Epigenetic Correction and Early Brain Pharmacotherapy in Down Syndrome

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Abstract: Down syndrome (DS) is the most frequent autosomal aneuploidy. It refers to a genetic condition due to the triplication of human chromosome 21 (Hsa21). Disruption of the phenotype is thought to be the result of gene-dosage imbalance. This phenotype is always characterized by neurodevelopmental anomalies. Additionally, persons with DS have higher risks of several medical challenges. These include congenital heart disease, susceptibility to viruses and immune defects, metabolic changes, and hematopoietic abnormalities. In recent years, experimental work has been conducted with the aim of correcting overexpressed genes on chromosome 21 or silencing the extra chromosome 21 to normalize genetic expression. The paper examines the clinical feasibility of these attempts and identifies several caveats. Improving neurogenesis, dendritic density and synaptic connectivity with pharmacological substances is more at hand with the current technical knowledge and legal provisos.

Keywords: Down syndrome, trisomy 21, Hsa21, *DYRK1A*, epigenetics, epigallocatechin-3-gallate, chromosome silencing, *XIST* RNA (ribonucleic acid), neurogenesis, neuronal connectivity.

INTRODUCTION

Down syndrome (DS) has an average incidence of 1 case in every 800 living births [1]. It is caused by triplication of chromosome 21 (Hsa21), the smallest autosomic chromosome of the human genome, which in turn increases expression of the Hsa21 genes. This impairs development and functioning of the brain and various body organs [2]. There is an important inter-individual variability in persons with DS. In some rare cases, for example, language may be spared and function almost normally [3]. Intellectual disability, however, is the most common hallmark ranging from mild to severe retardation.

DS exists in several forms: (1) standard (complete) trisomy 21 - T21 - (95% of the cases; karyotype 47+21); (2) mosaic T21 (1 to 2 % of the cases) where only a portion of the cells carries one extra Hsa21; (3) Robertsonian (centric fusion; nonreciprocal) translocations involving C21 are: C21 with C21, C14, and C15: formulae 46 t (21;21) + 21, 46 t (14;21) +21, and 46 t (15;21) + 21, respectively, accounting for 3% of the cases; (4) partial T21 (less than 1% of the cases) results in only a segment of Hsa21 being triplicated.

DS maps to a region on the long arm of Hsa21 corresponding to band 21q22. Hsa21 contain 225 protein-coding genes (DNA, desoxyribonucleic acid) and some 400 non-coding genes (RNA, ribonucleic acid) regulating gene expression [4].

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Current work in molecular genetics and pharmacotherapy opens the door to a biomedical treatment conducive to major improvements of the phenotype of persons with DS. Research in cytogenetics suggests the possibility of correcting DS by removing one of the three copies of chromosome 21 at the embryonic stage. However, the practical difficulties and the legal and ethical questions in this area are numerous.

The paper covers these issues. It is divided in three sections: (1) Regulating gene expression; (2) Chromosome therapy; and (3) Improving neurogenesis and neuronal connectivity

REGULATING GENE EXPRESSION

Identifying the genes overexpressed on Hsa21 that contribute to alterations in brain, behavior, and health in persons with DS is essential. Ait Yahya-Graison *et al.* [5] counted 120 genes expressed in lymphoblastic cells derived from persons with DS. Twenty-two percent of these genes are overexpressed in correspondence with the gene dosage effect (50%) and 7% amplified beyond that level.

The overexpressed genes in T21 include: *APP* (amyloid-beta precursor protein), *SOD1* (superoxide dismutase-1), *DYRK1A* (dual specificity tyrosine Y-regulation kinase 1A), *EURL* (betacateninsignaling modulator), *CBS* (cystathionine-beta synthase), *OLIG1* and *OLIG2* (oligodendrocyte transcription), *IFNAR* (alpha-interferon receptor), *CBR1* and *CBR2* (carbonylereductase), *S100B* (glial function in neurons), *ERG* (regulator of hemato-immune cells), *DSCR1* (inhibitor of calcineurin-mediated signaling),

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RCAN1 (calcineurin regulator), and *ETS2* (encoding a transcription factor).

DYRK1A, *APP*, *EURL* are involved in various cell functions and structural aspects of neurogenesis. *OLIG1/2* are responsible for myelinating axons and for oligodendrocyte differentiation. *ERG* and *RCAN1* are regulators of the central nervous system. When overexpressed these genes are among the most noxious mechanisms in brain etiopathology. For instance, *DYRK1A* transgenic mice exhibit neurogenesis alterations, brain, and behavioral abnormalities comparable to those of human beings with DS [6]. Overexpression of *DYRK1A* increases the number of spines on oblique dendrites of pyramidal neurons in the prefrontal brain of adult mice transgenic for gene DYRK1A [7]. Perturbation of *EURL* mRNA levels in mice C57BL/6 impairs progenitor proliferation and neuronal differentiation, and reduces the dendritic spine densities of cortical neurons [8]. Similar features are observed in tissue samples from human fetuses between 16-19 weeks of gestation. Overexpression of *OLIG1* and *OLIG2* in the forebrain of mice Ts65Dn leads to defective neurogenesis [9]. *OLIG2* gene dosage alters cerebral cortical interneuron development and contributes to cognitive disability in mice [10]. *ERG* gene triplication contributes to dysregulation of the homeostatic proportion of the populations of immune cells in the embryonic brain and decreases prenatal cortical neurogenesis in a mouse model [11].

Mouse models of DS are most useful in genetic research although DS in humans is orders-of-magnitude more complex. Genes in mice orthologous to Hsa21 are distributed and syntenically conserved on chromosomes 10 (39 genes), 16 (112 genes), and 17 (19 genes). Ts65Dn mice have genes corresponding to 60% of the genes harbored by Hsa21. Dp(16)1/Yey mice duplicate a 23.3-megabyte-segment of Hsa21 (119 genes). These models partially mimic DS in humans including developmental delay, learning and memory deficits [12]. Mice with full T21, i.e., with all their genes orthologous to those on Hsa21, can be created by complex crossing of genetic but they are short lived [13].

The genetic “scissor” CRISPR-Cas9 can perform precise cutting on the DNA and RNA ribbons and excise specific portions of a chromosome with enzymes that have the capacity to catalyze large DNA or RNA molecules. One could imagine removing triplicated genes on Hsa21 starting with the most noxious ones. The milder phenotype typical of persons with mosaic DS suggests that even partial corrections may have positive effects. At present, its use is forbidden in human embryos. Even safe and ethically approved, a CRISPR-Cas9 intervention involving a number of dosage-sensitive genes on chromosome 21 would be of an extreme complexity. Ongoing research with mouse models of DS will help clarifying the matter [14].

Epigenetics offers reasonable hope for improving organic development in individuals with DS by regulating gene expression. Epigallocatechin-3-gallate (EGCG) is generating much interest. It is a polyphenol of green tea with antioxidant properties that has the capacity of inhibiting the expression of the kinase encoded by gene *DYRK1A*. Experiments with genetically modified mice have showed that EGCG is efficient in rescuing various aspects of neurogenesis particularly when administered early in development [15,16]. Controlling product dosage and duration of treatment is fundamental. Chronic administration of doses of 100mg EGCG/kg/daily from embryonic times to early postnatal days in Ts64Dn mice has detrimental effects on craniofacial development. Instead, doses of 30mg/kg/day improve the facial skeleton of the animals [17].

In humans, De la Torre *et al.* [18] tested the effect of a daily treatment with EGCG green tea extracts, 9mg per kilo of weight daily, administered orally and coupled with cognitive training. The sample included 84 adolescents and adults with DS, aged 16 to 34 years. They were divided into two groups: one treated with green tea extract containing EGCG and undergoing cognitive training, the other receiving a placebo and the same cognitive training as the first group. A neuropsychological testing was made at the end of the study. Participants treated with green tea extract containing EGCG enrolled in cognitive training demonstrated a significant superiority in memory, visual recognition, and in daily routines abilities. A retest 16 months later showed partial persistence of the effects of the intervention.

Cross-sectional observations suggest that parents‘ administration of EGCG to their children with DS (dosages not available) reduces facial dysmorphies when the treatment is applied between birth and three years of age. Between 3 and 12 years, the effects of the product are milder and variable. Beyond 12 years, EGCG has no longer any effect on facial dysmorphies [17].

Parents supplementing their young children with DS with EGCG amount to 18% in the USA and EGCG dietary supplements are currently given to young children with DS at an average dose of 351mg/day [19,20]. Such uptakes should be monitored for possible side effects. In his doctoral thesis, Llambrich Ferré [21] observed indeed that chronic administration of 30mg/kg/day of EGCG from embryonic stages to TS65Dn mice although successful in improving the facial skeleton of the animals, reduces mineral density in longer bones. Extrapolating to humans, this means that chronic administration of EGCG at corresponding doses could fragilize skeletal development in children with DS. Additional studies are needed to clarify the matter.

More genes located on chromosome 21 than *DYRK1A* are overexpresed in DS. However, natural variation in gene expression may also modulate the outcome of gene-dosage imbalance [22]. As indicated earlier, Ait Yahya-Graison *et al.* [5] identified one-hundred and twenty genes expressed in lymphoblastoid cells derived from individuals with DS and controls. In individuals with DS, about one third of these genes are overexpressed either in proportion of the dosage effect expected in a trisomy (i.e., 1.5) or amplified beyond this level. These authors suggest that the remaining sequences genes are compensated post-transcriptionnally or are highly variable between individuals. If correct, that would be good news in a therapeutic perspective as fewer genes on chromosome 21 would need to be downregulated. Several reports suggest, indeed, that a proportion of genes on chromosome 21 are compensated back toward typical dosage expression levels. In contrast, other reports suggest that gene dosage compensation is not a common mechanism in DS. It may depend on the aneuploid chromosome, the tissue analyzed, and the stage of development [23].

The prevalent theory for the causes underlying the abnormalities in DS has been and is still largely that the individual phenotypes are determined by dysregulated expression of a number (unspecified yet) of the 225 protein-coding genes located on Hsa21. However, the triplication of chromosome 21 itself can simultaneously determine a general transcriptome disequilibrium partially responsible for the DS phenotype.

Examining fibroblasts from the tissues of monozygotic twins discordant for trisomy 21 and from Ts65Dn mice, Letourneau *et al.* [24, 25] reported the existence of patterns of alternating segments of decreased and increased gene expression affecting all chromosomes. They suggest that trisomy has widespread consequences on the chromatin environment throughout the genome. However, Do *et al.* [26] in a reanalysis of the human and mice data of the Letourneau group failed to confirm the presence of dysregulated sequences of gene expression across the genome. The question remains open. It is complicated by the natural variation in genomic expression.

CHROMOSOME THERAPY

Eliminating or inactivating one chromosome 21 in trisomic cells has been realized *in vitro*. Induced pluripotent stem cells (iPSCs) can be generated from adult human dermal fibroblasts through genetic engineering using transcription factors. Pluripotent stem cells are capable of differentiating into a limited set of specialized cells such as blood, liver, heart, or brain cells, but not all types of cells as is the case of embryonic or so-called omnipotent or multipotent stem cells originating from the inner mass of the embryonic blastocyst.

Li *et al.* [27] generated iPSCs from fibroblasts obtained from adults with DS. They introduced a *TKNEO* fusion transgene carried by a modified adenovirus at the locus 21q21.3 of the gene *APP* (amyloid-beta precursor protein) into one copy of Hsa21. A transgene is foreign DNA inserted into a genome. The operation resulted in spontaneous loss of an entire copy of Hsa21 in a large majority of the clones treated. No damage to other chromosomes was observed. Disomic cells proliferated faster in a co-culture than their trisomic counterparts doubling their population on average in about 37 ± 0.7 hours against 45 ± .09 hours for trisomic counterparts.

Else, nature has evolved a mechanism to compensate for the difference in dosage of X-linked gene copies between mammalian females and males. In humans, the formulae for the sex chromosomes are XY for males and XX for females. The Y chromosome is much smaller than its X counterpart is. It contains only a few dozen genes compared to about 3000 for the X. Natural X dosage reduction in females is driven by a large non-coding RNA, named *XIST* (for X-inactive specific transcript), produced from the inactive X chromosome. This RNA inactivates the DNA of this chromosome through methylation and chromatin modification turning it into a Barr body. Another RNA in mammalian females antagonist of *XIST* is named *TSIX* (anagram for *XIST*). *XIST* and *TSIX* neutralize each

other on the X chromosome that remains active, whereas the expression of *TSIX* is stopped on the inactivated X chromosome.

Jiang *et al.* [28] reprogrammed fibroblasts obtained from human males with DS into iPSCs. They inserted a transgene *XIST* at locus 21q22 of the gene *DYRK1A* in one of the three Hsa21. This silenced this chromosome in 85% of the clones treated. Silencing of a dozen genes on the inactivated Hsa21 was confirmed. No alterations of the other chromosomes were observed. As in the preceding experiment, disomic cells exhibited a capacity for *in vitro* proliferation above trisomy counterparts. This warrants that the maneuver is not toxic and has beneficial effects on cell proliferation and viability. The authors reported that the global expressivity of the two active Hsa21 was reduced by 20, 15, and 19%, respectively in the three clones tested, which is close to the 22 % usually observed in disomic aspics that lack the third Hsa21 altogether. This suggests that the *XIST* RNA inserted in the extra Hsa21 covers key regions of this chromosome preventing transcription factors from reading the sequence of nucleic acids.

These are remarkable achievements. Jeanne Lawrence, head of the team at the University of Massachusetts who published the Jiang *et al.* report [28] indicated in an interview to The Guardian International Edition (Wednesday 17 July 2013) that her team was starting *in vivo* research to prevent DS in genetically modified mice by silencing one extra chromosome 21 in early stage embryo, which should correct the whole mouse. She acknowledged, however, that it would not be practical in humans.

Besides legal, ethical and further technical questions, there is indeed a delicate time knot. Embryo cleavage kinetics routinely checked in the context of *in vitro* fertilization is assessed at day 1 (early cleavage, 25–27 hours after insemination of the oocyte), at day 2 (4 cells), and at day 3 (8 cells) [29]. Embryo quality assessment can also be founded on genetic analysis as first gene expression occurs between the first four- and eight-cell stages [30]. Preimplantation genetic tests are performed for detecting aneuploidies. They require a biopsy of the embryo aged 3 days for removing one or two cells. At day 5 or 6, trophectoderm (the external layer of the embryo) biopsy is now recommended as the result of the tests is more accurate due to a better implantation potential of the embryo [31].

Complete chromosome correction requires blastomeric biopsy and genetic analysis to ascertain T21 before inserting the biologic agent able to normalize the aneuploidy. The treated cell is then reimplanted into the embryo expecting cell proliferation to proceed normally from there on. The theoretical and technical knowledge needed for practicing such delicate maneuvers is not presently available. Binding legislations in most countries limit fundamental research on human embryos and embryonic stem cells as they involve destroying the embryos.

In order to normalize embryonic development all of the 8 cells at day 3 post-insemination would have to undergo chromosome correction. At this stage, embryonic cells are multipotent. An intervention on fewer stem cells would induce a mosaicism of cells with the normal number of chromosomes and others trisomic. Chromosome correction at day 2 after insemination would not need to be performed on the 4 blastomeres as these earlier stem cells are totipotent, which means that each one is capable of generating a complete organism (eliminating spontaneously the other three blastomeres in the process). However, whether performed at day 2 or 3 embryonic life, there would not be enough time between insemination, genome assessment, and chromosome editing for rendering the intervention practical.

However, a recent study by Czerminsky and Lawrence [32] suggest that contrary to prior belief the epigenetic plasticity of DS iPSCs is retained at least 35 days beyond the pluripotent stage. At this time, it is still strong enough to initiate chromosome-wide repression in neural stem cells (NSCs) differentiating into neurons. The neural cells need to express *XIST* RNA longer to silence most genes. In pluripotent stem cells, *XIST* silences the chromosome in three days but in differentiated cells it takes one or two weeks.

This finding opens the way to epigenetic chromosome correction beyond early stages of embryo development. Correcting a deficiency in the process of differentiation of trisomic neural stem cells into neurons appears to be possible by inducing *XIST* at different stages in neurogenesis. A further study by the same group of researchers [33] shows that the same strategy can be applied to iPSCs differentiating into other cell lines in fetal development. They showed in an *in vitro* model of human fetal hematopoiesis that *XIST* induction reliably corrects overproduction of megakaryocytes and erythrocytes (linked to DS myeloproliferative disorder) in transgenic clones. This suggests that inducible trisomy silencing could be envisaged for other cell types during fetal development

even without identification of the pathogenic genes. If there are cell-type specific effects with clear clinical relevance, that would also mean that trisomy silencing is able to correct an entire developmental program and not just some global aspects such as overproduction stress.

IMPROVING NEUROGENESIS AND NEURONAL CONNECTIVITY

Neurogenesis impairment during the fetal stage in DS [34] has two causes: (1) abnormalities during neural differentiation of iPSCs into NSCs and cell cycle alterations reducing proliferation of NPCs and leading to brain hypotrophy (between 10 and 30% reduction in weight, size, and volume); (2) augmented differentiation of NPSc into glial elements (oligodendrocytes and astrocytes) at the expense of their differentiation into neural cells. Neuron maturation is also abnormal with reduced dendritic areas, dendritic spine density, and reduced neuronal connectivity [35].

Stagni and Bartesaghi [36] have identified a series of genes responsible for neurogenesis impairment in DS, among which *DYRK1A* and *APP*, highly expressed during early development, and several of the genes mentioned in the first section of this paper. A few genes involved in neuron maturation anomalies have also been identified. They include *DSCAM* (Down syndrome cell adhesion molecule) that plays a role in dendritic and synaptic development and *C21orf91* (early undifferentiated retina and lens), which in excessive level concurs to reduce spine density.

The same authors have reviewed 40 prenatal and neonatal pharmacological attempts to improve neurogenesis, dendritic and connectivity in mice using either natural (e.g., rapamycin, melatonin, EGCG, curcumin, cyclosporine, oleic acid) or non-natural substances (e.g., fluoxetine, clenbuterol, salmeterol). The resulting picture varies with the product and the developmental aspect considered. The studies that targeted neural progenitor cell proliferation with natural substances (except melatonin) showed a short-term positive effect. Fluoxetine was the only product to have a longer-term effect. As to dendritic development, the effects of all substances tended to disapear with time.

Natural substances, except oleic acid, administered during the first two postnatal weeks (corresponding to the third semester of gestation in humans), have only a short-term effect on neurogenesis, hippocampal proliferation, and dendritic development. In contrast, the studies with non-natural substances show longer-term efficiency.

The neurobiology of DS results also in a reduction of synaptic plasticity. Gotti *et al.* [37] have reviewed a series of studies with mouse models of DS showing that different neurotransmission systems are dysregulated in the hippocampus and the frontal cortex.

Major brain neurotransmission systems are the cholinergic system (excitatory, neurotransmitter acetylcholine), the noradrenergic system (mostly excitatory, neurotransmitter noradrenaline), the glutamate system (excitatory, neurotransmitter glutamate), the GABAergic system (inhibitory, neurotransmitter GABA standing for gamma-aminobutyric acid), and the serotoninergic system (also inhibitory, neurotransmitter serotonin). The synthesis of the neurotransmitter occurs within the presynaptic nerve terminal. It is stored in secretory vesicles before being released in the synaptic space between the pre- and the post-synaptic neurons.

Drugs are being developed for reducing the neurotransmission deficits caused by DS. An efficient strategy is to inhibit the enzymatic cleavage of the neurotransmitter in the synaptic space. Bartesaghi *et al.* [38] have analyzed the results of a series of experimental and clinical attemps to improve the cholinergic, the glutamatergic, and the GABAergic systems in DS. They found no solid empirical support for the treatments aiming at increasing acetylcholine recapture in children and young adults with DS. In contrast, treatment with the NMDA receptor antagonist memantine (NMDA, for N-methyl-D-aspartic acid, is a molecule that mimics the action of neurotransmitter glutamate) improves cognitive measures and learning in TS65Dn mice and young adults with DS.

CONCLUSION

The latest decades have witnessed remarkable advances in biomedical research that open the door to an improvement of the genetic and physiological conditions of persons affected by DS. Treatment at early embryonic stage would cure the whole organism but is prevented by a series of obstacles of various sorts. Fetal intervention may be more practical pending a series of additional research and clinical work. Epigenetic gene correction is possible but the number of genes to be corrected is important. One could silence the most noxious genes in T21 in priority. That

would help normalizing the DS phenotype to some extent.

Pharmacotherapy in DS is still in its beginning stage. Clinical advances have been rare so far. However, the perspectives regarding the possibility of improving neurogenesis and neuronal connectivity are real.

LIST OF ABBREVIATIONS

APP = amyloid-beta precursor protein

CRISPR-Cas9 = clustered regularly interspaced short palindromic repeats associated Cas9 protein nuclease

DNA = desoxyribonucleic acid

DS = Down syndrome

EGCG = epigallocatechin-3-gallate

GABA = gamma-aminobutyric acid

Hsa21 = human chromosome 21

iPSCs = induced pluripotent stem cells

NMDA = N-methyl-D-aspartic acid

NSCs = neural stem cells

RNA = ribonucleic acid

T21 = trisomy 21

XIST = X-inactivating specific transcript

CONFLICT OF INTEREST

The author declares no conflict of interest.

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