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Characterization of the role of H_2S in neuronal differentiation in Trisomy 21

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MSc of Biomedical Sciences – Mechanisms of Disease

This work was done under the supervision of

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Declaração de autoria de trabalho

Declaro ser o autor deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam na listagem de referências incluída.

Vera Mascarenhas Marques

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ABSTRACT

Down syndrome (DS) is caused by trisomy of chromosome 21 and is one of the most prevalent aneuploidies compatible with life. The characteristics of DS include congenital heart defects, craniofacial abnormalities, gastrointestinal anomalies, leukemia, seizures, early onset of Alzheimer´s disease, and cognitive impairment among others. Disturbances in the neurological signal processing during critical stages of neurogenesis can affect proliferation, migration, and differentiation of stem cells which may be responsible for the mental impairment of these individuals. These features are important to understanding how DS's brain development is affected. The gene coding for cystathionine-beta-synthase (CBS) is present in chromosome 21 with an extra copy in individuals with DS. CBS is one of the enzymes responsible for the cellular production of hydrogen sulfide (H_2S). This is a ubiquitous small gaseous signaling molecule that plays an important role in many physiological processes. However, the contribution of H_2S to the abnormal neurodevelopment of DS individuals has not been addressed and is currently under investigation under the Araújo lab. In this study, we aimed to evaluate the contribution of H_2S in DS fibroblasts prior to reprogramming these cells into induced pluripotent stem cells (iPSC) to be used in the future as a human cellular model to address the role of H_2S in neuronal differentiation in DS. To accomplish this objective, fibroblasts collected from Down Syndrome patients and healthy donors were obtained and H_2S production was assessed by time-lapse imaging using a fluorescent probe selective for H_2S. The fibroblasts were afterward reprogrammed into iPS cells. The levels of intracellular H_2S were higher in the DS cell line when compared to the healthy donor cell line. iPS cells from the DS individuals and the healthy donor fibroblasts were reprogrammed with success and both cell lines expressed the main pluripotency markers Sox2, Nanog, and Oct4 observed by immunocytochemistry and flow cytometry analysis. The data obtained in this work and the iPS lines developed will allow in the future the establishment of an important cellular model to study how H_2S affects neurodevelopment in DS.

RESUMO

Síndrome de Down (SD) é causado pela trissomia do cromossoma 21 e é uma das aneuploidias mais prevalentes que são compatíveis com a vida. Os sintomas característicos de SD são defeitos cardíacos congénitos, defeitos craniofaciais, anomalias gastrointestinais, leucemia, convulsões, início precoce de Alzheimer, diminuição do défice cognitivo entre outros. Distúrbios no processo de sinalização neurológica durante as fases críticas da neurogénese podem afetar a proliferação, migração e diferenciação das células estaminais, podendo ser responsável pelo défice cognitivo. Estas características são de grande importância para entender como se desenvolve o cérebro afetado por SD. O gene que codifica para a cistationina-beta-sintase (CBS) está presente no cromossoma 21 com uma cópia extra presente nos indivíduos com SD. CBS é uma das enzimas responsáveis pela produção celular de sulfureto de hidrogénio (H_2S). É uma pequena molécula gasosa sinalizadora e ubíqua que desempenha um papel importante em muitos processos fisiológicos. Contudo, a contribuição do H_2S para o anormal desenvolvimento neuronal em indivíduos com SD ainda não foi abordada e está neste momento sob investigação no laboratório da professora Inês Araújo. Neste estudo, o nosso objectivo era avaliar previamente a produção de H_2S em fibroblastos com SD antes de os reprogramar em células estaminais pluripotentes induzidas, para serem usados no futuro como um modelo celular humano de maneira a abordar o papel do H_2S na diferenciação neuronal no SD. Para concretizar esse objectivo, fibroblastos colhidos de pacientes com SD e de dadores saudáveis foram obtidos e a produção de H_2S foi avaliada por imagiologia de lapso temporal usando uma sonda selectiva para H_2S. Os fibroblastos foram posteriormente reprogramados em células iPS. Os níveis intracelulares de H_2S foram mais elevados na linha celular com SD quando comparada com a linha celular de dador saudável. As células iPS de paciente com SD e de dador saudável foram reprogramadas com sucesso e ambas as linhas expressam os principais marcadores de pluripotência Sox2, Nanog e Oct4 que foram observados através de imunocitoquímica e citometria de fluxo. Os dados obtidos neste trabalho e as linhas iPS desenvolvidas irão permitir no futuro o estabelecimento de um importante modelo celular para o estudo de como o H_2S afeta o neurodesenvolvimento no SD.

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LIST OF ABBREVIATIONS

AAP – American Academy Pediatrics

AD – Alzheimer's Disease

ADA – Adenosine deaminase

AFSC – Amniotic fluid stem cells

AFU – Arbitrary fluorescence units

ASD – Atrial septal defect

AOAA – Aminooxyacetic acid

APP – Amyloid beta precursor protein

ATP – Adenosine triphosphate

ATP12A - ATPase H^+/K^+ Transporting Non-Gastric Alpha2 Subunit

ATP5F1E – ATP Synthase F1 Subunit Epsilon

ATP5PF – ATP synthase peripheral stalk subunit F6

ATP5PO – ATP Synthase Peripheral Stalk Subunit OSCP

ATP5MC1 – ATP Synthase Membrane Subunit C Locus 1

ATP6V1H – ATPase H^+ Transporting V1 Subunit H

ATP6V1B1 – ATPase H^+ Transporting V1 Subunit B1

AVSDs – Atrioventricular septal defects

BACE – β-site APP enzyme

bFGF – basic fibroblast growth factor

BMD – Bone mass density

BSA – Bovine serum albumin

CAT – Cysteine aminotransferase

CBS – Cystathionine beta synthase

CELSR3 – Cadherin EGF LAG seven pass G-type receptor 3

CH – Congenital hypothyroidism

CHD – Congenital heart defect

CPT – Cell proliferation tube

CSE – Cystathione gamma-lyase

CO – Carbon monoxide

CoA – Coarctation of the aorta

COA1 - Cytochrome C Oxidase Assembly Factor 1

COX7C – Cytochrome C Oxidase Subunit 7C

COX15 - Cytochrome C Oxidase Assembly Homolog COX15

CP – Capping protein

DCSR – Down syndrome chromosomal (or critical) region

DNA – Deoxyribonucleic acid

DPBS – Dulbecco´s Phosphate-Buffered Saline

DMSO – Dimethyl Sulfoxide

DS – Down syndrome

DSM 5 – Diagnostical and Statistical Manual of Mental Disorders, 5th edition

DTT – Dithiothreitol

DYRK1 – Dual specificity tyrosine phosphorylation regulate kinase 1

DMEM – Dulbecco´s modified eagle medium

DNMT3L – DNA methyltransferase like 3

DPBS – Dulbecco´s phosphate buffer saline

ER – Endoplasmatic Reticulum

ERK 1/2 – Extracellular signal-regulated kinase 1/2

ESC – Embryonic stem cells

EUROCAT - European Concerted Action on Congenital Anomalies and Twins

FBS – Fetal bovine serum

GAPDH – Glyceraldehyde-3-phosphate dehydrogenase

GFAP – Glial fibrillary acidic protein

GD – Gestational day

GrD – Grave Disease

GSH – Glutathione

GW – Gestational week

HAFF – Human adult foreskin fibroblast

HD – Hashimoto Disease

HIF – Hypoxia induced factor

hiPSCs – human induced pluripotent stem cells

HSA21 – human chromosome 21

H_2S – Hydrogen sulfide

ICAM1 – intercellular adhesion molecule-1

ID – Intellectual disability

KEAP – Kelch-like ECH-associated protein 1

LFA1 – Lymphocyte function-associated antigen-1

LTP – Long term potentiation

MAPK – Mitogen-Activated Protein Kinase 1

MRI – Magnetic resonance imaging

NaHS – Sodium hydrosulfide

NDUFA10 – NADH:Ubiquinone Oxidoreductase Subunit A10

NDUFS1 – NADH:Ubiquinone Oxidoreductase Core Subunit S1

NDUFS2 – NADH:Ubiquinone Oxidoreductase Core Subunit S2~

NDUFV3 – NADH:Ubiquinone Oxidoreductase Subunit V3

NEAA – Non-essential amino acids

NF – Nuclear factor

NFAT – Nuclear factor of activated T cell

NMDA – N-methyl-D-aspartate

NRF2 – NF-E2 p45 related factor 2

NO – Nitric oxide

OLIG 1/2 – Oligodendrocyte transcription factor 1/2

OSKM – Oct4, Sox2, Klf4 and c-Myc

PA – Pulmonary artery

PBS – Phosphate buffered saline

PBMCs – Peripheral blood mononuclear cells

PCDHG – Protocadherin gamma cluster

PDA – Patent ductus

PFA – Paraformaldehyde

PTCH - Patched

PLP – Pyridoxidal-5´-phosphate

RCAN1 – Regulator of calcineurin 1

REST – Restrictive silencer factor

RNA – Ribonucleic acid

RNS – Reactive nitrogen species

ROS – Reactive oxigen species

RSS – Reactive sulfur species

SAH – S-adenosylhomocysteine

SAHH – S-adenosylhomocysteine hidrolase

SAM – S-adenosyl-methionine

SCH – Subclinical hypothyroidism

SeV – Sendai virus

SF7-AM – Sulfidefluor-7-acetoxymethyl ester

SHH – Sonic Hedgehog

SMO – Smothened

SOD – Superoxide dismutase

SSEA-1 – Stage specific embryonic antigen-1

STR – Short tandem repeats

ToF – Tetralogy of Fallot

TRA-1-60 - T cell receptor alpha locus

T3 – Triiodothyronine

T4 – Thyroxine

TSH – Thyroid stimulating hormone

USA – United States of America

USF 1 – Upstream stimulatory factor 1

USP 16 – Ubiquitin Specific Peptidase 16

VSD – Ventricular septal defect

WHO – World Health Organization

XIST – X inactive specific transcript

3 MST – 3 mercaptopyruvate sulfurtransferase

1 INTRODUCTION

The clinical aspects of Down Syndrome (DS) were first described by the English physician John Langdon Down in 1866 (Down, 1995). He described DS as a distinct form of mental disability. Due to his perception that children with Down syndrome shared facial similarities with those of Blumenbach's Mongolian race, John Langdon Down used the term "mongoloid". In 1961, a group of scientists suggested that "mongolism" had "misleading connotations" and had become "an embarrassing term". The World Health Organization (WHO) dropped the term in 1965 after a request by the delegation from the Mongolian People's Republic while the term (also mongolism, Mongolian imbecility, or idiocy) continued to be used until the early 1980s, it is now considered unacceptable and is no longer in common use. The DS phenotype involves manifestations that affect multiple body systems, although it may vary, the musculoskeletal, neurological, and cardiovascular systems are normally affected. Individuals with DS commonly have short stature, muscle hypotonia, small brachycephalic head, epicanthal folds, flat nasal bridge, upward-slanting palpebral fissures, Brushfield spots, small mouth and ears, single transverse palmar crease, short fifth finger with clinodactyly, wide spacing between the first and second toes, often with a deep plantar groove, atlantoaxial instability, reduced neuronal density, cerebellar hypoplasia, intellectual disability, and congenital heart defects (CHDs) in particularly atrioventricular septal defects (AVSDs), representing ~45-50% of all newborns with DS (Antonarakis et al., 2004, 2020; Roizen & Patterson, 2003). Individuals with DS are also more likely to develop certain health conditions, including hypothyroidism, autoimmune diseases, obstructive sleep apnea, epilepsy, hearing and vision problems, hematological disorders (including leukemia), recurrent infections, anxiety disorders and early-onset Alzheimer's disease (AD) (Figure 1) (Antonarakis et al., 2020). Other conditions, such as most solid tumor types, show inverse comorbidity and seem to be less common in individuals with DS than in the general population, unfortunately, the mechanisms are still poorly understood. The discovery of a link between an extra chromosome 21 and the DS phenotype was first reported in 1959 by a French pediatrician and geneticist Professor LeJeune and was an important landmark for the development of genetic medicine (Lejeune et al., 1959). The complete nucleotide sequence of the long arm of HSA21 was published in 2000 by a multinational consortium of investigators (Hattori et al., 2000) which was a landmark paper on the sequencing of the long arm of HSA21 and substantial progress has been made in understanding the molecular pathophysiology of the different phenotypic manifestations of DS, which is currently considered a disorder of gene expression dysregulation (Patterson, 2009; Roizen & Patterson, 2003). In addition, widely used screening methods have been introduced for the prenatal detection of DS. The management of the different symptoms and the quality of life of individuals with DS have improved. However, enormous challenges remain, including understanding the precise biological mechanism of each phenotypic component of the syndrome; treatment of the

different symptoms, including cognitive dysfunction; and integration of individuals with DS into society in different parts of the world.

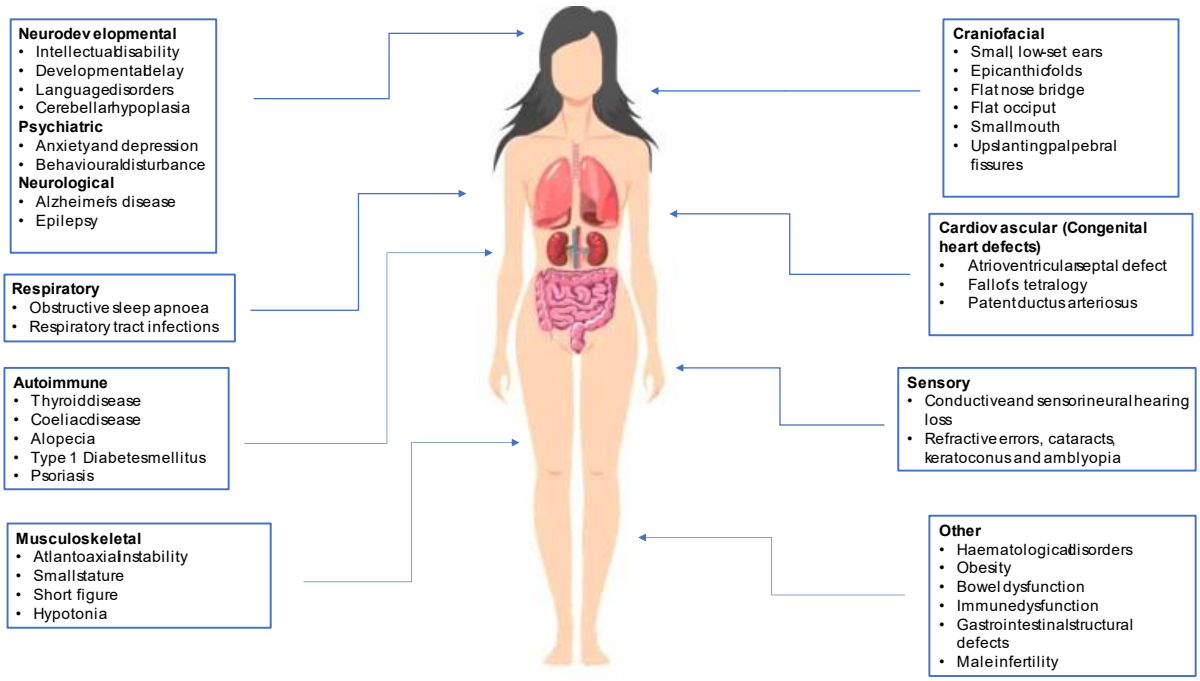

Figure 1 - Symptoms and manifestations of Down Syndrome

1.1 Epidemiology

DS occurs in all populations, but differences in maternal age at conception between countries, ethnicities, and sociocultural factors influence the number of live births. DS incidence, without major variations, around the world is 1 in every 700-800 gestation and it is estimated that 6-8 million people worldwide have DS (Neiva, 2021). The lifetime prevalence of DS is increasing substantially as the global population grows. In the USA, the prevalence of DS in the population increased from ~50,000 in 1950 (3.3 per 10,000 individuals) to ~212,000 in 2013 (6.7 per 10,000 individuals) (de Graaf et al., 2022) mostly due to improvements in childhood survival of individuals with DS. Starting in the 1950´s life expectancy increased considerably, for example in the US in 1991 the life expectancy was 56 years and in Australia, in the year 2000 it was 60 years. According to EUROCAT, in Portugal, the life birth prevalence was 3.06 per 10.000 individuals in 2019. According to unpublished data, about 95% of fetuses with DS are detected *in uterus* and about 95% of mothers opt to terminate the pregnancy which has been allowed since 1995 in these cases (Neiva, 2021). Although the probability of having a baby with DS increases with the maternal age, about 80% of children with DS have mothers less than 35 years old, this fact can be explained by the high fertility rate in younger women. The number of pregnancies that are electively terminated is influenced by the availability and accuracy of screening tests within each country, also the number of people choosing prenatal screening and subsequently prenatal

testing, as well as parental decisions once a prenatal diagnosis of DS is made. Between 2010-2015 in Europe, it was estimated a 54% reduction in gestations due to prenatal screening. Studies from EUROCAT regions, although there are substantial regional differences, have shown that increasing maternal age has counterbalanced the uptake in prenatal screening, resulting in a stable or slightly decreasing prevalence of DS in live births from the 1990s until now. In southern Europe, DS prevalence in live births almost halved in the period 1980–2015 (Lanzoni et al., 2019). In terms of mortality, heart and lung diseases are the main causes of death. DS children over 3 years old have an increased frequency of respiratory tract infections and acute respiratory distress syndrome which accounts for 54% of hospital admissions in Down Syndrome (Watts & Vyas, 2013). The other main cause is CHD, especially AVSD, surgical correction has greatly contributed survival rate in neonates, when uncorrected septal defects may lead to shunting of systemic blood to the pulmonary circuit, increased blood flow, and pulmonary artery hypertension (PAH), which may persist even after correction (Weijerman et al., 2010).

1.2 Risk Factors of DS

Advanced maternal age at conception is a major risk factor for trisomy 21, it is true for all human autosomal trisomies. This risk is associated with the non-disjunction of homologous chromosomes or chromatids occurring during the meiotic divisions that occur in the formation of oocytes (Hattori et al., 2000). Advanced maternal age has been associated with HSA21 segregation errors in both maternal meiosis I and meiosis II. In addition, specific altered recombination patterns have been observed for these types of maternal errors, only some of which are associated with maternal age (Hassold & Hunt, 2001). An interesting fact is that the frequency of DS is much higher at conception, approximately up to 75% and 50% of DS fetuses identified during the first and second trimester, respectively, are lost before term (Antonarakis et al., 2020). Thus, it is essential to acknowledge that in studying any DS-associated condition, only the combinations of genetic variants that allow the embryo to survive, to term and manifest the phenotype of interest are being considered. Several investigations addressing missegregation of HSA21 conducted in peripheral lymphocytes from young women (< 35 years) that gave birth to DS children revealed several markers of genome instability and premature aging, including an increased frequency of micronuclei, short telomeres, and global changes in DNA methylation patterns (Coppedè, 2016). Other risk factor associated with DS is the maternal occupancy, due to the increasing role of the woman in the labor market, pregnant women tend to work, and may be exposed to stress and environmental toxicity during pregnancy (Keen et al., 2020). Maternal obesity may be considered a risk factor as well when associated with an endocrinologic or cytogenic disorder (Hildebrand et al., 2014). The consanguineous may also have an impact as risk factor associated with DS, since both parents share portions of DNA there is a low renovation of the genetic pool which may lead to genetic errors (Ray et al., 2018).

1.3 Pathophysiology

Partial or complete trisomy 21 is the genomic cause of DS. Partial trisomy occurs from the duplication of a delimited segment of HSA21 and in complete trisomy 21, which is present in 95% of individuals with DS, results from a non-disjunction event in maternal meiosis I or II. This is perhaps not surprising, because the first division in females is initiated prenatally and is not completed until the time of ovulation and involves unique chromosome behaviors to segregate homologous chromosomes rather than sister chromatids (Hassold & Hunt, 2001). Several studies show that diminished recombination is a factor in all human trisomic conditions. Partial trisomy may also occur in paternal meiosis I or II or mitosis, after the formation of the zygote. Translocations affect ~5% of individuals, it results when one chromosome 21 attaches to another acrocentric chromosome, commonly 14 or 21 and less common chromosome 22. In t(14;21) one of every three cases involves a parental carrier, and in 90% of the corresponding cases, the carrier is the mother; in t(21;21) one of every fourteen cases involves a parental carrier being 50% of such cases the father (Bull, 2020). Mosaicism for trisomy 21 can occur in ~2% of DS patients (National Center on Birth Defects and Developmental Disabilities, 2019) and occurs when the extra copy of HSA21 is present in some cells of the individuals. Partial trisomy 21 is rare and is associated with a range of symptoms that vary according to the length of the partial triplication (Patterson, 2009) giving each DS individual a unique set of phenotypic traits, however, most patients have similar morphological features. Two different hypotheses have been proposed to explain the phenotype of DS: “developmental instability” (loss of chromosomal balance) and “gene-dosage effect.” According to the developmental instability hypothesis, the presence of a supernumerary chromosome globally disturbs the correct balance of gene expression in DS cells during development. Nevertheless, this hypothesis has been weakened by the fact that other autosomal trisomy syndromes do not lead to the same clinical pattern. In addition, correlations between genotype and phenotype in DS patients with partial trisomies indicate that a restricted region in 21q22.2 is associated with the main features of DS, including hypotonia, short stature, facial dysmorphies, and mental retardation. This DS chromosomal region (DSCR) supports the alternative gene dosage effect hypothesis, which postulates that the restricted number of genes from chromosome 21 that are overexpressed in patients with segmental trisomies contributes to the phenotypic abnormalities (Aït Yahya-Graison et al., 2007). Common findings among these studies emerged, first, the expression of various HSA21 genes is increased and it will be important to identify the mechanisms that underlie these different expression levels, which may include negative feedback, dosage compensation, and epigenetic alterations. Second, in many affected individuals, the expression of some non-HSA21 genes is also altered, which suggests that trisomy 21 leads to perturbation of downstream transcription and signaling networks. Although it has been shown that DSCR is necessary for the DS phenotype, its role in structural and functional features

of hippocampal impairment is not sufficient (Olson et al., 2007). Regarding neurogenesis, previous studies suggest that the number of neurons is reduced while that of astrocytes and oligodendrocytes remain unchanged or increased. This implies deregulation of the genetic mechanisms that control cell cycle progression and cell fate. Inside HSA21 the best candidates are DYRK1A (dual specificity tyrosine phosphorylation regulate kinase 1A); APP (amyloid beta precursor protein), RCAN1 (regulator of calcineurin 1), DNMT3L (DNA methyltransferase Like 3), OLIG 1/2 (Oligodendrocyte Transcription Factor 1 and Factor 2) and USP16 (Ubiquitin Specific Peptidase 16). Triplication of DYRK1A leads to overexpression of DYRK1A in the brain of DS fetuses, DYRK1A impairs the proliferation of neuronal precursor cells (NPCs) by reducing the activity of different cyclin-cyclin dependent kinases (CDKs). Fibroblasts derived from individuals with DS exhibit impaired proliferation due to elongation of the G1 phase of the cell cycle (J.-Y. Chen et al., 2013). Cyclin D1 is an important cell cycle protein that promotes G1-to-S phase transition and is required for cell proliferation. Cyclin D1 is one of the targets of DYRK1A, it phosphorilates cyclin D1 at Thr(286). Cyclin D1 phosphorylation causes its nuclear export, followed by degradation through a ubiquitin-dependent mechanism (Stagni et al., 2018). One of the mechanisms underlying the reduced proliferation potency of the DS brain may be the DYRK1A/cyclin D1-dependent precocious exit from the cell cycle which leads to a lengthening of the G1 cell cycle phase. This alteration promotes a reduction in the number of divisions producing neurons, leading to a deficit in cortical neurons that persists at postnatal life stages (Stagni et al., 2018). In addition to reducing nuclear levels of cyclin D1, overexpression of DYRK1A has been shown to increase the transcriptional levels of the antiproliferative cyclin-dependent kinase inhibitor $p27^{KIP}$ (Hämmerle et al., 2011) and promote its stability through phosphorylation. The parallel action of DYRK1A overexpression on cyclin D1 and $p27^{KIP1}$ may promote cell cycle exit into G0 and cause subsequent premature neuronal differentiation. It is well-established that DYRK1A phosphorylates p53 and that DYRK1A-induced p53 phosphorylation leads to the induction of p53 target genes and impairs G1/G0-S phase transition, resulting in attenuated proliferation. The major target of p53 is cyclin-dependent kinase inhibitor 1, $p21^{CIP}$, which promotes p53 transcription (Stagni et al., 2018). Increased levels of DYRK1A, p53, and $p21^{CIP1}$ have been observed in the frontal cortex of fetuses and adults with DS (J. Park et al., 2010). The neuron restrictive silencer factor (REST) is a key regulator of pluripotency and neuronal differentiation, and the transcriptional levels of REST are reduced in neural stem cells and progenitor cells from the cortex of fetuses with DS (Bahn et al., 2002). Target genes of REST play an important part in brain development, neuronal plasticity, and synapse formation. Embryonic stem cells that contain an extra copy of chromosome 21 have reduced levels of REST and that reduction is specifically due to DYRK1A overexpression. The reduced REST levels are accompanied by reduced levels of the key regulators of pluripotency, resulting in aberrant premature expression of differentiation-driving transcription factors. DYRK1A overexpression in DS may cause a premature differentiation which in turn causes a depletion of the pool of neuronal

progenitors by reducing the expression levels of REST (Canzonetta et al., 2008). Concerning the role of APP, several studies show high levels of APP in DS fetal brain (Stagni et al., 2018). These findings indicate that APP overexpression regulates the expression of numerous genes on HSA21, nine upregulated and eleven downregulated, all of them involved in some manner with cell cycle, cell proliferation, and/or cell differentiation, e.g., p53 (Wu et al., 2016). A major part of APP is predominantly cleaved by α-secretase, generating an N-terminal secreted soluble APPα and a C-terminal 83-amino acid fragment (C83). C83 is further cleaved by γ-secretase that releases a 3 kDa fragment (P3) and the APP intracellular domain (AICD). The minority of APP is cleaved by β-site APP cleaving enzyme 1 (BACE1), it then generates a C-terminal fragment of 99 amino acids or 89 amino acids (C99 or C89), which will be further cleaved to generate Aβ or truncated Aβ and AICD by γ-secretase (Wu et al., 2016). AICD migrates to the nucleus and modifies the transcription of various genes, including PTCH1 (PATCHED 1). PTCH1 is a receptor of SHH (Sonic Hedgehog) that binds to a second receptor, SMO (SMOOTHENED), thereby inhibiting the activity of the SHH pathway (Guidi et al., 2017). Increased levels of AICD due to APP triplication may enhance PTCH1 transcription, which leads to excessive inhibition of the SHH pathway, suggesting that early inhibition of the SHH pathway may be a key factor supporting the neurogenesis reduction that characterizes DS. RCAN1 also named Down syndrome cortical region 1 (DSCR1) is overexpressed in the embryonic brain of DS models (Antonarakis et al., 2020). Is a member of the family of calcineurin binding proteins and is highly expressed in neuroproliferative areas during brain development and in various brain regions postnatally (Harris et al., 2005). The protein encoded by this gene interacts with Calcineurin A and inhibits Calcineurin-dependent signaling pathways. Calcineurin is a calcium and calmodulin-dependent serine/threonine protein phosphatase. Calcineurin activates the transcription factor nuclear factor of activated T cell (NFAT) being then translocated into the nucleus, where it upregulates the expression of Interleukin 2 (IL-2), which, in turn, stimulates the growth and differentiation of T cell response (Serrano-Pérez et al., 2015). Although NFAT activation was originally described as a key event in the immune response, NFATs have been shown to be involved in several processes such as stimulation of axonal growth in neurons, maturation of immune system cells, heart valve formation, and differentiation of skeletal muscle and bone (Graef et al., 2001). Inhibition of NFAT activation reduces the percentage of cells in the G0/1 phase of the cell cycle and causes cell cycle elongation, suggesting an important role of NFAT signaling in the regulation of NPC proliferation (Stagni et al., 2018). DNMT3L is overexpressed in the DS frontal cortex (Antonarakis et al., 2020). It is an epigenetic regulator, which resulted in global CpG and CpG island hypermethylation as well as thousands of differentially methylated regions. The work of Lu and colleagues on DNMT3L, which is a gene overexpressed in DS neural progenitors, showed that DNMT3L activates DNMT3A/B, two proteins that alter neurogenesis and synaptic plasticity through methylation, mainly through the p53 pathway which in turn is associated with APP (J. Lu et al., 2016). Another candidate gene is OLIG 1/2, both implicated in oligodendrogenesis and

neurogenesis, OLIG2 overexpression inhibits neural progenitor proliferation contributing to the reduced neuronal number and brain size in DS (Chakrabarti et al., 2010). USP16 is a deubiquitination enzyme involved in chromatin remodeling and cell cycle progression, it reduces proliferation impairing self-renewal of hematopoietic stem cells and neural progenitors (Adorno et al., 2013). As mentioned above, apart from genes present in HSA21 there are many genes throughout the genome differentially expressed in DS, especially in the brain. Many of them are hypermethylated which means that their transcription is repressed (Stagni et al., 2018). Concerning genes not present in HSA21 there are two main candidates; protocadherin gamma cluster (PCDHG) located on Chr5 (5q31) which is crucial for neural circuit formation in the developing brain and cadherin EGF LAG seven pass G-type receptor 3 (CELSR3) located on Chr3 (3p.21.31) which is a plasma membrane protein involved in the regulation of contact-dependent neurite growth. Both genes are hypermethylated in DS and are top REST target sites. Both belong to the cadherin superfamily which is involved in cell-cell adhesion (El Hajj et al., 2016).

1.4 Neurodevelopmental Impairment in DS

As previously mentioned, the main focus of this work is the study of one of the most affected organs by increased expression of HSA21, the brain. While some symptoms may not be present in all individuals with DS, brain hypotrophy and intellectual disability (ID) are two hallmarks. ID is a neurodevelopmental condition that appears early in childhood and impairs the development of personal, social, academic, and/or occupational functioning. They typically involve difficulties with the acquisition, retention, or application of specific skills or sets of information. Neurodevelopmental disorders may involve dysfunction in one or more of the following: attention, memory, perception, language, problem-solving, or social interaction (Stephen Brian Sulkes, 2022). According to DSM 5 (Diagnostical and Statistical Manual of Mental Disorders, fifth edition), ID is approximately two standard deviations below the population, which equals an IQ score of about 70 or below. ID scores range from moderate (IQ of 50–70) to severe (IQ of 20–35). Although it is still not currently possible to identify the exact causes of the ID in DS, the scientific record points to structural changes that generate functional alterations in the brains of people with DS. Post-mortem and magnetic resonance imaging (MRI) studies consistently show that the overall size of the brain is decreased compared to age-matched controls. In particular, the cerebral cortical hemispheres, cerebellum, and hippocampal formations are significantly reduced in size (Klein & Haydar, 2022). A reduction in the number of neurons forming the brain and in brain size are typical phenotypic features of DS starting from prenatal life stages. Thus, it is very likely that the reduced number of neurons in the DS brain is a key determinant of the ID that characterizes this pathology. The reduction in neuron number in DS is not due to neuronal degeneration but to impairment in the process of neurogenesis during fetal life, the critical period during which almost

all neurons that form the brain are generated. In previous studies, various evidence available from the last 2/3 of the second trimester, shows that neurogenesis reduction in DS is due to two main causes: (i) cell cycle alterations leading to reduced proliferation of neural stem cells (NPCs), a reduction in the cell cycle (longer cell cycle) or premature exit from the cell cycle (fewer rounds of division) (Contestabile et al., 2007; Sharma et al., 2022) and (ii) augmented differentiation of the daughter cells into (mainly) astrocytical cells through asymmetrical division (Stagni et al., 2018). Neurogenesis begins in the embryonic period (that goes from the day of conception to GW8) and continues to mid-gestation (from GW13 to GW27). The NPCs appear during gastrulation, a process that takes place between GD14 and GD21. The first brain structure is the neural tube, a hollow cavity that begins to form at GD20 through GD27 and will subsequently give origin to the different parts of the nervous system. The inner surface of the neural tube is lined with NPCs. This region is called the ventricular zone (VZ) because the cavity of the neural tube will give origin to the cerebral ventricles. The VZ is gradually replaced by the subventricular zone (SVZ). From the end of gastrulation through approximately GD42 in humans, the NPCs of the VZ constitute a homogeneous pseudo-stratified epithelium. These cells have radial processes and divide “symmetrically” producing two identical NPCs. Various rounds of symmetrical cell division augment the size of the NPC pool and cause a surface expansion of the cerebral cortex. The NPCs generate subsequently radial glial cells (RGCs) which share some molecular characteristics with earlier NPCs (Bystron et al., 2008). RGCs undergo “asymmetrical” divisions thereby producing one progenitor and one neuron. The former remains in the proliferative niche, whereas the latter migrates to its final location in the developing brain. In the case of cortical neurogenesis, the first neurons that abandon the proliferative zone form a structure called the preplate (PP), a largely transient structure that comprises various cell types, most of which are destined to die (Bystron et al., 2008). Once the PP is complete, the next wave of migrating neurons splits the PP into two regions, the marginal zone (MZ) and the subplate (SP), beginning to form a new region interposed between the MZ and the SP, the cortical plate, which will become the cortex. The MZ and the SP are two transient laminar compartments populated by diverse cell types that have a major role in the development of the cortex but that are largely eliminated by the end of the fetal period (that goes from GW9 to birth) (Stagni & Bartesaghi, 2022). The MZ contains an important class of cells, the Cajal–Retzius cells, that control the positioning of neurons into the correct layers of the cortex and will become layer I of the mature cortex. The SP contains multipolar neurons that play a functional role in setting up connections between the cortex and thalamus during development. The SP in humans reaches its maximum thickness roughly two-thirds of the way through gestation (Bystron et al., 2008). Its size then gradually decreases, leaving only a thin layer with scattered cells in the white matter in the late fetal period. At the beginning of cortical development, neurons migrate through a process called somal translocation. During later stages, this process is no longer possible, due to brain growth, and so neurons migrate to the cortex along the shafts of RGCs. Cohorts of postmitotic neurons follow radial glial

scaffolding to form arragements of minicolumns. The larger the number of columns, the larger the cortical surface. Neurons that arrive first settle in the prospective layer VI, while later migrating neurons settle to successively more superficial layers. This pattern of migration is called inside-out and causes an expansion in cortical thickness (Stagni & Bartesaghi, 2022). Formation of NPCs starts at GD28 and gradually shifts at GD42 to neuron producing asymmetrical divisions (Bystron et al., 2008). Cortical neurogenesis starts at GW9 and is completed at GW24-25 (Kostović & Judaš, 2010; Mrzljak et al., 1992). Cerebellar and hippocampus neurogenesis starts at GW12 and continues beyond birth (ten Donkelaar et al., 2003). Cerebellar granule cell production stops at the fifth postnatal month. (Ábrahám et al., 2001). Hippocampal neurogenesis is largely completed by the first year but continues very slowly throughout life (Seress et al., 2001). NPCs give rise to neuronal-restricted progenitors early in development, and glial-restricted progenitors only later. Around GW16–GW18, radial glia slowly begin to form astrocytes and oligodendrocytes, a process by which the same progenitor domain switches the developmental program from neuron production to astrocyte or oligodendrocyte production. The neurogenic to gliogenic cell fate transition of radial glia is called the “gliogenic switch” (Rowitch & Kriegstein, 2010). Gliogenesis extends beyond birth. Neurons emit dendritic processes starting from GW13, but the dendritic spurts take place much later at GW26, associated with the appearance of dendritic spines. Dendritic maturation happens shortly before birth at GW34 and is largely completed after the second postnatal year (D. Lu et al., 2013) (Figure 2). In infants with DS, astrocytes exhibit a deficit of interlaminar processes, suggesting impairment in their maturation (Colombo et al., 2005), DS astrocytes contribute to the reduced neurogenesis of DS NPCs and induce cell death of DS neurons. DS astrocytes also fail to promote maturation and synapse formation of DS neurons (C. Chen et al., 2014). Moreover, because the process of neuron maturation in early infancy is also impaired it causes defective synaptic plasticity. Brain hypotrophy in fetuses with DS might be due to a reduction in the number of cells forming the brain and/or a reduction in the extension of their dendritic (and axonal) processes. Considering that brain hypotrophy has been detected well before the spurt of dendritic growth, it seems very likely that it is due (or mainly due) to a lack of cellular elements. Indeed, a reduction in cellularity has been documented in several brain regions of DS fetuses in the period GW17–GW21 (earlier evidence is missing) (Stagni & Bartesaghi, 2022). Studies in fetuses with DS show that at GW17–GW21, in various areas of the brain the majority of cells (~75–95%) are neurons (NeuN+ cells), which is fully consistent with the delayed timing of gliogenesis in comparison with neurogenesis (Stagni et al., 2018).

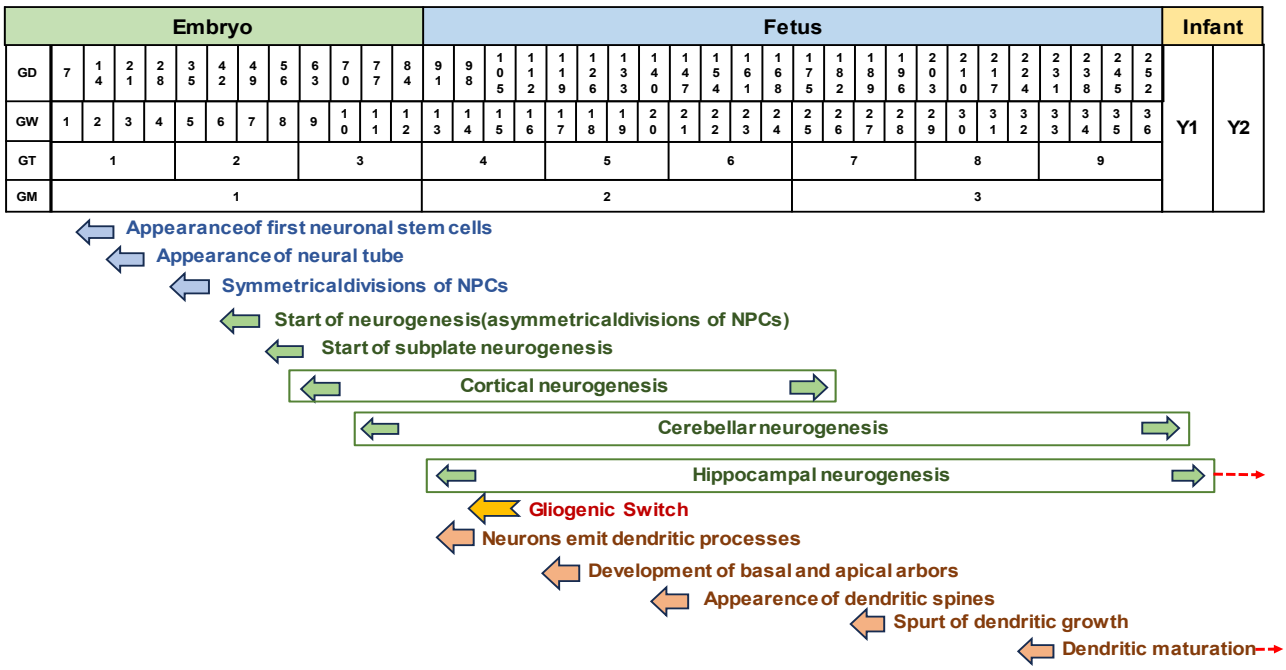

Figure 2- Timeline of neurogenesis, gliogenesis and neuron maturation in the human brain

1.5 Hydrogen Sulfide (H_2S)

H_2S is part of the reactive sulfur species (RSS) which includes thiols, S-nitrosothiols, sulfenic acids, and sulfite, and it plays a vital role in regulating intracellular redox status (Paul & Snyder, 2018). Is a colorless, pungent gas with a smell of rotten eggs. H_2S does not form hydrogen bonds and is lipophilic, allowing it to pass through biological membranes and act as a paracrine signaling molecule (Powell et al., 2018). Although well known as a toxic gas is now recognized as a significant mammalian biological regulator in health and disease. Is a ubiquitous small signaling molecule, playing an important role in many physiological processes including vasodilation, angiogenesis, oxygen sensing, apoptosis, inflammation, and neuromodulation, and it can also protect against ischemia/reperfusion injury (Kashfi, 2018). There are three main routes by which H_2S exerts its biological effects: metal center interactions (binding to heme iron groups, for example, cytochrome oxidase c) (Pietri et al., 2010), reactive oxygen species (ROS)/reactive nitrogen species (RNS) scavenging (for example, induction of antioxidative signaling) (Spassov et al., 2017), and S-persulfidation (for example, open of K^+ATP channels or influence of NF-κB translocation) (L. Li et al., 2011). Although the other mechanisms are important, S-persulfidation is accepted as the key process by which H_2S acts in a signaling capacity. S-Persulfidation is the process in which a thiol (R–SH) is converted into a perthiol (R–SSH, also called a persulfide). S-persulfidation modulates the biological activity of proteins due to the decrease in pKa and increase

in nucleophilicity of perthiols concerning thiols (Lippert et al., 2011; Powell et al., 2018). The three main routes of action allow H_2S to have numerous properties. H_2S has been demonstrated to act as a significant regulator of gene expression, several groups have demonstrated that exposure of biological organisms to exogenous H_2S or modulation of endogenous H_2S production results in significant and broad changes in the transcriptome. Also, exogenous H_2S can induce reprogramming of gene expression, which in turn can affect the most fundamental functions of the cells, from signal transduction and metabolism to DNA repair and cell cycle regulation (Sobol et al., 2019). Generally, the way these mechanisms work is by cellular mRNA levels which are determined by RNA polymerases and their stability (a complex process, which can involve elements in both the coding and the untranslated regions of an mRNA transcript). Moreover, there are several processes, known as epigenetics, that can change gene expression, for example, by affecting DNA methylation or changes in histones that, in turn, globally regulate the access of RNA polymerases to the DNA (Cirino et al., 2022). Whether H_2S can affect these fundamental processes is largely unknown at present. Given the ability of H_2S or polysulfides to react with various regulatory cysteines, which are common in the proteins that regulate DNA- and RNA-related processes, a potential role of H_2S in regulating the various enzymes involved in these processes is possible but remains to be elucidated (Cirino et al., 2022). H_2S is readily soluble in lipid membranes, in addition to passive diffusion, H_2S is also known to utilize various transport systems for transmembrane transport. The resting membrane potential is principally maintained by the Na^+-K^+-ATPase and in specialized conditions, cellular Na^+ transport can be affected by H_2S, for example, exogenous H_2S donation (via NaHS) inhibited Na^+ transport and decreased basolateral Na^+-K^+-ATPase current (Althaus et al., 2012; Tang et al., 2010). Studies show that these effects of H_2S are more pronounced under hypoxic conditions, the pharmacological inhibition of CBS and CSE indicates that in hypoxic epithelial cells endogenously produced H_2S may, in fact, contribute to the inhibition of Na^+-K^+-ATPase currents and the hypoxic inhibition of Na^+ absorption by pulmonary epithelial cells (Krause et al., 2016). Concerning the potential role of H_2S in cell division and cell proliferation, in most cell types, low concentrations of exogenous H_2S donors stimulate proliferation, whereas higher concentrations inhibit it, cell proliferation is intimately connected to cellular bioenergetics, DNA integrity, DNA division, DNA repair, and cellular redox status and all these processes are also known to be regulated by H_2S in a bell-shaped fashion. The underlying mechanisms are related to an acceleration of cell cycle progression (e.g., decreases the G0–G1 population and increases the S-phase cell population) at lower concentrations of H_2S, in line with its stimulatory effect on cell division and cell proliferation (Ma et al., 2015). At higher concentrations, however, exogenously administered H_2S can induce cell cycle arrest, in either G0/G1 or S phase. These effects are linked to the regulation of various intracellular effector pathways such as the downregulation of cyclin D1, upregulation of the CDK inhibitor $p21^{Cip1}$, upregulation of TP53, and downregulation of c-Myc (Cai et al., 2010; Deplancke & Gaskins, 2003). H_2S has recently been also found to regulate ATP kinase

(Shackelford et al., 2021); this enzyme is an important regulator of the cell cycle. Regarding potential changes in endogenous H_2S levels during the cell cycle, 20 years ago Jan Kraus's group made the seminal observation that "CBS is coordinately regulated with proliferation" (Maclean et al., 2002). In both yeast models and a variety of human cell lines (hepatoma, neuroepithelioma, and normal fibroblast), proliferation is associated with high CBS expression/activity, but when the cells reach confluence CBS is downregulated. The level of regulation occurs at the CBS-1b promoter, most likely via the stimulation of the transcription factor Sp1, the quiescent, contact-inhibited, and serum-deprived cells all have a comparable ~80% suppression in CBS expression compared with proliferating, nonconfluent cells. Although the above study did not investigate cause-effect relationships (i.e., CBS upregulation could be a cause or a consequence of increased cell proliferation), it was interesting to notice that basic FGF (bFGF) was capable of reproducing the stimulating effect of serum on proliferation, as well as on CBS induction. Part of the stimulation of CBS activity during proliferation was attributed to high levels of SAM, its allosteric activator (Cirino et al., 2022). In general, the physiological role of H_2S in the regulation of cell differentiation is the stimulation/maintenance of this process. This function is in line with the pleiotropic role of H_2S in the regulation of gene expression. In fact, exogenously applied H_2S was shown to stimulate various forms of cell differentiation, for instance, differentiation of different neuronal cell types (Fukami & Kawabata, 2015; Tarui et al., 2010) that will be discussed in the next section. During endocytosis, a substance or particle is captured from outside the cell by engulfment with the cell membrane, followed by its internalization. During exocytosis, intracellularly formed vesicles fuse with the plasma membrane and release their contents to the outside of the cell. Since both processes require ATP and rearrangements of the actin cytoskeleton, it would be expected that H_2S, would have an effect via the modulation of bioenergetics and cytoskeletal processes (Cirino et al., 2022) which could explain the cause of hypotonia and low energy levels in DS patients. The role of the H_2S system in the regulation of ER clusters is around three main areas:1) protein transcription/translation;2) the regulation of ER's calcium handling function, and 3) the regulation of ER stress. There are different studies around this subject, i.e., in cultured microglial cells H_2S donors induce an intracellular Ca^{2+} mobilization response, which is, at least in part, due to Ca^{2+} mobilization from the ER (Lee et al., 2006), the participation of H_2S in Ca^{2+} homeostasis regulation is crucial for neuronal synaptic plasticity. H_2S joins nitric oxide (NO) and carbon monoxide (CO) in the group of signaling agents termed gasotransmitters whose production and metabolism are primarily enzymatically regulated. The term gasotransmitter is applied to small molecules of gas and meets specific criteria: (i) can freely permeate cell membranes; (ii) regulate their endogenous production and have specific signaling functions for cellular and molecular targets (Wang R. 2002). Only two of the twenty amino acids normally present in proteins contain sulfur, namely methionine and cysteine. Methionine is an essential amino acid because it cannot be synthesized by the human body and cysteine is termed as semi essential amino acid due to humans synthesize it from methionine to

a limited extent (Hu et al., 2010). In case of dietary excess intake, cysteine can be stored endogenously as glutathione (GSH), a tripeptide containing cysteine, glycine, and glutamate. Once the GSH pool is replenished it can be converted to taurine or oxidized to sulfate (Figure 3). The availability of cysteine appears to be the rate-limiting factor for GSH biosynthesis from glutamate, glycine, and cysteine. H_2S also modifies cysteines in target proteins, forming persulfide bonds, by a process designated persulfihydration (Mustafa, Gadalla, Sen, et al., 2009). The "sulfane sulfur" pool which is a divalent sulfur atom bonded to another sulfur that is very reactive and labile, performs an essential function in the brain, upon neuron excitation the bound sulfane sulfur releases H_2S. H_2S formation from sulfane sulfur likely requires reduced GSH as both hydrogen and electron donors. In the brain, H_2S is produced mainly in astrocytes, which contain larger amounts of GSH than in the neurons. H_2S is mainly catalyzed by three enzymes: cystathionine beta-synthase (CBS), cystathionine gamma-lyase (CSE), and 3-mercaptopyruvate sulfurtransferase (3-MST) (Predmore et al., 2012) that will be discussed forward in this work, especially CBS. H_2S regulates inflammation and cell death, possibly exerting its beneficial effects through action on ATP-sensitive K+ channels (Tang et al., 2010) inhibition of activation of NF-κB and p38 MAPK, scavenging of oxidants, upregulation of intracellular cAMP, and inhibition of caspase-3 cleavage (Wallace, 2009). The induction of adhesive interactions between circulating leukocytes and the vascular endothelium is the initial step in the process of extravasation of leukocytes and their migration to sites of injury or infection. An important role of H_2S as a tonic regulator of this process was suggested by the observation that inhibition of H_2S synthesis in rats resulted in a rapid increase in leukocyte adherence in mesenteric venules (Zanardo et al., 2006). When H_2S synthesis is inhibited, an increase in the expression of adhesion molecules on the endothelium (P-selectin, ICAM-1) and leukocytes (LFA-1) can be detected (Fiorucci et al., 2005). Concerning H_2S role in the brain, it has a neuromodulator effect, disulfide bonds play an important role in modulating the functions of many proteins including N-methyl-D-aspartate (NMDA) receptors responses, it appears that H_2S interacts with disulfide bonds and free thiols in NMDA receptors to facilitate the induction of long-term potentiation (LTP) in the hippocampus increasing synaptic plasticity, although at higher concentrations it inhibits synaptic transmission(Abe & Kimura, 1996; H. Kimura, 2000). H_2S might also serve as a neuroprotectant, Glutamate neurotoxicity in brain cultures involves, at least in part, inhibition of cystine uptake (Y. Kimura & Kimura, 2004). The cystine/glutamate antiporter couples the influx of cystine, an H_2S precursor, with efflux of glutamate. H_2S can inhibit monoamine oxidase, leading to norepinephrine elevations in the hippocampus, striatum, and brainstem but not in the cortex and cerebellum (Warenycia et al., 1989). H_2S appears to signal predominantly by persulfhydrating cysteines in its target proteins, analogous to S-nitrosylation by NO (Mustafa, Gadalla, & Snyder, 2009). For instance, GAPDH, whose catalytic activity is abolished by nitrosylation at C150, is sulfhydrated at the same position, with persulfhydration resulting in a 700% enhancement of catalytic activity (Mustafa, Gadalla, & Snyder, 2009). Actin is also persulfhydrated physiologically. H_2S donors augment actin

polymerization by more than 30%, an effect reversed by dithiothreitol (DTT), which abolishes persulfhydration. Persulfhydration also leads to rearrangement of the actin cytoskeleton (Mustafa, Gadalla, & Snyder, 2009). These actions could regulate the influence of actin on synaptic dynamics. H_2S is emerging as an important mediator of human physiology and pathology but remains difficult to study. It became a major challenge to monitor such a small molecule in part due to the lack of methods that measure changes in H_2S endogenous fluxes, especially in living biological systems. The current techniques for such detection often require post-mortem processing and/or destruction of tissues or cell lysates. The development of fluorescent probes with appropriate sensitivity for H_2S in living cells is a considerable breakthrough that exploits the H_2S-mediated reduction of azides to fluorescent amines (Lin et al., 2013). They are a new reaction-based fluorescent type of probes that respond with a turn-on fluorescence signal enhancement and display high selectivity for H_2S over other biologically relevant reactive sulfur, oxygen, and nitrogen species. Azides and other oxidized nitrogen species can be reduced to amines by H_2S faster than by GSH and other thiols, signifying a promising method for H_2S detection. Upon reduction by H_2S, the electron-withdrawing azido group will be converted into an electron-donating amino group. Therefore, exploiting the electron-donating ability of different substituent groups will result in versatile fluorescent probes. The fluorescent probes employing the photoinduced electron transfer (PET) mechanism are typically constructed by connecting an electron donor/acceptor recognition group to a fluorophore through a space bridge. The design principles of such probes are clear, and the resulting processes will suppress or increase their fluorescence (Lippert et al., 2011). The fluorescent probes that adopt an internal charge transfer mechanism typically contain a strong push–pull system, wherein the electron-donating and the electron-withdrawing are conjugated to the fluorophore (Yu et al., 2014). Here we report Sulfidefluor-7 acetoxymethyl ester (SF7-AM), which offers the unique capability to image H_2S generated at physiological signaling levels allowing for direct, real-time visualization possessing visible excitation and emission profiles that are compatible with commonly available microscopy filter sets (Lippert et al., 2011). The main strategy used for H_2S detection in living biological specimens relies on monitoring this small molecule through chemoselective reactions that are bioorthogonal, which means that can occur inside a living system without interfering with its own native biochemical processes. SF7-AM displays cytosolic and nucleus localization and is able to retain its brightness for 60 minutes after replacing the cellular media. This enhanced trappability provides a notable increase in the sensitivity of this reporter for cellular imaging by maintaining a greater concentration of dye within the cell (Lin et al., 2013).

1.6 CBS and Down Syndrome

Another important gene encoded by HSA 21 is cystathionine β-synthase (CBS). Human CBS is a cytosolic homotetramer that is composed of four identical polypeptide monomers of

63kDa, each monomer consists of three domains the heme-binding domain (N-terminal amino acids), the catalytic core domain (the central amino acids), and the regulatory domain (C-terminal amino acids). It binds two cofactors, pyridoxal-5´-phosphate (PLP) (is the active form of vitamin B6 and is involved in the formation of neurotransmitters, among other functions) and its N-terminal containing heme-binding site, one of the features that distinguishes CBS from the other PLP-dependent enzymes. The function of the heme-binding site remains vague because it´s not directly involved in catalysis but still influences folding and is sensitive to the redox status of its environment (Zuhra et al., 2020). Each CBS subunit of 551 amino acid residues binds two substrates (homocysteine and serine) and is further regulated by S-adenosylmethionine (SAM also called as AdoMet) (Meier et al., 2001). A recent study suggested that the first 40 residues of the human CBS N-terminal constitute an intrinsically disordered region, which transiently binds heme via a second binding site, the CP (capping protein)-based motif with Cys15 and His22 as axial ligands. While the function of this additional heme-binding site is not fully understood, the CBS Cys15Ser mutant is unable to bind heme at this second binding site and is ~30% less active compared to the WT variant (Kumar et al., 2018). The full-length CBS has a C-terminal regulatory domain with a tandem of CBS domains, CBS1 and CBS2, which are associated in dimeric assembly form a called Bateman module (Ereño-Orbea et al., 2013). Each CBS domain comprises a three-stranded β-sheet and two α-helices, and together they play an autoinhibitory role by blocking the active site. SAM acts as an allosteric activator of CBS by binding into the cleft within the Bateman module followed by domain rearrangement and release of intrasteric block from the catalytic site. In addition to the regulatory role, the C-terminal domain is also involved in the formation of the CBS homotetramer. This conclusion is supported by the observation that the truncated CBS, which lacks the entire C-terminal regulatory domain (about 140 residues), forms dimers. CBS dimers are also formed when just 10 residues from the CBS2 domain of the regulatory domain are removed, which facilitates the successful crystallization of a full-length human CBS (Ereño-Orbea et al., 2013). The CBS gene is located on human chromosome 21 in the subtelomeric region q.22.3 (Münke et al., 1988) and its entire sequencing is of 23 exons (Kraus et al., 1998), with 15 of them coding for the CBS polypeptide. The two promoters −1a and −1b are found to be mainly used. They are rich in GC and their regions contain numerous putative binding sites for transcription factors but there is no TATA box, as well as an estrogen receptor binding site. Some of those possible bindings have been confirmed to regulate CBS basal transcription, such as specific protein Sp1 and Sp3, upstream stimulatory factor 1 (USF-1), and nuclear factor (NF) −Y on −1b promoter (Ge et al., 2001). Evidence has been presented that Sp1/Sp3 transactivates the -1b promoter, mainly Sp3 can repress transcription driven by Sp1 (Bouwman & Philipsen, 2002). The transcription factor NF-E2 p45-related factor-2 (Nrf2) was also shown to induce the CBS gene, when stabilized by H_2S which inhibits Nrf2 repressor, Kelch-like ECH-associated protein-1 (Keap1) (Hourihan et al., 2012). The common pathogenic c.833T>C(p.Ile278Thr) mutation observed in CBS deficiency is often found in combination with

68-bp insertion (844_845ins68), which is an exact duplication of the intron-exon boundary of exon 8 (Sperandeo et al., 1996). Interestingly, this variant can be skipped by alternative splicing, leading to the formation of normal mRNA and enzyme activity, but the output of its transcription is considerably reduced, indicating that proper regulation of CBS may depend on this region (Zuhra et al., 2020). Various factors can upregulate CBS, e.g., the active form of vitamin D ($1,25(OH)_2D3$) strongly induces gene transcription (Kriebitzsch et al., 2011); CBS is upregulated during pregnancy in uterine artery endothelium and smooth muscle cells compared to the menstrual cycle (through estrogen receptor agonists which increase CBS mRNA and protein levels) (Sheibani et al., 2017); also testosterone has been proposed to be a CBS regulator (Vitvitsky et al., 2007). CBS mRNA and protein are primarily found in the liver, kidney, pancreas, and brain. In the brain, all regions express CBS in various amounts but the hippocampus, cerebellum, and cerebral cortex seem to have the higher expression. Importantly, CBS is expressed in neuronal stem cells where it appears to regulate their proliferation and differentiation (Wang et al., 2013). Embryonic changes in CBS gene expression during differentiation were detected by *in situ hybridization* (Quéré et al., 1999). Different CBS enzymatic activity was observed in different parts of the brain with the highest activity in the cerebellum cortex which may suggest that this high CBS enzymatic activity in the cerebellum cortex is at least partially due to substantial CBS enzyme synthesis resulting from a high level of CBS gene transcription (Quéré et al., 1999). Early expression of the CBS gene in neural development suggests that endogenous H_2S may play critical roles in neural lineage restriction, Wang and coworkers demonstrated that exposure to L-cysteine induced the activation of ERK1/2 (Wang et al., 2013). It has been suggested that H_2S is a novel inducer of neuronal differentiation in NG108-15 cells, characterized by neuritogenesis (Tarui et al., 2010). The endogenous production of H_2S results from direct enzymatic depersulfhydration of cysteine which is catalyzed by CSE and CBS and indirect desulfhydration catalyzed by 3-MST in the presence of reductants. As mentioned above, CBS is present mostly in the central nervous system and the liver, while CSE is primarily responsible for H_2S production in the cardiovascular system. 3-MST is located predominantly in the mitochondria and produces H_2S in concert with cysteine aminotransferase (CAT) (Powell et al., 2018). Along with the generation of H_2S, CBS is also responsible for the metabolism of homocysteine. Homocystinuria is characterized by the accumulation of homocysteine in the urine. It causes defects in the methionine metabolism and insufficient production of vitamin B12. CBS is the first and rate-limiting enzyme in the transsulfuration pathway, it plays a central role in sulfur metabolism and redox regulation in cells. There are two transsulfuration pathways, the forward transsulfuration pathway involves the transfer of sulfur from cysteine to homocysteine and is mainly present in prokaryotes, fungi, and plants. The reverse transsulfuration pathway occurs in mammals and performs in the opposite direction, transferring sulfur from homocysteine to cysteine via cystathionine (Figure 3). CBS catalyzes the first step of the pathway by condensing serine with homocysteine to generate cystathionine although it can also act on two molecules of cysteine

(Sbodio et al., 2019). CBS is a cell- or tissue-specific constitutively expressed enzyme; its expression and activity are primarily regulated by post-transcriptional modifications under normal physiological conditions. However, modifications in CBS mRNA (and consequently CBS protein) expression are observed in certain physiological changes and pathophysiological states. Therefore, due to its diverse distribution and potential regulators such as H_2S, CBS regulation is extremely complex. While H_2S has many distinct molecular targets and can regulate many different proteins and second messenger systems, one important biological role of CBS produced H_2S is that can regulate the mitochondria (Panagaki et al., 2019). At lower concentrations, H_2S exerts mostly regulatory, beneficial, or protective effects by stimulating mitochondrial function. These effects involve several different mechanisms, including direct electron donation at the level of mitochondrial Complex II, inhibition of mitochondrial protein kinase A, stimulation of mitochondrial ATP synthase, formation of an antioxidant 'cloud' within the mitochondria, and stimulation of mitochondrial DNA repair via the facilitation of the assembly of mitochondrial DNA repair protein complexes, in short, H_2S stimulates oxidative phosphorylation and increases ATP production but at higher concentrations can cause deleterious and cytotoxic effects mainly through the inhibition of cytochrome c oxidase (COX) and consequent inhibition of mitochondrial complex IV (Panagaki et al., 2019; Pun et al., 2010). Panagaki and her group investigated the role of the H_2S pathway on the alterations in the brain metabolic and electrophysiological activity and cognitive function in a novel rat model of DS and demonstrated that CBS has an increased expression in DS brain, especially cortex and hippocampus, also DS is associated with the upregulation of CBS and 3-MST enzymes in a brain region-specific manner and CBS expression in the DS brain is localized in astrocytes and vascular tissues surrounded by astrocytic end-feet (Panagaki et al., 2022). The over-expression of CBS may cause developmental abnormality in cognition in DS children and that may lead to AD in DS adults (Ichinohe et al., 2005). The hypothesis that CBS-derived overproduction of H_2S may be responsible for some of the pathophysiological aspects of DS was first put forward by P. Kamoun almost two decades ago, based on his clinical findings demonstrating markedly increased circulating levels of sulfhemoglobin (a product of the reaction of hemoglobin with H_2S) and increased (approximately two-fold over healthy controls) urinary excretion of thiosulfate (a stable degradation product of H_2S) in individuals with DS. According to the 'Kamoun Hypothesis', excess H_2S—at least, in part, via inhibition of mitochondrial Complex IV— may be responsible for the metabolic and neuropathological features of DS (Szabo, 2020). This suppression of aerobic ATP generation is, at least in part, compensated by an upregulation of aerobic glycolysis, which "makes up" for part of ATP generation, but also produces excess lactate (Pecze et al., 2020). Such compensation reveals cellular ATP content is significantly lower in DS individuals, in addition, there was a tendency for an increase in inorganic phosphate (Pi) levels (Pecze et al., 2020). As a result of the "gene dosage effect", CBS is overexpressed in DS, and this has been long suspected to be a 'master switch' in the pathophysiology of DS (Szabo, 2020). As mentioned above, it has been

shown that an increased CBS activity contributes to mitochondrial sulfur metabolism via the transsulfuration pathway (Figure 3). Hence, an understanding of the mechanism that leads to hypertranssulfuration activity in DS children and its harmful effects is important to finding a strategy to alleviate the clinical DS phenotype. The S-adenosyl homocysteine hydrolase (SAHH) hydrolyzes S-adenosylhomocysteine (SAH) to homocysteine and adenosine. This homocysteine is converted to cystathionine, cysteine, and H_2S via CBS/CSE/3MST pathways. Briefly, in DS patients, the adenosine with the help of adenosine deaminase (ADA) is converted to inosine which is then converted to uric acid. Clinically, this tends to be high in the DS patients (Pogribna et al., 2001). The children born with DS exhibit elevated levels of CBS, CSE, 3MST, SOD, cystathionine, cysteine, and H_2S. Therefore, it appears that an increase in the activity of epigenetic gene writers together with a concomitant decrease in gene erasers cause folic acid exhaustion which eventually leads to an increase in the transsulfuration by the combined action of CBS, CSE, 3MST, and SOD pathway (Pushpakumar et al., 2023). The folic acid pathway plays a critical role in regulating several intracellular homeostatic mechanisms that also include the lowering of homocysteine concentrations through the regeneration of methionine in enzymatic reactions that involve vitamin B12 (Zinellu & Mangoni, 2023). Among the many interactions that occur between CBS and other proteins, with DYRK1A there is an interesting relation. Previous studies had reported the DYRK1A decreased expression observed in the liver of CBS-deficient mice (Hamelet et al., 2009); increased DYRK1A in the brain of CBS-deficient mice (Planque et al., 2013), and more recently the role of CBS and DYRK1A overdosage in mice models which report that the overexpression of DYRK1A restored memory recognition (Marechal et al., 2019), suggesting that CBS and DYRK1A only interact in specific regions of the adult brain.

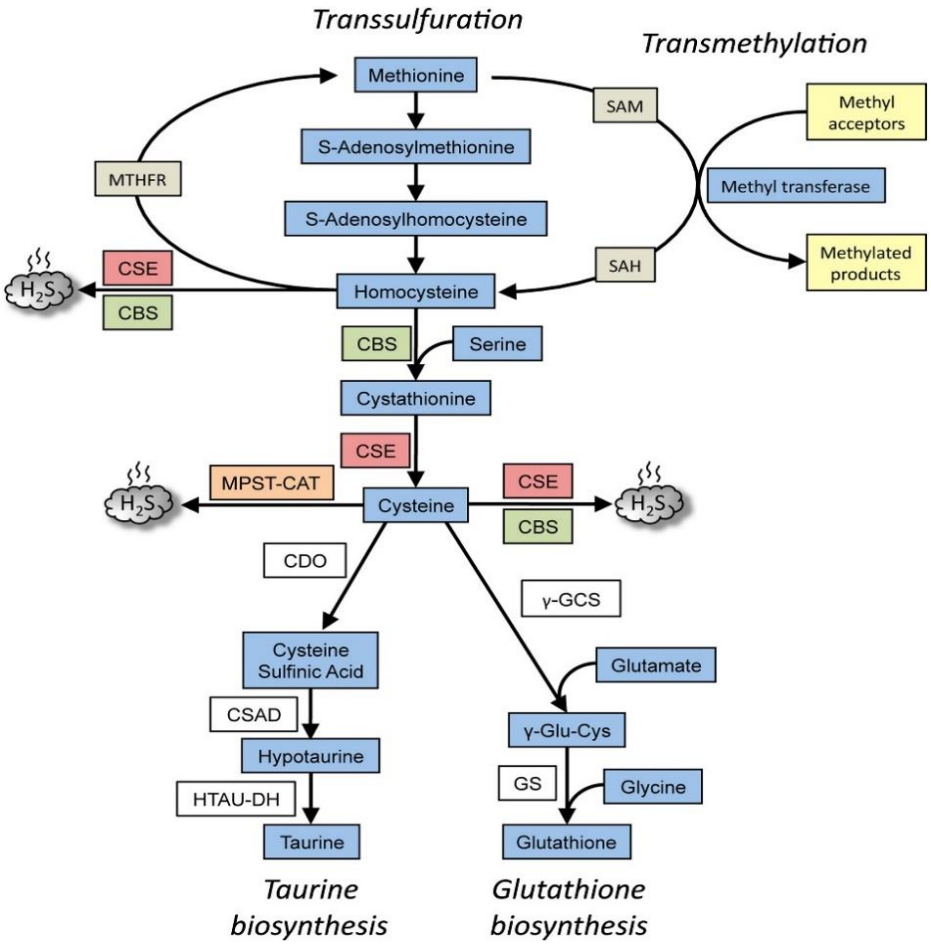

Figure 3 - Transsulfuration pathway

1.7 Available Therapies for DS

Currently, there is no cure for DS, however, as a result of longer life expectancy, these patients can live a healthy and relatively independent life. The main goal of the treatments is to reduce morbidity and maximize basic functions in life. With that in mind, several areas require ongoing assessment throughout childhood and should be reviewed at every health supervision visit and at least annually. These areas include personal support available to family; participation in a family-centered medical home; age-specific Down syndrome-related medical and developmental conditions; financial and medical support programs and long-term financial planning for which the child and family may be eligible; injury and abuse prevention, with special consideration of developmental skills and intellectual ability; and nutrition and activity to maintain appropriate weight (Bull et al., 2022). The improvement in developmental status and behavior is highly dependent upon the associated comorbidities, socioeconomic status, home environment, and the education level of the parents. Early stimulation therapy and behavioral intervention remain the foundation of management for children with DS and improve the long-term outcome.

The aim is to involve parents in effectively teaching the relevant skills in different areas of development to individualize the training program, identify and manage the specific deficit in the behavior, maximize self-help skills, and enhance independence. It promotes the overall development of children during the early years. These programs include speech therapy, physiotherapy, and occupational therapy. As previously reported, it is very common for DS children to have CHD, different forms of CHD can appear such as AVSD, atrial septal defect (ASD), patent ductus arteriosus (PDA), Tetralogy of Fallot (ToF), ventricular septal defect (VSD) and coarctation of the aorta (CoA). The first step to address these anomalies is surgical, for example, the optimal timing and type of repair of ASD, VSD, and PDA depends on the size of the defect and associated comorbidities. Some infants with post tricuspid shunts may require pulmonary artery (PA) banding before complete repair because of mitigating factors, such as prematurity. As with AVSD, repair of ToF is usually performed early (within 4 to 6 months of birth), even though children with a well-balanced circulation can be repaired at a later stage. A surgical systemic-to-PA shunt or transcatheter stent placement may be required before complete repair to augment pulmonary blood flow in infants with excessive systemic desaturation and to enhance PA development. Individuals with single ventricle physiology require multiple surgeries over their lifetime (Dimopoulos et al., 2023). People with DS have a higher lifetime risk of pulmonary hypertension (PH), In DS with CHD, especially large post-tricuspid (eg, VSD or PDA) or combined pre tricuspid and post tricuspid shunts (eg, AVSD), PAH commonly develops within the first year of life (Mourato et al., 2014). The mechanism responsible for the earlier onset in people with DS and CHD remains unclear and may be related to the genetic syndrome itself but also to common comorbidities. Respiratory issues are also very common, due to characteristics of the phenotype that include a flattened nasal bridge, large protruding tongue, small mouth, shallow hypopharyngeal dimensions, and airway malacia. These factors, together with the presence of reduced muscle tone, including pharyngeal muscles, and physiological enlargement of tonsillar and adenoid tissue increase the likelihood of proximal airway obstruction (Pandit & Fitzgerald, 2012). One example may include obstructive sleep apnoea, approximately 50% of children with Down syndrome will develop obstructive sleep apnoea, in a study demonstrated in 33 children with DS by Fitzgerald *et al*, 97% of children with DS who snore had obstructive sleep apnoea (Fitzgerald et al., 2007), a polysomnography (sleep study) may be advised in some cases. There exists a higher frequency of airway anomalies in children with DS when compared with children without DS but with the same recurrent symptoms. Antibiotic therapy or inhaled bronchodilators may be needed to treat some respiratory problems related to DS. Endocrine manifestations are also prevalent in patients with DS, thyroid dysfunctions are frequent in these patients, subclinical hypothyroidism (SCH) is the prevailing condition, congenital hypothyroidism (CH), and thyroid autoimmunities such as Hashimoto's Disease (HD) or Grave's Disease (GrD) are also seen. The American Academy of Pediatrics (AAP) recommends thyroid screening to be performed at birth, at 6 months, and then annually beginning at 1 year old (Whooten et al., 2018). Approximately half

of children with DS may have an elevated TSH level with normal T_3 and T_4 levels, which suggests subclinical hypothyroidism. A delay in maturation of the hypothalamic–pituitary–thyroid axis has been hypothesized as the probable cause, as TSH responses to TSH-releasing hormone tests were more exaggerated in patients with DS than in controls (Hawli et al., 2009). In cases of CH and SCH patients with elevated TSH, levothyroxine may be considered if elevation is mild (Whooten et al., 2018). In terms of development, bone growth is a complex process impaired by obesity, low physical activity, low calcium, low vitamin D, decreased muscle mass, decreased sun exposure, malabsorption syndromes, and anti-epileptic medication use. Patients with DS have an increased prevalence of these factors, increasing their risk for poor bone mineral density (BMD). There is conflicting research regarding whether BMD is reduced in individuals with DS. The specific causes of decreased BMD in this population are an area of active research. A clinical trial performed by Reza S. *et al* showed a beneficial effect of physical training and calcium intake on the development of BMD in children with DS (Reza et al., 2013), these findings enhance the importance of physical activity and nutrition especially because it is very common to find hypothyroidism accompanied by obesity, low muscular strength, and type 1 diabetes in these patients. As previously seen, individuals with DS have a greater risk of developing Alzheimer's-like dementia earlier in life compared to individuals without DS. Rivastigmine, galantamine, memantine, and donepezil have been used to treat DS associated dementia, several studies showed low or no improvement of the symptoms and one of the studies revealed elevated frequencies of adverse effects (Hefti & Blanco, 2017). In more recent years, research around genetic therapies have been improving, one line of investigation is regarding XIST (X inactivation gene), focusing on the gene dosage theory and in terms of the possibility of controlling the gene imbalance some researchers are advancing in a genetic correction of the overdose of genes across a whole extra chromosome in trisomic cells. The main goal is to create a trisomy silencing system by inserting a single gene that can epigenetically silence the whole 21 chromosome. XIST is a ~17kb non-coding RNA produced by the inactive X. During early development, the XIST RNA induces numerous heterochromatin modifications and architectural changes which transcriptionally silence the inactive X and manifest as a condensed Barr Body (Jiang et al., 2013). XIST covers the inactive X, XIST RNA accumulation has two components, a minor one associated with transcription and processing, and a spliced major component, which stably associates with the inactive X. After transcriptional inhibition the major spliced component remains in the nucleus and often encircles the noticeable heterochromatic Barr body (Clemson et al., 1996). In the case of DS, XIST expression fully corrects trisomy 21 dosage in neural cells, and dosage correction by XIST promotes the differentiation of trisomic NSCs to neurons (Czermiński & Lawrence, 2020).

1.8 DS available rodent models

Animal models of DS, especially mouse models, have been instrumental in advancing DS research. However, one needs to be very cautious when studying these models because DS is a human condition that cannot be precisely replicated in other species. Nonetheless, insights into the mechanisms that underlie the developmental and functional consequences of trisomy 21 have been obtained by overexpression of groups of HSA21 genes or orthologues of HSA21 genes from other species (Antonarakis et al., 2020). As previously mentioned, the phenotypes observed in DS are likely to arise because of the dosage sensitivity of HSA21 genes and associated gene-environment interactions and/or a global effect of the extra chromosome on chromatin regulation and methylation. rodent models of DS, have been essential for the determination of genotype-phenotype relationships for this syndrome. Owing to the genetic tractability of this animal, the most useful DS models to date have been derived from genetic engineering, due to their high susceptibility, one can generate precisely defined large genomic segmental duplications to model chromosomal disorders. Mouse models have also provided platforms for testing interactions between cell and tissue types, responses in the organism, and candidate therapeutics for DS (Herault et al., 2017). HSA21 has three orthologous regions on mouse chromosomes (MMU) 10,16 and 17 in which gene order and orientation are conserved (Davisson et al., 2001; Herault et al., 2017). Gropp *et al* tried modeling by studying mice with full trisomy of mouse chromosome 16, although these animals had numerous defects, including, for example, cardiac septation deficits, they did not model DS because the majority of genes that were triplicated in this model were from regions of MMU16 without homology to HSA21, furthermore, these animals died at birth which mean it was not possible to give insight into processes beyond this stage. Around 1990, Davisson *et al* developed the Ts65Dn model, this was the first viable segmental trisomy mouse model. It has a translocation that results in an extra-small chromosome made up of a fusion of the App-Zbtb21 region orthologous to HSA21 found on MMU16 with the centromeric region of MMU17 thus, the mouse shows aneuploidy. Despite being one of the most used models in DS, it has limitations: (i) it is trisomic for 125 human protein coding orthologs but only 90 of these are HSA21 orthologs, (ii) it lacks trisomy for ~75 HSA21 orthologs (Gupta et al., 2016). The Ts65Dn model exhibits cognitive deficits, altered brain morphology, and impaired synaptic plasticity. It has been crucial in understanding the genetic basis of DS-related cognitive impairments. Another well-known model of partial trisomy 16, is the Ts1Cje (trisomic for the Sod1-Zbtb21 region) displays cognitive deficits and certain phenotypic features resembling aspects of DS, making them valuable for exploring specific genetic contributions to DS-associated traits, and the Ts2Cje (harbors a Robertsonian translocation between the extra chromosome in Ts65Dn and MMU12) which exhibit cognitive impairments, altered synaptic plasticity, and changes in brain morphology. This model allows the investigation of the effects of a more precisely defined chromosomal duplication on DS-related phenotypes. Both have made important contributions to our understanding of DS. Nevertheless, like Ts65Dn, these models were generated by chance

rather than design, and carry additional genetic modifications that could have an impact on phenotypes, the MMU16 telomeric location facilitated the creation of new models, testis of male mice were irradiated to fragment chromosomes, offspring of these mice were crossbred with unirradiated females and screened cytogenetically to identify those carrying chromosomal rearrangements that produced Ts16, which is a mouse model for trisomy of the telomere of MMU16 (Gupta et al., 2016; Herault et al., 2017). The Ts1Rhr was the first DS model created using the Cre/lox technique of chromosomal engineering and was designed to replicate trisomy of the DSCR (Olson et al., 2007) showing in fact that trisomy of this region was not sufficient to replicate all structural and functional abnormalities seen in the Ts65Dn, further discrediting the concept of the DSCR. Using the same method, various models were next developed, for example, Dp(16)1Yey which carries a duplication of the entire MMU16 syntenic segment (Z. Li et al., 2007), Ts1Yah which has a trisomy of partial and complete syntenic segments of MMU17 (Pereira et al., 2009), Dp(10)1Yey has the complete MMU10 segment and Ts3Yah with an additional subregion of MMU16 (Brault et al., 2015). All these models allow the introduction of defined chromosomal rearrangements, such as deletions, duplications, inversions, and translocations, into the mouse genome by engineering them in embryonic stem cells to help in the study of dosage effects of specific genes within the duplicated region and their contributions to DS phenotypes, focusing on neurodevelopmental aspects. A different kind of mouse model is Tc1, is a transchromosomic mouse that carries an almost complete human chromosome and performs features seen in humans with DS and in other DS mouse models, including changes in behavior, synaptic plasticity, cerebellar neuronal number, heart development, and mandible size (O'Doherty et al., 2005). In contrast with transgenic methods, this approach reflects more closely the 3:2 dosage difference present between trisomic and disomic individuals but unfortunately, subsequent DNA sequencing revealed numerous deletions and rearrangements within the chromosome, caused by methods used in construction. Furthermore, being a chromosome with a human centromere in a mouse background, the Tc1 mice are mosaics meaning that a variable number of cells in any tissue carry the HSA21q, making each mouse unique. In 2020, Kazuki *et al* developed a new Tc DS model, TcMAC21, which is not mosaic and contains 93% of HSA21q protein coding genes that are expressed and regulatable. TcMAC21 recapitulates many DS phenotypes including anomalies in the heart, craniofacial skeleton, and brain, molecular/cellular pathologies, and impairments in learning, memory, and synaptic plasticity. TcMAC21 is the most complete genetic mouse model of DS extant and has the potential to support a wide range of basic and preclinical research (Kazuki et al., 2020). Each of these mouse models has its advantages and characteristics, allowing investigation into different aspects of DS pathology, including cognitive impairments, altered brain development, synaptic dysfunction, and behavioral phenotypes. By utilizing these models, it is possible to gain insights into the molecular and cellular mechanisms underlying DS and explore potential therapeutic interventions.

1.9 Human Induced Pluripotent Stem Cells

iPSCs are a type of pluripotent stem cells that exhibit the ability of self-renewal and have the potential to reprogram somatic cells into different cell types. The main advantage over embryonic stem cells (ESCs) is the lack of need for embryo destruction since they are collected from the patient itself which means it can be used not only for research but also for therapeutic purposes. They induce an embryonic-like state of pluripotency with the help of specific transcription factors such as master sa et of regulators for cell identity that can change cell fate (Karami et al., 2023). Takahashi and Yamanaka (Takahashi & Yamanaka, 2006) hypothesized that genes specifically expressed in ESCs contribute to the capacity of differentiation and proliferation. They identified 24 candidate genes for the reprogramming of somatic cells to the pluripotent state. Ectopically expressing those 24 genes in the nucleus of a fibroblast cell reprogrammed the state of the cell to one that morphologically and functionally mimics ESCs. Further experiments verified that the ectopic expression of only four genes, OCT 3/4, SOX 2, Klf4, and c-Myc (OSKM) were necessary for the reprogramming. Over the years, several models have been proposed to explain the events that reprogram the cell to the pluripotent state. Cell reprogramming remains with relatively low efficiency, only a small minority of cells successfully complete the process. Initially, two models dominated as an explanation for the low efficiency. Elite model – only elite cells, such as progenitors and stem cells that exist in a somatic population can be reprogrammed into iPSCs. Somatic populations normally are heterogenous, and lineage tracing studies have demonstrated that iPSCs can be generated from terminally differentiated cells, such as T or B lymphocytes, pancreatic β cells, and others suggesting that the proportion of cells sensitive to the induction of pluripotency is much larger than the elite model. The other more accepted model – the reprogramming factors (OSKM) model can reprogram all transduced somatic cells but only a small number will be successfully reprogrammed. The reprogramming mechanism is separated into phases, the early stochastic phase is characterized by down regulation of lineage specific genes, initiation of mesenchymal to epithelial transition, proliferation, expression of stage specific embryonic antigen-1 (SSEA-1) for mice, and T cell receptor alpha locus (TRA-1-60) for humans and metabolism changes from oxidative phosphorylation to glycolysis. The late deterministic phase is characterized by bivalent methylation of H3K4me3 and H3K27me3 on CpG promotor regions, sequential activation of pluripotency genes, and cells with a flat and round morphology. Phenomena such as DNA damage or apoptosis can disrupt the reprogramming process (Karagiannis et al., 2018). For the establishment of human cell lines, accessible cell sources have been thought that didn´t involve invasive surgical procedures. Peripheral blood cells such as T cells, B cells, hematopoietic stem cells, or fibrocytes are a good source for reprogramming. An interesting topic for consideration regarding the selection of the original cell source is that iPSCs could maintain part of the original epigenetic information even after reprogramming. Even though this methylation signature can be erased through prolonged culture it can affect the differentiation capacity of the iPSCs (Karami et al., 2023; Kim et al., 2010).

Normally, the pluripotency of iPSCs is evaluated by pluripotency related genes, colony morphology, differentiation in the three germ layers *in vitro,* and teratoma formation. Different methods can be distinguished by reprogramming efficiency, technical difficulty, and genomic integration (Table 1 and Figure 4). Retrovirus and lentivirus vectors have superior transduction efficiency and cloning capacity because chromosomal integration allows stable genome expression but has higher mutagenesis probability due to the same reason, conversely, adenovirus vectors are non-integrative which means that the risk of mutagenesis is lower but also less efficient.

Table 1 - Different reprogramming methods

Method	Type	Efficiency	Delivery method
Retrovirus	Virus	+++	Integrative
Lentivirus	Virus	+++	Integrative
Sendai virus	Virus	+++	Nonintegrative
Adenovirus	Virus	+	Nonintegrative
Plasmid	DNA	+	Nonintegrative
Episomal plasmid	DNA	++	Nonintegrative
PiggyBac transposon	DNA	++	Nonintegrative
Minicircle DNA	DNA	+	Nonintegrative
Synthetic RNA	RNA	+++	Nonintegrative
Recombinant protein	Protein	+	Nonintegrative

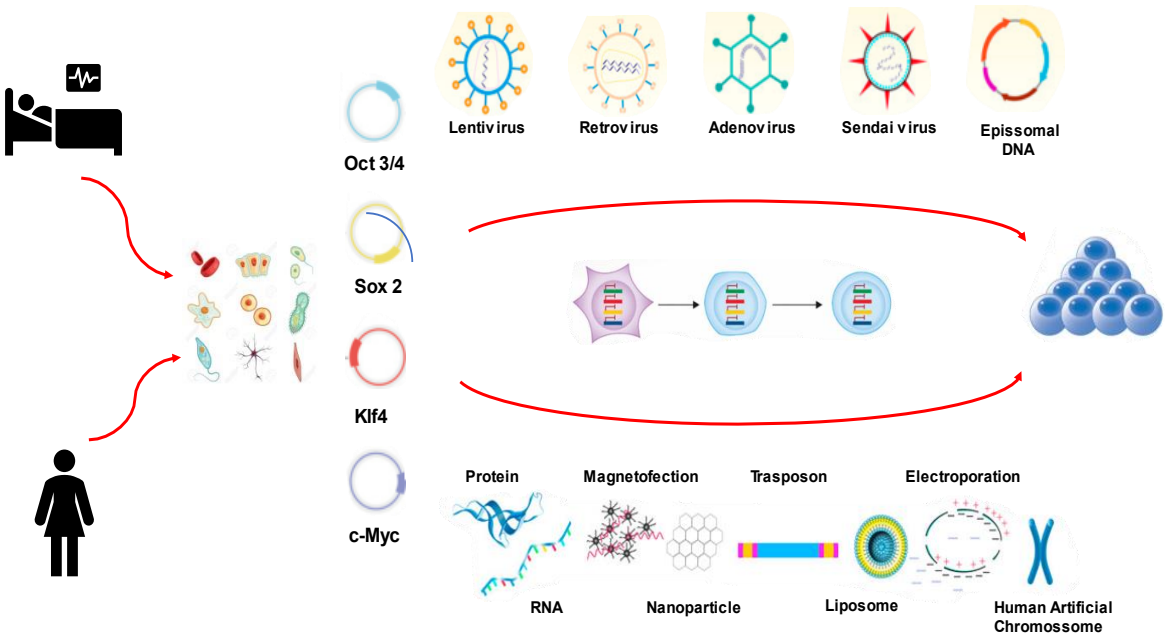

Figure 4 - Most commonly used reprogramming methods

Sendai virus (SeV) is a respiratory virus in mice and rats, it belongs to the *Paramoxyviridae* family. Is an enveloped virus of 150-250nm in diameter whose genome is a single strand of RNA. Due to the SeV infection mechanism through the sialic acid receptor present on the surface of many different cells, it can infect a wide range of cell types of various animal species. It conducts its replication outside the nucleus (Figure 5) so it is considered the safest viral reprogramming method (Nishimura et al., 2011). Regarding the non-viral methods, synthetic RNA is considered a safe and efficient method with a highly controllable procedure, it consists of the repeated administration of synthetic mRNAs incorporating modifications designed to bypass innate antiviral responses to reprogram human cells although is a very complex technique it has a low mutagenic risk (Warren et al., 2010) Overall, non-viral methods have lower mutagenic risk but also lower efficiency. Depending on the purpose of the iPSCs, the chosen method, research purposes, or clinical trials, should be based on the efficiency and safety of the procedure.

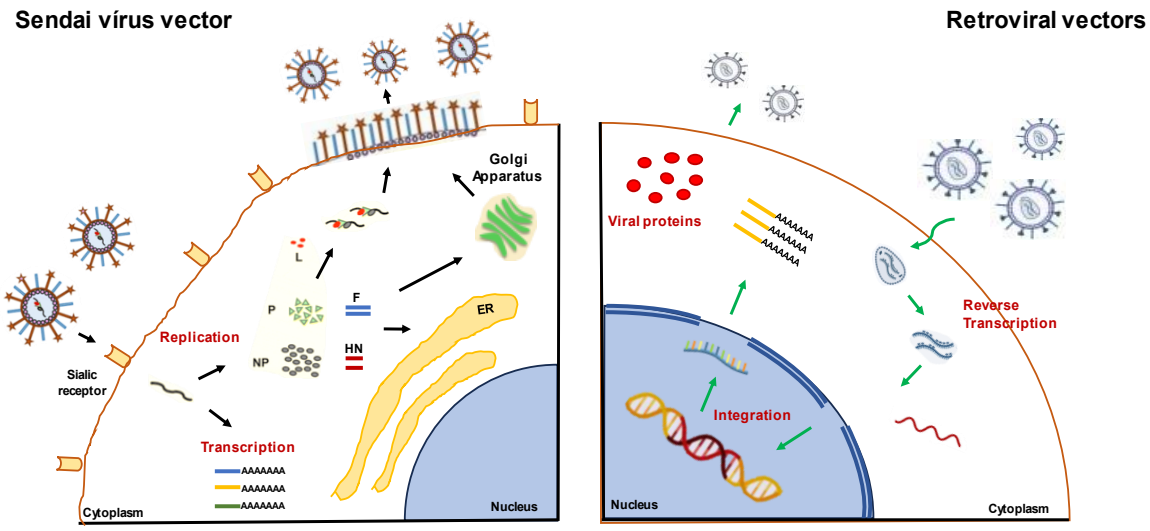

Figure 5 - Sendai virus transfection method compared to other viral vectors, adapted from CytoTune 2.0 Reprogramming Kit

The use of iPSCs revolutionized the way how to study different pathologies and DS is no exception, iPSCs derived from individuals with DS have been produced to study cellular and molecular foundations with a human genetic background in a way that has not been possible before due to the limitations of human tissue (Klein & Haydar, 2022). The first iPS cell line reprogrammed from an individual with DS was reported by Park and colleagues in 2008 (I.-H. Park et al., 2008) from then, multiple lines have been created from multiple different individuals (iPSCs studies recapitulated in Table 2). The current cell lines isogenic cell lines and age and sex-matched cell lines. The isogenic lines are created with genetically identical cell populations through DNA techniques, meaning that both populations express the same genes. They are a great tool because they eliminate any interference that may arise when comparing cells with different backgrounds. It is still not fully understood how different allelic combinations affect the severity of the various phenotypes in DS, hence eliminating that variability is essential to identify and understand underlying cellular and molecular neurodevelopmental changes. Age and sex-matched cell lines are also important since the cellular phenotypes that rise from the genetic variability elucidate on the particular phenotypes that are likely prevalent and impactful in DS. iPSCs derived from people with DS have been differentiated into many types of neuronal and glial cells and can summarize many of the phenotypes seen in studies with human tissue. Recent advances in 3D culture technology allowed the different types of stem cells to display their self-organizing properties into organoids. These *in vitro* cell cultures are valuable research tools that reflect the structural and functional properties of many organs, such as kidneys, lung, brain, and many others. The human brain is a complex organ that contains multiple cell and tissue types that interact between them through complex pathways. The differentiation of iPSCs into various types of neuronal and glial cells contributed significantly to the understanding of neurodegenerative

diseases pathogenesis but these systems are only able to model a single brain structure. The development of different organoid protocols allowed a better vision of the complexities of neural structure and function, thus providing a more realistic 3D environment for the cells (Clevers, 2016; Corrò et al., 2020).

Table 2 - DS iPS cell line studies

Research Group	Cell Source	Reprogramming Method	iPS cell line characteristics	Topic of investigation
(I.-H. Park et al., 2008)	Fibroblasts from young males	Lentivirus	Full trisomy 21	Hematopoiesis & Neurogenesis
(Mou et al., 2012)	Fibroblasts from young males	Lentivirus	Trisomy with translocation Full trisomy 21	DS cellular models and DS pathogenesis
(Chou et al., 2012)	Fibroblasts	Retrovirus	Full trisomy	Hematopoiesis & erythropoiesis
(Briggs et al., 2013)	Fibroblasts from male	Episomal DNA	Full trisomy	Neurogenesis, Gliogenic shift & Oxidative stress
(C. Chen et al., 2014)	Fibroblasts from two male and one female	Retrovirus	Full trisomy	Gene expression profiling, Oxidative stress & Astrogliogenesis
(H.-E. Lu et al., 2013a)	2nd Trimester amniotic fluid stem cells (AFSC)	Lentivirus	Translocation Full trisomy	Neurogenesis
(Pipino et al., 2014)	2nd AFSC	Lentivirus	Full trisomy Translocation	Hematopoiesis & Neurogenesis
iPSCs with isogenic controls				
(Maclean et al., 2002)	Fibroblasts from adult	iPSC T21 - Retrovirus iPSC euploid control - Culture-induced spontaneous loss of HSA21	Full trisomy	Gene expression profiling & Hematopoiesis
(Weick et al., 2013)	Fibroblasts from young male	Retrovirus & SeV	Full trisomy	Gene expression changes susceptibility to oxidative stress Neurogenesis Synaptic activity
(Hibaoui et al., 2014)	Fetal skin fibroblasts	Lentivirus	Euploid Full trisomy	Transcriptome analysis Neurogenesis
(Real et al., 2018)	Fibroblasts	SeV & iPSCs clone – induced spontaneous loss of HSA21	Full trisomy	Neurogenesis Synaptic function

(Murray et al., 2015)	Fibroblasts from young adult	SeV	Mosaicism	Neurodegeneration Mitochondrial Abnormalities & DNA double-strand breaks in neurons
Genetically modified iPSCs				
Research Group	Cell Source	Reprogramming method	Gene editing	Topics of Investigation
(L. B. Li et al., 2012)	Fibroblasts from adult	Lentivirus	TKNEO transgene insertion into HSA21 via Adenovirus. TKNEO induced spontaneous loss of HSA21	
(Jiang et al., 2013)	Fibroblasts from adult	Lentivirus	Insertion of XIST via ZFN to silence the additional copy of HSA2	Neural proliferation & neural rosette formation
(Ovchinnikov et al., 2018)	Fibroblasts from adult	Episomal DNA	CRISPR/Cas9 to inactivate a a single copy of APP	Transcriptome analysis

1.10 Objectives

This study aims to evaluate the intracellular production of H_2S in fibroblasts collected from Down Syndrome patients and healthy donors. Additionally, fibroblasts collected from these sources will be reprogrammed into iPCs by using Sendai viruses, in order to have a cellular model to study the contribution of H_2S to the neurodevelopment impairment of DS patients. To accomplish this objective, H_2S will be measured using a H_2S sensitive fluorescent probe and its levels will be modulated in both cell lines by using specific H_2S donors and enzymatic CBS inhibitors.

2 MATERIALS AND METHODS

2.1 Reprogramming Fibroblasts

Healthy young donor (Euploid) (GM01652) and Down Syndrome patient (AG06922) fibroblasts purchased from Coriell (coriell.org) were reprogrammed using CytoTune®-iPS 2.0 Sendai Reprogramming Kit – Feeder Dependent Method (Invitrogen) at a MOI 5–5-3 (hKOS, hc-Myc, hKlF4, respectively). After 24h of incubation, the medium was replaced with fresh fibroblast medium Dulbecco´s modified Eagle medium (DMEM) with high glucose; 10% fetal bovine serum (FBS); 1% non-essential amino acids (NEAA) and 1% penicillin/streptomycin/amphotericin B and 50μM β-mercaptoethanol (BioConcept). For the next 6 days, the spent medium was changed with fresh fibroblast medium. At day 7, cells were transferred onto a 60mm plate coated with Geltrex LDEV-Free Reduced Growth Factor Basement Membrane Matrix (Gibco) and inactivated HAFF. On day 8, the transition to mTeSR medium (StemCellTM Technologies) was made. When iPSC clones achieved the right confluency, they were cultured and expanded onto 6-well plates coated with Geltrex LDEV-Free Reduced Growth Factor Basement Membrane Matrix and inactivated HAFF, using a slip ratio of 1:5 and were maintained at 37 ◦C in a humidified atmosphere containing 5% CO_2. After some passages, the transition of iPSC clones was made to plates only coated with Geltrex LDEV-Free Reduced Growth Factor Basement Membrane Matrix (Gibco) (Figure 6).

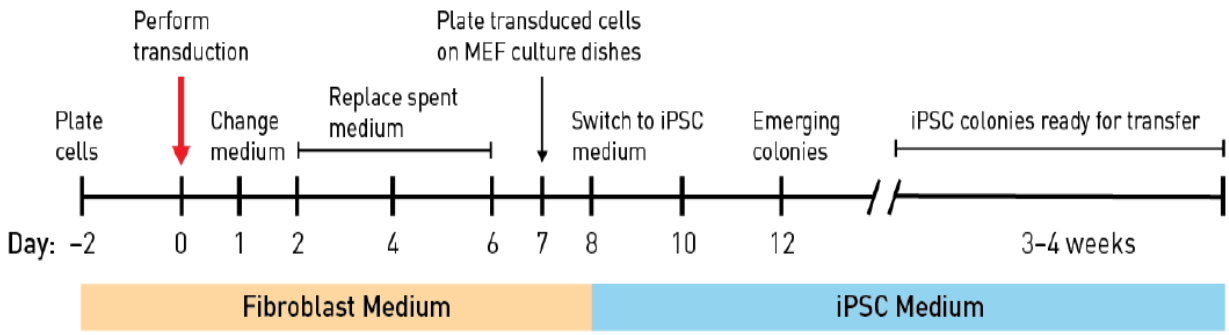

Figure 6 - Workflow for Reprogramming Fibroblasts Feeder Dependent Method

2.2 Reprogramming Feeder Cells

Two days before transduction, human adult foreskin fibroblast cells were plated onto a cell culture T75 flask (VWR) at the appropriate density to achieve between 2 × 10^5–3 × 10^5 cells on the day of transduction (Day 0) (Figure 6). Firstly, a working solution of Mitomycin C (Stem Cell) was prepared for a final concentration of 10ug/μL in DMEM high glucose (BioConcept). The fibroblast medium was discarded, and the cells were washed with Dulbecco´s Phosphate Buffered Saline (DPBS) without Ca^{2+} and Mg^{2+}. The mitomycin solution was added to the cell culture T75 flask and maintained at 37 ◦C in a humidified atmosphere containing 5% CO_2 for 3h. After 3h

mitomycin solution was discarded for a special residue container and cells were rinsed with PBS 1x several times until all mitomycin residues were no longer present. 0.05% trypsin solution (Gibco) was added to the cells and incubated for 3-5min at 37 ◦C in a humidified atmosphere containing 5% CO_2 until detachment from the T-flask was visible. After this, 10mL of fresh fibroblast medium DMEM High glucose (BioConcept) was added, the cells were transferred to a 15mL conical centrifuge tube and cells were centrifuged at ~200g for 5min. Afterward, the supernatant was discarded and 4mL of fresh fibroblast medium DMEM High glucose was added, and the cell pellet was resuspended. Subsequently, cells were counted with a Neubauer chamber. A 6-well cell culture plate was plated at different cell densities, between $10x10^4$ to $25x10^4$ cells per well.

2.3 Coating with Geltrex Matrix

Aliquots of 100µl Geltrex LDEV-Free Reduced Growth Factor Basement Membrane Matrix (Gibco), subsequently abbreviated as Geltrex, stored at -80ºC were used. They were taken out 1h before the procedure and put on ice. Firstly, a working solution was prepared for a final concentration of 0.01%. in DMEM F12 (PAN-Biotech). After, 1mL of the solution was added to a 6-well plate and 35mm cell culture dishes and incubated at 37 ºC in a humidified atmosphere containing 5% CO_2 for 1 hour.

2.4 Immunocytochemistry

Cells were allowed to grow in glass coverslips coated with Geltrex until between 60 to 80% confluency was reached. Then cells were washed in ice-cold PBS before fixation in 4% PFA, for 15 min. Fixed cells were washed twice in PBS and placed in a blocking solution (2% bovine serum albumin (BSA/0.2% Triton-X100/PBS) for 1 h, at room temperature. Cells were incubated for 1 h at room temperature with the primary antibody (Table 3). After incubation, the cells were washed 3 times with BSA/0.2% Triton-X100/PBS, and incubated with the secondary antibodies for 1 h, at room temperature (Table 2). Cells were then dyed with Hoechst 33342 (2µg/ml) on PBS 1x for 10 minutes at room temperature. Three final PBS 1x washes were made before mounting the glass coverslips with Dako mounting media. Slides were allowed to dry and then were kept at 4 ºC until visualization. The slides were then visualized and analyzed on an Axioimager Z2 fluorescence microscope (Carl Zeiss).

2.5 Flow Cytometry

Cells were grown on a 6-well plate until reached 90% of confluency. They were detached with TrypLE Express (Gibco) and transferred to a 1.5 mL Eppendorf™ tube. Afterward, the cells were centrifuged at 300g for 5 minutes at 4ºC. The supernatant was discarded, and the cell pellet was resuspended in cold 0.5% PFA for 20 minutes at room temperature. After this, 1mL of cold 0.5% BSA/0.1% Triton X100/PBS 1x was added, and the cells were again centrifuged at 300g for 5 minutes at 4ºC. The supernatant was discarded, and the cells were washed again with 0.5% BSA/0.1% Triton X100/PBS 1x. Another centrifugation was performed at 300g for 5 minutes at 4ºC and the cells were resuspended with 0.5% BSA/0.1% Triton X100/PBS 1x containing the primary antibody (Table 4) and incubated for 1h on ice. The cells were again washed two times with 0.5% BSA/0.1% Triton X100/PBS 1x, centrifuged, and resuspended in 0.5% BSA/0.1% Triton X100/PBS 1x containing the secondary antibody (Table 3) and incubated for 1h on ice. Two final rinses were done before resuspending the iPSC in PBS 1x for analysis on a flow cytometer (BD FACSCalibur). The software used to acquire the data was Cell Quest Pro and the one used for data analysis was FlowJo.

Table 3 - Antibodies for Immunocytochemistry and Flow Cytometry

	Primary Antibody (Dilution)	**Secondary Antibody (Dilution)**
Pluripotency Markers (Immunocytochemistry)	Rabbit anti-Oct4 (1:400) Cell Signaling Technology Cat# 2840	Goat Anti-Rabbit (1:1000) Invitrogen
	Rabbit anti-NANOG (1:400) Cell Signaling Technology Cat# 4903	
	Rabbit anti-SOX2 (1:400) Cell Signaling Technology Cat# 3579	
	Mouse anti-SSEA-4 (1:100) BD Biosciences Cat# 560,073	Goat Anti-Mouse (1:1000) Invitrogen
Pluripotency Markers (Flow cytometry)	Rabbit anti-NANOG (1:400) Cell Signaling Technology Cat# 4903	Goat Anti-Rabbit (1:1000) Invitrogen
	Rabbit anti-SOX2 (1:200) Cell Signaling Technology Cat# 3579	
	Mouse anti TRA-1-60 (1:50) Bio Legend Cat#330609	PE Conjugated
Differentiation Markers (Immunocytochemistry)	Mouse anti-TUJ1(1:500) BioLegend Cat#801213	Goat Anti-Mouse (1:200) Invitrogen
	Mouse anti-ASM (1:100) Sigma-Aldrich Cat#A5228	
	Rabbit anti-AFP (1:200) Dako Cat# A0008	Goat Anti-Rabbit (1:200) Invitrogen
Nuclear Marker	Hoescht 33342	1:1000

2.6 Alkaline Phosphatase Activity

Alkaline phosphatase staining was carried out using Alkaline Phosphatase Staining Kit II (Stemgent, MA)

2.7 Karyotype Analysis

The genome integrity of the iPSC was evaluated by G-banding at 400–550 band resolution, with a minimum of 30 metaphase spreads analyzed (Genomed, Lisbon, Portugal).

2.8 RT-PCR for Viral Clearance

To ensure that the cells were free of Sendai Virus, we performed RT-PCR after more than 10 passages. The total RNA from 50 × 10^5 iPSCs was extracted using the NZY Total RNA Isolation kit (Nzytech) following the instructions provided with the reagent. As a positive control, was used cells set aside during the reprogramming procedure (lower than 10 passages). The reverse transcription reaction was carried out using 1µg of RNA, previously isolated, using the NZY First-Strand cDNA Synthesis Kit (Nzytech). Then, the PCR was done using 1µL of cDNA from the reverse transcription reaction, using the NZYTaq II 2x Green Master Mix (Nzytech). The RT-PCR primer sequences and the expected product size, refer to Table 3.

Table 4 - Primers used for RT-PCR

	Target	**Size of band**	**Forward/Reverse primer (5'-3')**
Episomal Plasmids (RT-PCR)	SeV Plasmid	181bp	GGATCACTAGGTGATATCGAGC/ ACCAGACAAGAGTTTAAGAGATATGTATC
	hKLF4 Plasmid	410bp	TTCCTGCATGCCAGAGGAGCCC/ AATGTATCGAAGGTGCTCAA
	hKOS Plasmid	528bp	ATGCACCGCTACGACGTGAGCGC/ ACCTTGACAATCCTGATGTGG
	hC-MYC Plasmid	532bp	TAACTGACTAGCAGGCTTGTCG/ TCCACATACAGTCCTGGATGATGATG

2.9 STR Analysis

The fingerprinting analysis was made by STR analysis. Genomic DNA from blood and iPSC were extracted using the NZY Tissue gDNA Isolation kit (Nzytech). Fingerprinting analysis was performed using Promega's PowerPlex 16 kit and analyzed on ABI PRISM 3100 using GeneMapper (Thermo Fisher) by STABVida, Lisbon, Portugal.

2.10 *In vitro* Differentiation Assay

In vitro, differentiation was performed by EB formation. iPSC colonies were cultured in non-adherent conditions in mTeSR medium (StemCellTM Technologies), containing 0.4% polyvinyl alcohol, for 48h. Thereafter, the EBs were seeded on glass coverslips coated with Geltrex in a 6-well plate and cultured for 3 weeks in differentiation medium (DMEM, 10% FBS, 1% Pen/Strep, 1% GlutaMAX, 1% MEM-NEAA). The coverslips were fixed with 4% paraformaldehyde (PFA) for 15 min and analyzed by immunofluorescence on an Axioimager Z2/Apotome fluorescence microscope (Carl Zeiss)

2.11 Cell Incubation with SF7-AM

Fibroblasts ($10x10^3$ per well) were grown in 8-well glass chambered cover slides (ibidi ™) coated with Poly-L-Lysine 24h prior to probe incubation. SF7-AM probe (1.25 μM) was prepared in DMEM medium and loaded onto fibroblasts for 30 min. During this time cells were maintained a 37 ºC in a humidified atmosphere containing 5% CO_2. Fresh made stock solution for (Aminooxyacetic acid; a CBS inhibitor) AOAA (10mM) was prepared with H_2O milliQ. A working solution of 3 mM AOAA was prepared and loaded onto fibroblasts for 1h, prior to SF7-AM probe incubation. During this time cells were maintained at 37 ºC in a humidified atmosphere containing 5% CO_2.. Fresh made stock solution for NaHS (an H_2S donor) (10mM) was prepared with H_2O milliQ. Two working solutions for this reagent were prepared: 25 and 100 μM. These incubations were performed upon SF7-AM incubation. After cell treatment, cells were washed with Krebs medium buffer (132 mM NaCl, 4 mM KCl, 1.4 mM $MgCl_2$, 1 mM $CaCl_2$, 6 mM glucose, 10 mM Hepes-Na, pH 7.4), and imaging acquisition was also performed on this media in the presence of CBS inhibitor and donor.

2.12 Cell Imaging

Images were acquired at intervals of 5 min during 1h in a laser scanning microscope Zeiss LSM 710 Confocal with Airyscan with excitation 498nm/emission 526nm at 37 ºC. Two positions per well were acquired, on average 39 cells per well were counted in a total of n=13 experiments made for the control group, n=.5 for the AOAA group, and n=5 for the NaHS group. Absolute and relative frequency were used to calculate fluorescence that was measured in arbitrary fluorescence units (AFI). Images were analyzed, and the fluorescence intensity was quantified with the ZEISS ZEN software.

2.13 Statistics

Quantitative analyses were presented as the mean ± SEM of at least five independent experiments. Graphics were set up using GraphPad Prism and statistical analysis was carried out using a t-student paired test and ANOVA two-way test (CBS inhibitor and CBS Donor) with Dunnet´s multiple comparisons test or no multiple comparisons.

3 RESULTS

3.1 Basal levels of H_2S are higher in the T21 cell line than in the euploid cell line.

To determine the levels of H_2S in both DS fibroblasts and control fibroblasts, we incubated both cell lines with SF7-AM, which is a very sensitive probe with high cellular trappability that allows visualization of real-time imaging of endogenous H_2S that is produced within living cells. After 30 minutes incubation period, the cells were visualized in real-time imaging for 1 hour. Then cells were counted and fluorescence was measured. Images revealed an increase in the fluorescence levels and the results exhibit a statistical difference between the levels of H_2S DS fibroblasts when compared to the control fibroblasts (Figures 7 and 8).

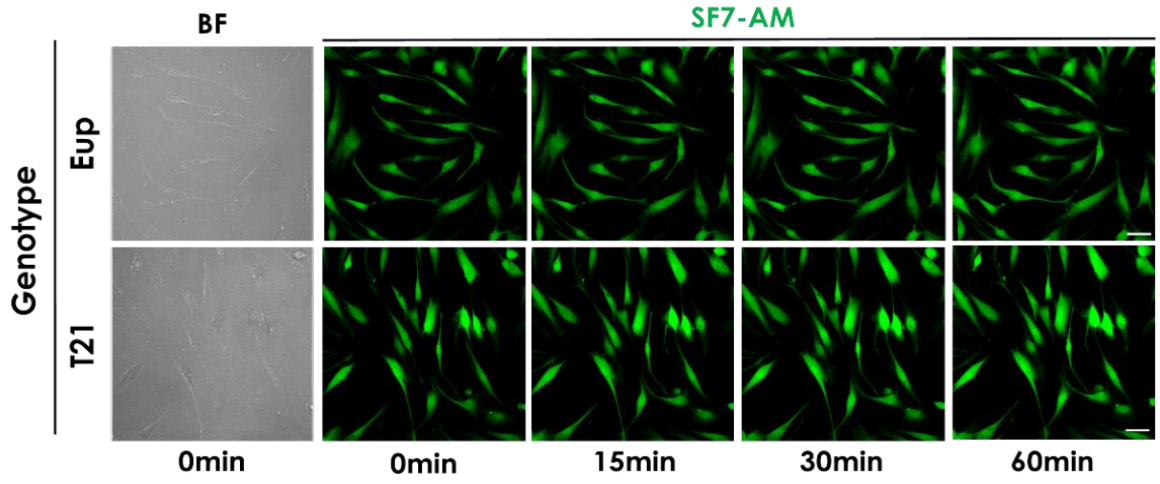

Figure 7 - Immunofluorescence of euploid and T21 cell lines incubated with SF7-AM. Scale bar represents 50µm.

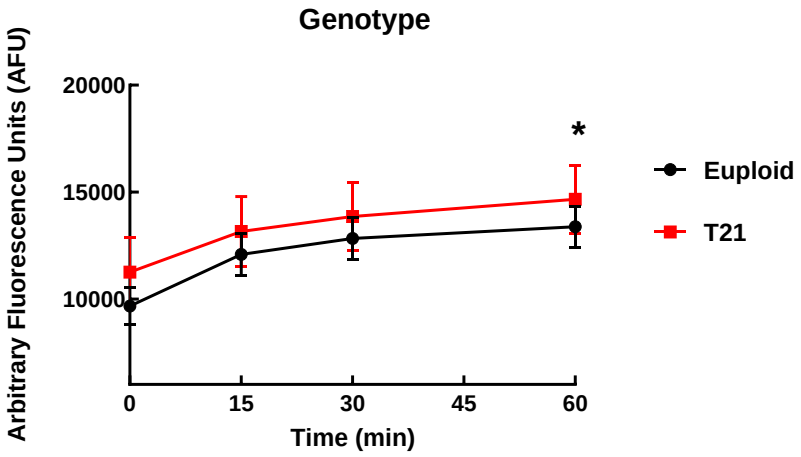

Figure 8 - Graphic of T21 and Euploid cell lines absolute fluorescence levels. Units in arbitrary fluorescence units (AFU) and time in minutes (min)

3.2 CBS Inhibitor decreased H_2S production levels in T21 cell line.

To test whether the H_2S production was affected by CBS, we incubated both cell lines with the fluorescent probe SF7-AM and AOAA (n=5) and SF7-AM alone (n=13) and measured the H_2S levels. A weaker fluorescence appeared in the T21 cell line with AOAA, revealing that less H_2S was produced. (Figures 9 and 10) which indicates that AOAA decreased H_2S production levels in the T21 cells.

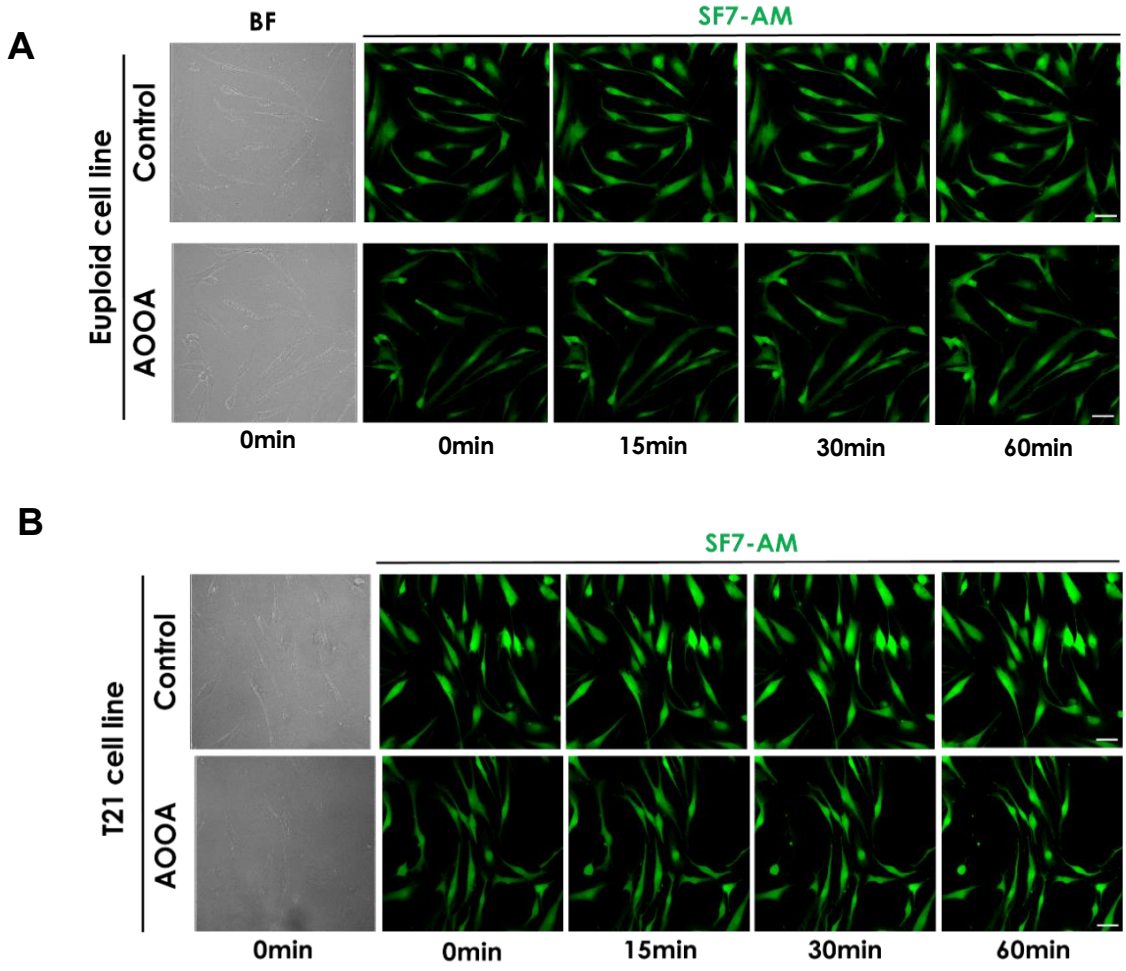

Figure 9 - Immunofluorescence of Euploid (A) and T21 (B) incubated with SF7-AM (1,25µL) and AOAA (3mM). Scale bar represents 50µm.

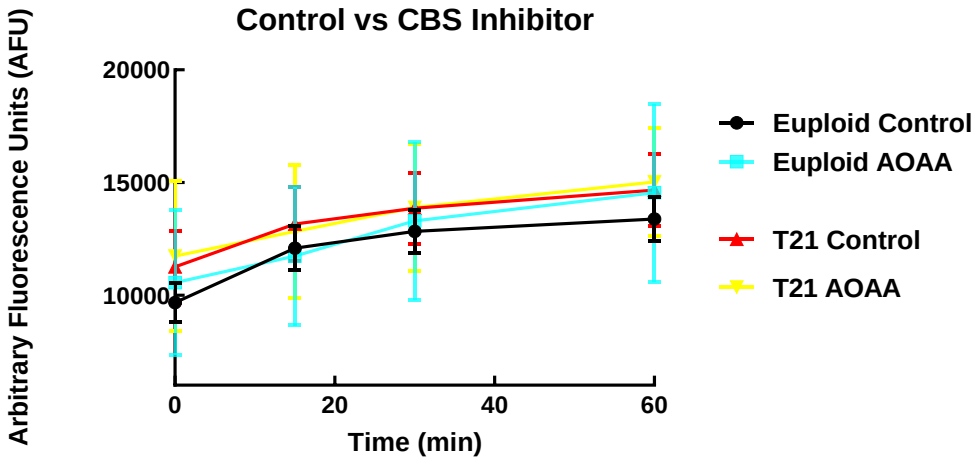

Figure 10 - Graphic of H_2S levels in T21 and euploid cell lines. Units in arbitrary fluorescence units (AFU) and time in minutes (min)

3.3 CBS donor altered the H_2S production levels in T21 cell line.

To test if CBS caused any difference in H_2S levels in the trisomic cell line, we incubated the fibroblasts with NaHS at two different concentrations, one concentration of 25μM along with SF7-AM (n=5) and another concentration of 100μM along with SF7-AM and as a control the fibroblasts from both cell lines were incubated only with the fluorescent probe and measured the H_2S levels in the cells. At the treatment with NaHS of 25μM concentration, the fibroblasts did not produce detectable levels of H_2S (Figures 11 and 12) and the same happened with fibroblasts treated with a100μM concentration. In fact, an opposite effect was detected (Figures 13 and 14) meaning that at 100μM concentration, fibroblasts exhibited a decrease in H_2S production (Fig. 14). Interestingly, at a concentration of 25μM, we did observe an increase in H_2S production in both cell lines (Fig.1), along the 60 minutes of imaging.

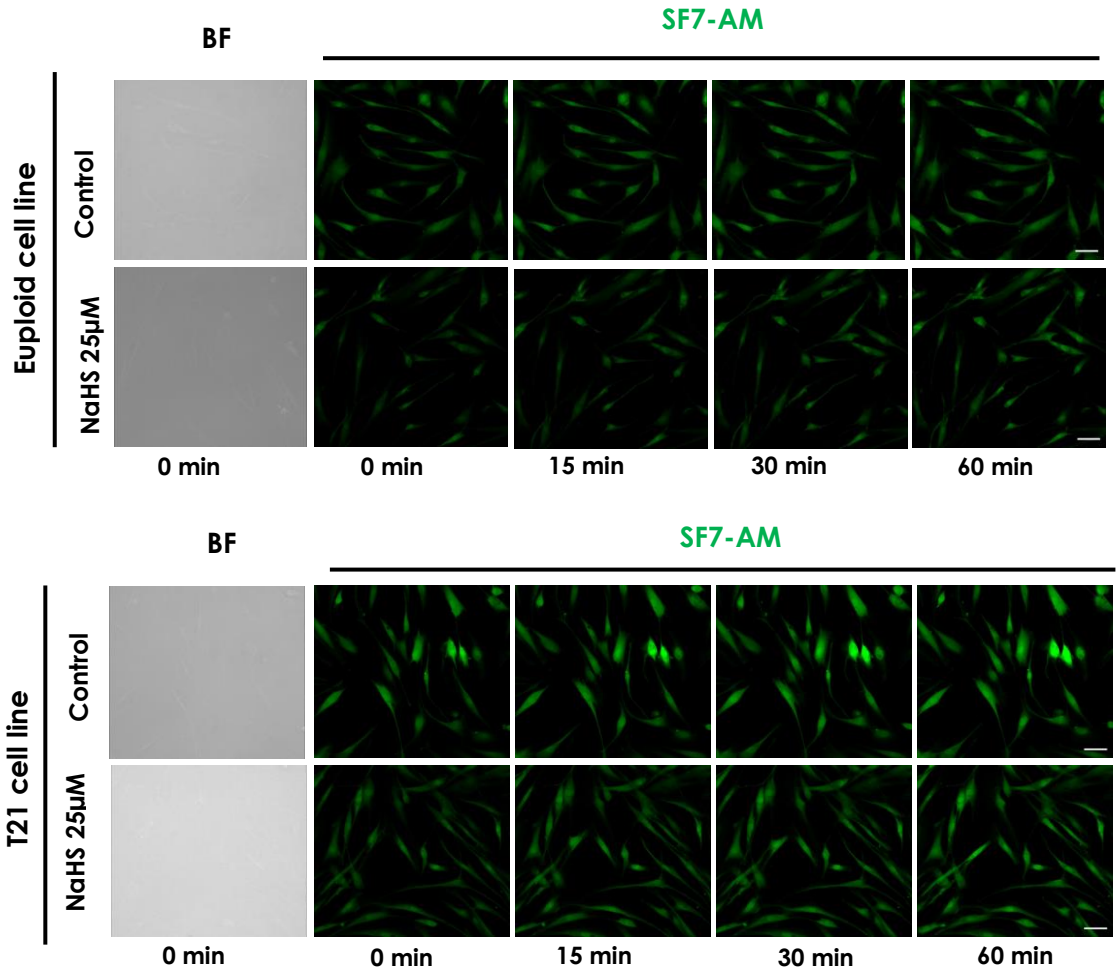

Figure 11 - Immunofluorescence of euploid (A) and T21 (B) cell lines incubated with SF7-AM and NaHS (25μM). Scale bar represents 50μm.

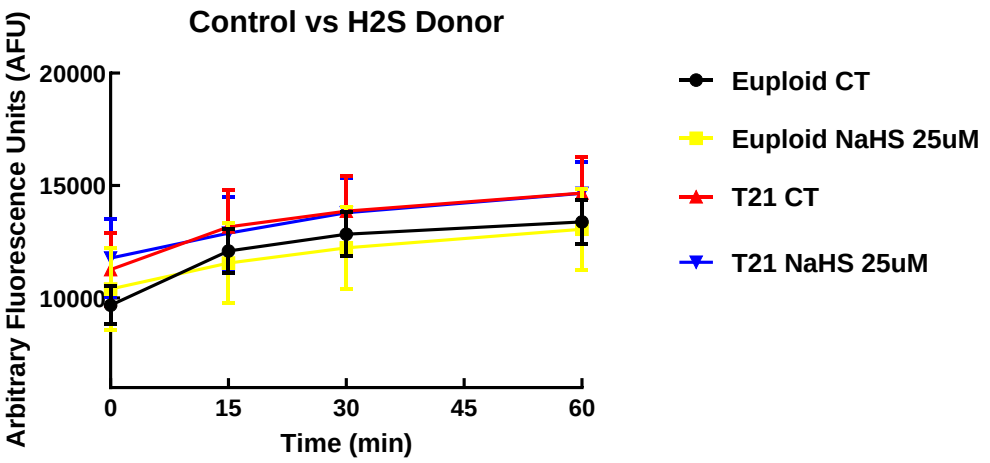

Figure 12 - Graphic representing H_2S production levels in euploid and T21 cell lines. Units in arbitrary fluorescence units (AFU) and time in minutes (min)

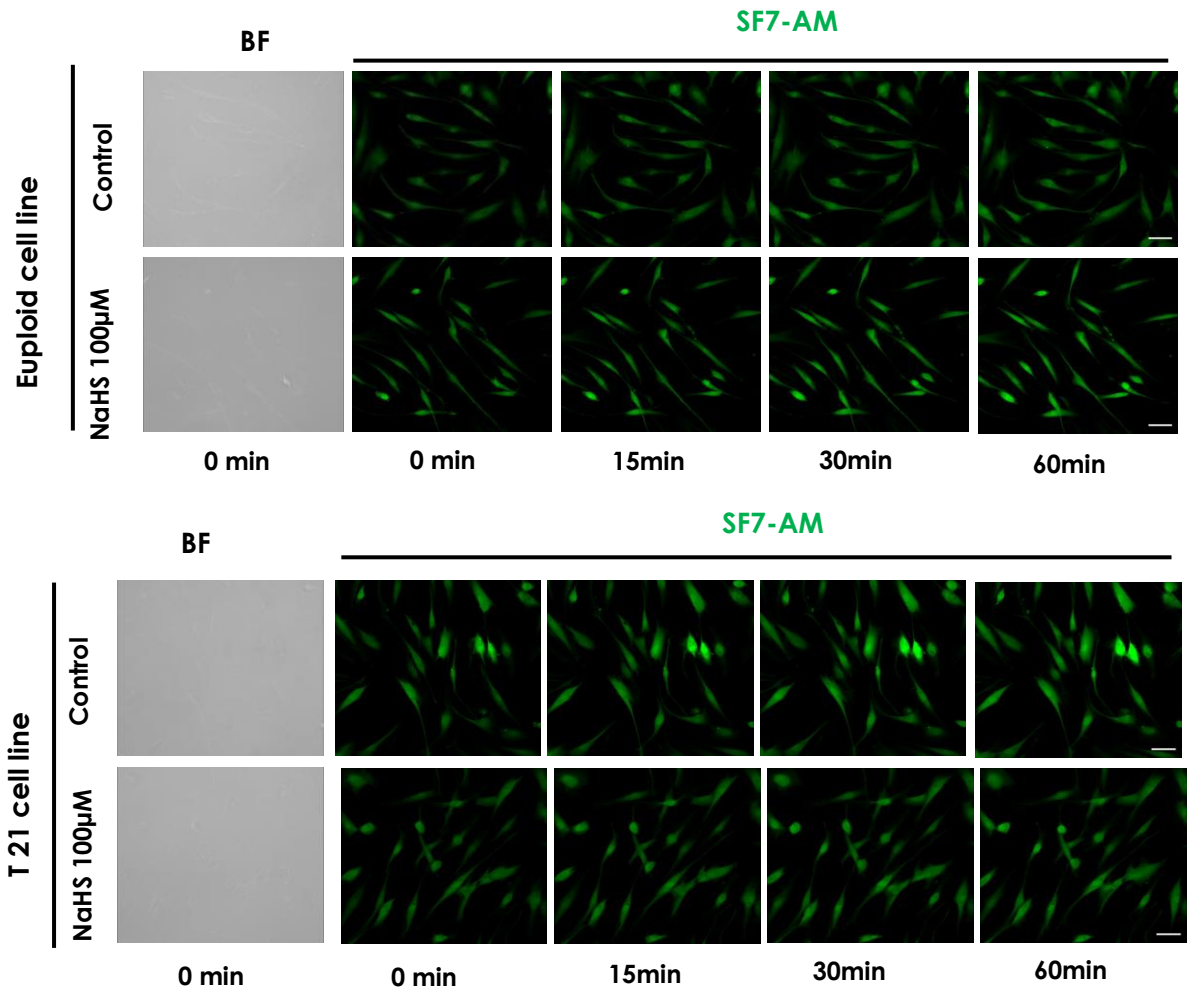

Figure 13 - Immunofluorescence of euploid (A) and T21 (B) cell lines incubated with SF7-AM and NaHS (100µM). The scale bar represents 50µm.

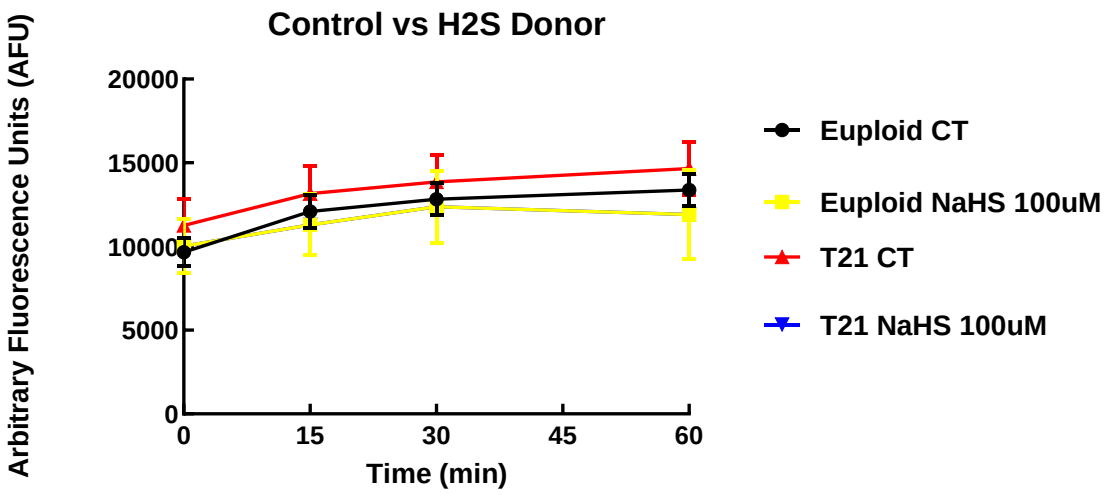

Figure 14 - Graphic of H_2S production levels in euploid and T21 cell lines. Units in arbitrary fluorescence units (AFU) and time in minutes (min) Note: Although is not visible, T21 NaHS 100μM is under the euploid NaHS 100μM.

3.4 Generation of human iPS cells from DS and EUPLOID fibroblasts

The DS fibroblast cell lines were reprogrammed using nonintegrative SeV transduction of OSKM. Starting day 6, colonies expanded well with a typical human ESC-like morphology, with a single nucleus and round shape, and manually picked select colonies for further growth and characterization on day 10 post-transduction.

3.4.1 iPS cell line AG06922 expressed pluripotency markers determined by immunocytochemistry.

Qualitative analysis of the pluripotency state of iPS cell colonies was performed by immunofluorescence (Figure 15). As shown in Figure 15, iPS cells expressed the pluripotency markers Sox2, Nanog, and SSEA-4, evidenced by the green staining. The presence of these specific proteins indicates that the colonies are in a dedifferentiated state.

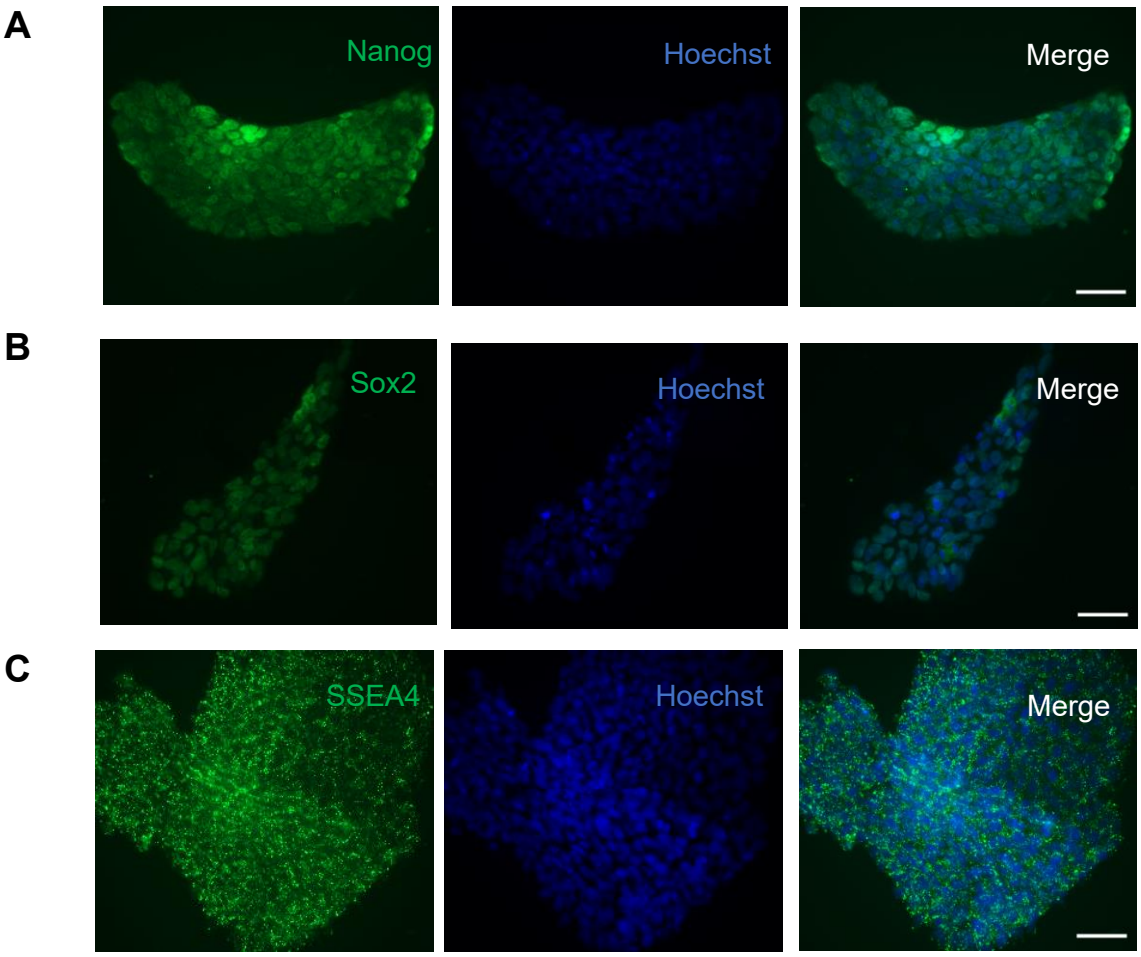

Figure 15 - Immunocytochemistry of nuclear (NANOG (A) and Sox2 (B)) and surface (SSEA-4 (C)) pluripotency markers in iPS cells from DS patients. Nuclei were counterstained with Hoescht (blue). Scale bars represent 50 µm.

3.4.2 iPS cell line GM01652 expressed pluripotency markers determined by immunocytochemistry.

Qualitative analysis of the pluripotency state of iPS cell colonies was performed by immunofluorescence (Figure 16). As shown in Figure 16, iPS cells expressed the pluripotency markers, Nanog, Oct4; Sox2, and SSEA-4, evidenced by red staining. The presence of these specific proteins indicates that the colonies are in a dedifferentiated state.

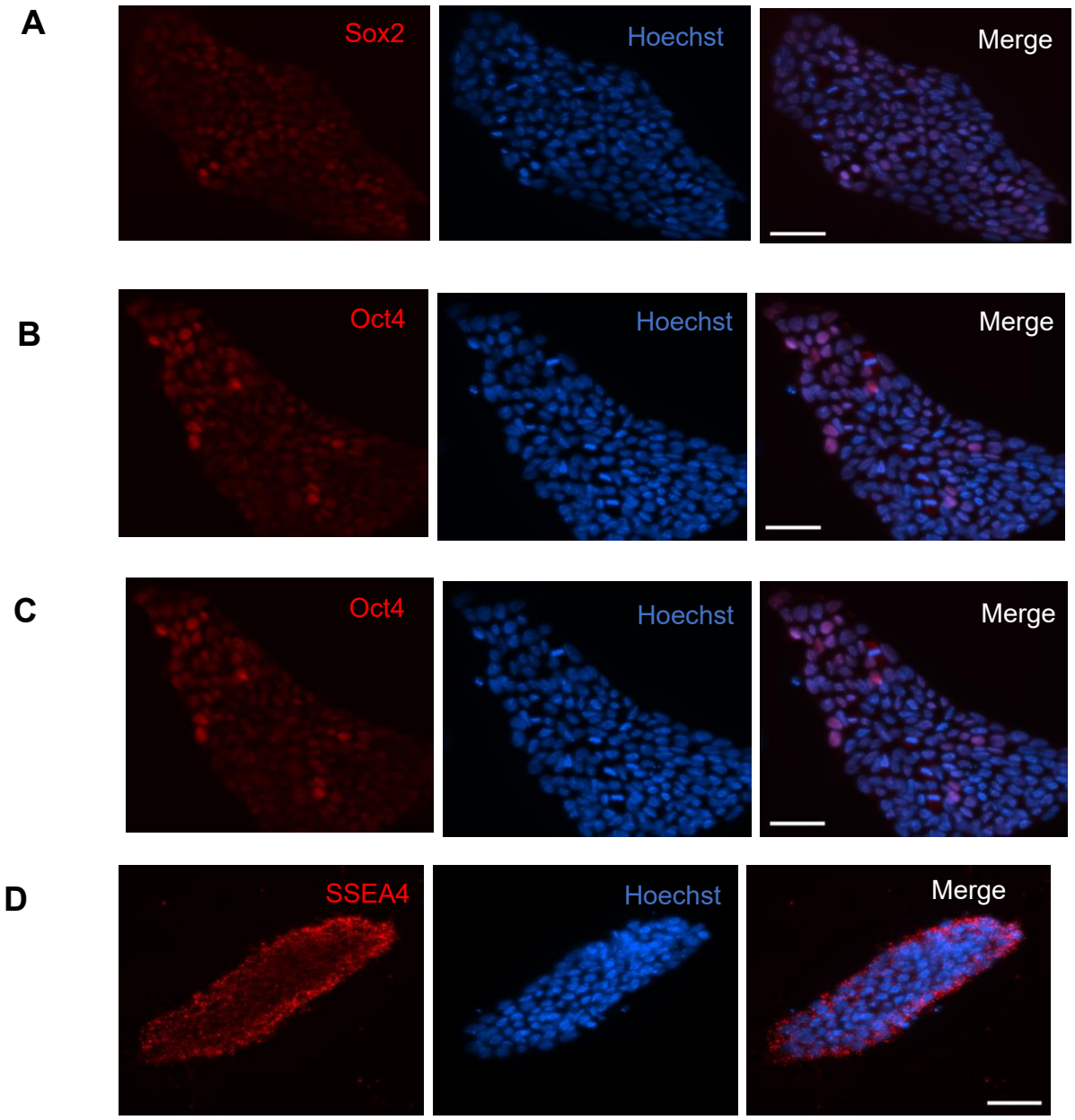

Figure 16 - Immunocytochemistry of nuclear (Sox2 (A), Nanog (B)), and Oct4 (C) and surface (SSEA-4 (D) pluripotency markers in iPS cells from a healthy donor. Nuclei were counterstained with Hoescht (blue). Scale bars represent 50 µm.

3.4.3 iPS AG06922 cell line expressed pluripotency markers determined by flow cytometry.

Quantitative analysis of the pluripotency state of iPS in the cell population from the AG06922 cell line was performed by flow cytometry (Figure 17). These results revealed that 87,6% of cells were positive for Nanog, 91,5% of cells were positive for Sox2 and 98,7% were positive for TRA-1-60 (Figure 8). Indicating that most of the cells present in the colony are pluripotent.

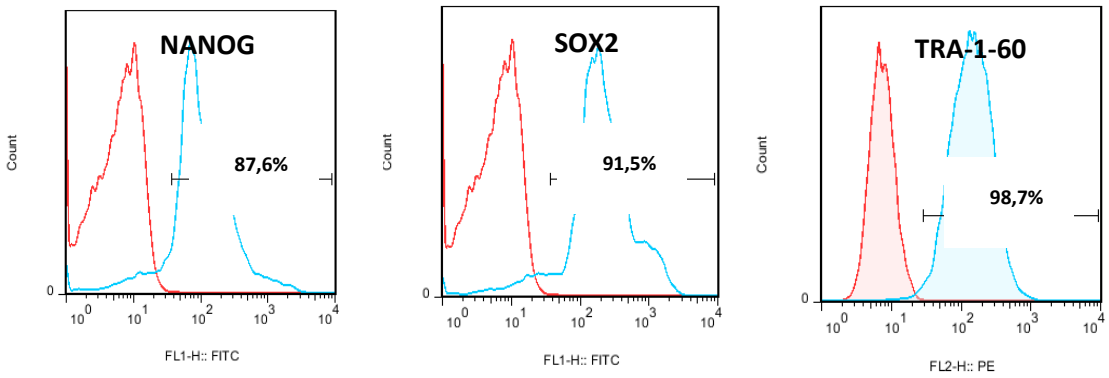

Figure 17 - Flow Cytometry analysis shows presence of pluripotency markers on AG06922 cell line iPSCs: 87,6% of iPSCs have NANOG, 91,5% of iPSCs have Sox2 and 98,7% of iPSCs have surface marker TRA-1-60. Control group (red) was unlabeled, thus showing no fluorescence, and was used for comparison with each of the other markers

3.4.4 Alkaline phosphatase positive staining in AG06922 cell line

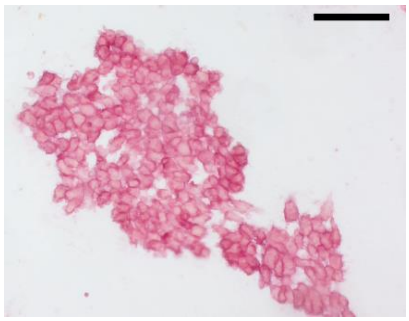

Figure 18 - Alkaline Phosphatase positive staining. Scale bar represents 100µm.

Usually, iPSCs have high AP activity. The generated colonies revealed the characteristics of AP positive (Figure 18), which was visible by the appearance of the pink color after the staining of iPS cells with the AP protocol.

3.4.5 RT-PCR analysis did not detect viral presence in the AG06922 cell line.

Sev iPSCs induction has been shown to maintain genome integrity with minimal level of genomic aberration. Validation of SeV clearance is an important quality control tool. In RT-PCR analysis we did not observe the presence of SeV in cells after 10 passages, (Figure 19) indicating that the colonies were clear from viruses. The original fibroblasts that were freshly transduced were used as a positive control.

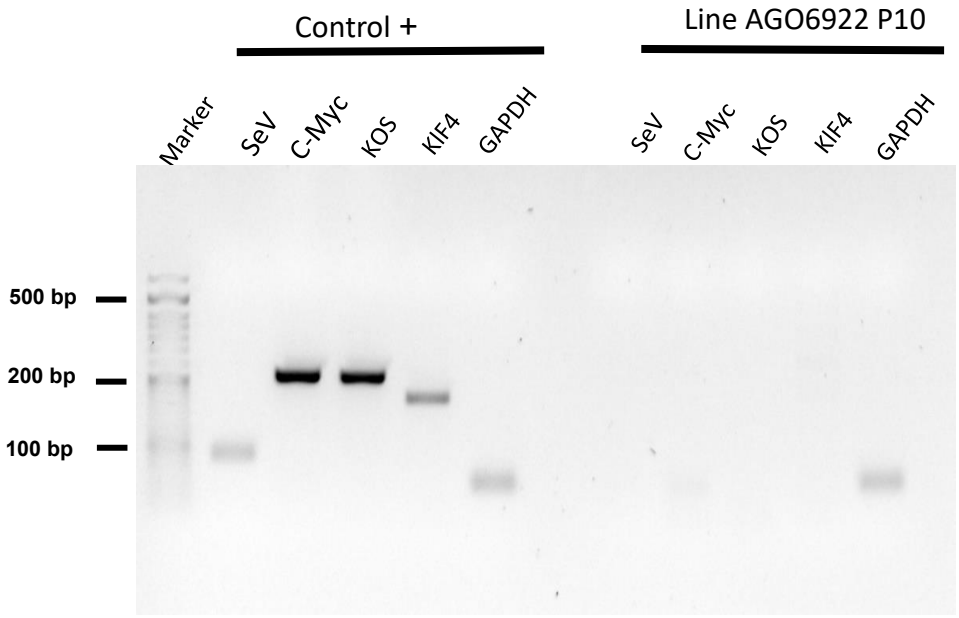

Figure 19 - RT-PCR of SeV clearance of reprogrammed transgenes for AG0622 iPS cell line. Positive control were cells in 0 passage. SeV (181bp), c-Myc (532bp); KOS (528bp) and GAPDH as a housekeeping gene.

3.4.6 RT-PCR analysis did not detect viral presence in the GM01652 cell line.

In RT-PCR analysis we did not observe the presence of SeV in cells after 11 passages, (Figure 20) indicating that the colonies were clear from viruses. The original fibroblasts that were freshly transduced were used as a positive control.

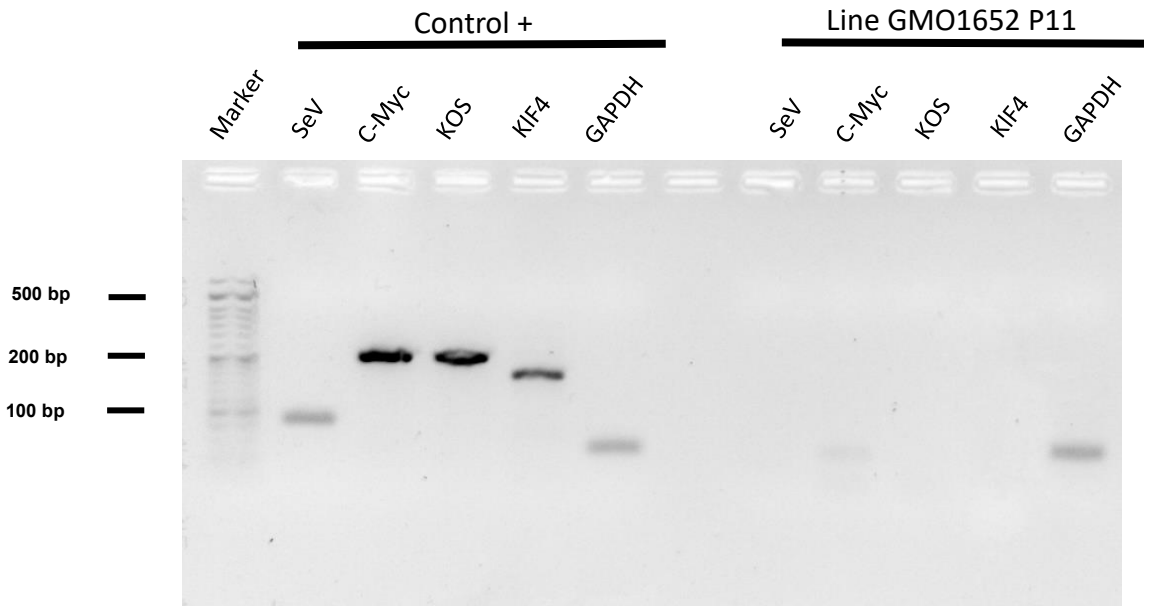

Figure 20 - RT-PCR of SeV clearance of reprogrammed transgenes for GM91652 iPS cell line. Positive control were cells in 0 passage. SeV (181bp), c-Myc (532bp); KOS (528bp) and GAPDH as a housekeeping gene.

3.4.7 EB Formation

The formation of EBs allowed the spontaneous differentiation of iPSCs into the embryonic three germ layers *in vitro*, expressed by the representative makers for ectoderm (Tuj1), mesodermal (α-ASM), and endoderm (AFP) (Figure 20).

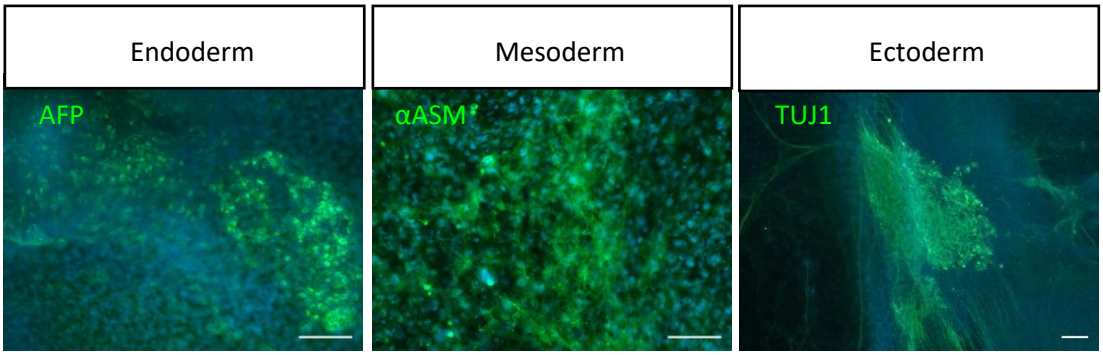

Figure 21 - Immunocytochemistry for endodermal (AFP), mesodermal (αASM), and ectodermal (Tuj1). Nuclei were counterstained with Hoescht (blue).

3.4.8 STR Profile

STR profile matched that of the original fibroblasts for both cell lines (Figures 22A,22B, 23A, and 23B).

A

PowerPlex 16 Loci	Sample PG07 - 1	
D3S1358	14	18
TH01	7	8
D21S11	29	30
D18S51	14	17
Penta E	9	11
D5S818	11	12
D13S317	12	12
D7S820	8	8
D16S539	11	11
CSF1PO	12	13
Penta D	9	14
Amelogenin	X	X
vWA	17	18
D8S1179	13	14
TPOX	8	9
FGA	19	23

B

PowerPlex 16 Loci	Sample PG08 - 2	
D3S1358	14	18
TH01	7	8
D21S11	29	30
D18S51	14	17
Penta E	9	11
D5S818	11	12
D13S317	12	12
D7S820	8	8
D16S539	11	11
CSF1PO	12	13
Penta D	9	14
Amelogenin	X	X
vWA	17	18
D8S1179	13	14
TPOX	8	9
FGA	19	23

Figure 22 - STR profile for GM01652 cell line. A - Original fibroblast cell line; B - Reprogrammed iPSCs GM0 1652 cell line

A

PowerPlex 16 Loci	Sample PG03 - 1 (CT)		
D3S1358	16	19	
TH01	6	8	
D21S11	29	31.2	32.2
D18S51	15	17	
Penta E	10	11	
D5S818	10	12	
D13S317	12	12	
D7S820	11	12	
D16S539	11	11	
CSF1PO	11	12	
Penta D	10	12	13
Amelogenin	X	Y	
vWA	15	17	
D8S1179	13	14	
TPOX	8	11	
FGA	22	24	

B

PowerPlex 16 Loci	Sample PG04 - 2 (ITS)		
D3S1358	16	19	
TH01	6	8	
D21S11	29	31.2	32.2
D18S51	15	17	
Penta E	10	11	
D5S818	10	12	
D13S317	12	12	
D7S820	11	12	
D16S539	11	11	
CSF1PO	11	12	
Penta D	10	12	13
Amelogenin	X	Y	
vWA	15	17	
D8S1179	13	14	
TPOX	8	11	
FGA	22	24	

Figure 23 - STR profile for AG06922 cell line. A - Original fibroblast cell line; B - Reprogrammed iPSCs AG06922 cell line

To assess the genotype the karyotyping test was made but until the conclusion of this work, we did not receive the report. Overall, the presented results demonstrated that iPSCs generated from DS and euploid fibroblasts exhibited the expression of pluripotency and general differentiation markers.

4 DISCUSSION

In this work, we investigated the role of an extra copy of the CBS gene in fibroblasts of DS patients through measurements of the production of H_2S. CBS activity is increased by about 150% in fibroblasts from DS patients compared to normal subjects (Chadefaux et al., 1985). A polymorphism of the CBS allele is significantly under-represented in children with high IQ, suggesting that the level of CBS may influence cognitive functions (Kamoun, 2019). We first assessed the basal levels of H_2S in DS fibroblasts that according to previous studies (Abdel-Salam et al., 2013; Caracausi et al., 2018; Pecze et al., 2020) were significantly higher than in the control cells. This increase in H_2S production may be due to the presence of a supernumerary CBS. There was a marked and uniform increase in H_2S generation in DS cells as demonstrated by live cell imaging with H_2S fluoroprobe SF-7AM. Based on the work made by Lin *et al* we selected SF-7AM as a probe to measure intracellular H_2S, due to its ability to produce a 20-fold increase in fluorescence intensity and also because this probe can retain brightness for 60min after replacing cellular media, This enhanced trappability provides a notable increase in the sensitivity of this reporter for cellular imaging by maintaining a greater concentration of dye within the cell. *In vitro* experiments demonstrated that this probe can detect submicromolar concentrations of H_2S up to 500nM. Moreover, it revealed a high selectivity for H_2S over RSS, RNS, ROS, and glutathione (Lin et al., 2013). H_2S overproduction along with CBS overdosage is re-emerging as one of the potential causes in the pathogenesis of DS and neurocognitive deficits associated with the pathology in the central nervous system (CNS) (Szabo, 2020). CBS overexpression and H_2S overproduction may occur mainly by two mechanisms (Kamoun, 2019): one is the dysregulation of the transsulfuration pathway causing a low availability of homocysteine and methionine, which consequently increments several metabolites including cysteine and cystathionine that are consistent with an increase on CBS activity (Pogribna et al., 2001). These findings may suggest dysfunctions in protein synthesis as well as antibodies and peptide hormone synthesis, one example is the overexpression of RCAN protein, which acts to prevent the nuclear occupancy of transcription factors responsible for calcineurin expression. Calcineurin plays a critical role in regulating T cell development and activation. In activated T lymphocytes, calcineurin dephosphorylates the nuclear factor of activated T cells and promotes its translocation into the nucleus and the up-regulation of early T-lymphocyte genes such as the Th1-cytokine interleukin IL-2 (Abdel-Salam et al., 2013). The other mechanism of overproduction concerns H_2S biogenesis, especially in the brain, attributing the accumulation of H_2S to possible inhibition of cytochrome c oxidase present in the Complex IV of the mitochondria such that suppresses mitochondrial function, being the reason for the failure in mitochondrial activity (but reversible) and consequent electron transportation, oxygen consumption, and ATP generation (Panagaki et al., 2019). Interestingly note that the heme group of CBS enzyme binds to the heme group of cytochrome c oxidase causing inhibition by competition. Mitochondrial metabolism impairment leads to the accumulation of several metabolites, such as pyruvate which connects the Krebs

cycle to glycolysis; fumarate and succinate intermediates of the Krebs cycle; lactate, the end product of anaerobic glycolysis; formate, involved in mitochondrial one-carbon metabolism; and creatine, involved in the process of energy-dependent muscle activity (Caracausi et al., 2018). Plasma samples collected from DS patients show an increase in plasma lactate which supports the hypothesis that in DS cells oxidative phosphorylation is impaired, and DS cells activate glycolysis for their energy demands (Caracausi et al., 2018). In terms of gene expression, there are five genes related to oxidative phosphorylation upregulated in DS patients and these were the following: NADH:ubiquinone oxidoreductase subunit V3 (NDUFV3), cytochrome c oxidase assembly homolog COX15 (COX15), ATPase H^+ transporting V1 subunit H (ATP5PF), ATPase H^+ transporting V1 subunit H (ATP6V1H) and ATP synthase peripheral stalk subunit OSCP (ATP5PO). Out of these five genes, three of them are mapped to chromosome 21 (ATP5PF, NDUFV3, and ATP5PO). Nine genes related to oxidative phosphorylation were identified to be downregulated: NADH:ubiquinone oxidoreductase core subunit S2 (NDUFS2), NADH:ubiquinone oxidoreductase core subunit S1 (NDUFS1), NADH:ubiquinone oxidoreductase subunit A10 (NDUFA10), cytochrome c oxidase subunit 7C (COX7C), cytochrome c oxidase assembly factor 1 homolog (COA1), ATPase H^+ transporting V1 subunit B1 (ATP6V1B1), ATP synthase membrane subunit c locus 1 (ATP5MC1), ATP synthase F1 subunit epsilon (ATP5F1E) and ATPase H^+/K^+ transporting non-gastric alpha2 subunit (ATP12A). The above gene products are involved in oxidative phosphorylation; they are essential constituents of the mitochondrial electron transport complexes, namely Complex I (NADH dehydrogenase), Complex II (Succinate dehydrogenase), Complex III (Ubiquinol cytochrome C oxidoreductase), Complex IV (Cytochrome C oxidase) as well as of ATP synthase (which is not an electron transporter and its former, somewhat misleading designation is mitochondrial Complex V). The coordinated function of the four electron transport complexes and the subsequent function of ATP synthase are essential for aerobic ATP generation in the mitochondria of all mammalian cells. Dysregulation of these genes would predict significant disturbances in aerobic ATP generation (Pecze et al., 2020). These abnormalities may explain some of the neurological and neurocognitive deficits associated with DS because neurons heavily depend on ATP produced by oxidative phosphorylation (Panagaki et al., 2019). The CBS inhibitor, AOAA, is used to determine the significance of the endogenously generated H_2S from cysteine and serine but it is a general inhibitor of all PLP-dependent enzymes. The plasma of DS patients revealed depleted levels of serine and elevated levels of cysteine (Mircher et al., 1997) which means that the cellular metabolic rate and serine/cysteine ratio likely represent the key factors determining the efficacy of AOAA inhibiting CBS-derived H_2S biogenesis. Petrosino *et al* found that the serine/cysteine ratio is the main determinant of H_2S production by CBS in vivo. Together, intracellular concentrations, availability, and compartmentalization of competing for CBS substrates serine and cysteine play a critical regulatory role in determining H_2S-producing capacity and its response to pharmacological inhibition (Petrosino et al., 2022). Several studies demonstrated AOAA as a good method to reduce H_2S toxicity in mitochondrial dysfunction, we

based the dosage on the work of Panagaki and colleagues which demonstrated that in human skin fibroblasts, 3mM of AOAA was sufficient to shut down the CBS-derived H_2S biogenesis (Panagaki et al., 2019). Another example is the work of Du and colleagues, they injected rats intraperitoneally with 5mg/kg of AOAA and were able to increase mitochondrial ATPase activity and restore axonal regeneration, AOAA reduced the H_2S levels in the brain and protected the structure and function of the neurons. It improves learning and memory capacities in chronic alcoholism (Du et al., 2018). Our findings did detect a decrease in H_2S production in trisomic fibroblasts in the presence of AOAA although it was not significant, probably due to a low number of independent experiments. Some reasons for having such a low number were related to the accomplishment of the cell incubation protocol. Firstly, when seeding the fibroblasts into the ibidi™ microscope plate some of the wells did not disperse properly causing a low quantity of cells, being this the main reason for excluding several of the independent experiments. The concentrations for the H_2S donor, NaHS, were established based on the work of Lin and colleagues which demonstrated that 25 μM of NaHS had a dramatic improvement in fluorescent intensity of SF-7AM when compared to other fluorescent probes that required 100 μM to achieve a comparable increase in signal intensity (Lin et al., 2013). NaHS is shown to be an anti-inflammatory agent that reduces neuroinflammation (Donertas Ayaz et al., 2021), it has been demonstrated that NaHS enhances cAMP generation in primary cultures of cerebral cortex, cerebellum neurons, and glial cells in a concentration-dependent manner (Shefa et al., 2018), at a concentration of 25μM our results did not find alterations in the production of H_2S between T21 with SF7-AM and T21 with NaHS 25 μM+SF7-AM, once again related with the same issues previously reported. Despite the small number of experiments, it was sufficient to reveal that fibroblasts treated with NaHS at a concentration of 100μM reduced H_2S production when compared to the lower concentration. In addition, to having anti-inflammatory properties, NaHS can help to reduce different types of fibrosis such as idiopathic pulmonary fibrosis (Fang et al., 2009), liver fibrosis (Fiorucci et al., 2006), among others (Y. Chen et al., 2021), in terms of mechanism during the process of fibrosis, the activations of the TGF-β signaling pathway, oxidative stress, cellular senescence, and inflammatory response play crucial roles in the activation and proliferation of fibroblasts to generate excessive extracellular matrix. Despite the beneficial result in the trisomic fibroblasts, the employed concentration of 100μM cannot be applied to *in vivo* systems, as NaHS caused a very similar concentration-dependent decrease in cell survival (Baskar et al., 2007). The reasons for a beneficial effect on trisomic fibroblasts are still unknown hence could be a good topic for investigation. One of the main goals of this work was to characterize trisomic iPSCs in terms of H_2S production levels, unfortunately, this was not possible mainly for two reasons, the PBMCs were collected from DS individuals through cell preparation tubes (CPT) vacutainers which contain an anticoagulant plus a gel product that aids blood layer separation then the buffy coat is isolated from the whole blood sample due to higher concentration of PBMCs in these samples compared with whole blood. PBMCs are more laborious to reprogram due to high sensitivity to

the reprogramming method which can produce some cytotoxicity leading to cell death. Many factors can impact the quality of PBMCs, the time between sample donation and processing is important for the yield and viability of PBMCs isolated, a freshly processed blood sample will generate a high percentage of T-cells and a low percentage of granulocytes. If the processing time is delayed, the sample ages, cells begin to die, and granulocytes become activated. Another important factor that can affect sample quality is storage and transport temperature. It is important to perform PBMC isolation between 18-25°C prior to cryopreservation. A centrifuge with adjustable temperature is recommended. Low ionic strength, calcium/magnesium-free DPBS is generally the wash buffer of choice for protocols. A robust freezing protocol is important for preventing severe cell damage, osmotic injury, and intra- or extra-cellular water crystal formation. Cells should be frozen in cryopreservation vials in cryoprotectant with freezing gradually applied at a rate of ~1°C/min. The DMSO used during cryopreservation should be high quality with low levels of endotoxins (https://www.reprocell.com/blog/cls/what-is-pbmc). Another reason for not achieving the characterization was related to the microscope used for real-time cell imaging that suffered several technical issues which made us lose several weeks of work. Patient-derived induced pluripotent stem cells offer some advantages for cell and tissue replacement, engineering and studying self-renewal capacity, pluripotency, and ease of accessibility to donor tissues. By reprogramming cells from DS patients, it is possible to obtain new tissue with the same genetic background, offering a valuable tool for the study of this genetic disease and designing potential customized patient-specific stem cell therapies. For an efficient model, the new cell system must be able to reproduce the features of the disease (Brigida & Siniscalco, 2016). There are several lines of iPSCs already established, p.e., Park and colleagues originated cell lines from two young male DS individuals one year and one month of age (I.-H. Park et al., 2008), Ponroy Bally and colleagues designed four cell lines from DS male individuals, a newborn, a one-month baby, a nine-month baby and a 5-year-old (Ponroy Bally et al., 2020) and Weick generated one cell line from a one-year-old DS male (Weick et al., 2013). Despite this to study the neurodevelopment *in vitro* it is important to have reprogrammed iPSCs from our patients because they possess the genetic and phenotypic characteristics of the disease in the study. As previously mentioned, DS is a genetic disease thus to have a correct model of the disease it is best to create our proper cell lines. In addition, the differentiation of DS-derived iPS cells into neural cells still retains cellular dysfunctions, providing evidence that the iPS cell system is an efficient model to study the syndrome and to enable the discovery of the underlying causes of and pharmaceutical treatments for this disorder (Brigida & Siniscalco, 2016). Despite our use of fibroblasts in this work, other types of cells can be utilized to generate induced pluripotent stem cells, such as peripheral blood mononuclear cells (Banno et al., 2016), amniotic fluid cells (second trimester) (H.-E. Lu et al., 2013b), mononuclear, stromal and neonatal fibroblasts (Chou et al., 2012). In addition to different types of cells, different sources of fibroblasts can be used, for instance, fetal skin fibroblasts from monozygotic twins (Hibaoui et al., 2014). In this study, it was possible to reprogram two fibroblast

lines, from a healthy donor, GM01652, and from a DS individual, AG06922, both revealed most colonies with pluripotency markers. However, until the conclusion of our investigation, we did not receive the karyotype analysis report. Without the report for the karyotype analysis, we cannot say with certainty that the iPSCs are pluripotent. Unfortunately, we were not able to have the complete characterization of both lines due to several issues. In the quality analysis by immunocytochemistry, the protocol had to be adapted in several steps, both antibodies (primary and secondary) concentrations had to be regulated several times for the correct visualization under the microscope, which caused several delays. In the AG06922 cell line, in addition to the antibody concentration issue, we were not able to visualize the pluripotency marker Oct4, probably due to low cell permeabilization that did not allow the antibody entrance into the cell. Various adjustments to BSA quantity and antibody concentration had to be made but still no marker was visualized. In the quantitative analysis by flow cytometry, we had a great loss of cellular matter more than once, due to the numerous washings and aspirations that the protocol requires, causing accidental loss of cells in different steps of the protocol. Due to the long time consuming reprogramming method with SeV, which is approximately two months, we did not have many *in vitro* cell cultures to work thus causing a handful of delays in our experiment timelines.

5 CONCLUSION AND FUTURE PERSPECTIVES

Although the karyotype report did not arrive in time, we can affirm that overall, the characterization of iPS cells from a healthy donor, GM01652, and from a DS individual showed that the reprogramming was successful, meaning we accomplished a very important tool for the next step in our investigation, which is the differentiation of iPSCs in NPCs to evaluate the production of H_2S levels and effects in those cells. Meanwhile, our preliminary findings regarding H_2S production levels in fibroblasts show a significant increase in the trisomic cell line compared with a healthy donor, and the same cell line also revealed a slight decrease in H_2S production levels in the presence of a CBS inhibitor, AOAA. We did observe some differences, although not significant, in H_2S production at different concentrations of a CBS donor, but more experiments must be done to draw a robust conclusion. In the future it is important to analyze the H_2S production levels in the presence of a different CBS inhibitor, p.e. hydroxylamine as well as different CBS donors such as Na_2S, a sulfide salt that belongs to the same class as NaSH. It functions as a solid analog of the gas that provides direct access to H_2S. This class is widely used to evaluate the therapeutic potential of exogenous H_2S delivery (Powell et al., 2018); AP39, is a mitochondrially targeting H_2S donor (Szczesny et al., 2014); FW1256, is a slow-releasing compound with anti-inflammatory properties; (Huang et al., 2016) or GYY4137, a water-soluble molecule slow releasing H_2S donor with a prolonged effect (Powell et al., 2018). It should be interesting to evaluate the CBS protein levels by western blot since it is a cell and tissue-specific enzyme, more investigation on the location may enlighten the molecular mechanism of H_2S in

different tissues and types of cells. Since we can correlate the presence of an extra copy of CBS to the overproduction of H_2S in the various mitochondrial dysfunctions, it would be of great interest to investigate the role of the mitochondria and oxidative phosphorylation in Down syndrome. In addition to CBS, a complete investigation should be done into other H_2S-producing enzymes, such as 3-MST, various studies reported higher expression levels of this enzyme in DS models that may be associated with some of the clinical features.

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