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Realizing the potential of exploiting human IPSCs and their derivatives in research of Down syndrome

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Abstract: Down syndrome (DS) is a genetic condition characterized by intellectual disability, delayed brain development, and early onset Alzheimer's disease. The use of primary neural cells and tissues is important for understanding this disease, but there are ethical and practical issues, including availability from patients and experimental manipulability. Moreover, there are significant genetic and physiological differences between animal models and humans, which limits the translation of the findings in animal studies to humans. Advancements in induced pluripotent stem cells (iPSC) technology have revolutionized DS research by providing a valuable tool for studying the cellular and molecular pathologies associated with DS. Induced pluripotent stem cells derived from cells obtained from DS patients contain the patient's entire genome including trisomy 21. Trisomic iPSCs as well as their derived cells or organoids can be useful for disease modeling, investigating the molecular mechanisms, and developing potential strategies for treating or alleviating DS. In this review, we focus on the use of iPSCs and their derivatives obtained from DS individuals and healthy humans for DS research. We summarize the findings from the past decade of DS studies using iPSCs and their derivatives. We also discuss studies using iPSC technology to investigate DS-associated genes (e.g., APP, OLIG1, OLIG2, RUNX1, and DYRK1A) and abnormal phenotypes (e.g., dysregulated mitochondria and leukemia risk). Lastly, we review the different strategies for mitigating the limitations of iPSCs and their derivatives, for alleviating the phenotypes, and for developing therapies.

Introduction

Down syndrome (DS) is a genetic condition caused by either partial or full trisomy of human chromosome 21 (HSA21), which leads to delayed brain development, intellectual disability, and premature Alzheimer's disease (AD) (Esposito *et al.*, 2008; Liu *et al.*, 2023; Ma *et al.*, 2021; Ponroy Bally *et al.*, 2020). Individuals with DS also have smaller whole brain volume (Giffin-Rao *et al.*, 2022; Huo *et al.*, 2018), specifically, they have reduced numbers of calretinin-expressing interneurons in the cortex (Giffin-Rao *et al.*, 2022). The DS-associated intellectual disability has been linked to the various changes in early brain development, including altered proliferation and differentiation of neural progenitor cells and abnormal formation and maintenance of myelin (Klein *et al.*, 2021). Mitochondrial dysfunction and oxidative stress are also regarded as hallmarks of DS (Omori *et al.*, 2017; Prutton *et al.*, 2023). Trisomy 21 alters gene dosage and expression, which leads to abnormal neurogenesis and metabolism across various cell types including neurons and astrocytes (Banno *et al.*, 2016; Omori *et al.*, 2017). Transcriptomic analysis showed there was altered transcription, splicing, and RNA editing in DS patients compared to healthy individuals (Cypris *et al.*, 2020). However, what causes the abnormal phenotypes in DS remains largely unknown. Moreover, effective strategies for treating or ameliorating the deficits in DS are lacking.

Primary neural cells and tissues are useful for investigating the pathology and cellular mechanisms in DS. However, there are ethical and availability issues in using human tissues, as well as limitations in their experimental manipulability (Gough *et al.*, 2020). In the past decades, several murine models of DS have been developed that have helped us to understand the neurobiological changes

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responsible for the cognitive and behavioral phenotypes associated with DS (Rueda *et al.*, 2020). However, due to the genetic and physiological differences between animals and humans, the applicability of these preclinical models has been questioned, particularly in testing potential therapies (Rueda *et al.*, 2020).

Induced pluripotent stem cell (iPSC) technology has emerged as a valuable tool for studying the cellular and molecular pathologies in specific cell types affected by DS (Wu *et al.*, 2022b). These iPSCs can be derived from somatic cells such as fibroblasts from DS patients or healthy humans (Cypris *et al.*, 2020). The fibroblasts can be reprogrammed into iPSCs through the introduction of Yamanaka factors via retroviral integration or non-integrating methods such as Sendai virus, episomal vector, or mRNA (Banno *et al.*, 2016; Cypris *et al.*, 2020). The iPSCs derived from DS patients contain their complete genomic sequence including disease-causing genetic mutations. Hence, these cells could be used to study the disease, investigate the molecular mechanisms, develop treatments, and test potential drugs (Banno *et al.*, 2016). Here, we aim to utilize iPSC models to explore the molecular mechanisms underlying DS phenotypes, understand the genetic factors that contribute to pathogenesis, and discuss potential therapeutic targets for this condition.

IPSCs and Their Derivatives in DS Research

Over the past two decades, a series of iPSCs summarized in Table 1 and iPSC-derived cells or organoids summarized in Table 2 have been developed and utilized to delve into the fundamental mechanisms and characteristics of DS, thereby shedding light on the pathophysiology of this condition. DS iPSCs have undergone differentiation into various neuron types, including cortical neurons and interneurons (Sobol *et al.*, 2019; Curtis *et al.*, 2022). Sobol *et al.* (2019) conducted comprehensive transcriptomic and proteomic analyses on DS iPSCs and compared them to normal iPSCs, providing insights into DS and normal fetal brain development. These analyses revealed temporal dysregulation in genes, proteins, and pathways across 11 major functional clusters. Notable disruptions included impaired DNA replication, synaptic maturation, and neuroactive clusters during early differentiation, along with disturbances in the transition to the neural progenitor cell stage and reduced cellular growth. Additionally, other affected clusters encompassed differentiation, growth factors, extracellular matrix, oxidative phosphorylation, and glycolysis (Sobol *et al.*, 2019).

IPSCs

For generating iPSCs, human somatic cells such as fibroblasts can be reprogrammed using viral vectors such as polycistronic lentiviral vectors to deliver four transcription factors OCT4, SOX2, KLF4, and c-MYC, referred to as Yamanaka factors (Gonzales *et al.*, 2018; Grad *et al.*, 2011; Hibaoui *et al.*, 2014; Takahashi *et al.*, 2007) (Table 1). To eliminate the effects of genomic background variability, Hibaoui *et al.* (2014) generated iPSCs from monozygotic twins discordant for trisomy 21 by transducing parental fibroblasts (Twin normal and Twin-DS) with polycistronic lentiviral vectors expressing the Yamanaka factors (Table 1). They observed the DS iPSCs had altered cell architecture, density, and misexpression of genes involved in neurogenesis *in vivo* and *in vitro*. Transcriptomic analysis of the DS iPSCs showed there were significant changes in transcript accumulation, alternative splicing, and repetitive element transcripts associated with trisomy 21 (Gonzales *et al.*, 2018) (Table 1). These findings suggest that trisomy 21 may interfere with the maintenance of pluripotency rather than intrinsically limiting neuronal differentiation (Gonzales *et al.*, 2018) (Table 1).

Retroviruses expressing either four reprogramming factors (OCT4, SOX2, KLF4, and c-MYC) or three factors (without c-MYC) have also been used to generate DS iPSCs from fibroblasts (Park *et al.*, 2008; Shi *et al.*, 2012). Subsequent differentiation of these DS iPSCs into cortical neurons revealed significant characteristics (Shi *et al.*, 2012).

Non-integrating techniques, such as Sendai virus expressing the Yamanaka factors, have also been applied to generate iPSCs and isogenic euploid iPSCs from skin fibroblasts from DS patients (Nehra *et al.*, 2023; Schuster *et al.*, 2020) (Table 1). The DS iPSCs carried trisomy 21, whereas the isogenic euploid iPSCs had a typical karyotype. These DS iPSCs also displayed trilineage differentiation capacity (Nehra *et al.*, 2023) (Table 1). Episomal plasmids have also been applied in the transient expression of *OCT4, SOX2, KLF4* and *MYC* genes to reprogram fibroblasts into DP iPSC ACS-1003™ and normal hiPSC ATCC® ACS-1011™ (Teles *et al.*, 2023) (Table 1). Global gene analysis of DP iPSC ACS-1003™ and hiPSC ACS-1011™ revealed differentially expressed genes (DEGs) with alternative splicing events. These DEGs were enriched in biological processes including cell adhesion and immune response. Notably, candidate genes such as FN1 were identified to potentially play a significant role in DS (Wang *et al.*, 2021) (Table 1). Recently urine cells from DS patients and control subjects have also been reprogrammed into iPSCs *in vitro* (Teles *et al.*, 2023) (Table 1). These iPSCs were generated using episomal vectors with an oriP/EBNA-1(Epstein-Barr-1 nuclear antigen) backbone for the delivery of six reprogramming factors (Oct4, Sox2, Nanog, Lin28, Klf4, and L-Myc). These iPSCs were successfully differentiated into neurons and astrocytes in the form of monolayer cultures and 3D cerebral organoids. The generated cerebral organoids exhibited early features of human cortical development, providing a valuable tool for studying DS *in vitro* (Teles *et al.*, 2023) (Table 1).

IPSC-derived cells and organoids

The generated iPSCs can be further induced into neural progenitors, which can reveal their capacity to differentiate into specific neural cells. Giffin-Rao *et al.* (2022) showed DS iPSCs had a reduced capacity to generate COUP-TFII+ progenitors, which has been linked to decreased WNT signaling (Table 2). Activating WNT signaling partially restored the generation of COUP-TFII+ progenitors,

TABLE 1

IPSCs in DS research

Research object and resources	Findings	Reference
IPSC lines from skin fibroblasts of two males with DS	Down syndrome (DS) is caused by trisomy for chromosome 21 (T21). We generated two induced pluripotent stem cell (iPSC) lines from skin fibroblasts of two males with DS using Sendai virus delivery of OCT4, SOX2, KLF4, and c-MYC. Characterization of the two iPSC lines, UUIGPi013-A and UUIPGi014-A, showed that they are genetically stable with a 47,XY,+21 karyotype. Both lines displayed expression of pluripotency markers and trilineage differentiation capacity. These two iPSC lines provide a useful resource for DS modeling and pharmacological interventions.	Schuster *et al.* (2020)
iPSCs derived from an individual with DS	RNA-seq analysis was performed on iPSCs derived from a single individual with Down syndrome to investigate transcriptome changes resulting from trisomy of chromosome 21. The study identified significant changes in transcript accumulation, alternative splicing, and repetitive element transcripts associated with trisomy 21. Comparison with similar studies suggested that trisomy of chromosome 21 may interfere with pluripotency maintenance rather than intrinsically limit neuronal differentiation.	Gonzales *et al.* (2018)
Urine-derived iPSC from individuals with DS and euploid controls	This study aimed to generate induced pluripotent stem cells (iPSCs) from individuals with Down syndrome (DS) and euploid controls using urine-derived cells. The iPSCs were differentiated into neurons and astrocytes in monolayer cultures, as well as into three-dimensional cerebral organoids (COs) representing early cortical development. The urine-derived iPSC lines from both DS and control individuals successfully differentiated into neurons, astrocytes, and COs that mimicked human cortical development. These findings highlight the potential of urine-derived iPSC lines for modeling Down syndrome.	Teles *et al.* (2023)
hiPSCs from DS and healthy individuals	Using the GeneChip Human Transcriptome Array 2.0 (HTA 2.0), this study conducted a global gene analysis comparing human induced pluripotent stem cells (hiPSCs) from individuals with Down syndrome (DS) and healthy individuals. The analysis identified differentially expressed genes (DEGs) and focused on specific transcripts with alternative splicing (AS) events. Among these genes, 466 were up-regulated and 722 were down-regulated with AS events. Gene ontology (GO) analysis revealed enrichment in biological processes such as cell adhesion, cardiac muscle contraction, and immune response among the DEGs. Candidate genes like FN1 were further investigated for their potential role in DS. These findings shed light on the possible involvement of alternative splicing in Down syndrome.	Wang *et al.* (2021)
DS hiPSCs and isogenic euploid hiPSCs generated from DS and healthy individuals	In this study, a pair of human induced pluripotent stem cells (hiPSCs) was generatedlobal gene analysis comparing human induced pluripotent stem cells (hiPSCs) from individualsrom a euploid individual with a typical karyotype (46; XY). The hiPSC lines were generated using an integration-free Sendai viral vector system. Both hiPSC lines demonstrated pluripotency, as indicated by the expression of pluripotency markers and their ability to differentiate into the three germ layers.	Nehra *et al.* (2023)
DS iPSCs derived from monozygotic twins discordant for trisomy 21	DS iPSCs derived from monozygotic twins discordant for trisomy 21 were used to eliminate the effects of genomic background variability. The study observed alterations in genetic analysis at the iPSC level and abnormalities in neural differentiation both *in vivo* and *in vitro*. These defects were associated with changes in cell architecture, density, and misexpression of genes involved in neurogenesis. The dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1A (DYRK1A) gene on chromosome 21 likely contributed to these defects, and targeting DYRK1A led to considerable correction of the observed abnormalities.	Hibaoui *et al.* (2014)

suggesting WNT signaling could be a target for correcting differentiation deficits (Giffin-Rao *et al.*, 2022) (Table 2). Moreover, Curtis *et al.* (2022) found that DS iPSC-derived neurons developed age-dependent deficiency of retromer proteins, which regulate the retrieval and recycling of cargo from early endosomes, associated with beta-amyloid and tau accumulation (Table 2). These deficits were rescued by normalizing amyloid precursor protein (APP) and DYRK1A copy number in DS neurons via CRISPR-Cas9 editing, but not by normalizing RCAN1 and SYNJ1. They also showed that DS neurons had dysregulated axonal trafficking and enhanced synaptic retromer overexpression or stabilization,

TABLE 2

IPSC-derived cells and organoids in DS research

Research object and resources	Findings	Reference
Interneuron progenitors derived from Ts21 human iPSCs (trisomy 21 iPSCs) and isogenic controls	Ts21 progenitors showed reduced proliferation and generated fewer COUP-TFII+ progenitors compared to controls. The study also identified reduced WNT signaling in Ts21 progenitors and demonstrated that activation of WNT signaling partially restored the COUP-TFII+ progenitor population.	Giffin-Rao *et al.* (2022)
Neural Induced Pluripotent Stem Cells from Individuals with Down syndrome	Transcriptome and proteome signatures at two stages of differentiation revealed strong temporal dynamics of dysregulated genes, proteins and pathways belonging to 11 major functional clusters. DNA replication, synaptic maturation and neuroactive clusters were disturbed at the early differentiation time point accompanied by a skewed transition from the neural progenitor cell stage and reduced cellular growth. With differentiation, growth factor and extracellular matrix, oxidative phosphorylation and glycolysis emerged as major perturbed clusters.	Sobol *et al.* (2019)
hiPSC-derived cortical neurons	IPSC-derived cortical neurons, when transplanted into the adult mouse cortex, organized into large vascularized neuron-glia territories with complex cytoarchitecture. Longitudinal imaging revealed refined neuronal arbors, restructuring of synaptic networks, and oscillatory population activity mirroring fetal neural networks. The study also demonstrated altered synaptic stability and reduced oscillations in transplants from individuals with Down syndrome, showcasing the potential of iPSC-based *in vivo* modeling.	Real *et al.* (2018)
Neurons from gene-edited iPSCs	CRISPR-Cas9 gene editing followed by neuronal differentiation of iPSCs was performed to study dysregulated proteins in trisomy 21 (T21) postmitotic neurons. Normalization of copy number of APP and DYRK1A rescued elevated tau phosphorylation in T21 neurons. Proteomic analysis revealed disruptions in the axonal cytoskeletal network and presynaptic proteins, affecting axonal transport and synaptic vesicle cycling. Dysregulated axonal trafficking and enhanced synaptic vesicle release were observed in T21 neurons.	Wu *et al.* (2022a)
Human trisomic iPSCs and isogenic controls were differentiated into forebrain neurons	Dysfunction of the retromer complex system contributes to amyloid and tau pathology in Down syndrome. Trisomic neurons developed an age-dependent deficiency of retromer core proteins, leading to the accumulation of Aβ peptides and phosphorylated tau isoforms. Enhancing retromer function through overexpression or pharmacological stabilization reduced amyloid and tau pathology in trisomic neurons.	Curtis *et al.* (2022)
MSCs from human iPSCs	In this study, an optimized procedure for efficiently producing mesenchymal stem cells (MSCs) from human induced pluripotent stem cells (iPSCs) is presented. The protocol involves generating embryoid bodies (EBs) and treating them with transforming growth factor beta 1 (TGF-β1). The resulting MSCs can be purified based on the expression of CD73, CD105, and CD90 markers. These MSCs can then be expanded for multiple passages while retaining their characteristic properties.	McGarvey *et al.* (2022)
Transient Abnormal Myelopoiesis model generated from iPSC lines	Isogenic iPSC lines were used to model acute myeloid leukemia (AML) associated with Down syndrome. Approximately 1%–2% of children with DS develop AML before the age of 5 years. The isogenic iPSC lines provided a valuable tool for studying the disease and its mechanisms.	Barwe *et al.* (2020)
DS GABAergic progenitors derived from iPSCs	DS GABA neurons displayed smaller size, fewer neuronal processes, reduced proportion of calretinin over calbindin GABA neurons, and decreased neuronal migration capacity. Transplantation of DS GABAergic progenitors into the mouse medial septum replicated these phenotypes. Altered cell migratory pathways were identified through gene expression profiling, and correction of the PAK1 pathway mitigated the cell migration deficit.	Huo *et al.* (2018)
Human DS iPSC-Based Organoid	DS iPSC-derived cerebral organoids exhibited overproduction of OLIG2+ ventral forebrain neural progenitors. This led to excessive production of specific subclasses of GABAergic interneurons and impaired recognition memory in neuronal chimeric mice. Increased OLIG2 expression directly upregulated interneuron lineage-determining transcription factors. Knockdown of OLIG2 reversed abnormal gene expression, reduced interneuron production, and improved behavioral deficits.	Xu *et al.* (2019)

Table 2 (continued)

Research object and resources	Findings	Reference
Cortical spheroids (CS) generated from iPSCs: Isogenic iPSC lines (trisomic and euploid) used to generate cortical spheroids	Cortical spheroids derived from isogenic iPSC lines were used to model the impact of trisomy 21 on brain development. Single-cell RNA sequencing revealed cell type-specific transcriptomic changes, particularly in excitatory neuron populations. The study highlighted the most profound divergence in the developmental asynchrony between trisomic and euploid excitatory neurons and identified candidate genes driving this divergence. The findings emphasize the importance of cell type-specific analyses to evaluate cellular phenotypes in the context of Down syndrome.	Li *et al.* (2022)
Cortical neurons generated from human iPSC derived from patients with DS	Cortical neurons derived from Down syndrome (DS) patients' induced pluripotent stem cells (iPSCs) exhibited Alzheimer's disease (AD) pathologies in culture. These neurons processed the transmembrane APP protein, leading to the secretion of the pathogenic peptide fragment amyloid-β42 (Aβ42), which formed insoluble amyloid aggregates inside and outside the cells. The production of Aβ peptides was successfully blocked using a γ-secretase inhibitor. Additionally, the presence of hyperphosphorylated tau protein, a hallmark of AD, was observed in the cell bodies and dendrites of the iPSC-derived cortical neurons, resembling the later stages of AD progression.	Shi *et al.* (2012)
iPSCs-derived astroglia from DS patients	DS astroglia exhibited higher levels of reactive oxygen species, lower levels of synaptogenic molecules, and abnormal gene expression profiles. Astrocyte-conditioned medium from DS astroglia showed toxicity to neurons and failed to promote neuronal ion channel maturation and synapse formation. Treatment with minocycline partially corrected the pathological phenotypes of DS astroglia.	Chen *et al.* (2014)
Cellular model differentiated from DS-patient-derived iPSCs	DS astroglia exhibited more frequent spontaneous calcium fluctuations compared to control isogenic astroglia. This reduced the excitability of co-cultured neurons. Suppressed neuronal activity was rescued by blocking adenosine-mediated signaling or genetically targeting inositol triphosphate (IP3) receptors or S100B, a calcium-binding protein coded on HSA21.	Mizuno *et al.* (2018)
Astrocytes from newly produced DS hiPSCs	Chromosome 21 genes were upregulated in DS astrocytes, consistent up- and down-regulation of genes across the genome with a strong dysregulation of neurodevelopmental, cell adhesion and extracellular matrix molecules. ATAC (assay for transposase-accessible chromatin)-seq also revealed a global alteration in chromatin state in DS astrocytes, showing modified chromatin accessibility at promoters of cell adhesion and extracellular matrix genes.	Ponroy Bally *et al.* (2020)

indicating that retromer dysfunction may contribute to AD pathology in DS (Curtis *et al.*, 2022) (Table 2). Wu *et al.* (2022a) used DS hiPSCs to generate postmitotic neurons that showed synaptic vesicle release (Table 2). These findings provide insights into the initial changes in neurons that likely lead to neurodegeneration in DS.

Moreover, DS iPSC-derived cortical neurons transplanted in mouse cortex were found to organize into vascularized and cytoarchitecturally complex neuron-glia networks. Longitudinal imaging showed refined arbors and oscillatory activity similar to the fetal cortex. However, the DS transplants exhibited altered synaptic stability and reduced oscillations (Real *et al.*, 2018) (Table 2).

Other studies have differentiated DS iPSCs into astrocytes (Chen *et al.*, 2014; Mizuno *et al.*, 2018; Ponroy Bally *et al.*, 2020) (Table 2). Mizuno *et al.* (2018) found that DS iPSC-derived astrocytes had increased spontaneous calcium fluctuations and reduced neuronal excitability in neuron-astrocyte co-culture (Table 2). These abnormalities were rescued by blocking adenosine signaling, inositol triphosphate receptors, or S100B. Ponroy Bally *et al.* (2020) showed that DS iPSC-derived astrocytes had altered chromatin state, particularly open promoters of cell adhesion/extracellular matrix genes (Table 2). Furthermore, the conditioned media from DS iPSC-derived astrocytes was toxic to neurons and prevented channel/synapse maturation. Treatment with minocycline, an anti-oxidant and anti-inflammatory compound, partially corrected the pathological phenotypes in DS astroglia such as the decreased proliferation rate (Chen *et al.*, 2014) (Table 2).

Organoids derived from DS iPSCs were shown to overproduce ventral forebrain neural progenitors and GABAergic interneurons. This was associated with an increase in OLIG2 that upregulated interneuron transcription factors. Knockdown of OLIG2 rescued the organoid phenotypes and improved memory in chimeric mice (Xu *et al.*, 2019) (Table 2). Single-cell RNA sequencing of cortical spheroids derived from DS and control iPSCs revealed cell type-specific changes, particularly in excitatory neurons. A major difference between DS and controls was the asynchronous development of excitatory neurons. This highlights the need for cell type-specific analyses to fully understand DS brain phenotypes (Li *et al.*, 2022) (Table 2).

In summary, iPSC and iPSC-derived cells or organoids have been used in a series of DS investigations, providing

insights into the mechanisms underlying the abnormal development of specific cells in DS.

Insights on DS-Related Genes

DS carrying the extra full or partial copy of Chr 21 shows a number of dysregulated genes including APP, OLIG1, OLIG2, RUNX1, and DYRK1A (Sobol *et al.*, 2019). The generation of DS iPSCs through non-genomic integration showed not only the overexpression of Chr 21 genes, but also the dysregulation of thousands of other genes (Briggs *et al.*, 2013). Therefore, iPSCs or their derivatives could provide insights into the potential roles of genes on Chr 21 and genes on other chromosomes.

Chr 21 genes

- APP: The APP gene located on Chr 21 and its increased expression due to trisomy 21 is believed to contribute to the early onset of AD in DS patients. In the study by Shi *et al.* (2012), they found that cortical neurons derived from DS iPSCs displayed AD pathologies including the accumulation of amyloid-β42 and hyperphosphorylated tau. Deleting or overexpressing the APP gene in human iPSCs resulted in AD-related changes such as Aβ42/Aβ40 ratio and pyroglutamate aggregates in DS and control neurons, respectively (Ovchinnikov *et al.*, 2018). Moreover, transcriptomic and proteomic profiling revealed the early upregulation of APP in DS iPSCs, supporting the role of APP in the disturbed neurogenesis in DS (Sobol *et al.*, 2019). In a Down syndrome mouse model (Dp16), the levels of Aβ42 and Aβ40 were found to exceed those observed in AD. The products of APP processing were also increased in the Dp16 model (Sawa *et al.*, 2022). Further study revealed that the increased dose of the APP gene, which is triplicated in DS due to trisomy 21, was necessary for the loss of vulnerable neurons, tau pathology, and the activation of astrocytes and microglia in these DS model mice (Sawa *et al.*, 2022). In a mouse model of DS (Ts65Dn), *in vivo* treatment with Posiphen was well tolerated and did not show adverse effects on behavior. Posiphen treatment normalized the levels of full-length APP (fl-APP) and C-terminal fragments, slightly decreased Aβ species, restored Rab5 activity to normal levels, reduced phosphorylated tau (p-tau), and reversed the deficits in the activation of TrkB as well as Akt, ERK, and CREB signaling pathways. Remarkably, Posiphen treatment also restored the level of choline acetyltransferase protein to normal levels in disomic mice (2N) (Chen *et al.*, 2021).
- OLIG1, OLIG2, and RUNX1: Chr 21 genes of transcription factors OLIG1, OLIG2, and RUNX1 and their dysregulation observed in transcriptome and proteome profiling of DS iPSCs were linked to the abnormal myelination and neuronal differentiation in DS (Sobol *et al.*, 2019). Xu *et al.* (2019) further showed that increased OLIG2 expression in DS neural progenitors directly upregulated genes that promoted the production of specific subclasses of GABAergic interneuron lineages. Meanwhile, shRNA-mediated knockdown of OLIG2 in DS neural progenitors largely reversed the abnormal gene expression in early neural progenitors and decreased interneuron generation in DS organoids and chimeric mouse brains (Xu *et al.*, 2019). This indicates that OLIG2 drives interneuron production in DS organoids and the behavioral deficits in DS chimeric mice (Xu *et al.*, 2019). The expression of RUNX1 gene on Chr 21 was found to be higher in DS iPSCs compared to normal controls, which resulted in mitochondrial dysfunction in DS iPSCs via promoting apoptosis through the activation of the PI3K/Akt signaling pathway (Liu *et al.*, 2023). Therefore, reducing RUNX1 expression could improve mitochondrial function in DS iPSCs.
- DYRK1A: The DYRK1A gene located on Chr 21 is triplicated in DS, contributing to DS-associated intellectual disability (Guimera *et al.*, 1999). Studies using Twin-DS iPSCs-derived neural progenitor cells (NPCs) and neurons have consistently shown overexpression of DYRK1A in fetal DS (Hibaoui *et al.*, 2014). This gene's role in neural differentiation has been highlighted by the observation that pharmacological treatment with a DYRK1A inhibitor (EGCG) or RNA interference improved the proliferation and prevented apoptosis in Twin-DS iPSC-derived NPCs (Hibaoui *et al.*, 2014). Deleting one copy of DYRK1A in DS iPSCs improved the development of cerebral organoids (Caglayan, 2016), suggesting that normalizing DYRK1A in DS could correct neurodevelopmental phenotypes.
- CXADR: The triplication of CXADR on Chr 21 resulted in migration deficits in neural crest cells derived from DS iPSCs (Liu *et al.*, 2022).
- Long non-coding RNAs (lncRNAs): High-throughput sequencing and DEG screening of DS-derived iPSCs revealed the aberrant expression of lncRNAs, which are normally involved in epigenetic regulation (Qiu *et al.*, 2017). Enrichment analysis of lncRNAs in DS indicated their target genes were mainly associated with nervous and muscle development (Ma *et al.*, 2021).

Non-Chr 21 Genes

- Fibronectin 1 (FN1): Transcriptome analysis of DS iPSCs identified FN1 gene on Chr 21 was differentially expressed, indicating it has a potential role in DS (Wang *et al.*, 2021).

In summary, triplication of Chr 21 genes like APP, OLIG1/2, RUNX1, DYRK1A, and CXADR contributes to neurodevelopmental and mitochondrial abnormalities in DS models. Normalizing their expression could ameliorate certain DS phenotypes. Dysregulated non-Chr 21 genes like FN1 may also contribute to DS pathologies.

Insights on Mitochondrial Dysfunction and Oxidative Stress in DS

Mitochondrial dysfunction in DS

Mitochondrial dysfunction and oxidative stress observed in Down syndrome (DS), such as impaired mitochondrial dynamics, structural defects, and dysregulated bioenergetic

profiles linked to OXPHOS deficiency and reduced ATP production, can be attributed to the dosage-sensitive nuclear-encoded mitochondrial genes located on HSA21 (Tan *et al.*, 2023). In neural progenitors derived from the frontal cortex of DS patients at 19–21 weeks, genomic and functional profiling revealed dysregulated gene expression, particularly in genes related to cell death and oxidative stress (Esposito *et al.*, 2008). In fibroblast cells from the skin of DS patients, there was mild basal mitochondrial dysfunction, but the use of a cocktail of mitochondrial stressors revealed significant mitochondrial deficits (Anderson *et al.*, 2021). However, the underlying mechanisms of the mitochondrial dysfunction and oxidative stress in DS remain unclear. The use of iPSCs or their derivatives obtained from DS patients has provided helpful information on understanding the mitochondrial deficits in DS.

Molecular mechanisms of oxidative stress

Results from PCR-array and qRT-PCR analysis of DS iPSCs showed nearly all genes associated with mitochondria were downregulated, and a large number of lncRNAs were differentially expressed (Qiu *et al.*, 2017). These observations provide evidence for impaired mitochondrial function in DS iPSCs, which is consistent with the observations in DS cells (Qiu *et al.*, 2017). An RNA-seq study revealed there was significantly increased expression of RUNX1 in DS iPSCs compared to normal controls (Liu *et al.*, 2023). Overexpression of RUNX1 in control iPSCs resulted in mitochondrial dysfunction, whereas depletion of RCAN1 or inhibition of RUNX1 expression improved mitochondrial function in DS iPSCs (Liu *et al.*, 2023; Parra *et al.*, 2018). Global gene expression analysis indicated the overexpression of RUNX1 could promote the induction of apoptosis in DS iPSCs by activating the PI3K/Akt signaling pathway (Liu *et al.*, 2023). Compared to disomic iPSCs, mitochondria in trisomic iPSCs were more fused, had higher oxygen consumption, and exhibited compromised coupling efficiency and metabolic flexibility (Parra *et al.*, 2018).

iPSC-derived cells and organoids insights into mitochondrial abnormalities

Neurons differentiated from trisomic iPSCs derived from DS fibroblasts exhibited early mitochondrial alterations. Mitochondrial dysfunction was detected as early as day 7 of neuronal differentiation in DS iPSCs, indicating the early onset of mitochondrial abnormalities (Mollo *et al.*, 2021). Mitochondrial ROS production was also observed to impact the differentiation timing of DS iPSCs to NPCs, which could contribute to the developmental deficits in DS (Prutton *et al.*, 2023). Notably, trisomic NPCs were observed to have greater glial-like differentiation potential compared to euploid NPCs (Mollo *et al.*, 2021). Additionally, GABAergic interneurons and medial ganglionic eminence (MGE) organoids derived from DS iPSCs were shown to exhibit abnormal perinuclear clustering of mitochondria and impaired mitochondrial function, which was rescued by the inhibition of the DSCAM-PAK1 pathway by gene editing or treatment with a small molecule FRAX 486 (Xu *et al.*, 2022).

In summary, impaired mitochondrial function and oxidative stress have been implicated in DS. The use of iPSCs or their derivatives could help us reveal the underlying mechanisms of the neural and cognitive deficits involving mitochondrial dysfunction.

Insights on DS-Associated Leukemogenesis and Solid Tumor Resistance

Induced pluripotent stem cell (iPSC) models have provided valuable insights into the pathogenesis of leukemia in Down syndrome (DS). Studies using iPSCs with trisomy 21 have shown perturbations in hematopoiesis, such as increased hematopoietic progenitor populations, accelerated production of aberrant cells, and upregulation of genes that promote myeloid differentiation. Stepwise introduction of mutations found in DS-associated leukemia (such as GATA1, SMC3, and MPL mutations) into DS iPSCs has allowed researchers to model the stepwise development of acute megakaryoblastic leukemia (Evans and DeGregori, 2022). These iPSC models have reproduced cellular and molecular abnormalities seen in patients and have identified mechanisms by which trisomy 21 and recurrent mutations collaborate to drive leukemogenesis. iPSC models have also been used to study other aspects of DS pathophysiology, including musculoskeletal defects and solid tumor resistance.

Enhanced hematopoietic potential and genetic factors

Maclean *et al.* (2012) compared hematopoietic differentiation of disomic and trisomic subclones from the same parental iPSC line, which showed cells derived from trisomic iPSCs had increased multilineage colony-forming potential and exhibited a two- to five-fold increase in CD43(+) (Leukosialin)/CD235(+) (Glycophorin A) hematopoietic cell populations. Banno *et al.* (2016) and McNulty and Crispino (2016) showed that iPSCs with trisomy 21 and GATA1 mutations had perturbed hematopoiesis, in which the increased early hematopoietic progenitors and upregulated mutant GATA1 accelerated aberrant cell production. These effects were mediated by the increased expression of RUNX1, ETS2, and ERG on Chr 21, which suggest multiple genetic factors contribute to leukemogenesis (Banno *et al.*, 2016; McNulty and Crispino, 2016).

Modeling DS myeloid leukemia: altered hematopoietic pathways and proliferation

Barwe *et al.* (2022) developed a DS Myeloid Leukemia model via hematopoietic differentiation of gene-targeted iPSCs bearing trisomy 21. Mutations in GATA1 and STAG2, which are recurrent in this leukemia, were sequentially introduced into DS iPSCs. The introduction of GATA1 mutations downregulated megakaryocytic and erythrocytic differentiation pathways and interferon α/β signaling, and upregulated pathways promoting myeloid differentiation such as toll-like receptor cascade. Subsequent knockout of STAG2 partially reverted the expression of genes associated with myeloid differentiation, likely promoting leukemogenesis (Barwe *et al.*, 2022). Arkoun *et al.* (2022) performed stepwise gene editing of GATA1s, SMC3+/−, and MPLW515K into trisomy 21 and disomic iPSCs to model

megakaryocyte differentiation defects. Among the 20 different trisomic and disomic iPSC clones, they found GATA1s profoundly reshaped iPSC-derived hematopoietic architecture, with a gradual myeloid-to-megakaryocyte shift. The addition of SMC3 and MPL mutations also affected megakaryocyte differentiation, with changes in essential megakaryocyte differentiation genes, including downregulation of NFE2 (Arkoun *et al.*, 2022). They also found that trisomy 21 enhanced the proliferative phenotype, reproducing the cellular and molecular abnormalities in DS-associated AMKL.

Solid tumor resistance

Galat *et al.* (2020) showed DS iPSC-derived progenitor stromal cells had aberrant musculoskeletal development and resistance to solid tumors. Additionally, Perepitchka *et al.* (2020) found that DS iPSC-derived endothelial cells exhibited decreased proliferation, migration, and inflammatory response. They also reported a set of genes potentially associated with the unfavorable solid tumor microenvironment and with the elevated leukemia risk in DS, suggesting that these genes could be potential therapeutic targets in translational cancer research (Perepitchka *et al.*, 2020).

In summary, these studies illuminated the heightened hematopoietic potential and genetic intricacies associated with Down syndrome. Trisomic iPSCs display enhanced hematopoiesis, influenced by genetic mutations like GATA1 and STAG2, which may contribute to leukemia. Notable genes on Chr 21, including RUNX1, ETS2, and ERG, play a pivotal role. Modeling DS Myeloid Leukemia underscores the complexity of hematopoietic pathways and proliferation. Furthermore, DS iPSC-derived cells exhibit resistance to solid tumors, opening avenues for potential therapeutic targets. These findings deepen our understanding of DS-related hematological and solid tumor abnormalities, paving the way for further research and therapies.

Mechanisms of the Neural Deficits in DS

Down syndrome causes a variety of developmental deficits, such as disruptions in neural progenitor division, neurogenesis and gliogenesis, altered cortical architecture, and reduced cortical volume, leading to neural abnormalities and intellectual disability. The use of DS iPSCs and their derivatives including NPCs, neurons, astrocytes, and organoids can provide a platform to investigate the cellular mechanisms underlying these neural deficits.

Chen *et al.* (2014) showed that DS iPSC-derived astroglia lacked the ability to support neuronal ion channel maturation and synapse formation *in vitro*, and were unable to promote neurogenesis in endogenous neural stem cells *in vivo*. Moreover, DS astroglia exhibited aberrant calcium signaling that led to inhibited neuron excitability, which could be rescued by suppressing astrocytic calcium activity (Mizuno *et al.*, 2018).

GABAergic neurons differentiated from DS-derived iPSCs showed cellular/migratory deficits, calretinin/calbindin imbalance, and impaired migration in mouse brain (Huo *et al.*, 2018). Correcting the disrupted pathways mitigated the migration deficits in DS GABA progenitors *in vitro* (Huo *et al.*, 2018). Real *et al.* (2018) transplanted DS iPSC-derived cortical neurons into adult mouse cortex, which showed increased synaptic stability and reduced oscillations. DS iPSC-derived organoids and chimeric mouse brain showed overproduction of OLIG2(+) progenitors and excess GABAergic neurons (Xu *et al.*, 2019). The increase in OLIG2 in DS progenitors upregulated interneuron determinants, whereas suppressing OLIG2 reversed the gene expression changes, reduced excess neurons, and improved the behavior in mice (Xu *et al.*, 2019). Neurons derived from DS iPSCs also displayed widespread transcriptomic, proteomic, and cellular changes associated with neurodevelopment (Sobol *et al.*, 2019; Wu *et al.*, 2022a). The DS neurons also exhibited progressive retromer deficiency together with amyloid and tau pathologies (Curtis *et al.*, 2022). Enhancing retromer function in DS neurons alleviated these pathologies (Curtis *et al.*, 2022).

In summary, the disruption of neural development in DS involves astroglia, progenitors, and GABAergic/other neurons. Exploiting DS iPSCs and their derivatives could help to identify dysregulated pathways and provide insights in the neuropathological mechanisms.

Potential Targets for DS Treatments

Although there has been much research focused on elucidating neurodevelopmental and neurological alterations in DS, laboratory studies on DS are still relatively challenging. Murine models have been developed and revealed many changes similar to those observed in DS individuals, which can potentially provide insights into disease mechanisms and the functions of specific genes. For example, the Ts65Dn DS model has three copies of most of the Chr 16 genes, which are homologs of human Chr 21 genes. This model has been used to investigate how triplication of genes results in abnormalities in DS patients (Murakami *et al.*, 2023). Several phenotypes observed in this DS mouse model have yet to be confirmed in DS individuals. Nevertheless, human neurodevelopment and neuropathological processes are too complex to recapitulate in mouse model. Consequently, most of the tested drugs indicated by DS studies using animal models have not demonstrated any positive effect or very limited efficacy in clinical trials, leading to only few novel treatments for DS in the past decade (Rueda *et al.*, 2020).

Gene-specific targets

Current research using iPSCs and their derivatives obtained from DS patients revealed that the abnormalities in DS were associated with specific genes, suggesting these genes could be potential targets for treatments. Considering the presence of an extra full or partial copy of Chr 21 in DS, much effort has been made to normalize Chr 21 genes, suppress the expression of Chr 21 genes, or even completely remove this extra chromosome. For example, pharmacological targeting or use of shRNA to suppress DYRK1A on Chr 21 resulted in the significant correction of defects, such as the abnormal neural differentiation observed in DS iPSCs (Hibaoui *et al.*, 2014). Caglayan (2016) revealed that single-copy DYRK1A

knockout in DS iPSC-derived cerebral organoids could correct neurodevelopmental phenotypes such as impaired neocortical development. These findings implicate DYRK1A on Chr 21 as a strong target for ameliorating cognitive impairments in DS. Chen *et al.* (2014) showed that DS astroglia treated with minocycline, an FDA-approved antibiotic, suppressed the expression of S100B, GFAP, inducible nitric oxide synthase, and thrombospondins 1 and 2, leading to the partial correction of the pathological phenotypes in DS astroglia.

Pathway-specific targets

Recent studies have uncovered promising pathway-specific therapeutic targets for DS. Suppressing the DSCAM/PAK1 pathway in cerebral organoids formed from DS iPSCs reversed the neurogenesis deficits (Tang *et al.*, 2021), which could be a potential therapeutic strategy. A chemical chaperone 4-phenylbutyrate was able to decrease protein aggregates, prevent premature senescence, and reduce apoptotic cell death in trisomy 21 cells (Hirata *et al.*, 2020; Nawa *et al.*, 2019), which suggests targeting aneuploidy-associated stress could benefit human trisomies like DS. Activating Sonic hedgehog signaling rescued early defects in neural lineage specification of neural precursors that were differentiated from DS iPSCs (Klein *et al.*, 2021), highlighting the potential of modulating key pathways at specific developmental stages. Extracellular vesicle treatment alleviated neuropathology in a DS cortical spheroid model (Campbell *et al.*, 2023), suggesting the manipulation of intercellular communication could provide new treatment paradigms. Activating WNT signaling partially restored COUP-TFII+ progenitors, which could be targeted to correct differentiation deficits (Giffin-Rao *et al.*, 2022). Chang *et al.* (2015) showed that treatment with N-butylidenephthalide (Bdph), which was emulsified by pluronic F127, promoted Wnt signaling and attenuated AD-like pathologies including Aβ40 accumulation and total or hyperphosphorylated tau levels in DS neurons.

Targeting epigenetic dysregulation

Some studies have revealed epigenetic dysregulation as a potential therapeutic target in DS. Bioinformatic analyses showed that several microRNAs encoded on chromosome 21, including miR-99a, let-7c, miR-125b-2, miR-155 and miR-802, were overexpressed in DS fetal tissues and likely contribute to cognitive and cardiac defects (Chang *et al.*, 2009; Kuhn *et al.*, 2008). Additionally, epigenetic therapies like DNA methyltransferase inhibitors and HDAC inhibitors have shown promise in preclinical DS models. One study found that the combination of azacitidine and panobinostat significantly improved survival compared to chemotherapy in a DS-acute myeloid leukemia patient-derived xenograft model (Barwe *et al.*, 2019). Another study revealed synergy between the EZH2 inhibitor GSK126 and the HDAC inhibitor romidepsin in inhibiting DS-megakaryoblastic leukemia growth in cell and xenograft models (Cicek *et al.*, 2022). These findings highlight the potential of normalizing dysregulated epigenetic pathways through microRNA, DNA methylation, or histone modification targeting therapies. Epigenetic treatment strategies hold great promise for ameliorating diverse cellular and physiological defects in DS and warrant further evaluation. In line with this epigenetic approach, Czerminski and Lawrence (2020) used XIST RNA to silence trisomy 21, which induced neural stem cells to undergo neuronal differentiation, indicating this unique non-coding RNA could have translational potential for DS neurobiology. Furthermore, reprogramming DS fibroblasts to iPSCs resulted in the spontaneous loss of the extra chromosome in some cells, which generated disomic iPSC clones. This phenomenon could be further investigated as a potential strategy for trisomy rescue (Akutsu *et al.*, 2022).

Cell therapy

Cell therapy has emerged as a potential treatment option for Down syndrome (DS). Implanting murine neural stem cells (mNSCs) or murine neural progenitor cells (mNPCs) has shown promising results in enhancing cell survival and injury response in trisomic mice. Notably, these transplanted mNSCs/mNPCs exhibited migration to injury sites, offering neuroprotection and facilitating axon growth (Salamt *et al.*, 2021). However, it is important to note that cell therapy has not yielded significant findings with regard to improvements in the physical appearance, cognitive function, social skills, or behavior of individuals with DS (Salamt *et al.*, 2021).

In summary, the above studies elucidate promising treatment strategies for DS that are emerging from studies employing cell models derived from DS patients. These strategies aim to rectify molecular and cellular deficits associated with trisomy 21 through diverse approaches, such as normalizing the expression of chromosome 21 genes, modulating key signaling pathways disrupted in DS, reducing pathological abnormalities in DS neural cells, reversing neurodevelopmental impairments, and exploring cell therapy options. These findings hold promise, but their translation into effective clinical treatments necessitates systematic evaluation of risks, benefits, and ethical considerations through rigorous preclinical studies and clinical trials. Collectively, recent advances in DS research models are uncovering molecular details that could pave the way for tailored interventions at different developmental stages to improve outcomes for individuals with DS.

Conclusions and Perspectives

Studies employing DS iPSCs and their derivatives have revealed many insights into the cellular and molecular mechanisms of the mitochondrial dysfunction, neural deficits and leukemia risk associated with DS. Some promising strategies for ameliorating DS pathology include targeting dysregulated pathways, suppressing the effects of triplicate genes, and rescuing altered network dynamics (Akutsu *et al.*, 2022; Caglayan, 2016; Chen *et al.*, 2014; Czerminski and Lawrence, 2020).

However, translating the promising findings from preclinical studies to clinical trials poses substantial issues. Given the complexity of DS, including the cognitive impairment, early onset AD, and leukemia risk, and the involvement of genetic, epigenetic, and environmental factors across developmental stages (Asim *et al.*, 2015; Sobol *et al.*, 2019), recapitulating this disease in animal models is

challenging. Mouse models with engineered trisomies and neuronal defects can inform such factors, but cannot completely mimic DS (Murakami *et al.*, 2023). With advancements in iPSC technology, iPSC-based models that integrate multiple cell types, ages, brain regions, and genetic backgrounds can now be used to study the underlying mechanisms in DS. Using 3D organoids or assembloids to model intact neural circuitry and brain regions may yield more translatable insights (Caglayan, 2016).

In summary, although the mechanistic insights gained from *in vitro* modeling are invaluable, developing safe and effective treatments for DS requires long-term commitment with careful consideration of the risks and real-world contexts. Understanding the multifaceted relationships between genetic, developmental, and environment factors in DS may lead to novel therapies offering dignity and independence to those affected. Although there is still a long way to go, the first steps have already been taken.

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