HHS Public Access

Author manuscript

Trends Mol Med. Author manuscript; available in PMC 2021 February 01.

Published in final edited form as:

Trends Mol Med. 2020 February ; 26(2): 150–169. doi:10.1016/j.molmed.2019.10.001.

Challenges and Opportunities for Translation of Therapies to Improve Cognition in Down Syndrome

Sarah E Lee[1,*], **Monica Duran–Martinez**[1], **Sabina Khantsis**[1], **Diana W Bianchi**[1,2], **Faycal Guedj**[1,**]

[1]Medical Genetics Branch (Prenatal Genomic and Therapy Section), National Human Genome Research Institute, National Institutes of Health, Bethesda, Maryland, United States of America

[2]Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland, United States of America

Abstract

While preclinical studies have reported improvement of behavioral deficits in the Ts65Dn mouse model of Down syndrome (DS), translation to human clinical trials to improve cognition in individuals with DS has had a poor success record. Timing of the intervention, choice of animal models, strategy for drug selection, and lack of translational endpoints between animals and humans contributed to prior failures of human clinical trials. Here, we focus on *in vitro* cell models from humans with DS to identify the molecular mechanisms underlying the brain phenotype associated with DS. We emphasize the importance of using these cell models to screen for therapeutic molecules, followed by validating them in the most suitable animal models prior to initiating human clinical trials.

Lack of Success in Clinical Trials to Improve Cognition in Down Syndrome

Down syndrome (DS) is a complex genetic condition caused by an extra copy of chromosome 21 (***HSA*21,** see glossary) that results in dysregulation of many genes across the genome. The multigenic nature of DS not only complicates the understanding of its pathophysiology, but also the design of therapeutic interventions [1]. All individuals with DS exhibit significant hypoplasia of the frontal lobe, hippocampus, and cerebellum, and mild to severe intellectual disability [2, 3].

In the last two decades, improvement of cognition and prevention of neurodegeneration have been the focus of preclinical and clinical trials [4, 5]. Preclinically, more than 20 drugs have been shown to rescue neurobiological and/or cognitive defects in the **Ts65Dn** mouse model of DS [5-7]. The success of these preclinical studies opened a new research era and led to

[*]**Corresponding Authors:** Sarah E. Lee, PhD, 35 Convent Dr., Rm 2F143, Bethesda, MD 20892, sarah.lee2@nih.gov, Phone: 301–496–6703. [**]Faycal Guedj, PhD, 35 Convent Dr., Rm 2F220, Bethesda, MD 20892, faycal.guedj@nih.gov, Phone: 301–402–1009.
Sarah E. Lee, PhD, 35 Convent Dr., Rm 2F143, Bethesda, MD 20892, sarah.lee2@nih.gov, Phone: 301–496–6703
Faycal Guedj, PhD, 35 Convent Dr., Rm 2F220, Bethesda, MD 20892, faycal.guedj@nih.gov, Phone: 301–402–1009
Monica Duran-Martinez, 35 Convent Dr., Rm 2F125, Bethesda, MD 20892, monica.duranmartinez@nih.gov
Sabina Khantsis, 35 Convent Dr., Rm 2F125, Bethesda, MD 20892, sabina.khantsis@nih.gov
Diana W. Bianchi, M.D., 31 Center Dr., Rm 2A03, Bethesda MD 20814, diana.bianchi@nih.gov

The authors declare no competing financial interests.

the translation of several of these drugs to human clinical trials designed to improve cognitive outcomes. However, most of these clinical trials failed to show significant therapeutic effects. As we are witnessing a rapid increase in the number of preclinical drug trials, there is an urgent need to understand the challenges to translational research in DS and develop novel research strategies to overcome them.

PubMed and Clinicaltrials.gov report the use of 13 different pharmacological interventions in 25 completed and ongoing human clinical trials (Table 1) [8, 9]. Despite evidence of efficacy in preclinical mouse studies, the tested compounds have shown no improvement in cognitive performance with one exception, **epigallocatechin gallate** (EGCG). EGCG, when combined with cognitive enrichment, improved visual recognition memory, inhibitory control, and adaptive behavior in young adults with DS [10].

Here we address some of the reasons for lack of success in human clinical trials to date, including limitations in preclinical and clinical experimental study designs, timing of interventions, methods used for drug design and screening, adequacy of animal models, and the absence of human translational endpoints, all of which suggest the need for novel models and strategies to improve the likelihood of therapeutic success (see Clinician's Corner).

Challenges in Preclinical and Clinical Trials to Improve Cognition in Down Syndrome

Clinical trial failures stem from challenges related to the study design, drug design and pharmacokinetic properties and validity of preclinical data for humans. These challenges include (Figure 1):

The low number of participants and lack of standardized outcome measures

To date, most clinical trials have had very few participants, ranging from eight to 350 for the largest study (Table 1). Individuals with DS exhibit interindividual variability in cognitive impairment and other phenotypes, which may limit the usefulness of small sample size studies. To address this issue, the United States National Institutes of Health (NIH) has recently launched the INvestigation of Co–occurring conditions across the Lifespan to Understand Down syndromE (INCLUDE) project (https://www.nih.gov/include-project). One of its goals is to develop large cohorts of people with DS that can be rapidly mobilized to test new therapies. Moreover, clinical trial outcome measures are seldom standardized, making it difficult to compare results from different trials [9, 11]. To address this challenge, the NIH has established standard clinical trial outcome measures for children and adults with DS and other intellectual disabilities [11, 12].

Timing of intervention

Due to safety concerns, participation in clinical trials is typically limited to older children, adolescents, and adults. Only one study has been conducted in infants using leucovorin [13], and a pilot study is being conducted during the prenatal period using fluoxetine [8]. Because critical periods of brain development, including neurogenesis, synapse formation, and gliogenesis occur mainly during prenatal and early neonatal stages, there is a unique window

of opportunity to improve brain development and achieve maximum treatment efficacy [4, 6, 7, 14]. Although potential benefits of early intervention are now recognized, detailed pharmacological and toxicological studies *in vitro* and *in vivo* are necessary to ensure safety of potential candidate therapies for the affected fetus or infant and the mother [15, 16]. Ethical aspects of fetal therapy for DS have been discussed elsewhere [17].

The placebo effect

Complicating clinical study interpretation is the presence of a significant **placebo** effect observed in individuals with DS and other forms of genetically driven intellectual disabilities [9, 18]. Precise mechanisms for apparent placebo effects are unknown but may be due to increased caregiver support and positive clinician–patient interactions [18]. Expectation of a positive effect may be enough to elicit improvement in cognitive performance [19]. It is important to note that the placebo effect is apparent in subjective, such as caregiver or clinical staff observations, as well as objective measures of cognitive performance. The placebo effect is heightened in younger patients but absent in individuals with dementia [18]. Apparent placebo effects underscore the need for careful study design to understand the contribution of treatment versus placebo in future clinical trials. Trial measures should include multitest-retest sessions for objective measures. For subjective measures requiring experimenter scoring, the presence of a second reader may reduce placebo effect. Finally, multi-center clinical trials performed by different research groups using standardized procedures provide another potential avenue for treatment validation.

Strategy of drug design

Most clinical trials in humans with DS have evaluated repurposed drugs approved for other conditions, particularly Alzheimer disease (AD).

Drugs repurposed from AD and dementia.—The presence of ***APP*** on *HSA*21 and its important role in familial early onset AD, coupled with the accumulation of **amyloid plaques** in the brains of individuals with DS led to the use of medications designed for AD–related dementia to enhance cognition in DS [20]. These include memantine, rivastigmine and donepezil, which target **glutamatergic** and **cholinergic** signaling (Table 1). Memantine had significant therapeutic effects in Ts65Dn mice, while chronic treatment with donepezil failed to improve hippocampal spatial memory in this model [21, 22]. The effects of rivastigmine were not evaluated in a mouse model of DS prior to beginning a human clinical trial. Other studies include the completed scyllo–inositol and ongoing ACI–24 **liposomal vaccine** trials ([XII]) targeting amyloid plaques [23, 24].

Drugs targeting GABAergic signaling.—Although human studies in individuals with DS reported decreased **GABA** in the frontal and temporal cortices [25, 26], two drugs (basmisanil and pentylenetetrazol [PTZ]) were used to further block this signaling in humans with DS (Table 1). The choice of these two molecules stems from increased inhibitory neuronal transmission in the Ts65Dn mouse model [27, 28]. Both molecules were shown to improve multiple cellular, behavioral, and electrophysiological deficits in this mouse [29, 30]. In clinical trials, basmisanil ([XIV], [XV], [XVI], [XVII]) failed to show efficacy in improving cognition and functional abilities in individuals with DS (Table 1). In 2012, a

phase I human clinical trial with PTZ was initiated in young adults with DS in Australia (ACTRN12612000652875[XXIV]). To date, however, no results have been communicated on the safety or efficacy of PTZ in this population (Table 1). PTZ was revoked by the FDA in 1982 because of its strong epileptogenic and convulsive effects [31].

Bumetanide, a potent blocker of Na-K-Cl-solute cotransporters and modulator of GABA signaling, was reported to improve social communication and interactions in children and adolescents (two to 18 years old) with autism spectrum disorder [32].]. In 2016, a phase II clinical trial (2015–005780–16[XXV]) was initiated to evaluate the efficacy of three months of treatment with bumetanide in improving cognitive function in children and adolescents (10–16 years old) with DS. To date, no trial results have been published. Outcomes of these trials demonstrate the need to develop therapies that target specific altered mechanisms occurring in DS.

The choice of preclinical animal models—Rodents are the only small animals that share large syntenic regions with human *Hsa*21. Peclinical testing of DS treatments has almost exclusively relied on a single rodent model, the Ts65Dn mouse (See box 1 for a summary of DS mouse models). Studies in this model have identified cholinergic, adrenergic, and GABAergic neuronal network deficits as possible targets for improving cognition [33-35]. Compounds targeting these pathways induced significant improvement of behavioral and electrophysiological alterations in the Ts65Dn mouse. Despite their usefulness, engineered mice do not fully recapitulate the genotype and complex, cognitive phenotypes of humans with DS, which may curtail their relevance as indicators of drug efficacy. There is a need to develop models that better mimic the genotype and phenotypes of individuals with DS.

Interspecies differences in drug pharmacokinetic properties.

In preclinical trials, drug **pharmacokinetics** (**ADMET**), are as important as efficacy [36]. For an orally administered drug, ideal ADMET properties include good **bioavailability**, blood clearance and volume distribution allowing appropriate dosing, low potential for drug–drug interaction, and no toxicity in humans [37]. **Allometric scaling**, physiological models, and computational modeling allow for interspecies extrapolation of drug pharmacokinetics, and the use of these methods has improved dosing translation from small animal preclinical studies to human clinical trials [38, 39]. However, interspecies differences in drug metabolism further complicate the interpretation of animal data during drug discovery and limit their translation to humans [40]. Drug metabolism involves oxidation, reduction, and hydrolysis (Phase I), and conjugation (Phase II) reactions. Differences in isoforms of enzymes catalyzing these reactions, such as the Cytochrome P (CYP) protein family is a major contributor to interindividual and interspecies differences in metabolism [37, 41, 42]. Translation of pharmacokinetic parameters from animal models to humans remains a major hurdle for drug discovery in general, and DS in particular, because detailed pharmacokinetic studies are lacking for individuals with DS.

Lack of human–relevant endpoints in behavioral testing to evaluate therapeutic efficacy

The key to linking post-treatment rodent and human cognitive outcomes requires behavioral tests that reflect cognitive performance in humans (Table 2). Behavioral tests often suffer from a lack of method standardization across research groups, which limits comparison of results between studies [1, 43, 44]. Furthermore, the choice of the behavioral endpoints varies between investigators. Current standard murine behavioral tests, such as fear conditioning or the Morris water maze, are not relevant or reproducible in humans (Table 2). There is an unmet need to develop behavioral tests in animal models that directly translate from human clinical studies, including the NIH Toolbox, CANTAB and ACTB (Table 2) [11, 12, 45].

Human Cells as Potential Models to Design Effective Therapies for Trisomy 21

Existing mouse models of DS do not completely mirror the karyotype and phenotypes present in humans with DS; therefore, they cannot be used alone to design and test potential therapeutics (Figure 2) [46]. Additionally, lack of accessibility, problems with tissue integrity, and ethical concerns hinder the use of human post–mortem tissues to study the **pathophysiology** of DS. Development and use of human cell models may enable better understanding of the underlying mechanisms of DS in a human context, and spur identification of druggable targets for treatment [47]. Despite their lack of complexity, *in vitro* cell models derived from humans with trisomy 21 (T21) have the advantage of recapitulating the human genotype and **epigenetic** changes. They may also provide a fast and scalable tool to design and screen therapies for safety and efficacy prior to costly and time–consuming mouse preclinical and human clinical trials. They do not, however, allow for the analysis of complex interactions between different cell types or high cognitive functions that would occur in a whole organism.

The use of primary cells and cell lines in DS research

Primary cells (**amniocytes, chorionic villi (CV),** fibroblasts, CNS derived neurons and astrocytes) and **lymphoblastoid** cell lines have been used for diagnostic procedures or studying limited aspects of the phenotype. These cells exhibit commonalities in phenotypes and dysregulated signaling pathways/cellular processes (Table 3), which provide starting points for better understanding the molecular mechanisms of DS and identifying potential targets for therapy.

Chorionic villi and amniocytes.—Little is known about cellular and molecular phenotypes of CV. A single study described T21 villi as hypertrophic and hypovascular [48]. Another study identified differential patterns of DNA–methylation in T21 CV samples [49]. Amniotic fluid contains a mixed population of cells (amniocytes) in various states of differentiation with some retaining stem cell like properties and expression of pluripotency markers [50, 51]. Amniocytes from fetuses with T21 exhibit transcriptome dysregulation, reduced proliferation, earlier **senescence**, increased oxidative stress, shorter telomeres, and increased proteolysis (Table 3) [46, 52-55].

Fibroblasts.—Fibroblasts from individuals with DS exhibit reduced proliferation rates and earlier senescence, mitochondrial dysfunction with reduced ATP production and abnormal cristae morphology, increased oxidative stress, and lower levels of antioxidant molecules compared to euploid fibroblasts [56-58]. T21 fibroblasts also increased expression of pro–inflammatory molecules, particularly overactivation of interferon signaling [59]. Candidate molecules that have been tested on T21 fibroblasts include metformin and EGCG. Both molecules promoted mitochondrial biogenesis and restored mitochondrial function (Table 3) [56, 60].

CNS derived neurons and astrocytes.—*In vitro,* primary cortical neurons and astrocytes derived from fetuses with DS showed increased reactive oxygen species (ROS) and apoptosis [61], increased accumulation of Aβ intracellularly, and deficits in amyloidogenic processing [62]. Coculture of T21 primary astrocytes with rat primary cortical neurons resulted in altered dendritic spine morphology and abnormal synapse formation [63]. T21 primary astrocytes exhibited mitochondrial dysfunction and genome wide dysregulation of genes related to mitochondrial function that was rescued with antioxidant and mitochondrial cofactor treatment [63-66].

Lymphoblastoid cell lines.—Lymphoblastoid cell lines from individuals with DS have been used to analyze genome–wide dysregulation compared to euploid cells but also between individuals with and without heart defects [67]. In several studies, T21 lymphoblastoid cells exhibited decreased proliferation, higher oxidative stress, mitochondrial dysfunction, activation of interferon and NF–κB inflammatory pathways, increased proteasome activity, and enlarged endosomes similar to findings in T21 fibroblasts (Table 3) [59, 68].

Studies of T21 primary cells demonstrate their importance as valuable tools for studying dysregulated gene expression, molecular and cellular phenotypes, and most importantly for validation of new *in vitro* models; however, primary cells are limited in their growth and expansion capacity, excluding them from use in high-throughput drug screening assays. Furthermore, use of post-mortem tissue and human fetal brain derived neural cells, neurons and astrocytes is hindered by ethical, legal, and accessibility concerns.

Induced pluripotent stem cells for modeling DS

Since the advent of stem cell reprograming technology over a decade ago [69], **induced pluripotent stem cells** (iPSCs) have become the gold standard for disease modeling, gene editing and drug discovery *in vitro* [69]. iPSCs have been used in preclinical trials for different disorders with promising results [70, 71] leading to the initiation of several human clinical trials in the last decade. The diseases that have been targeted include macular degeneration, spinal cord injury, achondroplasia, amyotrophic lateral sclerosis, diabetes, severe heart failure, and AD [72, 73]. However, the cost and time to develop patient specific iPSC lines and differentiated cells, variable transformation efficiencies, limit the use of iPSC lines [74]. Outweighing their limitations are advantages of human iPSCs, such as their capacity for self–renewal and differentiation into other cell types including neural stem cells (NSC). NSCs give rise to neurons, astrocytes, and oligodendrocytes, allowing for detailed

examination of critical events associated with brain development in humans with T21 [75]. To date, few groups have explored this approach and shown its feasibility. Importantly, common cellular phenotypes were observed in cell types derived from iPSCs from people with T21 [76-79].

Neurons.—In humans with T21, the number of neurons is reduced in the particularly hypoplastic brain regions (frontal cortex, hippocampus, and cerebellum) [80-83]. Lamination and cell layer definition are altered, with diffuse cellular distribution, poorly defined layers, and less neuronal prominence in the visual cortex [84]. In developing brains of fetuses with T21, the number of cells in the subplate, intermediate zone, ventricular and subventricular zones is reduced by a third compared to euploid fetuses [82]. During infancy, the number of neurons in layers II and IV (granular layers) is reduced by 20–50%, along with a significant reduction of calbindin+ and parvalbumin+ interneurons in the prefrontal cortex [83, 85]. Similar observations are reported in the dentate gyrus, pre–subiculum, entorhinal cortex, and lateral parahippocampal gyrus [80]. In the cerebellum, a significant reduction of neuronal populations in the granular, molecular, and Purkinje cell layers is observed during the second trimester [80]. In addition to neurogenesis defects, neurons possess altered synapses and spines. Expanded dendritic arbors appear earlier in T21, but expansion ceases and atrophy occurs in cortical pyramidal neurons and Purkinje cells during infancy [86].

T21 iPSC derived neurons exhibit reductions in synapse formation, lower frequency of spontaneous post–synaptic currents (sPSCs) and fewer synapsin puncta [77, 86]. Oxidative stress and mitochondrial membrane potential were increased in T21 iPSC derived neurons, but there was no increase in apoptosis [77]. However, another study showed increased caspase 3 positive cells in T21 derived neurons along with decreased Ki–67 positive cells, implicating increased apoptosis and reduced proliferation [86]. EGCG rescued Ki–67 expression, reduced caspase–3 activity, and restored neurogenesis and expression of neural markers [86].

Astrocytes.—Compared to euploid fetuses, the number of radial glia increases between 18 and 19 weeks of gestation but decreases after 20 weeks in the frontal lobe of fetuses with T21 [87]. This early maturation of radial glia is accompanied by a significant increase in the number of GFAP+ mature astrocytes in fetuses with T21 [87], and twice as many S100β+ astrocytes throughout the lifespan (between 17 weeks of gestation and 68 years of age) in the cerebral cortex of individuals with T21 [88]. In the hippocampus, astrogliosis was present during gestation, infancy, and adulthood [89, 90]. Although the cerebellum is the most affected brain region in humans with T21, to date no studies have been conducted to investigate the cellular density of cerebellar radial astrocytes (Bergman glia) and other glial populations in individuals with DS.

Several studies have shown an altered gliogenic pathology when T21 iPSCs differentiate spontaneously in the absence of neurogenic factors [76, 79, 86]. T21 iPSCs showed a two to three–fold increase in the production of glial cells. Ratios of neurons to glia were altered, but the timing of the neurogenic to gliogenic switch was similar in both T21 and euploid cells [76]. Studies of iPSCs derived from monozygotic twins discordant for T21 showed

alterations in neuronal differentiation based on decreased expression of the neuronal markers β–Tubulin and MAP2, and an increased expression of the glial markers GFAP and OLIG2 [86]. T21 iPSC derived NSCs demonstrated a preference toward astrocytic rather than neurogenic differentiation following spontaneous differentiation [79]. These astrocytes show increased expression of S100β and GFAP. No reduction or delay in differentiation of T21 iPSCs to neural progenitors and neurons occurs following directed differentiation protocols [77]. However, production of reactive oxygen species in T21 iPSC derived astrocytes is significantly higher through overactivation of inducible nitric oxide synthase and subsequent NO production and release. Paradoxically, these cells do not exhibit increased apoptosis, but rather, higher proliferative rates than controls [79]. Conditioned media from T21 iPSC derived astrocytes leads to decreased neurogenesis, increased apoptosis, and altered functional properties in control and T21 neurons [79].

Oligodendrocytes.—Myelination begins late in the prenatal period and continues into adulthood with peak activity occurring between six months and two years of age [91]. Imaging, histological, and molecular studies have reported that individuals with T21 show decreased myelination and reduced density of OLIG2+ cells throughout the lifespan [92-94]. Molecular and cellular mechanisms leading to myelination defects in humans with DS are poorly understood, but delayed maturation of oligodendrocytes progenitors is thought to play a major role [94]. To date, no studies have investigated these mechanisms in T21 iPSCs derived oligodendrocytes.

Microglia.—Microglia are important regulators of the inflammatory response and are essential for proper brain development through synapse pruning, neurogenesis, and differentiation [95]. In humans with DS, the number of microglia is increased; they possess an activated and ramified morphology [96]. Understanding the role of astrocytes and microglia in the exacerbated inflammatory status in the brain of individuals with DS is key to improving overall brain function in this population. This can be achieved through the study of iPSCs derived microglia [97].

Patient-derived iPSC models of DS provide a promising strategy for understanding mechanisms underlying neurodevelopmental alterations occurring in DS and provide potential methods to identify new therapies. Unlike primary cells, iPSC models are capable of large scale, self-renewal for use in high-throughput screening assays. Studies of T21 iPSC-derived cells show that these models reflect molecular and cellular phenotypes observed in primary cells and in individuals with DS. Growth and differentiation conditions can be controlled and directed in iPSCs providing a larger degree of standardization than primary cells; however, inherent variability due to genetic background remains in iPSCs. Patient specific iPSCs generated from skin biopsies or prenatally from amniocytes may allow researchers to generate biobanks of iPSC lines to evaluate therapeutic efficacy in drug screening assays.

Perspectives and Future Directions in Drug Development for Down Syndrome

Despite major advances in our understanding of the mechanisms underlying developmental alterations occurring in DS, no effective treatments are available to date (see outstanding questions). New paradigms for translational and clinical research are needed for the development of novel, targeted, and effective therapies to improve cognitive abilities and independent life skills in individuals with DS (Figure 2A). Each currently available model has limitations that reduce the ability to translate results from that model toward development of human therapies. Going forward it will be important to tackle the challenges presented in this review through the integration of multidisciplinary approaches.

First and foremost is the identification of relevant endpoint and phenotypic targets that can be easily translated from humans with DS to mouse models, and vice–versa. This will require the use of human/murine integrated approaches throughout the lifespan and identification of the most adequate human cell and mouse models that recapitulate the genotype/phenotype of individuals with DS (Figure 2B-D). Availability of primary cells from humans with DS and advances in iPSC development make it possible to generate patient–specific multipotent cells that can be differentiated and used for phenotyping and drug screening (Figure 2C). T21 iPSC derived cells share several phenotypic alterations that have been observed in humans with DS, which may allow for further identification of DS–specific molecular signatures and initial screening of candidate therapies prior to costly mouse preclinical and human clinical trials (Figure 2E). T21 iPSC derived models have a distinct advantage in that they are more accessible, not limited in their growth potential, and can produce large cell populations for medium and high–throughput phenotyping and drug screening. T21 iPSC models can provide important preliminary information about possible toxicity, efficacy, and mechanisms of action for targeted therapeutics; however, *in vitro* models alone are not sufficient for translational studies (Figure 2F). The use of *in vivo* models will provide complementary information not only about the drug efficacy but also the ADMET profiles of candidate therapeutic molecules in a complex system (Figure 2F).

Selection of appropriate animal models and testing regimens is equally important. In generating new or validating existing mouse models, it is critical to consider the human DS karyotype (Figure 2D). In 95% of cases, DS is caused by the presence of a third freely segregating chromosome 21. Additionally, there is need to re–evaluate the current cognitive tests that are being used in the DS clinical research field and to translate those tests for use in animal models. The use of common, standardized behavioral and cognitive screening protocols is essential for ensuring harmonization of results across institutions and research groups.

The timing of therapeutic interventions is critical for maximum beneficial impact in DS. Currently, most research studies are focused on understanding and developing interventions for early neurodegeneration in adults with DS. In light of the evidence of early phenotypic alterations in fetuses, infants, and young children with DS, it is crucial to direct similar or more attention and resources to early and safe interventions that could not only improve

cognition but might by the same mechanisms prevent or minimize early neurodegeneration in DS (Figure 2G).

Finally, the creation of multi–center patient cohorts and national and international consortia will bring multidisciplinary experts together to define strategic goals, promote resource and material sharing as well as standardization of preclinical and clinical protocols, encourage collaboration and cooperation, nurture and create funding opportunities for young investigators, and accelerate the discovery of therapies for neurodevelopmental and neurodegenerative sequelae that are associated with DS. In the last few years, several consortia have been established for other conditions, including the Academic Drug Discovery Consortium (ADDC), the International Rare Disease Research Consortium (IRDiRC), and the PsychENCODE consortium to only cite a few [98-100].

Concluding Remarks

In the last two decades, many attempts have been made to ameliorate the cognitive aspects of DS using mouse models. Although animal studies resulted in beneficial effects, translation to humans was unsuccessful. The lack of success can be linked to many challenges both in preclinical studies (strategy for drug design, choice of adequate models, and lack of human translational endpoints to test therapies) and clinical trials (low numbers of participants, lack of standardized outcome measures, timing of intervention, and placebo effects).

To tackle these challenges, standardized preclinical and clinical studies across the lifespan are needed that can improve the reproducibility of results and the likelihood of identification of successful therapeutic interventions. Combining human cell studies with the best available animal models may improve the success rates of clinical trials in DS. Developing a multidisciplinary approach using human translational, measurable, and relevant endpoints across all levels from gene expression and cellular functioning to the systems level behavioral tasks in appropriate animal models will enable real progress towards the identification of effective therapeutics that will improve cognition and enhance the independent life skills of people with DS.

Clinician’s Corner

The life expectancy of individuals with DS has increased considerably due to the development of surgical and pharmacological treatments for the non–neurological complications of DS, including congenital heart disease and hypothyroidism. With the increase in life expectancy, intellectual disability, and early neurodegeneration are the two biggest handicaps that hinder the development of independent life skills in individuals with DS.

Due to safety concerns, almost all clinical trials to date have been performed in older children or adults. Abnormalities in brain growth and development, however, occur prenatally. Compared to other syndromes associated with intellectual disability, DS is unique because in many countries pregnant women are offered blood-based noninvasive screening for trisomy 21. Positive screens are confirmed via diagnostic procedures such as

amniocentesis or CV sampling. Both of these procedures produce fetal or placental cells upon which to directly test the efficacy and safety of different molecules, effectively providing fetal personalized medicine to improve neurocognition. Advances in fetal brain imaging enable objective assessment of treatment effects on the developing brain.

To realize the vision of treatment of DS to improve independent life skills, further work needs to be done. Importantly, the way forward will include the use of both human iPSCs and differentiated neural cells lines to screen candidate therapies, with more thorough testing in animal models.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported in part by the National Human Genome Research Institute's Intramural Research Program, National Institutes of Health, Bethesda, Maryland through grant Z1A HG200399-04 (Investigator: Diana Bianchi). The authors also wish to acknowledge the help and support of Julia Fekecs and the NHGRI Medical Illustration Team.

Appendix

Resources

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Glossary

Allometric scaling
understanding how organisms change relative to size and development

Amniocytes
Cells obtained from amniotic fluid for routine prenatal clinical diagnosis of trisomy 21 (T21).

Amyloid plaques
Accumulation of Aβ peptides occurs due to overproduction of type Aβ-42, a less soluble, neurotoxic form of Aβ believed to underlie neurodegeneration in AD.

APP
A gene that codes for amyloid precursor protein. APP is cleaved by enzymes to produce soluble amyloid precursor protein and amyloid-β (Aβ). APP mutations are linked to early-onset Alzheimer disease (AD).

Bioavailability
The amount of administered dose that reaches the systemic circulation and varies by route of administration (e.g. oral vs intravenous).

Cholinergic
Molecules that modulate acetylcholine signaling. Potentiation of acetylcholine signaling is used to improve cognition in individuals with dementia.

Chorionic villi (CV)
Projections of placental tissue that contain cells produced by the fetus. CV are primarily used for diagnosis of T21 or other aneuploidies.

DYRK1A
Dual Specificity Tyrosine Phosphorylation Regulated Kinase 1A is an enzyme that catalyzes autophosphorylation on serine/threonine and tyrosine residues that may be involved in regulating cell proliferation and brain development.

Epigallocatechin gallate (EGCG)

is a polyphenol with antioxidant activity found in green tea that acts as a DYRK1A kinase inhibitor.

GABA
gamma-aminobutyric acid is the primary inhibitory neurotransmitter in the central nervous system (CNS).

Glutamatergic
Molecules that modulate the excitatory glutamate signaling pathway. Inhibition of NMDA type glutamate receptors is used to improve cognition in individuals with dementia.

Hsa21
Human chromosome 21 is one of the smallest autosomes and contains over 200 protein coding genes present in three copies in Down syndrome (DS).

Liposomal vaccine
In AD, phosphorylated tau accumulates in response to increased Aβ levels causing neurotoxicity. The vaccine elicits an immune response, targeting phosphorylated form of microtubule associated protein Tau to reduce tauopathy.

Lymphoblastoid
B–lymphocytes transformed using Epstein Bar virus to confer immortality.

Microglia
Small cells that act as the resident phagocyte within the CNS.

Pharmacokinetics/ADMET
What the body does to a drug, including absorption, distribution, metabolism, and excretion (ADME). Adding the study of toxicity becomes the acronym ADMET.

Placebo
A substance or procedure that mimics treatment but lacks any active ingredients, used as a control to determine the efficacy of treatment.

Senescence
The period when cells exit the cell cycle and cease to divide but continue to carry out physiological activities.

Ts65Dn
A mouse model of DS with a small freely segregating chromosome trisomy of the centromeric region (50 genes non-orthologous to Hsa21) of Mmu17 and distal region (104 genes orthologous to Hsa21) of Mmu16.

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Box 1: Most Commonly Used Mouse Models of Down Syndrome

A recent study of the phenotypes of the three most widely used mouse models of DS (Dp(16)1/Yey, Ts65Dn and Ts1Cje), at three different stages in the lifespan, reported not only differences in the genotypes of these mice, but also major differences in gene expression, neurogenesis, and behavioral profiles [43]. Although Ts65Dn mice demonstrated aberrant neurogenesis and learning/memory deficits, they do not recapitulate the typical phenotypic findings in people with DS, such as microcephaly [43]. Furthermore, Ts65Dn mice are trisomic for a segment of only half of the *Hsa*21 orthologous genes and are trisomic for a set of *Mmu*17 triplicated genes that are not orthologous to *Hsa*21. The contribution of these genes to the Ts65Dn phenotypes remains unknown [43].

Dp(16)1/Yey mice contain an elongation of the full *Mmu*16 orthologous region, which theoretically represents the best model for DS [139]. However, the engineering of this strain as an elongation and not a freely segregating chromosome (as is the case in humans with DS) likely results in different phenotypic abnormalities than in individuals with DS. Indeed, this model lacks gene expression and neurogenesis defects in the embryonic brain, in contrast to what is observed in human fetuses with DS. The Ts1Cje mouse model has a smaller segmental trisomy compared to Ts65Dn. Its milder phenotype may be the result of its genotype (translocation instead of freely segregating chromosome) and/or early neonatal mortality of the most severely affected pups [140].

Two research groups have attempted to generate trans–species mouse models, including ES(#21)–10 and Tc1, that harbor the entire human chromosome 21 in addition to the mouse diploid orthologous genes [141, 142]. Although they exhibit behavioral and electrophysiological alterations, both mouse models exhibit varying degrees of mosaicism between different mice and different organs (90–95% and 53% in the brains of ES(#21)–10 and Tc1 mice, respectively). Novel gene editing methods utilizing CRISPER/Cas9 may improve generation of additional animal models such as rat or non-human primate models [143]. These models may better reflect genetic and underlying molecular mechanisms of DS and as more complex models, allow for better translation of behavioral tasks to human cognitive performance.

Highlights

- Prenatal screening for Down syndrome (DS) creates unique opportunities for antenatal treatments to optimize neurocognitive development of affected individuals.
- Ts65Dn mice have been used in preclinical drug studies despite carrying three copies of genes that are non-orthologous to chromosome 21. Phenotypes in Ts65Dn and other mouse models of DS are significantly different from one another. Preclinical treatment studies have shown beneficial effects in Ts65Dn mice, yet translation to human studies has been unsuccessful.
- Primary human cells and pluripotent stem cells are important models for understanding and validating mechanisms of DS, but are limited by ethical, legal, and accessibility issues. Both primary cell types and iPSCs from humans with DS are valuable tools that can be used long-term for large scale drug screening to identify treatments for DS.

Outstanding Questions

Fetuses with Down syndrome exhibit microcephaly, delayed neurogenesis and abnormal synaptogenesis. Will prenatal treatment have a greater positive impact on cognition and independent life skills than later intervention in childhood or adulthood?

What are the most relevant cellular and molecular endpoints that can be used to identify and test the efficacy of therapeutics in vitro and in vivo?

Can the use of patient derived iPSCs and iPSC-derived neuronal and glial cells bridge the translational gap and improve the success rate of human clinical trials?

Many segmental trisomic mouse models have been generated, however, none of these mimic the human DS karyotype and phenotype. Which animal model recapitulates genetic and phenotypic changes that occur in DS and accurately predicts response to treatment or is there a need to explore other species such as rat and marmoset? If alternative models are needed, what is the role of newer gene editing techniques for improving animal models?

Preclinical mouse studies use conventional rodent behavioral tests to evaluate therapies. Can we effectively translate the human CANTAB and ARIZONA cognitive batteries, and use them to investigate cognitive delay and treatment efficacy instead of traditional rodent behavioral paradigms?

Can standardization, reproducibility, and resource sharing across investigative groups be improved through harmonization of preclinical and clinical research guidelines, study design, data collection and data sharing?

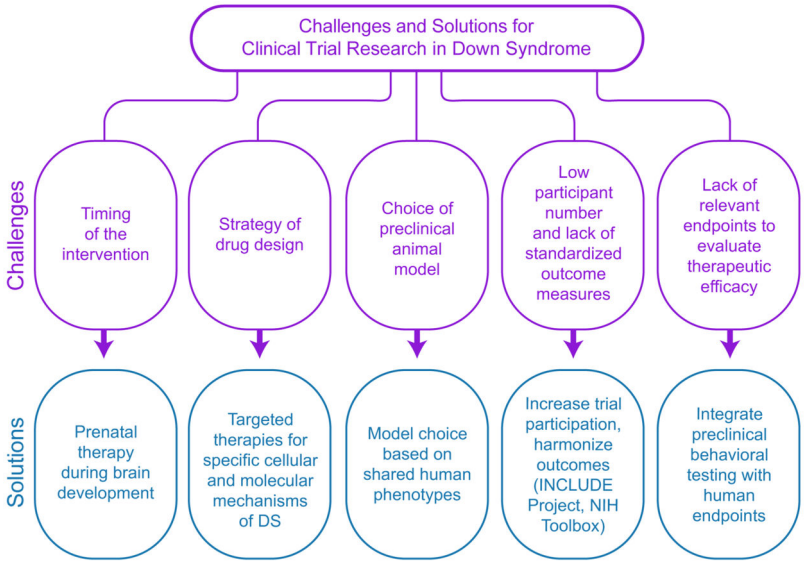

Figure 1: Challenges and Solutions to Improve Clinical Trial Research Success in Down Syndrome (DS).

Schematic representation of the major challenges and specific solutions to increase the likelihood of success in human clinical trials to improve cognitive outcomes in individuals with DS.

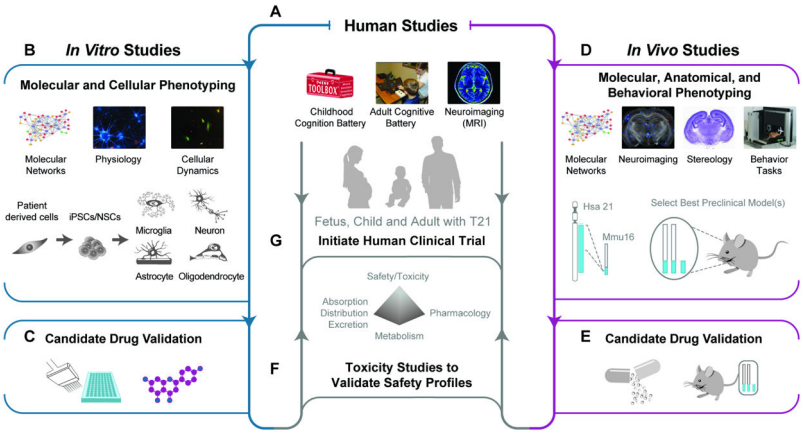

Figure 2: The Human/Murine Multidisciplinary Translational Approach to Develop Successful Therapeutics for Intellectual Disability in Down Syndrome (DS).

(A) Standardized outcome measures in human clinical studies must be applied throughout the lifespan in individuals with DS to define the most affected brain regions and cognitive domains that should be targeted for therapy. **(B)** The use of human *in vitro* models, including iPSCs and iPSC-derived neural stem cells and differentiated central nervous system cell populations will provide valuable information on the cellular and molecular phenotypes associated with DS. Human DS-specific molecular signatures should be the basis for candidate drug selection and screening (**C**). **(D)** In parallel with *in vitro* studies, *in vivo* studies should prioritize the creation of rodent models that mimic the human DS karyotype (free segregating extra-chromosome) and genotype (triplication of only Hsa21 orthologous genes). To phenotype these models, the endpoint measures (molecular, neuroimaging and behavioral) as well as area of phenotyping (target brain region and cognitive domains) should be translated from the human studies in individuals with DS. (**E**) These outcome measures can be then used to evaluate the efficacy of candidate therapeutic molecules defined in the *in vitro* drug screening phase. (**F**) During these *in vitro* and *in vivo* preclinical studies, efficacy, toxicity, and safety profiles of the most potent drug candidates are evaluated prior to starting human clinical trials in fetuses, newborns, or adults with DS (**G**).

Table 1:

Clinical trials conducted to evaluate safety and efficacy of therapies to improve cognitive deficits in Down syndrome

Treatment	Trial Id#	Phase	Participants	Ages	Use/MOA	Pre–clinical Data	Results	References
Rivastigmine	[i] [II]	N/A 1, 2	14 42	8+, 10–17 yrs.	AD/ Cholinesterase inhibitor	Derivative or Physostigmine/ Galantamine–Ts65Dn improve 4–arm maze, olfactory test	No significant improvement in aspects of cognition, language, or overall function	[101-103]
Donepezil/ E2020	[III] [IV] [V] [VI] [VII]	2 3 3 2 2	128 8 9 36 150	6–10, 11–17, 15–39 yrs.	AD/ Cholinesterase inhibitor	Ts65Dn– no change in MWM, Altered cholinergic system in DS	Terminated due to lack of efficacy	[104]
Memantine	[VIII] [IX] [X]	4 N/A 2	42 180 200	18–32, 15–32, 40+ yrs.	AD/ NMDA uncompetitive antagonist	Evidence of cognitive improvement/ partial improvement in Ts65Dn–Contextual fear conditioning, MWM, WRAM, NOR	No improvement in primary measures, $p<0.10$ improved CVLT–II scores, PAL Stages, DAS–II Recall of Digits	[22, 105]
ELND005/scyllo–inositol	[XI]	2	23	18–45 yrs.	AD/Prevent/reduce amyloid aggregation, increase amyloid–β clearance and myo–inositol regulation to improve cognitive function	Amyloid anti–aggregation effects in vitro, protective effects on oligomer–induced neuronal toxicity, and positive effects on learning in animal models of AD	No differences in cognitive or behavioral measures, and there were no SAEs or deaths in the study, high dose groups discontinued	[23]
ACI–24	[XII]	1	24	25–46 yrs.	AD/ liposomal vaccine–induce anti–Aβ antibodies	Ts65Dn non–significant reduction in brain Aβ levels, improved NOR and CFC, reduction of cholinergic neuron atrophy	Ongoing	[24]
Nicotine–transdermal	[XIII]	1, 2	15	25+ yrs.	Nicotinic Cholinergic	No studies performed	Ongoing	
Basmisanil/ RO5186582/ RG1662	[XIV] [XV] [XVI] [XVII]	2 1 1 2	173 35 13 45	6–11, 12–30, 18–30, 18–40 yrs.	Inverse agonist/negative allosteric modulator of GABAAR	Antagonism of GABA in Ts65Dn–improvement in NOR, MWM, Y–maze	Terminated due to lack of efficacy.	[29, 104]
BTD–001/ pentylenetetrazole/PTZ	ACTRN12612000652875[XXIV]	1	88	13–35 yrs.	Non–competitive GABAAR antagonist	Chronic treatment improved NOR, MWM, persisted after cessation of treatment	Ongoing	[21, 104]
Bumetanide	2015–005780–16[XXV]	2	24	10–16 yrs.	Autism & Seizure trials/ Sulfamyl loop diuretic for heart failure/ blocks the NKCC1 cation–chloride co–transporter,	Rescue of LTP, NOR, OL in Ts65Dn	Ongoing	[32, 106]

Treatment	Trial Id#	Phase	Participants	Ages	Use/MOA	Pre–clinical Data	Results	References
					decreases neuronal intracellular Cl–			
EGCG	XX XXI	2 2	31 87	14–29, 14–39 yrs.	DYRK1A inhibitor, lipid lowering properties, antioxidant	Inhibition by EGCG–showed improved MWM, NOR, Y–maze,	Some improvement in cognitive performance with EGCG and training	[10, 107]
Folic Acid/ Folic Acid + Thyroid hormone	XXII XXIII	2, 3 3	120 175	3–30 mos., 6–18 mos.	Vitamin– does not require dihydrofolate reductase for its conversion	Genes related to folate metabolism are located on chromosome 21–leading to abnormal metabolism & low folate levels	Some low–level positive impact on developmental age	[13]
Vitamin E	XXIV XXV	3 3	350 349	50+ yrs.	α–tocopherol, a lipophilic chain–breaking antioxidant, acts as an inhibitor of lipid peroxidation	Ts65Dn improvement in EP–maze, MWM, R–maze	Did not slow cognitive deterioration in older individuals with DS	[108]

AD: Alzheimer disease, GABAAR-GABA-A receptor, NKCC1-Na-K-Cl-Cotransporter, MWM: Morris water maze, WRAM: water radial arm maze, NOR: novel object recognition, CFC: conditioned fear conditioning. LTP: long-term potentiation of synaptic activity, CVLT-II: California Verbal Learning Test 2nd ed., PAL: Paired Associates Learning task, DAS-II: Differential Ability Scales-II, SAE: Significant Adverse Effect

Trends Mol Med. Author manuscript; available in PMC 2021 February 01.

Table 2:

Human cognitive behavioral tasks and related animal tests

Stage	Common Human Cognitive Task Used	Cognitive Domain Tested	Mouse Analogous Tasks	Non-Analogous Mouse Cognitive Tasks Used in Preclinical Studies	References
Infancy (0–3 years)	**1** CDC Developmental Milestones Tracker **2** Age and Stage Questionnaire, 3rd ed	• Gross and fine motor skills • Language and communication • Problem solving • Adaptive behavior • Personal–social skills	None	Fox scale was used to analyze developmental milestones in Dp(16)1/Yey, Ts65Dn and Ts1Cje mouse models	[43, 109, 110]
	Bayley Scale for Infant and Toddler Development–III (Birth to 3·5 Years)	• Cognitive • Language (Receptive and Expressive) • Gross and Fine Motor • Socio–emotional • Adaptive behavior	None	None	[111, 112]
	Speech Development/Vocalization	• Early infancy communication	Ultrasonic vocalization	Ultrasonic Vocalization was used to analyze Ts65Dn and Ts1Cje pup vocalization	[110, 113-115]
	Griffiths Scale of Child Development–III (Birth to 6 years)	• Foundation of learning • Language and communication • Eye and hand coordination • Personal–social–emotional • Gross motor	None	None	[116, 117]
	Piagetian Object Concept Task (Birth to 2 Years)	• Sensorimotor development	None	None	[118]
	Peabody Development Motor Scales–II (Birth to 5 Years)	• Gross and fine motor skills	None	None	[119]
	1 Test of Infant Motor Performance (TIMP) (Birth to 4 months) **2** Harris Infant Neuro–motor Test (HINT) (2·5 to 12·5 months)	• Spontaneous movements • Postural control and movement	None	None	[120-122]

Stage	Common Human Cognitive Task Used	Cognitive Domain Tested	Mouse Analogous Tasks	Non-Analogous Mouse Cognitive Tasks Used in Preclinical Studies	References
	3 Alberta Infant Motor Skills (AIMS) (Birth to 2 years)				
Childhood (3–11 years)	NIH Toolbox (3 to 85 years)	• Cognition • Motor function • Emotion • Sensation	None	None	[12]
	Stanford–Binet Intelligence Test (2 to 89 years)	• Fluid reasoning • Knowledge • Quantitative reasoning • Visual–spatial processing • Working memory	None	None	[123, 124]
	Leiter International Scale (3 to 75 years)	• Fluid reasoning • Visual–spatial processing • Working memory • Attention	None	None	[125, 126]
Adolescence-Adulthood (12–20 years)	Touch–Screen Based Cambridge Neuropsychological Test Automated Battery (CANTAB)	• General memory and learning • Working memory • Visual memory • Attention • Verbal memory • Decision making	Only one published study analyzed visual discrimination in Ts65Dn mice using touchscreen chambers	Most studies in mouse models of DS used the following conventional rodent behavioral tasks: • Morris water maze (spatial memory) • Novel object recognition (short and long–term memory) • Fear conditioning (long–term memory) • T maze (working memory) • Open field (locomotor activity) • Rotarod and treadmill (motor coordination)	[11, 127]
	Touch–Screen Based ARIZONA Cognitive Test Battery (ACTB)	• Verbal comprehension • Working memory • Perceptual organization • Processing speed	Only one study used non–touchscreen tasks to translate the ARIZONA battery		[45, 128, 129]

Table 3:

Advantages and disadvantages of T21 cellular models, relevant phenotypic findings, and responses to treatment

Cell type	Advantages	Disadvantages	Findings in Cells from Individuals with Down Syndrome	Effects of Therapeutic Molecules	References
Amniocytes & villi	• Obtained from amniocentesis or chorionic villus sampling during the first & second trimester • Express stem cell markers • Considered maternal tissue, no major ethical issues concerning the fetus • Can be grown & expanded *in vitro* • May carry signatures that give insights of disease state	• Heterogenous, mixed population of cell types • More limited access & requires IRB protocols & maternal consent • For amniocytes, gene expression profile changes with fetal age, which may impact experimental outcome • Limited growth capacity which limits use in high throughput screening	• Genome–wide gene expression dysregulation • Decreased cell proliferation • High prevalence of cell senescence • Shorter telomeres in amniocytes • Increased proteolytic activity in amniocytes • Reduced gene expression of mitochondrial ATPase 6 & TFAM in amniocytes • Hypertrophic & hypovascular villi	• Apigenin treatment reduces oxidative stress, improves anti–oxidant capacity, induces the expression of genes that are important for G2/M transition & decreases the expression of pro–inflammatory markers	[46, 53-55]
Fibroblasts	• Obtained from humans with DS throughout lifespan (fetal, infants, children, adolescents & adults) • Available in cell biobanks • Retain genetic defects & systemic phenotypes • Can be grown & expanded *in vitro* • Can be used for reprogramming to generate iPSCs • Carry signatures that give insights of disease state	• Not CNS cells; not representative of diseased tissue or CNS dysfunction • Limited growth capacity reduces usefulness in high throughput drug screening. After certain number of passages, cells enter senescence. • Properties change with extended time in culture	• Genome–wide gene expression dysregulation • Decreased cell proliferation • Increased senescence • Shorter telomeres • Increased ROS production • Reduced mitochondrial membrane potential, impaired ATP production, irregular shapes & increased mitochondrial mass. • Mitochondrial dysfunction & oxidative stress exacerbated in T21 fibroblasts from individuals with heart defects • Disrupted proteostasis network: increased	• EGCG increases mitochondria biogenesis & rescues mitochondrial complex I, improved ATP synthase activity & suppressed oxidative stress • Metformin activates PGC1α, promotes mitochondrial biogenesis, mitochondrial activity & ATP production • JAK inhibitor, ruxolitinib, induced phosphorylation of STAT1, promotes cell proliferation	[56-60, 130, 131]

Cell type	Advantages	Disadvantages	Findings in Cells from Individuals with Down Syndrome	Effects of Therapeutic Molecules	References
			endoplasmic reticulum (ER) stress, protein ubiquitination, & disrupted proteasome activity • Increased exosome secretion via increased expression of CD63 to mitigate endosomal abnormalities • Overactivation of interferon signaling response through JAK/STAT signaling • Defective repair of ROS–induced DNA damage, activation of DNA damage response, & increased p53 protein expression		
CNS-derived Primary Cells	• Obtained from post-mortem tissues • Can be grown in vitro • Reflect CNS phenotypes • Carry signatures that give insights of disease state	• Limited growth capacity • Limited access to tissues • Variable and/or poor quality of post-mortem tissues	• Genome-wide gene expression dysregulation • Increased oxidative stress and apoptosis • Increased ROS production • Mitochondrial dysfunction, abnormal mitochondrial morphology, and networks	• Antioxidant treatment improved viability of T21 neurons	[61, 62, 64, 65]
Lymphoblastoids:	• Arise from B–lymphocytes transformed with Epstein Barr virus (EBV) • Can be expanded for many months (unlike lymphocytes) without genetic rearrangements common in other immortalized cell lines • Have been used in expression studies of whole genome dysregulations in T21 • Reliable, inexpensive & easily accessible	• Lymphoid phenotype makes them inconvenient for CNS research • Cell are immortalized using EBV virus	• Genome–wide gene expression dysregulation • Impaired proliferative capacity & increased sensitivity to genotoxic stress • Mitochondrial dysfunction reduced mitochondrial membrane potential, reduced ATP production & increased ROS production • Disrupted proteostasis network, reduced protein ubiquitination & increased proteasome activity • Impaired autophagy enlarged early endosomes	Have not been evaluated	[59, 68, 132]

Cell type	Advantages	Disadvantages	Findings in Cells from Individuals with Down Syndrome	Effects of Therapeutic Molecules	References
	• Can be used to detect biologically plausible correlations between candidate genes & various genetically–induced diseases • May carry signatures that give insights of disease state		& enhanced exosome secretion • Overactivation of interferon signaling		
iPSC, NSC, & iPSC–derived cells	• Obtained by reprogramming of somatic cells using Yamanaka factors. Somatic cells are easy to obtain from patients or cell biobanks. • Can be grown & expanded *in vitro* indefinitely making them ideal material for high throughput drug screening • Can be used to generate the three embryonic layers lineages (ectoderm, mesoderm & endoderm) to study developmental events • Even after reprogramming, cells retain genetic defects/ chromosomal abnormalities • Neural stem cells generated from iPSCs are multipotent & give rise to CNS cell types • May carry signatures that give insights of disease state.	• Require reprogramming using integrative or non–integrative methods • Transformation efficiency varies although it has been improved nonintegrating methods • High maintenance, time consuming, expensive • Cultures may be heterogeneous • Genetic instability reported with increased passaging requiring regular karyotyping • Retain immature, fetal phenotypes • Do not recapitulate complex neuronal circuits as in in vivo animal models	• Global transcriptome dysregulation • Increased oxidative stress • Mitochondrial dysfunction • Gliogenic shift with spontaneous differentiation (more astrocytes than neurons) in T21 cells • Altered astrocyte–neuron communication. Hyperactivation of Akt/ mTOR signaling is thought to be the trigger of this phenotype • T21 iPSCs–derived astrocytes appear to be in a reactive state with more branching, thicker branches. • Decreased neurite length in T21 iPSC derived neurons • Altered electrophysiological properties of iPSC derived neurons and astrocytes; alterations Ca2+ signaling & decreased spontaneous post synaptic currents • Decreased migration of GABAergic interneurons derived from T21 iPSCs	• Treatment with EGCG (DYRK1A inhibitor) or DYRK1A shRNA promotes neurogenesis in T21 iPSCs derived neural progenitors & improved neuronal maturation • Treatment with minocycline rescued the neuronal/gliogenic ratio, improved neurogenesis, suppressed apoptosis, & rescued neuronal electrophysiological properties in T21 derived neurons,	[77, 79, 133-138]

Cell type	Advantages	Disadvantages	Findings in Cells from Individuals with Down Syndrome	Effects of Therapeutic Molecules	References
			• Increased Aβ peptide generation in T21–iPSC cortical neurons		

IRB: Institutional Review Board, CNS: central nervous system, TFAM: Transcription Factor A, mitochondrial, ROS; reactive oxygen species, PGC1α: Peroxisome proliferator-activated receptor-γ coactivator