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Mitochondrial genome and epigenome: two sides of the same coin

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1. ABSTRACT

The involvement of mitochondrial content, structure and function as well as of the mitochondrial genome (mtDNA) in cell biology, by participating in the main processes occurring in the cells, has been a topic of intense interest for many years. More specifically, the progressive accumulation of variations in mtDNA of post-mitotic tissues represents a major contributing factor to both physiological and pathological phenotypes. Recently, an epigenetic overlay on mtDNA genetics is emerging, as demonstrated by the implication of the mitochondrial genome in the regulation of the intracellular epigenetic landscape being itself object of epigenetic modifications. Indeed, *in vitro* and population studies strongly suggest that, similarly to nuclear DNA, also mtDNA is subject to methylation and hydroxymethylation. It follows that the mitochondrial-nucleus cross talk and mitochondrial retrograde signaling in cellular properties require a concerted functional cooperation between genetic and epigenetic changes. The present paper aims to review the current advances in mitochondrial epigenetics studies and the increasing indication of mtDNA methylation status as an attractive biomarker for peculiar pathological phenotypes and environmental exposure.

2. INTRODUCTION

Mitochondria are the only animal organelles to have their own genome. They comprise a circular, histone- and intron-free 'chromosome' of 16.6. kb of DNA, present in one or more copies, which encodes tRNAs, rRNAs, and a few subunits of the oxidative phosphorylation (OXPHOS) system (1,2). Traditionally the high mutation rate made the mtDNA an excellent tool for the reconstruction of human population history, similarly to what was described for other organelles (3). Lately, impressive evidence has expanded research in considering it as regulator of a wide variety of phenotypic physiological and pathological outcomes. This regulation takes place either directly by influencing the efficiency in energy metabolism or indirectly by interacting with nuclear genes and by increasing the penetrance of the nuclear mutations (4,5). Being semiautonomous organelles, mitochondrial functionality requires a coordinated expression of genes encoded by both the nuclear and mitochondrial genome, and this is accomplished through a close network of bidirectional signals between the two genomes balancing the mitochondrial status, in terms of biogenesis and function, and the energetic needs (6). In this context, it has also come to light that mitochondria and mitochondrial genomes impact the establishment and maintenance of the whole cellular epigenome by both controlling the availability of the co-substrates of epigenetics enzymes and being itself target of methylation changes (7-10). Indeed, the availability of high-throughput sequencing technology significantly improved the sensitivity of methods applied to mtDNA to detect methylated cytosines (5-mC), definitively clarifying the forty-year dispute about the possible existence of epigenetic modifications at mtDNA level (11). Not only methylated and hydroxymethylated (5-hmC) cytosines were noted at both CpG and non-CpG sites of mtDNA but also the intra-mitochondrial traslocation of DNMT and

TET enzymes and their dynamic regulation according to specific physiological (or pathological) conditions and in response to peculiar environmental changes were recently attested (12-19).

In this review, we summarize the most salient aspects relative to mitochondrial DNA genetics and epigenetics, giving special attention to those significant correlations between mtDNA methylation changes and peculiar phenotypes, diseases as well as environmental exposure.

3. mtDNA FEATURES

3.1. The mitochondrial genome: structure, replication and transcription

Mitochondria contain many copies (1000-5000) of their own genome, the mitochondrial DNA (mtDNA), documented for the first time in 1955 and indicated as non-chromosomal genetic element rho (20). These organelle share many features with prokaryotes and are commonly thought to originate by endosymbionts in the ancestral eukaryote. The historical "endosymbiosis theory" has been modified over the years and the revised theory has been labeled as the "hydrogen hypothesis" that postulates that the eukaryotic nucleus and the mitochondria were created simultaneously through the fusion of a hydrogen-requiring methanogenic *Archaebacterium* and a hydrogen-producing alpha-proteobacterium (the symbiont). A recent phylogenomic study suggests a common origin of mitochondria and the SAR11 clade of *Alphaproteobacteria* as a sister group to the Rickettsiales (21-23).

Human mtDNA is a covalently double-stranded closed circular molecule that is ≈5 μm long, has a molecular mass of 10^7 daltons and it is approximately 16.6. kb in size (2, 24). It was completely sequenced in 1981 and a revision of this sequence was later carried out by Andrews *et al*. (1, 25). Similar to bacterial chromosomes, mtDNA is organized into nucleoprotein structures called nucleoides, firstly reported in *Saccharomyces cerevisiae* and then observed in human cells. Nucleoids carry 1-2 molecules of mtDNA and are dynamic structures which can be associated with the inner mitochondrial membrane or also distributed through the mitochondrial network, as demonstrated by Garrido *et al*. by time-lapse fluorescence microscopy in cells lacking mtDNA (rho^0 cells) (26-30). Wang and Bogenhagen have identified a series of proteins forming nucleoids, including mitochondrial single-stranded binding proteins (mtSSB), TWINKLE, mtDNA polymerase (POLG), mitochondrial RNA polymerase (POLRMT) and mitochondrial transcription factor A (TFAM), which are directly involved in mtDNA replication and transcription, as well as ANT1 and prohibitin, which are proteins associated with mitochondrial membranes (31). According to some hypothesis, nucleoids replicate as discrete units, giving rise to daughter nucleoids identical to the parental, which can themselves segregate freely. Alternatively, nucleoids can reorganize, under nuclear genetic control, and dynamically exchange with each other mtDNA molecules (32, 33). It is likely that both the two models occur in cell and tissues in alternative conditions.

Human mtDNA has a compact structure characterized by very few intergenic spacers, except for one regulatory region, lacking of introns, overlapping genes and part of the termination codons are generated post-transcriptionally by polyadenylation of the mRNAs. It consists of two strands, a guanine-rich heavy (H) and a cytosine-rich light (L) strand, historically so named on the basis of the nucleotide composition and the separation in denaturing cesium chloride gradient. mtDNA contains 37 genes encoding for 13 essential subunits of the oxidative phosphorylation (OXPHOS) system, including seven subunits of complex I (ND1-6 and ND4L), one subunit of complex III (Cyt*b*), three subunits of complex IV (COI-III) and two subunits of complex V (ATP6-8), 2 rRNAs (12S and 16S) and 22 tRNAs. The remaining mitochondrial OXPHOS proteins, metabolic enzymes, DNA and RNA polymerases, ribosomal proteins and mtDNA regulatory factors are encoded by nuclear genes, synthesized on cytosolic ribosomes, usually with a N-terminal mitochondrial targeting sequence, and then imported into the organelle by translocases located in the mitochondrial compartments (34-37). The genetic code of human mtDNA differs from that of nuclear DNA. More specifically, in mitochondria TGA is not a termination codon but codes for tryptofan, ATA codes for methionine rather than for isoleucine and, finally, AGG or AGA code for a stop codon instead of arginine. Within mtDNA there is only one non-coding region, the displacement loop (D-loop), a region of about 1.1. kb, spanning between the phenylalanine and proline tRNA genes, that contains the origin of the H-strand replication (O_H) and the promoters of L- and H-strand transcription (LSP and HSP) as well as regulatory elements for both mitochondrial replication and transcription (38). The D-loop contains two hypervariable regions, HVRI (16024-16383 nt) and HVRII (57-372 nt), used especially in human population genetic and ancestry studies, and three highly conserved regions, CSBI-III, mainly involved in mtDNA replication. The loop is a stable triple-stranded structure that originated from the incorporation of a linear third DNA strand of approximately 650 nt, named 7S DNA, based upon its sedimentation properties, and originates from a premature termination of mtDNA replication from O_H (39). Beyond the historically recognized function to maintain an open structure that facilitates mtDNA replication, an involvement of the D-loop in maintenance of DNA topology, DNA recombination and structural association with membrane has been proposed (40).

MtDNA replicates independently of the cell cycle. To date, several models of replication have been proposed (41-43). Regarding the firstly characterized model, known as strand-displacement model (SDM), mammalian mtDNA molecules replicate unidirectionally from two distinct strand-specific origins, which are O_H and O_L, located in the D-loop region and in a cluster of five tRNAs, respectively. A round of replication begins at O_H and continues along the parental L-strand to produce a full H-strand circle. Only after the replication fork has passed the second replication origin, O_L,the synthesis of the L-strand initiates proceeding in the opposite direction (44-49). More recently, a second model, known as RITOLS (RNA incorporated throughout the lagging strand), has suggested a strand-coupled replication with a leading-strand synthesis proceeding with the simultaneous incorporation of RNA on the lagging strand and its following conversion to DNA when lagging-strand synthesis is started (50, 51).

MtDNA transcription occurs in opposite directions around the entire genome starting from the two strand-specific promoters, HSP and LSP, which are functionally independent, and generate poly-cistronic transcripts, subsequently processed to give mature mRNAs, rRNAs and tRNAs through a maturation process involving different enzymatic activities (38, 52-54). The H-strand encodes the majority of the genes through two transcription starting sites. The first is located 16 bp upstream of the tRNA phenylalanine gene and produces a transcript which terminates at the 3' end of the 16S rRNA gene, while the second is close to the 5' end of the 12S rRNA gene and produces a polycistronic molecule corresponding to most of the H strand. The L-strand, whose transcription start site is located within the L-strand promoter, encodes only eight genes including tRNAs and the ND6 subunit. Different *cis*- and *trans*- acting sequences and factors, respectively, involved in the replication and transcription processes, have now been identified and characterized (39, 55, 56). These factors include DNA polymerase gamma, mitochondrial RNA polymerase, mitochondrial SSB, TFAM, mitochondrial DNA ligase, RNA processing enzymes and different helicases and topoisomerases, TFB1M, TFB2M and mTERF. Some of these carry out their activity in both replication and transcription processes, such as the TFAM factor, that plays an overlapping role since it appears crucial for the regulation of genome copy number, although it is not a component of the basic replication machinery, and for mtDNA transcription, by unwinding the mtDNA helix and thus exposing the promoter region to the transcription machinery (57, 58). Other factors act as transcription regulators which can modulate the transcription of nuclear-encoded genes involved in various mitochondrial functions and biogenesis, such as nuclear respiratory factors 1 and 2 (NRF-1, NRF-2). Alternatively, they can be imported into mitochondria and alter transcription of the mitochondrial genome, for example the PPARγ coactivator 1α factor (PGC-1 gamma) (55, 59).

Mitochondrial DNA replication and transcription are constantly regulated by coordinated nuclear and mitochondrial pathways, during development and at both physiological and patho-physiological conditions, in order to ensure efficient biogenesis process, cell's energy demand, mitochondrial oxidative metabolism and proper cellular function (55, 59-62).

3.2. Genetics of mtDNA

Distinctive features of mtDNA make it particularly interesting for genetic studies as well as for the understanding of the aetiology of mitochondrial diseases. With rare exceptions reported in diverse eukaryotic taxa, it is inherited only maternally, through the oocyte cytoplasm, meanwhile mtDNA molecules in mammalian sperm, modified by ubiquitin labelling during spermatogenesis and degraded by the proteasomes and/or lysosomes after fertilization, are never transmitted to offspring, likely because they are highly damaged by ROS (Reactive Oxidative Species) produced during the spermatogenesis and the sperm swimming (63-68). For this reason, it has long been believed that mtDNA did not undergo recombination. However, over the last five years, some direct evidence concerning the existence of this process progressively emerging, taking into consideration that, in some cases, human paternal mtDNA may enter the egg and that mammalian mitochondria contain the enzymes necessary to promote homologous recombination (69-73).

Another form of mtDNA inheritance concerns the transfer of a large fraction of the mitochondrial genomic information to the nuclear genome, representing an important mechanism of genetic variation that helped to forge the prokaryote-to-eukaryote transition (74, 75). This transfer, that involves repair of double-stranded breaks by non-homologous end-joining, generates nuclear copies of mitochondrial DNA (NUMTs) which or are re-imported or, mostly, acquire novel functions. NUMTs account for a noticeable fraction of the nuclear genome. In the human genome at least 400 kb, in *Nasonia* 43 kb and in Apis over 230 kb of the nuclear DNA consists of NUMTs (76). In contrast, some genomes such as that of *Drosophila melanogaster* are nearly devoid of mitochondrial DNA (77). NUMTs appear to preferentially integrate into repetitive DNA sequences as well as into DNA regions with different GC content, thus suggesting that chromosomal structure might influence integration of NUMTs (78). Some NUMTs have accumulated many changes, and thus have resided in the nucleus for a long time, while others are similar to the reference human mtDNA, and thus must be recent. In humans, a dozen human loci are polymorphic for the presence of NUMTs, underscoring the rapid rate at which mitochondrial sequences reach the nucleus over evolutionary time. Overall, it is believed that

about a third of human-specific NUMTs is variable. In this context, NUMTs have been suggested as an interesting tool in primate phylogeny (79). Integration of NUMTs not only appears as neutral polymorphism but, more rarely, is also associated with human diseases, including severe plasma factor VII deficiency (bleeding disease), Pallister-Hall syndrome, mucolipidosis IV (80).

mtDNA has an evolutionary rate higher than that of the nuclear DNA, attributable to a high mutation rate due to both the lack of effective mitochondrial repair mechanisms and constant exposure of this DNA to oxygen free radicals which mostly derive from the mitochondrial oxidative phosphorylation system (OXPHOS). The accumulation of damages in mtDNA and the subsequent OXPHOS impairment over the course of life are at the basis of several aging and diseases models, in which mitochondria and mitochondrial genome play a central role (81-85).

Given the presence of multiple copies of mtDNA within each cell (polyplasmy), when a mutation occurs, both normal and mutated mtDNA can coexist in varying proportion, a condition known as heteroplasmy. The level of heteroplasmy can vary among cells, tissues, organs within the same individual, and among individuals in the same family as the result of a random distribution of both normal and mutant mtDNA molecules to the daughter cells (mitotic segregation). In human, many heteroplasmic mutations are associated to pathologies, in which biochemical defects in the respiratory chain can be detected only if the levels of mutations exceed a threshold value (about 60-80%), that is correlated to mutation type and to tissue's energy demand (86, 87). The occurrence of mutant and germinal mtDNAs in different organelles in the same cells may cause complementation, a process in which mitochondria fuse and mix their mtDNAs, so malfunctioning mitochondria can retrieve a wild-type mtDNA copy. This exchange should allow either the removal of the defective copy of the mtDNA or its repair by using a series of enzymes involved in this process. The mechanisms governing complementation, segregation and transmission of heteroplasmic mtDNA mutations depend on the dynamics of the mitochondrial compartment, the intra-mitochondrial organization and the mobility of mtDNA (33, 88, 89).

A substantial number of inherited mutations accumulate over time along radiating female lineages, that give rise to many types of mtDNA. These lineages differ with respect to mutations present in their ancestor and evolve independently. According to the presence/absence of specific variants at evolutionarily conserved positions, mtDNA types were categorized into haplogroups, which define a cluster of different mtDNA molecules sharing a common origin (90-93). The haplogroups have been identified by searching for population polymorphic sites, initially through the use of RFLP analysis and then by direct sequencing of both mtDNA coding and non coding regions. More recently, complete mtDNA sequencing allowed the subdivision of haplogroups into smaller groups, known as sub-haplogroups (94). The uniparental inheritance of mtDNA and associated lack of intermolecular recombination mean that these variants have remained restricted to specific ethnic groups and have been used by population geneticists to define the migration and colonization of the planet, supporting the 'out of Africa' hypothesis, which proposes that the human mtDNA had its origins in Africa before migrating out and populating the globe (87). The mtDNA variations were initially looked at as nearly neutral and used only for the reconstruction of human population history (95). The finding that these variations can have functional consequences and some of them are able to produce a spectrum of clinical symptoms in several mitochondrial diseases changed the view of their neutrality. In particular, such a view arose when it was shown that the different mtDNA lineages are qualitatively different from each other. The first evidence for this was provided by Ruiz-Pesini and co-workers, who reported that mtDNA molecules of H and T haplogroups displayed significant differences in the activity of OXPHOS Complex I and IV (96). More recently, significant associations have been found between mtDNA haplogroups and physiological phenotypes, including higher mitochondrial copy number, decreased reactive oxygen species production, mitochondrial metabolism, body fat mass, hearing loss, general cognitive ability, aging as well as with pathological traits, such as Parkinson and Alzheimer disease, diabetes, cardiovascular disease, schizophrenia, Leber's hereditary optic neuropathy and cancer, in which they were found to contribute to oncogenesis and metastatic spread (95, 97-103). In addition, specific mtDNA variants seem to contribute to climatic adaptation in human populations by regulating bio-energy processes (104-107). The above associations have been also explained considering that, although physically distinct, nucleus and mitochondria interact with each other through a bi-directional flow of information involving several signal transduction pathways (108, 109).

A significant improvement in understanding the influence of specific mtDNA variants on cellular phenotypes and, therefore, the relationship between these variants and complex traits has come from *in vitro* studies using cybrid cell lines, made by the repopulation of rho^0 cells lacking of mtDNA with mitochondria derived from enucleated cells harbouring particular types of mtDNA molecules, i.e. mutated DNAs, on a common nuclear genetic background (110-112). More specifically, mtDNA and mtRNA levels, mitochondrial protein synthesis, cytochrome oxidase levels and activity, normalized oxygen consumption, mitochondrial inner-membrane potential and growth capacity were found to be different in cybrids with mtDNA molecules harbouring the H haplogroup, representing about 41% of mtDNA types

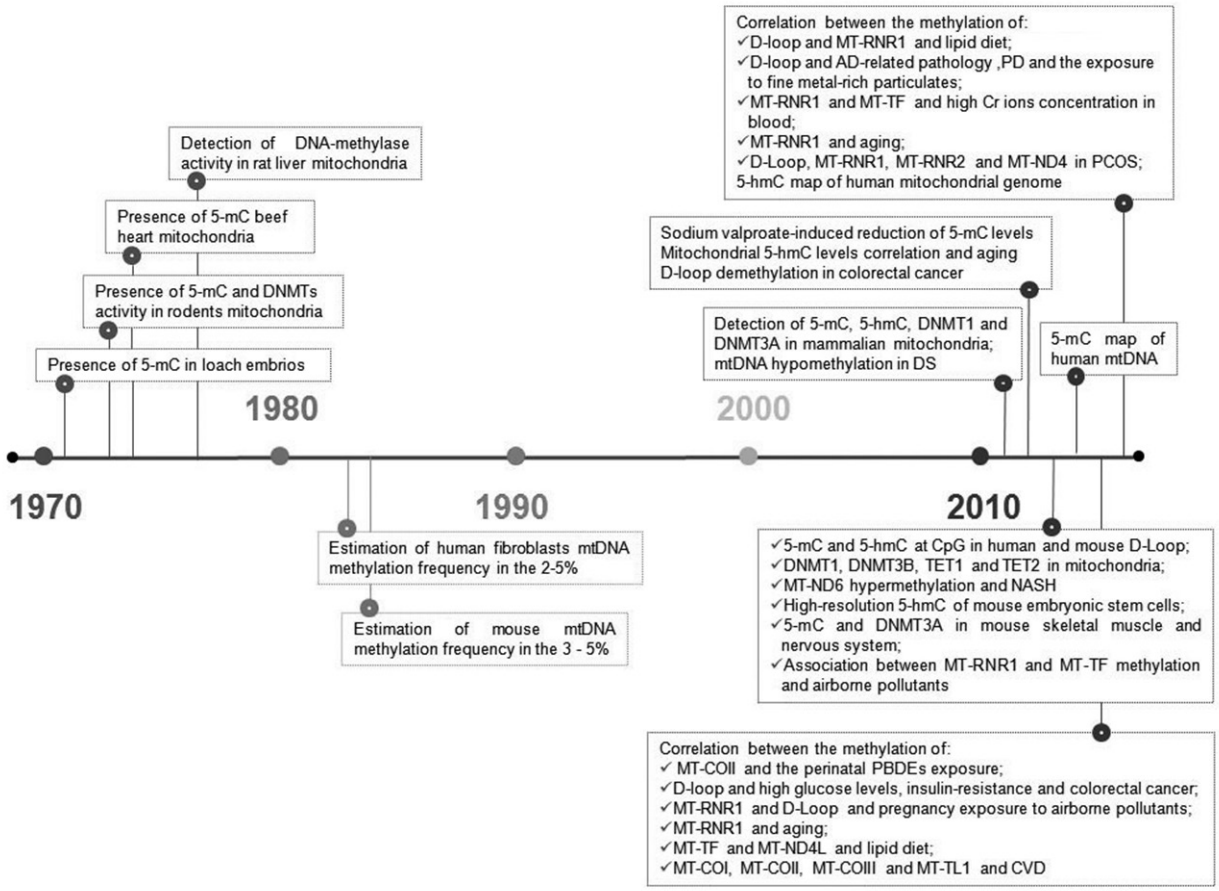

Figure 1. Timeline (years) of key discoveries supporting the existence of mitochondrial DNA methylation and hydroxymenthylation.

in our continent, when compared with those from the UK haplogroup, found in about 25% of Europeans (113). In addition, changes in expression levels of stress-responder genes as well as in intracellular ROS levels were ascribed to specific mtDNA sequences (114-116). It was also reported that mtDNA variants influence global DNA methylation most likely through a differential oxidative phosphorylation efficiency. In addition, Smiraglia *et al*. provided the first direct evidence that the absence/presence of mtDNA modulates the nuclear epigenetic modifications by influencing DNA methylation of several nuclear genes (7).

4. MITOCHONDRIAL DNA EPIGENETICS

4.1. Mitochondrial DNA methylation and hydroxymethylation

The possibility that the mitochondrial DNA can be the site of epigenetic modifications has long been the subject of intense discussion and controversies (Figure 1). The multicopy genome, the absence of canonical CpG islands as well as the lack of histones and their relative modifying enzymes prompted researchers to consider with skepticism the possibility that mitochondrial DNA could be a target of epigenetic modifications. Despite this, the under-representation of CpG dinucleotides within the mtDNA, with most of these co-locating with polymorphic variants, has suggested a susceptibility to mutation of these dinucleotides in the mitochondrial genome and, consequently, to methylation (117). The first attempt to identify traces of mtDNA methylation dates back to the early 1970s in loach embryos, beef heart and several mammalian mitochondria, that also showed the presence of DNA methylating enzymes within mitochondrial compartments (118-122). By contrast, roughly in the same period, there was no trace of cytosine methylation on mtDNA detected in yeast, *Ascomycete* fungi, rat, calf, frog, HeLa cells (123-125). A few years later, Shmookler Reis and Goldstein as well as Pollack *et al*. estimated a rate of mtDNA methylation equal to 3-5% in both human and mouse fibroblast (126, 127). Except for the failed attempt to find traces of mtDNA methylation in samples from gastric and colorectal cancer deployed by Maekawa *et al*. aimed at searching a mitochondrial epigenetic biomarker for cancer prediction and detection, the research in this field lapsed (128).

After nearly thirty years, the advent of more innovative and sensitive techniques, the discovery of DNA methyltransferase (DNMTs) family members in mitochondrial protein fractions and even more importantly the evidence proving the influence of mtDNA in nuclear epigenetics brought back the interest for mitochondrial epigenetics (7, 8, 13, 129-133). Initially, a mitochondrial isoform of DNMT1 was discovered in both human and mouse (13, 130, 134). Afterwards, also DNMT3A and DNMT3B were found localized within mitochondria, in a tissue-specific fashion (13, 131, 135). However, the persistence of methylated cytosines in the D-loop region of mouse ES cells that lacked for DNMT1, DNMT3A, and DNMT3B, although less marked than that of the wild type sample, suggested that a nonexclusive involvement of DNMTs in the establishment and maintenance of the mtDNA methylation patterns have to be regarded (13).

The 5-mC appeared to have a strand-specific role within the mitochondrial genome. Indeed, strand-specific bisulfite sequencing revealed that methylation is limited to the L-strand, with a prevalence in non-CpG sites (13). Moreover, asymmetrical effects on the transcripts expression from the heavy and light strands of mtDNA in mtDNMT1 overexpressed cells was observed, with increased levels of ND1, encoded by the H-strand and decreased levels of ND6, encoded by the L-strand (130). An inverse correlation between mt-ND2 and mt-ND6 expression was also independently observed in pathological phenotypes (14, 136).

An attractive hypothesis formulated to explain the functional relationship between methylation and gene expression of the mitochondrial genome has been detailed by Van der Wijst and Rots (137). These authors, indeed, suggested that mtDNA methylation regulates the affinity of TFAM binding and, thus, its action on mtDNA. This may result in an increased DNA compaction and in a reduced accessibility for POLRMT and TFB2B that may induce mitochondrial biogenesis rather that electron transport subunit transcription.

In addition, mtDNA methylation could be involved in the processing of mitochondrial polycistronic primary transcript (11,138-140). By alternative experimental approaches, such as 5-mC immunoprecipitation (Me-DIP), bisulfite sequencing, bisulfite pyrosequencing, bisulfite-next generation sequencing (NGS), Illumina MiSeq sequencing platform and liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS), the presence of methylated cytosines within the mitochondrial D-loop as well as in genes encoding for ND6, Cytb, COI, 12S rRNA, 16S rRNA, phenylalanine tRNA was evaluated (12-14, 134, 141-144). More recently, Ghosh *et al*., despite mitochondrial DNA polyploidy and its tissue- and developmental stage specific variable number, described a comprehensive mitochondrial methyl cytosine map. The authors pointing out that, except for some regions, such as *mt-ND6* and *mt-ATP6* that show methylation changes according to brain development, the pattern and the distribution of 5-mC across this genome appears quite constant (15). By contrast, only Hong *et al*. and Liu *et al*. have denied the existence of methylation within the mitochondrial genome, arguing this claim on the grounds that cytosine methylation within mtDNA is a very rare event and that such low levels cannot have any functional biological relevance (145, 146).

Next to cytosine methylation, cytosine hydroxymethylation (5-hmC) also recently raises significant interest and is regarded as a new mark. Although first discovered in 1972 in mouse and frog brain by Penn *et al*., a substantial boost in 5-hmC in evaluating this modified base has be provided the last 15 years, by experimental evidence reporting its presence in different tissues and cells and the occurrence of genome-wide changes during lineage commitment (147-152). In particular, the highest levels of 5-hmC has been detected in the central nervous system, comprising 0.6.% of the total nucleotides in Purkinje cells and 0.2.% in granule cells, while significantly variable levels were observed in other tissues as well as in cancer cells (149,153, 154). 5-hmC was initially considered as an intermediate of the oxidation of 5mC by the Ten-eleven traslocation (TET)- family of methylcytosine dioxygenases, in the active DNA demethylation pathway (148, 149, 155). However, considering its localization across the genome, mostly upstream of gene start site (GSS) regions and in gene body, its tissue-specific pattern as well as the deregulation of its levels, the 5-hmC appears to be closely implicated in the regulation of gene expression in both physiological and pathological conditions and in embryonic development (154, 156-159).

Recently, both mtDNA immunoprecipitation using antibodies directed against 5-hmC (hMe-DIP) and DNA-modification-dependent restriction endonuclease AbaSI coupled with sequencing (Aba-seq) showed a high density of 5-hmC within mitochondrial genome, indiscriminately at CpG and non-CpG sites (13, 130, 160, 161). These observations were supported by the finding of TET enzymes within the mitochondrial compartment (13, 160, 162). Very recently, Ghosh *et al*., through the use of genome scale datasets from 23 different cell lines and tissue types, designed a mitochondrial genome 5-hmC map. Interestingly, the mitochondrial hydroxymethylation profiles showed significant differences compared to the methylation once previously obtained by the same authors (15, 17). In particular, the 5-hmC density was lacking of conserved patterns across the mitochondrial genome, but they appeared mainly associated to cell and tissue-type, hinting towards a tissue-type specific role for 5-hmC. Moreover, similar to earlier reports, the regions comprising the gene start site (GSS) were characterized by a consistent

5-hmC density, with the highest levels in the GSS of tRNA genes of embryonic cell lines. Even though the latter observation suggests a possible involvement for this modified base during the developmental stages, the high density around the GSS did not show a significant correlation with the associate gene expression. Lastly, the presence of hydroxymethylation was evaluated in mtDNA control region (D-loop). In agreement with previous reports, this region is characterized by 5-hmC density, whose density appears to be closely linked to the cell and tissue type as well as to the developmental stage, with the embryonic cells displaying the highest density of 5-hmC (17). All the above described dynamic features of 5-hmC suggest that this mark may have a functional relevance, potentially regulating mitochondrial gene expression, but studies are still in infancy and many aspects have still to be elucidated.

5. mtDNA METHYLATION AS BIOMARKER OF AGING AND DISEASES

Once the existence of methylation/hydroxymethylation in the mitochondrial genome was confirmed, a significant number of reports have quickly provided that these two mitochondrial epigenetic marks exhibit significant correlation with environmental exposure, peculiar phenotypes and diseases, similar to what was observed for the methylation of the nuclear genome (Table 1).

5.1. mtDNA methylation and environmental exposures

A wide variety of environmental ubiquitous factors have been shown to influence epigenetic patterns in human and model organisms, resulting in both hypo and hypermethylation changes *in utero*, juvenile and adult life stages (11, 163-165). With regard to environmental-induced mitochondrial DNA methylation changes, interesting information has emerged from Byun *et al*. (12, 16) who analyzed the association between exposure to airborne pollutants and metal-rich particulates and blood mtDNA methylation. Authors observed that steel workers exposed to metal-rich particulate matter (measured as PM1) showed higher methylation levels of genes encoding for transfer RNA phenylalanine (*MT-TF*) and 12S ribosomal RNA (*MT-RNR1*) than low-exposed controls, while no significant differences were observed concerning the D-loop methylation. Conversely, the exposure to fine metal-rich particulates resulted in a significant reduction of the D-loop methylation levels and was significantly associated with markers of heart rate variability, but did not influence the *MT-TF* and *MT-RNR1* methylation. In addition, air benzene and traffic-derived EC exposure did not induce any effect on mtDNA methylation (12, 16). The analysis of chromate plating workers, displaying high concentration of Cr ions in their blood, were characterized by lower methylation levels in *MT-TF* and *MT-RNR1* genes (19). The apparent discrepancy between the two results was ascribed to the different exposure conditions, that might have different effects on mtDNA methylation.

It has been widely documented that the gestational environment stimuli received during prenatal life alter the global epigenome of placenta. Such stimuli could have both long-lasting effects on health span of an individual and transgenerational effects on fetal epigenomics reprogramming, according to still unclear mechanisms (166-169). In this context, a positive correlation has been demonstrated between placental mtDNA methylation of both *MT-RNR1* and D-loop, associated to a decrease in mtDNA content, and the pregnancy exposure to airborne particulate matter (PM) (170). Moreover, a reduction in the 5-mC levels of *MT-COII* was observed following the perinatal exposure of rats to Polybrominated diphenyl ethers (PBDEs), an organic chemical used as flame retardants in a variety of materials (171).

Beside the environmental pollutants, pharmacological agents were also found to have off-target effects on epigenetic signature (9, 172). Currently, the sole direct involvement of a mitochondrial epigenetics drug is represented by the anticonvulsant mood stabilizer sodium valproate (VPA), able to decrease the 5mC, but not the 5-hmC levels in mouse cultured cells (168). The potential role in mitochondrial epigenetics for a variety of drugs which regulate the intracellular epigenetic mechanisms and/or mitochondrial activity still needs to be clarified. A good candidate might be, for example, cocaine, which was associated on the one hand to the increase of both DNMT3A and DNMT3B gene expression, on the other to the d nuclear encoded mitochondrial genes (173-176).

Lastly, an mtDNA methylation involvement in the field of nutritional epigenetics studies has emerged very recently. Studies carried out on large yellow croakers (*Larimichthys crocea*) revealed effects on mtDNA methylation in fish fed different lipid sources. Indeed, in the liver, the methylation levels of *MT-TR* and *MT-ND4L* genes were significantly higher in fish fed with Perilla and olive oils, whereas those of *MT-RNR1* were lower in fish fed with olive oil with respect to the group fed with Fish and Sunflower oils. No changes in D-loop methylation were observed in all dietary treatments (177). In addition, with respect to the control group, fish fed a high- and a low-lipid diet were characterized by an increase of D-loop and *MT-RNR1* methylation, respectively (178).

5.2. mtDNA methylation and aging

The first evidence for a susceptibility to aging of mitochondrial epigenetic mechanisms was provided in 1983, with the observation of a hypermethylation of mtDNA in elderly cultured fibroblasts compared to those from younger donors (126). More recently, Dzitoyeva

Table 1. List of regions located within mitochondrial DNA displaying methylation changes according to specific phenotypes

Gene	Description	Methylation Change	Phenotype	Reference
D-loop	DNA control Region	↑	Pregnancy exposure to airborne particulate matter	159
		↑	Low-lipid diet in fish	166
		↑	AD-related pathology	17
		↑	Insulin resistance	171
		↑	High glucose	123
		↑	Polycystic ovarian syndrome	172
		↓	Parkinson Disease	17
		↓	Colorectal cancer	175
		↓	Exposure to fine metal-rich particulates	15
MT-RNR1	12S ribosomal RNA	↑	Exposure to high metal-rich PM1 exposure	11
		↑	Pregnancy exposure to airborne particulate matter	159
		↑	Low-lipid diet in fish	167
		↑	Aging	9
		↑	Polycystic ovarian syndrome	172
		↓	Aging	133
		↓	High Cr ion blood concentration	18
MT-RNR2	16S ribosomal RNA	↑	Polycystic ovarian syndrome	172
MT-TF	tRNA Phenylalanine	↑	Exposure to high metal-rich PM1 exposure	11
		↑	Perilla and olive oil diet in fish	166
		↓	High Cr ion blood concentration	18
MT-TL1	tRNA leucine 1	↑	Cardiovascular disease	173
MT-COI	Cytochrome c oxidase subunit I	↑	Cardiovascular disease	173
MT-COII	Cytochrome c oxidase subunit II	↓	Perinatal exposure to Polybrominated diphenyl ethers	160
		↑	Cardiovascular disease	173
MT-COIII	Cytochrome c oxidase subunit III	↑	Cardiovascular disease	173
MT-ND1	NADH dehydrogenase subunit 1	↓	AD-related pathology	17
MT-ND4	NADH dehydrogenase subunit 4	↑	Polycystic ovarian syndrome	172
MT-ND4L	NADH dehydrogenase subunit 4L	↑	Perilla and olive oil diet in fish	166
MT-ND6	NADH dehydrogenase subunit 6	↑	Nonalcoholic fatty liver disease	13

et al. analyzed different regions samples of brain from differently-aged mice, and observed not only the presence of both mitochondrial 5-mC and 5-hmC, but more interestingly, that progressive changes in these mitochondrial epigenetic marks occurs during lifespan in a region-specific manner (160). Particularly, Dzitoyeva *et al*. observed a decrease in 5-hmC but not in 5-mC levels and an increase in the expression of randomly selected mtDNA-encoded genes in the frontal cortex and in the cortex although no aging-associated changes in TET mRNAs, responsible for 5-hmC synthesis, were found. Conversely, no change in the levels of 5-hmc as well as in mRNA changes of mtDNA-encoded genes has been noted in the cerebellum, despite an increase of TET2 and TET3 expression. Furthermore, the expression of mtDNMT1 transcript in the brain and its modulation by aging was demonstrated (160). Moreover, D'Aquila *et al*., by analyzing human *MT-RNR1* and *MT-RNR2* genes,

encoding for 12S and 16S ribosomal RNA, respectively, revealed the presence of methylation at a CpG site of *MT-RNR1* (nucleotide position 932) as well as a positive increase of its methylation levels according to age and gender of analyzed samples (143). These findings, integrated by a survival analysis, reported that high methylation levels at the mtDNA 932 position significantly increase the mortality risk. This result suggested a still unclear functional role for *MT-RNR1* methylation that, ultimately may hamper individual survival chance. A significant role of *MT-RNR1* methylation in aging has also emerged from data obtained by Mawlood *et al*. which, by evaluating the methylation levels of 133 CpG sites in the mitochondrial genome by Illumina Sequencing, showed a stronger, negative correlation between two *MT-RNR1* CpG sites (nucleotide positions 1215 and 1313) and aging (144). The apparent discrepancy between the two previous results could be explained by the fact that the correlation between *MT-RNR1* methylation and aging could be site-specific. In addition the discrepancy could be strongly influenced by gender, environmental factors, nutrition and drugs, as also demonstrated for age-related nuclear epigenetic changes (12, 162, 179, 180).

5.3. mtDNA methylation and diseases

A mitochondrial involvement in the etio-pathogenesis of several diseases has been widely described and, only recently a role for mtDNA epigenetics is emerging. The first association between mtDNA methylation and disease was reported by Infantino *et al*., which observed a hypomethylation of mtDNA despite the increase of mtDNA content in Down Syndrome (DS) mtDNA (181). Afterwards, evidence from several publications has revealed that mitochondrial methylation may be regarded as a biomarker of neurodegenerative diseases. High levels of global 5-mC levels and of both DNMT1 and DNMT3A were detected in the mitochondria of neurons of patients with amyotrophic lateral sclerosis (ALS) (131). In addition, a significant abnormality in MT-RNR2 methylation and a severe loss of mitochondrial DNMT3A in skeletal muscle and spinal cords of ALS mouse models at presymptomatic or early symptomatic stages of disease were demonstrated (132). Similarly, alterations in mtDNA methylation were observed in Alzheimer Disease-related (AD) pathologies and Parkinson disease (PD). Blanch *et al*. observed, in both human samples and AD mouse models, increased methylation levels at both CpG and non-CpG sites in the D-loop region in AD-related pathology and a slight demethylation in the MT-ND1 gene, associated to an increase of ND1 expression. Conversely, a loss of D-loop methylation was observed in the substantia nigra of PD cases compared to the controls, whereas the 5-hmC levels in both AD-related pathology and PD remained unchanged (18).

It has also emerged that mtDNA methylation may be implicated in metabolic disorders. An *MT-ND6* hypermethylation, associated to a significant decrease in ND6 expression was observed in Nonalcoholic fatty liver disease (NASH) with respect to simple steatosis (SS) patients, thus suggesting an involvement of mtDNA methylation in the transformation from SS to NASH (14). A significant increase in D-loop methylation was also detected in obese and insulin-resistant individuals (182). In addition, compared to the control samples, high glucose in bovine and human retinal endothelial cells significantly increase the intra-mitochondrial DNMT1 levels and its binding to the mtDNA at both the D-loop and *Cytb* as well as the 5-hmC at the D-loop and *Cytb* regions of more than 3- and 2- fold, respectively. The increase in D-loop methylation was also associated to a decrease in mtDNA encoded gene expression, thus suggesting that in diabetes the mtDNA hypermethylation may result in dysfunctional mitochondria and promote capillary cell apoptosis (136). Lastly, by searching for the involvement of mtDNA epigenetics in the mitochondrial dysfunction occurring in Polycystic ovarian syndrome (PCOS), Jia *et al*. revealed that a hypermethylation in the D-loop, *MT-RNR1*, *MT-RNR2* and *MT-ND4* occurs in PCO oocytes in accordance with a down-regulated expression of mtDNA-encoded genes and impaired mitochondrial function (183).

The association between nuclear DNA methylation marks and cardiovascular disease (CVD) has been widely demonstrated. Analyses carried out in both CVD and healthy patients by bisulfite pyrosequencing demonstrated that cases displayed higher methylation levels than controls in *MT-COI*, *MT-COII*, *MT-COIII* and *MT-TL1* genes, meanwhile no significant difference in methylation was observed for *MT-ND6*, *MT-ATP6*, *MT-ATP8* and *MT-ND5* (184).

Limited data about the potential association between mitochondrial DNA methylation and cancer are currently available. A very low occurrence of mtDNA methylation in gastric, colorectal and cervix cancer was reported (141). In addition, a negative association between DNA copy number with cytosine methylation and hydroxymethylation was found in hepatocellular carcinoma cells (185). On the other hand, increased mtDNA copy number and ND2 levels associated with a markedly reduced methylation status of the D-loop were observed in colorectal cancer and associate to the clinic-pathological stages of the disease (136, 186).

6. CONCLUSIONS AND FUTURE PERSPECTIVES

The identification of epigenetic changes in the mitochondrial genome has led to extreme revision of previous knowledge on phenotypic implications of mtDNA genetic variations. Indeed, the association between mtDNA methylation and several phenotypes suggests an original scenario about the role of mitochondria in the

cell life and sheds light on the identification of a series of biomarkers implicated in the above phenotypes. However, understanding of the molecular mechanisms by which mtDNA methylation operates is still in its infancy. The predominance of methylated and/or hydroxymethylated cytosines within the D-loop and in the upstream of gene start sites suggest a possible regulatory role in mtDNA expression, that requires further investigation. Once the role of epigenetic marks in mtDNA have been clarified and the reversibility of epigenetic modifications are elucidated, we predict that exciting advances will prompt the search for mitochondrial-specific therapeutic agents able to restore the altered epigenetic equilibrium.

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Abbreviations: 5-hmC: Cytosine hydroxymethylation, 5-mC: Cytosine methylation, AD: Alzheimer Disease, ALS: Amyotrophic Lateral Sclerosis, COI-III: Cytochrome c oxidase subunits I-III, CSBI-III: Conserved Sequence Blocks I-III, CVD: CardioVascular Disease, Cytb: Cytochrome b, DNMT: DNA MethylTransferase, DS: Down Syndrome, ES cells: Embryonic Stem cells, GSS: Gene Start Site, hMe-DIP: h5-mC immunoprecipitation, HSP: Heavy-Strand Promoter, HVRI-II: Hyper Variable Region I and II, LC-ESI-MS: Liquid chromatography-electrospray ionization tandem mass spectrometry, LSP: Light-Strand Promoter, Me-DIP: 5-mC immunoprecipitation, MT-ATP6: ATP synthase F0 subunit 6, MT-ATP8: ATP synthase F0 subunit 8, mtDNA: Mitochondrial DNA, mTERF: Mitochondrial Transcription Termination Factor 1, MT-ND1: NADH dehydrogenase subunit 1, MT-ND4: NADH dehydrogenase subunit 4, MT-ND4L: NADH dehydrogenase subunit 4L, MT-ND5: NADH dehydrogenase subunit 5, MT-ND6: NADH dehydrogenase subunit 6, MT-RNR1: 12S Ribosomal RNA, MT-RNR2: 16S ribosomal RNA, mtSSB: Mitochondrial Single-Stranded Binding proteins, MT-TF: tRNA Phenylalanine, mitochondrial- encoded, MT-TL1: tRNA Leucine 1, mitochondrial- encoded, NASH: Nonalcoholic fatty liver disease, NGS: Next Generation Sequencing, OXPHOS: Oxidative Phosphorylation, PCOS: PolyCystic Ovarian Syndrome, PD: Parkinson Disease, PM: Particulate Matter, POLG: Mitochondrial DNA Polymerase Gamma, POLRMT: Mitochondrial RNA Polymerase, RFLP: Restriction Fragment Length Polymorphism, RITOLS: RNA Incorporated Throughout the Lagging Strand, ROS: Reactive Oxidative Species, rRNA: Ribosomal RNA, SDM: Strand-Displacement Model, SS: Simple Steatosis, TET: Ten-Eleven Traslocation (TET) enzyme, TFAM: Mitochondrial Transcription Factor A, TFB1M: Mitochondrial Transcription Factor B1, TFB2M: Mitochondrial Transcription Factor B2, tRNA: Trasfer RNA, VPA: Sodium Valproate

Key Words: mtDNA Genetics, mtDNA Epigenetics, mtDNA Methylation, mtDNA Hydroxymethylation, Aging, Diseases, Environmental Factors, Review

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