Chapter 12

Genetic and Epigenetic Mechanisms in Down Syndrome Brain

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1. Introduction

Down syndrome (DS) is the most common congenital disorder in children, affecting one in 800 live births. While the large number of contiguous genes from a trisomy of chromosome 21 (HSA21) is expected to broadly affect various organ systems during development, significant advances in medicine have been made in this disorder such that those with DS live fairly long life spans. Individuals with DS, however, uniformly demonstrate some degree of mental retardation. Arguably, cognitive disabilities are the more devastating aspect of DS disorder. Part of the cognitive dysfunction lies not only in the progressive neuronal degeneration/cell death and impaired neurogenesis seen in this developmental and degenerative disorder, but also in the reduction in dendrite formation and spine density, resulting in a disruption of synaptic function. These neurological endophenotypes seen in DS may not be merely due to genomic imbalance from triplication of HSA21 genes, but also to additive influences on associated genes within a given network or pathway and modification of gene expressions caused by epigenetic factors including DNA methylation.

Epigenetic factors regulate gene expression largely through DNA modification. Histones are alkaline proteins that package and order DNA into structural nucleosomes. Acetylation and deacetylation, as well as methylation, of histones can modify the density of chromatin and thereby regulate gene transcription through chromatin remodeling. In a parallel manner, biochemical modification of DNA can occur through DNA methylation. This process involves the addition of a methyl group to the 5 position of the cytosine pyrimidine ring or the number 6 nitrogen of the adenine purine ring. DNA methylation at the 5 position of cytosine has the specific effect of reducing gene expression by physically impeding the binding of transcriptional proteins to the gene itself, or by recruiting protein complexes including methyl-CpG-binding domain proteins (MBDs), histone deacetylases (HDACs) and other chromatin

remodeling proteins. Furthermore, environmental factors such as chemical toxins or oxidative stress can accumulate over time and effect gene transcription. Collectively, these processes modify DNA transcription and may affect many neurodevelopmental processes.

Recent advances in high throughput screening of both mRNA expression and DNA methylation have provided a means to examine changes in gene activation and expression, and to understand the integral relationship between gene clusters in effecting particular pathways. The following review begins by exploring the potential contribution of both genetic and epigenetic factors in regulation of various DS endophenotypes. More specifically, our prior work has examined changes in DS neural progenitor mRNA expression and has led us to identify several important pathways affected in this disorder, such as oxidative stress, mitochondrial dysfunction and gliogenesis. Ongoing studies suggest that changes in DNA methylation in DS may have an effect on oxidative phosphorylation, ubiquitin proteolysis and insulin signaling. The confirmation of mRNA and DNA methylation changes and the clarification of these possible causal pathways may have implications for impaired synaptic function and neurogenesis, which contribute to the cognitive impairment seen in DS. These ongoing studies may further provide informative targets for early pharmaceutical interference to ameliorate the symptoms of mental retardation (MR) in DS.

2. Genetic mechanisms underlying the DS phenotype

The triplication of genes on HSA21 causes a wide spectrum of neurological phenotypes in DS, including mental retardation. DS individual displays not only delayed linguistic skills and a relatively low IQ (Intelligent Quotient) but also behavioral issues such as attention-deficit disorder (sometimes with hyperactivity) and autism [1-5]. The cognitive impairments extend further after development, as individuals with DS are more prone to develop Alzheimer's type dementia [6]. In addition, individuals with DS are susceptible to epilepsy in the form of infantile spasms and tonic clonic seizures with myoclonus at early ages [7-9]. These pathological abnormalities in humans are, in part, replicated in DS animal models which show defects in learning, social interactions, memory, and seizures [10-14].

Several genes on HSA21 are implicated in the abnormal neurodevelopment in DS [15]. They can affect cellular function at every stage of neural development, such as proliferation and differentiation of neuroprogenitor cells, neuronal survival and death, synapse formation, maturation and plasticity, as well as myelination. Disruption of each of these pathways can conceptually contribute to the MR seen in DS. Moreover, HSA21 genes have global effects on other genes; a meta-analysis of heterogeneous DS data identified 324 genes with consistent dosage effects, 77 on HSA21 and 247 on non-HSA21 [16]. Therefore, the over-expression of a not so small group of genes on HSA21 may initiate cascades of other signaling pathways on other chromosomes thorough an interactive network. The combinatorial effects from activation of these processes may further contribute to the impairments seen during neurodevelopment in DS.

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2.1. Genetic mechanisms underlying oxidative stress in DS

Increased levels of oxidative stress and reactive oxygen species (ROS) have commonly been associated with the DS brain. Free radicals are thought to disrupt the mitochondrial respiratory system, induce apoptosis of neurons and stimulate gliosis, which can further promote neuronal damage. This cyclical pathway may contribute to neuronal losses during neurogenesis as well as neuronal degeneration in adulthood. Several HSA21 genes have been implicated in generation of ROS including *DYRK1A*, *DSCR1*, *SOD1*, *ETS2, S100B, APP* and *BACH1* [15, 17]. Additionally, more recent studies would suggest a synergistic role for various HSA21 genes in induction of this pathological process. For example, over-expression of HSA21 genes *APP* and *S100B* synergistically increase hydrogen peroxide levels and decrease membrane potential in the mitochondria of human DS neuroprogenitor cells. The combination of a loss of mitochondrial integrity and an increase of oxidative stress promotes apoptosis (changes in caspase and respiratory chain protein expression) and gliosis (increase of GFAP). S100B induction can occur through RAGE (Receptor for Advanced Glycation Endproducts) with consequent activation of JNK/p38 and JAK/STAT signaling. These stress response pathways are known to serve as downstream effectors potentially relevant to reactive gliosis, induction of S100B and glial associated aquaporin 4 [18, 19]. Increased levels of S100B and APP further enhance this cyclical cascade by promoting RAGE activation and inflammation with reactive gliosis. Lastly, multiple HSA21 genes have demonstrated enhanced APP-dependent toxic effects on the mitochondria whereas network prediction analyses have shown that four HSA21 proteins are components of the JAK/STAT pathway. These studies imply that an additional 19 HSA21 (among 2004 in total) proteins interact with components in this pathway [20]. These findings reiterate the large cascade of molecules that can be perturbed in a pathway following over-expression of a single gene.

Although oxidative stress in DS patients is considered to be a primary contributor of neurodegeneration such as Alzheimer's Disease (AD) in adult patients, evidences from both human and animal models suggest that these same processes could also affect neurodevelopment and cognitive function at a much earlier age [19, 21-23]. Oxidative stress could therefore not only alter neuronal numbers through degeneration and changes in synaptic plasticity through impaired mitochondrial function, but also affect the generation of neurons during development. In this respect, ongoing effects from over-expression of HSA21 genes likely promote the cognitive dysfunction in DS throughout the lifetime of an individual with this disorder.

2.2. Genetics mechanisms underlying neurogenesis in DS

The observation of reduced cortical volume and decreased neuronal numbers in DS patients and animal models could in part be attributed to a reduction in the generation of neurons [24-27]. Over-expression of several HSA21 genes has been implicated in neurogenesis by either altering the rate or proliferation or by changing cell fate specification. By over-expressing HSA21-associated OLIG2, we observed a phenotypic shift in the neural progenitor pool toward glial progenitor phenotypes, accompanied by a corresponding decrease in the number of neuronal progenitors. This change can partly be explained by OLIG2-dependent inhibition of the expression and activity of KCNA3 outward rectifying potassium channels whose activa-

tion stimulates proliferation of neural progenitors [28]. With respect to proliferation, APP overexpression can antagonistically compete with APPBP1, a protein required for the cell cycle progression from G1 to S phase [29]. Similarly, increased S100B levels stimulate p53 nuclear accumulation and inhibit proliferation [30]. DYRK1A has alternatively been shown to phosphorylate p53, impair G1/S phase transition and inhibit proliferation [31]. Finally, many HSA21 genes regulate neurogenesis through their effects on NGF, hedgehog, WNT, Notch and insulin signaling pathways [20]. Changes in expression of various HSA21 genes can also regulate subpopulations of progenitors. For example, microarray profiling of DS human neuroprogenitors implicated a defect in interneuron neurogenesis through increased expression of glial progenitor genes such as *OLIG1*, *OLIG2*, *OMG* and *COUP-TF1/NR2F1* and downregulation of the interneuron related genes *DLX1*, *DLX2* and *DLX5* [32].

2.3. Genetics mechanisms underlying synaptic formation, maturation and plasticity in DS

A reduction in brain volume in DS has been attributed to impaired dendritic and synaptic maturation. Dendritic branching and spine number are dramatically reduced in pyramidal neurons in the hippocampus, visual cortex and motor cortex after 4 months postnatal age in individuals with DS [33-35]. The decreased number of spines is usually accompanied by aberrant spine morphology including enlarged or irregular spine heads, and sparse, small, short stalks intermingled with unusually long spines [34, 36]. In addition, DS brains also show changes in expression levels of various synaptic proteins such as decreased SEPT6, SYN1, SNAP-25, SYP and increased SYNJ1 levels [37-41]. Similar morphological changes have been observed in DS animal models and correlate on a molecular level with synaptic protein level changes and functionally with synaptic plasticity defects, observed through LTP, LTD and imbalance of excitatory-inhibitory neurotransmission [42-50]. Many genes on HSA21 (*TINM1*, *SYNJ1*, *ITSN1*; *KCNJ6*, *KCNJ15*, *KCNE1*, *KCNE2*; *NRIP1*, *ETS2*, *PCP4*, *DSCR1*, *DYRK1A*, *S100B*, *APP*, *OLIG1*, *OLIG2*) have been implicated in the synaptic pathology in DS, and the resulting phenotype likely involves a complex interrelationship between these various genes and their direct or indirect effect on various synaptic proteins [15, 48]. For instance, Dyrk1A overexpression could impair synaptic vesicle endocytosis, reduce dendrite branching and spine density of neurons; these phenotypes might be attributed to Dyrk1A induced hyperphosphorylation of Tau and APP, or other synaptic proteins such as SYNJ1, resulting in impaired hippocampal-dependent learning [51-53]. Moreover, the multiple genetic interactions can additively promote the pathological DS synaptic endophenotype, as more severe defects were observed in Ts65dn mice than in Ts1Cje mice, the former of which contain a larger number of HSA21 associated genes [54].

3. Epigenetic mechanisms underlying the DS phenotype

DNA methylation refers to a process of DNA modification that involves the enzymatic transfer of a methyl group from a methyl donor S-adenosylmethionine to carbon 5 of cytosine at 5′-CpG-3′ sites. The enzymes carrying out this reaction are called DNA methyltransferases (DNMTs). There are five members in this family: DNMT1, DNMT2, DNMT3A, DNMT3B and

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DNMT3L. DNMT1 is responsible for DNA methylation maintenance while DNMT3A and DNMT3B are involved in *de novo* DNA methylation. DNMT2 is involved in RNA methylation. DNMT3L (DNA methyltransferase 3-like) does not have enzymatic activity but can stimulate DNMT3A and DNMT3B activation [55-57]. The addition of a methyl group to cytosine may physically impede the binding of transcriptional factors to the gene itself, or by recruiting protein complexes including methyl-CpG-binding protein 2 (MECP2), methyl-CpG-binding domain proteins (MBDs), HDACs and other chromatin remodeling proteins [58]. Alternatively, other enzymes involved in DNA demethylation can reverse this process. These molecules include cytidine deamination (AID, APOBEC) for deamination of cytosine and 5-methylcytosine and hydroxylation (TETs) for converting 5-methylcytosine to 5-hydroxymethylcytosine [59]. DNA modification, especially in the promoter region, by these various regulators may alter gene expression, and thereby affect many physiological processes [60]. In this context, proteins that affect the methylation machinery in DS are likely to alter gene expression and contribute to the DS phenotype.

Epigenetic modification is thought to be an important contributor to development and numerous diseases. Several disorders associated with cognitive impairment such as X-linked alpha-thalassemia mental retardation (ATRX) syndrome, Rett syndrome, and Rubinstein–Taybi Syndrome involve some level of disruption in gene regulation through epigenetic effects [61]. The pathology is medicated by different mechanisms including histone modification, chromosome remodeling, small RNAs (siRNA, miRNA and other non-coding RNA) regulation and DNA methylation. More directly, *DNMT3B* mutations are associated with Immunodeficiency, Centromere instability and Facial anomalies syndrome (ICF) with MR, suggesting that epigenetic alterations in the expression of genes regulating neurogenesis, axon branching, and neuronal migration such as IGF1 and ROBO1, contribute to cognitive impairment [62]. Certain features in DS may, in a similar fashion, be caused by epigenetic changes. For instance, HSA21 genes *DYRK1A*, *BRWD1* and *RUNX1* are associated with SWI/SNF complex, a chromatin remodeling complex that regulates the expression of subsets of genes such as *HDMTs*, *HMTs* and *HDACs*- histone modification proteins involved in controlling the expression of various interacting genes [63-65]. HSA21 genes *CHAF1B* and *HMGN1* express chromatin constitutive proteins involved in nucleosome assembly, which controls gene expression through DNA methylation and histone methylation or acetylation [66, 67]. Overexpression of HSA21 derived miRNA miR-155, miR802 in DS brain could also inhibit MECP2 expression, thereby mimicking *MECP2* loss of function in Rett syndrome with mental retardation. MECP2 transcriptionally activates and silences *CREB1* and *MEF2C*, genes that are critical in neurodevelopment [68-70]. DNA methylation is another extensively studied epigenetic regulator, being shown as impaired in many diseases. Although its importance has been recognized in cancers, its involvement in neurological disorders such as DS has not been well studied yet.

Several observations suggest that DNA methylation may play an important role in the DS endophenotype. Oxidative stress from over-expression of various HSA21 genes [15] could modulate DNA methylation directly through DNA damage or modification at the CpG sites, thereby preventing normal binding of DNMTs to DNA [71, 72]. DNMT3L is localized on

HSA21, and its triplication in DS suggests aberrant levels of expression. DNMT3L can form a heterotetramer with DNMT3A, and increased DNMT3L levels could potentially promote release of DNMT3A as well as increase its methylation activity [56]. DNMT3L can also stimulate DNMT3B activity directly [57, 73]. In addition, Dnmt3a modulates neurogenesis and synaptic plasticity in developing mouse neuroprogenitors and mature neurons by regulating related genes expression, such as *Bdnf, Reln, Dlx2, Gbx2, Sp8* and *Stat1* [74-77]. It remains to be seen whether other HSA21 genes in addition to DNMT3L can change the expression or activity of various epigenetic modifiers including the DNMTs, MBDs, HDACs or TETs. Overall, epigenetic modification provides an added layer of complexity to the interactive network established from over-expression of genes on HSA21. These modifiers also server as attractive candidates for targeting in DS given the broad effects they potentially have on a particular phenotype.

Next, we will discuss how DNA methylation could be involved in some important neurodevelopmental phenotypes in DS.

3.1. Epigenetic mechanisms underlying oxidative stress in DS

While excessive oxidative stress leading to mitochondrial dysfunction is a main feature of DS neurodevelopment, its effects on DNA methylation are not known. Currently no direct evidence demonstrates a role for oxidative stress in regulating DNA methylation changes in DS brain. However, DNA methylation studies from cancer seem to provide some clues. For instance, hydroxyl radicals generated from hydrogen peroxide can cause DNA damage including base modifications, deletions, and breakages, which could consequently interfere with normal function of DNMTs, leading to global hypomethylation in cancer cells [78]. 8-OHdG in CpG dinucleotides or the presence of O6-methylguanine could inhibit adjacent cytosine methylation [79-82] by inhibiting DNMTs or MBDs binding [83]. By extension, some of these same pathological mechanisms in cancer cells will likely be relevant in DS.

Methylation changes in the subset of DS genes involved in oxidative stress can contribute to similar phenotypes seen in DS development and disease. For instance, Dnmt1 conditional knockout in neural progenitor cells induced precocious astrogliogenesis through demethylation of *S100b*, *Gfap* and *Stat1* promoters and activation of the JAK-STAT pathway. Silencing of these genes occurs through Mecp2 mediated inactivation of chromatin remodeling [84], with demethylation resulting in an increase in S100B, GFAP and STAT1 expression. Enhanced expression of these genes further promotes oxidative stress, cell death and gliosis. HSA21 localized APP could also be regulated by promoter dependent DNA methylation. The methylation pattern in the APP promoter is different in different tissues and even in different brain areas [85]. Hypomethylation of APP is found in the cerebral cortex of aging people and AD patients [86, 87]; the methylation frequency of CpG sites on APP promoter in younger people (26%) is higher than that in older people (8%), suggesting an age related methylation difference [86]. Altered methylation patterns have also been implicated in deregulation of APP processing enzymes PS1 and BACE in AD [88]. Finally, APP can also regulate the expression of other genes such as *CTIF, NTX2* and *DDR2* through DNA methylation [89]. Overall, these studies suggest that DNMTs appear to play some role in regulation of neurogenesis and

neurodegeneration, and they do so by regulating several genes on HSA21 involved in oxidative stress. Moreover, HSA21 genes associated with oxidative stress can influence the methylation status of other genes.

3.2. Epigenetic mechanisms underlying neurogenesis in DS

DNA methylation regulates neurogenesis. Dnmts are broadly expressed in the brain and are dynamically regulated [90, 91]. For example, Dnmt1 is expressed in both dividing neuroprogenitors and postmitotic neurons [91, 92]. Dnmt3b is mainly expressed in neuroprogenitor cells during neurogenesis, whereas Dnmt3a is predominantly expressed in maturing brain (including neural precursors, neurons, astrocytes and oligodendrocytes). Dnmt3a expression peaks at three weeks after birth and then declines in adulthood [93, 94]. Dnmt3l directly regulates Dnmt3a and Dnmt3b but is weakly expressed in the brain and does not appear to disrupt normal cortical development. As for function, Hutnick et al used Emx1-cre to conditionally knockdown Dnmt1 exclusively in telencephalic precursors of mice, which induced hypomethylation in excitatory neurons and astrocytes of cortex and hippocampus. The methylation change increased neuronal apoptosis coupled with upregulation of apoptosis-related genes such as *Gadd45a*, *Casp4* and *Ngfr*. Loss of Dnmt1 also impaired neurogenesis, maturation, learning and memory and was associated with downregulation of layer specific gene such as *Lhx2*, neuronal channel genes such as *Kcnh5*, *Kcnj9* and *Scnn1a* [95]. Interestingly, Gadd45b could contribute to DNA demethylation of pro-neuronal genes such as *BDNF* and *FGF* [96]. Studies using postnatal neural stem cells (NSC) in Dnmt3a knockout mice suggest that Dnmt3a promotes non-promoter DNA methylation of neurogenesis genes such as *Dlx2*, *Gbx2* and *Sp8* by functionally antagonizing Polycomb repression, resulting in increased expression of these genes [77]. Finally, the expression pattern of Dnmt3b suggests that it may be important for the early phase of neurogenesis (Feng et al., 2005).

DNA methylation may directly effect neural progenitor development in DS. In normal development, Dnmt3l does not appear to have a significant phenotype in the developing mouse cerebral cortex, likely due to its relatively low expression levels in the brain [97, 98], (personal communications, Dr. Yi E. Sun, UCLA). DNMT3L, however, is located on chromosome 21 and its triplication results in aberrantly high levels of expression in DS neuroprogenitors (personal observations). Given that DNMT3L directly regulates both DNMT3A/B and both these proteins have been implicated in neural progenitor development, a pathological role for methylation genes such as DNMT3L in contributing to neurogenesis is likely.

3.3. Epigenetic mechanisms underlying synaptic formation, maturation and plasticity in DS

Several HSA21 genes can indirectly regulate epigenetic factors involved in synaptic function. For example, SWI/SNF (SWItch/Sucrose NonFermentable) is a nucleosome-remodeling complex that can destabilize histone-DNA interactions in an ATP-dependent manner. HSA21-localized *DYRK1A* binds the SWI/SNF complex and subsequently induces a coordinated deregulation of multiple genes that are responsible for dendritic growth [65]. Likewise, APP has been shown to alter CpG methylation in three target genes CTIF (CBP80/CBP20-dependent translation initiation factor), NXT2 (nuclear exporting factor 2), and hypermethylated DDR2

[89]. DDR2 is a tyrosine kinase that functions as a cell surface receptor for fibrillar collagen and regulates cell differentiation, remodeling of the extracellular matrix, cell migration, cell proliferation, and cell cycle progression. More evidences from DNA methylation changing synaptic function come from Dnmt transgenic mice. Dnmt1 and Dnmt3a knockout mice show reduced LTP, deficits in learning and memory and deregulated genes expression associated with synaptic plasticity [74]. Dnmt3a overexpression increases spine density in nucleus accumbens [75]. DNMT3B is the gene mutated in ICF syndrome. Its mutation in lymphoblastoid cell line from patients led to altered genes expression of several systems including regulators of neurogenesis and synaptic function, such as ROBO1, JPH4, FRY, MAP4K4, PCDHGC3, IGF1, SNCA, GABRA4 and BCHE [62]. Methyl-CpG binding protein 1 (MBD1), a member of the methylated DNA-binding protein family, whose mutation leads to reduced neurogenesis, decreased LTP and impaired spatial learning [99]. The involvement of Dnmts and Hdacs in synaptic function is further supported by pharmacological manipulations [100-102]. For instance, Dnmt inhibitors zebularine and 5-aza-2-deoxycytidine can alter DNA methylation at promoters for Reln and Bdnf, and block the induction of LTP in synapses of mouse hippocampus [103].

4. Global effects of DNA methylation in causing DS phenotypes

Several reports have shown global DNA methylation changes in DS [104, 105]. For example, individual proteins on HSA21 such as beta amyloid (the protein encoded by HSA21 localized APP) can induce global hypomethylation [106, 107]. Comparison of normal and DS methylation in DS leukocytes and T lymphocytes using microarray-based profiling (MSNP (single nucleotide polymorphism (SNP) chip-based method for profiling DNA methylation) identified a small subset of genes with altered methylation, specific to the DS cell population [104]. Among the genes identified, five candidates (*TMEM131*, *CD3Z*, *NOD2* and *NPDC1*) showed correlation with RNA expression, and the methylation changes could be recapitulated by exposing normal lymphocytes to the demethylation drug 5-aza-cytidine. These genes have known or predicted roles in lymphocyte development. In order to gain some insights into the DNA methylation deregulation in DS brain, we have performed some preliminary studies by comparing the methylation profiles of control (CON) and DS frontal cortex from 18 gestational weeks' fetal brain using Illumina 450 Infinium Beadchip assay. Approximately 4% of the CpG sites showed significant changes at the methylation level. When compared to CON baseline methylated and unmethylated states, more CON unmethylated CpG sites became methylated in DS than CON methylated states that became unmethylated. Moreover, there was overall greater global hyper versus hypomethylation in DS compared to CON across all chromosomes, except on HSA21. Chromosome 21 actually demonstrated a greater degree of hypo versus hypermethylation in DS (unpublished data). Hypomethylation generally results in increased gene transcription, whereas hypermethylation leads to the converse. Cross comparison of DNA methylation states with the differential mRNA expression genes from previous microarray studies, suggested epigenetic effects on several specific pathways (oxidative phosphorylation, insulin signaling and ubiquitination).

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4.1. Oxidative phosphorylation

Oxidative phosphorylation involves cellular metabolism through oxidation to produce ATP. The broad methylation and gene expression changes in this pathway suggest its role as a primary consequence of DS genes' overdose effects. Plasma membrane NADPH oxidase is considered a major producer of ROS in neurons or astrocytes in brain and is activated by S100B through a RAGE-dependent pathway [108-111]. Over-expression of HSA21 genes such as *S100B* and *APP* likely promote this pathway and cause cell death in DS neurons [19]. Small amounts of superoxide anion and peroxide are also produced by the electron transport chain in mitochondria [112-114]. The global deregulation of enzymes in this mitochondrial pathway could thus disrupt the balance between oxidant generation and ATP production, result in enhanced ROS generation and lead to diminished ATP levels [115, 116]. Several DS genes have been implicated in this process. For instance, three HSA21 genes, *ATP5J*, *ATP5O* and *NDUFV3* are components of ATP synthase and NADH dehydrogenase, though their expression and regulation in DS brain are not known yet. In addition, other HSA21 genes may indirectly affect this pathway. Alternatively, HSA21 gene *S100B* may target mitochondrial proteins such as p53 and ATPase ATAD3A, thereby assisting the cytoplasmic processing of proteins for proper folding and subcellular localization [117-121]. Another HSA21 gene APP and its product beta amyloid can interact with import receptors to gain entry into mitochondrial compartment, where they accumulate and affect the normal function of this pathway [122, 123]. Finally, gene expression in mitochondrial oxidative phosphorylation may be modulated by DNA methylation. For instance, prenatal protein diet excess or restriction leads to hypomethylation of CpG sites in the cytochrome C *CYCS* gene promoter, including those representing putative transcription factor-binding sites. Elevation of this protein can alter electron transport chain function in mitochondria and initiate apoptosis [124]. Our preliminary studies suggest there is a broad change of DNA methylation and genes expression in this oxidative phosphorylation pathway. Given the importance of ATP/ROS metabolism in mitochondrial function, further studies will be needed to understand the epigenetic contribution to this pathway.

4.2. Insulin signaling

The insulin/insulin growth factor (IGF)-I pathway is a conserved pathway required for neurogenesis and neuroprotection. It acts through IR/IGF-IR, IRS, and RAS/MAPK or PI3K/AKT in regulating neurogenic cell fate [125]. Decreased levels of IGF-I have been found to associate with growth retardation in DS patients, which could be rescued by GH therapy [126, 127]. In addition, the insulin receptor knockout mouse suggests that neurons without insulin receptor exhibit significant reduction of Akt and Gsk3beta and increased tau hyperphosphorylation, characteristics of neurotoxicity in DS and AD [128]. Inhibition of the brain insulin signaling pathways have been report in AD brain, with decreased expression of IR, IRS1, IRS2, PI3K and AKT [129, 130]. This deficiency may, in part, involve DNA methylation changes, given reports of co-localization of Hdac2 with insulin signaling components (Ir, Irs) in postsynaptic glutamatergic neurons of the mouse hippocampus [131]. DNA methylation changes in human DS progenitors (personal observations Lu and Sheen) also suggest that the insulin-associated pathways may contribute to the DS endophenotype during development.

4.3. Ubiquitin proteolysis

The ubiquitin proteasome/lysome system (UPLS) is responsible for the removal of excessive proteins from multiple cellular compartments (especially mitochondria and synapses) in order to maintain normal cellular function [132, 133]. Progression in DS cognitive impairment is associated with accumulation of NF plaques and tangles, which have been shown to contain ubiquitin [134]. Dystrophic neurites in DS also contain ubiquitin and the UPLS-associated molecules PSMA5 and USP5 are upregulated in DS fetal brain [135]. Beta amyloid could regulate synaptic protein degradation and function through ubiquitin pathway [136, 137]. Moreover, several E3 ubiquitin ligases have been shown to promote APP degradation [138, 139]. Additionally, HSA21 located genes *AIRE* and *UBE2G2* are directly involved in the ubiquitin pathway and could contribute to the phenotype. Taken in this context, disruption of mitochondrial function (i.e. through S100B, APP, OLIG2 or disruption of the oxidative phosphorylation pathway) might consequently impair ubiquitin-dependent lysosomal and proteosomal clearance, because it is an ATP-dependent process. Finally, our preliminary studies suggest that DNA methylation may also directly impair ubiquitin function. Loss of ubiquitin function would have direct effects on synaptic function and structure (through beta amyloid or synaptic proteins) but would also possibly enhance oxidative stress and mitochondrial dysfunction. It is interesting to note that the high throughput DNA methylation screen in DS invoked changes in methylation involving three networks (oxidative phosphorylation, insulin signaling, and ubiquitin function), which are highly dependent on one another.

5. Possible functions of *DNMT3L* in DS

Given that DNMT3A and DNMT3B are involved in neurogenesis and synaptic plasticity, HSA21 localized *DNMT3L* regulates activities of *DNMT3A*/*3B*, suggesting that over-expression of this gene will have pathological implications in methylation patterns involved in neural development. Moreover, DNMT3L represses transcription by recruiting HDACs, which may also affect the neurodevelopment [140, 141]. *Dnmt3l* null mice do not demonstrate a neurological phenotype due to low levels of expression but rather exhibits defects in reproductive organs where it is highly expressed and leads to imprinting and differentiation defect in early stages of embryonic development [97, 98]. *DNMT3L (R271Q)* variant is associated with significant DNA hypomethylation at the subtelomeric region in healthy human, though it does not seem to cause any diseases [142]. On the other hand, over-expression of DNMT3L in Hela cells mimics the characteristics of iPS cells and carcinogenesis by upregulating SOX2, HOX genes and DNMTs including DNMT1 and DNMT3B expression, suggesting that DNMT3L over-expression may change the DNA methylation profile in later stages of embryo development through activating DNMT3A/DNMT3B when neurogenesis and synapse formation happen [143]. Interestingly, a recently developed DS model Dp(10)1Yey/+ mice harboring a duplication spanning the entire HSA21 syntenic region on mouse chromosome 10 (Mmu10), which contains Dnmt3l and S100b, did not show alterations in cognitive behaviors or hippocampal LTP [144]. However, other mouse transgenic studies with over-expression of select HSA21 genes (i.e. APP and S100b) have shown combinatorial effects in contributing to AD

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features in DS and neuronal survival [19, 145]. These observations would suggest combinatorial and interactive effects between these genes in contributing to the MR seen in DS. It remains to be seen whether DNMT3L effects on DNMT3A/B are responsible for the part of the preliminary methylation defects seen in the several pathways discussed above. It is also not known how the trisomy of HSA21 genes will effect methylation, but it is highly likely that DNMT3L alters at least a subset of genes. In this respect, it will be important to identify the causative methylation defects due to this single gene, as it will have implications for other DS phenotypes.

6. Possible targets for pharmaceutical interference

The epigenetic screens in DS predict involvement of several mutually interactive pathways in contributing to the neurological endophenotype in this disorder: oxidative phosphorylation, insulin signaling, and ubiquitination. Approaches for therapeutic intervention possibly involve either altering the methylation patterns or directly targeting specific pathways.

If global hypermethylation in DS neuroprogenitors is confirmed, then inhibition of DNMT or DNA deamination could be used to rescue or treat the pathological phenotypes. There are two clinical licensed DNMT inhibitors currently used in myelodysplastic syndrome, where they relieve the repression of tumor suppressor genes: 5-aza-cytidine (Vidaza®) and 5-aza-2′-deoxycytidine (Dacogen®) [59]. In addition, because of the occurrence of hypomethylation, especially on HSA21, it would be desirable to develop a more specific methylation inhibitor/activator or deamination activator/inhibitor in order to target specific promoters of genes in important pathways.

Dysfunction of the UPLS system causes protein accumulation or over-degradation in cellular organelles. Thus developing activator or inhibitor of proteasomes would have therapeutic meaning. Most currently available activators/inhibitors of the ubiquitin-proteasome pathway directly target the subunits of proteasome, the core of the proteolysis machinery, instead of targeting upstream ubiquitination and recognition of ubiquitinated protein substrates by more specific E3 ubiquitin ligases. Proteasome inhibitors such as Bortezomib, (Velcade®) are in clinical treatment for multiple myeloma [146, 147]. Proteasome activators including 11s activator, Blm10/PA200, and 19s activator are still under research.

Preservation of oxidative phosphorylation pathway and mitochondrial function can be achieved through a new investigational drug EPI-743, currently in phase 2B/3 pivotal clinical trials in Inherited Mitochondrial Respiratory Chain Disease [148]. EPI-743 is an orally absorbable small molecule that readily crosses into the central nervous system. It works by targeting an enzyme NADPH quinone oxidoreductase 1 (NQO1). Its mode of action is to synchronize energy generation in mitochondria with the need to counter cellular redox stress [149].

7. Conclusion

DS is a contiguous gene syndrome which gives rise to MR, dementia, and seizures. These clinical outcomes are mirrored by endophenotypes including increased oxidative stress, decreased neurogenesis and synaptic dysfunction. While these characteristics have largely been attributed to HSA21 gene dosage effects, recent progresses in epigenetic studies have raised the high likelihood that DNA methylation have significant effects on DS neurodevelopment. Methylome screening suggests disruption of pathways involving oxidative phosphorylation, ubiquitination and insulin signaling in DS. Candidate gene analyses suggest that DNMT3L is over-expressed in DS given its location on chromosome 21. Alternatively, other studies have implicated several HSA21 genes in altering methylation sites on genes involved in these same pathways. The pathways invoked through epigenetic regulation contribute directly to known pathological mechanisms identified on prior gene expression profiling such as oxidative stress, gliosis, and mitochondrial dysfunction. In this respect, the DS brain endophenotypes likely arise from the integration of various genetic and epigenetic factors on chromosome 21.

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