support development (Houghton *et al.* 1996, Gardner & Wale 2013). In contrast, with the activation of the embryonic genome and the increased energy demand required for blastocoel formation, the blastocyst stage embryo exhibits both high levels of glycolysis and oxygen consumption (reviewed Mills & Brinster 1967, Wales 1986, Houghton *et al.* 1996, Thompson *et al.* 1996, by Gardner & Harvey 2015). These changing energy requirements therefore necessitate dynamic and coordinated regulation of metabolism to support ongoing development.

In further support of a requirement for mitochondrial ATP production during development, perturbations in mitochondrial activity have been found to impact embryo development and implantation potential (Van Blerkom *et al.* 1995), as well as foetal and placental growth (Wakefield *et al.* 2011). Oocytes containing higher levels of ATP result in significantly higher fertilisation rates and increased blastocyst development. Conversely, those deficient in ATP result in compromised development (Stojkovic *et al.* 2001, Nagano *et al.* 2006), reflecting the need for sufficient ATP to support embryo development. A reduced capacity of oocytes to generate ATP has been observed in aged mouse and hamster oocytes (Simsek-Duran *et al.* 2013) and has been associated with abnormal nuclear spindle and chaotic chromosomal distribution in humans (Wilding *et al.* 2003) with oocyte mitochondrial activity in particular known to decrease linearly with increasing age (Wilding *et al.* 2001), and vary with stimulation regimen (Cree *et al.* 2015). Alterations in ATP content manifest not only as poor or abnormal human oocyte quality but also as reduced embryo development and pregnancy rates

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modulation of the epigenetic landscape. Indeed, oocyte ageing is accompanied by reduced 5mC and DNMT expression levels (Yue *et al.* 2012), H4K12 and H4K16 acetylation (Manosalva & Gonzalez 2009) and histone methylation (Manosalva & Gonzalez 2010). Taken together, it is plausible that alterations in metabolic activity mediate the changes in the epigenetic landscape of oocytes and/or the early embryo, impacting subsequent developmental competence. Surprisingly, relatively few

studies have investigated specific reproductive tract metabolite levels associated with ageing or dietary changes. Alterations in human follicular fluid lipid profiles have been associated with ageing (Cordeiro *et al.* 2018), as well as with embryos that fail to cleave (O’Gorman *et al.* 2013), however whether epigenetic change is elicited by these environments remains unclear. Ageing likewise alters cumulus cell function, with a significant impact on transcripts (Al-Edani *et al.* 2014) and proteins (McReynolds *et al.* 2012) involved in metabolism, possibly reflecting a reduced ability to support the metabolic and epigenetic requirements of the oocyte. As acetylation has recently been shown to be regulated by lipids and lipid-derived acetyl-CoA (McDonnell *et al.* 2016), and circulating maternal lipids have been shown to programme the developing foetal DNA methylation landscape (Marchlewicz *et al.* 2016), alterations in follicular fluid lipid content with ageing may perturb the oocyte epigenetic landscape. Indeed, lipid-derived acetyl-CoA is more readily incorporated into histones than that derived from glucose (McDonnell *et al.* 2016), emphasising that metabolic plasticity results in epigenetic remodelling in response to nutrient availability. Oocyte NADH/FAD levels have also been correlated with ageing (Babayev *et al.* 2016), and mitochondrial dysfunction has been associated with reduced NADH/NADPH in human zygotes (Wilding *et al.* 2001, 2002, 2003), yet whether such changes impact oocytes from young individuals, or the regulation of the sirtuin NAD⁺-dependent deacetylases, remains to be determined. The effects of ageing on the levels of other metabolites within follicular or reproductive tract fluid, including acetyl-CoA, have similarly not been investigated. It will therefore be essential to examine metabolite perturbations associated with poor developmental competence and outcomes, along with consequences for long-term offspring health, to establish whether specific changes in metabolite content associated with ageing and nutrient availability result in epigenetic priming that plays a role in the reduction in developmental competence.

Mitochondrial morphology in the oocyte and preimplantation embryo

During preimplantation embryo development, mitochondria undergo dynamic, functional and structural changes, morphing from spherical organelles containing dense matrices and few peripheral arched cristae to long filamentous organelles with numerous transverse cristae that maximise the surface area available for OXPHOS (Zick *et al.* 2009). This contrasts with the reticular, elongated, electron- and cristae-rich morphology of somatic cells. A spherical morphology is also characteristic of ESC (St John *et al.* 2005, Cho *et al.* 2006, Chung *et al.* 2007), suggesting that it underpins

the pluripotency of these cells (reviewed by Harvey *et al.* 2013). These mitochondrial changes therefore reflect the developmental stage, and metabolic requirements, of the embryo (reviewed by Bavister & Squirrell 2000, Lees *et al.* 2017), but more significantly likely also facilitate mitochondrial signalling and crosstalk.

The primary human oocyte contains many spherical mitochondria with few peripheral arched cristae dispersed throughout the cytoplasm (Motta *et al.* 2000). Mitochondria remain primarily spherical during folliculogenesis, with pale matrices and small vesicular cristae, clustered around the nucleus (Stern *et al.* 1971, Sathananthan & Trounson 2000, Van Blerkom 2004). While primordial oocytes contain approximately 10,000 mitochondria, this increases to around 100,000 in mature oocytes (Lanzavecchia & Mangioni 1964, Sathananthan *et al.* 1986, Jansen & de Boer 1998). By ovulation, mitochondria are the most prominent organelle in the oocyte cytoplasm (Motta *et al.* 2000). During early cleavage, elongated mitochondria with well-developed transverse cristae begin to emerge (Piko & Matsumoto 1976, Motta *et al.* 2000). As development proceeds, the proportion of elongated, cristate-rich mitochondria increases, such that by the blastocyst stage in the human and mouse, blastomeres contain a mixed population of predominantly spherical and elongated mitochondria in the ICM and trophectoderm respectively (Nadijcka & Hillman 1974, Mohr & Trounson 1982, Motta *et al.* 2000, Sathananthan & Trounson 2000, Lees *et al.* 2017).

Despite the perceived ‘immature’ morphology, oocyte mitochondria are highly active, producing the majority of energy required to support development. The structural changes are however essential for preimplantation embryo development to proceed (Van Blerkom 1989) and are consistent with a progressive activation of mitochondrial respiratory activity, inferred from increased oxygen consumption and ATP production via OXPHOS (Houghton *et al.* 1996, Thompson *et al.* 1996, Trimarchi *et al.* 2000, Sturmey & Leese 2003) and changes in cristae shape (Mohr & Trounson 1982, Sathananthan & Trounson 2000, Cogliati *et al.* 2013). Interestingly, human apical trophectoderm cells are characterised by elongated mitochondria with transverse cristae, largely located around the periphery (Mohr & Trounson 1982). This morphological difference in both mitochondrial architecture and activity likely reflects differences in energy demand, but may also facilitate the process of implantation or may play a role in regulating cell specification, plausibly by differential metabolite availability regulating the epigenome. Metabolites are increasingly recognised as regulators of pluripotency and cell fate (Chung *et al.* 2007, Shyh-Chang *et al.* 2013b, Shiraki *et al.* 2014, Moussaieff *et al.* 2015), with mitochondria at the heart of sensing and regulating lineage decisions and establishing cellular memory (reviewed by Harvey *et al.* 2016, Lees *et al.* 2017).

It is tempting to speculate that mitochondria may be involved in the regulation of the earliest embryonic specification event, the establishment of a polarised epithelium, in a manner similar to that observed when hepatocytes initiate polarisation, necessitating mitochondrial elongation (Fu *et al.* 2013). Notably, ATP generated via OXPHOS is required for this process (Fu *et al.* 2013). Further, the Hippo pathway, which mediates the differentiation of the trophectoderm from the ICM (reviewed by Manzanares & Rodriguez 2013), also regulates mitochondria fusion and size (Nagaraj *et al.* 2012). These data suggest that mitochondrial structure and activity, and cell polarisation, may be intricately linked, such that deficiencies in mitochondrial dynamics and activity during early development may have a significant impact on cell allocation and developmental capacity beyond the provision of ATP.

Regulation of mitochondrial morphology

Mitochondrial morphology adapts to fluctuating cellular requirements by modulating changes in shape through fission and fusion (Frederick & Shaw 2007), continually dividing and fusing to form a dynamic interconnecting network necessary to maintain mitochondrial and mtDNA integrity (Suen *et al.* 2008). Fission and fusion events are mediated by a number of nuclear encoded proteins (reviewed by Wai & Langer 2016). Mitochondrial fusion not only enables the creation of extensive mitochondrial networks to increase energy supply (Chen *et al.* 2005) but also the complementation, and the mixing of mitochondrial content, between healthy and defective mitochondria (Youle & van der Bliek 2012). The outer mitochondrial membrane GTPase required for mitochondrial fusion; fuzzy onions (fzo; Hales & Fuller 1997) was identified as an essential regulator of spermatogenesis. The human homologs, mitofusin 1 and 2 (MFN1, MFN2 respectively) likewise display GTPase-dependent activity and mediate mitochondrial fusion (Santel & Fuller 2001, Chen & Chan 2004). Cells deficient in either Mfn1 or Mfn2 display considerably reduced levels of mitochondrial fusion and distinct types of fragmented mitochondria (Chen *et al.* 2003, 2005) and increased ROS (Lee *et al.* 2014a). Beyond their role in spermatogenesis, they also contribute to ongoing development. Mice deficient in either Mfn1 or Mfn2 die around embryonic day (E) 11.5. Mfn1 mutants are significantly smaller with a pronounced developmental delay prior to resorption, while Mfn2 mutant embryos display a severe disruption of the placental trophoblast giant cell layer (Chen *et al.* 2003). Mfn2 knockdown in oocytes reduces oocyte maturation and fertilisation and alters mitochondrial distribution and spindle morphology (Liu *et al.* 2016a), further implicating mitochondria in the regulation of chromosome segregation in the oocyte. Moreover, reduced Mfn2 expression following siRNA attenuates mouse blastocyst formation and significantly

reduces the number of embryos progressing through the third cell division (Zhao *et al.* 2015). As this coincides with the activation of the embryonic genome, active mitochondrial fusion is likely required to support this milestone. Embryos that survive Mfn2 knockdown, exhibit decreased ATP and mtDNA levels, reduced mitochondrial membrane potential and increased levels of apoptosis (Zhao *et al.* 2015), further suggesting that adequate control of mitochondrial dynamics is necessary for continued development, however whether these knockout phenotypes were accompanied by alterations in the epigenome, particularly at the time of EGA, were not examined.

A third protein, Optic Atrophy 1 (OPA1), is also essential for inner mitochondrial membrane fusion (Olichon *et al.* 2003). Opa1 knockout is similarly embryonically lethal by E13.5 in the mouse, further supporting a role for mitochondrial dynamics in regulating development (Davies *et al.* 2007). Loss of OPA1 results in cristae widening, thereby playing a role in the regulation of mitochondrial cristae shape (Patten *et al.* 2014). Significantly, low ADP concentrations, characteristic of the late cleavage and morula stage embryo (Leese *et al.* 1984), were reported to modify cristae shape to a more expanded, less dense matrix more than 50 years ago (Hackenbrock 1966), however only recently has cristae shape and volume been found to modulate the rates of several TCA cycle enzymes (Lizana *et al.* 2008), as well as the mitochondrial membrane, redox-sensitive protein ROS modulator 1 (ROMO1; Norton *et al.* 2014) and cardiolipin (Ikou & Ryan 2017, reviewed by Quintana-Cabrera *et al.* 2018). How these pathways are regulated by changes in nutrient availability, particularly during embryo development, remains to be determined. Given the redox-sensitive nature of OPA1 (Garcia *et al.* 2018) and ROMO1, ROS, NAD⁺:NADH and ATP:ADP ratios, which are impacted by *in vitro* and *in vivo* nutrient availability, are likely important signalling molecules that link mitochondrial metabolism, cristae morphology and cell signalling. Conceivably, any form of oxidative stress or metabolic dysfunction induced by assisted reproductive technologies, including culture, may therefore interfere with mitochondrial dynamics.

Conversely, the process of mitochondrial fission is mediated in part by dynamin-related protein 1 (Drp1), a large GTPase and fission 1 (Fis1). Drp1 is a large dynamin-related GTPase located primarily in the cytoplasm, with punctuate localisation marking future sites of fission (Smirnova *et al.* 2001). Downregulation of Drp1 results in loss of mitochondrial DNA, decreased mitochondrial respiration and increased ROS production in HeLa cells (Parone *et al.* 2003), as well as reduced cell proliferation and cellular ATP. Knockout of Drp1 in mice leads to embryonic lethality at E11.5 accompanied by a reduction in size, plausibly attributable to the loss of placental giant cells (Ishihara

et al. 2009, Wakabayashi *et al.* 2009). Perturbations in placental structure would contribute to reduced nutrient transport, thereby implicating mitochondria in regulating placental cell differentiation and fate. In addition, oocyte specific Drp1 knockout results in infertility (Udagawa *et al.* 2014), and both decreased expression of Drp1 and increased Mfn2 mRNA levels, favouring mitochondrial fusion, have been associated with fragmented human day 3 embryos (Otasevic *et al.* 2016), although whether this is a cause or consequence is unclear. Further, inhibition of mitochondrial fission with the specific Drp1 inhibitor Mdivi-1 reduces porcine blastocyst formation accompanied by reduced mitochondrial membrane potential and increased ROS (Yeon *et al.* 2015), with increasing expression during early porcine cleavage stages indicative of higher quality embryos (Yang *et al.* 2018). Significantly, disruption of mitochondrial fission via Drp1 siRNA results in chromosomal instability and over-amplification of centromeres in a number of cell lines (Qian *et al.* 2012), suggesting that the incidence of aneuploidy may reflect compromised mitochondrial dynamics, again linking mitochondrial function to chromosomal segregation. Additionally, ROS are also known to directly alter the epigenetic landscape (Hitchler & Domann 2009), such that conditions that result in the overproduction of ROS, including through the disruption of mitochondrial fission/fusion, may result in ROS-mediated changes to the epigenetic landscape. A second key component of mitochondrial fission is Fis1 (Fission 1), a transmembrane protein localised to the outer mitochondrial membrane, recruits Drp1 to areas of mitochondrial constriction, resulting in membrane fission (Stojanovski *et al.* 2004). However, no studies have addressed the role of Fis1 during mammalian embryo development.

Collectively, these data reveal that a balance between mitochondrial fission and fusion is required to regulate developmental progression and embryo viability, likely to ensure segregation of mitochondria between cells and ensure appropriate regulation of chromosome segregation, but also support appropriate mitochondrial activity and ROS levels. This balance can be perturbed by changes in nutrient availability, leading to mitochondrial fragmentation or hyperfusion and is dependent on mitochondrial membrane potential (Ishihara *et al.* 2003). Whether active fission and fusion events are altered in *in vitro* cultured embryos, or by alterations in nutrient availability, have not been examined. While knockout studies highlight the significance of regulators of mitochondrial dynamics during *in vivo* development, few studies have addressed changes that may be induced by loss of these proteins prior to implantation that may correlate with reduced potential or implantation failure. Equally, studies examining modulation of mitochondrial dynamics have not assessed whether changes in methylation or acetylation occur.

The dynamic nature of mitochondria: relocalisation to facilitate stage-specific epigenetic programming?

Mitochondrial localisation also changes significantly throughout oocyte and early embryo development. Given the role of mitochondrially derived metabolic intermediates in regulating the epigenome, mitochondrial trafficking may serve to not only transport mitochondria to sites of energy demand, and facilitate communication and interaction with other organelles to support embryo development, but also to maintain metabolite/signalling gradients with the nucleus.

Although subtle species differences exist, significant mitochondrial (re)distribution occurs in the developing oocyte (Takahashi *et al.* 2016), with distinct patterns of localisation in pronuclear oocytes and early cleavage stage embryos correlated with developmental potential (Wang *et al.* 2009c, Igosheva *et al.* 2010, Yu *et al.* 2010). In mouse and human oocytes, highly polarised mitochondria cluster around the oocyte cortex (Van Blerkom *et al.* 2003, Van Blerkom & Davis 2006, 2007), proposed to maintain sufficient ATP for fertilisation, but also to support Ca²⁺ regulation upon fertilisation. In contrast, mitochondria with low membrane potential are homogeneously distributed throughout the cytoplasm. The cortical localisation of active mitochondria may conceivably be required to support oocyte metabolism through close proximity of mitochondria to the source of pyruvate (the surrounding cumulus cells) given that the oocyte is unable to metabolise glucose (Sutton-McDowall *et al.* 2010) or establish nutrient gradients to modulate pronuclear dynamics. Localised ATP bursts have also been observed during mouse oocyte maturation, correlating with the redistribution of mitochondria (Yu *et al.* 2010), but may equally be indicative of fluctuations in other mitochondrially derived metabolites in a pulsatile manner.

Following fertilisation, mitochondria translocate to cluster around the two pronuclei (Motta *et al.* 2000, Squirrell *et al.* 2003). Mitochondrial translocation to a perinuclear arrangement has been observed in hamster (Barnett *et al.* 1996), mouse (Van Blerkom & Runner 1984), pig (Sun *et al.* 2001), rhesus macaque (Squirrell *et al.* 2003) and human (Noto *et al.* 1993, Van Blerkom *et al.* 2000) zygotes, providing ATP and other intermediates to facilitate syngamy and ensure an even organelle distribution between resultant blastomeres. Intriguingly, the relocation of mitochondria coincides with differential modulation of the paternal and maternal epigenomes, such that clustering likely establishes crosstalk between the nuclear and mitochondrial genomes, and may establish local metabolite domains to regulate epigenetic modifiers. Interestingly, nuclear expression of mitochondrial TCA enzymes, necessary to produce metabolites required for epigenetic remodelling at the time of EGA, has recently been described in mouse embryos (Nagaraj *et al.*

2017), and relies on pyruvate availability, indicating a need for temporal nutrient requirements to regulate the preimplantation epigenetic landscape. The dynamic changes to the epigenetic landscape that occur at syngamy may therefore necessitate translocation of mitochondria to the pronuclei to provide essential metabolites, including α -KG and NAD^+ to support demethylase activity. Dumollard *et al.* (2007) reported that NAD(P)H levels significantly decreased following fertilisation, an inverse indicator of an increase in NAD^+ availability, supporting this hypothesis. However, temporal changes in NAD(P)H levels during syngamy remains relatively unexplored. Significantly, a lack of mitochondrial organisation at this stage has been correlated with reduced implantation (Wang *et al.* 2009b), with mitochondrial aggregation characteristic of oocytes with reduced developmental potential (Igosheva *et al.* 2010), suggesting that these dynamic properties of mitochondria are essential for embryo development. Further, ovarian stimulation regimen has a substantial impact on mitochondrial distribution in the mouse (Ge *et al.* 2012) and human oocyte (Dell'Aquila *et al.* 2009), indicating that stimulation can significantly alter mitochondrial dynamics, which may have consequences for implantation, the induction of aneuploidies, viability and long-term health through the modulation of epigenetics.

Perturbations in mitochondrial movement and recruitment are also likely to lead to subcellular ATP depletion and interfere with mitochondrial Ca^{2+} signalling. Mitochondria play a role in Ca^{2+} storage and homeostasis (Duchen 2000), with sustained Ca^{2+} oscillations following fertilisation necessary for oocyte activation (Kline & Kline 1992) and embryo development (Stricker 1999). Mitochondrial localisation, and activity, interplays with Ca^{2+} signalling at fertilisation, such that mitochondrial ATP production is required for maintaining Ca^{2+} levels in the oocyte (Wakai *et al.* 2013) and for sustaining Ca^{2+} oscillations post-fertilisation (Dumollard *et al.* 2004). Significantly, mitochondrial movement along microtubule tracks is regulated by Ca^{2+} sensitive microtubule associated proteins, including kinesins, dyneins and myosins that drive motor-based movement (Varadi *et al.* 2004, Lawrence *et al.* 2016), suggesting that mitochondrial distribution relies on their own activity, as well as the integrity of the cytoskeletal network, to sustain localised signalling. Indeed, inhibition of microtubule polymerisation in human oocytes reduces germinal vesicle breakdown (GVBD) and prevents the completion of maturation, accompanied by the loss of mitochondrial redistribution (Takahashi *et al.* 2016), and cytoskeletal disruption in mouse eggs alters mitochondrial ATP production (Yu *et al.* 2010). Consequently, altered mitochondrial activity may reduce their ability to modulate Ca^{2+} dependent interactions with the cytoskeleton.

Connectivity and communication between mitochondria, the nucleus and other organelles, as well as the cytoskeleton, is therefore essential to the maintenance of cellular homeostasis in the oocyte and developing embryo, integrating diverse environmental signals as in other cell types (reviewed by Anesti & Scorrano 2006, Rowland & Voeltz 2012, Marchi *et al.* 2014) and eliciting signalling cascades that support preimplantation development. Loss, or misregulation, of these close interactions may therefore negatively impact the embryo's ability to respond to stress and regulate mitochondrial function, or lead to precocious or delayed modification of the epigenetic landscape, particularly in response to changes in nutrient availability, thereby impacting long-term viability and health.

Limitations to assessing mitochondrial DNA content in isolation

While mitochondrial health and functionality relies on metabolic activity, distribution, morphology and density, an often-overlooked characteristic that separates mitochondria from other organelles is that each mitochondrion contains numerous copies of its own double stranded genome (mitochondrial DNA; mtDNA). mtDNA encodes 13 proteins involved in the respiratory chain, along with 22 transfer RNAs and 2 ribosomal RNAs (Anderson *et al.* 1981, Taanman 1999, Clayton 2000); however a significant proportion of genes required for mitochondrial biogenesis, homeostasis and regulation of OXPHOS are encoded by the nuclear genome. Coordinated signalling between the nucleus and mitochondrion is therefore essential to regulate mitochondrial function (reviewed elsewhere by St John *et al.* 2010, Harvey *et al.* 2011), and metabolic intermediate levels, and therefore the epigenome. Indeed, a role for mtDNA, and therefore mitochondrial activity, in regulating the nuclear epigenetic landscape is evidenced by a study examining mtDNA depletion which results in aberrant CpG methylation that can be reversed following repletion of mtDNA (Smiraglia *et al.* 2008). Thus, deficiencies in mtDNA may be paralleled by changes to the epigenome that reduce developmental competence.

The mature human and mouse oocyte contains an estimated 20,000 to 800,000 mtDNA copies (Steuerwald *et al.* 2000, Reynier *et al.* 2001, Barritt *et al.* 2002) and between 10,000 to 650,000 copies (Piko & Taylor 1987, Steuerwald *et al.* 2000, Aiken *et al.* 2008, Cree *et al.* 2008) respectively, with mtDNA levels remaining low following fertilisation, only increasing around the time of implantation (Thundathil *et al.* 2005, May-Panloup *et al.* 2005b, Bowles *et al.* 2007, Spikings *et al.* 2007, Aiken *et al.* 2008, Mtango *et al.* 2008). A threshold of 40,000 to 50,000 mtDNA copies in a mature mouse oocyte has been proposed as critical to support development

post-implantation (Wai *et al.* 2010). While blastocysts still develop with as few as 4000 mtDNA copies, extrapolated from 8-cell embryos, those with low mtDNA levels failed to develop post-implantation (Wai *et al.* 2010). Likewise, while unfertilised human oocytes and their associated somatic cells harbour higher levels of mtDNA mutations and deletions (Keefe *et al.* 1995, Seifer *et al.* 2002, Hsieh *et al.* 2004), associated with reduced expression of mitochondrial genes (Hsieh *et al.* 2002), and oocytes from older females being more likely to possess deletions (Keefe *et al.* 1995, Jacobs *et al.* 2007, Hammond *et al.* 2016), how this relates to oocytes within the cohort that fertilise is difficult to determine. Significantly, levels vary considerably within one individual, and can be altered by ovarian stimulation regimen (Cree *et al.* 2015). Moreover, mtDNA copy number is often assumed to be an indicator of mitochondrial number, yet each mitochondrion can contain a varying number of mtDNA nucleoids (Gillkerson *et al.* 2013). Combined with species' differences, and the use of discarded and poor-quality human samples, the utility and specificity of these thresholds is potentially limited.

Despite these limitations, mtDNA defects have been associated with ageing and the failure of mature oocytes to fertilise. mtDNA levels decline in oocytes with advancing maternal age (de Boar *et al.* 1999, Murakoshi *et al.* 2013) and unfertilised oocytes contain significantly fewer copies of mtDNA, suggesting that mtDNA copy number may be indicative of fertilisation potential (Reynier *et al.* 2001, Santos *et al.* 2006, Wai *et al.* 2010). Ovarian insufficiency has also been characterised by a significantly lower mtDNA content in oocytes (May-Panloup *et al.* 2005a), as well as accompanying cumulus cells (Boucret *et al.* 2015). Further, blastocysts from high fat diet (HFD) fed mice contain fewer mtDNA copy numbers and give rise to heavier fetuses in which both liver and kidney mtDNA content is significantly reduced (Wu *et al.* 2015), indicating that altered nutrition prior to conception can disrupt mitochondrial transmission. In addition to a lowered capacity for ATP generation, reduced mtDNA levels likely result in a diminished pool of TCA metabolites that are preferentially shunted toward ATP production to support cell survival in lieu of histone modification, predisposing the developing embryo to compensatory changes in physiology and growth. Indeed, mitochondrial DNA depletion has been shown to result in alterations in one-carbon metabolism (Bao *et al.* 2016) and TCA activity, altering transcription of genes involved in acetyl-CoA metabolism, and modifies amino acid metabolism, particularly that of methionine, consequently impacting on methylation (Lozoya *et al.* 2018). Conversely, enhanced mitochondrial OXPHOS capacity with increased mtDNA content may result in an increased availability of mitochondrial metabolites that results in hypermethylation and/or acetylation, likewise impacting downstream growth and physiology.

Accumulating evidence suggests that epigenetic changes also occur within mtDNA. While low levels (<5%) of mtDNA methylation were reported in mouse and human cells in the 1980s (Shmookler Reis & Goldstein 1983, Pollack *et al.* 1984), mitochondria were believed to lack histones, the major component of chromatin for epigenetic modification, such that the existence of mtDNA methylation was, and remains, controversial. While speculation remains around potential sample contamination, histones have been detected in the mitochondrial membrane (Choi *et al.* 2011). Combined with the identification of mtDNA methyltransferase enzyme 1 (mtDNMT1), associated with mitochondrial cytosine methylation (Shock *et al.* 2011) and the isolation of DNMT3a from mitochondria (Wong *et al.* 2013), it is possible that methylation actively takes place within mitochondria. In support of this, mitochondrial targeted expression of DNA methyltransferases is sufficient to induce mtDNA methylation (van der Wijst *et al.* 2017) and (de)methylation of the D-loop has been associated with a reduction in mtDNA copy number (Tong *et al.* 2017), which would reduce mitochondrial gene expression. The mtDNA methylation may consequently alter the availability of the co-factors necessary for the activity of nuclear epigenetic modifiers, through the modulation of mtDNA encoded genes and therefore OXPHOS activity, although direct evidence for this, and how mitochondrial methyltransferases are regulated, is currently lacking. Similarly, given that mitochondria, and mtDNA, are more susceptible to damage, and rely on cellular antioxidant systems to prevent damage, the mtDNA epigenome may be even more vulnerable than the nuclear epigenetic landscape. Consequently, it will be important to investigate the effects of not only culture, but other aspects of assisted reproductive technologies, particularly mitochondrial replacement, on the oocyte and embryo nuclear and mtDNA epigenome, particularly given the potential for altering offspring health.

Controversially, studies have emerged suggesting that a higher mtDNA copy number (e.g. Mitoscore >97; Diez-Juan *et al.* 2015), in blastomere or trophectoderm biopsies, or in cumulus cells, is an indicator of poor blastocyst quality and/or implantation success (Diez-Juan *et al.* 2015, Fragouli *et al.* 2015, Ogino *et al.* 2016, Desquret-Dumas *et al.* 2017, Ravichandran *et al.* 2017), with the increase suggestive of underlying stress and compensatory mitochondrial biogenesis activated in an attempt to rescue development. This is however at odds with the metabolic requirements of the blastocyst stage embryo (Gardner & Harvey 2015). Moreover, these scores rely on an artificial reference threshold, and do not account for differences in mtDNA levels that exist between individual cells or cell types, which has been documented within the embryo (Cree *et al.* 2008). Indeed, mtDNA segregation has recently been shown to be non-random, rather relating to the level of OXPHOS

and mitochondrial membrane potential (Otten *et al.* 2018), the activity of which differs considerably between the trophectoderm and the ICM (reviewed by Gardner & Harvey 2015). However, a direct determination of neither whether a consistent relationship exists in both the trophectoderm and ICM nor whether defective mitochondria are preferentially segregated into the trophectoderm relative to the ICM, or whether trophoblast biopsies presenting with higher mtDNA levels reflect a significant reduction in ICM levels that contribute to their demise has been established.

Conversely, studies have shown mtDNA content to have no correlation with implanting and non-implanting embryos (Treff *et al.* 2017, Victor *et al.* 2017), although significant mathematical correction may preclude the identification of a relationship. Further, there appears to be little predictive value at moderate (e.g. 75th percentile) mtDNA levels (Klimczak *et al.* 2018), with considerable range overlap apparent across all studies to date. Methodological differences between species and clinics, including but not limited to stimulation regimens and maternal age (Cree *et al.* 2015, Fragouli *et al.* 2015), developmental stage relating to cell number and volume and developmental kinetics (Ho *et al.* 2018), but also culture conditions that alter nutrient availability, likely impact the determination of mtDNA copy number, although oxygen concentration does not appear to be a factor (de Los Santos *et al.* 2018). Consequently, the utility of blastocyst mtDNA copy number as a biomarker of embryo viability, in isolation, is questionable and will result in embryos that would produce a viable pregnancy being classified as non-viable; a travesty for those with limited embryo numbers and opportunities to become pregnant. While the use of mtDNA copy number is likely to be somewhat informative, it presents an oversimplistic view of the potential significance of mitochondrial function as a biomarker, particularly in light of their role in mediating the epigenome. Rather, mitochondrial, and mtDNA, integrity, taking into account activity, content (both mitochondrial and mtDNA heteroplasmy and mutational load) and mitochondrial dynamics (including morphology and distribution) will need to be integrated, together with existing markers and epigenetic analyses, in order to be of predictive value and to establish a correlation with post-transfer outcomes.

Mitochondrial replacement therapy: proceed with caution

Mitochondrial replacement therapy (MRT), approved for use in the United Kingdom in 2015, has been proposed as a viable option to prevent the transmission of debilitating mitochondrial diseases. The technique involves the removal of donor nuclear material, and replacement with the diseased individual's DNA, either via pronuclear, spindle or polar body transfer. However,

the procedure is only applicable to cases where the mitochondrial disease is encoded by mtDNA, excluding the vast majority of cases in which mitochondrial disease is nuclear encoded. MRT pregnancies are currently ongoing, although controversially, the first baby born using the technique was reported in 2016 (Zhang *et al.* 2017).

However, the technology brings with it the potential for long-term health effects, particularly given the plethora of roles mitochondria have in regulating not only metabolism, but also localised signalling cascades and intracellular interactions as noted herein. Of further concern is the potential for the retention of diseased maternal mtDNA, resulting in heteroplasmy, which can remain undetected (Tachibana *et al.* 2013), or be detected at levels of up to 10% in foetal tissues (Zhang *et al.* 2017). Sustained heteroplasmy has been reported following cytoplasmic transfer into human oocytes (Brenner *et al.* 2000) with varying proportions of mtDNA observed in different tissues (Barritt *et al.* 2001), although whether the technique and/or heteroplasmy has directly affected offspring health and disease risk is unclear. Heteroplasmy has also been reported in cloned animals, with proportions ranging from 0.5 to 40% (Hiendleder *et al.* 1999, Takeda *et al.* 1999, 2003, Steinborn *et al.* 2000, Hiendleder *et al.* 2003), although these figures are likely underestimates given the lack of sensitivity of the techniques used (Hammond *et al.* 2016). Ma *et al.* (2015) reported that mitochondrial deficits could be corrected with somatic cell nuclear transfer and genetic manipulation, however varying (0–100%) levels of heteroplasmy remained detectable in these lines. While heteroplasmy can occur in normal individuals, and varies across tissues, it has been associated with mitochondrial disease and metabolic perturbations in mice (Acton *et al.* 2007, Sharpley *et al.* 2012) with severity depending on the threshold level of diseased mitochondria. Significantly, a disease phenotype can occur even at very low (<5%) levels of heteroplasmy for certain deficits (Chinnery *et al.* 1997, Sacconi *et al.* 2008). While the birth of live macaque offspring (Tachibana *et al.* 2009) facilitated the development and adoption of this technique, longitudinal, whole systems studies of the health of resultant offspring is warranted, combined with care to ensure the absence of mitochondrial carry over from the patient.

Beyond the potential for heteroplasmy, the technique neglects the requirement for coordinated communication between mitochondria and the nucleus, along with the dynamic regulation of mitochondrial movement during oocyte maturation and fertilisation. As proposed, the recruitment of mitochondria to the pronuclei preceding syngamy (Barnett *et al.* 1996, Squirrell *et al.* 2003) may be required to modulate the local metabolite environment to facilitate syngamy, subsequent cytokinesis and differentially modify the parental genomes, a process that may well be initiated prior to

pronuclear migration. Further, given the significance of microtubules to mitochondrial distribution and chromosome segregation as noted above, it is therefore likely that a loss in microtubule network integrity will impact on further development via impairment of mitochondrial trafficking. Mitochondrial distribution following cytoplasmic transfer has been shown to depend on oocyte stage (Fulka 2004), possibly reflecting stage-specific changes in microtubule dynamics. Further, asymmetrical mitochondrial distribution at the pronuclear stage results in blastomeres with reduced mitochondrial inheritance and diminished ATP generating capacity (Van Blerkom *et al.* 2000), and plausibly altered paternal/maternal methylation characteristics that contribute to the observed reduction in developmental competence. Therefore, studies will need to address whether MRT methodologies alter mitochondrial and cellular metabolic regulation, as well as the embryo transcriptome and epigenome, to ensure minimal risk to resultant offspring health and the absence of transgenerational effects.

Conclusions

The involvement of mitochondria in regulating oocyte and embryo development goes beyond their role in generating ATP to support energy demanding processes. It is increasingly recognised that mitochondria are dynamic signalling organelles, trafficking to specific regions within the cell and communicating not only with the nucleus but also other organelles; interactions that facilitate signalling cascades and impact chromosome segregation, cytokinesis and lineage specification. Loss of mitochondrial function, including activity, organelle interactions and trafficking, and the mechanisms that modulate mitochondrial and mtDNA integrity, manifests as reduced development, and can result in failure to develop post-implantation. Combined with our evolving understanding of how metabolite availability and metabolic and mitochondrial pathway flux can drastically affect the availability of co-factors required for the activity of epigenetic modifiers, mitochondria are emerging as sensors that integrate not only metabolic and cell signalling cues but may in part explain the origins of poor developmental outcomes through heritable changes that lead to disease susceptibility. Therefore, while the developing embryo exhibits a degree of plasticity to changes in its extracellular environment, enabling development to the blastocyst stage to continue, it is nonetheless uniquely sensitive to perturbations in nutrient availability at a time when the epigenetic landscape is being established. The complexity of their diverse roles is of particular significance given the adoption of assisted reproductive technologies that detect or manipulate mitochondria or mtDNA numbers and potentially disrupt mitochondrial-organelle and -cytoskeletal communication and trafficking.

This complexity necessitates the determination of mitochondrial characteristics in combination with epigenetic change in order to provide a more accurate understanding of the impact of mitochondrial deficits and manipulation on oocyte, embryo and offspring health, as well as their utility as biomarkers.

Declaration of interest

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

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