Chapter 7

Mitochondrial Abnormalities in Down Syndrome: Pathogenesis, Effects and Therapeutic Approaches

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Abstract

Down syndrome (DS) consists of a complex phenotype with constant features, such as mental retardation and hypotonia, and variable features, including heart defects and susceptibility to Alzheimer's disease, type 2 diabetes, obesity and immune disorders. Overexpression of genes mapping to chromosome 21 (Hsa21) is directly or indirectly responsible for pathogenesis of DS phenotypic features, as overexpressed Hsa21 genes dysregulate several other genes mapping to different chromosomes. Many of these genes are involved in mitochondrial function. Recent studies highlight a link between mitochondrial dysfunction, consistently observed in DS subjects, and DS phenotype. In this review, we first provide a basic overview of mitochondrial alterations in DS in terms of mitochondrial bioenergetics, biogenesis and morphology. We then discuss how mitochondrial malfunction may contribute to the pathogenesis of clinical manifestations and how specific Hsa21 genes may cause the disruption of mitochondrial phenotype. Finally, we focus on drugs, which affect mitochondrial function and network to propose possible therapeutic approaches aimed at improving and/or preventing various aspects of the DS phenotype. Our working hypothesis is that correcting the mitochondrial defect might improve the neurological phenotype and prevent DS-associated pathologies, thus providing a better quality of life for DS individuals and their families.

Keywords: Down syndrome, trisomy 21, mitochondrial dysfunction, mitochondrial dynamics, Down syndrome/therapy

1. Introduction

Down syndrome (DS) (OMIM 190685), caused by the trisomy of chromosome 21 (TS21), is the most common autosomal aneuploidy compatible with postnatal survival with a prevalence of

1 in 700 newborns. Its phenotype is highly complex showing constant features, such as mental retardation, dysmorphic traits and hypotonia, and variable features, including heart defects, susceptibility to Alzheimer's disease (AD), type 2 diabetes, obesity and immune disorders. DS is also a risk factor for a number of diseases, such as thyroid dysfunction, leukemia and various other congenital malformations. The mechanisms causing the DS phenotype are still largely unknown and little progress has been registered so far in the therapeutic approach to ameliorate the life of DS subjects.

Overexpression of genes mapping to chromosome 21 (Hsa21) is clearly responsible for pathogenesis of DS phenotypic features either in a direct or indirect manner, as overexpressed Hsa21 genes affect the regulation of several other genes mapping to different chromosomes. Many of these genes are involved in oxidative phosphorylation (OXPHOS) and more generally in the mitochondrial function [1].

As fully described in the following paragraphs, the mitochondrial dysfunction together with the disruption of the mitochondrial network might concur to determine DS phenotypic traits. This suggests that correcting the mitochondrial defect might affect the severity of DS phenotype.

This review provides first a basic overview of mitochondrial alterations in terms of mitochondrial bioenergetics, biogenesis and morphology in DS. The latest theories are reported about: (i) how mitochondrial malfunction may contribute to the pathogenesis of clinical manifestations of DS and (ii) how specific Hsa21 genes may be involved in determining the pathogenesis of mitochondrial dysfunction in DS. Finally, we focus on drugs that target genes and/or pathways involved in mitochondrial function and mitochondrial network to examine potential therapeutic approaches.

2. Mitochondrial abnormalities in DS

Increasing evidences, widely documented in scientific literature, highlight that there is a link between mitochondrial damages and the complex DS phenotype. The downregulation of nuclear-encoded mitochondrial genes (NEMGs) is a hallmark of TS21 in human fetal hearts [1] and brains [2]. Transcriptome analysis of fetal heart tissues showed that more than 400 genes located on chromosomes other than 21 were differentially expressed, either upregulated or downregulated, in trisomic versus non-trisomic hearts [1]. Functional class scoring of these genes revealed a global downregulation of NEMGs. Together with the downregulation of genes involved in mitochondrial pathways, we demonstrated, in trisomic fetal fibroblasts of the same subjects, that mitochondria exhibited morphological abnormalities like increased size, irregular shape and evident breaks, mainly of inner membranes. Mitochondria with concentric and longitudinal cristae were significantly more abundant. Stereological analysis demonstrated that mean mitochondrial volume was significantly lower in DS cells [3, 4]. All indices of mitochondrial respiratory functions were decreased and a significant alteration in the redox homeostasis was observed, highlighted by an increased production of reactive oxygen species (ROS) and a

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higher steady level of intra-mitochondrial Ca^{2+} [3]. DS fibroblasts also showed a deficit of whole energy status as demonstrated by a decrease of basal ATP content and of mitochondrial membrane potential (**Figure 1**) [4].

Representative confocal microscopy live cell imaging of TMRM fluorescence in euploid and trisomic fibroblasts. A significant decrease in fluorescence intensity is observed in trisomic samples when compared with euploid ones.

These results were in agreement with different studies that demonstrated a less efficient mitochondrial energy production apparatus in fibroblasts from DS subjects due to the impairment of mitochondrial respiratory chain complex I, ATP synthase, ADP/ATP translocator and adenylate kinase activities [5, 6].

The protein expression of mitochondrial electron transport enzyme subunits has been found decreased in the brain of people affected by DS [7]. Decreased mitochondrial redox activity and membrane potential have also been observed both in DS astrocytes [8, 9] and in the brain of the Ts1Cje mouse model [10]. In neural progenitor cells (NPCs) isolated from the hippocampus of Ts65Dn mice, another widely used model of DS, a severe impairment of mitochondrial bioenergetics and biogenesis and reduced NPCs proliferation were demonstrated [11]. Furthermore, microarray analysis revealed that numerous pathways were altered in Ts65Dn muscle, including pathways involved in ATP biosynthesis [12].

Together with mitochondrial function alterations, a significant disruption of mitochondrial dynamics has been observed in trisomic cells. An increased fragmentation of the mitochondrial network was demonstrated in primary cultures of TS21 astrocytes and neurons [13] and in trisomic fetal fibroblasts [4] (**Figure 2**). In agreement with the impairment of mitochondrial network towards the fragmentation, the expression of *MFN2* and *OPA1*, two fusion-inducing genes, was decreased in the same cells.

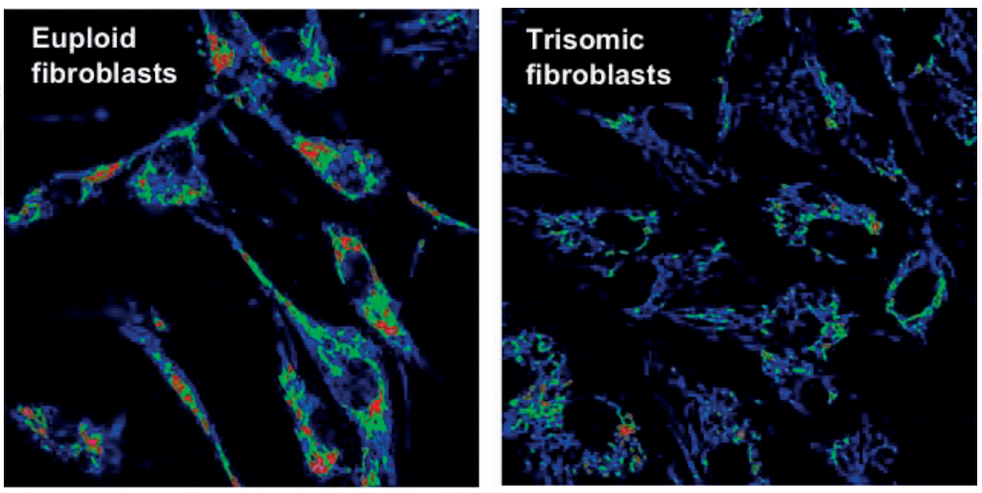

Figure 1. A significant decrease of fluorescence intensity demonstrates that membrane potential is reduced in DS fibroblasts.

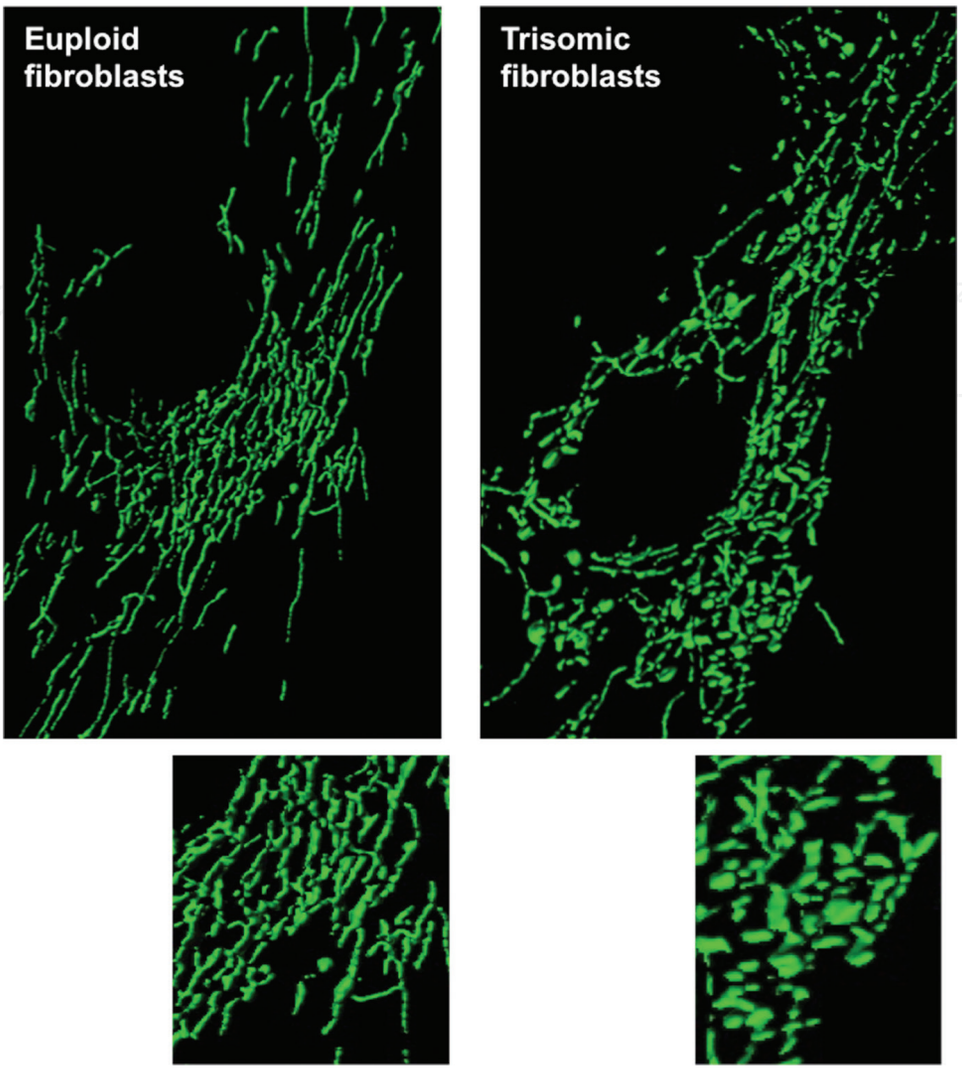

Figure 2. Mitochondrial network is fragmented in DS fibroblasts.

Representative images showing that the mitochondrial network is less fragmented in euploid fibroblasts than in trisomic ones. Magnifications of intracellular selected details show that the number of mitochondria is significantly higher in trisomic cells compared with non-trisomic cells.

A link between mitophagy and mitochondrial dynamics has been recently demonstrated [14, 15], as mitochondrial fusion and fission play a significant role in disease-related processes, such as mitophagy and apoptosis. Dysfunctional and damaged mitochondria are removed from the mitochondrial network via mitophagy processes. The segregation of impaired mitochondria due either to fission or to inhibition of fusion mechanism is hypothesized to be a requirement for this mitophagic degradation [16]. Mitophagy impairments are involved in the development of several neurodegenerative diseases [17].

The knowledge of molecular bases of mitochondrial dysfunction is allowing to set-up most appropriate therapeutic solutions to counteract it, as more fully described in the following paragraphs.

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3. Pathogenesis of mitochondrial dysfunction in DS

3.1. *PGC-1α* is a key modulator of mitochondrial biogenesis and respiratory function

A common denominator of most of the events that affect mitochondrial function is the transcriptional coactivator *PGC-1α*/*PPARGC-1α* (peroxisome proliferator-activated receptor gamma coactivator 1alpha), a master regulator of mitochondrial activity [4–6]. *PGC-1α*, through the interaction with transcriptional partners, such as *NRF1*, *ERRa*, *PPARs* and *YY1*, promotes mitochondrial biogenesis and regulates mitochondrial respiratory capacity [18, 19]. Also these *PGC-1α* transcriptional partners, as well as many NEMGs, have been found downregulated in DS fetal heart tissue [1] and fibroblasts [3]. *PGC-1α* knockout mice manifest a reduction of mitochondrial number and of respiratory capacity in skeletal muscle [18].

PGC-1α transcription and activity are positively regulated by Ca^{2+} signaling and negatively regulated by the Hsa21-coded corepressor *NRIP1* (nuclear receptor interacting protein 1) [19]. Indeed, *PGC-1α* has been found hypoexpressed at the transcriptional and protein levels in TS21 fetal fibroblasts, directly correlated with the amount of mtDNA, while *NRIP1* was upregulated [3]. PGC-1α activity was also found decreased in the hippocampus of DS patients, as well as in Alzheimer's, Huntington's (HD) and Parkinson's (PD) disease patients [20].

3.2. Role of Hsa21 genes in mitochondrial dysfunction

Little is known about the mechanisms by which trisomy 21 causes the abnormal features typical of the DS phenotype, apart the knowledge that the dosage imbalance of genes on Hsa21 and the resulting dysregulation of genes mapping to different chromosomes share the responsibility for molecular dysfunctions in DS.

Hsa21 gene expression was found globally upregulated 1.5-fold in trisomic samples [1, 2], in full agreement with a gene-dosage effect. A comprehensive meta-analysis from 45 DS gene expression studies [21] identified 77 Hsa21 genes mostly upregulated across all the studies, which are likely involved in the DS phenotype. Six of the genes included in this list, namely *NRIP1*, *SUMO3*, *DYRK1A*, *DSCR1*/*RCAN1*, *SOD1* and *APP*, are directly or indirectly involved in mitochondrial function. Other Hsa21 genes not included in the Vilardell's list, such as *ETS-2*, *ITSN1*, *PKNOX1*/*PREP1*, *BACH1* and S100B, were found to be involved in apoptotic events and/or to contribute to the regulation of oxidative stress when overexpressed [22]. The dysregulation of one or more of these genes, listed in **Table 1**, might account for mitochondrial alterations observed in DS, as discussed below.

3.2.1. NRIP1

We recently demonstrated that *NRIP1* overexpression is responsible for decreased respiratory efficiency and altered morphology of mitochondria in DS [23]. *NRIP1* is a corepressor that interacts with nuclear receptors and regulates the expression of genes that control metabolic processes such as energy homeostasis [24–27]. Its activity on mitochondrial pathways is

Genes and transcripts	Effects on mitochondrial phenotype
NRIP1/RIP140—nuclear receptor interacting protein 1	Decreases respiratory efficiency and alters morphology of mitochondria
APP—amyloid beta precursor protein	Induces mitochondrial oxidative stress and mitochondrial dysfunction
SUMO3—small ubiquitin-like modifier 3	Modulates *NRIP1* repressive activity and attenuates the transcriptional activity of *PGC-1α*
DYRK1A—dual-specificity tyrosine phosphorylation-regulated kinase 1A	Controls *PGC-1α* via the *calcineurin/NFAT* pathway
DSCR1/RCAN1—Down Syndrome critical region gene 1	Controls *PGC-1α* via the *calcineurin/NFAT* pathway and is associated with calcium overloading
SOD1—superoxide dismutase 1	Is associated with oxidative stress
ETS-2—V-ETS avian erythroblastosis virus E26 oncogene homolog 2	Promotes the activation of a mitochondrial death pathway
ITSN1—Intersectin 1	Regulates the mitochondrial apoptotic pathway
PREP1—PBX-regualting protein 1	Inhibits the OXPHOS negatively regulating *PGC-1α* and mitochondrial fusion genes *OPA1* and *MFN2*
BACH1—BTB domain and CNC homolog 1	Contributes to the early increase of oxidative stress in DS through the inhibition of the *HO-1/BVR-A* axis
S100B—S100 calcium-binding protein, beta	Overexpression induces ROS formation, activation of stress response kinases and increased programmed cell death
hsa-mir-155	Affects mitochondrial biogenesis by targeting *TFAM*
hsa-let-7c	May affect mitochondrial function by targeting *ANT1*

Table 1. Hsa21 genes and transcripts involved in mitochondrial function.

mainly exerted through the repressive control of *PGC-1α* [19]; the two proteins have mutually antagonizing roles in NEMG regulation. In neonatal rat cardiomyocytes, it was demonstrated that overexpressed *NRIP1* abrogates *PGC-1α*-mediated induction of mitochondrial membrane potential and mitochondrial biogenesis [25]. Furthermore, at least 1/3 of NEMGs upregulated after *PGC-1α* induction in human osteoblast-like cells [28] were found to be *NRIP1* targets [23].

To assess that, among the Hsa21 transcription regulators, *NRIP1* was indeed the main dysregulator of mitochondrial gene expression, the Gene Expression Omnibus (GEO) repository (http://www.ncbi.nlm.nih.gov/geo) was screened for gene expression data related to the modulation of Hsa21 genes. The functional class scoring of the lists of genes dysregulated, when Hsa21 genes were individually overexpressed (GSE19836 experiment [29]), demonstrated that, among the Hsa21 transcription factors or regulators, only *NRIP1* was able to affect NEMG regulation with a cluster of 37 NEMGs downregulated after *NRIP1* overexpression [23].

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We then demonstrated that *NRIP1* attenuation by siRNA in DS fibroblasts affected NEMG expression, increased *PGC-1α* expression and counteracted mitochondrial dysfunction in terms of ROS production, mitochondrial activity, mitochondrial calcium and ATP content [23].

These findings indicate that the Hsa21 gene *NRIP1* strongly contributes to the mitochondrial dysfunction observed in DS and suggest that the *NRIP1-PGC-1α* axis might represent a potential therapeutic target for restoring altered mitochondrial function in DS.

3.2.2. APP

Mitochondrial abnormalities and a decreased COX activity might also be induced by overproduction of beta-*APP* [30], although the TS1Cje mouse model, in which *APP* is not triplicated, also shows decreased mitochondrial function and ATP production [10]. Overexpression of APP induces mitochondrial oxidative stress and activates the intrinsic apoptotic cascade [31]. In addition, amyloid-β fragments, particularly Aβ42, exert direct toxic effects on cells, including Ca^{2+} dysregulation, mitochondrial dysfunction and induction of oxidative stress [32, 33]. APP protein has been demonstrated to progressively accumulate within mitochondrial matrix leading to increased free radicals and impaired mitochondrial metabolism [34]. In addition, APP have been shown to translocate into mitochondria when overexpressed in a human cortical neuronal cell line [35]. *APP* exerts synergistic effects with other Hsa21 genes [36].

3.2.3. SUMO3

It may affect mitochondrial function by modulating the *NRIP1* repressive activity [37]. SUMOylation also attenuates the transcriptional activity of *PGC-1α*, possibly by enhancing the interaction between *PGC-1α* and the corepressor *NRIP1* that alters its nuclear distribution [38]. *SUMO3* overexpression in DS could therefore be responsible for a concurrent improvement of *NRIP1* function and decrease of *PGC-1α* activity.

3.2.4. DYRK1A and DSCR1/RCAN1

The protein products of these genes interact functionally. Their increased dosage cooperatively leads to dysregulation of the signaling pathways that are controlled by *NFAT* family of transcription factors, with potential consequences for several organs and systems that are affected in DS individuals [39]. The two genes control *PGC-1α* activity via the calcineurin/NFAT pathway [40], namely through the binding of *NFATc* genes to the *PGC-1α* promoter [41]. Calcineurin is involved in the regulation of many cellular processes, including cardiac hypertrophy, skeletal-muscle development, synaptic plasticity and T-cell activation [39].

RCAN1, also known as calcipressin, has been found chronically overexpressed in the brain of both DS patients and sporadic AD patients [42]. *RCAN1* overexpression has been linked to oxidative stress and mitochondrial dysfunction [42–45] and is strictly related to calcium overloading [46], as it affects mitochondrial permeability transition pore (mPTP) function. *RCAN1*-induced mPTP opening leads to a series of consequences, including Ca^{2+} retention

incapability, massive swelling of mitochondria and rupture of the outer membrane [46]. In agreement with these data, deregulation of Ca^{2+} homeostasis and Ca^{2+}-mediated signaling has been described in cells derived from trisomic patients or in murine models of DS [47]. Mitochondrial Ca^{2+} concentration was found significantly higher in fibroblasts from DS feti [3], which also show swelled mitochondria with damaged membranes [4].

The overexpression of the brain-specific *RCAN1.1S* isoform in mice promotes dysregulation of dynamin-related protein 1 (*DRP1*), a protein that promotes mitochondrial fission [48]. Accordingly, *RCAN1* was found to induce mitochondrial autophagy in cardiomyocytes [49].

3.2.5. SOD1

The redox imbalance in DS has been long attributed to overexpression of Cu/Zn superoxide dismutase *SOD1*, whose levels are approximately 50% greater in cells from DS patients than in normal ones [50]. *SOD1*, the major *SOD* in mammalian cells, catalyzes the dismutation of superoxide radicals to H_2O_2 and O_2 and is an important antioxidant defense system [51].

3.2.6. ETS-2

Studies in transgenic mice showed that *ETS-2* overexpression induces apoptosis of thymus, spleen and brain cells [52]. Furthermore, ETS-2 promotes the activation of a mitochondrial death pathway in DS neurons. Overexpression of *ETS-2* induces cytochrome c translocation to the cytoplasm and apoptotic features in normal human cortical neurons [53].

3.2.7. ITSN1

This gene regulates the mitochondrial apoptotic pathway in endothelial cells [54].

3.2.8. PKNOX1/PREP1

This gene, which encodes for a homeodomain transcription factor, is involved in embryonic development regulating the homeobox protein Pbx activity [55]. DS human fibroblasts that express higher levels of *PREP1* are more sensitive to genotoxic stress. *PREP1* demonstrated to regulate mitochondrial oxidative phosphorylation components. It directly binds to the promoter region of genes encoding mitochondrial components [56] and indirectly controls the stability of p160 Myb-binding protein, a powerful negative regulator of *PGC-1α* activity [57]. In the muscle of *Prep1* ablated mice, *Pgc-1α* expression was increased with consequent increasing in abundance of mitochondrial OXPHOS proteins and in citrate synthase activity together with an improved maximal oxidative capacity. Most important, Prep1 ablation significantly increased the abundance of *Opa1* and *Mfn2*, two genes inducing mitochondrial fusion [56]. These results suggest that *PREP1* negatively regulates OXPHOS and mitochondrial network.

3.2.9. BACH1

This gene is a transcriptional regulator, which acts as hypoxia regulator by binding to antioxidant response elements of DNA thus inhibiting the transcription of specific genes involved

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in cell stress response, including heme oxygenase-1 (HO-1). HO-1 and its partner, biliverdin reductase-A (BVR-A), are upregulated in response to oxidative stress. BACH1 protein was found decreased in DS brains, coupled with reduced induction of brain HO-1. This supports the hypothesis that the dysregulation of HO-1/BVR-A system contributes to the early increase of oxidative stress in DS and provides potential mechanistic pathways involved in the neurodegenerative process and AD development [58].

3.2.10. S100B

This gene codes for the b subunit of S100 proteins, a large family of calcium-binding proteins. The S100B homodimer is the major form in the mammalian brain. It can stimulate neurite extension [59] and plays a role in synaptogenesis [60], dendritic branching [61] and apoptosis [62]. S100B protein has long been suggested to be involved in glial cell activation and neuroinflammation [63]. Elevated brain *S100B* expression occurs in various disease states, including AD and DS. *S100B* plays an important role in neuroinflammation and in the regulation and maintenance of the serotonergic nervous system, with a particular focus on the hippocampus [64].

In vitro studies of DS fetal human neural precursors (HNP) demonstrated that *S100B* is constitutively overexpressed in these cells and that overexpression leads to increased ROS formation, activation of stress response kinases and increased programmed cell death. Further studies demonstrated that DS HNPs adopt more gliocentric progenitor phenotypes, if compared with euploid controls, with a consequent reduction in neuronogenesis [65].

3.3. Hsa21 miRNAs involved in mitochondrial phenotype

Hsa21 encodes several classes of non-coding RNAs, the most enriched being long non-coding RNAs, while miRNAs are the less represented [66]. The most recent annotation of miRNA database (miRBase, release 21) reports 29 miRNAs mapping to Hsa21. At least two of them, miR-155-5p and let-7c-5p, are possibly involved in mitochondrial function.

It was recently reported that the Hsa21 miR-155-5p affects mitochondrial biogenesis by targeting the mitochondrial transcription factor A (*TFAM*) [67], a gene that was found downregulated in trisomic hearts [1]. TFAM is a nuclear-encoded protein that controls the transcription and maintenance of mtDNA and therefore mitochondrial biogenesis.

Another Hsa21 miRNA potential candidate for mitochondrial anomalies is let-7c. By bioinformatics analysis, it appears to have several targets among genes that were found downregulated in trisomic fetal hearts and involved in mitochondrial function. Among these targets, *SLC25A4*/*ANT1* [68] appeared as a potential candidate for both mitochondrial dysfunction and congenital heart defects in DS. This gene functions as a gated pore that translocates ADP and ATP between cytoplasm and mitochondria, regulating the intracellular energetic balance. Furthermore, its dysregulation has been associated to mitochondrial cardiomyopathies [69] and its genetic inactivation results in mtDNA damage and ROS increase [8].

4. How mitochondrial dysfunction might affect DS clinical phenotype?

4.1. Muscle hypotonia

DS patients suffer from muscle hypotonia and altered motor coordination. In theTs65Dn mice, the ultrastructural analysis of myofibrils showed mitochondrial structural changes [12, 70], whereas microarray analysis revealed that pathways involved in ATP biosynthesis, proteolysis, glucose and fat metabolism and neuromuscular transmission were dysregulated [12].

Skeletal muscle is particularly vulnerable to oxidative stress. The disruption of mitochondrial network towards fragmentation, together with mitochondrial dysfunction, is an essential step of the muscular atrophy programme in adult animals [71]. Conversely, inhibition of the mitochondrial fission inhibits muscle loss [72]. Furthermore, changes in mitochondrial morphology have been implicated in apoptosis as well as in the regulation of muscle metabolism [73].

It is worth noting that patients with DS have features of premature aging [74, 75] and exhibit a decrement in muscle strength if compared with euploid subjects, similar to what occurs in aged versus young persons [76]. It is, therefore, possible that muscle hypotonia and motor dysfunction in DS share some basic mechanisms with the progressive age-related decrease in skeletal muscle mass, strength and quality known as sarcopenia [77].

4.2. Intellectual disability and neurodegeneration

Increasing evidences are demonstrating that mitochondrial function is a key actor in the events that lead to intellectual disability and neurodegeneration in DS. Development of the DS brain is associated with decreased neuronal number and abnormal neuronal differentiation [78]. Patients with DS show higher levels of oxidative stress at all ages and apoptosis and generation of ROS are increased in human fetal DS neurons if compared with the general population [78, 79]. DS astrocytes and neuronal cultures [8, 9] as well as the brain of the Ts1Cje mouse model [10] show a decrease of mitochondrial membrane potential, ATP production and an increase of reactive oxygen species [10]. Mitochondrial bioenergetics and biogenesis are impaired during neural progenitor cell (NPC) proliferation in Ts65Dn cells [11]. Mitochondrial morphology was found consistently altered in TS21 astrocytes and neurons, which exhibit increased fragmentation of the mitochondrial network [13]. Mitochondrial function, fission-fusion mechanisms, biogenesis and degradation are critical for synaptogenesis, Ca^{2+} buffering, axonal transport and bioenergetics [80]. Functionally and structurally damaged mitochondria do not produce sufficient ATP and are more prone in producing pro-apoptotic factors and ROS [81], which also represent an early stage in neurodegenerative process [82]. An increased risk for AD manifests in most of DS individuals starting from 40 years of age [83, 84]. The similarity of neurodegenerative processes between DS and Alzheimer disease (AD) and the high prevalence of AD in DS patients suggest that AD and DS share common brain alterations possibly due to similar molecular pathways involved in the pathogenesis, such as mitochondrial dysfunction and oxidative stress [85]. Energy depletion and

oxidative stress can also induce amyloidogenic changes in AβPP processing [86]. Busciglio et al. [9] demonstrated that there is a marked alteration in AβPP processing and Aβ trafficking in cortical DS astrocytes and neurons, similar to those induced in normal human astrocytes by inhibition of mitochondrial energy metabolism.

It is important to note that neurodegenerative diseases, such as AD, PD and HD, show alterations of mitochondrial function and fusion and/or fission processes very similar to those observed in DS [82, 87] as well as a similar dysregulation of mitochondria-related genes most of which are target of the *NRIP1/PGC-1α* axis [23].

4.3. Heart defects

DS is a major cause of congenital heart defects (CHD), mostly derived from endocardial cushion anomalies, such as atrioventricular septal defects, ventricular septal defects and tetralogy of Fallot [88, 89].

Transcriptome analysis of human fetal heart tissues from DS subjects has shown a global significant downregulation of NEMGs. Genes from all five complexes were downregulated, suggesting that the corresponding proteins and enzymatic activities might be reduced and that the mitochondrial function could be consequently impaired [1]. When mitochondrial phenotype was analyzed in fibroblasts from the same subjects, a more pronounced chronic pro-oxidative state was demonstrated in DS fetuses with congenital heart defects if compared with feti without cardiopathy [3]. Significant differences in mitochondrial respiration, complex I activity and ROS production were observed, suggesting a relationship between mitochondrial function and cardiac phenotype [3]. These alterations might be harbingers of the heart defects associated with Hsa21 trisomy, which could be based on elusive mechanisms involving genetic variability, environmental factors and/or stochastic events [1].

Searching for a link between heart development and mitochondria, the focus falls on the Hsa21 genes *DYRK1A* and *RCAN1*, which play a role in the calcineurin/NFAT pathway [40] and are believed to affect both mitochondrial activity and morphology during heart development [90, 91]. *DYRK1A* and *RCAN1* are involved in regulating the levels of NFATc phosphorylation. The calcineurin/NFAT signaling pathway is known to be a critical regulator of organogenesis [92] and the NFATc transcription factors are transiently expressed in the endocardial cushions during heart septation [91]. The *DSCR1* and *DYRK1A* genes, both mapping on Hsa21 within the critical region for DS, act synergistically to prevent nuclear translocation of *NFATc* transcription factors and may cause their downregulation [40]. Even modest overexpression of *DYRK1A* decreases NFATc protein activity and levels and may induce vascular and cardiac defects [40]. The inhibition of the mitochondrial activity in Nfatc3$^{-/-}$Nfatc4$^{-/-}$ cardiomyocytes [90] suggests that the calcineurin/NFAT pathway affects mitochondrial activity during heart development. *Nfatc*-null mice show phenotypic anomalies that resemble those observed in human DS and 65% of *Nfat*c1–4-null mice have endocardial cushion defects [40]. In human DS fetal fibroblasts and hearts, *NFAT*c3 and *NFAT*c4 were found significantly downregulated, whereas *DYRK1A* and *RCAN1* were overexpressed possibly due to dosage effect [1, 3].

In addition to congenital heart defects, DS subject may develop ventricular hypertrophy during the post-natal life possibly as a result of reduced mitochondrial electron-transport chain activity and oxygen consumption. Alterations in mitochondrial function observed in right ventricular cardiac hypertrophy are mainly attributed to complex I dysfunction [93]. *NRIP1*-dependent repression of mitochondria related genes may be involved in the pathogenesis of this defect. The overexpression of this gene in a transgenic mouse demonstrated to cause cardiac hypertrophy [94].

Also the Hsa21 miR-155, a known repressor of *TFAM* gene [67], was uncovered as an inducer of pathological cardiomyocyte hypertrophy, suggesting that inhibition of endogenous miR-155 might have clinical potential to suppress cardiac hypertrophy and heart failure [95]. MiR-155 is overexpressed in fetal heart tissue possibly due to dosage effect [68].

4.4. Type 2 diabetes and obesity

Children with DS have an increased risk of developing endocrine disorders such as type 2 diabetes and obesity [96]. The hypothesis that prominent features of type 2 diabetes and the condition of obesity are caused by mitochondrial dysfunction and by an impaired bioenergetics capacity is definitively emerging [97]. Given the important role that mitochondria play for bioenergetics support of signal transduction, fat oxidation and substrate transport, an impairment of electron transport chain activity may have particular relevance to the pathogenesis of insulin resistance in type 2 diabetes [98]. This hypothesis is substantiated by two evidences: (i) a disproportionately large reduction of electron transport chain activity has been observed in the subsarcolemmal mitochondrial fraction in type 2 diabetic and obese subjects if compared with unaffected volunteers and (ii) mitochondria from human skeletal muscle were found to be smaller and to have reduced activity of complex I in both type 2 diabetes and obesity [99].

Interestingly, the Hsa21 corepressor gene *NRIP1* and its target *PGC-1α* play key roles in the transcriptional regulation of genes involved in energy homeostasis. The expression and promoter activity of CIDEA, an important regulatory factor in adipose cell function and obesity, is repressed by *NRIP1* and induced by *PGC-1α* [100]. These genes are also involved in glucose uptake by affecting the regulation of both transcription and subcellular localization of the insulin-sensitive glucose transporter *GLUT4* [101]. This evidence suggested that *NRIP1* might be a potential therapeutic target in the treatment of insulin resistance in obese and type 2 diabetic patients [101]. Accordingly, mice lacking *Nrip1* are lean, show resistance to high-fat diet-induced obesity and have increased oxygen consumption [102].

Some evidences support the role of an altered mitochondrial dynamics in obesity. It is known that obesity in both humans and mice is associated with reduced *Mfn2* expression and therefore with a defective mitochondrial fusion machinery [103]. Furthermore, an altered proteolytic processing of the GTPase *OPA1* in humans is associated with insulin resistance [104].

4.5. Immune disorders

Children with DS demonstrate an increased susceptibility to infections, usually of the upper respiratory tract [105–107], and autoimmune disorders, including hypothyroidism [108] and

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celiac disease [109, 110]. The abnormalities of the immune system associated with DS include alteration of B and T-cell number, with marked decrease of naive lymphocytes; abnormal thymus functions and development; impaired mitogen-induced T cell proliferation; reduced specific antibody responses to immunizations and defects of neutrophil chemotaxis [111, 112]. The rates of lymphocyte respiration in the children with DS were found slower than in the control group [113].

Important roles of mitochondrial dynamics in the immune system physiopathology have been recently demonstrated. The first evidence is that mitochondria transportation during lymphocyte migration requires mitochondrial fission [114]. The second is that mitochondrial remodeling works as a signaling mechanism that instructs T cell metabolic programming [115]. This theory arises from the demonstration that T effector (TE) cells show a fragmented network with punctuate mitochondria, whereas T memory (TM) cells show fused networks. Accordingly, in transgenic $Opa1^{-/-}$ mice, TM lymphocytes show a decreased survival associated with altered cristae structure and decreased spare respiratory capacity. In addition, TE cells could be shifted to a TM fate depending upon changes of mitochondrial dynamics. These data suggest that, by altering cristae morphology, fusion in TM cells configures electron transport chain (ETC) complex associations favoring OXPHOS and fatty acid oxidation, whereas fission in TE cells leads to cristae expansion, reducing ETC efficiency and promoting aerobic glycolysis [115].

5. Therapeutic approaches to improve mitochondrial function in DS

5.1. Possible therapeutic targets

As mitochondrial dysfunction might concur to determine DS mental retardation and other health problems, we might expect that counteracting the mitochondrial defects will improve and/or prevent some aspects of the DS phenotype.

The few clinical trials so far undertaken to restore mitochondrial function in DS subjects using antioxidants and nutraceutics have yielded either poor or discordant outcomes [116, 117]. Better results were obtained on learning and memory in the mouse model Ts65Dn using pentylenetetrazole, memantine, fluoxetine, lithium, epigallocatechin-3-gallate (EGCG) and antioxidants such as vitamin E [118]. Also in this case, the clinical trials have not yielded the expected results.

The key role of *PGC-1α* as a modulator of mitochondrial biogenesis and respiratory function suggests that therapeutic approach on mitochondrial dysfunction in DS could be based either on *PGC-1α* activators, which have been tested in mouse models for other disease [119–122], or on PPARg agonists, which demonstrated to attenuate mitochondrial dysfunction in AD mouse models [123–126].

It is known that *PGC-1α* activity is mainly controlled by *PPARs*, AMP-activated kinases (*AMPKs*) and the NAD-dependent deacetylase SIRT1 [127]. Direct phosphorylation by AMPK promotes *PGC-1α*-dependent induction at the *PGC-1α* promoter level [122], whereas *SIRT1*

stimulates *PGC-1α* activity through deacetylation, thereby inducing mitochondrial biogenesis [119]. Pharmacological activators for these proteins, such as metformin, via *AMPK* induction, and resveratrol, via *SIRT1* induction, have been tested in mouse models for neurodegenerative diseases in which mitochondrial alterations play a central role such as AD, Parkinson's disease and Huntington's disease [120–122].

A comprehensive analysis was performed to evaluate in vitro the effects of potential *PGC-1α* activating drugs [128]. The authors pharmaceutically targeted the PPARs (bezafibrate, rosiglitazone), AMPK (AICAR, metformin) and SIRT1 (resveratrol) pathways in HeLa cells, neuronal cells and *PGC-1α*-deficient MEFs demonstrating tissue-specific effects of these drugs in modulating mitochondrial processes and cellular stress programs. All the observed effects were clearly dependent on *PGC-1α* modulation.

5.2. Advances in preclinical and clinical therapeutic approaches

5.2.1. Antioxidants

Most of the clinical trials so far undertaken in DS patients are based on antioxidant nutrients or vitamin administration to scavenge oxygen-derived free radicals [129, 130].

A study in which a mixture of antioxidants (selenium, zinc, vitamin A, vitamin E and vitamin C) and/or folinic acid was administered as supplementation to children with DS aged under 7 months for about 18 months provided no evidence to support the use of these supplements as this supplementation did not affect oxidative stress [131]. An interesting comment to this study was "This is perhaps not surprising because differences between foetuses with Down's syndrome and unaffected foetuses can be identified after only 11 weeks gestation, implying that by 7 months of age, any damage may already have been done" [132]. Vitamin E administration in a recent study did not demonstrate to slow the progression of cognitive deterioration in older individuals with DS [133].

Coenzyme Q_{10} (CoQ_{10}) is a bioactive quinone ubiquitous in the organism, involved in mitochondrial bioenergetics, with a known role as a lipophilic antioxidant [134]. CoQ_{10} supplementation to 10 patients with TS21 for 3 months demonstrated that the pro-oxidant state in plasma of children with trisomy 21, as assessed by ubiquinol-10:total CoQ_{10} ratio, may be normalized with ubiquinol-10 supplementation [130]. The authors concluded that further studies would be needed to determine whether correction of this oxidant imbalance improves clinical outcomes of children with trisomy 21 but no results in this direction have yet been communicated.

Overall, these results show that antioxidant supplementation is safe but it does neither improve the cognitive performance nor dementia in DS patients.

5.2.2. Melatonin

The antioxidant properties of melatonin induced to study plasma melatonin concentrations in a small group of children with DS [135]. Plasma melatonin concentrations were lower in DS

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subjects than in age-matched controls. The authors concluded that this constituted an added oxidative risk to children with DS. Melatonin treatment has demonstrated to induce antioxidant and antiaging effects in the hippocampus of adult Ts65Dn mice [136]. Unfortunately, even though this treatment attenuated the oxidative damage and cellular senescence in the brain [136], pre-and post-natal melatonin administration in an additional study partially regulated brain oxidative stress but did not demonstrated to improve cognitive or histological alterations in the same DS mouse model [137].

5.2.3. Epigallocatechin-3-gallate (EGCG)

EGCG—a member of a natural polyphenol family—is a mitochondrial-targeted molecule displaying a selective antiapoptotic effect against inducers of mitochondrial oxidative stress in a variety of neuronal cell types [138]. EGCG has been found to prevent mitochondrial deterioration in aged rat brain [139], reduce cerebral amyloidosis [140] and correct amyloid-induced mitochondrial dysfunction in a transgenic mice model of Alzheimer disease [141].

EGCG modulates key regulators of mitochondrial metabolism such as Sirt1 activity [142] and cAMP levels [143, 144] in addition to being a specific and safe inhibitor of the Hsa21 gene *DYRK1A*, a kinase protein involved in brain development and in the control of synaptic plasticity [145]. This makes EGCG an interesting candidate drug for the treatment of DS phenotype.

A therapeutic benefit on mitochondrial activity by EGCG has been demonstrated in cellular and murine model of DS. Indeed EGCG treatment renews the capacity of DS cells to produce energy by mitochondrial OXPHOS and to prevent mitochondrial ROS overproduction [146]. The treatment of neural progenitor cells, isolated from the hippocampus of Ts65Dn, by EGCG reactivates mitochondria bioenergetics and biogenesis and promotes neural progenitor cell proliferation [11]. On the other hand, in vivo studies demonstrated that young adults with DS treated with EGCG exhibit some cognitive benefits, although these effects disappear with time [147]. Furthermore, the treatment carried out in the mouse model Ts65dn in the neonatal period rescues numerous trisomy-linked brain alterations. However, even during this critical time window for hippocampal development, EGCG does not elicit enduring effects on the hippocampal physiology [148].

A further study showed that a temporally specific prenatal EGCG treatment improved some craniofacial dysmorphology associated with DS in Ts65Dn embryos and mice. EGCG in particular improved neural crest cells (NCC)-related deficits in proliferation and migration in vitro in mandibular precursor cells from Ts65Dn E9.5 embryos. In vivo treatment with EGCG at E7 and E8, around the time of the developing NCC deficit, appeared to improve some of the NCC embryonic dysmorphology in Ts65Dn E9.5 embryos [149]. However, a long-lasting EGCG treatment at a lower dose (E0–E9) did not have the same corrective effects.

More recently, the same authors demonstrated that a higher dose of EGCG inTs65Dn mice and euploid littermates failed to improve cognitive deficits; EGCG also produced several detrimental effects on skeleton in both genotypes [150].

In conclusion, EGCG stimulates mitochondrial biogenesis and promotes oxidative phosphorylation through cAMP/PKA- and sirtuin-dependent mechanism [146], and also, at higher concentrations, it promotes apoptosis through mitochondrial damage, membrane depolarization and cytochrome c release [151, 152]. All these results suggest that timing and dosage of EGCG treatment are important and have to be optimized in treating DS-related phenotypes.

5.2.4. Resveratrol

Resveratrol (RSV), a natural polyphenolic compound found in a wide variety of plant species, induces expression of genes involved in mitochondrial biogenesis, oxidative phosphorylation and endogenous antioxidant defense by modulation of cell signaling pathways that control cell homeostasis. RSV treatment protected mice against diet-induced obesity and insulin resistance. This effect was largely explained by an RSV-mediated decrease in *PGC-1α* acetylation and an increase in *PGC-1α* activity [153]. RSV increased the mean life expectancy and maximal lifespan in a mouse model of sporadic and age-related AD. RSV-supplemented animals showed increased Sirt1 expression and consequent downregulation of apoptotic protein p53 in the cortex and hippocampus. Also, p-AMPK in the cortex and total AMPK in the hippocampus were increased [153]. Although thousands of research papers have been published related to RSV pharmacological activities in many diseases, only one study has been performed on the effect of this polyphenol in DS [11]. The authors of the study conclude that RSV can sustain and enhance mitochondrial functions by upregulating *PGC1α*/Sirt1/AMPK axis and promote neural precursor proliferation from Ts65Dn. They suggest resveratrol as a new drug to be tested in vivo as potential therapeutic tool to promote mitochondrial functions, accelerate neurogenesis and ultimately counteract some of the DS clinical features [11].

5.2.5. Metformin

The effects of the biguanide metformin on mitochondrial function have been investigated in human trisomic fibroblasts [4]. Metformin demonstrated to induce both the expression and the activity of *PGC-1α* and to upregulate its target genes *NRF-1* and *TFAM*, thus promoting mitochondrial biogenesis. The drug enhanced ATP production in treated cells and improved overall mitochondrial activity. Most interestingly, metformin treatment counteracted mitochondrial fission observed in trisomic fibroblasts, inducing the formation of a mitochondrial network with a branched and elongated tubular morphology (**Figure 2**) and regulating the expression of genes involved in the fission/fusion machinery, namely *OPA1* and *MFN2* [4].

Metformin has shown to improve cognition in patients with mild cognitive impairment and AD [154]. There were no serious adverse events related to its administration.

Metformin is a drug commonly used as a hypoglycemic agent in type 2 diabetes because it inactivates gluconeogenesis [155]. Metformin activates AMPK in the liver and muscles, causing the phosphorylation and the consequent activation of PGC-1α, and upregulates SIRT1 that in turn activates PGC-1α by deacetylation [155].

Moreover, it has been found that metformin promotes neurogenesis in rodent and human neural precursors and enhances spatial memory formation in normal adult mouse [156].

Metformin is an already registered drug with limited side effects that can be safely administered during pregnancy and crosses both the placental and blood-brain barrier [157, 158]. For these characteristics, it could be immediately introduced in human therapeutic protocols.

6. Conclusions

The study of candidate pathogenic mechanisms in DS is helping scientists to develop more appropriate therapeutic solutions for the treatment of this still untreatable genetic disorder.

A long way has been paved in this direction as we have already gained important knowledge about the importance of bioenergetics mechanisms in determining the DS phenotype and the roles played by Hsa21 genes in this scenario.

The working hypothesis is that counteracting the mitochondrial defect in DS may improve the neurological phenotype and prevent DS-associated pathologies, such as Alzheimer's disease, type 2 diabetes, obesity and hypertrophic cardiopathy, thus providing a better quality of life for DS individuals and their families.

Impaired energy metabolism, defect of mitochondrial enzyme activities and abnormalities of mitochondrial respiration are common characteristics of neurodegenerative conditions [20]. On these premises, restoring the mitochondrial function could represent also a promising strategy to limit the progression of neurodegenerative diseases and even to delay some common aging processes.

Should any of these drugs, already registered for different purposes, demonstrate to be effective, they could be immediately introduced in human therapeutic protocols possibly along with specific therapies aimed at restoring cognitive functions.

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