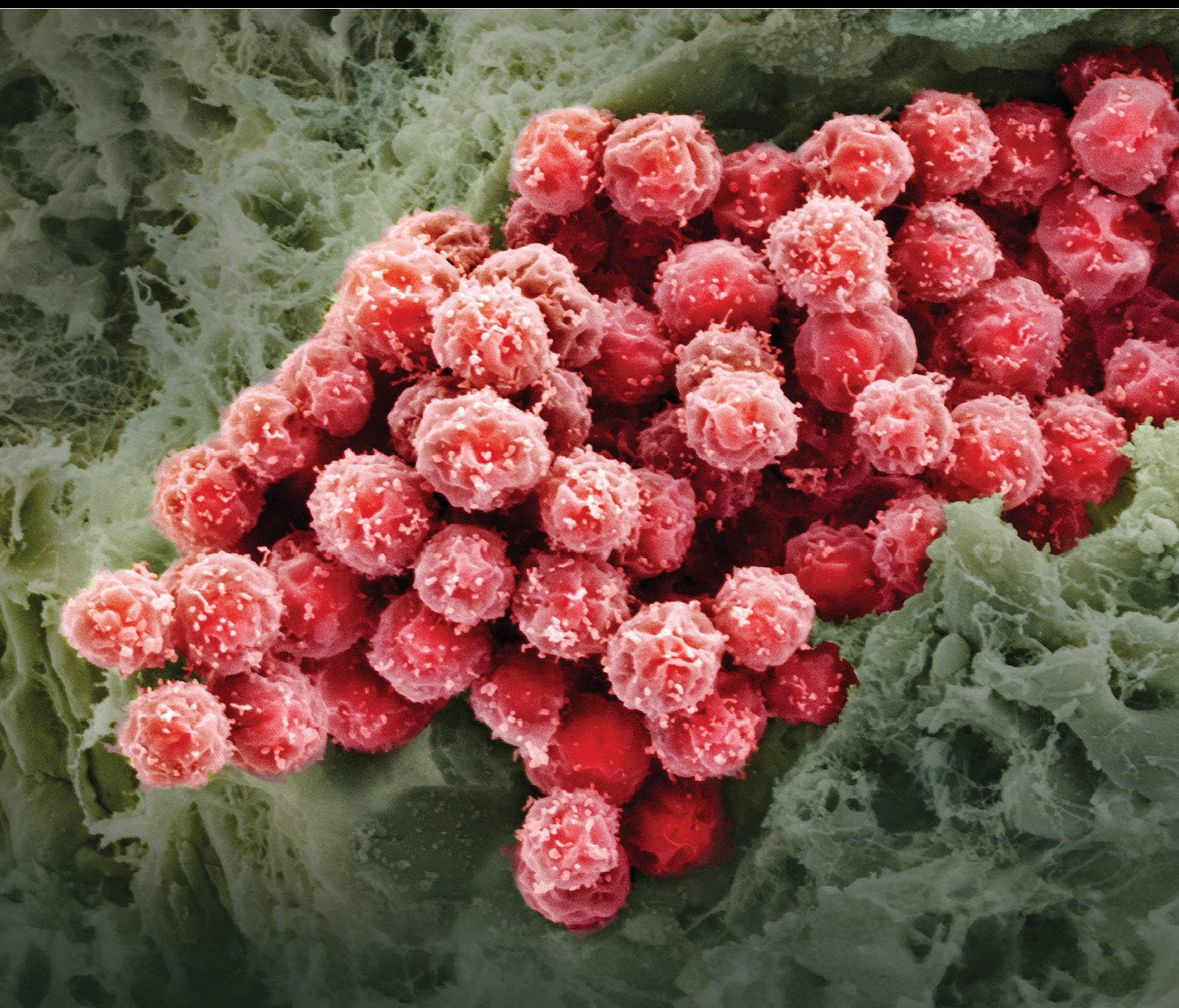
Stem Cells International

Mitochondria: More than Just "Power Plants" in Stem Cells

Lead Guest Editor: Martin Stimpfel
Guest Editors: Riikka Hämäläinen and Pascal May-Panloup



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Editorial Board

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Editorial

Mitochondria: More than Just "Power Plants" in Stem Cells

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In mammalian cells, mitochondria are the only organelles containing DNA besides the nucleus. Mitochondrial DNA (mtDNA) comprises 37 genes of which 13 encode proteins and the others encode RNA molecules involved in the translation of these proteins. Although the number of proteins encoded by mtDNA is very small compared to that encoded by nuclear DNA, mtDNA and mitochondria are of vital importance for proper cellular function. In addition to their main function of producing ATP through oxidative phosphorylation, mitochondria play several additional vital roles as illustrated by the articles in this special issue. Since mitochondrial function and properties vary according to cell type, a better understanding of mitochondrial properties is of considerable interest particularly in the case of stem cells that may be used in cell therapy or regenerative medicine. Since many stem cell types rely mostly on a glycolytic metabolism rather than oxidative phosphorylation for energy production, they were long thought to be almost independent of mitochondrial function. However, recent advances have shown that proper mitochondrial function in stem cells is essential to maintain their self-renewal and differentiation abilities.

This special issue sums up recent findings concerning the essential role of mitochondria in stem cells. It includes selected reviews and an original article discussing the role and properties of mitochondria in stem cells not only from the perspective of basic science but also from the perspective of therapy.

When using stem cells for cell therapy, their heterogeneity may represent a challenge. One source for this cellular heterogeneity are mitochondria and as reviewed by D. C. Woods, different subpopulations of mitochondria can be present even within a single cell. This mitochondrial diversity and heterogeneity is believed to respond to cellular metabolic demands, but the underlying mechanisms are not yet completely understood. Since various specialized cells have specific metabolic demands and mitochondrial properties, D. C. Woods suggests that stem cells may serve as a useful model for elucidating the phenomenon of mitochondrial differentiation and the mechanisms leading to mitochondrial diversity and heterogeneity.

J. G. Lees et al. focus on pluripotent stem cells and on the precise role played by mitochondrial metabolites in maintaining pluripotency. The authors discuss the role of mitochondria in the epigenetic modifications on chromatin and highlight the impact of hydrogen peroxide, an important by-product of mitochondrial respiration. In high concentration, hydrogen peroxide may stimulate proliferation of pluripotent stem cells via hypoxia-inducible factor α (HIFα) signaling, but this process is dependent on physiological oxygen level. Oxygen is therefore recognized as one of the key factors influencing the metabolism and behavior of pluripotent stem cells and has even been proposed to be used as a selective factor.

The mitochondria of specialized somatic cells differ substantially from those of pluripotent stem cells. Thus, when somatic cells are reprogrammed to the pluripotent state, the mitochondria undergo remodeling through mitochondrial fission and mitophagy. Several studies show that these processes are critically involved in nuclear reprogramming. J.

Prieto and J. Torres review these findings in normal cells and linked them with development of human malignancies.

Interestingly, mitochondria can be transferred between cells. The mechanisms of this phenomenon and its potential therapeutic applications are reviewed here in two articles by M.-L. Vignais et al. and A. Caicedo et al. Mitochondrial transfer has several physiological functions. It serves mainly in rescue operations with healthy cells donating mitochondria to damaged ones. Moreover, recent data have shown that mitochondrial transfer is also involved in mitochondrial degradation through transcellular mitophagy. Thus, it constitutes crucial mechanism for maintaining mitochondrial homeostasis in multicellular organisms. Natural mitochondrial transfer may occur through intracellular connections such as tunneling nanotubes or by the secretion of cellular bodies as in the case of microvesicles. Artificial mitochondrial transfer involves also other mechanisms such as the injection of mitochondria in recipient cells, the coincubation of mitochondria with recipient cells, or the use of various chemical compounds. With stem cell therapy, artificial mitochondrial transfer has been proposed for the treatment of mitochondrial retinopathies, muscular skeletal syndromes, and other mitochondrial diseases. However, since mitochondria contain DNA, such treatments raise ethical and legal questions that will need to be resolved before the technique comes into general use.

D. Yu et al. studied the methylation of mtDNA in human fetal heart mesenchymal stem cells (MSCs) during the process of senescence induced by chronic exposure to oxidative stress and low serum environment. The authors were able to identify for the first time the specific regions of mtDNA that were hypomethylated upon senescence. More precisely, COX1, which encodes a subunit of the cytochrome c oxidase complex, the main enzyme involved in mitochondrial oxidative phosphorylation, was hypomethylated and consequently upregulated. COX1 upregulation was also induced by knockdown of methyltransferases DNMT1, DNMT3a, and DNMT3b. However, the precise role of the upregulation of COX1 in senescence remains to be elucidated. The authors suggest that the hypomethylation of specific mtDNA regions could be used as a biomarker of the senescence of MSCs.

In summary, this special issue offers an overview of the major findings concerning the properties and the physiological roles of mitochondria in stem cells. These results should lead to new scientific insights into mitochondrial function in the context of potential therapeutic applications in the future.

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Review Article

Pluripotent Stem Cell Metabolism and Mitochondria: Beyond ATP

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Metabolism is central to embryonic stem cell (ESC) pluripotency and differentiation, with distinct profiles apparent under different nutrient milieu, and conditions that maintain alternate cell states. The significance of altered nutrient availability, particularly oxygen, and metabolic pathway activity has been highlighted by extensive studies of their impact on preimplantation embryo development, physiology, and viability. ESC similarly modulate their metabolism in response to altered metabolite levels, with changes in nutrient availability shown to have a lasting impact on derived cell identity through the regulation of the epigenetic landscape. Further, the preferential use of glucose and anaplerotic glutamine metabolism serves to not only support cell growth and proliferation but also minimise reactive oxygen species production. However, the perinuclear localisation of spherical, electron-poor mitochondria in ESC is proposed to sustain ESC nuclear-mitochondrial crosstalk and a mitochondrial-H_2O_2 presence, to facilitate signalling to support self-renewal through the stabilisation of HIFα, a process that may be favoured under physiological oxygen. The environment in which a cell is grown is therefore a critical regulator and determinant of cell fate, with metabolism, and particularly mitochondria, acting as an interface between the environment and the epigenome.

1. Introduction

Beyond roles in ATP production, metabolism and mitochondria lie at the nexus of cell signalling. A direct link between metabolic pathway activity and chromatin dynamics has recently been recognised, primarily because metabolic intermediates of cellular metabolism are required as cofactors for epigenetic modulators [1]. Changes in nutrient use have also been shown to modulate lineage specification [2–5], indicating that metabolism acts as a regulator of cell fate.

As the precursors to all adult cell types, the preimplantation embryo and derived embryonic stem cells (ESC) represent a nutrient-sensitive paradigm to understand the interaction between the nutrient environment and the regulation of development and differentiation. Studies on the impact of culture on preimplantation embryo development have highlighted the persistence of physiological perturbations induced by altered metabolism and nutrient availability during this short window of development [6–9]. ESC are similarly sensitive to nutrient availability in their environment, responding with significant shifts in primary metabolic pathways [10, 11].

Consequently, the significance of nutrient availability, particularly physiological oxygen (1–5%), and the role of the mitochondria and mitochondrial-derived reactive oxygen species (ROS), in regulating ESC physiology, cell state, cell fate, and the epigenome, are considered. Metabolism emerges as an interface between the environment and genome regulation, such that alterations in metabolic pathway activity disrupt the production and availability of cofactors required for epigenetic modifier activity, resulting in an altered epigenetic landscape.

2. Defining Pluripotent Stem Cell States In Vitro

ESC pluripotency represents a continuum of cell states, characterised by distinct cellular, metabolic, and epigenetic states. The capacity to maintain pluripotency relies on complex signalling networks that are regulated by the surrounding microenvironment; however, differing growth factor requirements and signalling in vitro between mouse and human ESC are presumed to reflect origins from different developmental stages within the embryo [12]. Mouse ESC derived from the

inner cell mass (ICM) of the blastocyst into serum/LIF conditions are representative of D4.5 ICM, a transitional stage within the pluripotency continuum that is functionally distinct from mouse ESC derived from a medium containing GSK and MEK inhibitors (2i), representative of day 3.5 ICM (naïve ESC) [13]. In contrast, human ESC rely on fiborblast growth factor (FGF) signalling to maintain pluripotency [14, 15], similar to mouse epiblast stem cells (EpiSC), representative of the postimplantation epiblast.

Numerous studies have focused on defining the molecular properties of ESC, particularly the transcription factor regulatory network, OCT4, NANOG, and SOX2, and the growth factor requirements of these populations (reviewed by [16]). Underpinning pluripotency are complex epigenetic mechanisms required for the progressive transitions during development which restrict cell potency and maintain cell fate decisions, silencing pluripotency genes and activating lineage-specific genes [17]. ESC are characterised by a euchromatic and highly dynamic chromatin landscape [18] and elevated global transcriptional activity [19]. Bivalent methylation, marked by a combination of active H3K4me3 and repressive H3K27me3 at a subset of developmental regulators, has been proposed to establish a primed epigenetic state, ready for activation prior to ESC differentiation [20], and to safeguard differentiation [21]. Progression through the early events of differentiation is accompanied by global changes in the epigenetic landscape, characterised by restricted gene expression and extensive regions of heterochromatin. Lineage-specific DNA methylation patterns are established, and repressive marks, such as H3K9me3, are upregulated within differentiated cells [22].

Establishment and maintenance of the epigenetic landscape rely on the activity of epigenetic modifiers that regulate DNA methylation, histone modification, and chromatin organisation. DNA methylation is regulated by DNA methyltransferases (DNMTs) that act as methyl donors for cytosine residues, restricting gene expression. Conversely, active demethylation is catalysed by ten-eleven translocation (TET) dioxygenases, responsible for the conversion of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) [23]. Methylation of arginine and lysine residues on histones H3 and H4 is catalysed by histone methyltransferases (HMTs), the modifications of which are associated with both transcriptional activation and repression. Histone acetylation, catalysed by histone acetyltransferases (HATs), is generally associated with a euchromatic state, permissive to transcription. Conversely, histone deacetylation, via histone deacetylases (HDACs), is associated with condensed heterochromatin resulting in transcriptional repression. Functionality of epigenetic modifiers requires specific metabolites and cofactors, serving to transduce changes in the microenvironment to alter chromatin state [24, 25]. Specifically, S-adenosylmethionine (SAM), generated through one carbon metabolism, integrating the folate and methionine cycles, acts as the primary methyl donor for DNA and histone methylation. Similarly, TET activity is dynamically regulated by alpha-ketoglutarate (αKG) and succinate, products of the tricarboxylic acid (TCA) cycle [26]. Acetylation transfers an acetyl group from acetyl coenzyme A (acetyl-CoA) to lysine residues, while HDAC activity is regulated by NAD^+-independent or NAD^+-dependent mechanisms [27]. Cellular fluctuations in metabolism in response to various physiological cues, including nutrient availability and metabolic pathway activity, therefore have the capacity to modulate the epigenome through the activity of these epigenetic modifiers.

Accompanying the transition from naïve pluripotency to a primed pluripotent state are changes in metabolism. Naïve mouse ESC are reliant on oxidative metabolism [28, 29], while EpiSC and human ESC metabolism is predominantly glycolytic [28, 30], accompanied by glutaminolysis [10]. Human naïve ESC have recently been obtained in culture, using a number of different protocols [31–35], with the transition accompanied by a similar metabolic remodelling towards a more oxidative [33] metabolism, although glycolysis remains important [36]. As metabolic change accompanies the transitions between cell states, including differentiation, recent studies have begun to elucidate the interplay between metabolites and the ESC epigenetic landscape, establishing a link between ESC metabolic state, epigenetics, and cell fate.

3. Nutrients in the In Vivo Stem Cell Niche

Until recently, little attention has been paid to the nutritional milieu within the stem cell niche. In vivo, the embryonic stem cell niche is comprised of a rich and complex mixture of proteins and metabolites, none of which are likely to be superfluous, and which maintain the viability of the developing embryo compared to the relatively simple composition of in vitro culture media. Mammalian reproductive tract fluids contain high levels of potassium, glucose, lactate, and pyruvate as energy sources, free amino acids including high levels of glycine, and proteins including albumin and immunoglobulin G, glycoproteins, prostaglandins, steroid hormones, and growth factors [37, 38]. This complex microenvironment changes composition dynamically throughout the estrus cycle and within different compartments of the tract [39, 40], indicating a tight regulatory mechanism to ensure proper embryo development.

Oxygen is a critical, but often overlooked, component within the stem cell niche. Vascularisation, and consequently the supply of oxygen, is tissue specific, ranging from ~9.5% in the human kidney to ~6.4% in bone marrow and ~4.7% oxygen in the brain [41]. Cellular oxygen ranges from 1.3 to 2.5%, while oxygen within the mitochondria is estimated to be <1.3% [41]. The mammalian reproductive tract, within which the preimplantation embryo develops, has been measured at 2–9% oxygen in the rat, rabbit, hamster, and rhesus monkey [42, 43]. The uterine environment ranges from 1.5 to 2.0% oxygen in the rhesus monkey and decreases from 5.3% to 3.5% in the rabbit and hamster, around the time of blastocyst formation and subsequent implantation [42, 44]. The precise oxygen concentration experienced by the inner cell mass of the human blastocyst is unknown, but likely approximates less than 5% oxygen [45, 46]. In spite of such physiological data on oxygen levels, atmospheric (20%) oxygen remains the predominant concentration used for cell culture, including stem cells and human embryos [47], with limited adoption of more physiological oxygen concentrations (1–5%). However, neither of these conditions sufficiently

capture the dynamic changes in oxygen concentration that occur during embryo and fetal development in vivo.

The significance of establishing an appropriate niche environment in vitro is apparent in the loss of embryo viability observed in vitro relative to in vivo conditions [48, 49]. The developing embryo is responsive to nutrient changes in its environment, where perturbations in nutrient availability alter metabolism through gene expression and altered gene imprinting status [50, 51]. While embryo metabolism appears relatively plastic in its response to suboptimal osmotic, pH, ionic, and nutrient changes in its environment, there is a significant loss in viability [52, 53]. ESC metabolism is plausibly similar in its plasticity, able to maintain proliferation under a range of suboptimal culture conditions.

4. Lessons Learned from the Preimplantation Embryo

Preimplantation embryo development represents a unique window of sensitivity during development encompassing the first lineage decisions and the most significant period of epigenetic programming that will persist in resultant daughter cells and their differentiated progeny [54]. Suboptimal embryo culture conditions, including atmospheric oxygen, serum supplementation, ammonium buildup, and the absence of necessary metabolites such as amino acids, have been shown to alter developmental kinetics, delay blastocyst development, and lower blastocyst numbers, mirrored by a loss of viability postimplantation [9, 55–57]. Culture in the presence of atmospheric oxygen is associated with retarded embryo development in several species ([58-60], reviewed by [61, 62]). Atmospheric oxygen delays mouse and human embryo cleavage prior to the 8-cell stage [63], resulting in a reduction in subsequent blastocyst quality [9]. Significantly, exposure to atmospheric oxygen during early cleavage is irreversible, as subsequent postcompaction culture at physiological oxygen is unable to restore blastocyst viability, highlighting the susceptibility of the early embryo to environmental stresses. Furthermore, the detrimental effects of atmospheric oxygen on gametes and embryos also manifest as changes in blastocyst gene expression [64, 65] and the proteome [66], perturbed metabolic activity, including loss of metabolic homeostasis and a reduced capacity for the transamination of waste products [7, 8, 48, 67], and a reduction in birth rates in humans by 10–15% [68, 69]. During this time, the most substantial epigenetic changes in the life of the organism occur (reviewed by [70]), thereby representing a sensitive window of development during which metabolic perturbations have the potential to alter the epigenetic landscape, impacting daughter cells. The sensitivity of the mammalian embryo to metabolite availability, and metabolic perturbations, infers that ESC, iPSCs, and potentially all in vitro-derived cell types may be similarly perturbed by nonphysiological culture conditions, with long-lasting/hereditary effects. Studies examining preimplantation embryo physiology, and the significance of metabolism and metabolic regulation during development, were instrumental in developing culture conditions capable of supporting embryo development [6] and highlight the need to understand ESC physiology, and how in vitro culture and nutrient availability impacts their functionality, particularly given the proposed use of these cells for clinical applications.

5. The Metabolic Framework of Pluripotent Stem Cells: The Relevance of Glucose and Glutamine Metabolism

Preimplantation embryo metabolism is characterised by a dependency on pyruvate, lactate, and aspartate, and a limited capacity for glucose, prior to compaction [48, 71], switching to an increasing need for glucose uptake and conversion to lactate [6], accompanied by an increase in oxygen consumption [72] postcompaction. This shift is driven in part by the exponential increase in cell number from the morula to the blastocyst stage, and by the energy required to generate and maintain the blastocoel (reviewed by [53]). While the trophectoderm, which forms the placenta, has the capacity to oxidise around half the glucose consumed, the ICM is predominantly glycolytic [73], converting approximately 100% of the glucose consumed to lactate, even in the presence of sufficient oxygen to support its complete oxidation [74].

Similar to the ICM, mouse and human ESC metabolism is characterised by a dependency upon glycolysis [11, 36, 75–78] (Figure 1), converting approximately 70–80% of the glucose consumed to lactate. Unlike oxidative phosphorylation (OXPHOS), which generates 36 ATP from the oxidation of glucose, glycolysis generates only 4 molecules of ATP. However, ATP can be generated quickly through glycolysis [79], such that equivalent levels of ATP can be generated provided there is a sufficient flux of glucose. The reliance of ESC on glycolysis is plausibly necessary to maintain a high cellular NADPH, allowing for rapid cell expansion through amino acid and nucleotide synthesis for proliferation [80]. Lactate generation, via lactate dehydrogenase (LDH), facilitates the regeneration of cytosolic NAD^+ required for the conversion of glyceraldehyde-3-phosphate to 1,3-biphosphoglycerate in glycolysis, ensuring continued glucose utilisation. Alternatively, glucose-derived pyruvate can be oxidised through the TCA cycle to provide lipids and carbon donors, such as acetyl-CoA necessary for membrane synthesis [81], and synthesis of the amino acids serine, glycine, cysteine, and alanine necessary for cell division. In human ESC, glucose-derived carbon metabolised through the oxidative pentose phosphate pathway (PPP), contributes between 50 and 70% of cytosolic NADPH [10], which is required for the constant reduction of antioxidants in order to keep them functional. Proliferation of both naïve mouse ESC and serum/LIF ESC is abolished in the absence of glucose [82, 83], and inhibition of glycolysis with nonmetabolisable 2-deoxyglucose significantly reduces mouse ESC self-renewal [76], demonstrating an absolute requirement for glucose in supporting self-renewal. The preferential metabolism of glucose through glycolysis also provides a means of generating ATP without the formation of ROS in pluripotent cells, allowing a level of control over the amount of ROS generated.

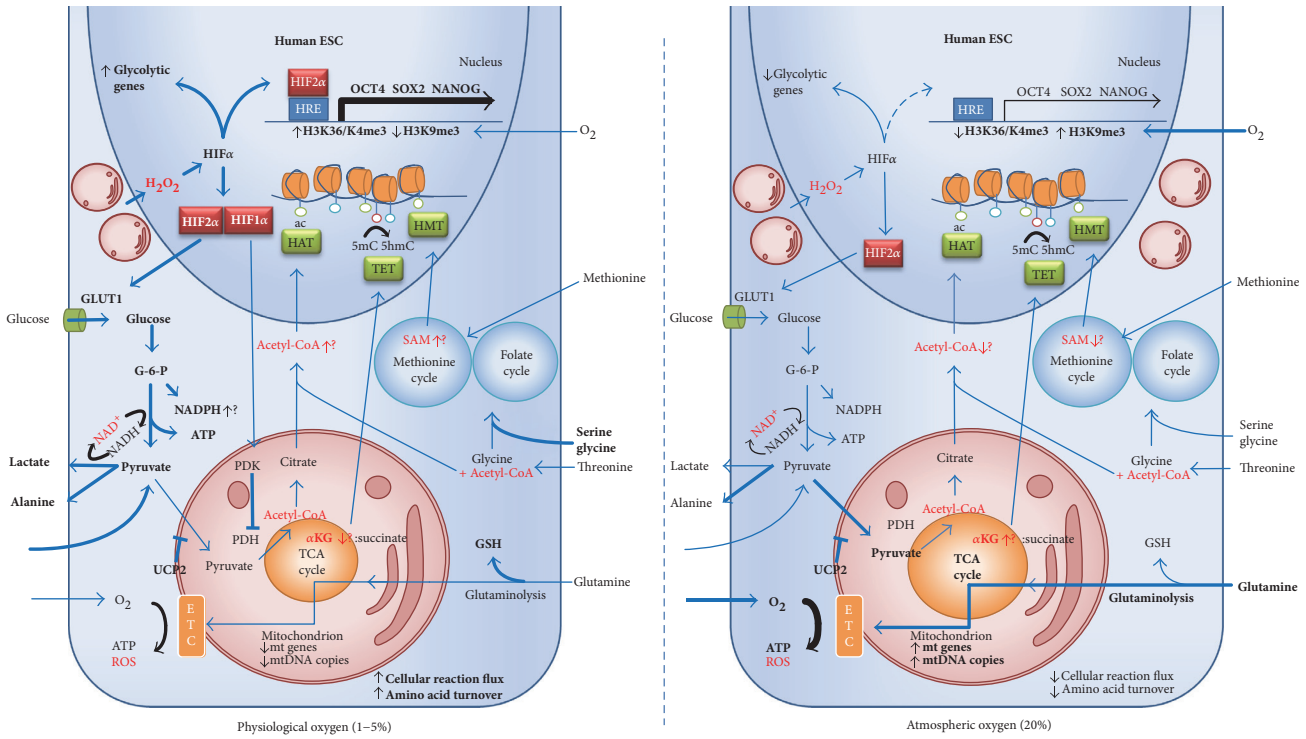

Figure 1: Oxygen regulation of ESC metabolism and epigenetic landscape. Relative to atmospheric oxygen (20%), physiological oxygen (1–5%) reduces the content of mitochondrial DNA (mtDNA) and mitochondrial electron transport chain (ETC) gene expression in pluripotent stem cells [11]. These mitochondria consume less oxygen and respire less than those at atmospheric oxygen generating less ATP through glucose-derived oxidative phosphorylation (OXPHOS). Mitochondrial OXPHOS from glutamine- and fatty acid-derived carbon is still an active pathway in pluripotent stem cells; atmospheric oxygen increases the consumption of glutamine and its oxidation in the mitochondria [77, 93]. Pluripotent stem cells rely heavily on glycolysis, followed by the conversion of pyruvate to lactate, which recycles the NAD^+ required for the rapid continuation of glycolysis. Per carbon, glycolysis is less efficient than OXPHOS at generating ATP; however, should there be a sufficient flux of glucose, then enough ATP can readily be formed. At physiological oxygen, glycolytic flux is increased relative to atmospheric oxygen resulting in significantly more lactate production [11, 77, 128]. Several mechanisms direct glucose-derived carbon towards either lactate or alanine and away from mitochondrial OXPHOS. Under physiological oxygen conditions, the hypoxic inducible factors (HIFs) are stabilised; targets of transcription factor HIF2α include glucose transporter 1 (GLUT1) [128] which increases glucose transport into the cell and pyruvate dehydrogenase kinase (PDK) which inhibits the conversion of pyruvate to acetyl-CoA by pyruvate dehydrogenase (PDH) in the mitochondrion. Uncoupling protein 2 (UCP2), an inner mitochondrial membrane protein, blocks the import of pyruvate into the mitochondria in human PSC [84]. Glutamine and fatty acids stimulate UCP2, decreasing pyruvate oxidation, which in turn facilitates glutamine and fatty acid oxidation and the maintenance of a rapid glycolytic flux [187, 188]. The flux of metabolic reactions in PSCs is increased at physiological oxygen [93] as is amino acid turnover [11, 189]. Increased serine and glycine consumption at physiological oxygen may feed into the folate and methionine cycles, collectively known as one carbon metabolism. One carbon metabolism, glycolysis, and the tricarboxlyic acid (TCA) cycle generate intermediate metabolites that act as cofactors for epigenetic modifying enzymes. Threonine and methionine metabolism in mouse [5] and human [4] PSCs, respectively, generate S-adenosylmethionine (SAM) which is a methyl donor for histone methyl transferases (HMT). Glucose-derived acetyl coenzyme A (acetyl-CoA), synthesised in the TCA cycle or from threonine metabolism [5], acts as a cofactor for histone acetyltransferases (HAT), modulating hESC histone acetylation and plausibly maintains pluripotency [88]. Glutamine metabolism increases the αKG:succinate ratio, leading to DNA demethylation by ten-eleven translocation (TET) activity, which then stimulates the mouse naïve pluripotency network [83]. In primed human ESC, an increased αKG:succinate ratio induces differentiation [100]. In human ESC, physiological oxygen causes a euchromatic state within *NANOG*, *OCT4*, and *SOX2* hypoxic response elements (HREs) allowing the binding of HIF2α and the upregulation of the pluripotency network [109]. HIFα is stabilised at physiological [160, 167] and atmospheric oxygen [170] due to the action of mitochondrial ROS [161, 168, 169]. Stabilised HIFα protein upregulates glycolytic flux through glycolytic gene expression [147], increases cellular glucose import, and upregulates pluripotency [109]. The proximity of the mitochondria to the nucleus facilitates a ROS-nucleus signalling axis in the form of H_2O_2, plausibly through the HIF family of transcription factors. Concurrently, antioxidant production is increased at physiological oxygen [175]. Glutathione (GSH) from glutaminolysis, and NADPH from either glutaminolysis or the pentose phosphate pathway, protect the cell from increased levels of ROS. Thick arrows and bold text indicate increased flux/transcription. Metabolic regulators of chromatin-modifying enzymes are highlighted in red. Circles attached to chromatin in the nucleus represent epigenetic modifications: acetylated (green); 5mC (red); 5hmC (blue).

Pyruvate flux in human ESC is in part regulated by the mitochondrial inner membrane protein uncoupling protein 2 (UCP2), which acts to shunt glucose-derived carbon away from mitochondrial oxidation and into the PPP [84] (Figure 1). Retinoic acid-induced human ESC differentiation results in reduced UCP2 expression, accompanied by

decreased glycolysis and increased OXPHOS [84]. Further, human ESC have a limited capacity to utilise citrate derived from pyruvate to generate ATP through OXPHOS, due to low levels of aconitase 2 and isocitrate dehydrogenase 2/3, concurrent with high expression of ATP-citrate lyase [85]. Significantly, inhibition of pyruvate oxidation stimulates anaplerotic glutamine metabolism in human ESC [85], and glutamine-derived acetyl-CoA production in human cancer cells [86, 87], which are similarly increased in ESC [88]. Plausibly, limited pyruvate oxidation may function to balance ROS production, enhance glutamine utilisation as an anaplerotic source, and stimulate NAD^+ recycling to maintain a high flux through glycolysis for rapid cellular growth and proliferation to support pluripotent self-renewal. In support of this, differentiation of mouse naïve ESC and human ESC alters the glycolytic:oxidative balance within 48 hours [30, 89–91].

Due to the principal requirement for glycolysis in ESC metabolism, the role of glutaminolysis has been relatively overlooked. However, after glucose, glutamine is the most highly consumed nutrient in human ESC culture [11, 77, 78] and is essential for human [10] and mouse [83] ESC proliferation. Other highly proliferative cell types, including tumour cells, use glutaminolysis to recycle NADPH for antioxidant reduction, fatty acid and nucleotide biosynthesis, and anaplerosis (synthesis of TCA cycle intermediates), while glucose-derived carbon is used for macromolecule synthesis [92]. Indeed, in mouse ESC cultured in the presence of glucose, virtually all glutamate, αKG, and malate in the TCA are derived from glutaminolysis [83]. In contrast, naïve mouse ESC are able to proliferate without exogenous glutamine, but only by using glucose to synthesise glutamate for anaplerosis [83]. Human ESC also make extensive use of glutaminolysis [10], which metabolic modelling suggests is likely used for ATP and synthesis of antioxidants (glutathione and NADPH), and anaplerotic pathways [93]. Glutamine-derived glutathione (GSH), a powerful cellular antioxidant, prevents the oxidation OCT4 cysteine residues and subsequent degradation, allowing OCT4 to bind DNA [94]. Combined, these data suggest that glucose and glutamine independently regulate metabolic pathway flux in ESC, and that nutrient availability can significantly impact metabolic pathway activity and cell state.

6. Nutrient Availability Modulates Pluripotency and the Epigenetic Landscape

The culture/nutrient environment in which a cell resides, in vivo or in vitro, and its resultant impact on the intracellular metabolite pool, plays a defining role in determining cellular phenotype. Metabolites can have a long-term impact on a cell through regulation of the epigenome, a relatively new field known as metaboloepigenetics, and their availability has been shown to impact ESC self-renewal and lineage specification (reviewed by [24, 25]).

ESC cell maintenance, cell fate, and DNA methylation have been shown to be regulated by the availability and utilisation of a number of amino acids. The first amino acid found to regulate pluripotent cell state was L-proline. Uptake of proline or ornithine drives mouse ESC differentiation to early primitive ectoderm [2, 95, 96]. This transition is accompanied by alterations in replication timing and H3K9/K36 methylation [97, 98]. Subsequent studies have identified the requirement for specific amino acids for the maintenance of pluripotency. Threonine is the only amino acid essential for the maintenance of pluripotency in mouse ESC and is responsible for maintaining a high cellular SAM level [5, 99]. Depletion of threonine leads to slowed mouse ESC growth, increased differentiation, and a reduction in SAM levels which leads to reduced H3K4me3 [5]. In a similar manner, human ESC require high levels of methionine [4]. Methionine deprivation causes a rapid reduction in SAM levels resulting in a rapid decrease in H3K4me3, while also decreasing NANOG, priming human ESC for differentiation [4].

Glutamine utilisation has been shown to contribute to αKG pools in mouse ESC, with naïve cells prioritising glutamine use to maintain αKG pools for active demethylation through the regulation of Jumonji and TET demethylases [83]. Glutamine depletion from 2i conditions leads to increased tri-methylation of H3K9, H3K27, H3K26, and H4K20 levels in naïve mouse ESC, which retain their ability to proliferate at a reduced rate. Naïve cells are capable of generating glutamine from glucose, while primed mouse ESC are unable to proliferate in the absence of glutamine [83]. Recently, supplementation with αKG during human ESC differentiation has been shown to accelerate the expression of neuroectoderm and endoderm markers [100]. In the presence of αKG, H3K4 and H3K27 trimethylation of differentiating human ESC increased, although an overall reduction in global methylation levels was observed [100]. Similarly, glucose-derived acetyl-CoA contributes to the modulation of the ESC epigenetic landscape, where differentiation, or the inhibition of glycolysis with 2-deoxyglucose, leads to a reduction in H3K9/K27 acetylation, which can be restored by supplementation of the acetyl-CoA precursor acetate [88]. These data highlight the changing metabolic requirements of the cell with progression through pluripotency and with differentiation and emphasise the need to customise nutrient conditions to support specific lineages.

Combined, these studies provide links between metabolism and pluripotency through chromatin state. It will be important to understand how, and if, metabolite presence/absence and abundance drives differentiation to more mature lineages through altered cell state, or whether metabolites select cell populations that are more receptive to differentiation. Indeed, cell type-specific metabolic requirements can be used to purify derivative populations. Human ESC-derived cardiomyocytes can be purified using a glucose-depleted, lactate-rich medium [101], or by sorting for high mitochondrial membrane potential [102], effectively eliminating undifferentiated ESC. Examination of metabolite compartmentalisation within cells, particularly the dynamic requirements that likely occur during cell differentiation, will also be crucial to understand the functional consequences of metabolic flux.

7. Oxygen Regulates ESC Pluripotency

Physiological oxygen conditions (~1–5%) have been reported to facilitate the maintenance of pluripotency, and reduce spontaneous differentiation in mouse [103] and human ESC [104–106]. Further, it has been shown to improve chromosome stability [107], preserve methylation status [108, 109], maintain 2 active X chromosomes [110], and facilitate the derivation of mouse [111] and human ESC [112]. Physiological oxygen increases pSmad2/3 levels, an indicator of TGFβ receptor activation, and decreases lineage markers in human ESC [113, 114], while increasing the efficiency of embryoid body (EB) formation [113]. In contrast, other studies have reported no benefits of low-oxygen culture on the expression of pluripotency markers [115, 116] or surface antigen expression [104] in human ESC. This lack of consensus has plausibly arisen from the many variations in culture conditions used, including the presence or absence of feeders which would respond to altered oxygen conditions [49], medium composition, type of protein supplement, osmolality, pH, or the considerable heterogeneity that exists between human ESC lines [117, 118]. Despite the significant body of evidence for the detrimental effect of atmospheric oxygen conditions from preimplantation embryo studies [49, 50, 61], and emerging evidence that oxygen and ROS [119] can impact the epigenome, ESC culture is predominantly performed under 20% oxygen conditions. In contrast, physiological oxygen levels have become mainstream for naïve cell generation and maintenance [31–33], primarily due to their stabilising effects.

Significantly, physiological oxygen has been shown to accelerate and improve the differentiation of mouse ESC to EpiSCs. Compared with atmospheric oxygen conditions, mouse EpiSCs exhibit a gene expression profile, methylation state, and cadherin profile more similar to in vivo EpiSCs under 5% oxygen [120]. Multiple stem cell types similarly display enhanced differentiation at physiological oxygen. Culture at 2% oxygen is highly beneficial for the derivation and expansion of human retinal progenitor cells [121], increasing population doublings by up to 25 times and enhancing their potential to form photoreceptors [122, 123]. 5% oxygen also facilitates human endothelial cell differentiation through increased expression of vascular endothelial cadherin, CD31, lectin binding, and rapid cord structure formation [124]. Significantly, 5% oxygen culture during the initial 3 days of a 6-day differentiation protocol generates two distinct cell populations, VEcad+ colonies surrounded by PDGFRβ+ pericytes, while 5% oxygen during the second half of differentiation blocks the emergence of these distinct populations. This suggests that there are specific windows of differentiation where oxygen interactions are critical in determining lineage specification. Targeted oxygenation regimes during differentiation likewise increase the yield and purity of neurons [125], definitive endoderm [126], and cardiac differentiation from ESC and iPSCs [127]. This is reminiscent of preimplantation embryo development, which requires precise control over the oxygen and metabolite environment [49]. Consequently, oxygen, and the concentration of other metabolites, will need to be modelled on in vivo niches to achieve the most efficient and viable differentiation outcomes.

8. Physiological Oxygen Underlies a More Active Metabolic State

ESC similarly elicit a conserved physiological response to culture under physiological oxygen conditions. When cultured under physiological oxygen conditions, human ESC increase the flux of glucose through glycolysis (Figure 1) [11, 77, 93, 128], accompanied by increased glycolytic gene expression [77, 116] and decreased oxidative gene expression [11]. Oxygen has also been shown to regulate human ESC mitochondrial activity and biogenesis [11, 128], as occurs in somatic cells [129]. Physiological oxygen increases the expression of glycolytic genes, while reducing human ESC mitochondrial DNA (mtDNA) levels, total cellular ATP, and mitochondrial mass and the expression of metabolic genes associated with mitochondrial activity and replication compared to 20% oxygen culture [11]. Physiological oxygen conditions therefore establish a metabolic state characterised by increased glycolytic flux and suppressed mitochondrial biogenesis and activity (Figure 1).

This conserved cellular response is mediated through the stabilisation of hypoxia-inducible factor (HIF) alpha subunits at physiological oxygen conditions (reviewed by [130]), with HIF activity increasing exponentially as oxygen concentrations decrease below 7% [131]. The human ESC response to physiological oxygen, as for the preimplantation embryo [64], is mediated primarily through HIF2α stabilisation, the silencing of which is accompanied by a reduction in OCT4, SOX2, and NANOG protein expression [105]. HIF2α also binds directly to the GLUT1 promoter increasing GLUT1 levels in human ESC at physiological oxygen [128] (Figure 1), associated with increased glucose consumption. The main HIF alpha subunit, HIF1α, is only transiently expressed in the nucleus of human ESC upon culture under 5% oxygen conditions, suppressed by the expression of the negative regulator HIF3α [105]. Interestingly, overexpression of HIF1α in naïve mouse ESC is sufficient to drive metabolic change from a bivalent oxidative and glycolytic metabolism to one primarily reliant on glycolysis, accompanied by a shift towards an activin/nodal-dependent EpiSC-like state [28], inferring that metabolic regulation alone is sufficient to drive cell state transitions.

Mathematical modelling suggests that ESC display a greater metabolite flux in 70% of modelled metabolic reactions with physiological oxygen culture [93], indicating that low oxygen conditions actually support a more active human ESC state. Cancer cell lines also demonstrate an increase in general metabolic activity under low oxygen, characterised by elevated intracellular levels of glucose, threonine, proline and glutamine, and fatty acid and phospholipid catabolic processes [132]. A higher metabolic turnover emerges as a shared feature of highly proliferative cell types. However, as proliferation is not increased at low oxygen in human ESC studies [11, 77], increased metabolic activity could therefore be underpinning pluripotency through the provision of

epigenetic modifiers. Plausibly, altered glycolytic, TCA flux, and amino acid metabolism will modulate the levels of αKG, NAD^+, and acetyl-coA, thereby regulating the activity of epigenetic modifiers. Indeed, physiological oxygen culture results in the methylation of the OCT4 hypoxia-response element (HRE) of human ESC, while at 20% oxygen, NANOG and SOX2 HREs display methylation marks characteristic of transcriptional silencing [109].

9. ESC Mitochondrial Morphology Is Reminiscent of the Preimplantation Embryo

Mitochondrial morphology is highly dynamic reflecting the developmental stage and metabolic requirements of the cell [133, 134] (Figure 2). In growing and maturing oocytes, mitochondria are primarily spherical, with pale matrices and small vesicular cristae, clustered around the nucleus [135]. By ovulation, mitochondria are the most prominent organelle in the oocyte cytoplasm [136], and the oocyte contains approximately an order of magnitude more mtDNA copies than most somatic cells (reviewed by [137]). Following fertilisation, mitochondria cluster around the 2 pronuclei [136, 138], plausibly, to meet increased energy demands and ensure an even distribution between dividing cells. During the 2- to 8-cell cleavage events of embryonic development, spherical mitochondria are partially replaced with elongated (height = ~3 × width) mitochondria with transverse cristae [136]; this further changes at the early blastocyst stage during differentiation into ICM and trophectoderm, expansion, and hatching of the blastocyst, when highly elongated mitochondria appear [135]. In human apical trophoblast cells, mitochondria are elongated, with transverse cristae, and are largely peripherally located [139], plausibly to facilitate the energetically costly process of blastocoel expansion and zona hatching [140]. Within the ICM and polar trophoblast cells, there is a mixed population of round/vacuolated and elongated/cristae-rich mitochondria that remain perinuclear [135, 139, 141].

In vitro human ESC mitochondria resemble those of in vivo ICM cells [135, 139] (Figure 2) and primordial germ cells (PGCs) [136], containing spherical mitochondria with clear matrices and few peripheral arched cristae (Lees et al. unpublished data; [30, 142, 143]), coincident with lower levels of mitochondrial DNA [11], oxygen consumption, and OXPHOS [84, 144, 145]. Comparatively, somatic cells typically contain filamentous, networked mitochondria with well-defined transverse cristae supporting a higher level of mitochondrial oxygen consumption and oxidative metabolism [30, 142]. The mitochondrial morphology of naïve human ESC is also suggestive of an earlier developmental time point as they display round, vacuolated mitochondria with few cristae compared to primed human ESC [31]. Significantly, naïve human ESC do not attain a mitochondrial morphology equivalent to that of in vivo human or mouse ICM cells, typified by a mixed mitochondrial complement [135]. This suggests that the conditions used to acquire or maintain pluripotency are insufficient for establishing an in vivo-like mitochondrial structure. This is not surprising given its considerable complexity; however, it suggests that only through a close physiological examination of in vivo cells can we hope to achieve in vitro counterparts with the same functionality. However, it is currently unclear at which precise developmental stage in vivo or in vitro all mitochondria take on a dispersed, reticulated, cristae-rich morphology, although it appears coincident with terminal differentiation, accompanied by an increased requirement for oxidative metabolism and a decreased requirement for self-renewal. This reticulated morphology has been observed after ~35 days of terminal neural differentiation to oligodendrocytes [89, 146] and upon terminal differentiation to cardiomyocytes [143]. Conversely, inhibition of mitochondrial fusion during reprogramming, forcibly fragmenting the mitochondrial network, facilitates the acquisition of pluripotency through a ROS-HIF-dependent mechanism [147], highlighting the requirement for dynamic modulation of mitochondrial structure across cell states.

10. Mitochondrial ROS and Perinuclear Localisation: A Requirement for ESC Proliferation?

In spite of the utilisation of aerobic glycolysis, ESC mitochondria, and mitochondrial function, are critical to maintaining pluripotency, self-renewal, and survival [148]. While inhibitors of mitochondrial metabolism increase glycolytic flux and the expression of pluripotent markers in ESC [148, 149], loss of mitochondrial function, following the knockdown of growth factor *erv1*-like (Gfer) [150], or mitochondrial polymerase PolG [145], or following mtDNA mutagenesis [151], result in mitochondrial fragmentation, reduced pluripotency, decreased cell survival and embryoid body forming potential, and the loss of pluripotency in mouse and human ESC. These data therefore highlight an absolute requirement for mitochondria, despite pluripotency being enhanced when OXPHOS is inhibited. Mitochondrial signalling (reviewed by [152]), independent of metabolic activity, may therefore have a role in regulating self-renewal.

In vivo, the location of mitochondria and their interaction with other organelles mark distinct developmental and cellular events. Mitochondria form complexes and localise strongly with other organelles, including the smooth endoplasmic reticulum and vesicles in the post-ovulation oocyte, plausibly generating cellular components in anticipation of fertilisation, as post-fertilisation; these complexes gradually recede [136] (Figure 2). Both in the embryo [136], mouse ESC [145] and human ESC [75, 143, 148], a perinuclear localisation of mitochondria is evident, and is typical of highly proliferative cell types [153], including cancers [154–157]. Expansion of mitochondria from the perinuclear space to a dispersed distribution occurs within 3–7 days of the initiation of ESC differentiation [143, 145, 148]. Significantly, dispersed mitochondria in somatic cells revert to a perinuclear localisation once reprogrammed to a stem-cell like state [75, 148], indicating that close contact with the nucleus is required for either pluripotency and/or self-renewal.

Several hypotheses have been proposed to explain perinuclear mitochondria including a requirement for crosstalk

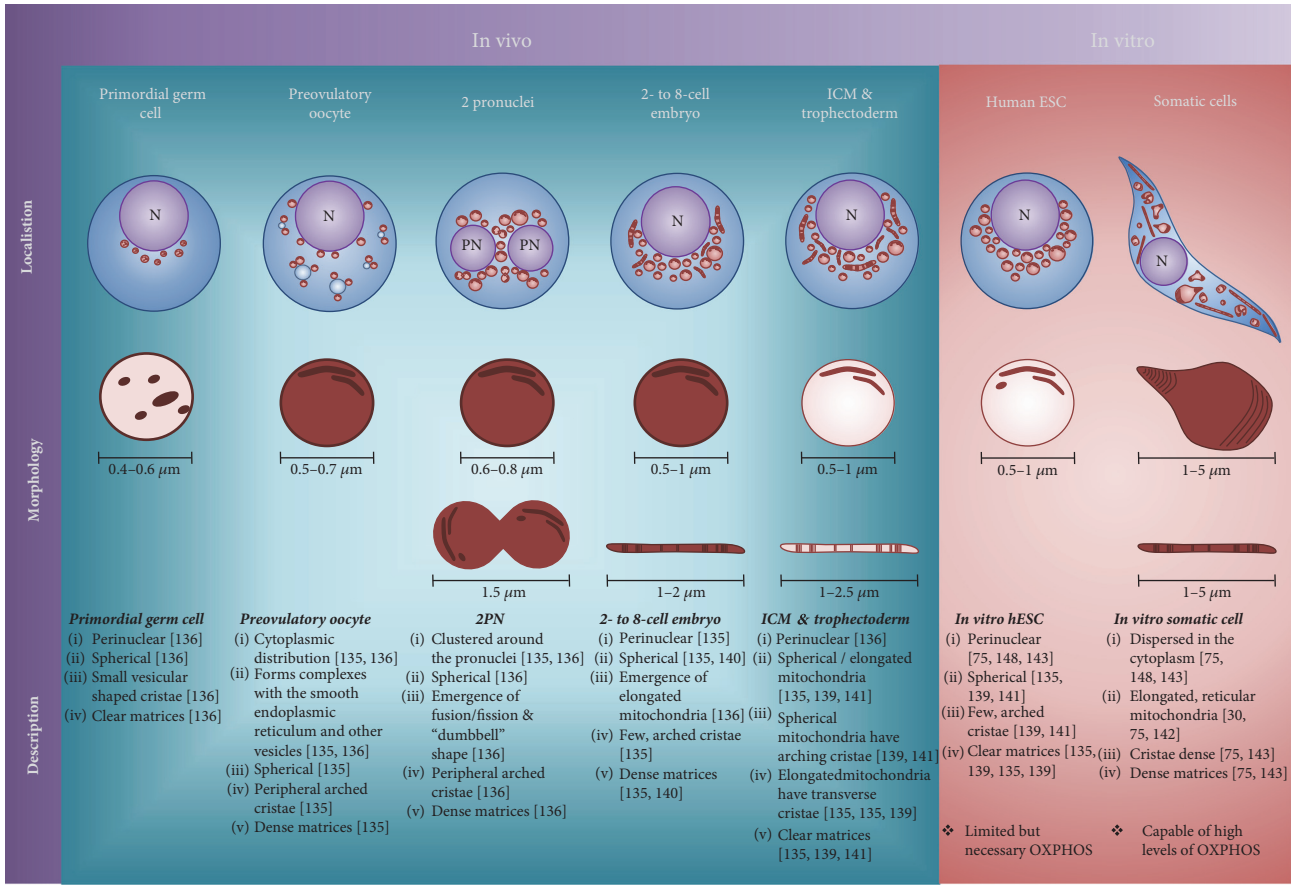

Figure 2: The dynamic localisation and morphology of mitochondria through human development and in culture. Mitochondrial morphology and localisation is determined by the developmental stage and metabolic requirements of the cell [133, 134]. Morphologies in the developing embryo range from spherical organelles with dense matrices and few peripheral arched cristae to long filamentous organelles with sparse matrices and many transverse cristae that maximise the surface area for OXPHOS. The mitochondria also localise strongly with the nucleus and other organelles throughout embryo development to provide ATP for growth and likely to maintain a signalling axis with the nucleus. In primordial germ cells (PGCs), both before and during migration to the gonadal ridge, the mitochondria localise strongly with the nucleus (perinuclear), maintaining a large, vacuous morphology, containing only small vesicular cristae and no transverse cristae [136]. The PGC mitochondrial matrix is clear, suggesting a low level of oxidative activity. During migration, mitochondria increase in number and overall mass. Nine weeks postfertilisation, the PGCs begin to differentiate into the oogonia; by 12 weeks, they begin expansion through mitotic divisions; and by 16 weeks, meiosis commences [190]. During the second stage of prophase in meiosis, zygotene (where the chromosomes closely associate), the mitochondria tightly envelop the nucleus. During the diplotene stage of prophase, when the chromosomes separate, the mitochondria and most other organelles localise to one side of the nucleus forming Balbiani's vitelline body [191, 192]. It is at this point that the human oocyte arrests until hormonal stimulation up to 50 years later [193]. Upon hormonal activation, the oocyte progresses through folliculogenesis. The primary oocyte contains many spherical mitochondria with very dense matrices and few peripheral arched cristae [135]. Notably, these mitochondria are dispersed throughout the cytoplasm and form complexes with the smooth endoplasmic reticulum (SER) and vesicles [136]. These complexes gradually dissipate throughout ovulation and fertilisation. At the 2 pronuclei (2PN) stage, the mitochondria cluster around the 2PN and the initial fission/fusion events take place giving rise to "dumbbell"-shaped mitochondria although the prevailing morphology is still spherical. During the initial cleavage events, elongated mitochondria begin to emerge approximately 2-3 times the length of the spherical mitochondria with well-developed transverse cristae. During the morula and early blastocyst stages, the ratio of elongated to spherical mitochondria increases, such that by the late blastocyst stage in vivo, there is an approximately even mix in both the inner cell mass (ICM) and trophectoderm cells [135, 136, 139]. This mix of mitochondrial morphologies is also observed in the mouse ICM and trophectoderm cells [141]. Notably, in the blastocyst, the mitochondrial matrix becomes clear while the perinuclear localisation and arching cristae phenotype is retained [135, 139]. In vitro hESC mitochondria are similarly perinuclear with few arching cristae and have clear matrices, although their morphology is almost exclusively spherical with a notable absence of the in vivo elongated mitochondria [30, 142]. After seven days of spontaneous differentiation, hESC take on the mixed mitochondrial population [142]. Somatic cell mitochondria are dispersed throughout the cytoplasm and are often highly elongated, reticulated, and bulbous. Their matrices are dense and their cristae are developed and transverse [30], likely a reflection of the more oxidative nature of somatic cell metabolism. N, nucleus (purple); cytoplasm (blue); electron dense mitochondrial matrix (red); electron sparse mitochondrial matrix (pink).

between the nuclear and mitochondrial genomes (reviewed by [137, 158]), buffering the nucleus from calcium fluctuations in the cytoplasm, and efficient energy transfer for transport of macromolecules across the nuclear membrane (reviewed by [159]). Indeed, in human ESC, mitochondria localise perinuclearly throughout mitosis, only moving to congregate around the cleavage furrow [148], likely providing energy to the contractile rings that cleave the cell in two. This is despite the fact that human ESC mitochondria maintain a relatively small inner mitochondrial membrane surface area for the assembly of respiratory chain complexes, accompanied by low levels of oxygen consumption even when working at maximal respiratory capacity [84, 144, 145].

Beyond the production of ATP via OXPHOS, mitochondrial respiration also generates ROS, in the form of hydrogen peroxide (H_2O_2) primarily generated from complex III of the ETC [160, 161]. ROS serve as signalling molecules within a physiological range, compared with their better known role in DNA damage when in excess [162]. ROS directly modulate numerous processes through the modification of kinases, transcription factor activity, and metabolic enzymes and proteins involved in nutrient-sensing pathways, and are capable of stimulating proliferation in a number of cell types [163]. Indeed, self-renewal in human and mouse ESC-derived neural stem cells relies upon high endogenous levels of ROS from cytoplasmic NOX activity [164, 165], supporting a role for endogenous ROS in regulating stemness. The acute proximity of the mitochondria to the nucleus in pluripotent stem cells is suggestive of a signalling axis whereby ROS, in the form of H_2O_2, may provide mitogenic signals [166], plausibly through regulation of the HIF family of transcription factors (Figure 1). This hypothesis is supported by evidence of a prolonged mitochondrial H_2O_2 presence stabilising HIFα proteins at both physiological [160, 161, 167–169] and atmospheric oxygen conditions [170]. As HIFs modulate OCT4 activity [171], and HIF2α both promotes and is necessary for self-renewal and the pluripotent transcription network in mouse and human ESC [105, 172], mitochondrial ROS signalling may underlie the acquisition and maintenance of pluripotency (Figure 1). Indeed, addition of N-acetylcysteine during reprogramming of somatic cells to a pluripotent-like state has been shown to decrease ROS-mediated stabilisation of HIFs [147], necessary for restructuring metabolism towards glycolysis to support pluripotency. Consequently, physiological oxygen would establish an ongoing H_2O_2 presence within a physiological range, capable of sustaining HIF2α activity with prolonged culture. In contrast, the increase in mitochondrial activity associated with culture under atmospheric oxygen likely generates supraphysiological levels of H_2O_2 and more damaging species. As such, increased mitochondrial activity under atmospheric oxygen, accompanied by increased glutathione recycling, may be required to generate sufficient H_20_2 to maintain HIF regulation under atmospheric conditions. Signalling by ROS may explain the maintenance of HIF2α under atmospheric conditions in ESC, albeit at lower levels compared with physiological oxygen [105]. Therefore, a precise balance between ROS production and neutralisation is likely necessary, dependent upon the prevailing oxygen conditions.

Superoxide (O_2^-) can rapidly be reduced to H_2O_2 in either the cytosol, the mitochondrial matrix, or the extracellular environment by superoxide dismutases (SODs) 1, 2, and 3, respectively. While SODs are highly expressed in human ESC [142], mitochondrial ROS generated from complex III cannot be reduced by SODs; instead, reduction to H_2O is carried out by the glutathione/glutathione peroxidase (GSH/GPX) system using the oxidation of NADPH to $NADP^+$ [168], which is also highly active in human iPSCs [173]. In addition to providing the cell with biosynthetic precursors, glutaminolysis also supports the de novo synthesis of glutathione and NADPH, which protect cells from potential damage by the buildup of excess ROS. Therefore, ESC maintain high levels of cytosolic and mitochondrial antioxidants and reducing agents in the forms of GSH, NADPH, and SOD2, to cope with damaging levels of ROS [142, 144, 173, 174]; and yet, ROS generated from the oxidative metabolism of glucose and glutamine are plausibly vital signalling molecules that play a pivotal role in human ESC metabolism and self-renewal.

Thus, an unconventional theory of ESC mitochondria and ROS emerges. The morphology and location of ESC mitochondria, strategically located around the nucleus in great numbers yet with limited OXPHOS capacity, suggest a metabolic strategy that may involve prioritising ATP supply for proliferation via glycolysis, coincident with regulated ROS levels adjacent to the nucleus to stimulate HIF-mediated proliferation (Figure 1). Substantial antioxidant production limits the damaging effects of the H_2O_2 while still enabling signalling. Interestingly, this metabolic strategy benefits from physiological oxygen, as reduced oxygen stimulates GSH production in human ESC [175] and has been shown to increase H_2O_2 production from complex III in human cancer cells [176, 177]. Mitochondrial H_2O_2 generated from physiological oxygen does not induce DNA damage, acting primarily as a signalling molecule [160]. Hence, a delicate ROS/antioxidant balance is struck, coordinated by metabolic pathway activity. Plausibly, persistent atmospheric oxygen used in culture will affect this balance, resulting in either suboptimal signalling levels or pathological levels of ROS and perturbed gene regulation.

11. The Emerging Complexity of Mitochondrial Epigenetic Regulation

In addition to their role in signalling, ROS have been shown to directly alter the epigenetic landscape (reviewed by [178]). Direct oxidative modification of the methyl group of 5-methylcytosine prevents DNMT1 methylation of the target cytosine [179]. Conversely, ROS have been shown to induce DNA methylation of the E-cadherin promoter in hepatocarcinoma cells, accompanied by HDAC1, DNMT1, and methyl-CpG-binding protein 2 (MeCP2) [180]. These data further support the need to modulate ROS within a tight physiological range. As mitochondrial metabolism also controls the levels of the key cofactors acetyl-CoA, αKG, and

NADH/NAD^+ and TCA intermediates including citrate and succinate, which, as discussed, act as substrates for epigenetic modifiers [181]. Metabolism, and particularly mitochondria, therefore acts as an interface between the environment and the nuclear epigenome. However, nuclear-mitochondrial cross-talk goes beyond the unidirectional regulation of cellular homeostasis, nuclear gene expression, and the nuclear epigenetic landscape. Mitochondrial DNA encodes 2 rRNAs, 22 tRNAs, and 13 subunits of the electron transport chain (reviewed by [137]) and was proposed to lack histones [182]. In 2011, Shock et al. identified translocation of nuclear DNMT1 to the mitochondrial matrix, regulated by a mitochondria targeting sequence [183], with translocation sensitive to overexpression of activators that respond to oxidative stress. Alterations in mtDNMT1 directly affected transcription from the light and heavy strands of mtDNA, suggesting epigenetic regulation of the mitochondrial genome [183]. Further studies have identified methylated cytosines within the control region of mtDNA [184], along with the existence of histones within the mitochondrial membrane [185].

Nuclear encoded genes contribute the majority of proteins required for mitochondrial regulation, and it is likely that others will be identified with roles in regulating mitochondrial epigenetics. The reciprocal relationship between the nucleus and mitochondria, both of which are responsive to changes in mitochondrial activity, and therefore nutrient availability, has implications for development, aging, and disease (reviewed by [186]). To this end, the sensitivity of the mitochondrial epigenome to changes in pluripotent, or differentiating, cell metabolism has not been studied. However, the significance of the dynamic nature, and apparent plasticity, of cellular metabolism is that a suboptimal nutrient environment is compensated metabolically, and a changed metabolism will result in a misregulated nuclear, and mitochondrial, epigenome. The impact of this may not be apparent in the pluripotent cell but is plausibly manifested in differentiated progeny through inheritance of aberrant epigenetic marks, modifying the expression of genes involved in signalling, growth and differentiation, and metabolism. While sufficient perturbation may be lethal, studies on the impact of embryo culture imply that cell plasticity enables ongoing development and differentiation. However, these compensatory changes likely establish lower cellular stress/environmental tolerance, manifest as susceptibility to disease. Hence, the environment in which a cell is grown becomes a critical regulator and determinant of cell fate.

12. Conclusions

The convention that metabolites are simply required for energy production is placed into a new context, in which metabolites are central to modifications of the epigenetic landscape, and a novel model explaining the previously unexplored phenomenon of human ESC mitochondrial morphology and localisation is presented. The mitochondrial signalling axis, possibly unique to highly proliferative cell types such as stem cells and the embryo, attempts to explain a hitherto undescribed facet of ESC metabolism in which numerous, vacuolated, perinuclear mitochondria may induce a H_2O_2-rich nuclear environment stimulating proliferation through HIF activity, a process that is plausibly facilitated by physiological oxygen. As metabolism and epigenetics intersect, metabolite and cofactor availability is hypothesised to have a significant impact on the chromatin landscape leading to persistent changes carried through lineage commitment. Pivotal studies in embryonic stem cells have established that oxygen, through its impact on metabolism and key transcription factors, modulates stem cell pluripotency and differentiation. Oxygen, as a nutrient in culture, is a signalling molecule capable of directing lineage decisions and remodelling metabolism. When used at the correct stage during in vitro development, and at the correct concentration, mimicking in vivo physiology, oxygen can exert significant selective pressures, generating larger numbers of more homogeneous populations. Metabolic pathway flux, encompassing fundamental metabolic pathway activity and network interaction, metabolic intermediate production, and ROS generation, therefore integrates nutrient availability with cell signalling and the epigenome. Consequently, the formulation of culture media has significant implications for stem cell maintenance, cell fate, and plausibly subsequent cell health and function.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Review Article

Mitochondrial Heterogeneity: Evaluating Mitochondrial Subpopulation Dynamics in Stem Cells

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Although traditionally viewed as the "powerhouse" of the cell, an accruing body of evidence in the rapidly growing field of mitochondrial biology supports additional roles of mitochondria as key participants in a multitude of cellular functions. While it has been well established that mitochondria in different tissues have distinctive ultrastructural features consistent with differential bioenergetic demands, recent and emerging technical advances in flow cytometry, imaging, and "-omics"-based bioinformatics have only just begun to explore the complex and divergent properties of mitochondria within tissues and cell types. Moreover, contemporary studies evaluating the role of mitochondria in pluripotent stem cells, cellular reprogramming, and differentiation point to a potential importance of mitochondrial subpopulations and heterogeneity in the field of stem cell biology. This review assesses the current literature regarding mitochondrial subpopulations within cell and tissue types and evaluates the current understanding of how mitochondrial diversity and heterogeneity might impact cell fate specification in pluripotent stem cells.

1. Mitochondrial Structure and Diversity

Mitochondria are ubiquitous across eukaryotic organisms and are critical for cellular bioenergetics. The classical mitochondrial ultrastructural features, consisting of an outer membrane and an inner membrane containing invaginations that comprise the matrix-rich cristae, are specifically modified in a tissue-specific manner in order to meet cellular-explicit energy demands. The differences in mitochondrial morphology between cell and tissue types as well as in localization and distribution within the cell have been well documented [1, 2], and more recently, it has been demonstrated that even within a single cell, mitochondrial features fluctuate rapidly in response to alterations in a metabolic state [3, 4]. Although the exact molecular mechanisms underlying ultrastructural remodeling of mitochondria are a topic of investigation with much remaining to be discovered, the diversity observed in a mitochondrial phenotype includes a range in size (from 0.1 micron to 1.0 microns in diameter), shape (from spherical to elongated and tubular), and cristae density (from essentially devoid of cristae to dense cristae). As a mitochondrial form relates to function, early studies postulated that mitochondrial cristae density serves to increase the surface area, thereby enhancing oxidative phosphorylation [5]. With subsequent efforts identifying the inner membrane as containing the components of the electron transport chain and the capacity for ATP generation positively correlated with the cristae surface area (reviewed in [6]), the relationship between energy production and mitochondrial morphology, with specific modifications in the inner membrane and cristae structure, has become increasingly clear. For example, ATP synthase dimerization has been demonstrated to govern biogenesis of the inner membrane and cristae formation, and oligomerization of F1FO-ATP synthase has been proposed to mediate the formation of cristae by modulating inner membrane curvature [7–10]. Additionally, a number of candidate proteins involved in modulation of cristae formation and inner membrane organization have been identified across model organisms and specifically correlated with pathophysiological disease states in humans (reviewed in [5]). Mitochondrial morphological dynamics are also dependent upon fission and fusion of inner and outer membranes, mediated by large GTPases [11–13], including the cytosolic DRP1 [14], the mitofusin (MFN)

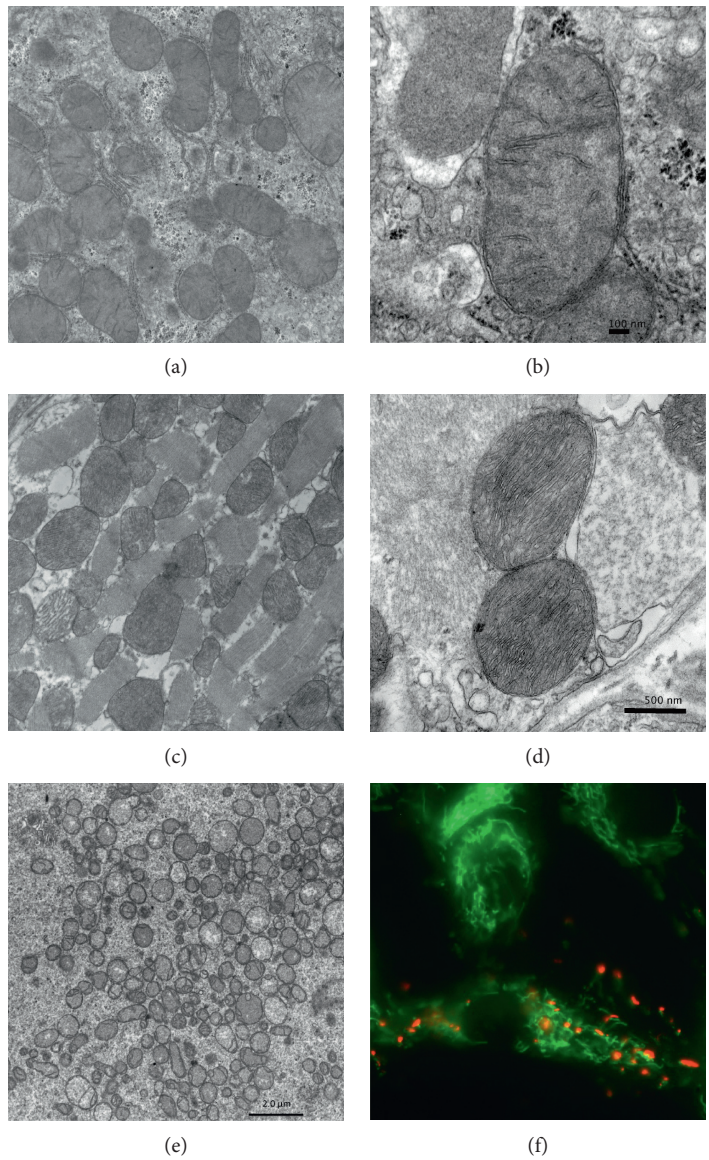

Figure 1: Mitochondrial heterogeneity between tissue types and within cells. (a)–(e) Transmission electron micrographs depicting mitochondrial features in mouse liver ((a), enlarged in (b)), mouse heart ((c), enlarged in (d)), and a primary-stage human oocyte (e). (f) A fluorescent micrograph depicting JC-1-labeled KGN cells demonstrates heterogeneity in mitochondrial membrane potential between cells and between individual subcellular mitochondrial populations. JC-1 monomers (green) indicate low $\Delta\psi$m and JC-1 aggregates (red) indicate high $\Delta\psi$m. Scale bars as marked.

proteins 1 and 2 (MFN1 and MFN2) [15], and optic atrophy 1 (OPA1) [16–19].

Although the specifics regarding how these nuclear-encoded proteins have been shown to participate in mitochondrial remodeling have been studied for almost two decades and extensively reviewed elsewhere [4, 20], far less is known regarding the cell signaling mechanisms that govern a mitochondrial phenotype and how this occurs on a tissue-specific basis during the process of differentiation. In undifferentiated embryonic stem cells (ESCs), mitochondria are characterized as having a globular shape, with few defined cristae and limited oxidative capacity [21]. This ovoid mitochondrial morphology and cristae arrangement are rather striking [21, 22] and also observed in oocytes (Figure 1), as well as in the inner cell mass of blastocysts—the originating source for ESCs [23]. The low matrix and cristae density are associated with slowly respiring state IV mitochondria, as compared to fast respiring state III mitochondria, which have a higher matrix and cristae density and a morphologically "condensed" ultrastructure [24–26]. Notably, the low cristae density and ovoid structure, along with distinctive perinuclear localization, are under consideration as features for cellular "stemness," having been detected in adult somatic stem cells and reprogrammed stem cells (i.e., iPSCs [21, 27, 28]), in addition to numerous ESC lines. Intriguingly, observations of the modification of mitochondrial patterning in a spontaneously differentiating rhesus monkey adult mesenchymal cell (MSC) line (ATSC line)

have demonstrated heterogeneity within stem cell populations and have led to the postulation that mitochondrial localization (deviation from a perinuclear region) may be a mechanism by which to monitor differentiation status, in vitro [28], although this remains to be firmly established, and applicability may be cell type- and status-dependent, as perinuclear localization can be induced in disease states and under conditions of hypoxia and apoptosis in nonstem cell lines [29–31]. A detailed analysis of several nonstem cell types, including HeLa, HUVECs, COS-7, cortical astrocytes, and primary hepatocytes, also demonstrated heterogeneity with respect to both distribution and functional properties [32]. Although in each cell type examined, mitochondria were distributed throughout the cell body, both cortical astrocytes and HUVECs demonstrated distinct perinuclear clustering, which was less pronounced in HeLa and COS-7 and absent in primary hepatocytes [32]. Functionally, it was noted that each cell type exhibited heterogeneity in mitochondrial membrane potential (potential ($\Delta\psi$m)), as each cell type examined contained distinct populations of mitochondria having both high or low $\Delta\psi$m, and no single mitochondrion contained regions of high and low $\Delta\psi$m. Quantitative analysis of $\Delta\psi$m in HeLa cells demonstrated that mitochondria localized to the perinuclear region had a greater percentage of mitochondria with low $\Delta\psi$m and were more closely associated with endoplasmic reticulum (ER) than their peripherally located counterparts [32].

Morphological changes in the physical appearance of mitochondria during the process of differentiation are not well characterized, although it is well understood that mitochondrial ultrastructure varies dramatically between tissue types ([1], Figure 1). In a recent study evaluating the changes in mitochondrial features that accompany progressive states of MSC differentiation into the endothelial lineage, Shin et al. assessed mitochondrial numbers, length, resident area per cell, and morphology. Consistent with the high energy demands required for differentiation followed by a subsequent decline as cells approach a terminally differentiated phenotype, the authors determined that mitochondrial number, area per cell, length, and morphological complexity decreased corresponding with progression of differentiation [33]. To specifically address the mitochondrial reconfiguration that occurs during the differentiation process, Forni et al. monitored changes in mitochondrial mass, morphology, dynamics, and bioenergetics during MSC differentiation into osteocyte, chondrocyte, and adipocyte lineages. Data indicate that mitochondrial elongation and increases in MFN1 and MFN2 occurred during the early stages of adipocyte and osteogenic differentiation, whereas chondrogenesis was associated with a fragmented mitochondrial phenotype. Strikingly, the differentiation ability of MSCs was inhibited following knockdown of Mfn2 in adipogenesis and osteogenesis, while dominant-negative Drp1 impeded the chondrogenesis differentiation capability [34]. Together, these data provide strong supporting evidence for a fundamental role for mitochondrial dynamics, including the modulation of mitochondrial ultrastructure, in the differentiation process.

Although metabolic and structural remodeling during cell differentiation represents emerging fields of study in mitochondrial and stem cell biology, mitochondrial heterogeneity as it pertains to specific mitochondrial subpopulations within a single tissue or cell remains a considerably under-characterized component in cell biology. It is well understood that under physiological conditions, cells possess a heterogeneous mitochondrial population based on differences in membrane potential ($\Delta\psi$m [32, 35, 36]). Mitochondria with a low $\Delta\psi$m are generally regarded as metabolically quiescent ("resting" or damaged), whereas those with a high $\Delta\psi$m are viewed as metabolically active ("respiring"). However, there are caveats to this generalization, including that $\Delta\psi$m is maintained by the balance between the electron transport chain ATP synthases. Accordingly, even well-coupled (respiring) mitochondria can have a decreased $\Delta\psi$m. The heterogeneity in $\Delta\psi$m both within individual cells and between neighboring cells can be observed in vitro using fluorometric compounds, such as JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide). JC-1 undergoes a shift in spectral fluorescence from green (in mitochondria that are metabolically inactive) to red-orange upon conversion from monomeric (green) to aggregate (red-orange) form in actively respiring mitochondria. Utilizing the aggregate fluorescent properties of JC-1 to detect differences in mitochondrial respiration status in undifferentiated human ESCs (H9), Kumagai et al. have recently demonstrated that shifts in $\Delta\psi$m (e.g., cells with more aggregate-state JC-1) within undifferentiated ESC colonies may serve as a simple visual predictive indicator of cells that are undergoing the earliest stages of differentiation [37]. Due to the predominant metabolic reliance on glycolysis during the undifferentiated state [38–41], the observed increase in $\Delta\psi$m associated with oxidative phosphorylation is one of the first markers to distinguish differentiating cells in a mixed culture. Accordingly, mitochondrial metabolic changes have been proposed as early markers of stem cell differentiation [38–41], although how these changes occur on a cell type-specific basis and the relationship of metabolic shifts to cell fate specification remains less well defined. However, it has been shown that mitochondrial activity and stem cell function are intimately linked. In isolating undifferentiated mouse ESCs based on differences in $\Delta\psi$m, Schieke et al. obtained two distinct populations of ESCs which were indistinguishable based on morphology, yet had remarkably different resting oxygen consumption rates (low $\Delta\psi$m, $\Delta\psi$mL; high $\Delta\psi$m, $\Delta\psi$mH). Analysis of differentiation potential revealed that in the presence of bone morphogenetic protein 4 (BMP-4) in the absence of leukemia inhibitory factor (LIF) to promote mesoderm specification, $\Delta\psi$mL ESCs exhibited a markedly greater mesodermal differentiation capacity (10-fold) than $\Delta\psi$mH ESCs, whereas $\Delta\psi$mH ESCs had a greater propensity for teratoma formation than $\Delta\psi$mL ESCs [42]. Furthermore, treatment with rapamycin, a potent inhibitor of mTOR, resulted in a decrease in $\Delta\psi$m and oxygen consumption in undifferentiated mouse ESCs and augmented BMP-4-induced mesodermal differentiation [42]. Together, these data suggest a strong link between intrinsic mitochondrial metabolic function and stem cell fate.

Alterations in $\Delta\psi$m in human ESCs have also been demonstrated as a consequence of in vitro age (e.g., passage

number). In undifferentiated H9 and PKU1 cell lines, $\Delta\psi$m increased significantly in late-passage cells as compared to their younger counterparts. The elevated $\Delta\psi$m also correlated with increases in total mitochondrial volume and generation of reactive oxygen species (ROS) [43]. Late-passage human ESCs also exhibited a reduced capacity for differentiation. While early-passage cells differentiated evenly into ectodermal, mesodermal, and endodermal lineages, high-passage cells preferentially differentiated into ectodermal lineage, although the authors acknowledge that the impact of prolonged duration on culture cannot be ruled out, rather than directly related to the passage number of the cell line [43]. Given that additional established markers of stemness, such as telomerase activity and pluripotency markers, were unaffected by passage number, the impact of in vitro age on mitochondrial function is striking and may represent an additional factor when screening stem cells for potential therapeutics. This type of in vitro aging has a similar impact on the mitochondria of iPSCs. In a direct comparison between iPSCs cultured for 1 month postcellular reprogramming (young iPSCs) and iPSCs cultured for over 1 year (aged iPSCs), H_2O_2-dependent $\Delta\psi$m depolarization occurred at a faster rate in aged iPSCs than that observed in their "younger" counterparts, demonstrating a diminished ability to counteract oxidant exposure with in vitro age. Moreover, the capacity for in vitro neurogenesis was diminished in the aged iPSCs versus the young iPSCs [44].

Although emerging evidence supports a role for $\Delta\psi$m in the maintenance of stemness, the impact of mitochondrial subcellular heterogeneity on cellular function, including differentiation, has not been evaluated. Based on a series of studies examining mitochondrial properties within individual cells [34, 35], Kuznetsov and Margreiter have neatly described mitochondrial heterogeneity as belonging to 4 major classifications (reviewed in [45]): (1) *ultrastructure*—mitochondria range in size (0.2 μm to 1.0 μm), shape (circular to elongated and tubular), and cristae density (no visible cristae/vacuole-like appearance to dense cristae) and can be found alone or physically networked; ultrastructure varies across tissue types, as well as within individual cells; and fission-fusion events, as well as respiratory status, also impact on morphology; (2) *functional properties*—mitochondria can differ in redox state, respiration, intramitochondrial Ca^{2+} and reactive oxygen species (ROS) levels, mitochondrial protein composition and content, and $\Delta\psi$m; (3) *behavior*—mitochondria respond differently to oxidative stress, starvation, apoptotic stimuli, and mitophagy signals and exhibit selective responses to toxins and substrates; (4) *dynamics*—cell type-specific intracellular localization, oscillatory movements, translocation events, filament extension and retraction, and fission and fusion events [45]. Additionally, new evidence also now demonstrates heteroplasmy in mitochondrial DNA (mtDNA) at the level of the single cell [46], bringing another layer into subcellular mitochondrial heterogeneity. However, despite the understanding that mitochondria within a cell may well serve different functions, precise characterization of subcellular mitochondrial subpopulations has proven challenging, due to difficulties in the isolation of specific mitochondrial subtypes for further analysis [45–47], and much remains to be discovered. In a discussion of mitochondrial heterogeneity pertaining to $\Delta\psi$m, Wikstrom et al. propose that low $\Delta\psi$m represents a mechanism by which mitochondria are selectively targeted for autophagy [36]. Although the dynamics that define mitophagy are unclear, it has been demonstrated that mitochondria depolarize prior to autophagy [48, 49] and join a preautophagic mitochondrial pool [49] characterized by small size and reduced levels of the mitochondrial fusion protein, OPA1 [49, 50]. It has also been proposed that mitochondrial heterogeneity may contribute to preservation, as reduced metabolic activity may serve to preserve genomic integrity [42].

2. Tissue- and Cell-Specific Mitochondrial Subpopulations

In addition to the differences in mitochondrial morphology mentioned above, mitochondria also differ functionally and are known to be involved in cellular processes beyond metabolism. Such functions are well characterized and ubiquitous, including cell death and differentiation, intracellular Ca^{2+} regulation, oxygen sensing, and ROS generation, while others are cell and tissue type-specific, such as steroid hormone biosynthesis, hormone signaling and responsiveness, thermogenesis, hemesynthesis, and processing of toxins [51]. These complex processes, in addition to those associated with metabolism, are carried out through bidirectional communication with the nucleus. In humans, 13 proteins, along with 2 rRNAs and 22 tRNAs, are encoded by the 37 genes contained within the small (16,569 bp) circular mitochondrial genome. The proteins serve as key constituents of mitochondrial electron transport chain (ETC) protein complexes I–IV that are embedded in the inner mitochondrial membrane. However, mitochondrial protein composition is estimated to approach 1500 proteins [52] encoded by the nuclear genome, presumably to execute the diverse array of functions performed by mitochondria. Of these, approximately 1000 proteins have been identified (although not functionally characterized) [53], with the vast majority predicted to localize to the mitochondrial inner membrane and the matrix [54, 55]. However, mitochondrial protein composition is not fixed, nor is it consistent between tissue types. A well-executed proteomic study using ultrapurified mitochondrial preparations revealed striking compositional differences between brain, kidney, liver, and heart mitochondria, determining that only 57% of mitochondrial proteins identified were consistently expressed between the examined tissues [55]. With nearly half of the protein composition of mitochondria differing between tissue types, the cell type-specific differences in mitochondrial function are also underscored. Although mitochondria are highly morphologically heterogeneous between tissues [1, 56], details regarding proteomic profiles on mitochondrial subpopulations within a single cell or tissue type are lacking, as is information regarding the ESC mitochondrial proteome [57]. Mitochondrial proteomic analyses in pluripotent stem cells as they undergo transformational changes related to reprogramming and differentiation would likely provide important information

pertaining to the utility of stem cells in research and, as directed differentiation strategies improve, therapeutics and regenerative medicine.

Given the complexity and diversity of mitochondrial proteomic profiles amongst mitochondrial subtypes, it is not surprising that mitochondria serve to perform a diverse spectrum of functions—with many more remaining to be discovered. Among the more well-characterized mitochondrial types within a single tissue are two cardiac mitochondrial subpopulations, subsarcolemmal mitochondria (SSM) and interfibrillar mitochondria (IFM). Initially characterized by ultrastructural differences observed between the two populations located beneath the sarcolemma and between the myofibrils, respectively, physical isolation of each mitochondrial subtype revealed distinct biochemical functional properties [58]. Specifically, succinate dehydrogenase and citrate synthase activities were elevated in the IFM population as compared to SSM, and oxidation of substrates was found to proceed 1.5× faster in IFM isolates than SSM preparations [58]. Subsequently, the Ca^{2+} uptake capability of IFM and SSM was comparatively demonstrated to differ, as a further indicator of mitochondrial heterogeneity between the two subpopulations [59], although differences in isolation strategies may have impacted some of these conclusions. Additionally, IFM demonstrated higher protein import rates for the precursors to malate dehydrogenase and ornithine carbamoyltransferase [60]. However, due to the technically difficult nature of the isolation procedures, in-depth comparative experimental characterization between the two subtypes has been impeded. More recently, Hatano et al. have developed a three-dimensional computational model, integrating electrophysiology, metabolism, and mechanics with subcellular structure. Using this intriguing multifaceted simulation approach, the authors demonstrate that the impact of the subcellular environment modulated mitochondrial function [61]. Although individual intrinsic functional differences between mitochondrial populations could not be examined in the study, this work highlights that mitochondria work within a subcellular "niche," in which microenvironmental cues can govern function. It is intriguing to think of mitochondrial subpopulations as respondents to the microenvironment (similar to stem cells within a niche). As a first step toward evaluating "mitochondrial heteroplasmy" in a single mitochondrion, Pham et al. developed a microrespirometer to monitor mitochondrial respiration on individual organelles. Using this novel technological approach, the authors confirmed differences in respiration between coupled and uncoupled mitochondria [47]. Additionally, our own data utilizing a nanoparticle-sorting platform for the isolation of mitochondrial subpopulations similarly revealed differences in the ATP-generating capability between coupled and uncoupled mitochondria; however, a subset of uncoupled mitochondria could be induced to generate ATP when the microenvironment was altered [62]. As more information becomes available regarding intrinsic mitochondrial differences, computational models along with emerging technologies to evaluate mitochondrial subpopulations such as this will likely prove invaluable in the development of experimental paradigms and testable hypotheses.

3. Conclusion

The current understanding of mitochondrial function in stem cells is limited in scope as compared to the broader field of mitochondrial biology. Given the highly specialized features of mitochondria in differentiated cell types, it stands to reason that a separate field of study dedicated to mitochondrial fate specification and differentiation might coevolve with the stem cell field. In this way, stem cells serve as an excellent model to study "mitochondrial differentiation." As each mitochondrion contains DNA encoding for only 13 proteins, yet contains a subtype-specific proteomic profile of up to 1500 nuclear-encoded proteins, the significance of nuclear communication in the regulation of mitochondrial function becomes increasingly clear. How the mitochondria and nucleus communicate on a per organelle basis (i.e., why some are activated while others remain resting, how cell type-specific functions are executed) remains to be determined. As nanoscaled technologies emerge for the study of subcellular organelles, the mechanisms that govern mitochondrial heterogeneity and function will be elucidated and perhaps provide additional platforms and metrics for stem cell reprogramming and differentiation.

Conflicts of Interest

D. C. Woods declares no conflict of interest regarding the information contained herein.

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Review Article

Artificial Mitochondria Transfer: Current Challenges, Advances, and Future Applications

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The objective of this review is to outline existing artificial mitochondria transfer techniques and to describe the future steps necessary to develop new therapeutic applications in medicine. Inspired by the symbiotic origin of mitochondria and by the cell's capacity to transfer these organelles to damaged neighbors, many researchers have developed procedures to artificially transfer mitochondria from one cell to another. The techniques currently in use today range from simple coincubations of isolated mitochondria and recipient cells to the use of physical approaches to induce integration. These methods mimic natural mitochondria transfer. In order to use mitochondrial transfer in medicine, we must answer key questions about how to replicate aspects of natural transport processes to improve current artificial transfer methods. Another priority is to determine the optimum quantity and cell/tissue source of the mitochondria in order to induce cell reprogramming or tissue repair, in both in vitro and in vivo applications. Additionally, it is important that the field explores how artificial mitochondria transfer techniques can be used to treat different diseases and how to navigate the ethical issues in such procedures. Without a doubt, mitochondria are more than mere cell power plants, as we continue to discover their potential to be used in medicine.

1. Introduction

Mitochondria are cell organelles descended from an alphaproteobacterial endosymbiont [1] and play a fundamental role in growth, differentiation, and survival beyond sustaining the energetics of the cell [2, 3]. Diseases, tissue damage, and aging challenge the cell and its mitochondria, thereby affecting their integrity, function, and homeostasis [4, 5]. Cells naturally have the capacity to exchange intracellular material and especially mitochondria through different processes such as cell-to-cell contact, microvesicles, nanotubular structures, and other mechanisms [6–8]. Clark and Shay pioneered the artificial mitochondria transfer (AMT), which involved transferring mitochondria with antibiotic-resistant genes into sensitive cells, thereby enabling them to survive in a selective medium [9] and opening this new field of research. Since the work of Clark and Shay, the process of artificial transfer has and continues to mimic aspects of naturally occurring cell transport, especially in the mechanisms cells naturally use to rescue other damaged cells. The

AMT restores and increases respiration and proliferation and completes other cellular processes [5, 10–16].

This review will consider key advances necessary to improve the current knowledge about the artificial transfer of mitochondria and how these techniques could be used therapeutically. We will provide an overview of the features of the mitochondrial structure that are important in maintaining its integrity throughout artificial transfer [13, 14]. Next, we will discuss how a cell naturally protects the mitochondria during their transport by using intercellular bridges or microvesicles and the effects of the transferred mitochondria in the receiver cell [6, 17, 18]. The in vivo artificial transfer of mitochondria was carried out at the same time as many in vitro assays [5, 7, 12, 13, 16, 19]. These approaches will be covered in the third section. For example, those assays performed by McCully in 2009 [16] and recently by Huang et al. in 2016 [19] raised questions about the best source of mitochondria, what kinds of stress during their transfer could affect mitochondrial function or prevent their arrival to the target tissue, among other questions. The key to developing new lines of research in this field is determining the diseases in which AMT could be effective as well as the potential advantages of such therapeutic treatments over others. Taking this into account, it is essential that we further study the effectiveness of different donor sources of mitochondria in repairing recipient cells and determine how such findings can help to establish ethical guidelines that will facilitate future safety research and enable the development of new medical applications of AMT. Without a doubt, more advances are needed to better understand and improve AMT and lay the foundation for its safe use in treating mitochondrial damage and related diseases.

2. Structural and Functional Characteristics of Mitochondria for a Successful Artificial Transfer

The mitochondrion is an organelle present in most of eukaryotic cells; it is in charge of ATP synthesis via oxidative phosphorylation (OX-PHOS), calcium metabolism, and the control of the apoptotic intrinsic pathway, among other functions. At present, the mitochondrion is recognized as an endosymbiotic organism, whose noneukaryotic origin could facilitate its ability to be transferred from one cell to another. It has a double protective membrane and partial transcriptional independence from the nucleus, thereby making the mitochondria an item which can naturally be exchanged by microvesicles and nanotubes between cells [20–22]. Given that there is no cellular protection when performing AMT, it is important to conserve mitochondrial integrity after isolation when exposed to an extracellular environment. The isolation procedure and stressors present outside the cell or organism like temperature change and surrounding media would greatly modified the structural stability, function, and potential effects of the mitochondria in the receiver cell [23]. In this section, we will focus on key biological aspects that should be taken into consideration when the AMT to other cells is sought.

The mitochondria evolved from a prokaryotic organism, and when it colonized the first protoeukaryotic cell, it developed a system of close communication with the nucleus by exchanging its own mtDNA sequences with it [24, 25]. It is estimated that mitochondria need almost 2000 proteins to work properly, but in many species, mtDNA encodes barely 63 proteins or less [26, 27] and most of these proteins are synthesized in the cytoplasm by means of ribosomes encoded in the nucleus and not by those of the mitochondria, thereby making them partially independent [28]. The interaction between nuclear and mitochondrial genes is essential for the organelle transcription, translation of proteins, and respiration [29]. Considering this close relationship, the compatibility between the mitochondria of one cell or species interacting with the nucleus of another could potentially affect their crosstalk, thereby inhibiting cell respiration and function [29–32]. These specific differences in the nuclear and mitochondria genome between cells or species could cause incompatibility if the auto, allo, and xenogenic AMT is pursued [13].

The mitochondria's small size as well as its capacity to change its shape and length allows it to be transported by subcellular transporting mechanisms such as tunneling nanotubes (TNTs) and microvesicles (MVs) [33, 34]. Its diameter varies between 0.5 and 1.0 μm, and its length shows great variability, from 0.5 to 10 μm. Although its shape is defined as rounded or elongated, mitochondria can be very pleomorphic, or in other words, they may exhibit great morphological variations. Some mitochondria could be fused and interconnected in networks, in contrast to the classic bean shape that appears in most illustrations [35, 36]. This organelle is characterized by a double lipoprotein membrane, each of which are about 7 nm thick. The outer mitochondrial membrane is smooth, biochemically identical to the membranes of eukaryotic cells, and rich in cholesterol (possibly contributing to the cell capacity to internalize this organelle when it is free in external medium) [11].

Guaranteeing the integrity of the outer and inner membranes during any process of AMT between one cell to another is key to protecting this organelle's function and the effects on the receiver cell after transfer. The outer mitochondria membrane (OMM) serves as a barrier and a platform to exchange products between that cytoplasm and the intermembrane space [37, 38]. The OMM also protects the cell from any harmful product, like free radicals from the active metabolic processes carried out by the mitochondria [37, 39]. OMM permeabilization can be induced by toxins, *gamma* and/or UV irradiation, hypoxia, and growth factor deprivation causing irreparable mitochondria DNA (mtDNA) damage. These factors can lead to the activation of proapoptotic multidomain Bcl-2 proteins, such as Bax or Bak [40–43]. A permeabilized or fragile OMM would not be effectively internalized after AMT by the receiver cell or even could activate apoptotic processes instead of repairing or increasing cellular functions [11]. Further studies should be completed in order to fully understand the interactions between the OMM and the receiver cell membrane and to understand the process of uptake.

The inner mitochondrial membrane (IMM) is chemically similar to bacterial cell membranes and rich in cardiolipin, a

phospholipid made of 4 fatty acids that decreases this membrane's permeability to protons. The IMM's lack of proton permeability is essential because it allows the existence of differential concentrations between the mitochondrial compartments (intermembranous space and mitochondrial matrix). The IMM is composed of the inner boundary membrane (IBM) and cristae membrane (CM), where the IBM is opposed to the OMM and the CMs are extended protrusions of the IBM inside the matrix [44]. The CM's shape is created by multiple folds in the membrane. This allows more IMM to be packed into the organelle and thereby provides scaffolding for the electron transport chain complexes and ATP synthase which represent 80% of the protein mass of the inner membrane [45, 46]. The disruption of the IMM architecture could result in the alteration of the cristae dynamics in the mitochondria, consequentially affecting its capacity to fuse with other mitochondria and to produce ATP [37, 47, 48]. One of the therapeutic possibilities of AMT is enabling the exchange of mtDNA from exogenous healthy mitochondria to damaged receiver mitochondria thereby contributing to the ATP production in which maintaining the integrity of IMM could favor the process.

Mitochondrial fitness is essential to maintain the integrity and functioning of the cell. Many reactions take place inside the mitochondria and are the consequence of its good condition, among fatty acids β-oxidation, Kreb's cycle, urea cycle, heme biosynthesis, and part of the steroid, cardiolipin, and ubiquinone biosynthesis pathways. Genetic variations in mitochondria and the presence of deleterious mutations in their DNA can alter their structure, function, and integrity. Many crucial aspects of their physiology are still not fully understood which are necessary to understand how physiological changes or stressors, like subproducts of the electron transport chain (i.e., reactive oxygen species (ROS)) and others induced by the environment (contamination or age), can damage components of the mitochondria. In order to develop more efficient mechanisms and succeed the AMT, we must find ways to maintain their structural integrity during AMT, guaranteeing that the outer and inner membrane structures will be conserved and also that the mitochondria does not lose its function during the transfer, thus assuring the beneficial effects of the procedure [9, 14]. Previous work about the AMT evaluates mitochondrial function by fluorescent probes and electron microscopy being a key aspect of the transfer procedure [14, 16, 49, 50]. Picard et al. observed in 2011 that the isolation procedure of the mitochondria from cells or tissues induces the fragmentation of the organelle, modulates the permeability of the transition pore sensitivity to calcium, alters the respiration rates of oxygen consumption, and increases the mitochondrial stress-producing free radicals [23]. There is still no information about the absolute or relative number of damaged versus healthy mitochondria during the AMT process. Obtaining this information could contribute to a better evaluation and comparison of the different AMT methods discussed in this review.

Cells and mitochondria change during the process of differentiation. It has been described that stem cell mitochondria are in a dormant and immature state: they are small and favor anaerobic metabolism. Through the process of differentiation and loss of their pluripotency, mitochondria proliferate and the quantity of DNA, the rate of respiration, and the generation of ATP synthase increase. These changes cause the mitochondria to develop an elongated morphology and swollen cristae. Its matrix also becomes more dense, being relocated to a wider extent in the cells [51–54]. It has not been studied whether the isolated mitochondria show variations on their effects on the recipient cells depending on the differentiation states as mitochondria show strong differences on their structure and metabolic profiles. Questions like whether the cristae distribution change, ROS produced during the transfer, need to be answered and incorporated to the isolation and transfer protocols.

In the next section, we will describe key aspects of natural intercellular mitochondria transfer, a cellular function which protects other cells from damage or stress [7, 8, 33, 55]. During transport, mitochondria are enclosed and secured by membranes, thus protecting them from external damage. In order to achieve successful artificial transfer, these mechanisms will need to be recreated in order to protect the mitochondria.

3. Natural Intercellular Mitochondrial Transfer

To date, several groups have reported the horizontal transfer of mitochondria in different cell types in vitro and in vivo, describing a new cellular property [7, 8, 21, 56, 57]. Most of the work about mitochondrial delivery from one cell to another deals with the rescue of damaged cells by healthy ones, such mesenchymal stem cells (hMSCs) [8, 56, 58]. Additionally, other studies have linked this transfer process to MSCs' enhanced immune response to macrophages; this is just one example of the diverse effects this mechanism has on cells involved in the transfer [33]. Recently, this process was also observed occurring between astrocytes and neurons during focal cerebral ischemia [21]. Interestingly enough, in such cases, mitochondria from the retinal ganglion cell are transferred to astrocytes of the optic nerve head to be broken down and recycled [57]. From the first description of the transfer of intracellular material between cells in 2004 by Rustom et al. [6], the work of Spees et al. in 2006 [7] to the in vivo assays performed by Islam et al. in 2012 [8] and Jackson et al. in 2016 [33], most studies show that MSCs are the best cells to transfer mitochondria. Considering the potential benefits of natural mitochondria transfer, there is great urgency to better comprehend, facilitate, and artificially replicate this process.

The transport of mitochondria from one cell to another is part of the dialogue necessary to the development and maintenance of homeostasis in multicellular organisms (Figure 1) [59]. Mitochondria can travel from one cell to another by intercellular structures such as tunneling nanotubes (TNTs) and secreted cellular bodies, such as microvesicles [5, 20, 33, 60]. In 2004, Rustom et al. described TNTs as a structure that enables cell-to-cell interaction. Since then, a number of groups have studied the cells that produce TNTs and receive mitochondria and other intracellular cargo [5, 6, 33, 61]. Other reviews in this special issue and

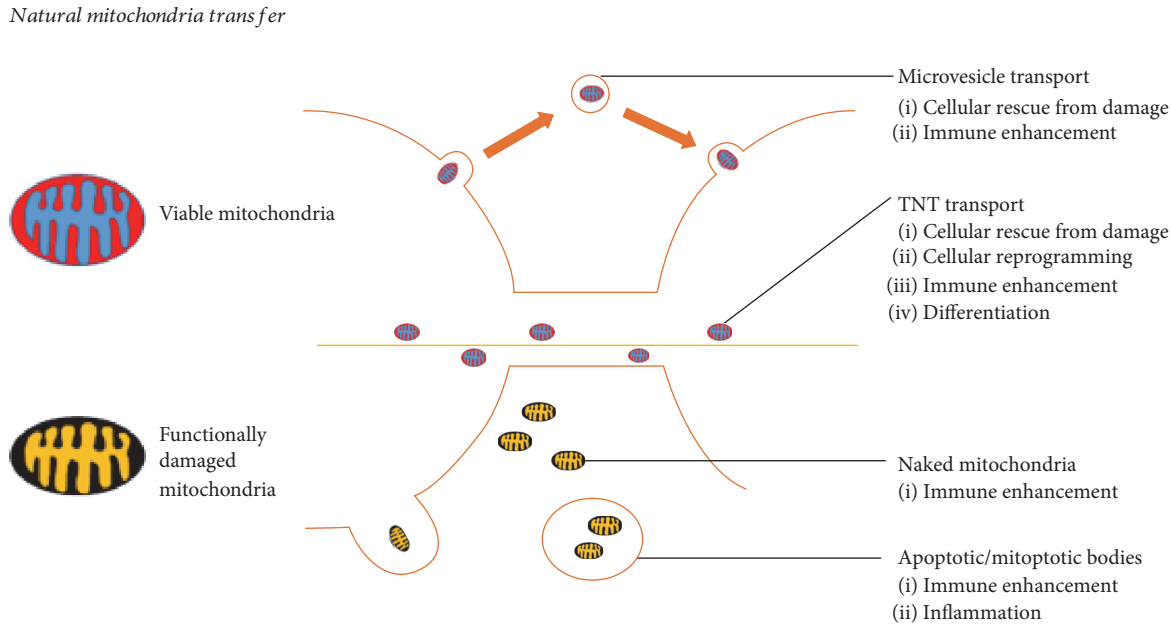

Figure 1: Natural mitochondria transfer. Viable and nonfunctional mitochondria can be shared by the cell inducing different cellular responses from cellular rescue to promoting inflammation. The first transfer mechanism is the microvesicle transport of mitochondria, it has been observed specially in MSCs in which the secreted microvesicles carrying mitochondria, once internalized by the recipient cells, induce its rescue from cellular damage and enhance the phagocytic properties of immune cells [182, 183]. The second way of transfer is by TNTs; many cells share the ability to produce them and transport mitochondria with proven effects in the rescue from cellular damage, metabolic reprogramming, and immune enhancement and it was also associated with its differentiation [65, 184]. During cellular stress, defective mitochondria can be released without being covered like in apoptotic or mitoapoptotic bodies and being naked promoting the immune response and inflammation [17, 77, 185].

recently published work recapitulate the details of TNT structure generation, characteristics, and mitochondria transfer [60, 62–64].

TNTs are produced by the outgrowth of filopodia-like cell membrane protrusions that connect with the target cell. The membrane from each cell extends to fuse together, thereby forming a tightly connected bridge which is independent from any substrate [22]. TNTs contain a skeleton mainly composed of F-actin and transport proteins like MIRO1 that facilitate the active transfer of cargo and mitochondria along these structures [58]. TNTs were first described in rat-cultured pheochromocytoma PC12 cells [6], and subsequent studies have shown that they connect a wide variety of cell types. These studies provide more evidence that TNTs are involved in mitochondrial transport between cells, the repair of cell damage, the activation of enhanced immune responses, and cell metabolic reprogramming [5, 8, 33, 61, 65].

The directionality of the transport of intracellular material and mitochondria through the TNTs is not fully understood. It is important to define what factors promote the donation of material and their effects on the recipient cells. Sun et al. observed that TNTs' growth is guided by the extracellular protein S100A4 and its putative receptor RAGE (receptor for advanced glycation end product). Stressed hippocampal neurons and astrocytes initiated the formation of TNTs after p53 activation. This signaling pathway triggered caspase 3, which decreased S100A4 in injured cells and caused cells with a high level of S100A4 to become receptor cells [66]. By these results, the authors proposed that damaged cells need to transfer cellular contents to healthy ones, in a process related to the spread of danger signals but no insights about mitochondrial participation were given. In contrast, Spees et al. in 2006 observed that MSCs transferred mitochondria to respiration-deficient cancer cells, but the direction of the transport or bidirectionality mode were difficult to determine due to the fact that the recipient cells were depleted of mitochondria, and it was not described whether the MSCs received any intracellular material from the cancer cells [7]. Koyanagi et al. in 2005 have shown that mitochondria are exclusively transported by TNTs from human endothelial progenitor cells to neonatal undifferentiated cardiomyocytes in a process intended to sustain their maturation [67]. Gao's team in 2016 used microfluidic channels while tracking TNTs' formation and exchange of material in coculture assays. Gao's team observed that MSCs were responsible of the TNT formation and of mitochondria transfer to cardiomyocytes, as opposite to fibroblasts (negative control of the interaction) [68]. Bidirectional transport of mitochondria is also plausible as it was observed between malignant mesothelioma cells. These cells produce more TNTs than normal mesothelioma cells, but interestingly, their proliferation was inversely correlated with TNT formation during their culture in low serum, hyperglycemic, acidic growth medium [69]. These represent just a few examples of the extensive literature about the exchange of intracellular material and mitochondria and its directionality. Yet, mitochondrial transport is not fully understood. For example, the field still needs to define the cell types that

produce TNTs and deliver cargo to recipient cells [68]. The determination of the directionality of and conditions necessary for mitochondria transfer between different cells is essential to understanding the potential role of this process in helping cells exposed to stress or during the transmission of danger warning signals among cells. Another important question that still remains unanswered is the reason why MSCs have a greater propensity to form TNTs compared with other cells.

Many groups of researchers that use lung disease models have corroborated that mitochondria can be transferred to other cells in vivo. Islam et al. in 2012 reported that bone marrow-derived stem cells (BMSCs) could be used to supply healthy mitochondria to alveolar epithelial cells in a mouse model of *E. coli* LPS-induced acute lung injury [8]. The delivery of mitochondria into injured cells increased ATP levels which in turn maintained cellular bioenergetics and recovered epithelium functions. A follow-up study in lung disease models (rotenone-induced lung injury and allergen-induced asthma) contributed to the understanding of the mechanisms involved in mitochondrial transfer through nanotubes, confirmed the protective effect of mitochondrial donation, and revealed a Miro1-regulated mitochondrial movement from MSC to damaged recipient epithelial lung cells [58]. All these data corroborate that mitochondrial delivery can rescue damaging cells. Furthermore, in a mouse model of *E. coli*-induced pneumonia and acute respiratory distress syndrome (ARDS), transfer of mitochondria from MSC toward innate immune cells by TNTs enhanced macrophage bacterial phagocytosis in the harmed tissue, thus improving the process of repair [33].

Extracellular vesicles (EVs) are also involved in the transport of intracellular cargo to other cells. EVs are spheroid structures surrounded by a lipid bilayer membrane [70] and are capable of transporting proteins, lipids, carbohydrates, metabolites, small RNAs [71], and mtDNA [17]. EVs are classified depending on their size and biogenesis. This classification includes exosomes (30 to 100 nm in diameter), microvesicles (100 nm to 1 μm in diameter), and apoptotic bodies (1 to 2 μm in diameter) [72]. Apoptotic bodies have been less studied due to their rapid elimination by phagocytic cells [70]. Lastly, exosomes and microvesicles are released by diverse cell types including, platelets, endothelial cells, and breast cancer cells [34]. Both mRNA and microRNA have been found in exosomes and could be transported to target cells [73]. Guescini et al. in 2010 also observed the delivery of mtDNA by exosomes. mtDNA can also be delivered via exosomes, as it was detected in glioblastoma cells and astrocytes [74]. The full understanding of the mechanisms of mitochondrial transfer by EVs and effects in receiver cells are still unclear.

It has been observed that the nervous system benefits from the transfer of mitochondria for different purposes. For example, the transfer of mitochondria allows cells to breakdown nonfunctional mitochondria and to transfer healthy mitochondria to stressed neurons. The process of mitochondrial transfer is not always meant to protect damaged cells but also to recycle these organelles in other cells in a process called transcellular degradation of mitochondria or transmitophagy [57]. The transmitophagy process is mediated by cellular evulsions containing mitochondria from neurons, in which these structures are embraced by astrocytes and then recycled [57]. The reason why transmitophagy takes place is still unknown, but it has been hypothesized that focal axon damage stimulates the process. Another theory posits that transporting damaged mitochondria back to the neuron soma is energetically disadvantageous and that there are specialized astrocytes to perform this task and clearance of unfunctional mitochondria [57]. Astrocytes are responsible for protecting and repairing damaged neurons through several mechanisms in which the transfer of mitochondria by extracellular MVs containing vascular endothelial growth factor (VEGF), fibroblast growth factor 2 (FGF-2), and mitochondria is vital to support cell recovery after stroke or cellular stress [21, 75]. Understanding transmitophagy and the natural transfer of mitochondria by microvesicles in the nervous system will allow us to find new therapeutic options in which this processes could mediate the recovery of neurons' homeostasis and function in degenerative diseases.

One of the multiple mechanisms by which MSCs exert their natural therapeutic effects is via EVs. In 2012, Lee et al. isolated the exosomes from mouse and human MSCs. Subsequently, they injected the MSC-derived exosomes into the murine model of hypoxic pulmonary hypertension (HPH) and observed the therapeutic effects of MSC action in the tissue [76]. The same study found that MSCs prompt depolarized mitochondria to move to the outer limits of the plasma membrane in response to a higher concentration of oxygen (21%). This movement is mediated by the arrestin domain with protein 1-mediated MVs larger than 100 nm. Finally, these MVs are secreted and fuse with macrophages, thus enhancing their oxygen consumption rate and most likely improving their therapeutic properties as well.

MSCs secrete exosomes with microRNAs, thus inhibiting the activation of the macrophages and repressing the TLR signaling. Phinney et al. in 2015 found an association between this response and a mechanism in which MSCs make macrophages more susceptible to acquiring exogenous vesicles and mitochondria [17]. In 2012, Cho et al. [56] replicated the assays performed by Spees in 2006 [7] in which he cultured MSCs with human osteosarcoma 143B cells, subsequently causing their mitochondria to become compromised or depleted [7]. Cho et al. observed that MSCs actively transferred healthy mitochondria by nanotubular structures to the 143B mitochondria-depleted cells [56]. Cho et al. treated the MSCs with rhodamine 6G in order to alter mitochondria activity but not mtDNA. The authors observed that fully functional mitochondria were needed to recover the loss of respiration of the 143B mitochondria free cells. In a crucial part of the experiment, they cocultured MSCs with cells carrying mtDNA mutations (A3243G mutation or 4977 bp deletion) and saw no recovery of function [56].

Cells tend to dispose of their mitochondria when they are unfit after exposure to stress conditions or when keeping them becomes harmful as mitochondria can produce large quantities of ROS [77]. Around 30 to 50% of the highly glycolytic HeLa cells were able to survive after their

mitochondria were damaged by ejecting them through selective elimination or mitoptosis [77]. During this process, the mitochondria were degraded by their inclusion in membrane vesicles and exocytosis. The presence of degraded mitochondria and especially of mtDNA in the extracellular space has been associated with a proinflammatory response and the presence of antimitochondria antibodies such as anticardiolipin and antisarcosine dehydrogenase, which are characteristic of sepsis and associated with negative patient outcomes [77].

The mtDNA and ROS released by eosinophils have been shown to provide antimicrobial protection; they also represent a key component of the innate immune response [78]. Stimulated LPS hepatocytes and mouse embryonic fibroblasts extrude mitochondrial material through autolysosomal exocytosis, thereby activating polymorphonuclear leukocytes [79]. The release of mitochondrial contents activated inflammatory responses [79]. Lastly, intact mitochondria from necroptotic cells may play a role in hazard signaling when they are ejected from cells during tumor necrosis factor alpha (TNF α) induced necroptosis. These mitochondria are engulfed by macrophages and dendritic cells, resulting in the secretion of proinflammatory cytokines by macrophages and dendritic cell maturation [80].

A handful of in vivo studies have shown that mitochondria can be released either naked or encapsulated by a membrane bilayer. Nakajima et al. in 2008 used a mouse model to confirm that naked mitochondria are released into the intercellular space after an anti-Fas antibody injection. In response to this treatment, cytoplasmic vacuoles engulfed fragmented mitochondria and extruded them from apoptotic hepatocytes [81]. Likewise, activated platelets released respiratory-competent mitochondria, both as free organelles and encapsulated within the microparticles. These extracellular mitochondria mediate inflammatory responses [82]. Elucidating the mechanisms involved in mitochondrial extrusion will lead to a better comprehension of the diseases produced by dysfunctional mitochondria and inflammatory disorders.

Apparently, cells, especially MSCs, use mitochondria as a direct reprogramming agent because the mitochondria are independent from receptors or coupled proteins to induce their effects. Cytokines, miRNAs, transcription factors, and other cell components require the activation of specific signal pathways in order to induce a response of proliferation, growth, or other in cells [83, 84]. However, we can speculate that the exogenous mitochondria, once inside the cell, start to breathe and fuse with other mitochondria. These characteristics or mechanisms make the transport of mitochondria through TNTs or vesicles important to their protection and ensure their integrity and stability. We do not know if mitochondria free in circulation or inside microvesicles have the same effects or which one could better induce proliferation, cell repair, or other [5, 8, 33, 67, 68]. Another issue is if the isolating protocol of mitochondria when applied to cells or tissues could damage its function and effects in cells [85].

The quest for the most efficient method to deliver mitochondria in vitro and in vivo remains ongoing. Based on currently available literature, it appears that achieving effective AMT will require us to preserve the integrity and effectiveness of the mitochondria by protecting them within membrane structures, such as microvesicles. Since the first description of the mitochondrial transfer from MSCs to mitochondria-depleted cells [7], numerous studies have been conducted in vivo and in vitro. The results of these studies have provided more evidence to this novel field of research. Understanding these cell properties opens a new avenue for the development of therapeutic strategies like AMT to the treatment of mitochondrial-related disorders.

4. Artificial Mitochondria Transfer (AMT)

Without a doubt, the mitochondrion is the master organelle of cell energetics, fueling multiple processes like proliferation, migration, differentiation, and stress resistance [86–89]. The transfer of mitochondria between cells through nanotubes or microvesicles stimulates these processes and also protects the recipient cells from stress-related injury. Several research teams are currently working on AMT in order to understand how to promote cellular repair in this context [5, 8, 10, 55]. Since the first formal mitochondrial transfer from one xenogeneic cell to another was completed by Clark and Shay in 1982 through coincubation [9, 19], this rapidly growing field has developed new AMT methods in order to observe its effects in recipient cell types and imagine new possible applications (Figure 2).

In 1982, Clark and Shay developed "Mitochondrial Transformation," the very first technique to transfer mitochondria from one cell to another [9]. In their model, they were able to transform around 30,000 recipient cells in just one procedure, making it a highly efficient method. They used the antibiotics chloramphenicol (CAP) and efrapeptin (EF), which inhibit the mitochondria's protein synthesis and ATPase function in order to kill sensitive mammalian cells. Cells resistant to CAP have mutations in their mtDNA located in one region of the mitochondrial large subunit rRNA gene [9]. They observed that the transfer of mitochondria from CAP and EF-resistant fibroblasts increased the survival of the recipient cells, which were sensitive to these antibiotics. Interestingly, they observed that when the mitochondria of sensitive cells are transferred to new cells, they did not confer resistance to the recipient cells. This provides evidence that a higher concentration of mitochondria in and of itself is not sufficient to protect cells from CAP or EF; rather, mitochondria can only survive by having the genes for antibiotic resistance. It was also apparent that mitochondria from murine fibroblasts which were resistant to CAP and EF did not increase the survival of sensitive human cells. This indicates that mixing endogenous and transferred mitochondria across different species could potentially be restricted. A crucial observation of this article is that the failure of AMT into murine cells by simple coincubation suggests that this process is not equally efficient among different cell types and that some cells may be more receptive than others [9]. Clark and Shay's observations and questions regarding the mechanism of mitochondrial transfer have opened up the path for further advances in the field.

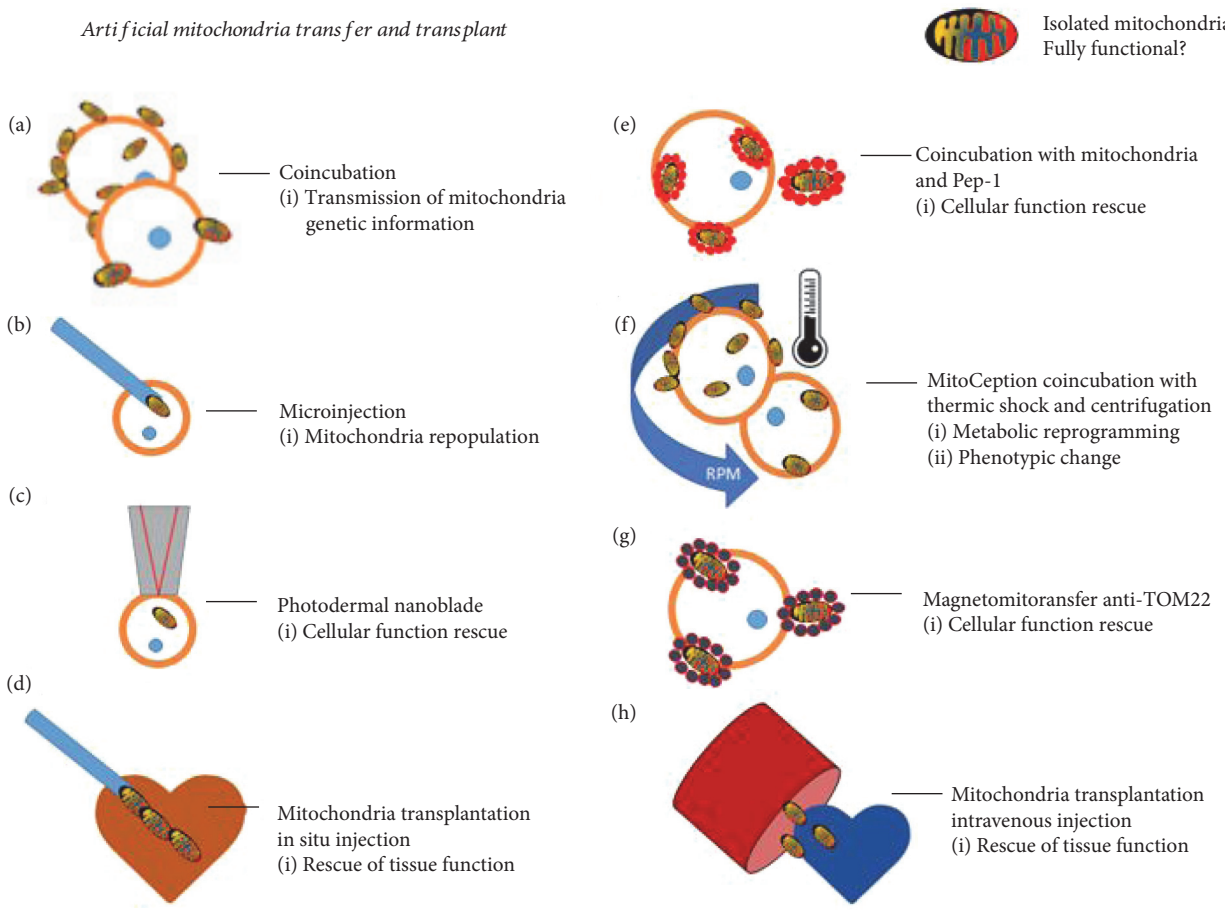

Figure 2: Artificial mitochondria transfer (AMT) and transplant. Different techniques emerged to mimic the natural transfer or mitochondria on its in vivo and in vitro applications. The coincubation technique was the first proposed in which the antibiotic resistance carried in the mitochondrial DNA was passed to sensitive cells [9], later after the technique was used to rescue respiratory deficient cells among other damaged cells [10, 11, 14, 63]. Microinjection of exogenous mitochondria was applied in assays to eliminate the endogenous copies of oocytes carrying mitochondrial diseases [90, 93]. The photothermal nanoblade effectively transferred isolated mitochondria inside the cell; even if they showed great effectiveness, its application is limited to small cell numbers [12]. Two different approaches were developed to facilitate the mitochondria internalization in the recipient cells, the first is by using Pep-1 and the other with magnetic beads (Magnetomitotransfer) designed to bind to TOM22 a receptor complex in the mitochondrial membrane. The MitoCeption technique uses a thermic shock and a centrifugation to improve the process of mitochondria uptake; first applied in cancer cells, this technique induces the metabolic reprogramming of these cells. The in vivo application of the mitochondria transfer applies two approaches: the first is to directly inject mitochondria to the harmed tissue and the other in the circulatory system close to the area of interest. Both of them have shown to restore tissue function but the in situ injection showed better results [16, 49, 50].

In 1988, King and Attardi then developed the first AMT technique using invasive instruments; they injected exogenous mitochondria isolated from CAP resistant cells into sensitive human cells [90]. Their method was less efficient than Clark and Shay's coincubation protocol because the technique limited the number of cells that could be transformed in each procedure and caused harm to the recipient cell. However, this study demonstrated that the injection of just one mitochondrion could very quickly repopulate a cell depleted of its endogenous mitochondria in just six to ten weeks. Additional techniques to perform AMT involving nanoblades and other invasive instruments have been developed, but all of them are less efficient than coincubation [12, 91].

Mitochondria carrying genetic mutations can cause diseases that can be transmitted to offspring through the oocyte [92]. To prevent and treat such diseases, experiments have utilized a variety of different AMT approaches, from microinjecting healthy mitochondria into oocytes [93] to transferring the nucleus of an unfertilized, mutation-carrying oocyte to a healthy enucleated ovule. Using King and Attardi's AMT technique, Pinkert et al. used microinjection to transfer mitochondria isolated from the livers of *Mus spretus* to fertilized oocytes taken from *Mus musculus* [93]. After 4.5 days in culture, Pinkert et al. detected xenogeneic mitochondria DNA sequences in the recipient cells, thereby demonstrating that xenogeneic mitochondria from closely related species are able to survive in recipient oocytes for at least a limited time

[93]. In 2007, Yi's team observed that zygotes that had received mitochondria transferred from the livers of young mice developed better through the blastocyst stage, as compared to old zygotes that did not undergo AMT [94]. In 2010, Takeda et al. used the mitochondria from bovine fibroblasts cultured in 10% and 0.5% serum [95]. Interestingly, the oocytes that received mitochondria isolated from cells at 0.5% serum showed a lower rate of development [95]. Recently, the transfer of mitochondria by microinjection has been replaced by enucleating the ovule of a healthy donor and adding the zygote nuclear material from a carrier of mitochondrial mutations in a pronuclear state and in metaphase II [96]. Although these techniques have been successful [97], they are still ethically controversial due to the amount of germ cells sacrificed in the procedure; these issues will be discussed in a later section of this review.

Clark and Shay's assay [9, 14] coincubates isolated mitochondria with the recipient cell, a technique which can be easily applied to many different types of cells. This procedure provides an opportunity to study the behavior and effects of artificially transferred exogenous mitochondria inside recipient cells. Despite other successes using this technique, in 2005, AMT unexpectedly failed when Spees et al. coincubated mitochondria isolated from hMSCs with human lung carcinoma recipient cells (A549) [7]. Before the procedure, A549 cells were pretreated with ethidium bromide in order to deplete their mtDNA and to make the cells unable to perform aerobic respiration, just as King and Attardi had done before [90]. When Prockop's group cocultured the depleted A549 cells with hMSCs, they observed the natural transfer of mitochondria between them. This transfer appeared to rescue respiration of the dysfunctional A549 cells. After their coincubation assay failed, they hypothesized that the transfer of mitochondria is mediated by active mechanisms such as the formation of nanotubular structures like TNTs or vesicles which transport these organelles to the interior of recipient cells [6]. Unknown details about temperature changes during the mitochondria isolation may have been instrumental to understanding the lack of passive transfer. Two years later, in a xenogeneic model, Weissig successfully transferred isolated mitochondria from mouse livers into human cancer cells, MDA-MB-231 and MCF-7. Katrangi et al. tested this technique in four models, using each type of cancer cell with and without mitochondria depletion by ethidium bromide [13]. The success of each of these models can be attributed to the methodology they used to isolate the mitochondria. The key of their methodology was maintaining the mitochondria at 4°C at all times in order to preserve their structure and function. They also maintained the cells in normal culture medium with uridine and pyruvate. The success of this protocol provides insights into the specific conditions that recipient cells may need in order to successfully internalize exogenous mitochondria and to prevent changes in cellular function and metabolism due to exposure to temperature variability. Another possibility is that some cells may be more receptive to accepting mitochondria. For instance, cancer cell lines such as MDA-MB-231 naturally incorporate more material from their surroundings in a process described as entosis or cell cannibalism [98].

Supporting Weissig's work (2007), the same year Yoon et al. observed that mitochondria from different species have the ability to fuse together. Their study did not use the mitochondria transfer technique, but instead they fused the cells with polyethylene glycol (PEG). They also labeled human and mice mitochondria differently (mtGFP and mtDsRed, resp.) and observed a mix of the two types of mitochondria 45 min after adding the PGE and fusion of the mitochondria of all hybrids at 4 h. The fusion of both human and mice mitochondria apparently occurs because of the homology of the sequence between the proteins responsible for this process, the mitofusins proteins (Mfn1 and Mfn2). Mfn1 and Mfn2 share a 90.7% and 94.8% homology between humans and mice. The formation of the mitofusin homodimers between the membranes of both types of mitochondria initiates the tethering and the fusion of the inner membrane [99]. However, even when mitochondria from both species fused, Yoon et al. were not able to create long-term cybrids from the mouse-human fusion, although they were able to achieve it from mouse-mouse fusions. This can be explained by the accumulation of differences between species, especially within the nuclear-coded mitochondrial genes and the dialogue between mitochondria and nuclei; it appears that long-term crosstalk between the nucleus and the xenomitochondria cannot be established [99, 100]. Yoon's work on the compatibility of xenomitochondria with human cells demonstrates that successful mitochondrial transfer may only be possible between cells and/or tissues of the same species.

In 2012, Elliott et al. coincubated mitochondria isolated from immortalized breast epithelial cells with their malignant counterparts MCF7, MDA-MB-231, and ADR-Res. They observed a decrease in their proliferative potential and a higher sensitivity to chemotherapeutic drugs such as doxorubicin, abraxane, and carboplatin [101]. Interestingly, they observed that only isolated mitochondria from the immortalized breast epithelial cells were able to enter the breast cancer cells, but not the original immortalized breast epithelial cells [101]. It was not further discussed in the article whether the characteristics of the immortalized cells' mitochondria are different from those of normal epithelial cells or if such differences could affect the process of their integration into the immortalized and cancer cells. To sustain the transfer of isolated mitochondria into recipient cells by coincubation, Kitani et al. demonstrated that this process can be performed autogeneically and xenogeneically. They documented the results of the transfer through real-time PCR and fluorescent imaging [11]. Nevertheless, although they obtained functional cells with integrated xenomitochondria, Yoon was unable to show the permanence of the exogenous mtDNA for longer than two weeks [99].

Assays to optimize transfers involving the use of chemical compounds and physical methods have been performed since 1988. In 2013, Liu and colleagues conjugated isolated mitochondria with penetrating peptides to foster their internalization. They used Pep-1, a cell-penetrating peptide that was originally developed to induce pores in the membrane to facilitate the delivery of molecules like oligonucleotides into the cell. The authors adapted Pep-1 to conjugate it with isolated mitochondria of human osteosarcoma 143B cells

[15]. The mix of Pep-1 and the isolated mitochondria promoted their internalization by fibroblasts involved in a model of the mitochondrial disease myoclonic epilepsy with ragged red fiber (MERRF) syndrome. They observed that the Pep-1-mediated transfer was more successful in facilitating the internalization of mitochondria than mitochondria alone [102]. Unfortunately, the authors did not describe the efficacy of or the rationale behind conjugating Pep-1 and mitochondria. They also did not describe the unexpected lack of internalization of the exogenous mitochondria not conjugated with Pep-1. Interestingly, in 2016, Liu's team used the Pep-1-conjugated allogeneic and xenogeneic mitochondria in an in vivo assay of a Parkinson disease model (PDM) [103]. They observed an increase of neuron survival and movement recovery in the animals of the experimental group as compared with the control. Yet, questions arise from Lui's technique, such as whether the Pep-1 peptide acts as a protective agent from environmental damage or whether it acts as an internalizing agent that facilitates the transfer of mitochondria to the affected tissues. Finally, defining the optimal quantity of the mitochondria administered to the PDM in rats will be important in studying the possibility of applying this technique to treat neurodegenerative disorders or any other disease.

In 2015, our group standardized the transfer mechanism of isolated mitochondria to cultured cells (MDA-MB-231, human breast cancer cells), adding two extra steps to the coincubation procedure: centrifugation and a thermic shock. We named the protocol *MitoCeption*. Our technique allowed the constant and reproducible increase of mitochondrial uptake by the recipient cells proportionally to the material added. Following these observations, we could prove an equivalent increase of respiration and ATP production in accordance with the supplementation of mitochondria. We witnessed a functional change in the mitocepted cancer cells: their proliferative and invasive potential increased. Interestingly, we also observed that a cancer cell cannot constantly incorporate mitochondria without harming their functional properties; the proliferation and invasive capacities of the cells diminished after increasing their mitochondria concentrations. Our work showed that it is useless to constantly improve the mitochondria transfer mechanism if there is a functional threshold of the internalized mitochondria. It is clear that this technique cannot be used for the in vivo transfer of mitochondria to organs or tissues, but cells can be mitocepted before being introduced into in a living organism with the purpose of reprogramming or repairing its metabolism and function [10].

The transfer of mitochondria by coincubation seems to depend on the viability and metabolic activity of the donor mitochondria and particularly on the integrity of the outer membrane, as this is the first structure organelle to interact with the receiver cell. Kesner et al. in 2016 further studied the coincubation transfer mechanism using isolated mitochondria from Hela cells and transferring them to other cancerous cell lines, healthy fibroblasts, and cells carrying mitochondrial mutations. They noted that the uptake was fast, with recipient cells uptaking the exogenous mitochondria just 10 minutes after the coincubation began. Kesner et al. also established that the mitochondrial transfer is principally mediated by macropinocytosis [14], which is a regulated form of endocytosis mainly involved in the uptake of molecules, nutrients, and other materials from the extracellular space [104, 105]. Furthermore, they found that perturbing the outer membrane of mitochondria with digitonin or other mitochondria-damaging molecules inhibits the uptake process. Their key observations provided insights about how mitochondria integrity is important to the success of the transferring process. They also importantly noted that membrane characteristics of the recipient cells may play a major role in the macropinocytosis of mitochondria.

Wu et al. developed a photothermal nanoblade to deliver cargo including mitochondria to the interior of mammalian cells, bypassing cell fusion and endocytosis [12]. Progressive resistance BTK-143 osteosarcoma and MDA-MB-453 p0 cells lacking mtDNA were treated with the photodermal nanoblade in order to deliver HEK293T-expressing mitochondria labeled with DsRed. The goal of this procedure was to rescue the metabolic function of the receiving cells. This technique effectively transferred exogenous mitochondria, but due to the technical expertise and equipment required to carry out this procedure, the results obtained in the experiment are difficult to replicate. Furthermore, as the authors mention, the transfer of mitochondria by the photothermal nanoblade is low throughput, meaning that the technique must be adapted in order to achieve the same efficiency as coincubation or MitoCeption techniques. Later the same year, Macheiner and colleagues [91] developed the use of anti-TOM22 magnetic beads to improve the purity of mitochondria isolates. They used a magnet to transfer the mitochondria coupled with the magnetic beads into host cells, naming this technique Magnetomitotransfer [91]. TOM22 is a multisubunit translocase embedded in the outer membrane of the mitochondria [106]. Coupling the mitochondria with anti-TOM22 beads increased the quantity of viable mitochondria able to be transferred. However, because the beads can also bind with nonfunctional mitochondria fragments, they may also inadvertently transfer them into recipient cells. According to the authors, a greater ratio of transfer was achieved using this technique after one to three days of culture as compared to passive transfer. By the same token, Kesner had already observed that the process of internalization of mitochondria via coincubation can occur in as little as 10 minutes [14]. This observation could put into question the need of the magnetic beads to accelerate the process. The authors also did not show whether the fusion of exogenous and endogenous mitochondria is affected in some way by the anti-TOM22 beads. Fusion is an essential process for the exchange of mtDNA between mitochondria; therefore, this method may not actually be effective if fusion is not facilitated by the anti-TOM22 beads. As they mentioned, further studies are needed to learn about the toxicity and changes in cell physiology after magnetic mitochondrial transfer, especially those associated with respiration and metabolic reprogramming. Both the photothermal nanoblades and Magnetomitotransfer are still limited in terms of the number of cells that they can reach, the damage they may cause, and the greater technical challenges involved in executing each procedure [10].

In 2009, McCully et al. proved that mitochondria can be used in vivo to repair damaged tissues [16]. Because ischemic damage affects the mitochondria in tissues, these authors hypothesized that the replacement of affected mitochondria with healthy ones would significantly improve the postischemic recovery. They induced ischemia in the heart of rabbits by occluding the left coronary artery using the Langendorff perfusion allowing to test the contractile strength and heart rate. Then, they injected either a vehicle, vehicle with mitochondria, or mitochondria alone which had been thawed after an overnight period at −20°C in the presence of the vehicle. These were injected directly into the ischemic zone just before reperfusion. Interestingly, they observed that the infarcted area was reduced and the functional recovery increased after injecting mitochondria combined with the vehicle. This was not observed in the tissue only injected with mitochondria isolates, meaning that mitochondria must be active in order to serve therapeutic functions, as described in a previous work in vitro [13]. The authors used healthy heart tissue from rabbits as a supply of mitochondria, which limited the impact of the study. This strategy has many translational limitations. The use of other sources, like unharmed tissue from the same donor or other nonvital tissue from other rabbits, would have provided greater evidence for further applications.

Later in 2013, Masuzawa et al. added further assays to sustain the efficacy of the transfer of mitochondria in the ischemic heart model in rabbits [49]. This time, they isolated mitochondria from the pectoral muscle of the same rabbit used for the ischemic shock (autologous transfer). After a follow-up of 28 days, the authors observed that the autologous transplantation of mitochondria was not proarrhythmic: infarct marker levels decreased and the generation of precursor metabolites for energy and cellular respiration increased. Interestingly, they also found that mitochondria were internalized by the cardiomyocytes 2 hours after transplantation, with cardioprotective effects after 28 days. The proposal of a mechanism of mitochondria transfer and their internalization in vitro based on macropinocytosis by Kitani et al. and Kesner et al. [11, 14] was not conclusive. In their latest contribution, McCully's team (2015) observed that the transfer of mitochondria and their in vivo internalization was mediated by actin-dependent endocytosis and not by macropinocytosis. The authors used different inhibitors to prevent the internalization of mitochondria. They used cytochalasin D to inhibit actin polymerization, methyl-β-cyclodextrin (MβCD) to stop endocytosis, and nocadozole to block tunneling nanotubes. They observed that the use of (MβCD) greatly inhibited the uptake process, inferring that internalization is mainly mediated by actin-dependent endocytosis [50]. Further assays need to be developed in vivo to fully understand the process of internalization related to AMT, possible heterogeneity across different tissues, and the effects of the transfer of mitochondria to harmed tissue. Such assays may also help to illuminate ways to improve AMT and address its limitations.

After McCully's experiments in 2013, Lin et al. applied the same procedure to replace damaged mitochondria with healthy ones to mitigate symptoms in the rat model of hepatic ischemia-reperfusion [107]. Both McCully and I-Rue Lai observed that the introduction of mitochondria to diseased ischemic tissue decreased damage and oxidative stress and improved recovery. Despite this success, these experiments were not able to make any conclusions about three factors which may be key to the success of mitochondria therapies: extract concentration, mitochondria viability, and organ-to-mitochondria ratio. With respect to the first factor, neither experiment demonstrated a dose response related to the concentration of isolated mitochondria introduced to the damaged tissue. McCully used $9.7 \times 10^6 \pm 1.5 \times 10^6$/ml of mitochondria isolated from healthy hearts in injections of 0.1 ml, eight times into the affected zone of the ischemic hearts [49]. In his study, I-Rue Lai used a concentration of $7.7 \times 10^6 \pm 1.5 \times 10^6$/ml of mitochondria isolated from healthy livers in one injection of 0.1 ml into the subcapsular region of the spleen poles. Although each concentration yielded therapeutic benefits, neither was established as the optimal injection concentration. Additionally, each study verified the viability of the isolated mitochondria before injecting them into the given tissue using fluorescent probes dependent on membrane potential, including CMTMRos [107], JC1, and respirometry [49]. However, it remains unclear how the mitochondria's state at the moment of injection affects the success of the therapy. We cannot assume that the mitochondria's optimal injection state is simply indicated by activity, because activity is not related to the coupling of the electron transport chain or ROS production, which can damage the mitochondria and the cell [108]. Additionally, these studies did not consider the impact of organ-to-mitochondria ratio, although this factor may have important implications in the successes of mitochondria therapy. McCully and I-Rue Lai's studies are highly important because they demonstrate that AMT in vivo can have therapeutic benefits to the damaged renal and cardiac tissues; perhaps even more impactfully, they have also opened new lines of investigation to help us understand how to optimize these procedures for clinical applications.

In 2014, Sun et al. transferred mitochondria to the damaged lung tissue of adult male rats affected by acute respiratory distress syndrome (ARDS) [109]. In this experiment, Hon-Yap Yip and colleagues transferred mitochondria, melatonin, and mitochondria in combination with melatonin to diseased lung tissue. Melatonin was used because it is a known anti-inflammatory molecule which is protective against lung injury disease [110]. Islam et al.'s previous experiment in which they observed the transfer of mitochondria from bone marrow-derived stromal cells (BMSCs) to harmed lung tissue served as the foundation for Hon-Yap Yip's work [8]. Islam et al. demonstrated that BMSC mitochondria transferred to the affected alveolar epithelia resulted in an increase of cell bioenergetics and had additional protective effects [8]. Taking into consideration the work of Islam et al. [8], Masuzawa et al. [49], and Lin et al. [107], Sun et al. [109] completed onetime intravenous injections of two different mitochondria concentrations (not coupled with melatonin) 750 μg and 1500 μg diluted in IBc buffer in rats 6 hours after inducing ARDS. They did not specify the total volume they injected. In other assays, the authors injected mitochondria in combination with melatonin and melatonin

alone. In both cases, the melatonin was injected in a concentration of 50 mg/kg, 6 and 24 hours after ARDS was induced. Sun et al. [109] observed that the treatment with just mitochondria and mitochondria plus melatonin decreased DNA damage, ROS generation, apoptosis, and the quantity of albumin in bronchoalveolar lavage (BAL), an indicator of capillary leakage in proteins and proinflammatory cytokines such as MMP-9, TNF-α, and NF-κB, [109]. The fact that this study requires a great quantity of mitochondria makes it less likely that it could be successfully applied in larger organisms. However, this transfer method could be better used in localized injections, as put into practice by McCully et al. in their studies [16, 49].

Huang et al. transferred mitochondria isolated from the kidney of young hamsters to rats that had suffered a cerebral stroke induced by middle cerebral artery occlusion (MCAO) [19, 109]. The authors discovered that the administration of the xenogenic mitochondria had protective effects and were associated with a faster recovery of motor performance in the rats. They injected 75 μg of mitochondria diluted in 10 μl of SEH solution (0.25 M sucrose, 0.5 mM ethylene glycol tetraacetic acid (EGTA), and 3 mM N-(2-hydroxyethyl) piperazine-N¢-ethanesulfonic acid (HEPES), pH 7.2) into the ischemic stratum and infused 750 μg in 100 μl into the femoral artery. Interestingly, the authors showed that the direct in situ injection of the isolated mitochondria were more effective in rescuing motor activity than the mitochondria injected through the artery. They also showed that the exogenous mitochondria have a low percentage of internalization in the harmed neural cells, but even so, the low internalization rate seems to be sufficient to exert their protective properties.

The application of the AMT in vivo should be further developed because all investigations to date have only used one or two mitochondria doses [16, 19, 107, 109]; the similarity of this aspect of research protocols limits our understanding of the effects and therapeutic applications of mitochondria. Hon-Yap Yip [109] injected isolated mitochondria intravenously and Hong Lin-Sun [19] injected mitochondria intra-arterially; they compared the limitations of the therapeutic effects of these methods with those of the in situ infusion. It will be important to further study if the regenerative properties of mitochondria injected into the circulatory system are comparable with direct infusion into the damaged site. Some possible limitations of the systemic infusion may include the loss of integrity of the mitochondrial membrane, the delay in arriving to the damaged site, and the possibility that others cell in the circulatory system endocytose the mitochondria, thus restricting the quantity of mitochondria that ultimately arrive to the harmed tissue.

Two trends in the study and application of the AMT between cells have emerged in the last 30 years. In 1982, Clark and Shay created the mitochondrial transformation technique, based on the simple coincubation of isolated mitochondria and cultured cells [9]. In 2009, McCully et al. innovated the direct in vivo approach [16]. These techniques established important questions to guide the future development of AMT. For example, they revealed that mitochondrial integration may not occur equally between cells and that it may be possible for cells with different membrane properties or tissue organization to be similarly transformed. Additionally, they brought into question how isolation techniques affect the mitochondria's functioning and integrity, and if these changes could influence the transfer itself. Lastly, they raised questions about how the genetic patrimony of donor mitochondria could influence the effects of the transferred mitochondria in the host [9]. These questions have been addressed by authors like Spees et al. [7], Katrangi et al. [13], Kitani et al. [11], Kesner et al. [14], Wu et al. [12], and Caicedo et al. [10]; however, work is still necessary in order to be able to apply AMT in clinical settings.

Many relevant questions in the field remain to be answered. For example, it is still unknown how mitochondria from different cells are able to improve or decrease cellular processes. Similarly, the field must also determine whether transferred mitochondria are able to fuse with endogenous ones and communicate correctly with the nucleus, which is essential to their long-term effectiveness. Caicedo et al. previously observed that the transfer of mitochondria from MSCs to cancer cells by MitoCeption in concentrations higher than 1.25 μg (measured in protein) was deleterious for cell proliferation and that even higher concentrations (2.5 μg>) were restrictive for invasion [10]. Recently, Kitani et al. [11] and Kesner et al. [14] observed that the coincubation of isolated mitochondria and cultured cells was sufficient to prompt the cells to internalize the mitochondria, thereby resulting in the repair of cellular function. However, more research is necessary to understand if additional procedures like thermic shock, centrifugation [10], cell penetration by peptides [15] with mitochondria-conjugated beads [91], or introduction by nanoblades [12] can optimize the AMT and induce the desired effects in the recipient cells.

To develop AMT procedures that are easily replicable and effective, it is important to define a mitochondria-isolating procedure standard that allows scientists to obtain pure mitochondria and analyze the effects of transfer. The isolation of mitochondria is based on the conditions and speed of centrifugation, the concentration of the sucrose solution, and some other factors which, when managed incorrectly, can contaminate the mitochondria concentrate and therefore negatively impact the therapeutic effectiveness of the AMT procedure. Contamination can happen because of the structural closeness and functional connections of the mitochondria with other organelles [85]. For example, mitochondria closely interact with the endoplasmic reticulum (ER), together forming the mitochondria-associated membranes (MAMs) which play a crucial role in calcium homeostasis, regulation of lipid metabolism, and autophagy [111, 112]. Due to the tight contact between the MAMs, contamination with ER may be common in most AMT protocols. Similarly, in other cell structures, mitochondria isolation may be contaminated with other organelles with which the mitochondria interact, including the nucleus, lysosomes, and peroxisomes [111, 113–116]. Clark and Shay [9], Katrangi et al. [13], Kitani et al. [11], Kesner et al. [14], Sun et al. [109], and Huang et al. [19] used the classic sucrose gradient with differential centrifugation, and then, Elliot et al.

[101], Chang et al. [102], Macheiner et al. [91], and Lin et al. [107] used experimental kits to guarantee the purity and viability of mitochondria for downstream applications. McCully et al. did not clearly define the type of protocol used for mitochondria isolation for his in vivo procedures [16, 49, 50], but in his latest review [117], he suggested two protocols by Gostimskaya and Galkin [118] and Claude [119] which can rapidly isolate mitochondria. In 2006, Spees et al. briefly mentioned a mitochondria isolation protocol that involves differential centrifugation; however, the article may not have mentioned important details of this protocol because the experiment did not yield any successful internalization of mitochondria in incubation with the recipient cells [7]. It is possible that this protocol unexpectedly failed because of contamination with MAMs, which may influence the internalization of isolated mitochondria, although no work to date has addressed this potential problem.

Mitochondrial tissue specificity or differentiation state should be taken into account when choosing the donor mitochondria. Mitochondria differ in their shape, size, energy production, and metabolic processes among cell types and states of differentiation [120, 121]. During cell proliferation, the mitochondria modifies its dynamics, segregates from others, and fuses with other mitochondria in a process mediated by the expression of Mfn1 and Mfn2 or dynamin-related protein 1 (DRP1) in the OM [122]. Interestingly, the growth factor erv1-like (Gfer) plays an important role in regulating DRP1 in embryonic stem cells (ESCs). When Gfer is not present, DRP1 is highly expressed driving mitochondrial network fragmentation and a decrease of pluripotency in ESCs [123, 124]. Isolating mitochondria from a cell in a specific state like proliferation could prime the mitochondria and influence their impact in recipient cells' mitochondria networks or even their capacity to fuse with endogenous mitochondria. Most of the studies regarding the transfer procedure were performed using mitochondria from differentiated cells like fibroblasts, liver cells, MSCs, and others [7, 9, 13, 90, 93]. Testing different cell states will be important to understand how exogenous mitochondria interact with the endogenous organelles, how the cell's phenotype changes after transfer, and if metabolic reprogramming is possible.

AMT has shown promising results in healing damaged or stressed cells in vitro and in vivo. Understanding their mechanisms of action inside the cell will allow us to explore and mix AMT with other techniques to repair dysfunctional mitochondria. The use of AMT could potentially repair endogenous and damaged mitochondria by introducing healthy copies to recipient cells and inducing a state of heteroplasmy. In heteroplasmy, altered or pathogenic mtDNA exists together with healthy or wild-type mtDNA [125]. A cell eliminates damaged mitochondria carrying altered mtDNA by mitophagy, a key process in maintaining the mitochondria pool quality [126]. Mitochondria pass through fission in which unhealthy copies carrying altered mtDNA, those with low membrane potential, and those with excessive ROS are eliminated mainly by the PTEN-induced kinase 1 (PINK1) and Parkin pathway [127]. In recent years, the field has gained interest in mitophagy because of its implications in maintaining cell viability and stemness [125, 126, 128–130]. The inhibition of rapamycin (mTOR) kinase activity activates mitophagy, thereby enhancing the selection against dysfunctional mitochondria. However, but healthy mitochondria must also be present to induce this process [126]. Using mitophagy inducers could improve mitochondrial function in mitochondrial diseases or other conditions in which the mitochondria metabolism may be altered [131]. Understanding how AMT may induce heteroplasmy in cells carrying mitochondria mutations and encourage the clearance of unhealthy mitochondria copies in order to support the quality control of mitochondria by mitophagy may reveal new therapeutic possibilities [126].

More studies are needed to understand the possible applications and challenges of AMT. Solid preclinical assays must be designed in order to select the best mitochondria donor cells to treat a specific disease. With this approach, tuning the delivery methods of AMT will further facilitate the reconceptualization of mitochondria not only as the powerhouse of the cell but also as active therapeutic agents.

5. Mitochondrial Diseases and Their Potential Treatment by AMT

The pathophysiology of mitochondrial diseases is complex considering that they can be caused by mitochondrial or nuclear genes involved in the correct biogenesis and function of this organelle. At present, medical approaches for the treatment of mitochondrial disease are only palliative. For this reason, mitochondrial transfer techniques could potentially play a curative role in the care of individuals at risk for or those already suffering from mitochondrial disease caused by mutations in their DNA. Primary mitochondria diseases (PMDs) are caused by mitochondrial mutations that are inherited and transmitted by one's maternal lineage. The transmission of these mutations to successive generations causes most of the known mitochondrial disorders such as Leigh syndrome. This syndrome can affect mtDNA and nuclear DNA (nuDNA), which are considered mitochondria-associated genes [132]. Mutations in the mtDNA can occur during life, and the expression of the disease depends on the quantity of mitochondria that are damaged in comparison with healthy copies in the cell. These diseases are secondary mitochondria diseases [47]. Once an individual's cells cross a certain threshold of damaged mitochondria, the disease will manifest [133]. It is in these cases that the artificial transfer of healthy mitochondria to damaged cells or tissue may help to treat the disease.

Although usually considered rare, mitochondrial diseases appear to be more common than ever thought. Recent work establishes population frequencies of at least 1 : 5000 [134, 135]. The first links between mtDNA alterations and mitochondrial disease were established in the year 1988 [136–138], and today, more than 260 disease-causing mutations and 120 mitochondrial genome rearrangements have been classified [139]. Furthermore, carriers of asymptomatic mtDNA mutations are estimated to be 1 : 200 [134], a frequency 25 times higher than that of people actually suffering from mitochondrial disease. Because healthy carriers of genes that cause mitochondrial

diseases likely do not know that they have these mutations, there is an increased risk of them passing on their potentially deleterious genes to their children.

Mitochondrial disorders are extremely difficult to diagnose because they appear with signs and symptoms common in many other non-mitochondrial-related diseases [140]. Therefore, they should be considered as syndromes. Clinical manifestations may affect any system in the body, but since mitochondria act as power stations, the most affected tissues or organs are those that manage and consume a great deal of energy. Accordingly, most common mitochondrial disorders affect the nervous system (including the sensory organs) and the musculoskeletal system. Because the reproductive system also has organs that require a lot of energy, it can also be affected by mitochondrial disease. However, such diseases have not received as much research attention as mitochondrial diseases of other systems, perhaps because they are not life-threatening. The severity of mitochondrial diseases and their negative impact on patients' quality of life emphasize the need for innovative management of these disorders.

With new therapeutic techniques, the manipulation of mitochondrial biology can allow mothers carrying mutations in their mtDNA to have healthy offspring. Used for the first time in humans in 2016, this set of technique is called mitochondria replacement (MRTs) and gives rise to "three-parent babies" [97, 141]. MRT techniques eliminate the majority of mutant mitochondria in the female germ line during very early developmental periods before the baby is even born, thereby preventing mitochondria-related morbidity entirely. One method, pronuclear transfer, transplants the nucleus from a zygote carrying mutant mitochondria to a donor-derived enucleated oocyte with healthy mitochondria [96]. The offspring receives its nuclear genome from the sperm and the nucleus from the original oocyte, which are transplanted into the donor-derived enucleated oocyte which contains cytoplasm and healthy mitochondria. Despite a few successful applications of MRT, these highly invasive techniques can cause difficulties in fertilization because of the high levels of manipulation involved. For example, maternal spindle transfer (MST) transplants the microtubular spindle system with all chromosomes attached (before pronucleus formation) into a healthy oocyte that has had its own spindle fibers removed during the same developmental stage; this technique has proven to be very effective in monkeys [142] but fertilization issues have been observed in some human spindle-transferred oocytes [143]. This technique requires that the embryo be pierced with micropipettes, which not only ruptures the cell membrane, but probably also disrupts cytoplasmic organelles and the cytoskeleton. This invasive procedure is required in all MRT techniques involving nuclear material transfer [143, 144]. Moreover, oocytes must undergo additional manipulation during the necessary ablation of the *zona pellucida* [142, 144]. During spindle transfer, several chemicals including the cancer drugs cytochalasin B and nocodazole are also applied to the cells involved, possibly damaging genetic material in the cells [142, 144]. Additionally, the use of MRTs is ethically controversial because embryos have to be destroyed during the procedure [145]. AMT offers a less invasive alternative to allow mothers with mitochondrial mutations to have healthy children, especially in cases in which there are only palliative treatments available for the given mitochondrial disease [146].

In the future, AMT could be used in conjunction with induced pluripotent stem cells (iPSCs) to model mitochondrial diseases or to generate healthy cells to be reintroduced into the patient. It is thought that diseased somatic cells could be used to generate iPSCs cells, which would then be forced to differentiate into lineage restricted adult stem cells of the specific diseased tissues. Once obtained, these cells would be inoculated with healthy mitochondria by AMT and then injected into the diseased tissue. This combinatory technique may have applications in diseases like mitochondrial retinopathy, which currently has no known curative treatments [147, 148]. It may be possible to combine AMT with currently available techniques used to derive specific retinal progenitors in order to introduce healthy mitochondria copies into diseased cells, thereby repairing the damaged mitochondrial pool and curing the disease [149].

Patients with skeletal muscular syndromes caused by mtDNA damage may also benefit from AMT. One therapeutic technique that may be able to treat such diseases involves the systemic injection of mitochondria to allow them to arrive to the target tissue. However, because the injection could become diluted in the circulatory system, a sufficient number of mitochondria probably would not reach the muscle tissue in order to heal it. The Magnetomitotransfer could offer an alternative to better guide mitochondria into the target tissue by using magnets [91]. Yet another option may be to inject a greater concentration of healthy mitochondria into small local arteries feeding specific muscles or directly into the muscle mass. This approach has been attempted in the heart, a muscle which suffers from mitochondrial damage after ischemic episodes during myocardial infarction [16, 117]. McCully et al. directly injected mitochondria into the infarcted areas and subsequently discovered that the procedure was beneficial to the recovery of the cardiac tissue [16].

The theoretical AMT procedures outlined here may have great therapeutic potential in a range of applications, from curing mitochondrial retinopathies to treating muscular skeletal syndromes [150]. However, in order to unlock the therapeutic potential of AMT, we must address a number of ethical concerns and technical challenges to safely use this techniques in humans. Significant preclinical experimentation must be performed before adapting AMT for clinical trials.

6. Ethical Issues

The UK has become the first country in the world to formally approve the use of mitochondrial donation, both MST and PTN [151]. This regulation was created in October 2015, under the licensing and regulation of the UK Human Fertilization and Embryology Authority (HFEA) [152]. This medical and legal advance gave families with serious mitochondrial diseases a range of possibilities to allow them to have their own genetically healthy children. In 2016, MRTs were deemed ethical by a panel of U.S. experts, provided that the procedures

adhered to certain guidelines. The panel further recommended that MRT only be used to produce male babies, thereby avoiding the transmission of the surrogate donor mitochondria to future generations [153].

Prior to the approval of the UK statutory instrument, there were several round table discussions involving the public which focused on the scientific and ethical implications of MRT. These well-scrutinized debates have engendered both widespread support and significant dismay. Ultimately, the common ground that helped pass this regulation was the understanding that mitochondrial donation can prevent a child from inheriting metabolic disease, thereby offering parents with mitochondrial disease an opportunity to have healthy children. As demonstrated in this case, good regulation helps science to advance, avoid setbacks, and, ultimately, reach patients within a reasonable time frame.

On the other hand, some constituents voiced great disagreement following this debate before and even after the final approval of MRT. The responsible regulatory agencies are now obligated to develop a robust case-by-case licensing protocol which takes into account the technical challenges and ethical complexities of this procedure. This is a crucial component of regulation to avoid setbacks, to facilitate the further development of the MRT, and to continue to provide families affected by mitochondrial disease a way to ensure the health of their babies. In practical terms, for a clinic to be able to carry out mitochondrial donation, it will need to follow a two-stage licensing process: first, it will need to apply for a license and then seek additional authorization to initiate the treatment in a particular case. Having recently concluded this lengthy legalization process, the first babies conceived using MRT following this protocol are expected to be born this year in the UK.

Many experts have recommended that families that use mitochondrial donation should be encouraged but not obligated to take part in long-term follow-up studies in order to monitor any possible effects on children conceived through this technique and on future generations. This post-procedure follow-up has been deemed crucial to ensuring that the development of this technique keeps pace with ethical advancements and the evolving sociopolitical climate of the UK and the wider world. However, by the same token, there are potential pitfalls looming on the horizon. For example, "medical tourism" from countries that lack the technology or the legal approval for such procedures may attract patients to the UK. This may significantly limit clinical follow-up of children conceived with MRT and the eventual identification of any related safety issues [154].

Most of the arguments against MRT are either scientific or ethical in nature. One question that dominates this debate is about the classification of MRT as a medical procedure. Should MRT be considered more similar to egg/sperm donation or tissue/organ donation? Given that mitochondrial donation involves the transfer of genetic but not nuclear material, this has led to uncertainty as to whether it should be regulated as egg or as tissue donation [155]. Many studies have concluded that the genes that contribute to personal characteristics and traits come solely from nuclear DNA [155–157]. In other words, traits arise from a child's mother and father, not the mitochondrial donor. Although interactions between mtDNA and cellular entities including nuclear DNA do exist, there is no evidence that nuclear DNA can be altered through epigenetic or translocation mechanisms. Considering the limited genetic contribution of mitochondrial donors, MRT is more likely to be classified as a procedure similar to tissue donation. The only confirmed traits that could arise from the donor mtDNA are related to energy production; these traits are considered minor in their overall impact on the organism. For example, variations in the mitochondrial genome have been associated with subtle differences in energy metabolism, such as the ability to cope at high altitudes.

Other studies consider the contribution of mtDNA to bioenergetics [158] to be highly important given that mitochondria-related metabolic processes in the brain play an essential role in neurotransmitter release and synaptic plasticity [159]. For this reason, the great metabolic demands of normal brain function make human cognition dependent upon mitochondrial function. Impaired mitochondrial function caused by mtDNA damage may render neurons more susceptible to oxidative injury [160] and thereby allow systemic or environmental factors to exert a noticeable effect on the brain. Certain allelic variants in mtDNA genes often lead to cognitive impairments, and it has even been hypothesized that "mitochondrial dementia" may exist [161]. Supporting this argument, a great deal of other evidence suggests that mitochondrial dysfunction may play a role in psychiatric disorders such as schizophrenia, bipolar disorder, and major depressive disorder. A study of mitochondrial dysfunction in a small cohort found that increased common deletions and decreased gene expression in mitochondria was associated with increased prevalence of psychiatric illnesses [162]. The role of mtDNA in human cognition and in the onset of degenerative diseases requires further clinical investigation in large cohorts.

In many countries, ethical review committees allow parents to decide if they wish to undergo the MRT technique. Although MRT has been successfully applied, there are ongoing questions about the cost benefits of this procedure, especially related to the stress and invasiveness of the technique on the potential mother [163]. An expert UK panel commissioned on evaluating the safety of MRT concluded that the techniques were "not unsafe" based on successful trials completed in mice and monkeys [142]. An important question remains as to whether additional rigorous preclinical safety testing is still required, although the technology has already been approved for use in humans. There is emerging evidence in recent literature which raises concerns regarding MRT. For example, a recent publication highlighted the gradual loss of donor mtDNA in embryonic stem cells (ES cells) derived from MRT embryos and a reversal to the maternal haplotype. The group identified a polymorphism within the conserved sequence box II region of the D-loop as a plausible cause behind the preferential replication of specific mtDNA haplotypes. In addition, they demonstrated that some haplotypes confer proliferative and growth advantages to cells [164]. Another group provides direct evidence of mtDNA involvement in cognitive functioning. In fact, the

Table 1: A comparative description of MST and PNT techniques and ethical concerns.

	MST	PNT
Technical approach	Transfer of nuclear DNA before fertilization	Transfer of nuclear DNA postfertilization
mtDNA carry-over	Lower (1%)	Higher (1-2%)
Risk of chromosomal abnormalities	Higher	Lower
Operator dependent	Yes	Yes
Proof-of-concept In macaque	Yes ($n = 4$)	No
Ethical issues		
Approval (UK)	Yes	Yes
Approach	Selective reproduction	Therapy based on embryo modification
Manipulation and destruction of	Oocytes	Fertilized eggs
Occurs	Preconception	Postconception

association between mtDNA and the functioning of the nervous system during neural development and synaptic activity involves mitochondrial genes. The total substitution of mtDNA modified learning, exploration, and sensory development as well as the anatomy of the brain; all of these changes persisted with age. These findings demonstrate that mitochondrial polymorphisms are not as insignificant as previously believed [165].

Additionally, new evidence has emerged that shows that even low levels of heteroplasmy introduced into human oocytes by mitochondrial carry-over during nuclear transfer often vanish. Low levels of heteroplasmy can sometimes instead result in mtDNA genotypic drift and reversion to the original genotype [166]. It is important not to overinterpret these results, as most of the corresponding experiments were performed in mice. Furthermore, this evidence should not be used as an argument to rethink the approval of MRT, but rather to be considered when performing the recommended follow-up of children born following MRT intervention [164].

It is important to mention that both PNT (pronuclear transfer) and MST were approved by the Human Fertilization and Embryology Authority based on successful studies completed with rodents and nonhuman primates. However, they may not be equally safe because only MST has been tested in large animals. When MST was tested in mature nonhuman primate oocytes (*Macaca mulatta*), it showed normal fertilization, embryo development, and production healthy offspring [142]. A point-by-point comparison between MST and PNT is detailed in Table 1 [167]. The chart details a lower carry-over of mtDNA but higher potential risk of chromosomal abnormalities for MST [168].

AMT which does not involve any kind of nuclear transfer has great potential to satisfy both ethical and safety concerns. Mitoception involves the transferring of mitochondria from a donor to a recipient cell or tissue and does not involve the passing on of nuclear material. Nevertheless, this technique requires a thorough ethical analysis before considering clinical implementation. One central issue is the origin of donor mitochondria. This requires individual analyses of autotransfer, allotransfer, and xenotransfer because each donor source may have unique ethical and biological implications. In autotransfer, the mitochondria from a tissue with a low mtDNA mutation risk could be used to treat a highly compromised organ of the same person. This donor source poses few ethical concerns but entails great biological challenges that require further complex experimentation and the use of animal models to develop. Allotransfer would use mitochondria donations from genetically close family members. Ideally, the human donor and recipient should share the same haplotype [169]. Alternatively, if no close relatives are available, haplotype matching could be considered [170]. Another ethically tricky option for allotransfer is the potential to use the still-viable mitochondria from a dead human relative in treatment [171, 172]. The final donor source option, xenotransfer, involves the transfer of mitochondria from another species to humans [173]. As unorthodox as it may appear, experiments involving this variation of the AMT did not show apparent mismatch effects in the animal-to-animal models [174]. If xenotransfer of mitochondria between animals and humans were to be successfully executed in vitro or in vivo, numerous ethical concerns would need to be addressed before considering any potential clinical applications.

The use of AMT techniques not involving the transfer of nuclear material raises a number of ethical and safety concerns but may also provide new therapeutic options. One of the key ethical debates related to MST involves the birth of babies with "three parents"; AMT may provide a way to solve this debate by allowing the father's mitochondria and not the donor's to populate the zygote. Another relevant concern in biomedical sciences today deals with the donation of organs and tissues from other people or animals for therapeutic purposes. AMT may allow us to bypass these debates by transferring only microscopic organelles rather than the entire cells or organs to deal and treat certain conditions. The applications of AMT ought to be further explored given these possibilities; however, it is crucial to note that moving from in vitro to in vivo and later to clinical applications AMT implies even greater ethical and biosafety hurdles.

Adequately framing the ethical challenges of cutting edge biomedical procedures like AMT is extremely difficult because these debates are, at least in part, anchored in the sociocultural contexts in which they arise [175]. For example,

the UK recently took a major step forward in permitting and regulating the therapeutic applications of MRT by developing a guideline for its use [151]. It should be noted that the UK has been fertile ground for scientific development and that many other countries have not even begun to consider these techniques even for experimentation because of localized ethical and safety concerns [176]. The only way to appropriately address the myriad of ethical and safety issues that impede the global development of this technology is by gathering further evidence on these procedures and facilitating national and international dialogues on these subjects.

7. Conclusions

This review analyzes all current AMT techniques and describes the future steps necessary to develop better in vitro, in vivo, and clinical applications. We focus in providing the first academic work known to globally analyze and summarize the field of AMT. To our knowledge, no other publication has compared and contrasted investigators working in in vitro and in vivo applications of AMT. Our review provides a comprehensive summary of the impact of the mitochondria in the cell, the mechanisms through which it is naturally and artificially transferred, and the ethical implications related to its potential clinical applications. This review gives a concise yet detailed overview of the past, present, and future of AMT, which we hope will orient scientists within and outside of this field and to help them contribute to the progress of this technology.

Keeping in mind our goal to orient other scientists, the authors would like to point out three final observations important to the advancement of this field. With respect to the development of AMT techniques, in vitro and in vivo procedures have evolved in a parallel, rather than in a sequential manner [177, 178]. This is unusual in the biomedical field in which techniques are usually first perfected in vitro and then later further developed in in vivo and clinical applications. While this somewhat unorthodox trajectory has certainly produced valuable knowledge, it is also important to recognize that by "skipping steps" scientists may have left important gaps in our knowledge about AMT.

Another potential avenue for scientific investigation not covered in this review is the study of genetic modification of mitochondria before artificially transferring them. Such modifications could range from slight alterations of the mtDNA in order to better facilitate specific cellular processes to the creation of completely artificial "super" mitochondria [179, 180]. As is the case with other genetically modified organisms, this line of investigation not only raises a whole host of ethical, legal, and biosafety questions but also has the potential to greatly benefit humanity if developed correctly.

Finally, the authors would like to critique the terminology used within the field of AMT in service of better elucidating the uniqueness of AMT and its possible therapeutic applications. Investigators currently use the terms mitochondria transformation [9], transfer [93], and transplant [117, 181] interchangeably to designate the artificial transport of mitochondria from one cell to another via diverse methodologies. According to our criteria, the term that best defines this process is *transfer*. Mitochondria can be effectively transferred in vitro and in vivo via a variety of processes such as MitoCeption [10] and Magnetomitotransfer [91]. Mitochondrial "transformation" engenders misunderstandings because the cell is transformed, not the mitochondria. Additionally, because the term "transplant" has come to be associated with tissue and organ transport between donors and recipients, transfer should be used to denote the transport of subcellular components such as mitochondria from one cell to another in order to distinguish the ethical, legal, and biomedical nuances associated with such procedures and to facilitate more exact discussion of these issues.

In order to continue to advance the development of AMT, it is essential that scientists answer questions key to the functioning of these techniques, troubleshoot the challenges for clinical applications, and resolve the ethical, legal, and biosecurity concerns that will determine if this technology is logistically applicable around the world. Addressing these hurdles will enable our generation to unlock the transformative potential of an organelle that was once merely considered the cell power plant.

Abbreviations

AMT: Artificial mitochondria transfer
AIF: Apoptosis-inducing factor
ARDS: Acute respiratory distress syndrome
BAL: Bronchoalveolar lavage
BBB: Blood-brain barrier
BMSCs: Bone marrow-derived stem cells
CAP: Chloramphenicol
CM: Cristae membrane
DRP1: Dynamin-related protein 1
EF: Efrapeptin
EGTA: Ethylene glycol tetraacetic acid
ER: Endoplasmic reticulum
ESCs: Embryonic stem cells
EVs: Extracellular vesicles
FGF-2: Fibroblast growth factor 2
Gfer: Growth factor erv1-like
HEPES: N-(2-hydroxyethyl) piperazine-N′-ethanesulfonic acid
HFEA: Human Fertilization and Embryology Authority
hMSCs: Human mesenchymal stem cells
HPH: Hypoxic pulmonary hypertension
IBM: Inner boundary membrane
IMM: Inner mitochondrial membrane
iPSCs: Induce pluripotent stem cells
LPS: Lipopolysaccharide
MAMs: Mitochondria-associated membranes
MCAO: Middle cerebral artery occlusion
MERRF: Myoclonic epilepsy with ragged red fiber syndrome
Mfn1: Mitofusin 1
Mfn2: Mitofusin 2
MMP-9: Matrix metalloproteinase-9
MRTs: Mitochondria replacements

MSCs: Mesenchymal stem cells
MST: Maternal spindle transfer
mtDNA: Mitochondrial DNA
mTOR: Mammalian target of rapamycin
MVs: Microvesicles
MβCD: Methyl-β-cyclodextrin
NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells
nuDNA: Nuclear DNA
OM: Outer membrane
OMM: Outer mitochondria membrane
OX-PHOS: Oxidative phosphorylation
PEG: Polyethylene glycol
PDM: Parkinson disease model
PINK1: PTEN-induced kinase 1
PMDS: Primary mitochondria diseases
PNT: Pronuclear transfer
RAGE: Receptor for advanced glycation end product
ROS: Reactive oxygen species
TNF α: Tumor necrosis factor alpha
TNTs: Tunneling nanotubes
UV: Ultraviolet
VEGF: Vascular endothelial growth factor.

Conflicts of Interest

The authors declare no conflict of interests.

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Review Article

Cell Connections by Tunneling Nanotubes: Effects of Mitochondrial Trafficking on Target Cell Metabolism, Homeostasis, and Response to Therapy

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Intercellular communications play a major role in tissue homeostasis and responses to external cues. Novel structures for this communication have recently been described. These tunneling nanotubes (TNTs) consist of thin-extended membrane protrusions that connect cells together. TNTs allow the cell-to-cell transfer of various cellular components, including proteins, RNAs, viruses, and organelles, such as mitochondria. Mesenchymal stem cells (MSCs) are both naturally present and recruited to many different tissues where their interaction with resident cells via secreted factors has been largely documented. Their immunosuppressive and repairing capacities constitute the basis for many current clinical trials. MSCs recruited to the tumor microenvironment also play an important role in tumor progression and resistance to therapy. MSCs are now the focus of intense scrutiny due to their capacity to form TNTs and transfer mitochondria to target cells, either in normal physiological or in pathological conditions, leading to changes in cell energy metabolism and functions, as described in this review.

1. Introduction: TNTs, What Are They? How Were They Discovered?

Cell communication is essential for tissue homeostasis, specific cell functions, and response to external cues. Indeed, during development and self-repair, tissues constantly need to adapt to changing biological conditions in order to reach physiological homeostasis. For this, their constituting cells constantly interact with target cells that reside in their close vicinity or alternatively, they can reach out to cells much further away, without necessarily involving the close-by surrounding cells. This cell-to-cell communication can be achieved by various processes including diffusible factors like cytokines and chemokines, secreted microvesicles, or direct passage through gap junctions. Long-distance diffusible factors can target different cell types, depending on the expression, by these cells, of the relevant receptors.

Another impressive means of communication cells devised to allow long-distance cell-to-cell contacts are the formation of tunneling nanotubes (TNTs) between these cells, as initially reported in the rat pheochromocytoma- (PC12-) derived cells and in immune cells [1, 2]. These are long tubular structures, with diameters between 50 and 1500 nm, that can span several tens to hundreds of microns, connecting two cells together [3]. In a characteristic manner, in 2D cultures, TNTs are not tethered to the extracellular matrix, rather floating in the culture medium. Microscopy imaging, either of live or of fixed cultures, proved very useful to characterize these cellular structures [3–10]. The tunneling nanotubes allow a continuity in plasma membrane and cytoplasm between the connecting cells, thus allowing trafficking of a number of cellular components from one cell to the other. This trafficking can rely on cytoskeleton fibers, of either actin, microtubules, or both (Figure 1 and [3]).

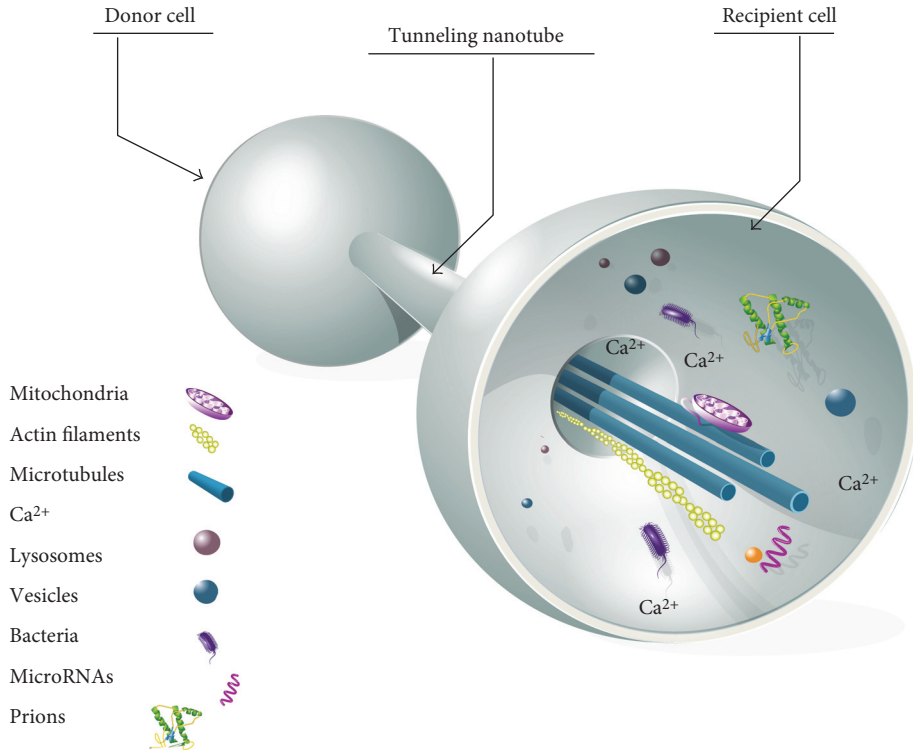

Figure 1: Tunneling nanotube (TNT). Tunneling nanotubes can connect many different cells together, using cytoskeleton actin microfilaments, microtubules, or both. TNTs allow the trafficking, from donor to recipient cells, of cargoes including organelles, proteins, miRNAs, and ions.

In the past few years, a number of studies reported this capacity of cells, from an ever increasing number of cell types, to connect to one another. Interestingly, these TNTs also allow the trafficking of a number of different cargos between the connected cells, therefore increasing the combinatorial complexity of these cell-to-cell connections and their biological outcome, as summarized in Table 1. In this review, we provide a general overview of what is currently known about tunneling nanotubes, the cells involved, the cargoes transported within TNTs, and the regulation of these processes. We further focus on the specific capacity of mesenchymal stem cells (MSCs) to connect to target cells through such TNT structures and to transfer mitochondria to the targeted cells, emphasizing the modifications in the energetic metabolism and the biological functions the MSC mitochondria generate in these cells. Due to space constraints, we do apologize in advance for articles we could not cite.

2. How Are TNTs Formed? What Are the TNT-Connected Cell Partners?

2.1. Cell Types Involved in TNT Connections (Table 1). Cells involved in connections through nanotubes can be of the same or of different types. Many cell types appear endowed with the capacity to form TNTs with one another. TNTs were observed among rat pheochromocytoma (PC12) cells [8, 11], renal proximal tubular epithelial cells (RPTEC) [12], rat kidney cells [13], and retinal pigment epithelial cells [14]. Tunneling nanotube formation was also reported between endothelial progenitor cells and endothelial cells [15], between endothelial progenitor cells and cardiac myocytes [16], and between immature hippocampal neurons and astrocytes [17]. Noteworthily, even though tunneling nanotubes have been largely described in human and murine systems, they were also reported in bacteria, connecting *B. subtilis* cells together and *B. subtilis* with the distantly related *E. coli* [18], in Drosophila where they contribute to niche-germline stem cell signaling [19] and in the zebrafish during gastrulation [20].

Cells of the immune system, notably macrophages, dendritic cells (DCs), NK, and B cells, extensively use TNTs to communicate [6, 21–27]. Shortly after the discovery of TNTs in PC12 cells, these structures were also identified between DCs and monocytes [28]. The transfer of antigenic information from migratory DCs to lymph node-residing DCs through TNTs was recently shown to be critical for the induction of immune responses [24]. TNT formation was also described in neural CAD cells (mouse cell line of catecholaminergic origin) and from bone marrow-derived dendritic cells to primary neurons [6, 25, 26].

As it will be further described below, mesenchymal stem cells (MSCs) actively use TNTs to deliver cargos to renal tubular cells [29], cardiomyocytes [30], bronchial epithelial cells [31, 32], macrophages [33], endothelial cells [34], and breast cancer cells [35, 36]. Reciprocally, MSCs can receive cargos from TNT-connected cells as in the case of human vascular smooth muscle cells (VSMCs) [9].

Formation of TNTs has been observed for a number of cancer cells, either connecting cancer cells together or

Table 1

Authors	TNT donor cells	TNT receiver cells	Transported cargoes	References
Onfelt et al. (2004)	Human NK cells	Human EBV-transformed human B cells	GFP-tagged cell surface class I MHC	[2]
	Human macrophages	Same cells		
	Human EBV-transformed human B cells	Same cells		
	Murine J774 macrophages	Same cells		
Rustom et al. (2004)	Rat pheochromocytoma PC12	Same cells	Microvesicles	[1]
	Human embryonic kidney (HEK)	Same cells	Organelles	
	Normal rat kidney (NRK)	Same cells		
Castro et al. (2005)	Colon carcinoma cell line SW620	Same cells	ND	[41]
Koyanagi et al. (2005)	Human endothelial progenitor (EPC)	Neonatal rat cardiomyocytes (CM)	Mitochondria	[16]
Watkins et al. (2005)	Human dendritic cells	Same cells and THP-1 cells	Calcium flux	[23]
	Human THP-1 monocytes	Same cells	Major histocompatibility proteins (MHC class I)	
Chinnery et al. (2008)	Murine MHC class II dendritic cells	Same cells	ND	[21]
Gurke et al. (2008)	Normal rat kidney cells (NRK)	Same cells	Endocytic organelles	[13]
Onfelt et al. (2006)	Human macrophages	Same cells	Bacteria Mitochondria Vesicles (endosomes, lysosomes)	[28]
Sowinski et al. (2008)	Jurkat T cells	Same cells and primary T cells	HIV viral particles	[57]
	Primary T cells	Same cells		
Bukoreshtliev et al. (2009)	PC12 cells	PC12 cells	Intracellular organelle transfer	[11]
Eugenin et al. (2009)	Human macrophages	Same cells	HIV viral particles	[58]
Plotnikov et al. (2010)	Human mesenchymal multipotent stromal cells (MMSC)	Rat renal tubular cells (RTC)	Mitochondria	[29]
Acquistapace et al. (2011)	Human mesenchymal stem cells (MSCs)	Cardiomyocytes	Mitochondria and intracellular material	[30]
Domhan et al. (2011)	Human proximal tubular epithelial cells (RPTEC)	Same cells	Microvesicles	[12]
Wang et al. (2011)	Rat hippocampal astrocytes	Same cells and rat hippocampal neurons	Endoplasmic reticulum	[49]
	Rat hippocampal neurons	Same cells and rat hippocampal astrocytes	Mitochondria Golgi fragments Endosomes Amyloid β	
Yasuda et al. (2011)	Human umbilical vein endothelial cells (HUVEC)	Stressed HUVEC	Lysosomes Mitochondria	[15]
Islam et al. (2012)	Murine MSCs	Murine alveoli	Mitochondria	[32]
Lou et al. (2012)	Human primary cancer cells	Same cells	Mitochondria	[39]
	Human mesothelial lines (MSTO-211H, VAMT, H-Meso)	Same cells		
Schiller et al. 2012	HeLa	Same cells	Transmembrane HLA-A2-EGFP protein	[43]

Table 1: Continued.

Authors	TNT donor cells	TNT receiver cells	Transported cargoes	References
Vallabhaneni et al. (2012)	Human MSCs	Human vascular smooth muscle cells (VSMCs)	Mitochondria	[9]
Wittig et al. (2012)	Human retinal pigment epithelial (ARP-19) cells	Same cells		[14]
Costanzo et al. (2013)	CAD cells	Same cells and with transfected CADs	Htt aggregates	[26]
	Primary cerebellar granule neurons (CGNs)	Same cells and with transfected CGNs		
Pasquier et al. (2013)	Human mesenchymal stem cells (MSCs)	Same cells and ovarian and cancer cell lines	Mitochondria	[36]
	Human endothelial cells (HECs)	Same cells and ovarian and cancer cell lines		
	Human ovarian cancer cells (SKOV3, OVCAR3, HTB-161)	Same cells		
	Human breast cancer cells (MDA-MB231 and MCF7)	Same cells		
Rainy et al. (2013)	Human B cells	Human T cells	Plasma membrane-associated proteins (H-Ras)	[27]
Ady et al. (2014)	VAMT (sarcomatoid mesothelioma cell line)	Same cells	ND	[38]
	H2052 (mesothelioma cell line)	Same cells		
	MSTO-211H (derived from mesothelioma patient)	Same cells		
	Met5A (immortalized mesothelioma cell line)	Same cells		
Ahmad et al. (2014)	Murine MSCs	Murine lung epithelial cells	Mitochondria	[31]
Liu et al. (2014)	Human MSCs	Human umbilical vein endothelial cell (HUVEC)	Mitochondria	[34]
Thayanithy et al. (2014)	Murine osteosarcoma K7M2 cells	Same cells and MC3T3 murine osteoblasts	MicroRNAs (miR-199a)	[37]
	SKOV3 ovarian cancer cells	Nonmalignant ovarian epithelial cells		
Thayanithy et al. (2014)	Human biphasic mesothelioma MSTO-211H cells	Same cells	Exosomes from other cells	[40]
Biran et al. (2015)	Oncogene or DNA damage-induced senescent cells	NK cells	Proteins	[10]
Burtey et al. (2015)	HeLa	NRK fibroblasts	Tf-R (transferrin receptor), endosomes	[44]
Caicedo et al. (2015)	Human mesenchymal stem cells (MSCs)	Human breast cancer cell line MDA-MB-231	Mitochondria	[35]
Polak et al. (2015)	Bidirectional: human MSCs to human acute lymphoblastic leukemia cells (BCP-ALL cell line) Bidirectional: human MSCs to human B cell precursor of Leukemia Nalm6 (B-Other) Bidirectional: human MSCs to human acute lymphoblastic leukemia cells (TEL-AML1)		ND	[47]
Wang and Gerdes (2015)	PC12 cells (−/+ultraviolet light treatment)	PC12 cells (−/+ultraviolet light treatment)	Mitochondria	[8]

Table 1: Continued.

Authors	TNT donor cells	TNT receiver cells	Transported cargoes	References
Zhu et al. (2015)	CAD neuronal cells	Same cells	Prions Lysosomes Early endosomes	[54]
Hashimoto et al. (2016)	Monocyte-derived macrophages	Same cells	HIV-1	[56]
Hayakawa et al. (2016)	Astrocytes	Neurons	Mitochondria	[115]
Jackson et al. (2016)	Human MSCs	Human monocyte-derived macrophages Murine alveolar macrophages	Mitochondria	[33]
Lu et al. (2016)	Bladder cancer cells	Same cells	Mitochondria	[46]
Moschoi et al. (2016)	BM-MSCs	Acute myeloid leukemia cells	Mitochondria	[48]
Tardivel et al. (2016)	Neurons	Neurons	Tau protein	[7]
Victoria et al. (2016)	Astrocytes	Neurons	Prions	[55]
Zhang et al. (2016)	iPSC-MSCs and BM-MSCs	Cardiomyocytes	Mitochondria	[62]

connecting cancer cells with normal stromal cells, notably mesenchymal stem cells (MSCs). TNT formation was described in a diversity of different cancer cell types, including malignant mesothelial cells [37–40], colon carcinoma cells [41], MCF7 and MDA-MB-231 breast cancers, SKOV3 and OVCAR3 ovarian cancers [36], K7M2 murine osteosarcoma cells [37], laryngeal squamous cell carcinoma (LSCC) [42], HeLa cells [43, 44], astrocytoma cells [45], and bladder cancer cells [46]. TNT formation was also observed between normal and cancer cells, including between nonmalignant IOSE human ovarian epithelial cells and SKOV3 ovarian cancer cells [37], between stromal MC3T3 murine osteoblast cells and K7M2 osteosarcoma cells [37], and between HeLa cells and fibroblasts, in both directions [44]. A number of the connections between normal stromal cells and cancer cells were found to involve MSCs, as for instance in the following studies involving MDA-MB-231 breast cancer cells [35], B cell precursor acute lymphoblastic leukemia (ALL) cells [47], and acute myeloid leukemia cells [48].

The nanotubes formed between these different cell types do share some features, notably a continuity in cell membrane and cytoplasm between the connected cells, allowing the trafficking of biological cargos. However, with the accumulation of new TNT-related data, it appears that these structures will have properties, concerning the connecting modes, cargos transported, cytoskeleton-based molecular motors, and biological outcome that will underline the specificity of each cell system.

2.2. Cargos Transported within the TNTs

2.2.1. Mitochondria. Organelles such as mitochondria have now been described as trafficking entities in the tunneling nanotubes connecting many different cells types including renal proximal tubular epithelial cells (RPTEC) [12], astrocytes [49], astrocytomas [45], endothelial cells [50], neuronal CAD cells [25], laryngeal squamous cell carcinoma (LSCC) [42], and monocyte-derived macrophages [33]. This mitochondria transfer was also observed between endothelial and cancer cells [36], endothelial progenitor cells and cardiac myocytes [16], and from healthy to damaged (UV-treated) PC12 cells [8].

MSCs were shown to share mitochondria through a TNT-mediated process with number of target cells. These target cells include cardiomyocytes [30], endothelial cells [34], pulmonary alveolar epithelial cells [31, 32], renal tubular cells [29], macrophages [33], and acute myeloid leukemia cells [48] as well as breast cancer cells (Figure 2) [35, 36], leading to modifications of the functional properties of these cells. Interestingly, in this latter case, the MSC mitochondria transfer was observed to be of a higher extent for the leukemic $CD34^+$ myeloblasts than for the normal mononuclear $CD34^+$ cells [48]. Conversely, when MSCs were in coculture with vascular smooth muscle cells, the TNT-mediated mitochondrial trafficking resulted in the acquisition of the VSMC mitochondria by the MSCs [9].

On a technical point of view, detection of the transfer of mitochondria from donor to target cells is often performed based on the imaging of mitochondria prelabeled with fluorescently dyes such as MitoTrackers as shown in numerous reports, including [35, 51]. Detection of the transferred mitochondria can also be performed by genetically labeling the donor cells by the lentiviral-mediated expression of a GFP fusion protein with the mitochondrial cytochrome c oxidase subunit VIII (LV-Mito-GFP) [52]. Alternatively, heterologous systems, that is, human/mouse or human/rabbit, allow the use of antibodies like the monoclonal anti-human mitochondria antibody (MTC02) that specifically recognizes human mitochondria, but not mitochondria of mouse or rabbit origin, and can, thus, enable to discriminate between

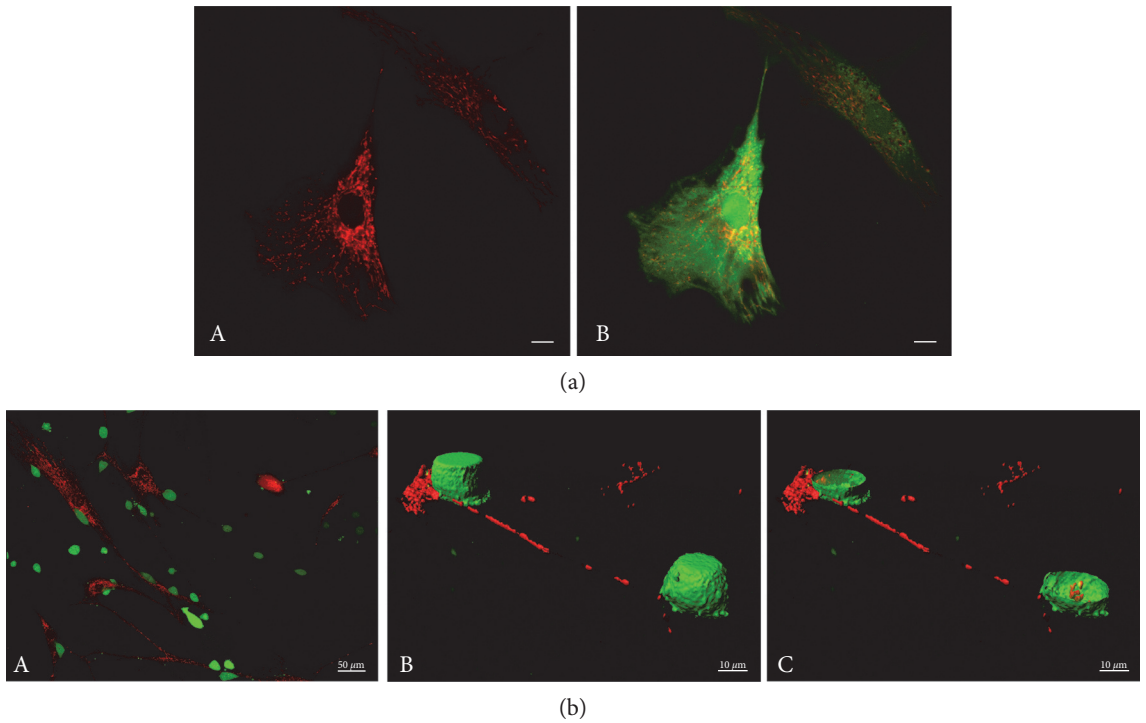

Figure 2: Mitochondrial trafficking from MSCs to MDA-MB-231 breast cancer cells. (a) MSC mitochondrial network. MSCs were labeled by MitoTracker Deep Red FM and Green CellTracker CMFDA. Scale bars, 10 μm. (b) Transfer of MSC mitochondria to MDA-MB-231 cells. Coculture (24 h) of human MSCs (MitoTracker Red CMXRos prestained) and MDA-MB-231 cells (Green CellTracker CMFDA prestained). (A) 2D view of the coculture, (B, C) 3D reconstruction of the cells from stacks of confocal images with the cell isosurface view (B), and *xy* plane section (C) (Imaris). Scales, (A) 50 μm, (B, C) 10 μm.

the transferred human mitochondria and the endogenous [35, 53]. Mitochondria contain their own mitochondrial DNA (mtDNA) that is transferred, as expected, concomitantly with the trafficking mitochondria. Because mitochondria from unrelated donors harbor mtDNA with specific SNPs, these punctual nucleotide differences in the mtDNA can also be used as a tool to distinguish and quantify the mtDNA of the transferred mitochondria in respects to the endogenous ones [35, 51].

As it will be discussed later in the review, this mitochondrial trafficking leads to notable effects in the target cells as mitochondria are involved in multiple cellular functions including the biosynthesis of ATP, through the electron transport chain, or that of lipids and amino acids. In addition, mitochondria are now recognized as signaling entities that can induce cell events such as autophagy and apoptosis.

2.2.2. Other Cargoes. Among organelles, lysosomes were also found to be transferred between progenitor and senescent endothelial cells and this transfer [15]. The TNTs formed between CAD cells (mouse neuronal cell line of catecholaminergic origin) were shown to allow the transfer of lysosomes and also of prions (PrP^{Sc}) from the infected to noninfected cells [25, 54]. A similar TNT-mediated PrP^{Sc} transfer was observed from bone marrow-derived dendritic cells to primary neurons providing a possible route for prions to the brain [25]. PrP^{Sc} was recently suggested to transfer, as well, from infected murine astrocytes to primary cerebellar granule neurons through tunneling nanotubes [55]. The protein Tau can also be transferred as a fibrillar protein between neurons, therefore possibly contributing to Tau pathologies [7].

Viruses also display the capacity to be transmitted through TNTs. This was shown for HIV, between infected T cells and noninfected T cells, thus eliminating the need for the infected cells to release a fully mature HIV virus in order to infect the neighboring cells [56, 57]. In addition, HIV-containing TNTs were described to be formed by infected macrophages, connecting them to other macrophages [58] and between noninfected and HIV-infected DCs [24].

TNTs also constitute a route for the transfer of microRNAs between cells, as shown for miR-19a among K7M2 murine osteosarcoma cells, [37]. The transport of miRNA was observed between cancer cells and the normal cells of the surrounding tumor microenvironment, as demonstrated between the K7M2 osteosarcoma and the stromal MC3T3 osteoblast cells and between the human SKOV3 ovarian cancer cells and nonmalignant IOSE ovarian epithelial cells [37]. In addition, the presence of lipid droplets was also detected in the TNTs connecting human microvascular endothelial cells (HMEC-1). The number of these lipid droplets was found to increase under angiogenic conditions (VEGF) and in response to arachidonic acid [50]. Ions like calcium (Ca^{2+}) can also be transmitted between TNT-connected cells, as shown from DCs to monocytes [23], for SH-SY5Y neuroblastoma and HEK cell lines [59], for ARPE-19 human retinal pigment epithelial cells [14], and for astrocytomas [45].

2.3. TNT Formation Mechanisms and Molecular Motors. Two major processes have been proposed for the formation of TNTs. Cells can extend filopodia-like protrusions that, in contact with target cells, can undergo plasma membrane fusions. Alternatively, cells that were initially in close contact with one another can move apart, remaining bound by the extending tunneling nanotube structure. Several proteins have now been identified for their role in nanotube formation, for the functional connection between the two interacting cells, and for the cargo trafficking within the connecting TNTs.

The role of connexin 43 (Cx43) gap junction marker has been documented for different cell systems. In the murine model of LPS-induced acute lung injury, gap junctions between the instillated murine bone marrow stromal cells (BMSCs) and the pulmonary alveolar epithelial cells depended on the expression of Cx43 by both cell types and occurred at sites of high-Cx43 expression. Cx43 was therefore proposed as essential for BMSC attachment to the alveolar cells, leading to the generation of TNTs between these cells [32]. A high expression of the Cx43 was also observed at the neuronal contact site of the TNTs formed between hippocampal neurons and astrocytes [17]. Expression of Cx43, and not that of other connexins, was reported for TNTs formed between astrocytoma cells [45]. Finally, among the different connexins expressed by human laryngeal squamous cell carcinoma (LSCC), Cx43 was the one required for gap junction and TNT formation among LSCCs [42].

The role of M-Sec/TNFaip2 and the exocyst complex has also been put forward in different studies [5, 56]. Interestingly, M-Sec-induced TNTs were found to contain actin filaments, but not microtubules [5]. Other factors, like LST1 (leukocyte-specific transcript 1), were found to contribute to the formation of nanotubes through the exocyst complex, by recruiting the small GTPase RalA to the plasma membrane and promoting its interaction with the exocyst complex [60]. In HeLa cells, TNT formation involved the action of the GTPase Rab8 [44].

Another small GTPase, Cdc42, was found to play a dual role in TNT formation. Cdc42 was demonstrated to play a role in the TNT elongation process in the Raw264.7 macrophage cell [5] and to favor protein trafficking from oncogene- or DNA damage-induced senescent cells to NK cells [10]. However, it was the GTPase RalA, and not Cdc42, that was shown to be important for LST1-induced nanotubes in HeLa cells [60]. On the other hand, Cdc42 (together with IRSp53) and VASP (vasodilator-stimulated phosphoprotein) were found to inhibit TNT formation in neuronal CAD cells [61].

The mitochondrial trafficking within these TNTs can rely on the Rho GTPase Miro1 (also called RhoT1/2), as shown for the transfer of mitochondria from mesenchymal stem cells to damaged alveolar epithelial cells in mouse models of airway injury [31]. Miro1 was also found to play a key role and be responsible for the differences in mitochondrial transfer efficacies observed between iPSC-MSCs and adult BM-MSCs [62]. As a matter of fact, mitochondrial transportation has been thoroughly studied in polarized cells, such as neurons, where mitochondrial production of ATP at distant sites from the cell body is crucial to meet local energy demands. The Rho GTPase Miro1 was shown to connect to the kinesin-1 molecular motor through the Milton adaptor protein (also called TRAK1/2 and OIP106/98), enabling mitochondrial transport along microtubules [63].

Other cytoskeleton motors can allow the transport of small molecules and organelles within the cells. In addition to the kinesin motor, the cytoplasmic dynein also moves along microtubules while, on the other hand, the family of myosins are actin-based cytoskeleton motors [64]. The molecular motor myosin-X (Myo10) was also proposed as a key regulator of tunneling nanotube formation in murine neuronal (CAD) cells, increasing the occurrence of TNTs and the transfer of vesicles in these TNTs [6].

Depending both on the types of cells connected and cargos transported, it is likely that TNTs will rely on different types of cytoskeletons, that is, microfilaments and/or microtubules, and therefore on different cytoskeleton motors to support the trafficking of these cargos. For instance, the protein Tau was reported to associate with both microtubules and the actin network and to contribute to the formation of TNTs, bridging neurons together [7]. On the other hand, mitochondria were found to traffic along microtubules in PC12 cell-connecting TNTs [8]. Interestingly, cytochalasin D, latrunculins A, and B, that are potent inhibitors of actin polymerization, were shown for instance to inhibit TNT formation between MSCs and vascular smooth muscle cells [9] or between senescent cells and NK cells [10].

Altogether, the diversity of factors involved in the formation of the TNTs and of the cargoes trafficking within these TNTs points to the complexity of the whole process of TNT-mediated cell-to-cell communication. New paradigms will be needed to allow to predict which cargoes might be transferred, using what type of cytoskeletal motor, for any given couple of cell types.

2.4. Regulation of TNT Formation. The formation of TNTs, as tested in 2D in vitro cultures, was observed to be controlled by several factors including serum and glucose concentrations, viral infection, or exposure to therapeutic agents, as detailed further below. Beyond the fact that this information is important to design experimental settings and collect in vitro data on TNTs, it also gives clues about how TNT formation might be regulated in vivo, by nutrient supply, infection, or therapy, and thus contribute to our understanding of the holistic organism responses.

In vitro, low-serum (2.5% FBS) and high-glucose concentrations (50 mM) were found to stimulate TNT formation, as observed between murine K7M2 osteosarcoma cells and MC3T3 osteoblast cells [37]. Low-serum, hyperglycemic, acidic growth medium was also used to stimulate both the formation of TNTs and the mitochondrial trafficking between malignant or between normal mesothelial cells [39]. Rat hippocampal astrocytes and neurons, as well as HEK293 kidney cells, produced more TNTs at low-serum concentration and upon H_20_2 stimulation [49]. Concerning the effects of glucose concentrations, it is worth mentioning that, for neuronal mitochondrial trafficking, high-glucose concentrations were shown to diminish mitochondrial motility, by a mechanism involving Milton and its O-GlcNAcylation by the O-GlcNAc

transferase (OGT) [65]. Given the apparent similarities between the processes of mitochondrial trafficking in neurons and in TNTs, a possible role of glucose-dependent OGT activation for mitochondrial trafficking within TNTs might be worth checking. Albeit the effects of high-glucose concentrations reported above, it is also worth noting that, in other cell systems, it is the glucose deprivation that was found to enhance the TNT-mediated mitochondrial transfer, as observed from MSCs to endothelial cells [34].

Cellular stress caused, for instance, by HIV infection in human macrophages was demonstrated to increase the number (but not the length) of TNTs formed by these macrophages towards other macrophages, in correlation with viral replication [58]. Chemotherapeutic agents have also been found to influence the occurrence of TNT formation and cargo trafficking. Zeocin is a DNA-intercalating agent related to bleomycin that induces cell death by causing double-strand breaks. Zeocin treatment of renal proximal tubular epithelial cells (RPTEC) was found to increase up to 10-fold (for 400 ng/ml Zeocin) the number of TNTs formed by these cells [12]. Cytarabine (ARA), a nucleoside analog used as a chemotherapeutic agent to treat AML, was found to increase physical interactions between AML cells and bone marrow-derived MSCs and mitochondrial incorporation by the AML cells [48]. This effect on mitochondrial uptake by AML cells was also observed following treatment with the topoisomerase II inhibitor etoposide and the anthracycline doxorubicin, but not for the microtubule-disrupting agent vincristine [48]. Finally, other mechanisms were also linked to TNT formation. They could involve cell activation by CD40L, a member of the TNF family, shown to activate DC TNTs [24]. Besides, the enhanced expression of p53 was shown to be important for TNT formation by MG63 osteosarcoma cell TNTs [49] while dispensable for TNT formation in other cell types as shown for PC12 cells, OCI-AML3 (acute myeloid leukemia) cells, human osteosarcoma cell line SAOS-2 (p53-null), and murine bone marrow-derived MSCs [66].

3. Mesenchymal Stem Cells: Characterization and Functional Properties

3.1. MSC Properties. Mesenchymal stem cells (MSCs) are characterized by their multilineage differentiation capacity, notably into osteocytes, adipocytes, and chondrocytes [67–71]. They also express specific cell surface markers that include CD105 (endoglin) [72], CD73 [73], CD90, CD19 [74], CD79 [75], CD14 [76], CD11b [77], and HLA-DR [70], but not the hematopoietic markers CD45 [78]. Additional MSC surface markers, like Stro-1, SSEA-4, CD271, and CD146, have also been proposed [79]. MSCs are found in nearly all tissues. Outside from the bone marrow-derived mesenchymal stem cells (BM-MSCs), MSCs have now been isolated from other tissues including the human placenta, umbilical cord, spleen, and adipose tissue-derived mesenchymal stem cells (AT-MSCs) [80, 81]. These MSCs share common properties but also exhibit differences in the expressed cell markers, in their differentiation potential and phenotypes [82–84]. As an example, both BM-MSCs and AT-MSCs show a high expression of CD271 and of the Stro-1 marker compared to MSCs from other tissues, while CD146 (or MCMAM (melanoma cell adhesion molecule)) appears specific to BM-MSCs [79]. Interestingly, CD146 is also a marker of pericytes, proposed to give rise to MSCs following blood vessel damage or inflammation [85–87]. It is worthwhile noting that, even when isolated from a single tissue, MSCs can form a heterogeneous population with diverse differentiation and immune regulatory capacities [79, 82, 88].

3.2. Role of MSCs in Immune Suppression. MSCs are attracted and activated by cytokines such as IFN-γ, TNF-α, IL-6, IL-8, IL-1, and TGF-β that are present at high concentrations in the inflammatory environment [89–91]. MSC exposure to this inflammatory environment contributes to their tissue repair and immunosuppressive properties. Once at the inflammation site, MSCs prevent cellular destruction and damage to surrounding tissues [90–92]. MSC immunosuppression is mediated by the secretion of soluble factors like indoleamine 2,3-dioxygenase (IDO), IL-10, TSG-6 (TNF-α-stimulated gene/protein 6), prostaglandin E2 (PGE2), TGF-β-1, inducible nitric oxide synthase (iNOS) and human leukocyte antigen (HLA-G) [93–97]. Interestingly, different mechanisms were proposed for the murine and human MSC immunoregulatory properties. For example, murine and human MSCs, respectively, produce inducible nitric oxidase synthase (iNOS) and indoleamine 2,3-dioxygenase (IDO), both iNOS and IDO contributing to the immunosuppressive functions of MSCs [98].

3.3. Role of MSCs in the Tumor Microenvironment. The tumor microenvironment is known to play an important role in tumor progression, metastasis, and resistance to therapy [99, 100]. Mesenchymal stem cells are recruited to the tumor microenvironment where they have the capacity to modify the growth and metastatic potential of the cancer cells [88-89, 99, 101–104]. The recruitment of MSCs to the tumor microenvironment depends on a number of cytokines and chemokines secreted by the tumor cells [105, 106] which can give rise to mutual cross talks between MSCs and cancer cells [102, 107]. The MSCs present in the tumor microenvironment also display the capacity to modify the response of nearby cancer cells to therapeutic agents [99] as exemplified for cisplatin [108] and paclitaxel [109]. Interestingly, multiple reports now establish that the response of the cancer cells to therapy is closely linked to the metabolic reprogramming of these cells [110, 111].

4. Capacity of MSCs to Connect to Target Cells via Nanotubes and Biological Outcomes

4.1. TNT Connections and Mitochondria Transfer between MSCs and Target Cells. MSCs interact with other cells, reprograming their function through the secretion of small molecules like growth factors, chemokines, cytokines, and molecular mediators (bioactive lipids, nucleotides, among others). The human mesenchymal stem cells (MSCs) have been shown to display the ability to connect to target cells

through tunneling nanotubes and to transfer the mitochondria through these TNTs. Prockop laboratory observed for the first time that functional mitochondria could be transferred between MSCs to tumor cells [112]. These target cells now include cardiomyocytes, endothelial cells, pulmonary alveolar epithelial cells, renal tubular cells, and cancer cells, leading to modifications of the functional properties of these cells [8, 29–32, 34–36, 48, 113].

4.2. Other Mechanisms for MSCs to Transfer Mitochondria. Mitochondria can also be transported from MSCs to the other cells by microvesicles (MVs). MSC mitochondria can be taken by arrestin domain-containing protein 1-mediated microvesicles (ARMMs) that range from 0.1 to 1 μm in diameter; therefore, fitting mitochondria whose average size is of the order of 0.5 μm. These microvesicles were shown to be engulfed by macrophages, resulting in increased macrophage mitochondrial bioenergetics [114]. This MV-dependent mitochondria transfer between MSCs and macrophages was thus proposed to be beneficial for both macrophages and MSCs, as it also decreased the MSC load of depolarized mitochondria [114]. Mitochondria were also reported to be released by astrocytes as mitochondria-containing particles, in a CD38-dependent process, and recaptured by neurons [115].

4.3. Mitochondria, Isolated beforehand from Cells, Can Be Transferred to Target Cells. Mitochondria, in an isolated form, can also be internalized by cells, notably cardiomyocytes [35, 51, 53, 116, 117]. This process has been proposed to depend on macropinocytosis [116, 117]. The quantitative transfer of isolated mitochondria to target cells by the technique of MitoCeption, based on this capacity of mitochondria to be internalized by living cells, was shown to be a tool of choice to determine the effects of the transferred mitochondria in the target cells [35]. Other techniques to transfer the target cells of mitochondria, isolated beforehand, include formation of transmitochondrial cybrids [118] and the use of photothermal nanoblades [119] as well as the direct cell injection of mitochondria, as performed in oocytes [120–122].

4.4. Mitochondrial TNT Transfer Observed In Vitro Also Occurs In Vivo. Islam and colleagues demonstrated the transfer of mitochondria in vivo from MSCs to pulmonary alveolar epithelial cells in a murine model of lipopolysaccharide- (LPS-) induced acute lung injury [32]. After their instillation in the lungs of mice with LPS-injured alveoli, MSCs were found to form gap junctions with the injured lung epithelial cells. This resulted in the transfer of MSC mitochondria to these cells and to the regeneration of the affected alveoli. Furthermore, this study pinpointed the role of connexin 43-containing gap junctions for the efficient in vivo transfer of the MSC mitochondria [32]. Likewise, Ahmad and colleagues used a mouse model of rotenone-induced acute lung injury that enabled them to demonstrate the role of MSCs and their transferred mitochondria in the rescue of the injured bronchial epithelial cells [31]. This in vivo model helped establish the role of the Rho-GTPase Miro1 in the mitochondrial intercellular trafficking. The TNT-mediated in vivo transfer of MSC mitochondria was also observed in a rat model of cigarette smoke-induced chronic obstructive pulmonary disease (COPD). In this model, human MSCs were demonstrated to protect from alveolar destruction through mitochondrial transfer to the rat airway epithelial cells, moreover, with a higher efficiency for the iPSC-MSCs than for the BM-MSCs [123].

Lung alveolar macrophages were also shown to acquire MSC mitochondria, which lead to an enhancement of their phagocytic activity and, thus, contributed to the MSC antimicrobial effect in a murine model of *E. coli*-induced pneumonia [33]. The in vivo mitochondrial transfer from the endogenous murine bone marrow stromal cells was demonstrated as well in a NSG immunodeficient murine model of xenograft of human AML cells, on the basis of the expression of the mtDNA-encoded murine Co2 RNA [48].

The transfer to target cells of mitochondria, isolated beforehand from cells, was also demonstrated in vivo in a rabbit model of regional ischemia [53]. The injection of autologous mitochondria (~10^7 mitochondria) at the site of ischemia lead to their internalization within 8 hours of their administration and resulted, among other phenotypes, in reduced apoptosis and infarct size, as detected 4 weeks later [53].

5. Functional Outcome of MSC Mitochondrial Transfer to Target Cells

5.1. Effect on Metabolism. The transfer of MSC mitochondria to A549 ρ° adenocarcinoma cells, following their coculture, led to the recovery of the mitochondrial function in these cells, including O_2 consumption [112]. The metabolic effects of the acquired mitochondria were demonstrated in a number of studies in vitro. The coculture of MSCs and endothelial cells (HUVEC) was found to lead to an increase of HUVEC basal and maximal oxygen consumption, while glycolysis and lactate production were concomitantly reduced [34]. A similar effect (increase in OXPHOS, decrease in glycolysis) was observed for MDA-MB-231 breast cancer cells that had acquired human MSC mitochondria. These data were obtained using the technique of MitoCeption that allows a quantitative transfer of mitochondria, isolated beforehand from the MSCs. It showed a dose-response effect of the MSC mitochondria on the cancer cell metabolism [35]. In addition, the concentrations of both the endogenous mitochondrial DNA and the produced ATP were increased [35]. Similar increases in ATP concentrations were also reported in acute myeloid cells following their coculture with bone marrow-derived MSCs (both human and murine) [48]. In addition, a beautiful study of Islam and collaborators demonstrated in vivo, in a LPS-induced lung injury model and using single-cell ATP determination, that ATP concentrations increased in the alveoli cells that had received MSC mitochondria and, thereafter, spread to adjacent alveoli [32].

5.2. Effect on Cell Function and Survival in Response to Therapy. The acquisition of human vascular smooth muscle

cell (VSMC) mitochondria by human MSCs resulted in the increase of MSC proliferation rate [9]. Such an enhancement of the capacity of cellular proliferation, as well as invasion, was also observed for the MDA-MB-231 breast cancer cells after acquisition of human MSC mitochondria [35]. Acquisition of MSC mitochondria induced cardiomyocyte reprogramming to a progenitor state, characterized notably by markers such as GATA-4, myocyte enhancer factor 2C, and Nkx2.5 [30].

In a rabbit ischemia model, the injection of autologous mitochondria at the site of ischemia resulted in their internalization by cardiomyocytes and in increased cell survival [53]. In the two mouse models of acute lung injury following LPS [32] or rotenone [31] treatments, airway instillation of MSCs and MSC mitochondrial transfer to alveoli resulted in alveoli functional rescue and mice survival. MCF7 breast cancer cells with acquired endothelial cell mitochondria were reported to display increased resistance to doxorubicin [36]. Acquisition of exogenous mitochondria by AML cell allowed them to maintain their overall mitochondrial membrane potential and increased their survival rate in response to ARA treatment [48]. This increased survival was also observed for the leukemia-initiating cells (LICs) that play a major role in AML relapse [48].

6. Conclusion and Perspectives

Tunneling nanotubes appear henceforth to constitute a widespread means of communication between cells that can lay close-by or far apart. This communication process is used by many cell types, allowing the trafficking of many different cargoes between these cells. This TNT-mediated cell-to-cell exchange can contribute to the cell homeostasis, to the spontaneous tissue repair, to the spreading of pathologies, and to the resistance to therapies.

As detailed in this review, mesenchymal stem cells are particularly prone to establish these TNT connections with target cells. Numerous studies reported and characterized effects that the mitochondrial trafficking in these TNTs can have on the target cells be at the metabolic or functional levels. On a therapeutic point of view, at a first glance, these effects can be beneficial, when they lead for instance to tissue repair, but also detrimental, when they contribute to acquired resistance to therapy. Obviously, further work will be necessary to find the tools to enhance the first while hindering the second. The fact that mitochondria can be transferred spontaneously between cells or from preparation of mitochondria, isolated beforehand, will obviously open new paradigms for the available options to treat patients.

Abbreviations

ALL: Acute lymphoblastic leukemia
AML: Acute myeloid leukemia
ARA: Cytarabine or cytosine arabinoside
AT-MSC: Adipose tissue-derived mesenchymal stem cells
BM-MSC: Bone marrow-derived mesenchymal stem cells
CM: Cardiomyocytes
COPD: Chronic obstructive pulmonary disease
Cx43: Connexin 43
DC: Dendritic cells
DOXO: Doxorubicin
EPC: Endothelial progenitor cells
ETO: Etoposide
FBS: Fetal bovine serum
GTPase: Guanosine triphosphatase
HIV: Human immunodeficiency virus
HMEC-1: Human microvascular endothelial cells
ICAM-1: Intercellular adhesion molecule 1
IDO: Indoleamine 2,3-dioxygenase
iNOS: Inducible nitric oxide synthase
IRSp53: Insulin receptor substrate of 53 kDa
LIC: Leukemia-initiating cell
LPS: Lipopolysaccharide
LSCC: Laryngeal squamous cell carcinoma
LST1: Leukocyte-specific transcript 1
MCMAM: Melanoma cell adhesion molecule
MSCs: Mesenchymal stem cells
Mt-Co2: Mitochondrially encoded cytochrome c oxidase II
mtDNA: Mitochondrial DNA
MVs: Microvesicles
Myo10: Myosin-X
NK: Natural killer
NOD: Nonobese diabetic
NRK: Normal rat kidney
NSG: NOD scid gamma
OGT: O-GlcNAc transferase
PC12: Rat pheochromocytoma
PGE2: Prostaglandin E2
RPTEC: Renal proximal tubular epithelial cells
SNP: Single-nucleotide polymorphism
TNFaip2: Tumor necrosis factor, alpha-induced protein 2
TNT: Tunneling nanotube
TSG-6: TNF-α-stimulated gene/protein 6
VASP: Vasodilator-stimulated phosphoprotein
VEGF: Vascular endothelial growth factor
VINCRI: Vincristine
VSMC: Vascular smooth muscle cell.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Review Article

Mitochondrial Dynamics: In Cell Reprogramming as It Is in Cancer

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Somatic cells can be reprogrammed into a pluripotent cellular state similar to that of embryonic stem cells. Given the significant physiological differences between the somatic and pluripotent cells, cell reprogramming is associated with a profound reorganization of the somatic phenotype at all levels. The remodeling of mitochondrial morphology is one of these dramatic changes that somatic cells have to undertake during cell reprogramming. Somatic cells transform their tubular and interconnected mitochondrial network to the fragmented and isolated organelles found in pluripotent stem cells early during cell reprogramming. Accordingly, mitochondrial fission, the process whereby the mitochondria divide, plays an important role in the cell reprogramming process. Here, we present an overview of the importance of mitochondrial fission in both cell reprogramming and cellular transformation.

1. Introduction

Mitochondria and their movement as organelles were described for the first time 100 years ago [1]. In addition to producing energy by oxidative phosphorylation (OXPHOS) of pyruvate and beta-oxidation of lipids, the mitochondria play important roles in the regulation of a wide variety of intracellular processes, such intracellular calcium homeostasis [2], iron-sulfur protein assemblage [3], or apoptosis [4] and innate immunity cell signaling pathways [5].

There is no de novo mitochondrial biogenesis; the mitochondria divide by fission and join by fusion [6, 7]. Fission-fusion balance allows the mitochondria to acquire different structures. When fission is higher than fusion, mitochondria become fragmented and isolated. When fusion is higher than fission, these organelles display a tubular and networked morphology. Cells can shift the fission/fusion balance in response to either intracellular or extracellular stimuli. And thus, mitochondrial fission is increased during (1) G2/M phase of cell cycle, to guarantee an accurate mitochondrial segregation between the two daughter cells during cell division [8, 9]; (2) mitochondrial transport in neurons, to facilitate their transport along the axons and dendrites [10]; (3) early phase of apoptosis, to facilitate cytochrome c release into the cytoplasm by inducing mitochondrial cristae remodeling [11, 12]; or (4) mitophagy, to eliminate dysfunctional mitochondria [13]. On the other hand, mitochondrial fusion is favored during (1) G1/S transition of cell cycle, to provide with the necessary energy for DNA synthesis [14]; (2) cell survival during starvation, to maximize energy production and protect themselves against mitophagy [15, 16]; (3) mitochondrial complementation, to avert the loss of mitochondrial functions caused by damaged components of these organelles [17, 18]; or (4) embryonic development, as in trophoblast or placenta formation [19, 20]. Regulation of mitochondrial dynamics is therefore crucial for the correct implementation of mitochondrial functions. In fact, mutations in the components that drive or regulate fusion and fission processes are associated with several human pathologies, such as optic atrophy (*Opa1* gene) or Charcot-Marie-Tooth disease (*MFN2* and *GDAP1* genes) [18].

The molecular machinery that controls the fission and fusion processes includes proteins that are either localized in mitochondrial membranes or recruited to the surface of these organelles in response to different stimuli. Three key

players of the fusion process are mitofusin (Mfn) 1 and 2 and optic atrophy protein 1 (Opa1), both of which are transmembrane proteins localized in the outer or inner mitochondrial membranes, respectively. Mfn1 and Mfn2 tether adjacent mitochondria by forming trans-hetero- or homocomplexes to promote the fusion of their outer membranes [17, 19]. It has been suggested that a heptad repeat region in Mfn1 adopts an antiparallel coiled coil conformation to tether neighboring mitochondria during the fusion process [21]. Cells that lack both Mfn1 and Mfn2 display fragmented mitochondria and fail in mitochondrial complementation [19, 22], which eventually leads to an accumulation of dysfunctional mitochondria [17]. Fusion of outer and inner mitochondrial membranes is a temporally linked, multistep process controlled by transmembrane adaptor proteins that span both membranes [23]. Mfn1 and Mfn2 interact with Opa1 [24], suggesting that the interaction of Mfn1/2 with Opa1 and/or other adapters physically connects both membranes to coordinate the fusion of these organelles [25]. The fission process is executed by dynamin-related protein 1 (Drp1), a cytosolic protein with GTPase activity [26, 27]. Drp1 is activated in the cytosol by posttranslational modifications in response to different stimuli and then recruited to the mitochondrial surface by its interaction with protein adapters [28, 29]. Mitochondria-recruited Drp1 oligomerizes on the external surface of mitochondria forming a ring-shaped structure around the organelle. Once a Drp1 spiral around the mitochondria is completed, the hydrolysis of GTP bound to Drp1 causes a conformational change in the protein that causes the constriction of the ring, eventually leading to the fragmentation of mitochondria in two different organelles [30, 31]. Different protein adapters for Drp1 have been described, including mitochondrial fission protein 1 (Fis1) [28], mitochondrial fission factor (Mff) [32], and mitochondrial dynamic proteins of 49 (Mid49) and 51 (Mid51) kDa [33, 34]. Recent work has shown that these Drp1 adapters could either operate together or be redundant in the recruitment of the GTPase to the mitochondria [35, 36].

Mitochondrial dynamics, in terms of the fission/fusion balance, is a highly regulated process where posttranslational modifications play a central role in the outcome of this equilibrium. Phosphorylation Mfn1 by extracellular regulated kinase 1/2 (Erk1/2) impairs its oligomerization properties and leads to decreased mitochondrial fusion [37]. Also, phosphorylation of Mfn2 by c-Jun N-terminal kinase (Jnk) results in its ubiquitin-mediated proteasomal degradation, leading to increased mitochondrial fragmentation [38]. Opa1 undergoes proteolytic processing by several proteases to produce short and long protein isoforms [39–41]; however, it is poorly understood how this proteolytic processing alters mitochondrial dynamics [42]. It is known that alterations in Opa1 proteolysis affect inner mitochondrial membrane dynamics and cristae structure [43]. Drp1 is target of several posttranslational modifications that affect its function: phosphorylation, ubiquitination, sumoylation, and nitrosylation [7]. Regarding its phosphorylation, only the phosphorylation in three Drp1 residues by different kinases has been well documented to play a role in the regulation of this protein: serine 579 (serine 616 in humans), serine 600 (serine 637 in humans), and serine 656 (serine 693 in humans). Phosphorylation of any of these three residues affects Drp1 protein-protein interactions and can either impair or favor the mitochondrial recruitment of Drp1. It has been described that Ser579 phosphorylation induces mitochondrial fission [9, 44–48]; Ser656 phosphorylation induces mitochondrial fusion [49]; and Ser600 phosphorylation induces either mitochondrial fission [50, 51] or fusion [15, 48, 52–55], depending on the cellular context (Figure 1). Recently, it has been described that AMP-activated protein kinase (Ampk) induces mitochondrial fission in response to energy stress through direct phosphorylation of Mff [56].

Endoplasmic reticulum (ER) also plays an important role during mitochondrial fission. It has been shown that ER projections wrap mitochondria around the areas where fragmentation of these organelles takes place. These ER-mitochondria contacts are not Drp1-dependent, but rather enhance the recruitment of the GTPase to these focal points [57, 58]. ER-associated inverted formin 2 (Inf2) plays an important role in mitochondrial fission by inducing the accumulation of actin filaments around the ER-mitochondria contact points. The distribution of actin filaments around the mitochondria at the ER contact points may drive an initial mitochondrial constriction to favor the action of mitochondrion-bound Drp1 [58]. Also, a profission role for ganglioside-induced differentiation-associated protein 1 (Gdap1) has been proposed, as Gdap1 favors the formation of ER-mitochondria contacts in certain neural cell types and its overexpression leads to fragmented mitochondria [59–61].

2. Mitochondrial Dynamics during Embryonic Development and Cell Differentiation

As the oocyte provides all the mitochondria to the zygote during fecundation, all these organelles are of maternal origin. During the first phase of embryonic development, mitochondrial biogenesis and mtDNA synthesis are not active and mitochondrial mass decays by half upon each cell division [62]. During the early stages of development, cells have a simple mitochondrial network: cristae-poor and fragmented mitochondria with low mtDNA copy number. Conversely, the mitochondrial network of somatic cells shows a complex structure: cristae-rich and tubular mitochondria with a dense mitochondrial matrix and high mtDNA copy number [62–66].

Cell differentiation during embryogenesis leads to a progressive increase in mtDNA copy number, mitochondrial mass, size, and complexity of these organelles [67–69]. For instance, the specification of cardiomyocyte [70, 71] or adipocyte [72] cell lineages is characterized by an increase in the elongation, matrix complexity, and functionality of mitochondria. Also, during cardiomyocyte differentiation, the closure of the mitochondrial permeability transition pore increases mitochondrial membrane potential and reduces reactive oxygen species (ROS) levels [71]. Mfn2 and Opa1 play an important role in this mitochondrial maturation

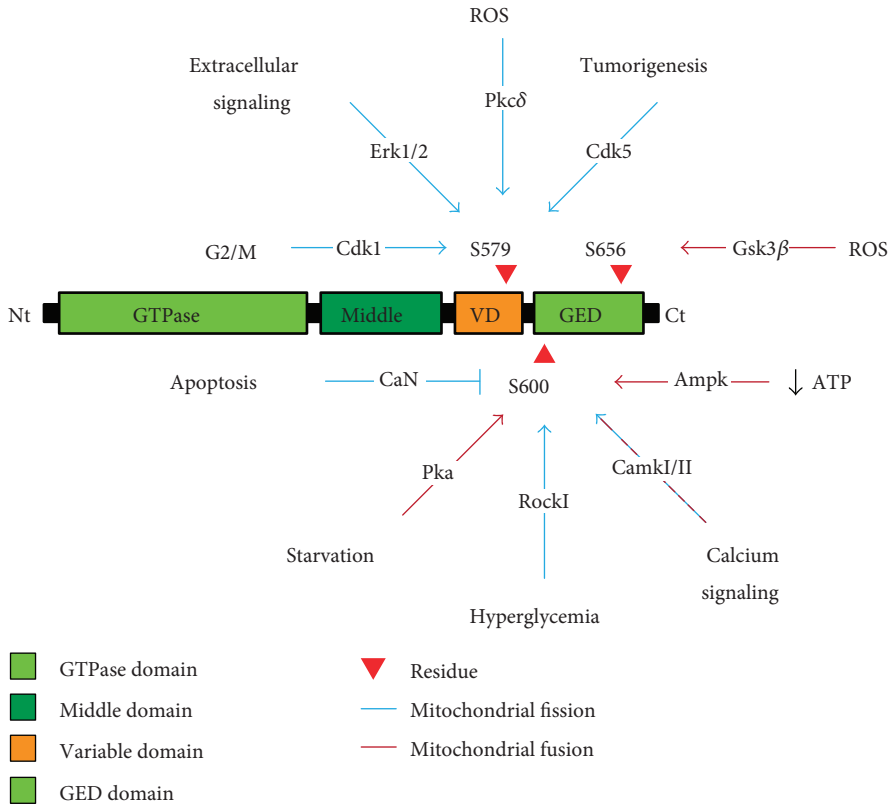

Figure 1: Drp1 regulation by phosphorylation. Phosphorylation of Ser579 by Cdk1 [9, 44], Pkcδ [45], Erk1/2 [46, 47], or Cdk5 [48] induces mitochondrial fission. Phosphorylation of Ser656 by Gsk3β [49] induces mitochondrial fusion. Phosphorylation of Ser600 by Pka [15, 52, 53], CamkII [48], or Ampk [55] induces mitochondrial fusion, and its dephosphorylation by calcineurin (CaN) [53, 54] induces mitochondrial fission, but, in some circumstances, phosphorylation of Ser600 by CamkI [50] or RockI [51] can induce mitochondrial fission.

process. Lack of *Mfn2* or *Opa1* prevents mitochondrial fusion leading to an increase in cytosolic calcium levels and calcineurin activation, which impairs efficient cardiomyocyte differentiation [73]. Mitochondrial integrity, in terms of energetics, Ca^{2+}-storage/buffering, neurotransmitter metabolism, or ROS signaling, plays a central role in neuronal physiology during both development and adulthood [74]. Interestingly, a wide range of neurodegenerative diseases, such as Charcot-Marie-Tooth disease, Parkinson's disease, or several ataxias, are linked to mutations in gene-encoding proteins involved in mitochondrial dynamics, underscoring the role of this equilibrium in maintaining neuronal homeostasis [75].

The low rate of mtDNA replication observed during the very early stages of embryonic development and in embryonic stem (ES) cells is associated with high methylation levels in the genes encoding DNA mitochondrial polymerase subunit gamma [76] and mitochondrial transcription factor A (*Tfam*) [77], which impairs their expression in cells of the early embryo. However, demethylation of these genes is induced upon implantation of the embryo, leading to an increase in their expression and mtDNA replication.

Despite the profound changes experimented by the mitochondrial network during cell differentiation, the regulation of mitochondrial dynamics in ES or adult stem cells is poorly understood. ES cells present a fragmented mitochondrial morphology [65, 78]. Surprisingly, downregulation of growth factor *erv1*-like (*Gfer*) in ES cells, which leads to an increase in Drp1 protein levels and mitochondrial fission rates, impairs pluripotency and induces apoptosis [79]. On the other hand, adult neural stem (NS) cells display tubular mitochondrial morphology and NS cells derived from *Mfn1/2*- or *Opa1*-null mice, which display increased mitochondrial fragmentation, show decreased self-renewal and a greater tendency to cell commitment associated with augmented ROS and Nfe2-related factor 2 (Nrf2) expression levels [80]. These evidences strongly suggest that a proper balance of mitochondrial fission and fusion is required to maintain a homogeneous and functional mitochondrial population in the cells.

3. Mitochondrial Dynamics in Cell Reprogramming

Somatic cells can be reprogrammed to a pluripotent state similar to that of ES cells by ectopic expression of Oct4, Sox2, Klf4, and c-Myc (OSKM hereinafter) [81]; chemical treatment [82]; or nuclear transfer [83–85]. The pluripotent nature of the resultant cells makes them a formidable tool for (1) studying embryonic development [86], (2) producing genetically modified animals [87, 88], (3) establishment of in vitro models of genetic diseases [89], and (4) developing

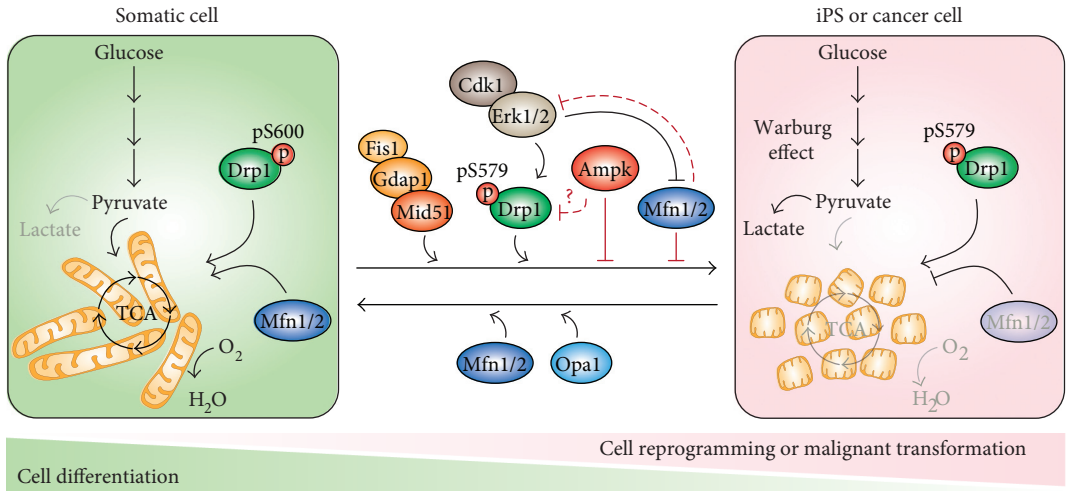

FIGURE 2: Mitochondrial dynamics in somatic, pluripotent, and cancer cells. Model illustrating the factors involved in the regulation of mitochondrial dynamics and metabolism in somatic, pluripotent (iPS), or cancer cells. The roles played by the indicated factors in favoring (forward arrow) or impairing (reverse arrow) cell reprogramming are also shown.

new therapies in regenerative medicine [90]. Among the different approaches, OSKM-induced somatic cell reprogramming has become the most widespread technique due to its high reproducibility, applicability to human samples, and simplicity of the process.

OSKM-induced cell reprogramming constitutes an organized sequence of events that starts with the downregulation of somatic cell markers [91]. Then, activation of cell proliferation [92], induction of a metabolic switch from OXPHOS to glycolysis [65], and a mesenchymal-to-epithelial transition (MET) [93, 94] follow. Finally, the process culminates with cellular immortalization [95–100] and upregulation of core pluripotency markers, such *Oct4* or *Nanog* [91, 101]. Also, there is a global erasure of the somatic epigenetic signature during the reprogramming process, which is undertaken by histone-modifying [102–104] and DNA-modifying [105] enzymes. It has been shown that this erasure of the somatic epigenetic marks is increased sequentially cycle after cycle during cell proliferation due to a dilution effect upon cell division [92, 106, 107].

Three seminal studies have demonstrated that the changes undertaken by somatic cells during OSKM-induced cell reprogramming are organized in two consecutive waves [108–110]. The first wave, called stochastic phase, is associated with changes in cell cycle, DNA replication, and MET. The second wave, named deterministic phase, is associated with the total reactivation of the transcriptional core of pluripotency. These studies have revealed that the low efficiency of the process is due to the fact that some of the starting somatic cells are refractory to cell reprogramming and remain trapped as cell intermediates.

In contrast to cell differentiation during embryogenesis, it has been suggested that mitochondrial dynamics follows a reverse pathway during cell reprogramming: mitochondria rejuvenate and become fragmented [64, 111], their functionality as energy-producing organelles is reduced [65, 112], and mtDNA replication is decreased [113]. Although it has been suggested that Drp1 does not play a role during OSKM-induced somatic cell reprogramming [114], several reports indicate that this protein plays a key role in this process [78, 115, 116]. In this regard, we have observed an increase in both Drp1 total protein and Drp1-S579 phosphorylation during the stochastic phase of cell reprogramming. During early reprogramming, Erk1 and Erk2 are activated as a consequence of the downregulation of the Dusp6 protein phosphatase. Activated Erk1/2 phosphorylate Drp1-S579, which induces its recruitment to mitochondria and triggers mitochondrial fission during the stochastic phase of cell reprogramming [78] (Figure 2). In addition to Erk1/2, cyclin-dependent kinase 1 (Cdk1) could also participate in the phosphorylation of Drp1-S579 during early cell reprogramming [9]. In fact, it has been observed that the transcriptional factor associated to pluripotency reduced expression protein 1 (REX1) activates *cyclin B* expression in human ES cells. This upregulation activates CDK1/cyclin B complex, leading to an increase in Drp1-S579 phosphorylation and mitochondrial fragmentation. *REX1*-null ES cells show tubular mitochondrial morphology and a decreased self-renewal capacity [116]. Also, pluripotent mouse ES cells lacking a functional *Drp1* gene have been derived by homologous recombination [117] (Figure 2). Although *Drp1* knockout mice show major defects in embryonic development and synapsis formation, *Drp1*-null ES cells maintain pluripotency and self-renewal capacities. *Drp1* knockout cells display a tubular mitochondrial morphology and a lower proliferation rate. Surprisingly, lack of *Drp1* gene does not affect cytokinesis. Given the central role played by Drp1 in mitochondrial fission and that this process is critical to assure an equal distribution of these organelles between the two daughter cells in each cell division, the results obtained by Ishihara and colleagues were puzzling. To circumvent this conundrum, Ishihara and colleagues suggested that unknown mechanical forces could play a role in the uneven segregation of mitochondria between the two daughter cells during cell division.

Conversely to the conventional idea of mitochondrial fission as a mechanism for assuring the equal distribution of mitochondria between the two daughter cells during cytokinesis, a recent report showing that mitochondrial fission also drives the asymmetrical distribution of these organelles during cell division of stem-like cells adds an additional layer of complexity to the physiological roles already ascribed to the fragmentation of these organelles. Interestingly, this asymmetrical distribution of mitochondria depends on the quality of the organelles and whereas aged or deficient organelles are segregated to the more differentiated daughter cell, healthy mitochondria are retained by the resultant stem-like cell upon cytokinesis [118]. Interestingly, this asymmetric segregation of mitochondria contributes to maintain a homogenous and healthy population of stem-like cells, which could be considered as a sort of selfish self-renewal. It would be interesting to investigate whether this unequal segregation of mitochondria takes places under normal and/or pathological conditions in vivo.

Compared to somatic cells, ES cells show low levels of *Mfn1/2* expression [78, 119]. Interestingly, *Mfn1/2* knockout cells display a faster and higher efficiency of cell reprogramming due to an increase of mitochondrial fragmentation and cell proliferation. Also lack of *Mfn1/2* favors Erk1/2 activation, which may improve Drp1-S579 phosphorylation by these MAP-kinases [119]. Furthermore, Erk1/2-mediated phosphorylation of Mfn1 causes its inactivation [37]. Thus, in addition to increase mitochondrial fission through Drp1 phosphorylation [78], Erk1/2 activation during early cell reprogramming may inhibit mitochondrial fusion through Mfn1 phosphorylation (Figure 2).

Recently, it has been described that other proteins involved in mitochondrial fission, such as Mid51 or Gdap1, are also important for cell reprogramming [120]. Downregulation of any of these two proteins reduces cell reprogramming efficiency. Interestingly, *Gdap1* knockout cells displayed a lower reprogramming efficiency due to a defect in triggering mitochondrial fragmentation during the process. The failure to undergo an efficient mitochondrial fission by *Gdap1*-null cells during cell reprogramming induced a DNA damage-independent G2/M arrest (Figure 2).

Important similarities between cell reprogramming and cellular transformation do exist [105]. In this regard, a similar role for mitochondrial fission in tumorigenesis has been proposed [121]. As it happens during cell reprogramming, some cellular transformation processes are associated with MET [122] and changes in mitochondrial morphology: from a tubular network to fragmented and isolated mitochondria. As observed in ES cells, lung [123], gastric [124], breast [125, 126], glioblastoma [127], colorectal [128], neuroblastoma [129], ovarian [130], pancreatic [46], and melanoma [47] cancer cells display high levels of *Drp1* and low amounts of *Mfn1/2* gene expression. Accordingly, inhibition of *Drp1* expression or overexpression of *Mfn1/2* results in a marked reduction of cancer cell proliferation and an increase in spontaneous apoptosis [123–126, 128]. Some other cancer cells are characterized by either a decrease or an increase in *Mfn1/2* and *Fis1* expression levels, respectively [131, 132] (Figure 2).

Furthermore, Drp1 regulation during cellular transformation seems to be similar to that of cell reprogramming. Erk1/2 inhibition in transformed cells decreases Drp1-S579 phosphorylation levels, elongates mitochondria, and reduces cell proliferation and the capability of tumor formation [46, 47]. In lymphoblastic leukemia cells, Erk1/2 triggers Drp1-dependent mitochondrial fission to reduce ROS and enhance glycolysis for protecting cells against chemotherapeutic agents. In this regard, activation of ERK signaling by constitutive expression of a constitutively active K-Ras mutation confers on cells a large degree of phenotypic plasticity that promotes their neoplastic transformation and the acquisition of stem cell-like characteristics [133]. Brain tumor-initiating (BTI) cells display fragmented mitochondria. BTI cells show high levels of DRP1-S579 phosphorylation and targeting DRP1 using RNA interference or pharmacologic inhibition induced apoptosis in BTI cells and inhibited tumor growth [48]. Finally, and similar to *Gdap1*-null cells during cell reprogramming, a knockdown of *Drp1* in lung and breast cancer cells induces a DNA damage-dependent G2/M arrest [134] (Figure 2).

4. Mitochondrial Fission and Mitophagy in Cell Reprogramming

Mitochondrial fission is necessary for mitophagy, whereas mitochondrial fusion impairs this mitochondrial-specific form of autophagy [135]. As it has been described in a mitochondrial clearance during cell reprogramming [111, 136, 137], some studies have suggested that mitophagy could be involved in this reduction in mitochondrial mass and therefore play a positive role in the reprogramming process [138, 139]. Accordingly, an induction of autophagy has been shown to increase cell reprogramming efficiency [140] and an early and transitory activation of this process has been observed to take place very early in cell reprogramming to reduce mitochondrial mass [137]. However, new studies have put into question these results. Work by three different laboratories demonstrated that Lc3b/Atg5-dependent autophagy is not responsible for the mitochondrial clearance observed during cell reprogramming [78, 141, 142]. Furthermore, observations by two additional laboratories showed that ES cells have a mitochondrial mass/total protein ratio similar to that of somatic cells [143, 144]. Thus, it seems improbable that an active reduction of mitochondrial mass by mitophagy is taking place during the cell reprogramming process. Altogether, these observations suggest that, beyond its role in the constant turnover of dysfunctional mitochondria, mitophagy seems not necessary for cell reprogramming. It is nonetheless possible that the absolute reduction of mitochondrial mass could be due to an adaptive process to the new culture conditions required to maintain pluripotency [78] or through a Lc3b/Atg5-independent autophagic pathway [141]. Paradoxically, the hypothesis of a passive mitochondrial clearance during successive rounds of cell division would require the generation of new mitochondria to maintain a proper distribution of these organelles between the two daughter cells. In keeping with this idea, it has been reported that mitochondrial biogenesis markers are induced during cell

reprogramming [78, 111]. The role of autophagy, in general, and mitophagy, in particular, in the maintenance of pluripotency is poorly understood; however, some studies point that autophagy activation is more important during cell differentiation than during the acquisition of the pluripotent state [145]. Further, chemical activation of Ampk reduced cell reprogramming efficiency [146], and induction of autophagy is associated with an increase in Ampk activation [147]. Interestingly, it has been described that Ampk phosphorylates Drp1-S600, which impairs Drp1 function and mitochondrial fission [55, 148, 149]. Proteomic analysis of Drp1 in mouse ES cells revealed the absence of this posttranslational modification in both mitochondrial and cytosolic fractions of the protein under self-renewal culture conditions [78]. Although it has not been described a role for this phosphorylation in cell reprogramming, it may be similar to that found in cancer cells, where dephosphorylation of Drp1-S600 has been associated to tumor progression [48]. In agreement with this, Ampk activation seems to inhibit cancer progression [150–152]. Further research may shed light into the role of Ampk-mediated Drp1-S600 phosphorylation during the early stages of cell reprogramming (Figure 2).

5. Conclusions

In addition to changes in mitochondrial morphology, cell reprogramming induces a metabolic switch: from an oxidative-somatic state to a glycolytic-pluripotent state [153]. This metabolic remodeling presents several similarities with the Warburg effect observed in cancer cells [154]. In fact, there are many processes in which an increase of mitochondrial fission goes along with an activation of glycolysis and a decrease of OXPHOS [155]. The observed changes in both mitochondrial morphology and metabolism seem to be key for cell reprogramming and during the early events of tumorigenesis. Altogether, published data suggest a close parallelism between the stochastic phase of cell reprogramming and cellular transformation [105]. The similarities between both processes reveal that any advance in the control of induced pluripotency will not only help to manage properly this powerful tool for its biomedical application but also to better understand the early events that take place during the development of human malignancies. Interestingly, in vivo cell reprogramming is emerging as an alternative approach to regenerative medicine that does not require cell transplantation [156–159]. Given the importance of mitochondrial dynamics for somatic cell differentiation and dedifferentiation, this mitochondrial process is likely to play a key role in cell fate remodeling during in vivo cell reprogramming. It is therefore possible that the discovery of new techniques to locally modulate mitochondrial dynamics in a specific set of cells, combined with partial in vivo cell reprogramming, will set the grounds for developing novel mitochondria-based therapeutic approaches to improve human welfare.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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Research Article

Mitochondrial DNA Hypomethylation Is a Biomarker Associated with Induced Senescence in Human Fetal Heart Mesenchymal Stem Cells

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Background. Fetal heart can regenerate to restore its normal anatomy and function in response to injury, but this regenerative capacity is lost within the first week of postnatal life. Although the specific molecular mechanisms remain to be defined, it is presumed that aging of cardiac stem or progenitor cells may contribute to the loss of regenerative potential. *Methods*. To study this aging-related dysfunction, we cultured mesenchymal stem cells (MSCs) from human fetal heart tissues. Senescence was induced by exposing cells to chronic oxidative stress/low serum. Mitochondrial DNA methylation was examined during the period of senescence. *Results*. Senescent MSCs exhibited flattened and enlarged morphology and were positive for the senescence-associated beta-galactosidase (SA-β-Gal). By scanning the entire mitochondrial genome, we found that four CpG islands were hypomethylated in close association with senescence in MSCs. The mitochondrial COX1 gene, which encodes the main subunit of the cytochrome c oxidase complex and contains the differentially methylated CpG island 4, was upregulated in MSCs in parallel with the onset of senescence. Knockdown of DNA methyltransferases (DNMT1, DNMT3a, and DNMT3B) also upregulated COX1 expression and induced cellular senescence in MSCs. *Conclusions*. This study demonstrates that mitochondrial CpG hypomethylation may serve as a critical biomarker associated with cellular senescence induced by chronic oxidative stress.

1. Introduction

The adult mammalian heart has traditionally been viewed as a nonregenerative organ as it retains very minimal regenerative potential. Following cardiac injury, as in the case of myocardial infarction, the heart fails to replace the vast majority of lost or damaged cardiomyocytes. Instead, the heart heals with scar tissue, leading to a contractile defect of the organ and ultimately heart failure [1]. However, the hearts of one-day-old mouse retain full regenerative potential and are able to restore normal anatomy and function after cardiac injury [2]. This regenerative capacity of the neonatal mouse heart, however, becomes lost within the first week of postnatal life. Decreases in cardiac stem or progenitor cells could be one of the determining factors for the loss of regenerative capacity in this aging process [3].

Mesenchymal stem cells (MSCs) have attracted great interest as a promising regenerative therapeutic for many human diseases, primarily because of their capacity for self-renewal, multilineage differentiation, and immune modulation [9–13]. MSCs derived from fetal heart (c-MSCs, HMSCs) have the potential to differentiate into cardiomyocytes, endothelial cells, and smooth muscle cells [3, 14–16]. Engraftment of fetal cardiac MSCs improves cardiac function and can repair myocardium in a rat model of myocardial infarction [15]. However, to serve as a useful regenerative therapy, MSCs isolated from patients need to be expanded ex vivo [17]. MSCs have a limited lifespan in cell culture,

and after a few passages of expansion, the cells enter the senescent state, leading to the reduction in self-renewal ability and differentiation potential [18, 19]. Moreover, the self-renewal potential of MSCs is significantly reduced with aging. Like many primary cells in culture, MSCs show decreased proliferation and increased apoptosis as the age of the donor animal or human [20–24]. As MSCs approach senescence, their proliferation slows significantly, leading to a decrease in differentiation potential [25]. Therefore, it is critical to understand the mechanisms that control replicative senescence in MSCs.

Replicative senescence, a process that places cells in permanent proliferative arrest in response to various stressors, is a potentially important contributor to aging and age-related disease. It is clear that cellular senescence is associated with an array of epigenetic modifications that may be responsible for changes in gene expression that ultimately lead to catabolic and degenerative processes. Mitochondrial DNA (mtDNA) damage has long been implicated in the aging process [26]. A number of mitochondrial signaling pathways can induce cellular senescence [27]. However, the role of mitochondrial epigenetics has largely been unexplored. In fact, the importance and relevance of mitochondrial epigenetics in aging has been controversial [28], primarily because of the difficulty of studying the relatively small mitochondrial genome in the context of the far larger nuclear genome.

Methylation of mtDNA in a variety of tissues varies with aging, disease states, environmental exposure, and certain drugs. In addition to 5-methylcytosine, a so-called "sixth base," 5-hydroxymethylcytosine has also been identified in mtDNA, where its abundance changes during aging independently of 5-methylcytosine levels [29]. It has been suggested that mtDNA methylation might become a next-generation biomarker for aging [30]. Currently, we know very little about the role of mtDNA methylation in the aging-related dysfunction of cardiac stem or progenitor cells. To gain insight regarding the role of mitochondrial epigenetics in senescence, we induced senescence in MSCs cultured from human fetal heart tissues and examined epigenetic mechanisms that may be associated with cellular aging in MSCs.

2. Materials and Methods

2.1. Isolation of MSCs. Isolation of MSCs from fetal heart tissues was performed following the method as described previously [3, 16] with some modifications. Briefly, fetal heart tissues from days 45–67 of embryos were obtained from Human Tissue Network (Central Laboratory for Human Embryology Tissue, University of Washington, WA) in phosphate-buffered saline (PBS). Upon arrival, the tissues were minced with a razor into small pieces. To avoid destroying the cardiac stem cell niche, we did not digest the tissue with collagenase. Instead, the minced heart tissues were directly seeded on a 10 cm plate with a small amount of DMEM (2-3 ml) containing 10% fetal calf serum (FCS) and antibiotics and were cultured at 37°C in a 5% CO_2 humidified atmosphere. After the heart tissue became attached to the plate, a small amount of fresh medium was added to the plate without disturbing the tissues on a daily basis. After 5–7 days, MSCs began to migrate out from the heart explants and the MSCs were collected for further expansion. MSCs at passages 3–5 were used for analysis. MSCs from fetal skin tissues were isolated using the same approach and were used in parallel with HMSCs in the study. The experimental protocol was approved by the Institutional Review Board/Stem Cell Research Oversight Panel at Stanford University.

2.2. Characterization of MSCs. The MSCs cultured from fetal heart (HMSCs) were characterized by flow cytometry using stem cell markers as previously described [31]. Cells were collected at a concentration of 1×10^6 cells/ml in phosphate-buffered saline (PBS) containing 0.1% BSA. Cells were incubated at 4°C with antibodies against MSC-markers (CD90, CD73, and CD105), hematopoietic cell markers (CD45, CD34, CD14, and CD19), and receptors for extracellular matrix (CD29, CD44) and major histocompatibility (HLA-DR) (all from BD Biosciences, CA). Thirty minutes after antibody incubation, cells were washed and suspended in 300 μL PBS. Flow cytometry was performed using FACSCAria III Cell Sorter (BD Biosciences, CA) [32–35].

2.3. Differentiation of MSCs. The differential potential of the isolated MSCs into adipogenic and osteogenic lineages was performed as described previously [32–35]. After induction, cells were stained with the Oil Red O and Alizarin Red (Sigma, USA) to detect the presence of neutral lipid vacuoles in differentiated adipocytes and calcium deposition in osteocytes, respectively.

2.4. Induced Senescence in MSCs. To mimic the replicative senescence seen in MSCs [36, 37], we adopted a new approach by chronically exposing cells to low oxidative stress and a low serum environment. Specifically, HMSCs at passages 3–5 at approximately 50% confluence were continuously exposed to low concentration of hydrogen peroxide (50 μM H_2O_2) in DMEM supplemented with 5% FBS. Using this approach, MSCs exhibited a typical senescence-like morphology after 2-3 passages. Cells were collected for RNA-Seq analysis (Beijing Honor Tech Co., Ltd., Beijing, China).

2.5. Staining of Senescence-Associated β-Galactosidase. Cellular senescence was quantitated by measuring the activity of SA-β-Gal as previously described [38, 39]. MSCs were collected and fixed using 3% formaldehyde for 3–5 min at room temperature. After washing with PBS, cells were incubated at 37°C with freshly prepared senescence-associated SA-β-Gal staining solution: 1 mg/ml 5-bromo-4-chloro-3-indolyl P3-D-galactoside (X-Gal), 40 mM citric acid/sodium phosphate, pH 6.0, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, and 2 mM MgCl2. After overnight incubation, cells with the SA-β-Gal staining were assessed using a microscope-mounted camera.

2.6. Detection of Telomere Length in Aging Cells. The relative length of telomeres was estimated by quantitative PCR as previously reported [40, 41]. Briefly, genomic DNA was extracted with Qiagen DNeasy Blood & Tissue Kit (Qiagen,

CA, USA). After dilution, 35 ng/μl DNA was heated at 95°C for 5 min and chilled on ice for 5 min. The DNA samples were placed in a 20 μl real-time qPCR reaction system containing 10 μl 2 × SYBR premixed buffer (Roche, Shanghai, China), 2 μl forward and reverse primers. The sequence of primers includes (1) telomere forward: 5′-GGTTTTTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGT-3′ (100 nM) and reverse: 5′-TCCCGACTATCCCTATCCCTTCCCTATCCCTATCCCTA-3′ (300 nM); and (2) β-globin forward: 5′- GCTTCTGACACAACTGTGTTCACTAGC-3′ (150 nM) and reverse: 5′-CACCAACTTCATCCACGTTCACC-3′ (150 nM). The PCR amplification process was one cycle at 95°C 10 min and 40 cycles at 95°C for 15 s, 56°C for 30 s, and 72°C 30 s (ABI SepOnePlus, Beijing, China). The telomere length was estimated by the relative ratio between the copies of telomere and the copies of β-globin (T/S). T/S value is calculated by $2^{-\Delta\Delta Ct}$ [40, 41].

2.7. Gene Expression of the Senescence and the Cardiac Development Pathways. Comparison of the senescence and cardiac development pathway genes was performed by RNA-Seq [42]. Total RNA was isolated using Qiazol (Qiagen, CA) and used for indexed library preparation using Illumina's TruSeq RNA Sample Prep Kit v2. Libraries were sequenced using a HiSeq4000 (Illumina) and yielded approximately 34 million reads with a length of 150 bp per sample. Gene counts were normalized to the values of Reads Per Kilobase of transcript per Million mapped reads (RPKM). KEGG pathways were selected as significantly regulated if the corrected P values were smaller than 0.05 (Beijing Honor Tech Co.,Ltd., Beijing, China).

2.8. Measurement of mtDNA Methylation by COBRA. Mitochondrial and total cellular DNAs were extracted by DNeasy Blood & Tissue Kit (Qiagen, CA) and treated by sodium bisulfite using EZ DNA Methylation™ Kit (Zymo, CA), following the protocol provided by the manufacturer. Initially, we obtained mtDNA from isolated mitochondria and treated the DNA with sodium bisulfite for cloning sequencing. Later, we found that the protocol could be greatly simplified by simply using total genomic DNA with PCR primers that are specific for mitochondrial DNA.

Bisulfite-treated DNA was amplified by polymerase chain reaction (PCR) under liquid wax in a 6 μl reaction containing 2 μl of 3 × Klen-Taq I Mix, 2 μl template DNA, and 1 μl of each 2.5 μM primer. After incubation at 95°C for 5 min, DNA was amplified by 38 cycles of 95°C for 20 s, 62°C for 20 s of annealing, and 72°C for 20 s of extension and finally with extension at 72°C for 2 min. Methylation PCR primers sequences are listed in Table S1 (see Table S1 in the Supplementary Material available online at https://doi.org/10.1155/2017/1764549).

The status of mtDNA methylation was determined by restriction enzyme digestion. PCR DNAs were digested by Taq I at 65°C for 2 h or HpyCH4IV at 37°C for 2 h and separated on 3% agarose gel. Taq I recognizes the TCGA site and HpyCH4IV digests the ACGT site. After treatment with sodium bisulfate, unmethylated cytosines were converted to uracils [43, 44]. As a result, methylated mtDNA will be digested by the restriction enzymes. In contrast, unmethylated mtDNAs will be converted to TTGA and ATGT, which are not digested by Taq I and HpyCH4IV, respectively. The methylated and unmethylated bands were scanned for quantitation.

2.9. Knockdown of DNMTs by RNA Interference Lentiviruses. Two different RNA interference oligonucleotides targeting each DNMT gene were driven by H1 and U6 promoters, respectively, and were jointly subcloned into pGreenpuro lentiviral vector by PCR. The oligonucleotide sequences targeting DNMT1, DNMT3a, and DNMT3b are as follows: DNMT1 (A): 5′-GCCCAATGAGACTGACATCAA-3′; DNMT1 (B): 5′-GGAACCAAGCAAGAAGTGA-3′; DNMT3a (A): 5′-AGCGGGCAAAGAACAGAAG-3′; DNMT3a (B): 5′-CCAGATGTTCTTCGCTAATAA-3′; DNMT3b (A): 5′-CCTGTCATTGTTTGATGGCAT-3′; DNMT3b (B): 5′-CCATGCAACGATCTCTCAAAT-3′. The scramble control sequence is 5′-GCTTCAATTCGCGCACCTA-3′.

For viral packaging, 293 T cells were transfected with 2 μg of each lentiviral expression construct. Transfections were done in six-well plates using Lipofectamine 2000 (Invitrogen, USA). Viral supernatants were collected at 24 and 48 h after transfection. After addition of polybrene (8 μg ml^{-1}), the supernatants were placed on the cultured HMSCs cells. Cells were transfected twice to increase transfection efficiency.

2.10. RT-PCR Analysis. RT-PCR was used to quantitate the expression of genes related to senescence. Total RNA was extracted by TRIzol reagent (Sigma, MO) and was converted to cDNA by reverse transcription reaction as previously described [45, 46]. We designed PCR primers as follows: *β-actin*: 5′-CAGGTCATCACCATTGGCAATGAGC-3′ (forward) and 5′-CGGATGTCCACGTCACACTTCATGA-3′ (reverse); *caveolin-1*: 5′-TCCCATCCGGGAACAGGGCAACAT-3′ (forward) and 5′-GTCCCTTCTGGTTCTGCAATC-3′ (reverse); *P16*: 5′-CGGATAATTCAAGAGCTAACAGGT-3' (forward) and 5′-GGCCTCCGACCGTAACTATTCGGT-3′ (reverse); *P21*: 5′-GTGGACCTGTCACTGTCTTGTAC-3′ (forward) and 5′-GCTTCCTCTTGGAGAAGATCAGC-3′ (reverse); apolipoprotein: 5′-GGTCTCWGACAATGAGCTCCA-3 (forward) and 5′-TCCCAGAGGGCCATCATGGTC-3′ (reverse); *COX1*: 5′-CAGCATGCCCCAGGATTTGTC-3′ (forward) and 5′-CAKGTCCTGCTCCAGGGCAGC-3′ (reverse, K = G/T). The PCR amplification was composed of 1 cycle at 95°C for 5 min and 33 cycles at 95°C 20 s, 62°C 15 s, and 72°C 15 s and ending with an extension cycle at 72°C 5 min.

2.11. Measurement of COX1 Enzyme Activity. COX1 activity was determined by cellular staining for cytochrome C oxidase. MSCs (1×10^6) were plated in 6-well plate and were cultured for 24 hrs. Cells were rinsed 3 times with PBS and were dried in air. Cells were incubated for 15 min at RT in the preincubation medium (50 mM Tris-HCl, pH 7.6; 0.29 M sucrose; 2.2 mM cobalt chloride) and were rinsed with buffer I (0.1 M sodium phosphate pH 7.6; 10% sucrose). Cells were incubated for 4 hrs at 37°C in 10 ml incubation

medium (pH 7.4, 0.1 M sodium phosphate pH 7.6, 10% sucrose, 10 mg cytochrome C (Sigma, MO), 10 mg DAB (3,3′-diaminobenzidine) hydrochloride (Sigma, MO), and 2.0 mg catalase (Sigma, MO)). Cells were rinsed once with buffer I and 5 min with PBS, washed with H_2O for 5 min, and observed under microscope.

2.12. Statistical Analysis. Data were analyzed using SPSS software (version 16.0; SPSS, Inc., IL). Student's t-test or one-way ANOVA (Bonferroni test) was used to compare statistical differences for variables among treatment groups. The data were expressed as mean ± SD. All experiments were performed in triplicate, and results were considered statistically significant at $P < 0.05$.

3. Results

3.1. Multilineage Differentiation of Fetal Heart Mesenchymal Stem Cells. MSCs isolated from different tissues, despite their molecular congruence, exhibit strong biases in gene and protein expression, pathway activity, and lineage differentiation, suggesting the presence of "molecular memory of tissue origin" [14]. These conserved organ-specific functions may potentially render them more appropriate as cellular therapeutic agents for their organ of origin, particularly in "*in situ* reprogramming" or "*in situ* differentiation" models. Of note, the murine neonatal heart can regenerate and restore damaged sections resulting in the restoration of normal anatomy and function without scar formation, but this capacity is lost after one week of age [2]. We were interested to learn if epigenetic alterations in mitochondria DNA were involved in this aging process.

To define the potential epigenetic mechanisms underlying this cardiac aging, we cultured MSCs from human fetal heart tissues, which are presumed to maintain full regenerative potential. Adherent MSCs grew ~8–12 days after the initial tissue seeding and were collected for phenotypic analysis using flow cytometry. We found that stem cell markers were universally expressed in isolated MSCs, including CD105, CD73, CD90, CD44, and CD29 (Figure 1(a)). Negative stem cell markers, including CD45, CD34, CD14, CD19, and HLA-DR, were expressed at very low levels. The isolated HMSCs could be differentiated into adipogenic and osteogenic lineages (Figure 1(b)). These data suggest that the MSCs cultured from fetal heart exhibited the potential of multilineage differentiation as previously reported [3, 14, 16].

As tissue MSCs exhibit strong "molecular memory of tissue origin" [14], we used RNA-Seq to examine the pathway genes that are associated with cardiac development [4–8]. We found that genes involved in cardiac programing were also expressed in HMSCs, including AKT pathway, GATA family, and TBX family genes (Figure 1(c)). These data suggest that these HMSCs may serve as an appropriate model to study cardiac aging.

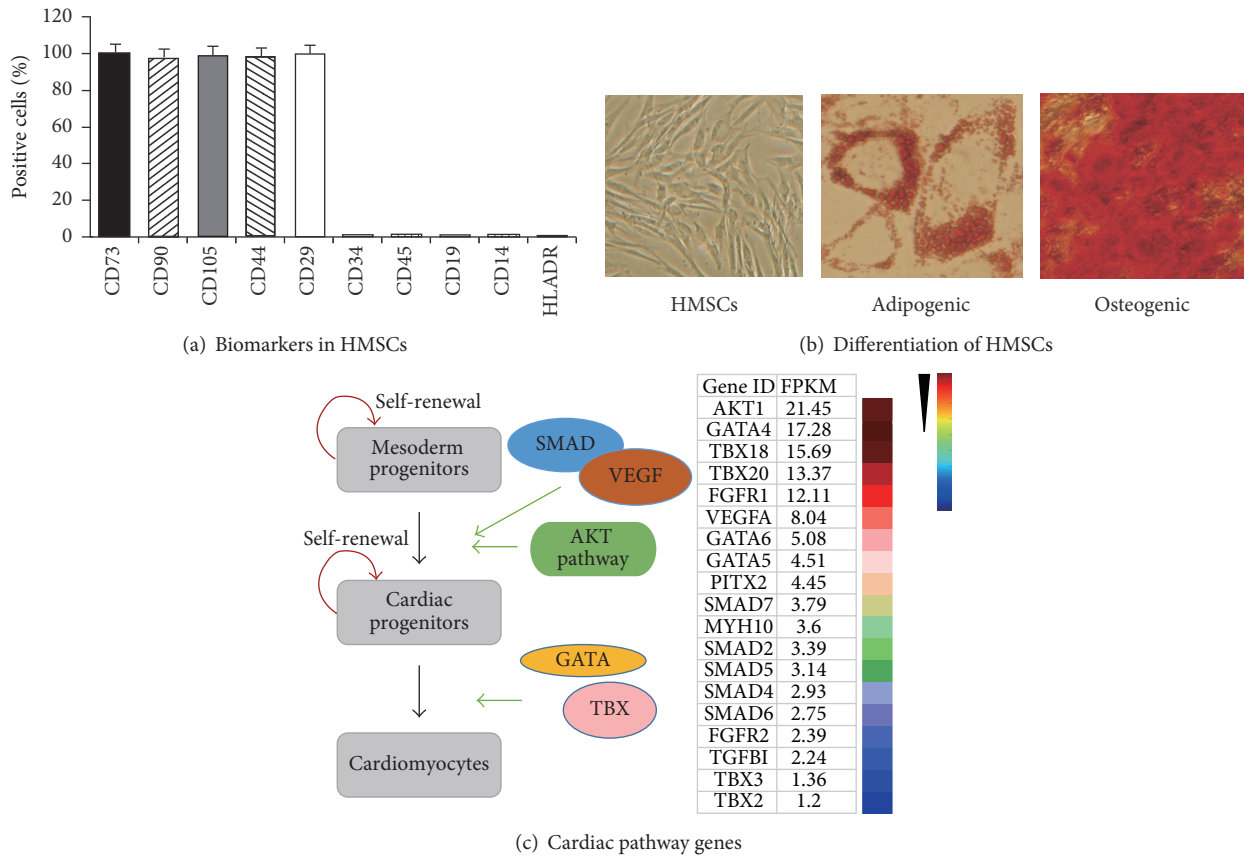
3.2. Induction of Chronic Senescence in Mesenchymal Stem Cells. Two types of induced senescence have commonly been used to study aging *in vitro*, including replicative senescence (RS) and stress-induced premature senescence (SIPS) [47–49]. RS is characterized by progressive telomere shortening, which occurs at every cell division. Although it is an excellent mimic of the natural aging process, induction of senescence is very time-consuming. On the other hand, SIPS is induced by exposure to subcytotoxic stress, and cells undergo premature senescence without telomere shortening. SIPS is the most frequently used model in studying aging. However, cellular toxicity is encountered in this model, particularly by high concentrations of hydrogen peroxide. Therefore, SIPS may not be an appropriate model of *in vivo* pathophysiological aging in cardiac stem cells.

In this study, we established a novel model by combining the advantages of both RS and SIPS. In this model, we facilitated the occurrence of replicative senescence by a new "two-hit" approach. Fetal heart-derived MSCs (HMSCs) were exposed simultaneously to low doses of hydrogen peroxide (50 μM) and to low serum (5%) in cell culture. It was presumed that exposure to low dose of hydrogen peroxide would generate much less toxicity than that usually encountered in SIPS. Meanwhile, low serum exposure would accelerate the development of senescence. After combined exposure for 2-3 passages, we found that HMSCs exhibited flattened morphology and decreased cell proliferation. The treated MSCs stained positive for senescence-associated beta-galactosidase (SA-β-Gal) activity (Figures 2(a) and 2(b)).

We then used PCR to quantitate senescence-related genes that have been previously reported [38, 39, 50]. We found that the caveolin-1 gene (CAV1) was upregulated in senescent MSCs (Figure 2(c)), although we did not detect a significant change in APO-J and OX1. P21 was also upregulated in parallel with cell senescence (Figure 2(d)). Future studies are needed to clarify the time point from the senescence-inducing stimulus when p53 and p21 start to be upregulated.

To further characterize the senescent cells, we measured telomere length using quantitative PCR. By comparing telomere length among the three models, it was clear that the senescent cells in our model had the telomere shortening similar to that seen in replicative senescent cells (Figure 2(e)). As expected, there was no significant change in telomere length in cells that were treated with high dose of hydrogen peroxide. In addition, using RNA-Seq we found that the pathways previously identified in replicative senescent cells [42, 51] were also activated in our senescent cells (Figures S1-S2). Together, these data suggest that our model carries a phenotype that is prone to replicative senescence.

3.3. Cellular Senescence Is Associated with Differential Methylation of mtDNA CpG Islands. To depict the epigenetic mechanism underlying cellular senescence, we used sodium bisulfite sequencing to assess DNA methylation in a total of 11 CpG islands throughout the mitochondrial genome (Figure 3(a)). Using restriction enzymes to distinguish the methylated and unmethylated CpGs, we found that the mitochondrial genome was primarily unmethylated. Three CpG islands exhibited considerable mtDNA hypomethylation in senescent HMSCs, including CpG islands 4, 1, and 2 (Figure 3(b)). Similar mtDNA methylation pattern was also



(a) Biomarkers in HMSCs

(b) Differentiation of HMSCs

(c) Cardiac pathway genes

Figure 1: Characterization of human fetal heart-derived mesenchymal stem cells (HMSCs). (a) The profile of stem cell markers in cultured HMSCs. Immunophenotypes of MSCs were determined by flow cytometry using labeled antibodies specific for the indicated human surface antigens. (b) Differentiation potential of HMSCs. Cells were stained by Alizarin Red for calcium deposits during osteogenic differentiation. Adipogenic differentiation was detected by Oil Red O staining (200x). (c) The "molecular memory of cardiac origin" of HMSCs. Left panel: schematic diagram of the published cardiac stem cell pathways [4–8]. Right panel: expression of pathway genes in HMSCs. Total RNAs were isolated from HMSCs for RNA-Seq using a HiSeq4000 (Illumina). Colors represent from high (red) to low (blue) expression based on normalized FPKM values for each gene.

observed in SMSCs (Figure 3(c)). The remaining CpG islands, however, showed fewer differences between the control and senescent MSCs (Figures S3–S5). We also noticed the difference in mtDNA methylation in three CpG islands (4, 2, and 1) between neonatal and adult skin fibroblasts (Figure 3(d)).

The degree of mitochondrial DNA methylation was quantitated by scanning the PCR band density. Clearly, significant mtDNA hypomethylation was observed in cells following senescence (Figure 3(e)).

3.4. Differential mtDNA Methylation Affects COX1 Expression. CpG island 4 is located within the 3′-region of the mitochondrial COX1 gene (Figure 4(a)), which encodes cytochrome c oxidase I, a key enzyme in aerobic metabolism. As the degree of mtDNA methylation at CpG 4 declines following senescence, we suspected that COX1 expression would be affected by this epigenetic regulation. We used RT-PCR to semiquantitate COX1 and found that a significant upregulation of COX1 was associated with senescence (Figure 4(b)). ND2, a second mitochondrial gene located downstream of CpG island 2, was slightly decreased in senescent MSCs. Similarly, the activity of the COX1 enzyme was also increased in senescent MSCs cultured from fetal heart and skin tissues (Figure 4(c)). Thus, altered mtDNA methylation may be accompanied by subtle changes in the activity of mitochondrial enzymes.

3.5. Knockdown of DNA Methyltransferases Upregulates Mitochondrial COX1. Since mtDNA becomes hypomethylated during senescence, we examined the role of three DNA methyltransferases (*DNMT1, DNMT3A,* and *DNMT3B*) that control DNA methylation. We found that all three enzymes were downregulated in senescent MSCs (Figure 5(a)).

To confirm the role of DNA methyltransferases in senescence, we knocked down the enzymes with shRNAs (Figure 5(b)). Interestingly, knockdown of these three enzymes induced senescence and inhibited cell proliferation in MSCs (Figure 5(c)), in parallel with the upregulation of COX1 (Figure 5(d)).

4. Discussion

The mechanisms underlying the loss of regenerative potential of the fetal heart during postnatal life remain to be illustrated.

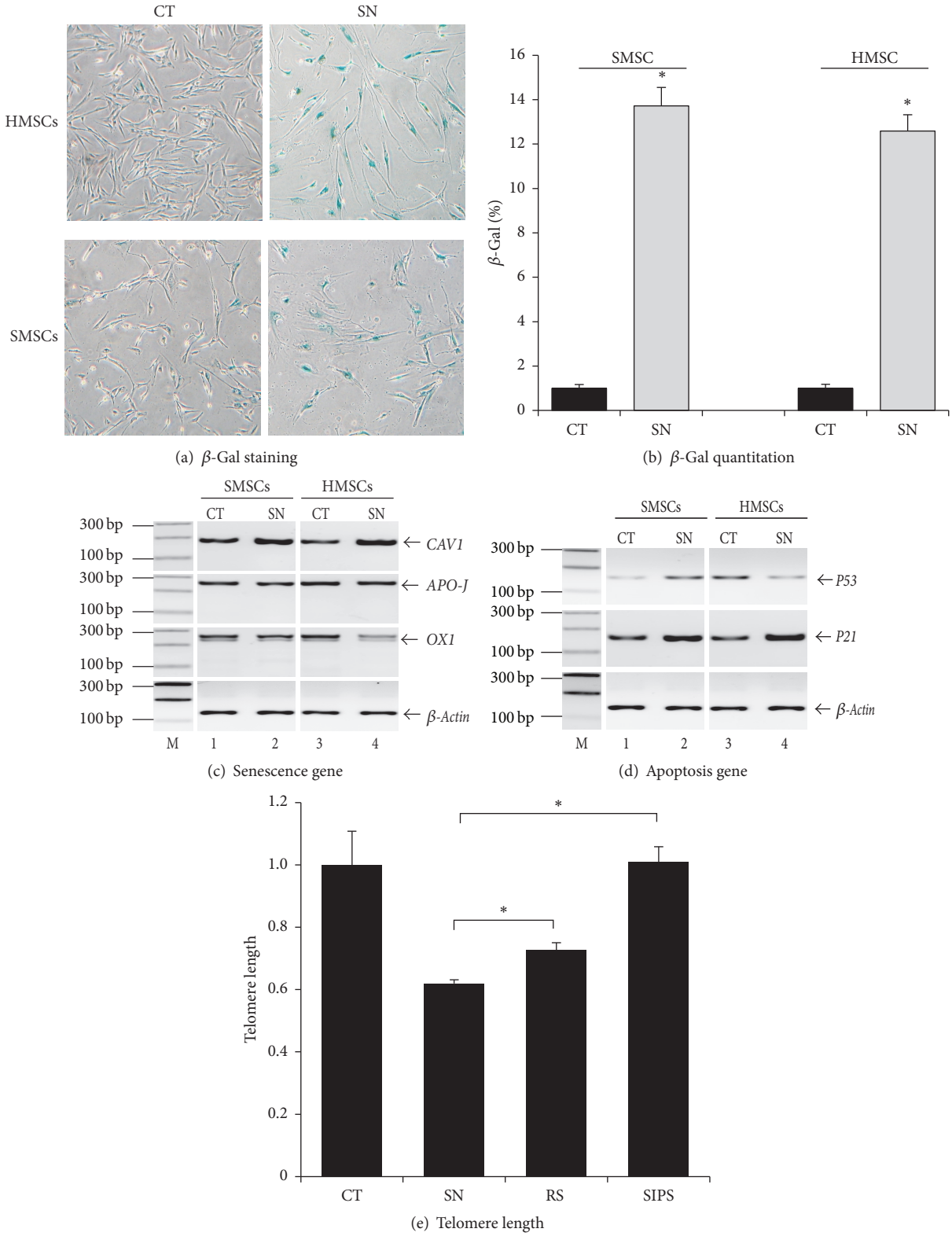


Figure 2: Induction of premature senescence in MSCs. (a) Induced senescence in MSCs. Senescence was induced by continuous exposure of HMSCs (heart-derived MSCs) and SMSCs (skin-derived MSCs) to a low dose of H_2O_2 (50 μM) and low serum (5% FBS) in the culturing medium. CT: control HMSCs treated with PBS; SN: senescent HMSCs treated with 50 μM H_2O_2 for 14 d (×10). (b) Quantitation of β-Gal cells. Cells were counted under microscopy. The results are expressed as the mean ± standard deviation of β-Gal positive cells per field. $^*P < 0.05$ as compared with the PBS control. (c) Senescent-related genes. Senescent HMSCs were harvested and RNA were extracted. RT-PCR was carried out to amplify senescence-related genes, including caveolin-1, apolipoprotein J, and OX 1. (d) Apoptosis-related genes. Expression of p53 and p21 genes was measured by RT-PCR. (e) Telomere length in senescent HMSCs. The relative length of telomere was estimated by qPCR as the ratio between the copies of telomere and the copies of β-globin (T/S). $^*P < 0.05$ as compared with the replicative senescence and high H_2O_2 groups.

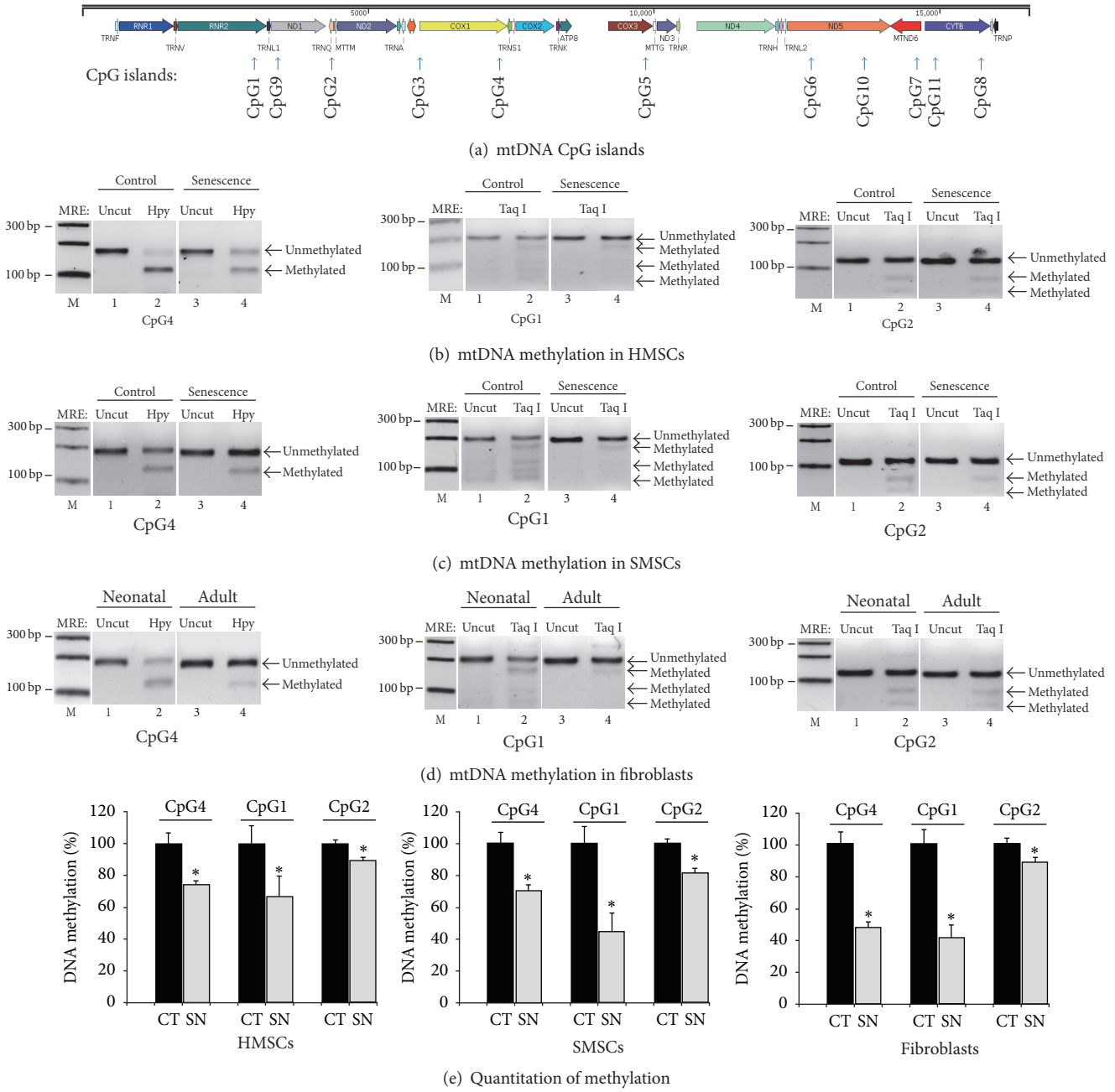


Figure 3: Altered mtDNA methylation in senescent MSCs. (a) Schematic diagram of the mitochondrial genes and the location of CpG islands. In order to detect mtDNA methylation in senescent cells, we designed 11 pairs of methylation-specific primers located on different genes on mitochondrion. (b) Comparison of mtDNA methylation between the control and senescent HMSCs. mtDNA methylation was measured by combined bisulfite restriction analysis (COBRA). PCR products from mtDNA of control and senescent HMSCs were digested by TaqI or HpyCH4IV (HPY) to separate the unmethylated and methylated DNAs. Taq I and HpyCH4IV recognize and digest the methylated ACGT and TCGA sites, respectively. After treatment with sodium bisulfate, unmethylated cytosines were converted to uracils, and the TTGA and ATGT sites are not digested by these two enzymes. After digestion, unmethylated and methylated DNA were separated on 3% agarose gels. Only the data for CpG islands 4, 2, and 1 are presented here. (c) Differential mtDNA methylation between the control and senescent SMSCs. (d) Altered mtDNA methylation in human neonatal and adult fibroblasts. (e) Quantitation of mtDNA CpG methylation. The methylated and unmethylated bands were scanned. The status of CpG methylation was calculated as the relative percentage of DNA methylation using the untreated MSCs as 100. $^{*}P < 0.05$ as compared with that in untreated MSC control cells. Note the decrease in mtDNA methylation in senescent MSCs.

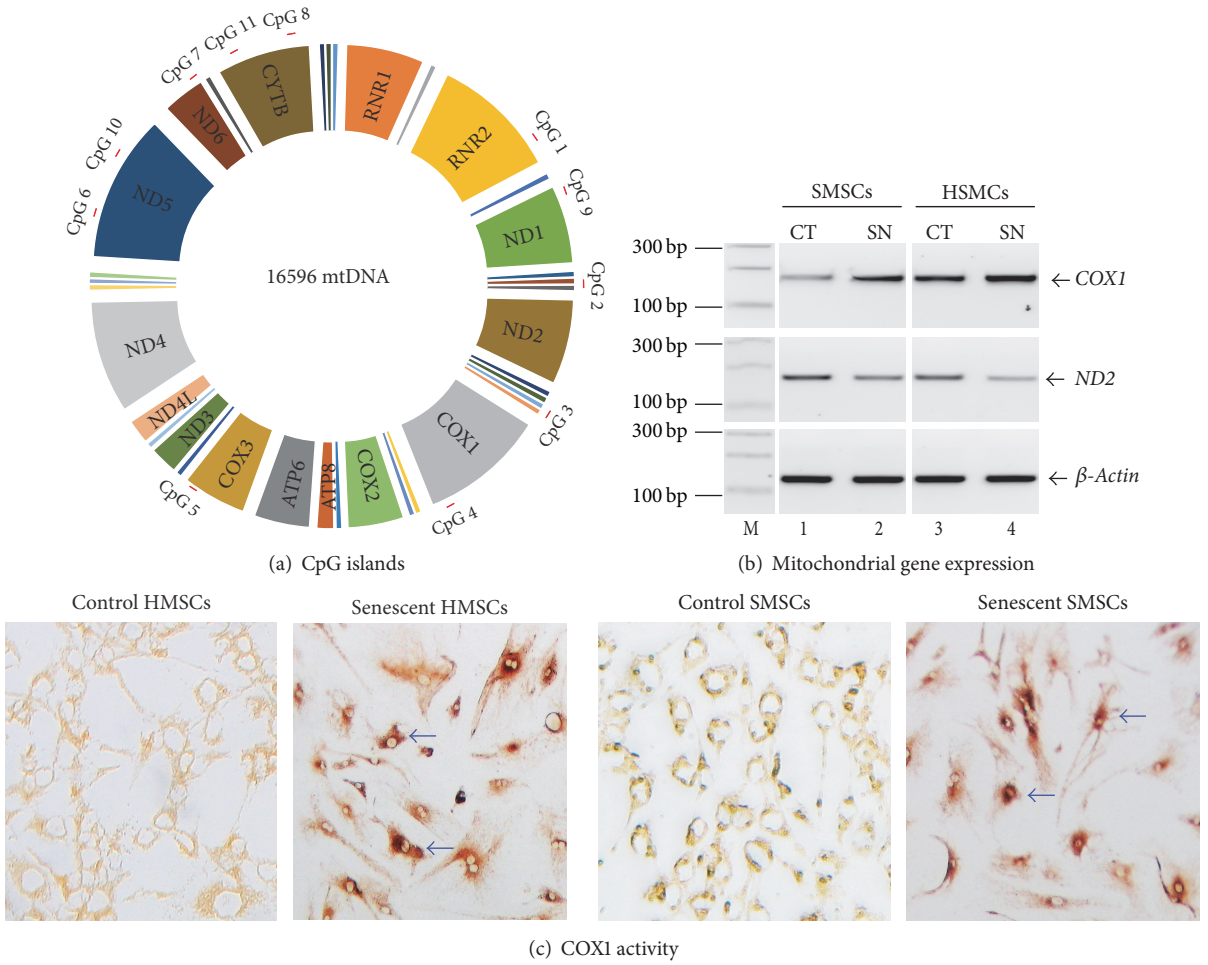


FIGURE 4: Alteration of mitochondrial genes during cellular senescence. (a) Location of CpG islands in the mitochondrial genome. (b) Altered gene expression of mitochondrial COX1 and ND2 genes in senescent MSCs. (c) The enzyme activity of COX1 in control and senescent MSCs.

In this study, we cultured multipotent MSCs from human fetal heart tissues and used a chronic oxidative stress/low serum approach to induce typical senescence. By scanning the status of CpG methylation in the whole mitochondrial genome, we demonstrated considerable mtDNA hypomethylation following senescence in MSCs. COX1, encoding the main subunit of the cytochrome c oxidase complex, was significantly upregulated in senescent MSCs. Knockdown of three DNA methyltransferases (DNMT1, DNMT3a, and DNMT3B) also upregulated COX1 and induced senescence in MSCs. Together, our data suggest that mitochondrial CpG hypomethylation may be a useful biomarker in association with cellular senescence in MSCs.

Significant progress has been made in deciphering the regulatory pathways that control cellular senescence. There are two distinct pathways that lead to the development of cellular senescence [47, 52, 53]. Replicative senescence (RS) depends on the dysfunction in biological clock that is caused by progressive shortening of repetitive DNA sequences (TTAGGG) in telomeres that cap the ends of each chromosome. This shortening eventually triggers DNA damage and initiates a program of cell cycle arrest. In contrast, stress-induced premature senescence (SIPS), although sharing many cellular and molecular features as those undergoing replicative senescence, is telomere-independent. In SIPS, ROS induces cellular dysfunctions, playing a critical role in age-related diseases [54]. In the SIPS senescence model, oxidative stress is often used as the inducer of SIPS [55]. The cells at early passages were exposed once or several times to acute, sublethal oxidative stress, such as H_2O_2 [56, 57]. In order to capture the characteristics of both replicative senescence and SIPS, we adopted a modified approach by exposing MSCs to a low concentration of H_2O_2 and a low serum supply. Using this strategy, we induced typical cellular senescence in MSCs within a short period of time, usually ~2-3 passages. Significantly, these senescent cells exhibit shortened telomeres. RNA-Seq confirms that the senescence is accompanied by the activation of the same pathways as seen in replicative senescence. Thus, this approach may provide an ideal model to study cellular senescence in MSCs. Future studies will be needed to comprehensively compare the gene- and protein-expression profiles of this model with two well-established SIPS and RS models.

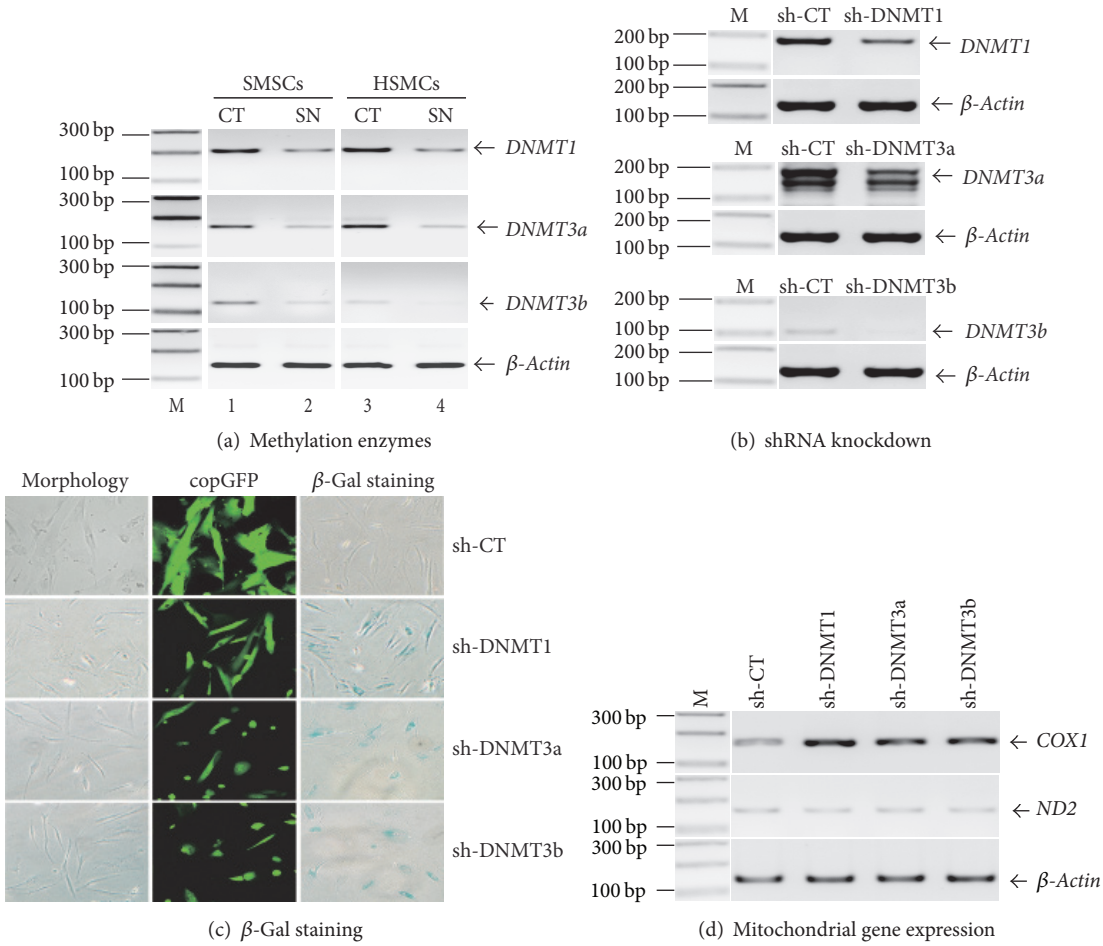


Figure 5: Knockdown of DNMTs induces senescence in MSCs. (a) Downregulation of DNMTs in senescent MSCs. After induction of cellular senescence, cells were harvested and the expression of DNMTs was determined by semiquantitative PCR. (b) Knockdown of DNMTs by shRNAs. Lentiviruses containing DNMT shRNAs (sh-DNMT1, sh-SNMT3a, and sh-DNMT3b) or scramble control (sh-CT) were transduced into HMSCs. After 72 h, transduction efficiency was assessed by the observation of GFP positive cells. Cells were harvested and RNA were extracted, and the expression of DNMTs was determined by RT-PCR. (c) Induction of cellular senescence by DNMT-shRNA knockdown. Left panel: HMSCs morphology taken 7 days after DNMT-shRNA transduction. Sh-CT: shRNA scramble control; sh-DNMTs: HMSCs were transducted by lentiviruses containing DNMT1, DNMT3a, and DNMT3b shRNAs. Middle panel: lentiviral transduction efficiency as shown by copGFP fluorescence of the shRNA vector. Right panel: senescent-associated β-Gal staining. After DNMT-shRNA knockdown, MSCs were stained for β-Gal activity. Note the occurrence of senescence in DNMT-knockdown MSCs in the absence of peroxide treatment. (d) Expression of COX and ND2 in DNMT-shRNA-treated MSCs. β-Actin was used as the internal control for PCR reaction. Cox1 was upregulated in DNMT-knockdown MSCs in parallel with cellular senescence.

Replicative senescence in culture expansion of MSC appears to be epigenetically controlled by DNA methylation and repressive histone marks at the genome DNA level [58]. However, epigenetic regulation of the mitochondrial genome in the aging of MSCs is poorly defined [30, 59]. Age-associated accumulation of mtDNA mutations has been proposed to be responsible for the age-associated mitochondrial respiration defects found in elderly human subjects. Aging phenotypes are reversible and controlled by epigenetic regulation. Reprogramming of elderly fibroblasts restores age-associated mitochondrial respiration defects [60]. We measured the status of mtDNA methylation in senescent MSCs cultured from human fetal heart tissues. Overall, the mtDNA is generally hypomethylated in senescent MSCs. This is in agreement with the extent of mtDNA methylation reported in human blood samples and lung tissues [61]. However, we noticed considerable changes in mtDNA methylation during cellular senescence. Particularly, mtDNA at CpG island 4 became more hypomethylated. This senescence-related mtDNA demethylation was also observed in fibroblasts cultured from human adult skin as compared with those from neonatal skin tissues. Clearly, the mitochondrial genome undergoes epigenetic alterations in cellular senescence.

Accompanying the alteration of mtDNA methylation, the mitochondrial COX1 gene, which includes CpG island 4, was upregulated in all aged MSCs, as shown at both mRNA and protein enzyme levels. COX1 encodes cytochrome c oxidase I, the main subunit of the cytochrome c oxidase complex, which is a key enzyme in aerobic metabolism

[62]. The proton pumping heme-copper oxidase represents the terminal, energy-transfer enzyme of respiratory chains in both prokaryotes and eukaryotes. Mitochondria generate adenosine 5′-triphosphate (ATP) but also produce potentially toxic reactive oxygen species (ROS) [59]. Gradual mitochondrial dysfunction is observed to accompany aging. Continuous accumulation of mtDNA damage may play a causal role in the aging process. However, it is unclear if the upregulated COX1 in our model is functionally involved in the initiation of senescence or it is just a sequela of the aging process.

Currently, we know little about the role that mtDNA methylation plays in the regulation of gene activity in our senescence model. For example, CpG island 4 is located in the 3′-region of the mitochondrial COX1 gene, rather than in the D-loop promoter region, where we might expect a CpG island to exert epigenetic control. However, several studies have suggested a role for gene body DNA methylation in gene regulation [63–65]. In addition, we also found that knockdown of DNA methyltransferases using shRNA induced the upregulation of COX1 in MSCs (Figure 5(d)), in parallel with cellular senescence (Figure 5(c)), in support of a role of mtDNA hypomethylation in the regulation of COX1. DNMT1, the most abundant DNA methyltransferase in mammalian cells, is the key methyltransferase required for the maintenance of DNA methylation in mammals. It predominantly catalyzes methylation at hemimethylated CpG di-nucleotides [66]. Homozygous deletion of DNMT1 is lethal for mouse embryos at 10-11 days of gestation [67]. Both DNMT3a and DNMT3b, on the other hand, are *de novo* methyltransferases [66]. All three enzymes are required for the establishment, maintenance, and erasure of epigenotypes, including genomic imprints, in mammalian development [68, 69]. In our induced senescence model, these three DNMTs were downregulated in senescent MSCs (Figure 5(a)). Importantly, knockdown of DNMTs by shRNA in the absence of the oxidative stress also induced cellular senescence in MSCs (Figure 5(c)). Our data thus suggest that all three DNMTs are involved in senescence of MSCs. It should be noted that knockdown of DNMTs by shRNA also induces global DNA demethylation, including genomic DNAs. In addition, it is not clear whether mtDNA hypomethylation is a specific biomarker for our model. Particularly, we do not know if it also might occur in the replicative senescence and SIPS models. Future studies need to be performed by comparing the epigenetics of mitochondrial DNA in these models. We can learn more about epigenetic control using site-specific de novo DNA methylation or demethylation that can be induced by the CRISPR Cas9-Sss1 epigenetic regulators [43].

5. Conclusions

In summary, by scanning DNA methylation in mitochondrial genome, we demonstrate the association of aberrant epigenetics with the occurrence of senescence in MSCs. The alteration of mtDNA methylation is accompanied by the upregulation of COX1 gene in both senescent MSCs and the DNMT-knocked down MSCs. This study thus implicates the mtDNA epigenotype as a critical biomarker in cellular senescence of MSCs.

Abbreviations

MSCs: Mesenchymal stem cells
mtDNA: Mitochondrial DNA
DNMT: DNA methyltransferase
CpG: Regions of DNA where a cytosine nucleotide is followed by a guanine nucleotide separated by only one phosphate.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

All authors read and approved the final manuscript.

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