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The mitochondrial side of epigenetics

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Epigenetic mechanisms constitute a further level of gene expression control beyond the gene sequence itself. These factors are capable of shaping gene expression in response to environmental changes (99, 100). For this reason epigenetics have been defined a flexible interface between the organism and its environment (100). DNA methylation, regulation of gene expression by noncoding RNA [microRNA (miRNA)], and post-translational histone modifications are the three main categories of epigenetic mechanisms, which not only modulate gene expression but also control development, differentiation, and aging (100). As environment plays a role in stimulating certain patterns of gene expression, nutrition is probably a crucial factor dictating epigenetic shaping of gene expression (69, 100). Being the cell’s powerhouse, mitochondria are destined to play an important role in providing signals of different nature that translate the organism response to external stimuli into epigenetic changes. On the other hand nuclear epigenetics are destined to condition mitochondrial function, as many of the mitochondrial proteins are nuclear-encoded. The fact that clinical manifestations of mitochondrial diseases correspond to those attributed to epigenomic changes provides insights into the epigenetic communication between nucleus and mitochondria (100). The recently confirmed finding that human mtDNA is methylated (8, 35, 45, 87) opens new frontiers in the cross talk between nucleus and mitochondrion, suggesting the interesting possibility that this subcompartamental epigenetic factor could represent an additional instrument and target of mitochondrial-nuclear reciprocal control.

This review summarizes the mechanisms of mutual influence between mitochondria and nucleus with respect to epigenetic factors, highlighting the role of mitochondrial genome and metabolism in shaping epigenetic modulation of gene expression. Additionally, potential regulatory mechanisms of mtDNA epigenetic modifications, including factors of nuclear and mitochondrial origin, are discussed.

**DNA METHYLATION TURNOVER: METHYL VS. HYDROXYMETHYLCYTOSINE**

Three cytidilic nucleotides, namely cytosine, 5-methylcytosine (5mC), and 5-hydroxymethylcytosine (5hmC) are present in both nDNA and mtDNA. DNA methylation pattern is very dynamic among cell types since it is subjected to reprogramming during embryogenesis and changes with aging, environmental stimuli, and diseases. The dynamic nature of this DNA modification implies that a rapid turnover mechanism must be taking place to equilibrate the methylation/demethylation levels. Studies performed on the nDNA methylation/demethylation processes highlight a variety of demethylation pathways...
Mitochondrial function requires strong input from nuclear DNA. All potential factors that regulate mtDNA replication, transcription, and nuclear-encoded RNAs processing flow from nucleus to mitochondria. Most of the mitochondrial proteins are imported into mitochondria. Although 13 of the key proteins that constitute the respiratory chain complexes derive from mtDNA, the majority is nDNA-encoded. Nuclear-encoded RNAs are also imported into mitochondria (88). Epigenetic factors are expected to play a role in this communication, but the mechanisms that regulate the epigenetic cross talk between the two genomes are not completely elucidated. Indeed a complete understanding of nuclear and mitochondrial factors, cofactors, and enzymes regulating this complex process is missing. In the next sections we summarize the known communication networks between nucleus and mitochondria with respect to epigenetics.

**Epigenetic Cross Talk at the DNA Level**

The bidirectional cross talk between nuclear and mitochondrial DNA is essential for cellular homeostasis and proper functioning (76). Coevolution of both DNAs is a fundamental process that preserves biological functionality and cell activity (51). Interaction between nDNA and mtDNA products is known to occur at different levels such as protein–protein interaction in oxidative phosphorylation (OXPHOS), protein–RNA interactions, and nuclear factor-mtDNA recognition sites interactions in the processes of replication and transcription (3, 63, 77). Epigenetic changes play a role in this cross talk being both instrument and recipient of informative flows between the two genomes.

**Role of epigenetic modifications of nuclear-encoded proteins on mtDNA**

Epigenetic regulation of nDNA has been shown to influence mtDNA. A significant example is provided by the nuclear encoded enzyme polymerase gamma (Pol-γA), a subunit of Pol-γ responsible for mtDNA replication, repair, and copy number. This gene is regulated by epigenetic mechanisms through DNA methylation of CpG islands within exon 2 leading to reduction of its expression. Additionally, steady-state levels of Pol-γA expression and mtDNA copy number are linearly correlated (48).

Nuclear-encoded proteins involved in maintaining the mitochondrial nucleotide pool and transcriptional and translational machinery are also potential candidates for epigenetic control. The thymidine kinase (TK2), a mitochondrial pyrimidine nucleoside kinase, is involved in the salvage pathway of deoxyribonucleotide synthesis in mitochondria (26). This protein has been demonstrated to play a role in facilitating the maintenance of nDNA integrity in the cells that are temporarily arrested in the quiescent state (58). Recently, hypermethylation of TK gene promoter in human hearts affected by dilated cardiomyopathy was correlated to a decrease in TK2 protein level, which is responsible for mtDNA depletion typical of this condition (55). Although no specific information is available yet, other potential nuclear-encoded mitochondrial candidates for epigenetically driven mtDNA control could be the human genes encoding ortholog 2 of the bacterial RNase Z (elaC), the mitochondrial RNase P proteins 1 (MRP1) and 3 (MRP3), and pentatricopeptide repeat domain protein 1 (PTCD1), which are involved in the processing of mitochondrial polycistrionic RNAs (81).

**Role of nuclear encoded miRNAs on mitochondrial transcripts.**

miRNAs are important regulators of gene expression. Among the hundreds of miRNAs that regulate cellular functions (5), some have been found in mitochondria both as pre- and as mature miRNA (92). miRNAs have also been associated with the outer mitochondrial membrane. A list of validated miRNAs enriched/associated with mitochondria from different cell lines and tissue has been reported (91). Additionally, few of the nuclear encoded ones have been shown to regulate mitochondrial transcripts. For example,
hsa-miR-133a targets ND1 complex 1 subunit (4, 57); miR-130 targets COX3 in rat liver mitochondria (57); has-miR-181c regulates the mitochondrial complex IV and the levels of reactive oxygen species (ROS) during myocardial infarction (28). Other miRNAs are more directly associated with the regulation of mitochondria metabolism. Indeed miR-210 modulates mitochondrial functions reducing the metabolic rate (20), whereas miR-30 regulates mitochondria fission (60). In rats a small pool of miRNAs associated with mitochondria are involved in the expression of genes related to apoptosis, cell proliferation, and differentiation (57). The expression pattern of miRNAs in mouse liver and its mitochondria are abnormal in Streptozotocin-induced Type 1 diabetes (10). Beyond these preliminary encouraging findings, several questions on the complexity of epigenetics-miRNA regulatory networks remain to be investigated (82). First of all, the mechanism by which miRNAs affect expression of DNA methyltransferase and histone acetylases (104), which could in turn regulate DNA epigenetic, is not completely understood. Second, the exact role of each identified miRNA has to be determined, especially with respect to the regulation of mitochondria metabolism, biogenesis, and mtDNA transcription/translation machinery. Their double location, both in the outer membrane and the matrix of mitochondria, leaves some unanswered questions with respect to their functional difference. Also the role of cell metabolism, origin and function, and interaction with other compartments in inducing miRNAs association to mitochondria is not clear. Elucidation of these aspects may help to define the signature of mitochondrial specific miRNAs and their cross talk with other organelles inside the cell (91). Additionally, the process of miRNA import system is completely unknown together with its origin. In this respect identification of 33 pre-miRNAs and 25 mature miRNAs from miRBase search (4), two miRNAs from HEK293 and HeLa, and 22 putative novel miRNAs from HEK293 aligned with mtDNA (91) suggests that these miRNAs might originate from mtDNA.

Role of mtDNA copy number and haplotype on nuclear epigenetics. The mtDNA copy number is one of the signals by which mitochondria affect nDNA methylation patterns. Experiments performed on cancer cell lines demonstrate that changes in mtDNA copy number, often observed in cancer, induce changes in nDNA methylation. Depletion of mtDNA in cancer cell lines produces significant changes in the methylation pattern of a number of genes, which are promptly reversed after reintroduction of mtDNA in cells (89). Since mtDNA depletion strongly affects cell function by changing the redox status, the membrane potential, and ATP levels together with the redistribution of metabolites between mitochondria and cytosol, it is conceivable that signals associated with mitochondrial dysfunction could be responsible for epigenetic changes. Studies on inhibition of the different steps of the OXPHOS or the transport activity of the identified mitochondrial carriers (72) may help us to understand the exact nature of the mitochondrial signals linked to the epigenetic response.

Among the nine different mtDNA haplotypes identified in Europe, the presence of the J haplotype is associated with higher levels of global nDNA methylation (7). MtDNA haplotypes also affect nDNA gene expression, cell differentiation, and mitochondrial metabolism. Interestingly, an mtDNA haplotype-specific expression of genes involved in pluripotency, differentiation, mitochondrial energy metabolism, and DNA methylation has been observed in undifferentiated and differentiating embryonic stem cells (49). Furthermore, mtDNA methylation and copy number are sensitive to some mitochondria toxicants (13), highlighting the role of mtDNA as an environmental biosensor. An interesting area of investigation could be the relationship between mtDNA copy number and mtDNA methylation changes in exposure-related human diseases and with respect to mtDNA haplotypes. Significantly, an association between cancer and mtDNA haplogroups has been reported (96, 97).

Epigenetic Cross Talk at the Metabolic Level

Mitochondrial function is considered fundamental for the maintenance of genomic DNA. Beyond energy requirements, a functional mitochondrial electron transport chain is required to produce orotic acid, which is the building block for de novo pyrimidines synthesis (69). Adverse products of the electron transport chain such as ROS can damage nDNA at different levels (100). Recently emerging findings have suggested that physiological or pathological changes in mitochondrial metabolism lead to accumulation of key intermediates capable of regulating nDNA epigenetic (100). The levels of these signaling molecules respond to many stimuli, the most important of which is caloric intake. Our discussion will cover the role of mitochondrial metabolism in nDNA epigenetics by focusing on specific intramitochondrially produced metabolites or their immediate products (see also Fig. 1).

Role of mitochondrial metabolism. Beyond the fundamental role of being the site of OXPHOS, mitochondria are at the crossovers of a variety of metabolic and signaling pathways and can be legitimately defined key regulatory organelles in various physiological and pathological events (36, 79). Mitochondria represent the machinery by which stimuli from the environment can translate into epigenetic regulation of gene expression (69). Mitochondrial metabolism controls the levels of key signaling molecules, such as ATP, acetyl-CoA, NADH, NAD+, and their ratio, S-adenosylmethionine (SAM) (99, 100), together with some TCA enzymes (isocitrate dehydrogenase, succinate dehydrogenase), TCA intermediates (citrate, oxoglutarate, fumarate, and succinate), or their derivatives [2-hydroxyglutarate (2-HG)] (80) (see Fig. 1). Therefore, mitochondrial dysfunctions can modify the expression of nuclear genes involved in metabolism, signaling, growth, differentiation, and apoptosis. In the next subsections we highlight how some mitochondrial metabolites can affect nDNA epigenetic.

ATP and the redox state. ATP is known to be involved in the modification of chromatin through phosphorylation of histones tails. Repulsion of negative charges among histone tails opens chromatin promoting transcription and replication, making mitochondrial energy flux strictly linked to nDNA gene expression. In conditions of energy depletion, reduction of ATP and acetyl-CoA levels lowers the levels of reducing equivalents, resulting in lower NADH/NAD+ (100). Increased NAD+ availability activates class III NAD+-dependent histone deacetylases (sirtuins), which deacetylate histones (27). ATP depletion also lowers the protein phosphorylating capacity, which is destined to affect DNA-protein interactions and signal transduction pathways. Conversely, the presence of high-energy substrates maintains DNA-bound proteins in a phosphorylated state, and high NADH/NAD+ ratio inactivates
sirtuins, opening chromatin structure for DNA transcription (100). The flux of reducing equivalents through mitochondria regulates the redox state of glutathione affecting the cysteine/cystine and thiol/disulfides ratio, leading to regulation of the activity of many proteins, including transcription and growth factors (50, 98).

**CITRATE.** Citrate, produced in the TCA cycle, is exported outside mitochondria into cytosol, where it is cleaved by ATP-citrate lyase (ACL) into acetyl-CoA and oxaloacetate (43). In addition to its metabolic role in the fatty acid biosynthesis, acetyl-CoA serves also for global histone acetylation by histone acetyl transferases (HAT) (102). Production of acetyl-CoA is strictly related to energy production. When energy production increases, acetyl-CoA accumulation triggers chromatin opening through histone modifications. Reduction of acetyl-CoA availability contributes to lower histone acetylation resulting in chromatin condensation. Furthermore, acetyl-CoA is a known acetyl donor for posttranslational modification of many classes of proteins (22). Since DNA methyltransferase (DNMT1) is a known target of acetylation (32, 54), which promotes its degradation (32), acetyl-CoA depletion might increase the level of DNA methylation. Interestingly, DNMT1 expression has been shown to be regulated by ACL, the cytosolic acetyl-CoA-producing enzyme from mitochondrial citrate (62). All these metabolic signals contribute to chromatin condensation, suppression of gene expression and replication, and proliferation.

**SAM.** SAM is the main cellular methyl donor for the versatile methylation processes of DNA and chromatin (42). SAM synthesis occurs at the intersection of the homocysteine cycle and the mitochondrial metabolism of folate (42). Mitochondrial metabolism regulates production of SAM through synthesis of ATP and folate. Folate cycle reactions occur in both the cytosol and mitochondria (42), with the serine-to-glycine exchange being the linking reaction between the two compartmentalized pathways. The amino acids are interconverted by the mitochondrial and cytosolic serine hydroxymethyltransferases (SHMT) through methylenetetrahydrofolate (MTHF). Mitochondrial MTHF availability dictates the switch between SAM and nucleotide synthesis through the action of the mitochondrial bifunctional enzyme (MBE), which is active in embryonic and cancer cells to promote nucleotide synthesis and is blocked in adult cells to promote SAM-dependent DNA methylation (69). Cytosolically synthesized SAM is trans-

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**Fig. 1.** Overview of the epigenetic cross talk between nucleus and mitochondria. Methylation of nDNA and mtDNA represents a mechanism of mutual control of mitochondrial and cellular function. From the nuclear side, many nuclear encoded mitochondrial proteins are epigenetically regulated, and this influences mitochondrial function. MicroRNAs (miRNAs) can also regulate mitochondrial function. Mitochondrial metabolism plays an important role in modulating nuclear epigenetics through ATP, NADH/NAD⁺, citrate, acetyl-CoA, and S-adenosylmethionine (SAM) levels and the mechanisms regulating SAM cellular availability through the methyl cycle. Mitochondrial metabolism controls mtDNA methylation by modulating the availability of intramitochondrial SAM and TCA intermediates, which can influence ten-eleven translocation (TET) activity and thus the equilibrium between 5-methylcytosine (5mC) and 5-hydroxymethylcytosine (5hmC).
ported into mitochondria by means of the specific mitochondrial carrier SAM carrier (SAMC), to provide the methyl moiety for all mitochondrial methylation processes (2).

2-OG. 2-OG is a product of TCA cycle that is transported outside mitochondria by specific carriers (71). It plays a role in the process of removal of the 5mc modification from DNA, being the necessary substrate of TET proteins. The most important role suggested for TET proteins is DNA demethylation, although a functional role of 5hmC cannot be excluded. Increasing evidence indicates that TET members might be regulators of chromatin dynamics. Recently, Chen et al. (19) and Deplus et al. (30) reported that TET2 and TET3 not only are involved in demethylation but also regulate histone glycosylation and methylation. These enzymes interact with O-linked N-acetylgalactosamine (O-GlcNAc) transferase (OGT) leading to increased histone H2B112 glycosylation and H3K4 methylation (30). Another study reported that TET1 can repress gene expression by binding to Sin3A and Mi-2/nuclear-some remodeling and deacetylase (NuRD) complexes (85). Glycosylation of TET1 itself by OGT leads to increased TET1 expression and 5mC-to-5hmC conversion (85). Furthermore, TETs and 5hmC might play a role in diseases and aging (17). Another family of demethylating enzymes, the Jumonji C (JmjC) domain-containing histone demethylases, involved in the methyl removal from lysine and arginine (80), use 2-OG together with Fe$^{2+}$ as cofactors to demethylate the repressive H3K27me3/2 (1).

Succinate and fumarate. Succinate and fumarate, metabolites of the TCA cycle, are competitive inhibitors of the JmjC domain-containing histone demethylases and the three TETs (80). Experiments performed on cell culture showed that accumulation of succinate through blockade of succinate dehydrogenase leads to increase of histone H3 methylation, which is reversed by overexpression of the histone demethylase proteins KDM3 (14). Impaired function of succinate dehydrogenase also promotes nDNA hypermethylation in different tumors (53). Screening studies have shown that succinate is a strong inducer of histone and DNA methylation during cancer development (53, 59). In addition to the inhibition of TETs, fumarate is an inducer of protein succination (68), a posttranslational modification that impairs function of many proteins.

2-Hydroxyglutarate. 2-Hydroxyglutarate (2-HG), an unconventional intermediate derived from citrate metabolism, is produced by mutated forms of isocitrate dehydrogenation (IDH). The mutation confers to IDH a novel enzymatic activity (gain of function) catalyzing the NADPH-dependent reduction of 2OG to 2HG (105). 2-HG has two enantiomers, R-2HG and S-2HG, which inhibit TET activity, although to a different extent (23). The reason of the more effective inhibitory activity of S-3HG with respect to R-2HG is not clear. It has been suggested that R-2HG selectively stimulates the activity of EgIT prolyl-hydroxylases, which modify the hypoxia-inducible transcription factor (HIF-1α) (56). Production of 2-HG has also been noticed in cells proliferating in hypoxia in the absence of IDH mutations (103). IDH mutations, inhibition of TET activity, and cancer appear to be strictly related, although the mechanism is not clear. Different studies reporting DNA hypermethylation in cancer patients bearing IDH mutations may be attributed to TET inhibition (6, 34, 46).

ROS. Mitochondrial generation of ROS can influence epigenetic signaling (31). ROS are produced as a byproduct of OXPHOS in the mitochondria. About 2–4% of the oxygen consumed during OXPHOS is converted to ROS. Besides mitochondria, NADPH-oxidase 1 (Nox1) also generates a significant amount of ROS in the cell. It has been recently demonstrated that mitochondria control Nox1 redox signaling, which may contribute to tumorigenesis (31).

Finally it should be mentioned that mutations or methylation of the mtDNA may promote aberrant expression of mitochondria-encoded subunits of the respiratory chain resulting in alteration of mitochondrial metabolism. Disruption of mitochondrial homeostasis may modify the landscape of cellular physiology, including nuclear epigenetics.

Although different questions need to be elucidated, these reported experimental observations support the view that the metabolic balance of mitochondrial reactions can affect the level of DNA and histone methylation and thus control gene expression. When this balance is lost, accumulating intermediates could gain a new function as signaling oncometabolites capable of inducing epigenetic changes of gene expression.

MITOCHONDRIAL DNA METHYLATION: NEW FRONTIER OF CROSS TALK

Numerous recent studies on mtDNA methylation (8, 35, 45, 87) and mitochondrial methyltransferase activities (21, 87) demonstrate the existence of mitochondrial epigenetic factors that have been clearly neglected or unaccepted until recently (39). Lack of mitochondrial histones and methylase activities together with some methodological limitations have prevented the literature from reaching a clear statement in this respect (64). Recently, Bellizzi et al. (8) showed that mtDNA samples from human and murine blood and cultured cells are methylated at very crucial regions such as the D-loop and outside the CpG sites. This explains why the traditionally used methods, which can identify DNA methylation exclusively at CpG islands, often failed in providing clear and conclusive evidence with respect to mtDNA methylation (see Ref. 4).

In line with nDNA methylation (40), abnormalities in mtDNA methylation patterns are dependent on different factors, such as diseases (67), exposure to environmental stimuli, and drugs (13, 18). Emerging studies on changes of mtDNA methylation in disease states provide some interesting clues in this respect. Our group has focused on the possible role of the methyl metabolism in regulating the extent of mtDNA methylation. Our study model was represented by immortalized cells isolated from Down syndrome (DS) patients. DS is the most common human autosomal trisomy, although overexpression of genes encoding for specific enzymes directly leads to biochemical aberrations typical of metabolic diseases (74). In DS the one-carbon metabolism is greatly affected by the overexpression of cystathionine-beta-synthase (CBS), located on chromosome 21, which withdraws homocysteine from the methyl cycle to produce cysteine (74). CBS overactivity produces changes in the levels of several intermediates of the cellular one-carbon metabolism in DS, among which is SAM. We detected lower levels of mtDNA methylation in DS compared with control samples and related the finding to lower SAM levels in the cytosol and mitochondria of DS patients (45). This highlights the importance of SAM availability, suggesting that its synthesis and transport to mitochondria could represent key regulatory steps of mtDNA methylation.
The methyl cycle transfers the methyl moiety from methionine thus producing homocysteine and SAM. Homocysteine is then converted back to methionine. This flow is characterized by different points of chemical withdrawal and supplementation that could modulate SAM availability. In particular, CBS withdraws homocysteine for cysteine production. Additionally SAMC removes SAM from the cytosol to supply mitochondria. Epigenetic tuning of their nuclear-encoded gene expression could represent an important mechanism of nuclear control of mitochondrial epigenetics through regulation of SAM availability into mitochondria. Conversely, methyl-THF/THF exchange, which supplements the methyl moiety for SAM synthesis, could represent the cross-talk point by which mitochondrial metabolism directly regulates mitochondrial epigenetics by acting on the folate metabolism.

Homocysteine, a key metabolite in the methyl cycle, is a promising molecule with respect to epigenetic regulation. Evidence from studies on hyperhomocysteinemia patients showed that inborn errors of metabolism caused by hyperhomocysteinemia may result in abnormal DNA methylation (44). Additionally neural tube defects, congenital heart defects, and nonsyndromic oral cleft, which appear to be related to hyperhomocysteinemia (15, 70, 95), might be characterized by epigenetic abnormalities. Recently a potential link between homocysteine, mitochondrial epigenetics, and bone remodeling was identified, suggesting the homocysteine synthesis could be another key regulatory point of epigenetic mechanisms (47, 94). However, the mechanisms of this relationship needs to be fully understood.

METHODS: FROM PAST LIMITATIONS TO FUTURE DEVELOPMENTS

Quantification of methylation on mtDNA is a challenge. Uneven distribution in the methylation pattern within a gene and among cells, together with the fact that DNA methylation cannot be amplified, requires challenging levels of method sensitivity (83), which for many years have hampered mtDNA methylation detection. The first approach on mtDNA methylation is actually not recent. Conflicting results were obtained in the 1970s and early 1980s, demonstrating from the very beginning that targeting mtDNA methylation displays fundamental problems at the method level. In its infancy, detection of mtDNA methylation relied on identification of methylated CpG dinucleotides located within recognized sequences of methylation-sensitive restriction enzymes (29, 37, 75, 86). Now we know that these approaches are not optimal for mtDNA, since the majority of the methylated cytosines have been shown to be located outside of CpG nucleotides in mtDNA (8).

The bisulfite method represents further evolution in the evaluation of the mtDNA methylation status since it allows for single base resolution. Recently it has been associated with innovative platforms to read the methylation signal (see Ref. 42) to the point that the whole genome shotgun bisulfite sequencing (WGsBs) has been achieved by using the Illumina Genome Analyzer platform for small eukaryotic genomes, such as Arabidopsis thaliana (25), and for mammalian DNA (61), providing single-base-pair resolution. Bisulfite sequencing has been successfully applied to mtDNA, although its application has sometimes failed to detect methylation on mtDNA (39, 64). In the case of the bisulfite method, several precautions need to be taken into account. Extraction of DNA must be carried out by using different methods and avoiding contamination of proteins that could impair bisulfite conversion. Additionally, control experiments are fundamental, such as assaying 1) fully unmethylated DNA samples to rule out any possible structural effect the analyzed DNA sample on the bisulfite conversion, and 2) DNA samples extracted from cells lacking mtDNA to exclude amplification of nuclear mitochondrial pseudogenes (NUMTs). Another important point regarding the bisulfite method is that it does not distinguish between 5mC and 5hmC. Since the product of bisulfite conversion of 5hmC tends to stall DNA polymerases during PCR, hydroxymethylated regions of DNA are destined to be underrepresented in quantitative methylated analyses (41). With this in mind, alternative methods have been developed. Enrichment of methylated regions by immunoprecipitation of denatured DNA with specific antibodies has been proven very useful in the detection of mtDNA methylation (17, 18, 87) and has been applied in combination with microarray hybridization [MeDIP (101) and mDIP (52)], leading to a fast and efficient evaluation of the methylation status. Other nonspecific (or global) measurements of DNA methylation provide an overall picture of cellular DNA methylation levels and are crucial for understanding the relationship between genome-wide alterations in DNA methylation, gene-specific methylation patterns, and genome stability (33). When applied to mtDNA sensitivity is crucial. Liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) offers a fundamental tool for the determination of genomic and mitochondrial DNA methylation (45, 90), providing clear separation and specific quantitation of 5mC and 5hmC without any sequence limitation. Future developments in the technical approaches to quantitate mtDNA methylation will be crucial. Nanopore sequencing offers the potential for direct sequencing of 5mC without bisulfite treatment (12, 24), and this might represent a revolution in high-throughput DNA methylation analysis.

PRESENT GAPS AND FUTURE PERSPECTIVES

As mitochondrial genome encodes for a fraction of the mitochondrial proteins, a strong input from nuclear DNA is required to produce a completely functioning organelle. Conversely, mitochondria are capable of controlling nuclear gene stability and expression to the point that the cellular fate depends on the organelle’s function. Our understanding of the complex levels of communication between mitochondria and nucleus is obtaining benefits from studies focused on the relationship between epigenetics and mitochondria. Accumulating evidence is elucidating the role of mitochondria in shaping nDNA expression through epigenetics, but more needs to be done with respect to mitochondrial epigenetic mechanisms. The renewed interest into the status of mtDNA methylation and the improved methodological approaches are expected to provide significant clues into the regulatory mechanisms conditioning mitochondrial genome expression in response to mitochondrial and nuclear signals (Fig. 1), legitimately rendering mtDNA epigenetics a new frontier of gene expression modulation in response to environmental stimuli. This is destined to improve our comprehension of mitochondrial signaling and metabolism with the perspective of identi-
fying new biomarkers of disease. However, our research needs to be addressed to elucidate several aspects that are still elusive, as the scientific community is paying the gap of many years of “neglecting” mtDNA methylation.

First of all, it is imperative to establish specific mtDNA methylation profiles in diseases, in particular neurodegenerative and in cancer, leading to the discovery of disease-specific levels of total methylated mtDNA or epigenetically modified mitochondrial genes as biomarkers. A further step in the evolution of the role of mtDNA methylation is to recognize that several mitochondrial enzymatic activities such as DNMT1 and TETs are crucial in regulating mammalian mtDNA methylation and that epigenetic regulation of mtDNA might respond to signaling pathway mechanisms, especially in the context of the intriguing biological significance of hmC. Future investigations will be aimed at understanding how mitochondrial expression of these enzymes is regulated and distributed among tissues in normal and pathological conditions. Similarly, mtDNA methylation appears to be conditioned by the availability of SAM together with the expression and activity of proteins involved in the regulation of its cytosolic and mitochondrial availability, such as CBS and SAMC. Expression and activity of these proteins, together with their tissue distribution and their nuclear epigenetic regulation, are expected to provide important clues on the mechanisms finely shaping mitochondrial and cell function. CBS and SAMC gene epigenetic modifications, coupled to the corresponding changes in their expression and activity, need to be mapped in different conditions to establish diagnostic tools, such as the methylation levels of their gene promoter, that could be related to the amount of mtDNA methylation. Similarly, the levels of intracellular SAM or other methyl metabolites could be related to the extent of mitochondrial epigenetic modifications, establishing simple diagnostic tools to assess a risk factor for developing a particular disease associated with abnormal mtDNA methylation.

Although no such research has been reported on mtDNA, it is conceivable that drugs capable of selectively crossing mitochondrial membrane could be targeted to modify mtDNMTs and TETs activities and consequently mtDNA methylation. Additionally, targeting methyl metabolism is a promising therapeutic tool to shape mtDNA methylation in such a way that new preventive strategies could be developed to modify mtDNA methylation levels into a “control” range in those individuals in which, for genetic or environmental reasons, the methyl levels on mtDNA are outside healthy ranges.

Crucial to these advances is the development of technologies for the analysis of epigenetic variations in mtDNA in physiological and pathological conditions. Even though global mtDNA methylation assessment can be performed today (78), accurate, large-scale mapping of mtDNA 5mC and 5hmC patterns obtained from blood samples is not yet available and still represents a challenge for next-generation sequencing technologies.

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