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Dosage Compensation of Trisomy 21 and Its Implications for Hematopoietic Pathogenesis in Down Syndrome

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DOSAGE COMPENSATION OF TRISOMY 21 AND ITS IMPLICATIONS FOR HEMATOPOIETIC PATHOGENESIS IN DOWN SYNDROME

A Dissertation Presented

By

JEN-CHIEH CHIANG

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This work was undertaken in the Graduate School of Biomedical Sciences

Cell Biology Program

The signature of the Thesis Advisor signifies validation of Dissertation content

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Abstract

Down Syndrome (DS), the most common aneuploidy seen in live-borns, is caused by trisomy for chromosome 21. DS imposes high risks for multiple health issues involving various systems of the body. The genetic complexity of trisomy 21 and natural variation between all individuals has impeded understanding of the specific cell pathologies and pathways involved. In addition, chromosomal disorders have been considered outside the hopeful progress in gene therapies for single-gene disorders. Here we test the feasibility of correcting imbalanced expression of genes across an extra chromosome by expression of a single gene, *XIST*, the key player in X chromosome inactivation. We targeted a large *XIST* transgene into one chromosome 21 in DS iPS cells, and demonstrated *XIST* RNA spreads and induces heterochromatin and gene silencing across that autosome *in cis*.

By making XIST inducible, this allows direct comparison of effects of trisomy 21 expression on cell function and phenotypes. Importantly, *XIST*-induction during *in vitro* hematopoiesis normalized excess production of differentiated blood cell types (megakaryocytes and erythrocytes), known to confer high risk for myeloproliferative disorder and leukemia. In contrast, trisomy silencing enhances production of iPS and neural stem cells, consistent with DS clinical features. Further analysis revealed that trisomy 21 initially impacts the endothelial hematopoietic transition (EHT) to generate excess $CD43^{+}$ progenitors, and also increases their colony forming potential. Furthermore, results provide evidence for a key role for enhanced *IGF* signaling, involving over-expression of non-chromosome 21 genes controlled by trisomy 21. Finally, experiments to examine trisomy effects on angiogenesis showed no effect on

production of endothelial cells, but it remains unclear whether trisomic cells may differ in ability to form vessels.

Collectively, this thesis demonstrates proof-of-principle for *XIST*-mediated "trisomy silencing". Phenotypic improvement of hematopoietic and neural stem cells demonstrates the value for research into DS pathogenesis, but also provides a foundation of potential for future development of "chromosome therapy" for DS patients.

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Jiang, J., Y. Jing, G.J. Cost, J.C. Chiang, **H.J. Kolpa**, A.M. Cotton, D.M. Carone, B.R. Carone, D.A. Shivak, D.Y. Guschin, J.R. Pearl, E.J. Rebar, M. Byron, P.D. Gregory, C.J. Brown, F.D. Urnov, L.L. Hall, and J.B. Lawrence. (2013) Translating dosage compensation to trisomy 21. *Nature.* **500**(7462):296-300.

List of Abbreviations

AGM	Aorta-gonad-mesonephros
ALL	Acute B-lymphoblastic leukemia
AMKL	Acute megakaryocytic leukemia
APP	Amyloid Beta Precursor Protein
AVSD	Atrioventricular septal defects
CHD	Congenital heart disease
CLP	Common lymphoid progenitors
CMP	Common myeloid progenitors
CNV	Copy number variations
DAPI	4',6-diamidino-2-phenylindole
DS	Down Syndrome
EHT	Endothelial-to-hematopoietic transition
EMP	Erythroid-myeloid progenitors
EOAD	Early-onset of Alzheimer disease
ESC	Embryonic stem cell
GMP	granulocyte-monocyte progenitors
HE	Hemogenic endothelium
HSA21	Human chromosome 21
HSC	Hematopoietic stem cell

HSPC	Hematopoietic stem and progenitor cell
iPSC	Induced pluripotent stem cell
IQ	Intelligence quotient
MEP	megakaryocyte-erythrocyte progenitors
MPP	multipotent progenitors
MRI	Magnetic resonance imaging
PGD	Pre-implantation genetic diagnosis
PGS	Pre-implantation genetic screening
PRC1	Polycomb repressive complex 1
PRC2	Polycomb repressive complex 2
PSC	Pluripotent stem cell
SNP	Single nucleotide polymorphisms
TMD	Transient myeloproliferative disorder
VSD	Ventricular septal defects
XCI	X chromosome inactivation
XIC	X chromosome inactivation center
XIST	X inactive specific transcript
ZFN	Zinc finger nucleases

Chapter I: Introduction

Down syndrome (DS) is the most frequently occurring chromosomal disorder and the leading cause of intellectual disability, resulting from the presence of an additional copy of chromosome 21. Besides the neurological system, DS individuals exhibit a variety of pathologies associated with many different body systems, caused by increased activities of chromosome 21 genes, including the hematopoietic and immune systems. One obvious approach for developing therapies for a genetic disorder is to understand the pathogenic mechanism and correct the dysregulated genes or pathways. However, since there are over four hundred coding and non-coding genes on chromosome 21, it is technically impractical to correct the dosage of these genes one by one. Additionally, simultaneous expression of multiple genes at the theoretical 1.5-fold disturbs many biochemical pathways in many organs, manifested by pathologies in many body systems in DS individuals, which makes it extremely difficult to identify and understand the pathogenic mechanisms. Moreover, genetic variations among individuals further complicate the identification of critical genes and pathways that cause symptoms. Therefore, the DS research field still has a relatively insufficient understanding about the detailed pathogenic mechanism involved (for most aspects of the syndrome), in order to devise an efficient therapy for DS patients. To avoid these complexities, we took a novel approach in this thesis work to transcriptionally silence one chromosome 21 by *XIST* RNA mediated chromosome inactivation, which we termed “trisomy silencing”. This system allows us to demonstrate normalization of DS related cellular phenotypes and to study the pathogenic mechanisms in the context of the hematopoietic system. Demonstrations of phenotypic improvement in DS iPSCs from silencing one

chromosome 21 are also included. In this chapter, introductions for various topics including Down syndrome, hematopoiesis, X chromosome inactivation, and disease modeling using induced pluripotent stem cells are provided as essential background knowledge for the research in this thesis.

The challenges of Down syndrome

Chromosomal abnormalities are a large part of the genetic burden in infants, impacting 1 in 140 live births, and Down syndrome is the most common viable human aneuploidy. Aneuploidy is present when cells contain an incorrect number of chromosomes, or another form of chromosomal imbalance due to translocations, duplications, and deletions. In most cases, aneuploidy for a whole chromosome causes death of the developing fetus, accounting for 50% or more of miscarriages. Loss of an autosome is embryonic lethal, but there are a few types of autosomal trisomy that are seen in newborns, including trisomy for 21, 18, and 13 (Driscoll and Gross 2009). The majority of trisomy 13 and 18 infants die within the first few years, however, individuals with trisomy 21, the cause of Down syndrome, can live into their 60's or beyond, but face several medical challenges characteristic of Down syndrome. While this thesis work focuses on Down syndrome, we note that the innovative approach to silence an extra autosome would also be applicable to translational research and possibly a therapeutic strategy for the other viable autosomal trisomies of chromosome 13 and 18.

Although the first description of Down Syndrome (DS) phenotypes was made by Langdon Down in 1866 (Down 1995), the cause of DS was not clear until scientists confirmed the correct number of chromosomes in human cells. In 1959, Lejeune, Gautier,

and Turpin were able to identify the consistent chromosomal abnormality in karyotypes prepared from DS individuals, trisomy 21. The most common cause of trisomy 21 is meiotic nondisjunction during maternal oogenesis (Figure 1.1). While trisomy 21 is generally not heritable, in a small subset of cases, DS can be heritable when a parent carries a balanced translocation in which part of chromosome 21 is attached to another chromosome, frequently chromosome 14. Patients who are mosaic have a mixture of euploid and trisomy 21 cells and noticeably many mosaic patients have much less severe phenotypes. Importantly, parents with isochromosome 21, in which one chromosome 21 is fused with another, will have 100% of viable children with DS.

The frequency of Down syndrome is approximately one in 800 newborns in the United States today. The most influential factor for DS pregnancy is maternal age, because the frequency of meiotic nondisjunction increases in women with age. Although women older than 35 have a substantially increased risk for DS pregnancy, most babies with Down syndrome are born to younger pregnant women, as younger women constitute the majority of pregnant women. Additionally, DS pregnancies are associated with an increased rate of miscarriage and stillbirth, with several studies suggesting that up to 70% of DS fetuses are lost, especially with increased maternal age (Savva et al. 2006).

With hundreds of genes being triplicated and overexpressed, DS individuals can display significant pathology in various systems of the body, but some features are more consistent than others. The syndrome is consistently characterized by dysmorphic facial features, delayed psychomotor development, mild to moderate intellectual disability and cognitive impairment. Early onset of Alzheimer’s disease is also highly common in DS individuals. Other pathologies that are commonly observed in subsets of DS patients

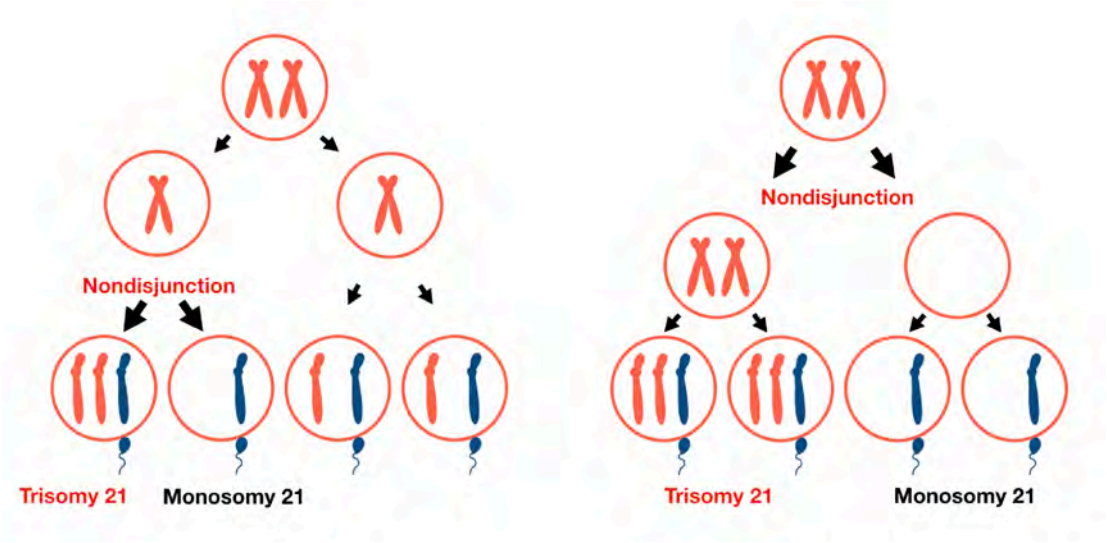


Figure 1.1 Meiotic nondisjunction causes trisomy 21.

Nondisjunction during meiosis contributes to most cases (~95%) of Down syndrome. Chromosomes colored in red and blue represent maternal and paternal chromosome 21, respectively. In this example, meiotic nondisjunction is illustrated in the eggs but these can also happen in the opposite way where the sperms have too many or too few copies of chromosome 21 due to meiotic nondisjunction.

include congenital heart disease, seen in ~40% of patients, acute lymphoblastic leukemia, 20 times more common, acute megakaryocytic leukemia, 500-fold more frequent than the general population, and higher incidence of respiratory infections (Figure 1.2) (Antonarakis 2017).

Impaired cognitive functioning is the consistent defect of DS individuals

Although clinical symptoms of DS vary from individual to individual, impaired cognition is recognized as the most consistent and pressing feature of DS. Almost all DS patients have difficulty in learning, memory, and language that lead to mild to severe intellectual disability. Although lower IQ scores are seen in DS individuals, children score as only "mildly" or "moderately" affected, but cognitive impairment can progress to "severe" in adults (Couzens et al. 2011; Couzens et al. 2012). Particularly, verbal processing and speech are often a weakness associated with DS individuals, with about half of DS children having difficulty in articulation of speech. In adults, the disabilities related to language skills can become more prominent with increasing age and other aspects of cognitive functions, such as learning and memory, are adversely affected as well. Although many DS patients are sociable and cooperative, and can form strong relationships, impaired cognitive functioning can also affect social development of DS individuals to some extent, which, combined with other pathologies of DS, significantly impact the quality of life of DS individuals and their families. MRI studies using post-mortem brains from DS patients revealed reduced brain size and brachycephaly (shorter skull), particularly the frontal and temporal areas that include the uncus and hippocampus are disproportionally smaller in DS brains. These anatomic abnormalities in brain are

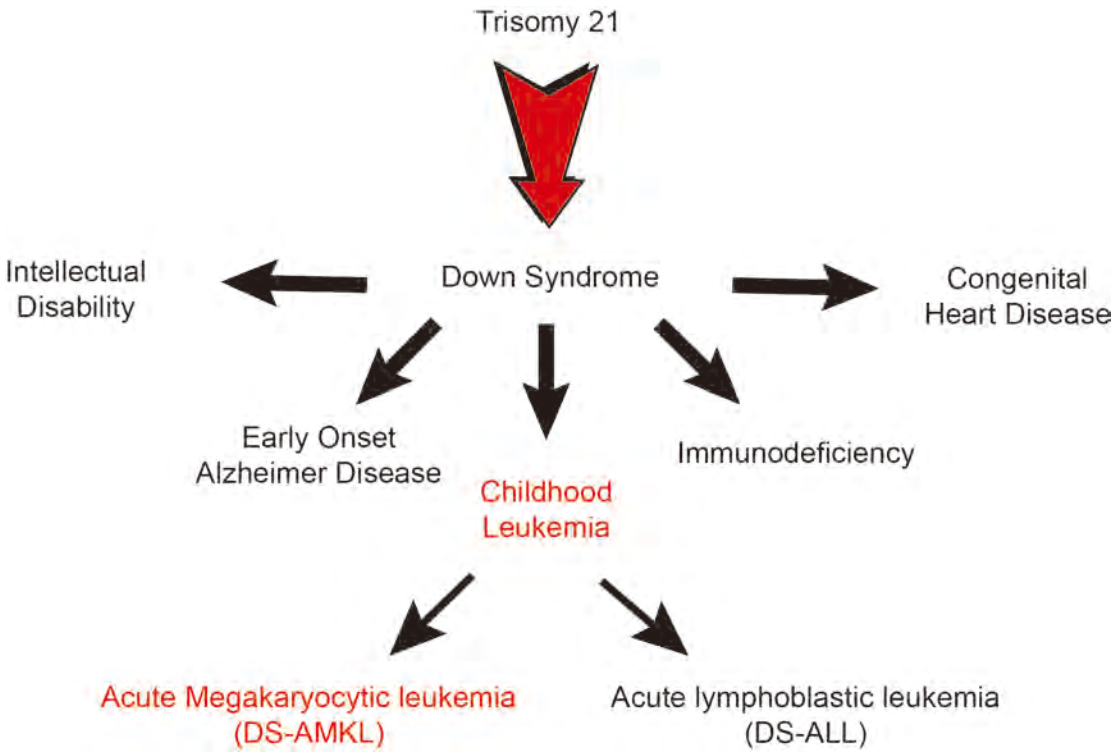


Figure 1.2 Major characteristics of Down syndrome.

Down syndrome, caused by trisomy 21, is characteristic by numerous defects within the body. A major portion of this thesis work will focus on the leukemia aspect of the syndrome, with emphasis on acute megakaryocytic leukemia.

likely the underlying causes of the impaired cognitive functioning in DS patients (Lott 2012).

DS individuals are at higher risk for early-onset of Alzheimer disease

Early-onset of Alzheimer disease (EOAD) is defined as Alzheimer disease diagnosed before the age of 65. With advances in medical care, life expectancy of DS patients has increased and it is now recognized that they have an increased risk of EOAD. While other genes on chromosome 21 may contribute, the presence of a third copy of the *APP* gene on chromosome 21 is thought to be the primary cause (Wiseman et al. 2015). *APP* encodes a protein called amyloid precursor protein that is naturally cleaved into several peptides, some of which are released outside the cell, including one called amyloid beta peptide. Since one of the observations from people with Alzheimer's disease is the accumulation of clumps of amyloid beta peptide, called amyloid plaques, in the brain, increased dosage of the *APP* gene in DS patients is widely thought to underlie the EOAD in DS (Wiseman et al. 2015). The accumulation of amyloid plaques may interact with subcellular organelles of the neurons, triggering neuronal dysfunction and apoptosis that ultimately lead to memory decline and dementia. Increased expression of *APP* in a mouse model of DS has been shown to cause disruption of nerve growth factor transportation and may result in cholinergic neuron degeneration in the forebrain (Salehi et al. 2006). This might be related to the disruption of the endocytosis pathway caused by APP mediated RAB5 activation that results in enlargement of endosomes (Xu et al. 2016). In addition, triplication of the *APP* gene alone in the normal non-DS population causes early-onset of Alzheimer's disease (Sleegers et al. 2006). Other genes on chromosome 21

involved in endocytosis pathway are also thought to contribute to EOAD in DS, including *CSTB* and *SYNJ1* (Cossec et al. 2012; Zhu et al. 2013). Therefore, DS is a good model for studying Alzheimer's disease and more detailed pathogenic mechanism remain to be explored.

Congenital heart disease is commonly associated with DS newborns

Congenital heart disease (CHD) is another major common phenotype in DS, clinically observed in about 40% of DS patients (Ferencz et al. 1989). The most common form of DS associated CHD is atrioventricular septal defects (AVSD), accounting for 43% of DS-CHD cases. Other forms of DS associated CHD includes ventricular septal defects (VSD), atrial septal defects (ASD), and tetralogy of Fallot that constitute 32%, 19%, and 6% of DS-CHD, respectively. By analyzing rare partial trisomy 21 cases (individuals with duplication of only a small region of chromosome 21) associated with CHD, one study suggests the segment of chromosome 21 required to develop CHD can be narrowed to a 1.77 Mb region between genes *DSCAM* and *ZBTB21* (Barlow et al. 2001; Korbel et al. 2009). Another study using multiple animal models further suggested a role for *DCSAM* and *COL6A2* overexpression in the development of ASD (Grossman et al. 2011). In addition to specific chromosome 21 genes, scientists also suggested single nucleotide polymorphisms (SNP) and copy number variations (CNV) are involved in determining the risk of developing CHDs in DS patients (Sailani et al. 2013). However, a detailed mechanism about the pathogenesis of DS-CHD is still lacking and more large-scale genomic analyses of DS-CHD and DS-non-CHD patients are needed.

DS-associated hematopoietic abnormalities cause increased chance of childhood leukemia and compromised immune functions

The hematopoietic system appears to be impacted by trisomy 21 as DS individuals suffer from multiple hematopoietic defects, including increased risks for childhood leukemia and for infections, partially attributed to immunodeficiency. Two major types of leukemia commonly seen in DS children are acute B-lymphoblastic leukemia and acute megakaryocytic leukemia. A significant portion of this thesis work will be dedicated to demonstrating correction of cellular phenotypes associated with acute megakaryocytic leukemia.

DS-associated acute B-lymphoblastic leukemia

The most frequent childhood leukemia among the general population is acute B-lymphoblastic leukemia (ALL) and the incidence of ALL is approximately 20-fold higher in DS children. DS children with ALL (DS-ALL) have poorer survivor rates than non-DS children with ALL (non-DS-ALL). Gene expression analysis of DS-ALL reveals that it is a highly heterogeneous disease for which it is difficult to elucidate the molecular causes of pathogenesis. However, more than half of the DS-ALL cases have overexpression of *CRFL2* caused by chromosomal rearrangements or deletions that create *P2RY8-CRFL2* chimeric transcripts (Mullighan et al. 2009). Additionally, aberrant *CRLF2* expression in DS-ALL cases are associated with mutations in either *JAK2* or *RAS* genes, suggesting cooperative effects of these two genetic events in the pathogenesis of DS-ALL (Mullighan et al. 2009; Hertzberg et al. 2010; Nikolaev et al. 2014). These genetic features of DS-ALL are likely to have adverse impacts on the prognosis for

chemotherapy and thus result in lowered overall survival rates compared to non-DS-ALL patients. Although none of these genes are located on chromosome 21, overexpression of *HMGN1*, a nucleosome remodeling gene encoded on chromosome 21, in B cells suppresses global level of H3k27me3 and promotes proliferation of B progenitor cells and development of ALL in T65Dn mice, a mouse model of DS (Figure 1.3) (Lane et al. 2014). Interestingly, the most frequent chromosomal abnormalities associated with non-DS-ALL cases are acquired trisomy 21 and tandem triplication and quadruplication of the long arm of chromosome 21 (Baialardo et al. 1996; Heerema et al. 2007). Notably, many of these cases involve additional copies of the *RUNX1* gene, a chromosome 21 gene commonly associated with translocation in multiple types of leukemia (Harewood et al. 2003). Therefore, these findings provide a rationale behind the increased risk of ALL in DS children.

DS-associated acute megakaryocytic leukemia

Another type of leukemia that has an increased incidence in DS children is acute megakaryocytic leukemia (AMKL), a subtype of acute myeloid leukemia, which is the main focus of this thesis work. While the frequency in the general population is very rare, DS children have a 500-fold increased risk for AMKL (Al-Kasim et al. 2002). AMKL is characterized by abnormal expansion of megakaryoblasts, the precursor of megakaryocytes (platelet-producing cells), in circulating blood and the bone marrow. The megakaryoblasts ultimately replace normal bone marrow elements, resulting in a decreased number of red blood cells, white blood cells, and platelets that further cause

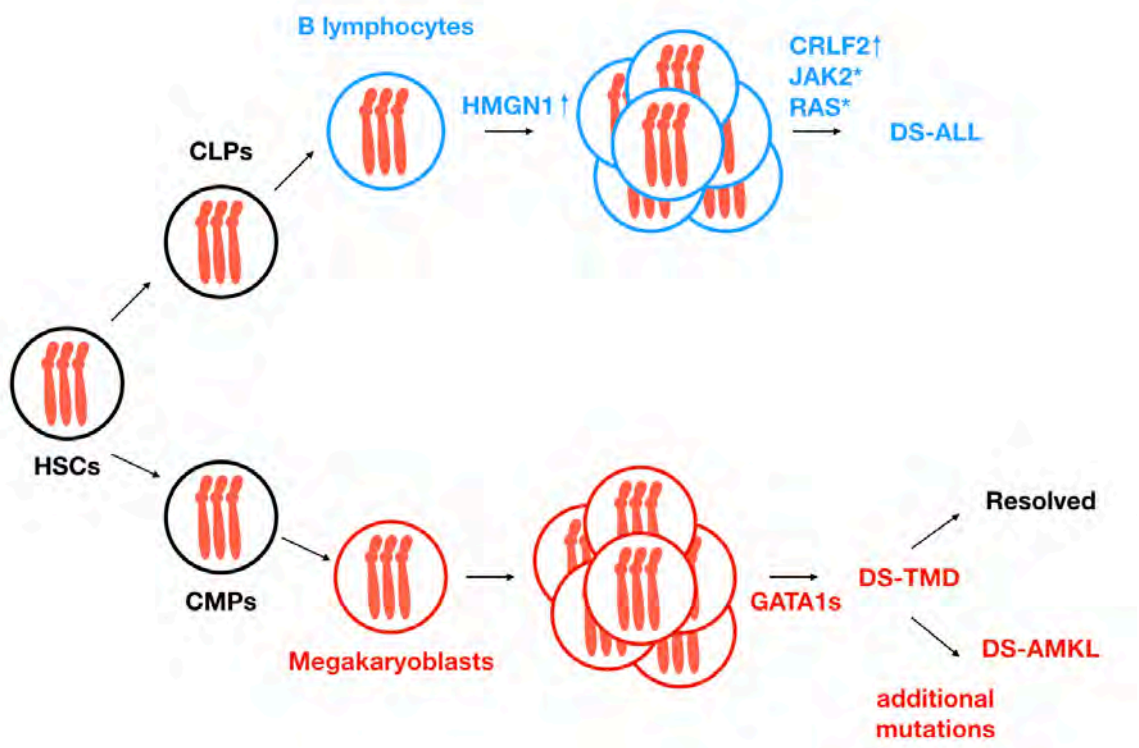


Figure 1.3 Pathogenesis in Down syndrome associated leukemia.

Hypothesized mechanisms for Down syndrome associated acute lymphblastic leukemia (DS-ALL) and acute megakaryocytic leukemia (DS-AMKL) are illustrated. For DS-ALL (upper part of the figure), the presence of three copies of *HMGN1* gene, which encodes nucleosome remodeling protein, promotes B cell proliferation and with additional driver mutations on either *JAK2* or *RAS* cause the progression to DS-ALL. For DS-AMKL (bottom part of the figure), trisomy 21 alone promotes over-proliferation of megakaryocytes and in combination with acquired somatic mutations on *GATA1* gene that causes exclusive expression of GATA1s, a shorter form of GATA1, result in transient myeloproliferative disorder (DS-TMD). Although a portion of DS-TMD cases resolves spontaneously, additional driver somatic mutations can drive the progression of some DS-TMD cases to DS-AMKL.

anemia, recurring infections, and bleeding disorders, respectively. DS-AMKL usually occurs before DS children reach the age of 4, and similar to non-DS-AMKL, DS-AMKL is associated with bone marrow fibrosis, presumably due to an increased number of megakaryocytes or megakaryoblasts (Roy et al. 2009).

The most unique characteristic of DS-AMKL is its association with a hematopoietic abnormality commonly seen in DS newborns, transient myeloproliferative disorder (TMD). TMD occurs in approximately 10-15% of all DS newborns and is characterized by increased circulating megakaryoblasts, which normally should be present in the bone marrow. Other characteristics include an increased amount of red blood cells, enlarged liver with megakaryocytic infiltration, and liver fibrosis. TMD can present substantial medical issues with approximately 20% mortality rate due to severe liver damage and complications in heart and lung. However, the majority of DS-TMD cases do not cause clinical symptoms in DS newborns despite massive numbers of megakaryoblasts in the blood. Interestingly, most DS-TMD cases recover spontaneously. However, a substantial fraction (about 20%) of DS-TMD cases further develop into DS-AMKL. Although more experimental evidence is needed for this hypothesis, the spontaneous recovery of DS-TMD is probably due to the fact that TMD is a fetal origin disorder resulting from dysregulation of megakaryopoiesis during fetal liver hematopoiesis, the change of environment for hematopoiesis from liver to bone marrow after birth resolves the disorder.

The evidence that DS-TMD as the precursor of DS-AMKL came from the finding that the blast cells (overproduced megakaryoblasts) in both disorders harbor the same somatic mutations in the *GATA1* gene that causes exclusive expression of *GATA1s*, the

shorter form of *GATA1* (Roberts et al. 2013). These mutations consist of various short deletions, insertions, and point mutations that cluster within exon 2, and results in an introduction of a premature stop codon. The use of a downstream alternative initiator codon results in translation of *GATA1s* that lacks the amino-terminal transcriptional activation domain (Wechsler et al. 2002). *GATA1* is located on the X chromosome and is an important hematopoietic transcriptional factor involved in erythropoiesis and megakaryopoiesis (Kuhl et al. 2005). Interestingly, these *GATA1s* mutations are only detected in blast cells of DS-TMD and DS-AMKL and not in any other somatic cells in DS individuals. AMKL patients in the non-DS population or DS patients who have other types of leukemia do not have these mutations either, suggesting *GATA1s* is an unique feature shared between DS-TMD and DS-AMKL. However, DS-AMKL blast cells are also usually associated with various additional mutations that are not detected in DS-TMD blast cells. These observations suggest that DS-AMKL blast cells are a clonal evolution of DS-TMD blast cells and additional genetic events are required for the progression from DS-TMD to DS-AMKL (Nikolaev et al. 2013; Yoshida et al. 2013). Although GATA1s mutations are thought to be the cause for both DS-TMD and DS-AMKL (Ahmed et al. 2004; Ge et al. 2006), excessive production of hematopoietic cells, particularly erythroid and megakaryocytic cells, have been observed as early as the second trimester in DS fetal liver, without detectable *GATA1* mutations that contribute to TMD and leukemia (Chou et al. 2008; Tunstall-Pedoe et al. 2008). Hence, this suggests that trisomy 21 alone may be sufficient to cause hematopoietic abnormalities in fetal liver hematopoiesis, a precursor for later hematopoietic abnormalities commonly developed in DS children. Additionally, GATA1s mutations alone without trisomy 21 fail to initiate

leukemia-like symptoms in both human and mouse models. Therefore, DS-AMKL is a multistep disease initially involving yet to be defined effects of trisomy 21 on hematopoiesis, followed by acquired GATA1s mutations that cause DS-TMD, and then multiple other mutations that ultimately result in DS-AMKL (Figure 1.3). A key point in understanding the contribution of trisomy 21 is the extent to which abnormalities in DS hematopoiesis arise prior to GATA1s mutations. Work in this thesis will bear on this question by studying hematopoiesis as a function of trisomy 21 without the GATA1s mutations. Use of the experimental system here allows us to investigate changes prior to GATA1s mutations as well as examine the potential of correction by "trisomy silencing" with *XIST* RNA (as further explained below).

Currently, the most effective treatment for DS-AMKL is chemotherapy. Interestingly, compared to non DS-AMKL, DS-AMKL have significantly higher event-free survival rates after cytosine arabinoside based chemotherapy. However, despite effective chemotherapy for DS-AMKL, DS-ALL patients usually don't respond as well and need more aggressive treatments than non-DS-ALL patients. Regardless, chemotherapy is still a difficult process for all DS children with leukemia and the results of this thesis work to serve as a potential alternative therapeutic approach in the future. Additionally, use of this experimental system may also help uncover mechanisms for improved chemotherapy responsiveness, which could be beneficial for non-DS-AMKL patients that are relatively difficult to treat by chemotherapy.

DS-associated immunodeficiency

In addition to the subset of DS children who have DS associated childhood leukemia, it is also important to note that less severe hematopoietic abnormalities are also seen in almost all DS patients, with potentially broad effects on health. A major one of these involves the immune system (Roizen and Amarose 1993; David et al. 1996; de Hingh et al. 2005; Bloemers et al. 2010).

DS children suffer from greatly increased infection rates compared to normal children, mainly in the upper respiratory tract. These frequent infections in DS patients are characterized by increased severity and a prolonged course of disease, and historically, respiratory infections had been the major cause of death. Several defects in immune compartments have been described and are postulated to contribute to increased infections in Down syndrome. For the innate immune system, defective neutrophil chemotaxis and low humoral immune response are most consistently reported to be associated with respiratory infections (Khan et al. 1975). For the adaptive immune system, the normal lymphocyte expansion in infancy is absent and mild to moderate B and T cell lymphopenia (abnormal low levels of lymphocytes in the blood) are observed in DS children (de Hingh et al. 2005). Additionally, the size of thymus, a lymphoid organ in which T cells mature, in DS children is smaller than age-matched controls (Prada et al. 2005). Despite major well documented abnormalities in the DS immune system, the genetic mechanisms determining these immunological deficits are still not well defined or not heavily studied.

Although research in DS has not focused on immunodeficiency, recent evidence suggesting involvement of immune responses in the development of neurological disorders provides a renewed interest and driving force for scientists to investigate the DS

immune system (Liewluck and Miravalle 2015). Since all immune players are derived from the hematopoietic system, abnormal hematopoiesis may well contribute to not only immunological but also neurological defects in DS. Therefore, correction of hematopoietic abnormalities may have broader implications for immunological and neurological aspects of DS, beyond the leukemia aspect studied here.

Efficient treatments for DS are still lacking due to insufficient understanding of DS pathogenesis

To cure a genetic disease, an obvious approach would be to identify the pathogenic responsible gene(s) or pathways and then pursue either genetic therapy or more traditional therapeutic agents, such as drugs, to correct the pathologies. However, this approach is not easily translated to DS due to the complexity of genes involved in trisomy 21. Chromosome 21 is the smallest human chromosome, which might in part explain why it is the most frequent trisomy seen in live births. Nonetheless, there is estimated to be up to four hundred genes, including about two hundred protein coding genes and numerous potential non-coding RNAs, encoded from chromosome 21. Given this number of overexpressed genes, it is very difficult to estimate how many would have an impact if expressed 50% more and thus be dosage sensitive. Investigations into this have been further perplexed by genetic variation among all individuals (including DS patients), which complicates interpretations of many studies. Therefore, there is currently no cure and no standard treatment for all DS individuals.

Current treatments are usually based on each DS individual's physical and intellectual needs. Proper treatments are also chosen according to their personal strength

and limitations. Depending on the symptoms, DS individuals may need immediate medical treatments, such as surgery and chemotherapy for congenital heart disease and leukemia, respectively, as well as lifelong treatments, such as a special diet for DS individuals with digestive problems. For intellectual disability, early interventions and educational therapies have been shown effective for helping DS individuals live independently and thus improve their outcomes and qualities of life (Guralnick 2010; Guralnick 2011). Additionally, drugs for improving the cognitive function of DS individuals are also under development, however, the effectiveness of these drugs remains unclear.

In sum, since the identification of Down syndrome, no cure has been developed and most of the therapies mentioned above are more sociological then medical. Although scientists have spent a tremendous amount of effort to try to determine the molecular mechanisms of DS pathogenesis, in order to identify therapeutic targets, the number of genes involved and genetic variation among DS individuals significantly complicate this task. Therefore, understanding for most pathogenic mechanisms of DS are still lacking.

This thesis work advances progress towards developing therapeutics for DS blood-related disorders in two ways. Instead of trying to figure out which dosage sensitive genes or pathways might cause DS symptoms, we attempted to shut down the extra chromosome 21 in order to correct the imbalanced transcriptome and reverse phenotypes caused by trisomy 21 in DS iPSCs. Additionally, the novel experimental system we developed can be utilized to help decipher the developmental steps in DS hematopoiesis pathways to understand the biology and to develop therapeutic drugs to treat or even prevent leukemia.

The process of normal Hematopoiesis

Since one of the major focuses in this thesis is to demonstrate the correction of DS-associated hematopoietic phenotypes, essential knowledge for normal hematopoiesis is provided in this section. Hematopoiesis is the process of generating different types of blood cells from a rare population of cells residing in the bone marrow called hematopoietic stem cells (HSC) that have self-renewal capacity (Hattori et al.). The hematopoietic development hierarchy is shown in Figure 1.4. HSCs can give rise to multipotent progenitors (MPPs), which only have limited capacity to self-renewal but can further differentiate into all types of hematopoietic cells. The hematopoietic system can be subdivided into the lymphoid and myeloid compartments, comprised of the progeny of common lymphoid progenitors (CLP) and common myeloid progenitors (CMP), respectively, and both CLP and CMP are derived from MPPs. CMPs can further differentiate into megakaryocyte-erythrocyte progenitors (MEPs), common progenitors for megakaryocytes and erythrocytes, and granulocyte-monocyte progenitors (GMPs), common progenitors for cells involved in the innate immune response. The lymphoid compartment contains T cells and B cells that are involved in the adaptive immune response. Hence the process of normal hematopoiesis has wide and varied effects on health and function of the whole body.

Hematopoiesis begins during early embryogenesis and throughout adulthood to generate and replenish the blood cells for the life of the organism. Despite its complexity, it is by far the most well understood developmental system. In addition, transplantation of bone marrow cells, which contain hematopoietic stem and progenitor cells, can cure

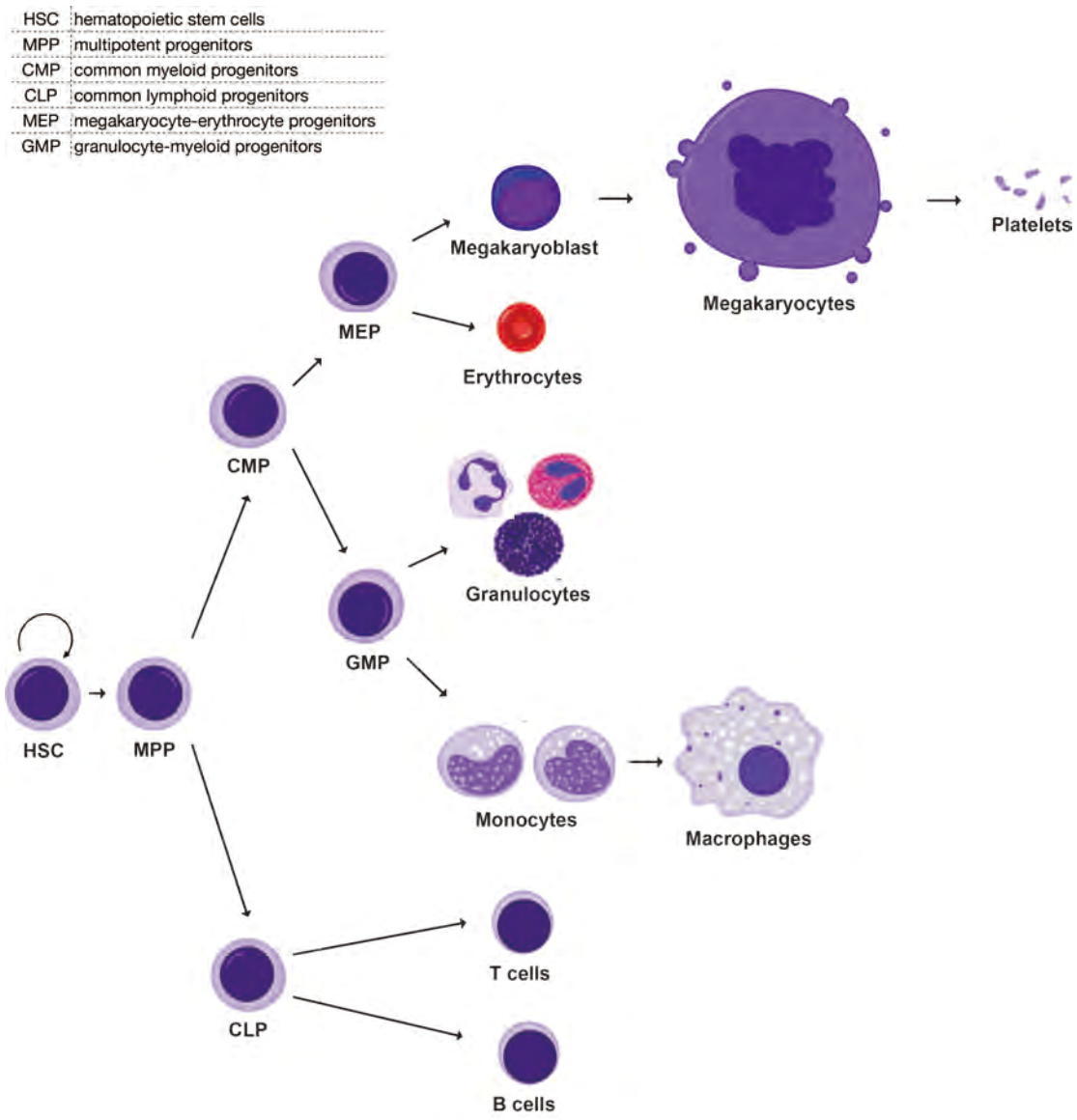


Figure 1.4 Development of the hematopoietic system.

This diagram shows the hematopoietic development hierarchy. Notably, DS-TMD, the precursor form of DS-AMKL, is featured by overproduction of erythrocytes and megakaryocytes.

several hematological disorders and is today the most widely deployed regenerative therapy clinically. However, finding a immunocomaptiable donor is usually time consuming and the procedure for bone marrow transplantation is difficult for both the donor and recipient. Hence, development of alternative approaches to obtain or produce healthy hematopoietic stem and progenitor cells is of great value for treating hematological disorders and these require in depth understanding of molecular mechanisms and cellular development involved in hematopoiesis. Since the hematopoietic abnormalities associated with DS individuals occur most in infancy and childhood, suggesting that the defects arise from fetal hematopoiesis, this section will focus on the process of prenatal hematopoiesis.

There are two waves of hematopoiesis during early embryogenesis

Embryos need oxygen to maintain proper development, therefore, the hematopoietic system is one of the very first to develop in early embryogenesis. Due to the inaccessibility of human embryos, current understandings about the process of hematopoietic development during early embryogenesis mostly come from experiments done in zebrafish and mice. There are two waves of hematopoiesis during early embryogenesis, the primitive and definitive hematopoiesis. Primitive hematopoiesis includes formation of erythroid progenitors that give rise to erythrocytes and macrophages during early embryonic development (Palis and Yoder 2001). These erythroid progenitors first appear in blood islands in the extra-embryonic yolk sac early in development and do not have self-renewal capacity (Paik and Zon 2010).The primary purpose of primitive hematopoiesis is to produce red blood cells facilitating tissue

oxygenation as the embryo is growing rapidly (Orkin and Zon 2008). Hematopoietic lineage specifications are tightly regulated by numerous hematopoietic transcriptional factors. *Gata1* and *Pu.1*, master regulators for erythrocyte and myeloid development, respectively, are two critical genes involved in primitive hematopoiesis (Scott et al. 1994; Cantor and Orkin 2002). *Gata1* knockout results in embryonic lethality due to failed development of mature erythrocytes. Additionally, *Gata1* knockdown experiments done in zebrafish demonstrated that blood cells switch to the myeloid fate with increased expression of myeloid related genes including *Pu.1*. In contrast, *Pu.1* knockdown causes increased Gata1 expressing cells and these cells later upregulate embryonic hemoglobin genes, suggesting their switch to erythroid fate (Rhodes et al. 2005). Based on the physical interaction between *Gata1* and *Pu.1*, the switch is hypothesized to occur as a result of competition for target genes (Cantor and Orkin 2002). Primitive hematopoiesis is very transitory and is rapidly replaced by definitive hematopoiesis that generates adult-type hematopoietic cells.

In most organisms, there is a transient wave of definitive hematopoiesis occurring in blood islands in the yolk sac that produces erythroid-myeloid progenitors (EMP) (Bertrand et al. 2007; McGrath et al. 2011). These progenitors later colonize the fetal liver before the emergence of HSCs. A second wave of definitive hematopoiesis involves the production of HSCs, which are multipotent and can give rise to all lineages of blood cells. In most vertebrates, this process occurs in the aorta-gonad-mesonephros (AGM) region of the developing embryo and the HSCs later migrate to and colonize the fetal liver (Cumano and Godin 2007). Interestingly, *Runx1*, a chromosome 21 gene, has been shown to be required for initiation of definitive hematopoiesis. Knocking down *Runx1* in

mice causes absent development of definitive erythroid, myeloid, and lymphoid cells (Wang et al. 1996). Similar observations have been shown in developing zebrafish embryos as well (Paik and Zon 2010). Fetal liver definitive HSCs are cycling cells but become quiescent after they further migrate to bone marrow, the site of adult hematopoiesis, later in development.

Understanding the mechanism for the formation of HSCs is critical because this will help the development of techniques that generate HSCs *in vitro* from iPSCs, which may serve as an alternative way to obtain patient-specific HSCs for cell therapies for hematological disorders. Additionally, since *Runx1* from chromosome 21 is required for definitive hematopoiesis to generate HSCs, trisomy 21 may well have effects on formation of HSCs, which may further cause abnormalities in the hematopoietic system associated with DS individuals.

HSCs are generated from hemogenic endothelium

Due to the clinical value of HSCs in regenerative medicine, the developmental process of HSCs has been a highly focused area of research. In early 1900s, a number of researchers observed clusters of cells with hematopoietic capacity present in close connection with the endothelium of the ventrolateral aspect of the developing aorta in vertebrates including bat, mongoose, chick, pig, rabbit, and human (Adamo and Garcia-Cardena 2012). The hypothesis that blood cells are developed from a specialized endothelium, termed hemogenic endothelium, was then proposed. Early evidence supporting this hypothesis includes shared markers between blood and vascular cells and lineage-tracing endothelium in developing chick embryo (Jaffredo et al. 1998; Jaffredo et

al. 2000). More recently, lineage-tracing experiments done in mouse further support this hypothesis (Zovein et al. 2008). The strongest evidence for this hypothesis came from time-lapse imaging studies that visualized the transition from endothelial to hematopoietic cells in real-time (Eilken et al. 2009; Bertrand et al. 2010; Boisset et al. 2010; Kissa and Herbomel 2010). The process of generating hematopoietic cells from endothelium, which involves a morphological change of the endothelial cells to form round hematopoietic cells, was named “endothelial to hematopoietic transition (EHT)” (Figure 1.5).

Hemogenic endothelium is a very transitory cell population that exists only in a very short developmental window and only small subsets of endothelium in sites of definitive hematopoiesis are hemogenic. This special subset of endothelium is defined by exhibiting endothelial morphology and phenotype while also the ability to produce hematopoietic cells and endothelial offspring. The co-expression of both endothelial and hematopoietic markers in this small subset of endothelium has made identifying and purifying hemogenic endothelium from endothelial cells and hematopoietic cells difficult.

To further understand the mechanism behind EHT, efforts have been made by researchers to identify essential genes that regulate this process. As an important gene expressed in definitive HSCs, *Runx1* was knocked out to study its normal function. Mouse embryos with homozygous mutations in *Runx1* resulted in an embryonic lethal with absent definitive hematopoiesis while primitive hematopoiesis was only marginally affected (Okuda et al. 1996; Sasaki et al. 1996; Wang et al. 1996). Being the first gene that upon mutated causes distinct effects separating primitive and definitive hematopoiesis, *Runx1* has since become the focus of research. *Runx1* expression was only

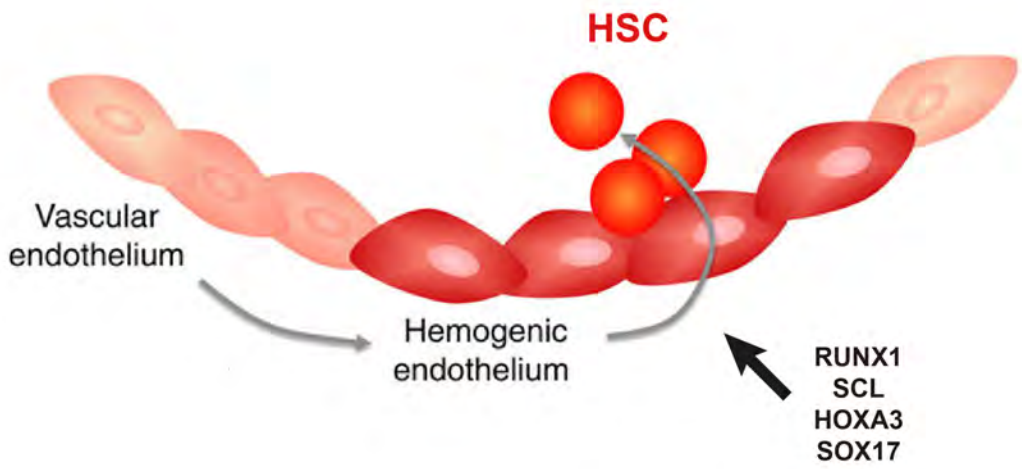


Figure 1.5 Endothelial to hematopoietic transition (EHT)

During definitive hematopoiesis, hematopoietic stem cells (HSCs) are generated from hemogenic endothelium, a small subset of endothelium that has the capacity to produce hematopoietic cells. Genes involved in this endothelial to hematopoietic transition includes *RUNX1*, *SCL*, *HOXA3*, and *SOX17*.

found in subsets of endothelial cells located in sites of definitive hematopoiesis, prior to the emergence of hematopoietic stem cells but not in endothelial cells elsewhere (North et al. 1999; Ottersbach and Dzierzak 2005; Rhodes et al. 2008). The temporal and spatial expression pattern of *Runx1* suggests its role in the formation of HSCs from hemogenic endothelium. Conditional deletion of *Runx1* in VE-Cadherin positive endothelial cells in developing mouse embryos causes failure to produce definitive HSCs, suggesting the role of *Runx1* in the endothelial to hematopoietic transition (Chen et al. 2009). Interestingly, in the absence of *Runx1*, hemogenic endothelium is still made but no definitive hematopoietic cells are formed.

In addition to *Runx1*, several other genes including *SCL*, *HoxA3*, and *Sox17* have also been reported involved in EHT. Knocking out *SCL* in mouse ES cells undergoing hematopoietic differentiation resulted in the absence of hemogenic endothelium and the formation of hematopoietic cells (Lancrin et al. 2009). *HoxA3* down-regulation within hemogenic endothelium correlates with the onset of *Runx1* expression and re-expression of *HoxA3* in nascent hematopoietic progenitors reestablishes the endothelial program (Iacovino et al. 2011). Overexpression of *Sox17* was also shown to result in expansion of a cell population with properties similar to hemogenic endothelium (Clarke et al. 2013; Nakajima-Takagi et al. 2013).

As mentioned earlier, hematopoietic abnormalities in DS individuals can be observed as early as in fetal hematopoiesis stage (Chou et al. 2008; Tunstall-Pedoe et al. 2008), therefore, it is of interest to identify at which developmental stage the trisomy 21 gene expression affects hematopoiesis. One study using an isogenic system to compare hematopoietic differentiation from trisoimc and disomic human pluripotent stem cells

(PSCs) revealed an increased production of early hematopoietic progenitor cells resulted from trisomy 21 (Maclean et al. 2012), which suggests that DS fetal hematopoietic abnormalities can be modeled by human PSCs. Consistent with its role in HSC formation and definitive hematopoiesis, the third copy of *RUNX1* on the extra chromosome 21 has been shown indispensible for overproduction of early hematopoietic progenitor cells during in vitro hematopoietic differentiation from DS iPSCs (Banno et al. 2016). In chapter III of this thesis study, I will demonstrate that our inducible trisomy silencing system can recapitulate hematopoietic phenotypes revealed by the isogenic trisomic and disomic system, and therefore useful for studying effects of trisomy 21 on early hematopoiesis.

Experimental approaches to model and study Down syndrome

Mouse models of DS

Based on the mouse strains developed by Gropp, A. et al. that allow generation of trisomy for any mouse chromosome (Gropp et al. 1974), the phenotypic similarities between trisomy 16 mouse and DS, and the synteny between human chromosome 21 (HSA21) and mouse chromosome 16, the trisomy 16 mouse (Ts16) was identified as a potential mouse model of DS (Polani and Adinolfi 1980; Miyabara et al. 1982). However, the embryonic lethality of Ts16 in mice makes it difficult to use in most DS related research. To overcome this, Davisson et al. created a mouse model termed Ts65Dn that has an extra chromosome that contains regions of mouse chromosome 16 homologous to HSA21, translocated to centromere of mouse chromosome 17 (Davisson et al. 1990; Davisson et al. 1993). Since Ts65Dn possesses several physical, behavioral, and

neurological features similar to those seen in DS individuals, it has since become the most widely used mouse model for DS research. As advances have been made on the techniques to manipulate the mouse chromosome, other mouse models with partial trisomy for HSA21 have also been developed. A mouse trisomy model for all syntenic regions of HSA21 was produced by appropriate crosses of several partial trisomy 21 mice (Yu et al. 2010). Although this mouse model has not been fully characterized for DS features, its availability provides the trisomy 21 community enthusiasm for new discoveries in the future. Another very widely used mouse model of DS is Tc1. Tc1 was constructed by introducing a copy of the human chromosome 21 (HSA21) in mice (Hernandez et al. 1999). Although the HSA21 in Tc1 has several deletions, rearrangements, and duplications due to previous irradiations, it is still widely used for DS research, especially for Alzheimer neuropathologies seen in DS.

Although most major hypotheses about the mechanisms behind DS biology and pathologies come from studies using various mouse models of DS, the species differences are still a barrier that limits DS research and conclusions about human biology. For example, all mouse models of DS don't recapitulate most of the hematopoietic abnormalities commonly seen in DS children and DS newborns (unless additional genetic modifications are introduced). This has led researchers seeking alternative models for DS.

New ways to model DS

Thanks to the invention of methods for generating induced pluripotent stem cells (iPSCs), iPSCs derived from DS patients have become an emerging new cellular model

for studies of human DS cell pathologies. Since iPSCs can be theoretically differentiated into all the cell types in the body, DS iPSCs can be valuable for studying various aspects of DS including neuronal, hematopoietic, and cardiac phenotypes. This will enhance our ability to determine which cell types show pathology and when in the developmental process the pathology arise, as I investigate here. Ultimately, genome editing techniques, such as zinc finger nuclease and CRISPR, will allow researchers to identify the contribution of specific genes or sets of genes to certain phenotypes in cell models that most naturally relate to DS individuals. Moreover, the combined efforts of genome editing and gene delivery techniques have accelerated the development of gene therapy for various genetic diseases.

However, although recent advances have been made in developing gene therapies for various genetic diseases, DS has not been considered as a candidate to develop gene therapy, due to the large amount of over-dosed genes from chromosome 21. To overcome this challenge, in this thesis, I applied the chromosome-wide silencing property of a long non-coding RNA, *XIST*, to silence one copy of chromosome 21. This approach simplifies the need for silencing hundreds of genes to the simple addition of a single gene, *XIST,* to "dosage compensate" trisomy 21 expression. More details for *XIST*-mediated chromosome silencing are explained in the next section.

XIST Mediated Chromosome Silencing

Discoveries of X-inactivation and a remarkable RNA

In most mammals, sex is determined by the XY sex-determination system in which females have two X chromosomes and males have one X and one Y chromosome.

This context induces an imbalance of X chromosome genes between two sexes and therefore a mechanism to dosage balance X-linked genes, X chromosome inactivation (XCI), is required. The first observation of the inactive X chromosome came from the discovery made by Barr and Bertram in 1949 (Barr and Bertram 1949). They noticed a small body near the nucleolus of cat neurons that could be stained with the same dye that stains chromosomes. While they had no clue as to the composition of this small body, they termed it “sex chromatin” because this small body was found only in female cells of mammals. The thought that X chromosomes might act differently than autosomes came with the discovery of the first mouse X-linked gene that, when mutated, caused an unusual pattern of white spotting in the coat. This was seen only in females and mutation of this gene in males caused embryonic lethality. Additionally, Welshons and Russell showed that mice with a single X chromosome and without the Y chromosome, termed XO, are viable and fertile females. This suggested that the Y chromosome was the male determining factor and one active X chromosome was sufficient for survival of female cells (Welshons and Russell 1959). Furthermore, in 1959, Susumu Ohno showed that the two X chromosomes in female cells appeared different, with one being autosome-like and another being condensed and heterochromatic, suggesting it was inactivated (Ohno et al. 1959). Given the above observations, Mary Lyon proposed a theory that one X chromosome is randomly selected to be inactivated during early embryogenesis in female mammalian cells (Lyon 1961).

In order to understand the mechanisms of X chromosome inactivation, analysis of both human and mouse rearranged X chromosomes revealed the existence of the X chromosome inactivation center (XIC), the region required for initiation of X

chromosome inactivation (Brown et al. 1991b). A major breakthrough came from the identification of a human 17 kb long non-coding RNA, XIST, and its mouse counterpart, Xist, mapped to XIC and expressed only from the inactivated X chromosome, suggesting its potential role in X chromosome inactivation (Brown et al. 1991a; Brown et al. 1992). Furthermore, the discovery that XIST/Xist RNA coats the inactivated X chromosome at interphase in female cells and the absence of X chromosome inactivation after deletion of Xist in mice confirmed the requirement of XIST/Xist for X chromosome inactivation in female cells (Clemson et al. 1996).

Known Mechanisms for XIST-mediated X-inactivation

Fifty years after the initial proposal of X chromosome inactivation, much progress has been made for understanding the mechanism of X chromosome inactivation (XCI). Due to the inaccessibility of human embryos, most research has been done in mouse models, thought to largely reflect XCI in other mammals, including humans.

Xist RNA is the critical player in XCI. During early embryogenesis, when cells are still in the pluripotent state, *Xist* RNA is expressed at low levels from both active X chromosomes in female cells. Upon differentiation, one of the two active X chromosomes is randomly selected to go through XCI and *Xist* RNA is up-regulated and stabilized from the chosen X chromosome. Although the mechanism of random choice is not understood yet, the *Xist* antisense transcription unit, *Tsix*, seems to play a critical role in the choice of *Xist* allele to be upregulated. The expression of *Tsix* is accompanied by the appearance of repressive chromatin modifications on the promoter of *Xist* and the deletion of *Tsix* in mouse leads to preferential upregulation of *Xist* in the deleted locus (Lee and Lu 1999).

However, this *Tsix* dependent mechanism is not well conserved among species and the regulation of initiating XCI in human early embryos is still not clear yet. The coincidence of down-regulated pluripotency factors and up-regulation of *Xist* during mouse ES cell differentiation suggested that pluripotency factors may play roles in the regulation of initiating XCI, although further evidence is still needed for this hypothesis since this does not explain why *Xist* is up-regulated only in female cells, but not in male cells. Recent findings showed that several X-linked loci might participate in female specific up-regulation of *Xist* in mice, including *Rnf12*, *Xpr*, and *Jpx* (Augui et al. 2007; Jonkers et al. 2009; Tian et al. 2010; Barakat et al. 2011).

Although the *Xist* mediated XCI is conserved among most mammals, the sequence of *Xist* is largely variable except for a series of conserved repeat motifs, termed repeat A to F (Brockdorff et al. 1992; Brown et al. 1992). Upon up-regulation from the randomly selected X chromosome, *Xist* RNA spreads across the whole X chromosome territory *in cis*, a critical process for silencing the X chromosome. While several proteins with both RNA and DNA binding domains (including *Saf-A* and *YY1*) have been suggested involved in *Xist* spreading across the X chromosome *in cis*, the precise mechanisms are still unclear. Additionally, deletion experiments showed that certain regions on the *Xist* transcript are required for different aspects of *Xist* function (Wutz et al. 2002). For example, repeat C is suggested to be required for proper *Xist* spread and localization. Interestingly, interaction between repeat C and the RGG domain (putative RNA binding domain) of *Saf-A* has been shown and disruption of *Saf-A* leads to mis-localization of Xist RNA in some cell types. However, the evidence that repeat C is required in XIST localization in human female cells is still lacking.

While the critical regions for *Xist* localization are still not clear, the region of the *Xist* transcript required for proper gene silencing (in both human and mouse female cells) is much more well-defined. Repeat A is the most conserved region on *Xist* transcript among species and its role in gene silencing is confirmed from multiple independent research groups. Importantly, *Xist* RNA lacking repeat A was capable of spreading across the entire X chromosome but did not result in proper chromosome silencing, suggesting its unique role in gene silencing (Wutz et al. 2002; Chow et al. 2010). The repeat A is composed of eight motifs that form inter-repeat double stranded duplexes, flanked by single stranded regions (Lu et al. 2016). This unique structure is thought critical for interaction with multiple RNA binding proteins, including SPEN, RBM15 and WTAP, recently identified as factors required for initiating the *Xist*-mediated chromosome silencing cascade in mouse ESCs (Chu et al. 2015; Moindrot et al. 2015; Monfort et al. 2015).

The interaction between SPEN and Xist is repeat A dependent (Chu et al. 2015). Once recruited to Xist, SPEN can interact with the SMRT corepressor deacetylase complex that contains HDAC3 for histone deacetylation of X-linked genes. Defective XCI can be observed with depletion of HDAC3, SMRT, or SPEN, suggesting a role for recruitment of SMRT-HDAC3 complex by SPEN in *Xist*-mediated transcriptional silencing of X-linked genes (Figure 1.6A) (Shi et al. 2001; You et al. 2013; McHugh et al. 2015). Additionally, a recent study conducted by Patil, D.P. et al. reported a functional link between N^6-Adenosine methylation (m^6A), a common post-transcriptional modification, and *Xist*-mediated gene silencing (Patil et al. 2016). RBM15 interacts with *Xist* RNA and recruits m^6A machinery through its interacting protein, WTAP, whose

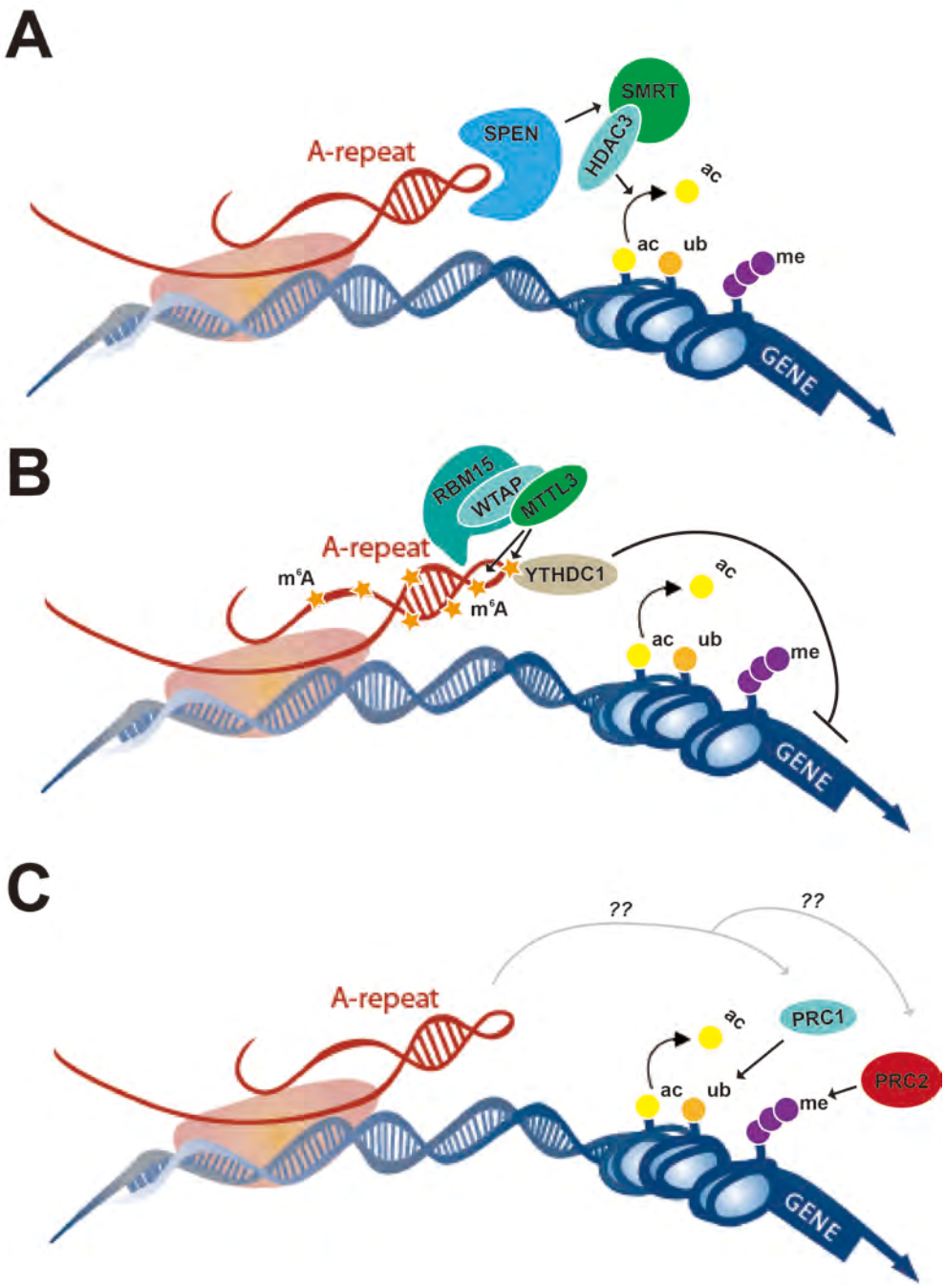


Figure 1.6 Mechanisms for Xist mediated X chromosome inactivation.

(A) SPEN is recruited by Xist through the A repeat. Further recruitment of SMRT complex that has HDAC3 results in deacetylation of X-linked genes. (B) RBM15 can bind to Xist and interact with MTTL3 through WTAP. MTTL3 methylates adenosine residues on Xist RNA and YTHDC1, an m^6A reader that binds to m^6A residues on Xist, is required for gene silencing. (C) PRC1 and PRC2 that put heterochromatic modifications on X-linked genes are recruited by Xist during XCI through yet unknown mechanisms.

interaction partner, MTTL3, then methylates adenosine residues on Xist RNA (Figure 1.6B) (Patil et al. 2016). Depletion of either RBM15 or WTAP resulted in decreased recruitment of MTTL3 and m^6A levels within *Xist* and knocking down either RBM15 or MTTL3 causes loss of *Xist*-medicated gene silencing (Patil et al. 2016). Additionally, YTHDC1, a known m^6A reader, was also found to bind to m^6A residues within Xist RNA (Patil et al. 2016). Knocking down *Ythdc1* in mouse ES cells disrupted Xist-mediated gene silencing and artificial tethering of YTHDC1 to Xist RNA can rescue Xist-meidated transcriptional silencing upon loss of m^6A machinery(Patil et al. 2016). Furthermore, although the mechanisms are not clear yet, PRC1 and PRC2 complexes are also recruited by Xist RNA for initializing XCI (Figure 1.6C). These recruitments will ultimately result in several heterochromatic modifications, such as H3K27, H3K9, and macroH2A, on the inactive X chromosome, which further leads to chromosome-wide transcription silencing and the formation of a heterochromatic nuclear structure called the Barr body. However, currently, the mechanisms for this Xist-mediated structural change of the inactive X chromosome remained unknown.

Because *XIST* RNA is highly effective in silencing an entire chromosome, the idea of applying XIST mediated chromosome silencing to aneuploidy evolves. Although the ability of *XIST* RNA to effectively inactivate an autosome is still unclear, several findings support this idea. While unbalanced X;autosome translocations would theoretically result in fetal lethality, the fact that individuals with aneuploidy of this type (most frequently unbalanced t(9;X) and t(14;X)) are mostly normal suggests that *XIST* is still functional in silencing the translocated autosome. In addition, our previous study showed that human *XIST* transgene inserted into chromosome 4 in a transformed cell line

can induce chromosome silencing (Hall et al. 2002a). Based on these evidence, we hypothesize that *XIST* RNA can silence an autosome. Therefore, in this thesis work, instead of trying to decipher mechanisms for *XIST* mediated chromosome silencing, we apply its chromosome silencing capacity to correct the number of active chromosome 21 in DS cells. Since induced pluripotent stem cells (iPSCs) are capable of differentiating into all cell types that are affected in DS and are one of the cell types known to support initiation of *XIST* mediated chromosome silencing, in order to take full advantage of *XIST* RNA and to maximize the value of the DS model being built in this thesis, induced pluripotent stem cells (iPSCs) derived from DS individuals with inducible *XIST* transgene targeted on one chromosome 21 is created and chosen as the cellular model. This model not only can demonstrate the effect of trisomy silencing on cellular phenotypes of DS but also will provide a correctable "disease in a dish" model to study DS pathologies.

Disease modeling using pluripotent stem cells

Advantages of disease modeling with pluripotent stem cells

It is necessary to have a comprehensive view of biological processes underlying human pathologies in order to devise strategies for disease treatments and even prevention. As the causes of most diseases can be narrowed down to specific genomic loci, studies performed on these single gene defects in a suitable context has been facilitating disease modeling and helping researchers understand more about these diseases. Due to some conservation among mammalian genomes, animal models such as mice, rats, and non-human primates have been valuable tools for disease modeling.

However, due to speciation and genetic differences, these animal models are not always applicable for modeling human diseases, and fail to recapitulate some phenotypes seen only in human patients. For example, current mouse models of DS, without any additional genetic modifications, fail to recapitulate most of the hematopoietic abnormalities seen in human DS individuals. Therefore, for these kinds of diseases, it is ideal to conduct biomedical research in humans or humane cells. Most of the time this is limited to *in vitro* systems where researchers perform experiments on cultured patient-derived primary cells. However, diseases that have phenotypes specific to cell types that are difficult or impossible to isolate are excluded from this kind of approach. Hence, a better human-based model is needed.

The availability of different types of human pluripotent stem cells (PSCs) such as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) provide the solution for the limitations of aforementioned disease modeling approaches. Human pluripotent stem cells outperform those approaches because they are a normal primary cell line, have self-renewal capacity that supports *in vitro* culture, and have the potential to differentiate into virtually any cell type found in the human body.

Strategies for generating disease models using human PSCs

The first example of using human PSCs for disease modeling came from a targeted mutation of the *HPRT* gene in human ESCs by homologous recombination, used to model Lesch-Nyhan syndrome (Urbach et al. 2004). Human embryos carrying specific genetic mutations or chromosomal aberrations that can be identified by pre-implantation genetic diagnosis (PGD) (Mateizel et al. 2006; Eiges et al. 2007) or pre-implantation

genetic screening (PGS) (Biancotti et al. 2010), respectively, are also valuable sources for isolating ESCs for disease modeling. However, a crucial limitation for these approaches is that it is only applicable for diseases that can be screened by these methods. Additionally, *in vitro* culture of normal ESCs can sometimes spontaneously generate aneuploid cells that are useful for modeling aneuploidy diseases such as Turner syndrome (monosomy X) (Urbach and Benvenisty 2009).

Despite the promising potential offered by human ESCs, the availability and ethical concerns for human ESCs are still crucial limitations for researchers. The revolutionary invention of technique to reprogram somatic cells back to their pluripotent state, as known as iPSCs, has opened a new horizon for diseases modeling using human PSCs (Takahashi et al. 2007). The ability to generate patient specific iPSCs from somatic cells not only avoids the problems mentioned previously for ESCs but also prevent the issues of immunocompatibility for developing personalized medicine and stem cell treatments. Additionally, recent improvements of nuclease-based genome editing techniques, such as zinc finger nucleases and CRIPSR, significantly facilitates the usage of human PSCs for creation of various kind of disease models (Kim and Kim 2014). The combination of these techniques allows generation of highly controlled modeling system, such as isogenic PSC lines that differ only in the mutated genomic locus, for researchers to elucidate the underlying pathologies for diseases to be studied.

Concerns for disease modeling with human PSCs

Although human PSCs outperform other approaches for disease modeling, several criteria should be taken into account. The most intuitive approach for disease modeling

using human PSCs is to identify phenotypes recapitulated from patients, find ways to correct the pathological genetic abnormalities, and compare the phenotypes between corrected and non-corrected cells. However, it is well known that clonal or even intra clonal variations exist or arise during the process of culturing human PSCs. Therefore, comparisons between corrected and non-corrected disease PSCs should be addressed more carefully. One way to avoid this is to create an "iso-epigenetic" system. For example, in this thesis work, our design that the targeted chromosome 21 can be silenced on demand provides us an iso-epigenetic system, a well-controlled system that avoids any source of variations that may potentially arise from the process of culturing corrected and non-corrected cells separately.

Human PSCs-based approaches are best for modeling monogenic disorders with clear cellular phenotypes. In contrast, it is relatively difficult to model complex diseases that are caused by mutations from multiple or undefined genomic loci. In addition, diseases with phenotypes that involve structural abnormalities in certain tissues are generally very difficult to model using human PSCs due to the requirement for three-dimensional tissue structures to observe the phenotypes, such as with congenital heart disease in DS. Moreover, the availability of an established differentiation protocol for particular cell types is also key when disease modeling with iPSCs. It is usually extremely time consuming to generate a protocol for differentiation of certain cell types from humans PSCs *de novo* and an animal model, if available, might be a better choice. Furthermore, since PSCs are "young" cells, it is more useful for modeling diseases with phenotypes in fetal development or early childhood, as it requires more efforts to

differentiate PSCs into more mature cell types to model diseases with phenotypes seen only in adulthood, or with advanced age.

Based on our discussion so far, Down syndrome, although being a complex disorder caused by abnormalities in hundreds of genes, can be easily modeled and with application of *XIST*-mediated chromosome silencing. Importantly, among several DS-associated pathologies, DS-AMKL has clear phenotypes (overproduction of megakaryocytes and erythrocytes) that can be observed in fetal development and, although being a very complex system, several established protocols for hematopoietic differentiation from iPSCs are available. Therefore, DS-AMKL is ideal for disease modeling using iPSCs derived from DS individuals.

In vitro hematopoietic differentiation from human PSCs

The techniques for growing human PSCs *in vitro* have made modeling early hematopoiesis *in vitro* feasible. In addition to research applications, the ability to generate clinically useful hematopoietic cells will be tremendously beneficial to patients with hematological diseases, especially for those caused by genetic abnormalities. Despite current success of bone marrow transplantation in curing hematopoietic disorders, limited supply of donors and the issues with immunocompatibility are still difficult for the patients and their families. Therefore, scientists have spent tremendous efforts trying to generate HSCs *in vitro,* which requires an efficient protocol for hematopoietic differentiation from pluripotent stem cells.

The most common approach for differentiating hematopoietic cells from human PSCs is through the formation of the embryoid body (EB). EBs are three-dimensional cell

aggregates of ES/iPSCs formed in suspension culture that mimics the developing embryo through enhancing cell-cell interactions to form three germ layers (Doetschman et al. 1985; Thomson et al. 1998). As hematopoietic stem cells are naturally present in a very small amount of population, cytokine cocktails that contains factors essential for hematopoietic differentiation in vivo, including BMP4, FGF, VEGF, SCF, IL3, IL6, and IL11, are usually contained in the medium in which EBs are differentiating. The first cell with hematopoietic capacity can be detected at day 3 to 4 in differentiating EBs (Kennedy et al. 2007). Differentiation of human iPSCs throughout 21-day EB formation demonstrated a sequential gene expression changes from BRACHYURY (mesodermal differentiation) to SCL, GATA-2, and RUNX1 (hematopoietic commitment), followed by the emergence of $CD34^{+}CD45^{+}$ cells that denote hematopoietic lineages (Lengerke et al. 2009). Other methods including co-culturing ES/iPSCs with feeder cells that support hematopoietic differentiation have also been successful, although it requires an additional purification step to separate out the differentiated hematopoietic cells from the feeders before further analysis.

The method to evaluate differentiated hematopoietic cells includes the colony-forming assay, which uses methylcellulose-based media, a system that supports preferentially the growth of cells with hematopoietic capacity, and engraftment into irradiated mouse for bone marrow reconstitution. Although generation of different lineages of hematopoietic cells has been very successful using various differentiation methods, *in vitro* derived hematopoietic stem cells from human PSCs fail to repopulate the bone marrow of irradiated mice, suggesting intrinsic differences are still present between hematopoietic stem cells generated *in vitro* and *in vivo*. This has been a major

obstacle for generating clinically applicable hematopoietic cells in vitro and tremendous amount of efforts have been put into this area. Recently, through introduction of seven transcription factors including *ERG, HOXA5, HOXA9, HOXA10, LCOR, RUNX1*, and *SPI1* into hemogenic endothelium differentiated *in vitro,* Sugimura et al demonstrated success of generating HSCs that are able to engraft irradiated mice (Sugimura et al. 2017). Interestingly, two of the five transcription factors they introduced into hemogenic endothelium are encoded on chromosome 21. There is no doubt that their research has overcome a major obstacle and will be valuable for this field of research.

Concluding Remarks

Although decades of efforts have been spent on DS research, due to its complexity, pathogeneses in DS is largely unknown except overexpression of chromosome 21 genes. Additionally, a better DS model is needed in the field in order to provide a more controlled system to compare the effect of trisomy 21 in various cell types. This thesis adopts a novel approach to establish a model system for DS where the expression of one chromosome 21 can be silenced on demand. This model provides an "iso-epigenetic" system, which would minimize any source of variations when comparing corrected and non-corrected cells and therefore provide more accurate and less confusing results. Additionally, this is the first demonstration of "chromosome therapy", in which the abnormalities associated with a whole chromosome is corrected. I also use this approach to demonstrate correction of known hematopoietic abnormalities associated with DS individuals and to study the contribution of trisomy 21 along the process of hematopoietic differentiation. Furthermore, possible effects of trisomy 21 on

angiogenesis, which are potentially related to tumorigenesis in DS individuals, are also tested using this model system. To sum up, this work provides the field with a novel tool to study DS and demonstrates a proof of principle for developing an innovative therapeutic approach for DS individuals in the future.

Chapter II: Translating *XIST* mediated chromosome silencing to trisomy 21 in DS induced pluripotent stem cells

Preface

Work presented in this chapter contributes to the larger effort by members of the Lawrence lab to demonstrate comprehensive transcriptional repression of one chromosome 21 in induced pluripotent stem cells derived from an individual with Down syndrome and it resulted in the following publication:

Jiang, J., Y. Jing, G.J. Cost, **J.C. Chiang**, H.J. Kolpa, A.M. Cotton, D.M. Carone, B.R. Carone, D.A. Shivak, D.Y. Guschin, J.R. Pearl, E.J. Rebar, M. Byron, P.D. Gregory, C.J. Brown, F.D. Urnov, L.L. Hall, and J.B. Lawrence (2013) Translating Dosage Compensation to Trisomy 21. Nature. **500**(7462):296-300. doi: 10.1038/nature12394

My contribution to this publication involved construction of the vector containing a transgene carrying the doxycycline control component (rtTA) targeted to the *AAVS1* locus on chromosome 19, isolation and characterization of two XIST-transgenic clones, analysis of allele specific silencing of APP gene on the targeted chromosome, SNP analysis for allele specific transgene insertion, cell culture for microarray analysis (clone 3), and the proliferation assay. This section includes most parts of the cited manuscript, including contributions of my own and others in order to keep the flow intact. At the time this work was done, insertion of a very large transgene (~21 kb) was novel and made possible by zinc finger nuclease (ZFN) technology. Sangamo Biosciences created the chromosome 21 specific ZFNs (for which seven authors were included). This work was supported by NIH grants GM053234, GM085548 and GM096400 RC4 to J.B.L.

Introduction

In the United States, about 1 in 300 live births carry a trisomy, half of which are for chromosome 21, which causes Down syndrome. Down syndrome is the leading genetic cause of intellectual disabilities and the millions of Down syndrome patients across the world also face multiple other health issues, including congenital heart defects, haematopoietic disorders and early-onset Alzheimer's disease (Megarbane et al. 2009; Gardiner 2010). Down syndrome researchers have sought to define the genes on chromosome 21 most closely associated with Down syndrome, but this has proven difficult due to high genetic complexity and phenotypic variability of Down syndrome, confounded by normal variation between individuals (Prandini et al. 2007; Megarbane et al. 2009; Gardiner 2010). Despite progress with mouse models for Down's syndrome (O'Doherty et al. 2005; Haydar and Reeves 2012), there remains a need for better ways to understand the underlying cell and developmental pathology of human Down's syndrome, key to therapeutic design of any kind (Gardiner 2010).

The last decade has seen great advances in strategies to correct single-gene defects of rare monogenic disorders, beginning with cells *in vitro* and in several cases advancing to *in vivo* and clinical trials (Lee and Davidson 2011). In contrast, genetic correction of the over-dosed genes across a whole extra chromosome in trisomic cells has remained outside the realm of possibility. Our effort was motivated by the idea that functional correction of living trisomic cells may be feasible by inserting a single gene that can epigenetically silence a whole chromosome. An inducible system for such 'trisomy silencing' would have immediate translational relevance as a resource to investigate the cellular pathology and gene pathways affected in Down's syndrome, in a

setting free from pervasive genetic or epigenetic variation that exists between individuals, sub-clones, or even isogenic cell isolates (Prandini et al. 2007; Hall et al. 2008; Nazor et al. 2012).

There is a natural mechanism to compensate the difference in dosage of X-linked gene copies between mammalian females (XX) and males (XY). This is driven by a large (~17 kilobases (kb) in human), non-coding RNA, *XIST*, which is produced exclusively from the inactive X chromosome (Brown et al. 1992), and 'paints' (accumulates across) the interphase chromosome structure (Clemson et al. 1996; Heard 2005). During early development, the *XIST* RNA induces numerous heterochromatin modifications and architectural changes which transcriptionally silence the inactive X chromosome and manifest cytologically as a condensed Barr body (reviewed in (Heard 2005; Hall and Lawrence 2010)). There is evidence for some DNA sequence specificity to *XIST* function, as certain human genes escape X-inactivation (Carrel and Willard 2005); however, autosomal chromatin has substantial capacity to be silenced (Lee et al. 1996; Hall et al. 2002a; Hall et al. 2002b). Understanding the full potential of an autosome to be silenced, however, requires examination under conditions that avoid creating a deleterious functional monosomy. The strategy pursued here meets that requirement and creates a tractable model to study the distinct biology of human chromosome inactivation.

As outlined in Figure 2.1a, we set out to determine whether the human X-inactivation gene, *XIST*, could be inserted into one copy of chromosome 21, and enact a chromosome-wide change in its epigenetic state. We pursued zinc finger nuclease (ZFN)-driven targeted addition (Moehle et al. 2007) of an inducible *XIST* transgene to the gene-

rich core of chromosome 21 in induced pluripotent stem (iPS) cells derived from a Down's syndrome patient. If accomplished, this milestone would provide a system to study Down's syndrome cell pathology and the first step towards a potential genetic/epigenetic approach to 'chromosome therapy'.

Results

Insertion of XIST into a trisomic chromosome 21 in DS iPSCs

Given its large size, neither the *XIST* gene nor its cDNA has previously been integrated in a targeted fashion. Based on our previous success on inserting a ~16-kb and a ~21 kb *XIST* transgenes in a transformed cell line (HT1080), using established ZFNs to the *AAVS1* locus on chromosome 19 (DeKelver et al. 2010) and to the intron 1 of *DYRK1A* locus on chromosome 21, respectively, we proceeded to determine whether this was achievable in technically challenging iPS cells, which have unique therapeutic and developmental potential to form various cell types, and thus would be important for any future *ex vivo* cellular therapy efforts. We used a male Down's syndrome iPS cell line (Park et al. 2008) which we confirmed maintains pluripotency markers and trisomy 21. Although a constitutively transcribed transgene could be used, we engineered an inducible system to maximize utility for investigating Down's syndrome biology. In one step, we integrated both the doxycycline-controlled *XIST* transgene into chromosome 21 (Figure 2.1b) and a transgene carrying the doxycycline control component (rtTA) into the *AAVS1* chromosome 19 safe harbour, disruption of which creates no known adverse effects (DeKelver et al. 2010) (Figure 2.1b).

We analysed 245 colonies from the pooled transformants by interphase RNA/DNA fluorescence *in situ* hybridization (FISH) (Figure 2.1c) to determine whether *XIST* was present and overlapped one of three *DYRK1A* alleles. Notably, 98.5% of *XIST* RNA-positive colonies carried *XIST* at this location on chromosome 21. Efficiency was sufficiently high that, through modifications to editing conditions, we obtained a few sub-clones with XIST integrated into two or even all three alleles of

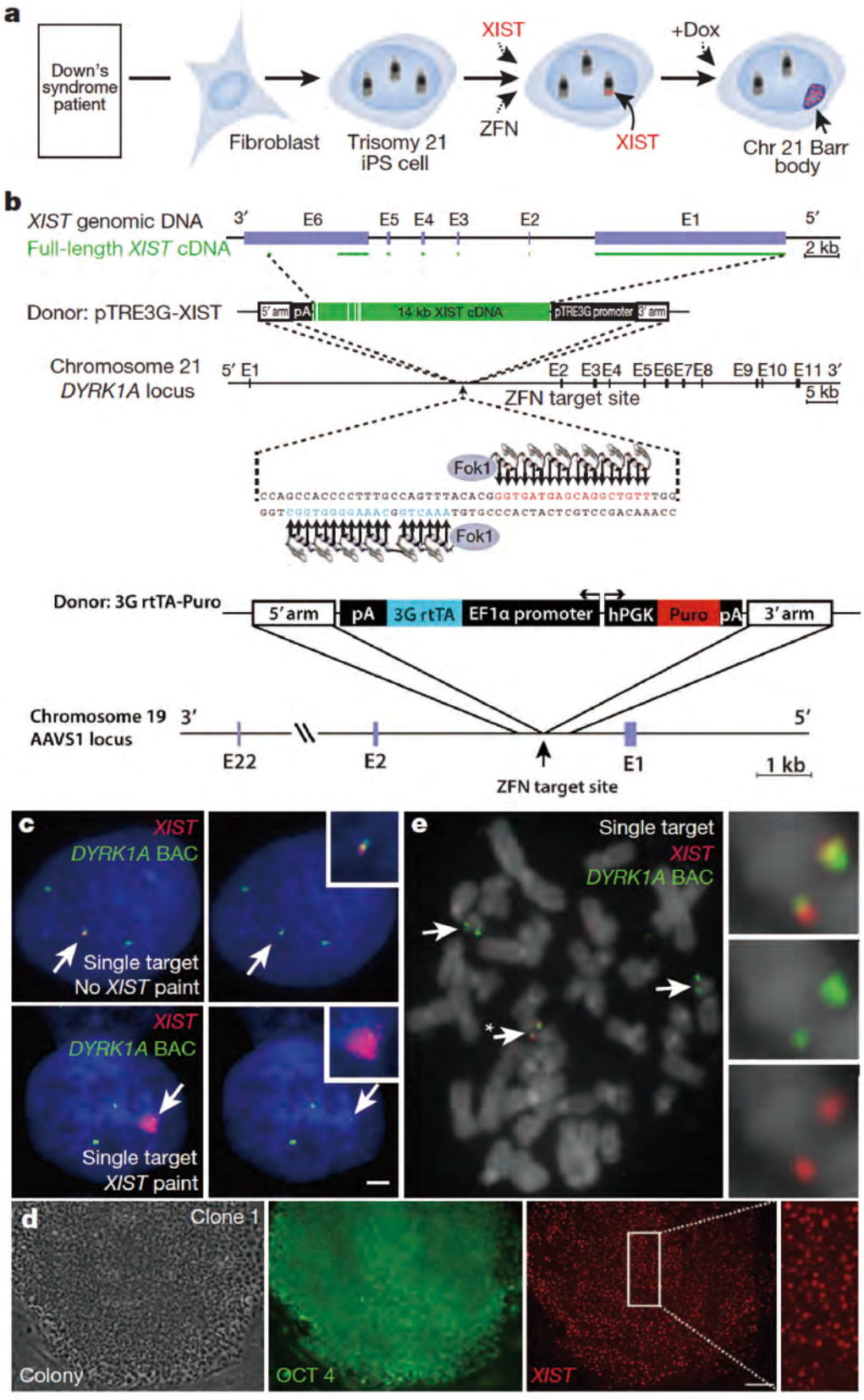

a
Down's syndrome patient
Fibroblast
Trisomy 21 iPS cell
XIST
ZFN
+Dox
XIST
Chr 21 Barr body
b
XIST genomic DNA
Full-length XIST cDNA
3′
E6
E5
E4
E3
E2
E1
5′
2 kb
Donor: pTRE3G-XIST
5′ arm
pA
14 kb XIST cDNA
pTRE3G promoter
3′ arm
Chromosome 21
DYRK1A locus
5′ E1
E2 E3E4 E5E6E7E8 E9 E10 E11 3′
5 kb
ZFN target site
Fok1
CCAGCCACCCCTTTGCCAGTTTACACGGGTGATGAGCAGGCTGTTTGC
GGTCGGTGGGGAAACGGTCAAATGTGCCCACTACTCGTCCGACAAACC
Fok1
Donor: 3G rtTA-Puro
5′arm
pA
3G rtTA
EF1α promoter
hPGK
Puro
pA
3′arm
Chromosome 19
AAVS1 locus
3′
5′
E22
E2
E1
ZFN target site
1 kb
c
XIST
DYRK1A BAC
Single target
No XIST paint
XIST
DYRK1A BAC
Single target
XIST paint
e
Single target
XIST
DYRK1A BAC
d
Clone 1
Colony
OCT 4
XIST

Figure 2.1 Genome editing integrates *XIST* into chromosome 21 in trisomic iPS cells.

(a) Concept for translating dosage compensation to trisomy 21. (b) The *XIST* construct (19 kb) contains two homologous arms and 14-kb *XIST* cDNA with inducible pTRE3G promoter. The rtTA-Puro construct contains a puromycin selection gene and rtTA cassette that is targeted to the *AAVS1* safe harbor locus on Chr19 by ZFNs. (c) DNA/RNA FISH in interphase Down's syndrome iPS cells shows that *XIST* overlaps one of three *DYRK1A* genes (left panels and insets) in a non-expressing cell (top, arrows), and a cell induced to express a large *XIST* RNA territory over the *DYRK1A* locus after 3 days in doxycycline (bottom, arrows). Right panels show green channel (*DYRK1A*) alone. Nuclear DNA is stained with 4′,6-diamidino-2-phenylindole (DAPI, blue). Scale bar, 2 μm. (d) OCT4 immunostaining and *XIST* RNA FISH in a transgenic colony: highly consistent *XIST* expression throughout the colony. Scale bar, 100 μm. (e) Metaphase DNA FISH shows one targeted chromosome 21. *XIST* gene (asterisk and close-up) overlaps one of three *DYRK1A* genes (arrows).

DYRK1A. Six independent sub-clones were chosen for further study based on: an *XIST* transgene on one of three chromosome 21 copies, pluripotent colony morphology, OCT4 (also called POU5F1) staining (Figure 2.1d) and formation of embryoid bodies. FISH to metaphase chromosomes (Figure 2.1e) confirmed the gene addition accuracy, with 47 chromosomes, for all six clones.

XIST RNA induces a chromosome 21 Barr body

In the panel of six independent genome-edited clones, we induced transgene expression and detected *XIST* RNA by FISH 3 days later. A localized *XIST* RNA 'territory' over one chromosome 21 (Figure 2.1c) was seen in over 85% of cells in all six clones (Figure 2.1d). This mirrored the unique behaviour of endogenous *XIST* RNA which 'paints' the inactive X chromosome nuclear territory (Clemson et al. 1996). The natural inactivated X chromosome forms a condensed Barr body which carries repressive histone marks (Heard 2005). Similarly, 5 days after *XIST* induction, the edited chromosome 21 became markedly enriched in all heterochromatin marks examined, including H3K27me3, UbH2A and H4K20me in 90–100% of cells and, later, with macroH2A (Figure 2.2a, b and Figure 2.3a). Figure 2.3b illustrates that H3K27me spread across the whole metaphase chromosome 21. Moreover, chromosome 21 DNA in many nuclei became notably condensed, further evidence that we successfully generated a heterochromatic chromosome 21 Barr body (Figure 2.2c).

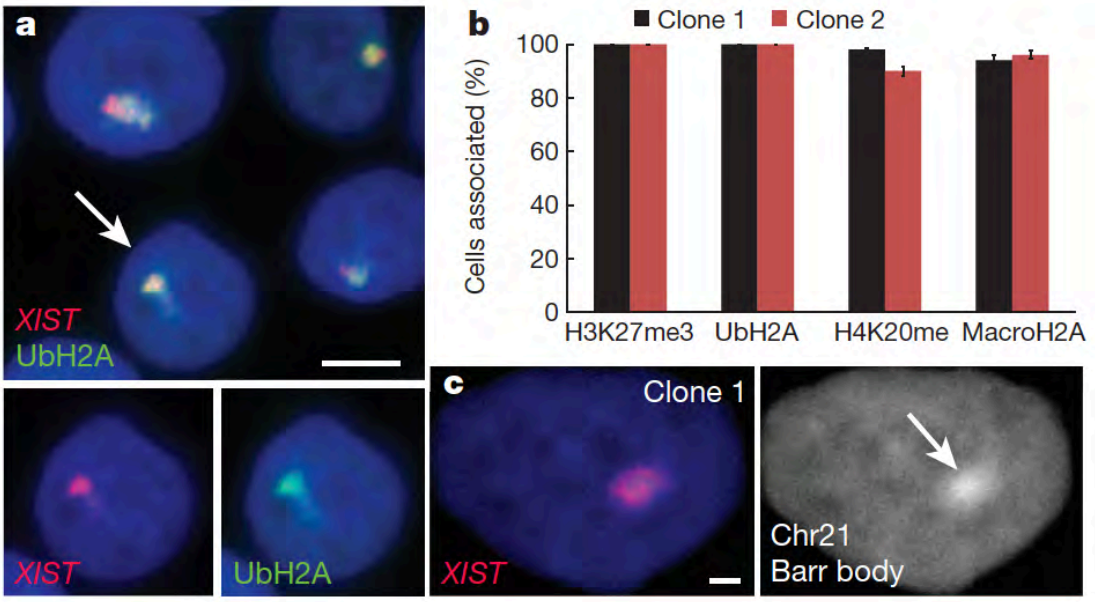


Figure 2.2 *XIST* induces heterochromatin modifications and condensed chromosome 21 Barr body.

(a) XIST RNA recruits heterochromatic epigenetic marks (for example, UbH2A). Channels are separated for cell indicated with an arrow. Scale bar, 5 mm. (b) Percentage of XIST territories with heterochromatin marks. Mean +/- standard error, 100 nuclei in ~5 colonies. (c) XIST RNA induces chromosome 21 Barr body visible by DAPI stain (arrow). Scale bar, 2 mm.

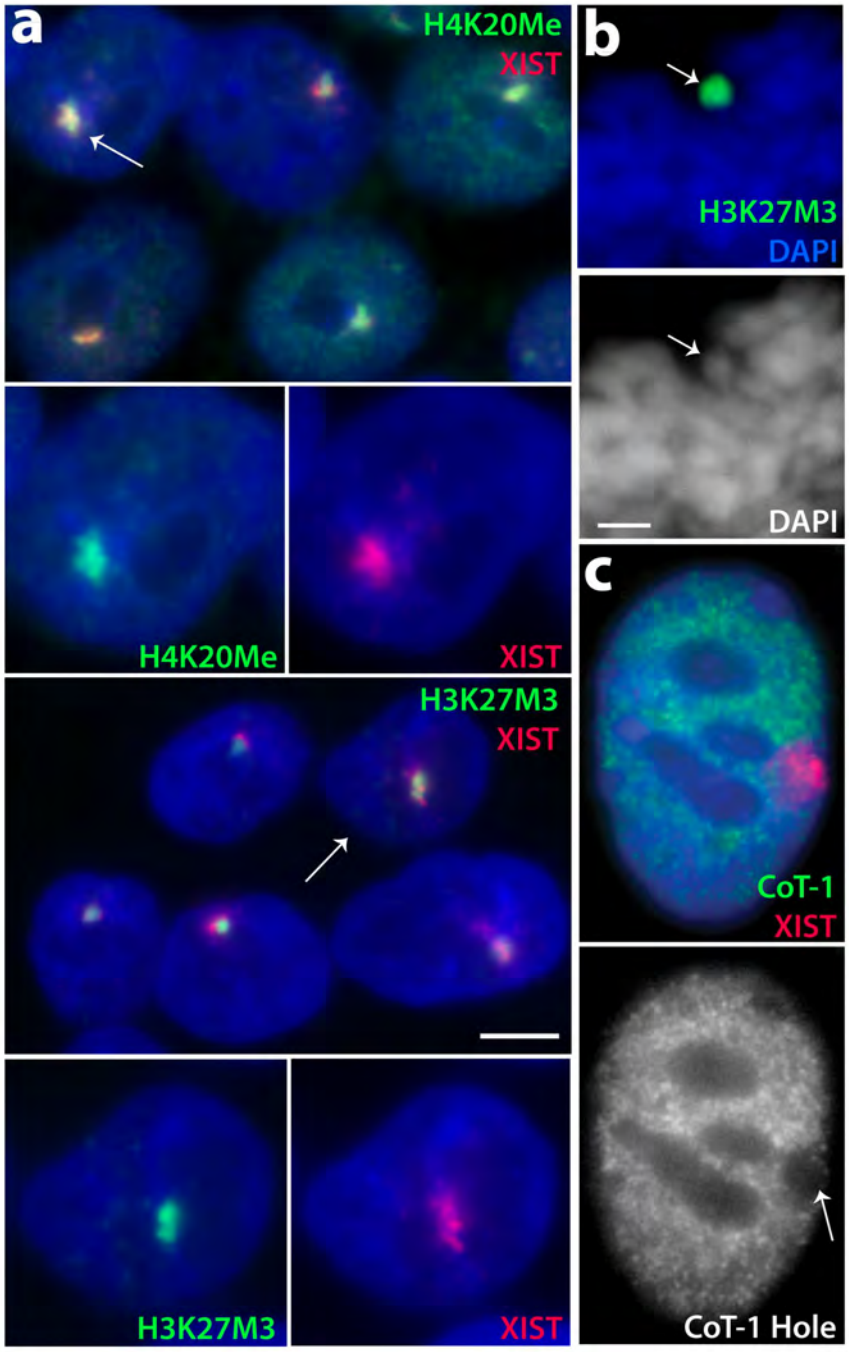


Figure 2.3 *XIST* RNA mediated recruitment of heterochromatic hallmarks to the targeted Chr21 in edited iPSCs.

(a) *XIST* RNA recruits a number of heterochromatic epigenetic marks to the inactivating chromosome, for example H4K20me (top) and H3K27M3 (bottom). Channels are separated for each indicated cell (arrows) at bottom. Scale: 5um. (b) H3K27M3 (green) coats the small chromosome 21 (arrows) in a mitotic cell line. Scale: 2 um. (c) *XIST* RNA territory delineates hnRNA (Cot-1) ”hole” (arrow), indicating Chr21 silencing.

Allele-specific silencing across chromosome 21

To measure overall transcription across the *XIST*-targeted chromosome 21, we used an approach that we developed to broadly assay heterogeneous nuclear RNA (hnRNA) expression and to distinguish inactive from active X chromosome (Hall et al. 2002a), on the basis of *in situ* hybridization to CoT-1 repeat RNA. This showed that the chromosome 21 *XIST* RNA territory was depleted for hnRNA detected by CoT-1 (Figure 2.3c), similar to the inactive X chromosome (Hall et al. 2002a).

We next used multi-colour RNA FISH to determine the presence of transcription foci at each allele for six specific chromosome 21 genes, an established approach that we earlier showed discriminates active versus silenced genes on inactive X chromosome (Clemson et al. 1996; Hall et al. 2002b). Without *XIST* expression, there are three bright transcription foci from each *DYRK1A* allele (Figure 2.1c, top), but after *XIST* expression, the targeted allele becomes weaker or undetectable, indicating repression of *DYRK1A* (Figure 2.1c, bottom).

The *APP* gene on chromosome 21 encodes β-amyloid precursor protein; mutations in *APP* which cause accumulation of β-amyloid lead to early-onset familial Alzheimer's disease, and *APP*overexpression is linked to the Alzheimer's disease characteristic of Down's syndrome (Megarbane et al. 2009). Initially, three bright RNA transcription foci are apparent (Figure 2.4a, top). Short-term *XIST* expression resulted in incomplete repression of the targeted allele (Figure 2.4a, middle), which after 20 days was completely silenced, as shown in two independent clones (Figure 2.4a, bottom, and Figure 2.4b).

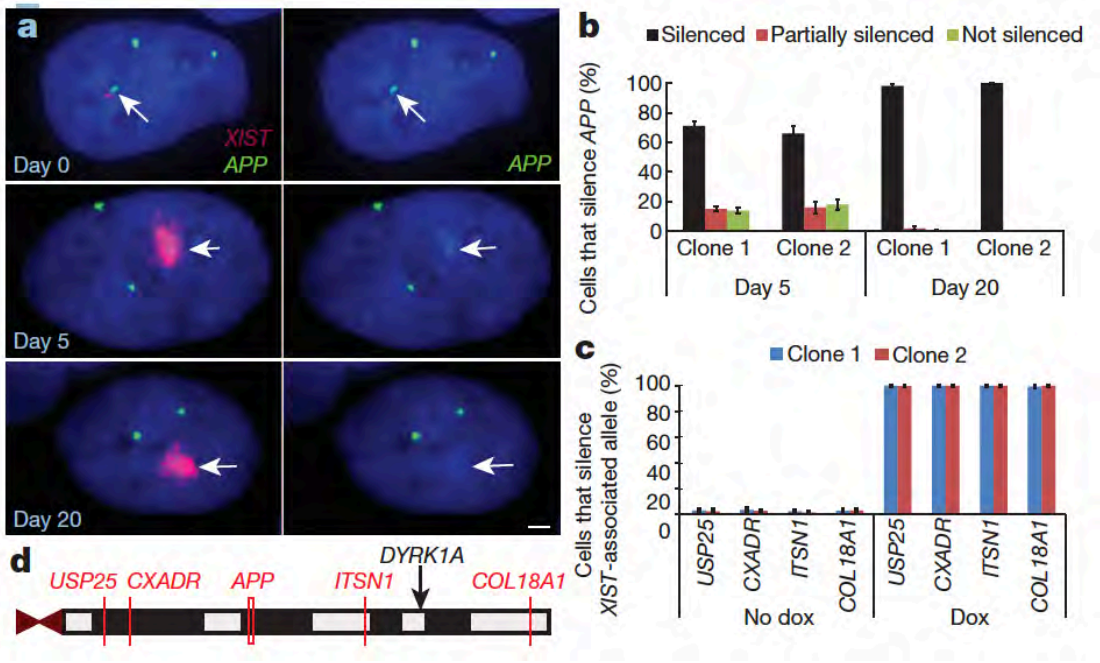


Figure 2.4 XIST induces long-range silencing in targeted iPS cells.

(a) RNA FISH. APP RNA transcribes from three loci in uninduced cells (day 0), and is progressively silenced after induction (targeted chromosome 21, arrows). Scale bar, 2 mm. (b) Quantification of APP silencing. Mean +/- standard error, 100 nuclei. (c) Silencing for four more chromosome-21-linked genes by RNA FISH. Mean +/- standard error from 100 nuclei. (d) Long-range silencing of chromosome 21 genes by XIST RNA. USP25 is ,21Mb from the XIST integration site (black arrow).

We examined four more loci, 3–21 megabases (Mb) from *XIST: ITSN1*, *USP25*, *CXADR* and *COL18A1*. Complete silencing of each allele on the edited chromosome 21 was seen in ~100% of cells accumulating *XIST* RNA (Figure 2.4c, d and Figure 2.5a). Allele-specific silencing was further validated using single nucleotide polymorphism (SNP) analysis. PCR with reverse transcription (RT–PCR) products for eight known polymorphic sites (in four genes) were sequenced (*ADAMTS1*, *ETS2*, *TIAM1* and *HSPA13*) (Figure 2.5b, c). Interestingly, clones 2 and 3 showed an identical pattern of eight SNP alleles repressed, whereas clone 1 showed an alternative pattern of SNPs repressed. As summarized in Figure 2.5c, this chromosome-wide pattern allows extrapolation of the haplotype for each of the three chromosome 21 homologues, and indirectly identifies for each clone which chromosome 21 was silenced by an *XIST* transgene.

We also examined clones carrying *XIST* on two or all three copies of chromosome 21 and found that after 20 days in doxycycline, most or all cells lost *XIST* localization or expression, and the targeted chromosomes did not silence the *APP* gene (Figure 2.6a, b). Thus, there is *in vitro* selection and epigenetic adaptation to circumvent creating a functional monosomy or nullisomy, consistent with observations that monosomic cells do not persist in mosaic patients.

Genome-wide silencing and methylation

Having demonstrated allele-specific repression for the ten genes examined above, we extended this to genome-wide expression profiling. We treated three transgenic clones and the parental line with doxycycline for 3 weeks, and compared their transcriptomes to

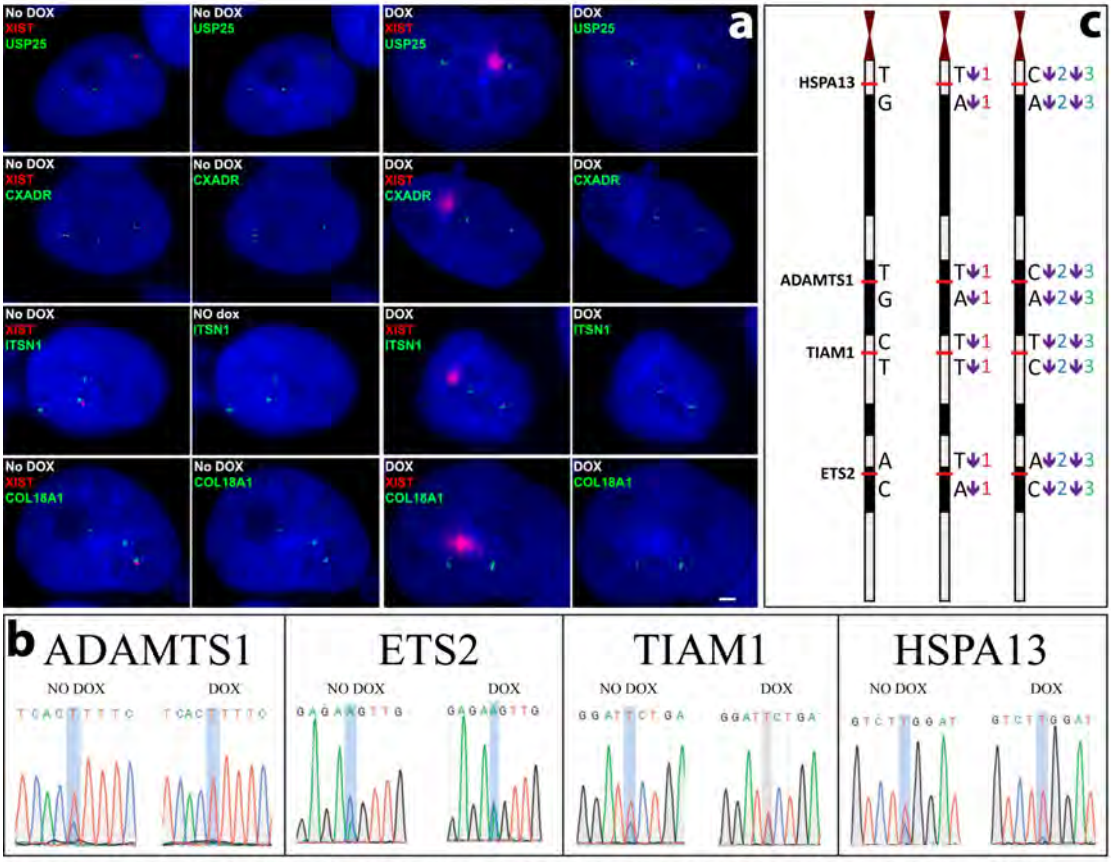


Figure 2.5 *XIST* RNA induces allele-specific gene silencing on the targeted Chr21 in all DS iPS clones.

(a) RNA FISH for four of the six Chr21-linked genes examined. RNA FISH for *APP* (Fig. 2.4a) and *DYRK1A* (Fig. 2.1c) are shown in other figures. In all no-dox treated cells, *XIST* RNA (red) is not expressed and all three transcription foci (green) of four Chr21 genes are visible (left panels). In dox treated cells, *XIST* RNA is expressed (red), paints the inactivating Chr21, and silences the transcription focus of that allele (right panels). Scale: 2 um. (b) Allele-specific SNP analysis for four Chr21 genes. *ADAMTS1* goes from TTC to TT, *ETS2* from CCA to CA, *TIAM1* from TTC to TC, and *HSPA13* from TTC to TT. (c) Alignment of eight Chr21 SNP alleles repressed in clones 1-3. Both clone 2 and 3 silence the fart right chromosome and the center chromosome is silenced in clone 1.

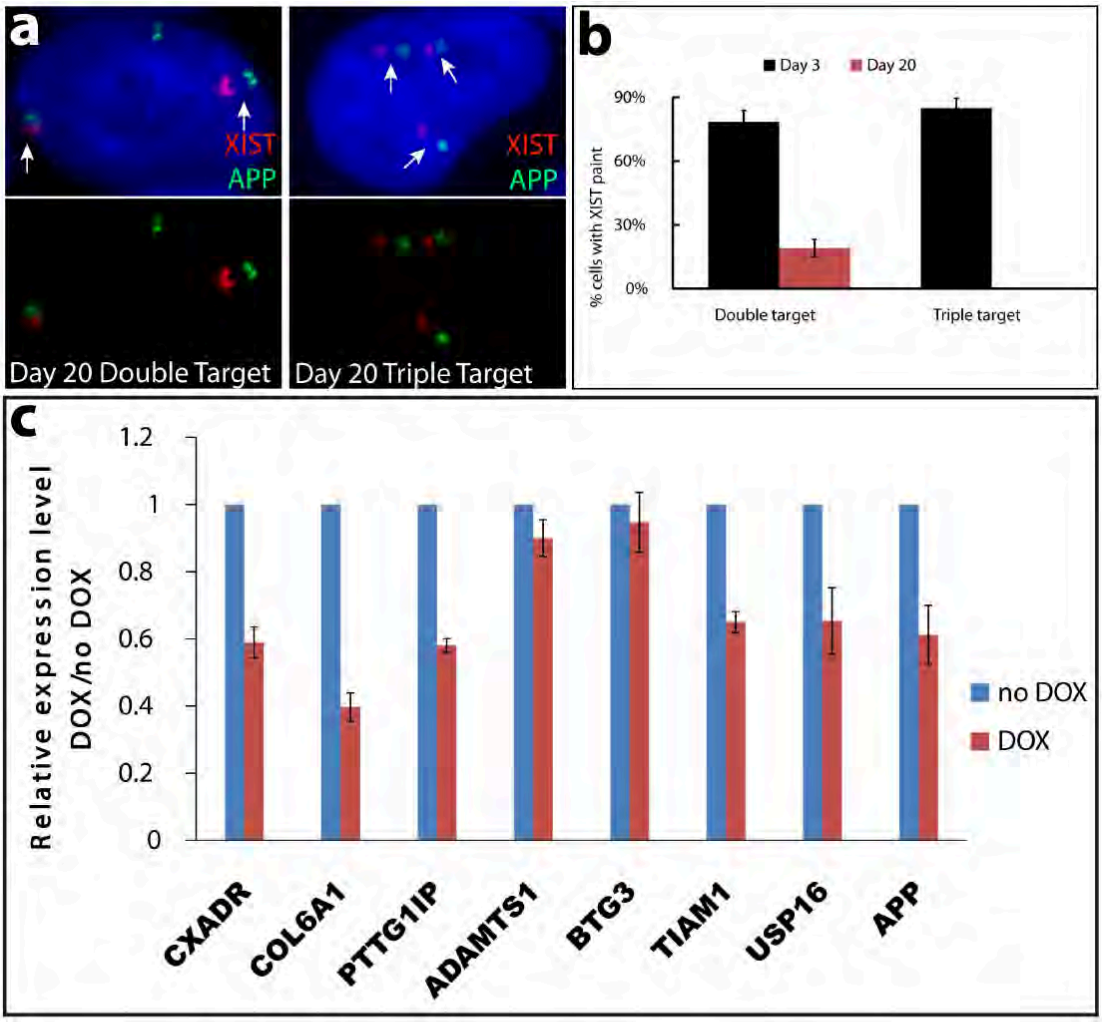


Figure 2.6 Selection against silencing in double targeted and triple targeted clones, and qRT-PCR validation for microarray silencing.

(a) RNA FISH shows *XIST* expression is silenced during long-term culture (20 days) in double targeted (arrows, left) and triple targeted (arrows, right) iPSCs. The *APP* gene is not silencing by the low level *XIST* RNA expressed in these cells. (b) Percentage of *XIST* paint on day 3 and 20 after dox induction. Although *XIST* RNA is robustly expressed in early time points (s days) in the double and triple targeted clones, *XIST* becomes almost entirely silenced in later time points (20 days). Mean +/- SE from 100 nuclei. (c) qRT-PCR for eight Chr21 linked genes for Clone 3. Mean +/- SE from triplicate samples.

parallel cultures without *XIST* transcription, all in triplicate. Only on chromosome 21 is there overwhelming change, in all three clones (Figue 2.7a), with ~95% of significantly expressed genes becoming repressed (Table 2.1).

Dosage compensation corrects chromosome 21 expression to near normal disomic levels, based on the change in total output of expressed genes per chromosome after *XIST* is induced. Because evidence suggests that many chromosome 21 genes are not increased the theoretical 1.5-fold in trisomy (Ait Yahya-Graison et al. 2007; Biancotti et al. 2010), we also directly compared trisomic to disomic cells. This provides a baseline for evaluating the degree to which chromosome 21 overexpression is corrected by *XIST*. After *XIST* induction, overall chromosome 21 expression is reduced by 20%, 15% and 19% for clones 1, 2 and 3, respectively; this mirrors very well the 22% reduction for disomic iPS cells that lack the third chromosome 21 altogether (Figure 2.7a). This disomic line is representative, as a similar difference (21%) was seen for an isogenic disomic sub-clone that we isolated from the trisomic parental iPS cells (not shown). Individual genes repressed by *XIST* are distributed throughout chromosome 21, as do genes overexpressed in trisomic versus disomic cells (Figure 2.7b). In addition, qRT–PCR confirmed repression for individually examined genes (Figure 2.6c). Clearly, *XIST* induces robust dosage compensation of most chromosome 21 genes overexpressed in trisomy.

Trisomy 21 may have an impact on genome-wide expression pathways, but differences attributable to trisomy 21 are confounded by genetic and epigenetic variability (Ait Yahya-Graison et al. 2007). This inducible trisomy silencing system provides a new foothold into this important question. For example, even the three

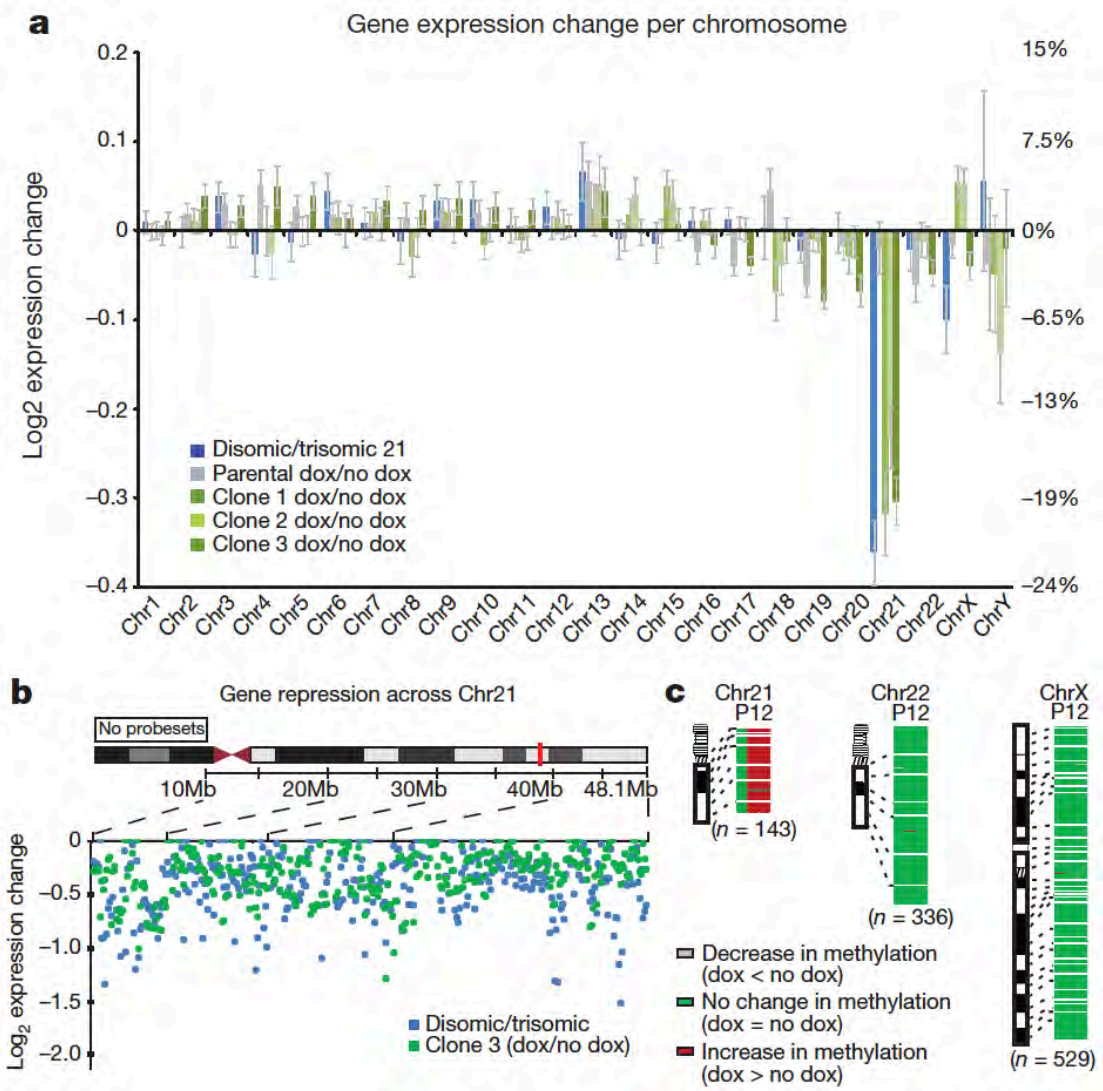


Figure 2.7 Genomic expression and methylation reveal widespread silencing of chromosome 21.

(a) Microarray: expression difference for three transgenic clones in doxycycline (dox) versus no doxycycline, compared to disomic line versus trisomic parental line. Total change in gene expression (n53) per chromosome shows chromosome 21 'correction' near disomic levels, with only limited changes on other chromosomes. The right y axis is scaled for per cent gene expression change. Mean +/- standard deviation, in triplicate. (b) Distribution of individual gene repression across chromosome 21.
c, Methylation of CpGisland promoters. In treated clones, 97% of chromosome 21 genes increased by at least 5% (2-fold greater than average), compared to none in the parental line. P, parental line; 1, clone 1; 2, clone 2.

Table 2.1 Percentage of down regulated genes ($p < 0.01$) by microarray

	Chr 21	whole genome
Disomic/Trisomic	93.5	42.2
Parental line (DOX/no DOX)	41.0	36.6
Clone 1 (DOX/no DOX)	96.3	51.2
Clone 2 (DOX/no DOX)	94.5	49.4
Clone 3 (DOX/no DOX)	98.1	49.5

Of genes with significant change in expression ($p<0.01$), ~95% were repressed on Chr21, with balanced (~50%) changes (up and down) on other chromosomes in three transgenic clones. Repression in dox-treated clones reflects repression seen if the third Chr21 was lost (Disomic/Trisomic).

isogenic transgenic sub-clones show many expression differences (>1,000), but upon *XIST* induction, ~200 genes throughout the genome change in all three clones (but not the doxycycline-treated parental), most probably directly due to chromosome 21 trisomy. Therefore, 'trisomy correction in a dish' has promise as a means to identify genome-wide pathways perturbed by trisomy 21.

In addition to transcriptional silencing, X-inactivation is stabilized by hypermethylation of promoter CpG islands (Csankovszki et al. 2001; Cotton et al. 2011), which occurs late in the silencing process. Therefore, we also examined the promoter methylome in two genome-edited clones 3 weeks after *XIST* induction and found it largely unaltered, with one striking exception, genes on chromosome 21 (*P* value $<2.2 \times 10^{-16}$) (Figure 2.7c(Cotton et al. 2011)). Here, 97% of CpG-island-containing genes exhibited a robust increase in promoter DNA methylation, within the range of that seen for the inactive X chromosome (Cotton et al. 2011) (adjusted for active/inactive chromosomes; see Methods). This change swept the entire chromosome, with the interesting exception of a few genes that 'escape' methylation in both clones.
In summary, data from eight different approaches demonstrate impressive competence of most chromosome 21 genes to undergo epigenetic modification and silencing in response to an RNA that evolved to silence the X chromosome.

Phenotypic correction *in vitro*

Dosage compensation of chromosome imbalance presents a new paradigm, with opportunities to advance Down's syndrome research in multiple directions, including a new means to investigate human Down's syndrome cellular pathologies, which are

largely unknown. Inducing trisomy silencing in parallel cultures of otherwise identical cells may reveal cellular pathologies due to trisomy 21, which could be obscured by differences between cell isolates. We examined cell proliferation and neural rosette formation to look for an impact on cell phenotype.

There is some evidence of proliferative impairment in Down's syndrome brains (Guidi et al. 2011; Haydar and Reeves 2012); however, we observed that this varied *in vitro* between our Down's syndrome fibroblast samples, and this would be highly sensitive to culture history. A clear answer emerged from comparing identical cell cultures, grown with or without doxycycline for 1 week. *XIST* induction in six independent transgenic sub-clones rapidly and consistently resulted in larger, more numerous and tightly packed colonies in just 7 days (Figure 2.8a), with 18–34% more cells (Figure 2.8b). Doxycycline did not enhance growth of the parental Down's syndrome cells or sub-clone (Figure 2.8b). Thus, a proliferative impairment linked to chromosome 21 overexpression can be rapidly ameliorated by dosage compensation.

We next examined differentiation of targeted Down's syndrome iPS cells into neural progenitor cells. In 11–12 days after neural induction of already confluent cultures, all three *XIST*-expressing cultures began to form neural rosettes, and in 1–2 days were replete with neural rosettes (Figure 2.8c), a signature of neural progenitors. Notably, even at day 14, parallel uninduced cultures remained devoid of rosettes (Figure 2.8c). Uncorrected cultures required 4–5 more days in neural-induction media to fill with neural rosettes of similar size and number, which they did on day 17 (Figure 2.8d). There was no effect of doxycycline on neurogenesis in the parental line (Figure 2.8c). This marked delay in neural differentiation seems to be primarily independent of cell proliferation

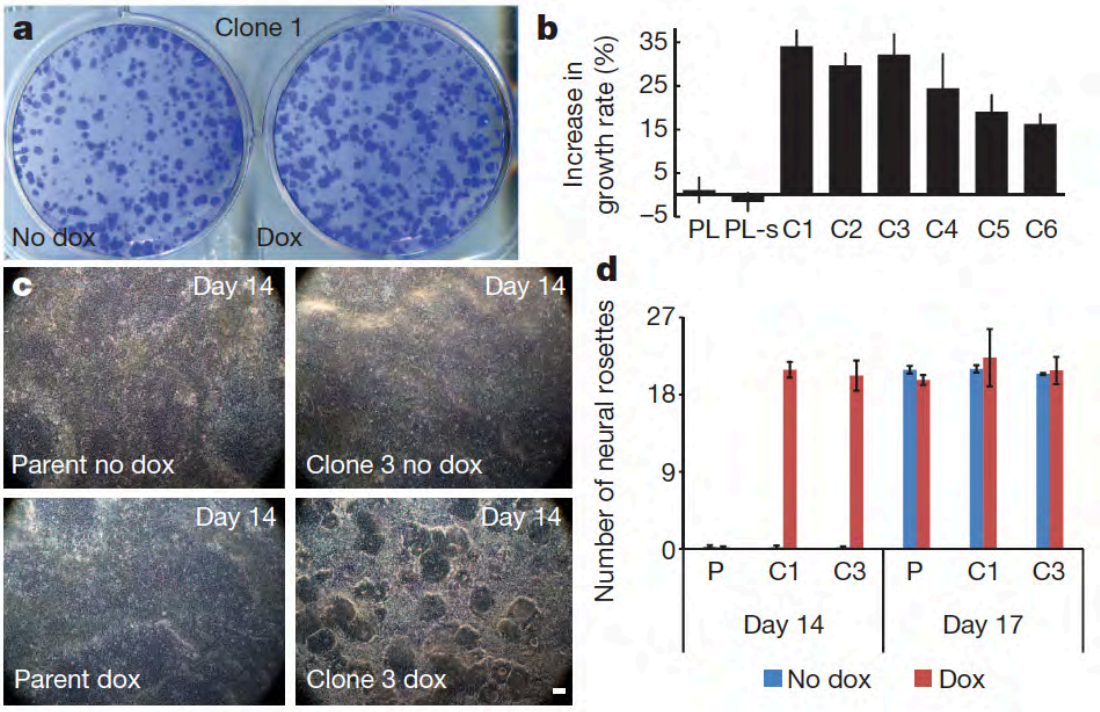


Figure 2.8 'Trisomy correction' affects cell proliferation and neurogenesis.

(a) One week of XIST expression resulted in larger, more numerous colonies (representative sample). (b) Changes in cell number for parental line (PL), parental line subclone (PL-s), and six transgenic clones (C1–C6). Mean +/- s.e. (n=4–6). (c) Corrected cultures formed neural rosettes by day 14; trisomic (parental and non-induced) cultures took longer (17 days). Scale bar, 100 mm. (d) Number of rosettes formed on day 14 and day 17. Mean +/- standard error, 10–12 random fields in triplicate. P, parental; C1, clone 1; C3, clone 3.

(Methods). Variability in the kinetics of neural differentiation between various iPS cell lines can obscure differences due to trisomy 21 (Shi et al. 2012). We circumvented this using parallel cultures and on-demand chromosome 21 silencing, which made clear these important phenotypic differences. This highlights the potential of this new experimental model to illuminate cellular pathologies directly attributable to chromosome 21 overexpression in iPS cells and their differentiated progeny.

Towards future applications

There are two significant points relevant to potential applications and therapeutic strategies. First, we show that heterochromatic silencing is stably maintained, even upon removal of doxycycline and *XIST* expression (Figure 2.9a, b), consistent with previous studies (Csankovszki et al. 2001). Second, although not investigated extensively, we targeted *XIST* in non-immortalized fibroblasts from a female Down's syndrome patient, which generated many cells carrying *XIST* (and some heterochromatin marks) on chromosome 21 (Figure 2.9c, d). Finally, we note that our *XIST* transgene lacks X-chromosome 'counting' sequences, and thus is compatible with natural female X inactivation.

Discussion

We set out to bridge the basic biology of X-chromosome dosage compensation with the pathology of chromosomal dosage disorders, particularly Down's syndrome. In so doing, the present work yields advances that have an impact on three important areas: one basic and two translational.

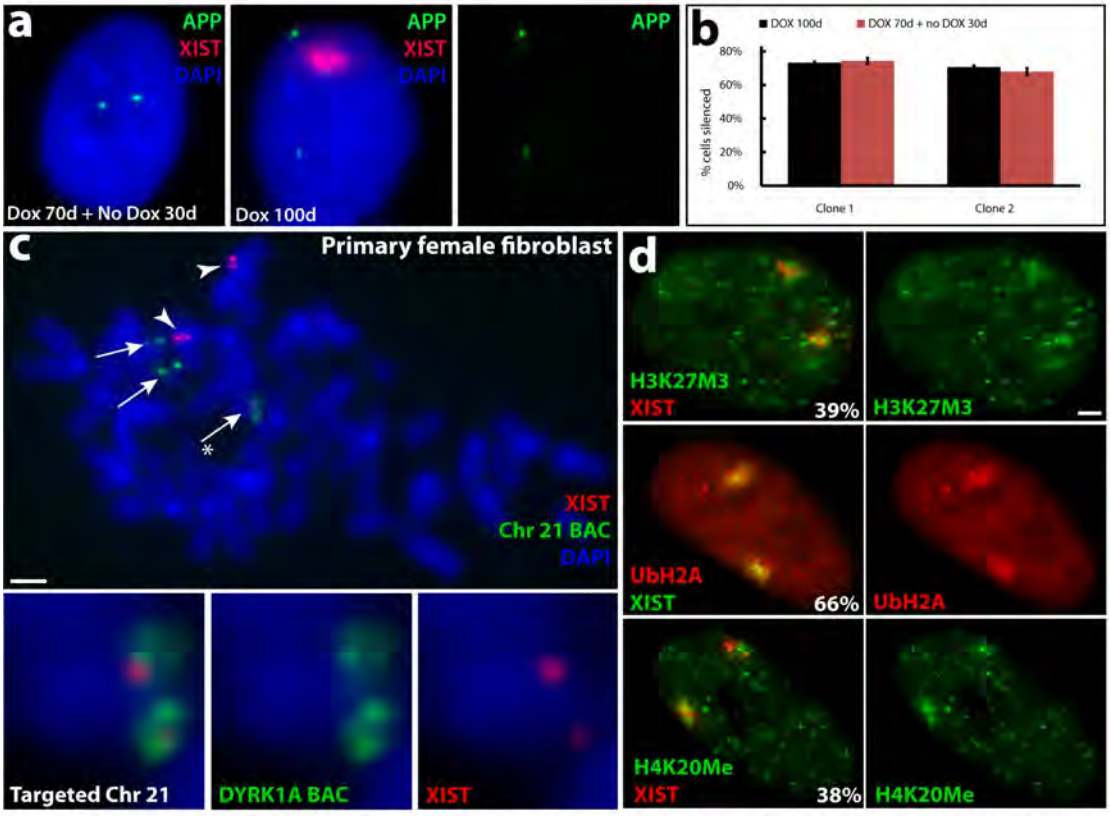


Figure 2.9 Stable silencing in cortical neurons and targeted addition in primary fibroblasts.

(a) RNA FISH in differentiated cortical neurons. The left cell shows the third *APP* transcript locus (green) remains silenced after the cells was treated with dox for 70 days and then dox was removed for 30 days, as similarly shown in the cell (middle and right) that was treated with dox 100 days. This is consistent with other evidence that multi-layered chromatin modifications triggered by *XIST* maintain a largely irreversible silent state. (b) Quantification of *APP* silencing by RNA FISH. *APP* silencing is stable upon withdraw of *XIST* RNA. Mean +/- SE from 100 nuclei. (c) Metaphase DNA FISH in human DS primary fibroblasts. Long arrows indicate three Chr21s, and asterisk indicates *XIST* targeted Chr21 that is enlarged at the bottom panel. Arrowheads indicate endogenous *XIST* genes on the two X-chromosomes in the female cell. Scale: 5um. (d) Immunostaining for targeted primary fibroblasts indicates enrichment of H3K27me3, UbH2A, and H4K20me on both the targeted Chr21 and endogenous Xi in many cells (percentages shown on figures). Scale: 2um. This is consistent with evidence from our lab and the Wutz lab that chromosome silencing does not necessarily require the optimal pluripotent cell context. Future studies will be required to assess the ability of various somatic cell contexts to support chromosome silencing.

Although not our primary focus here, a significant impact of this work is that we have created a tractable, inducible system to study human chromosome silencing. Importantly, unlike random integration into a diploid cell, silencing a trisomic autosome avoids selection against full autosomal silencing, and this demonstrated remarkably robust competence of chromosome 21 to be silenced. Thus, *XIST* RNA evolved for the X chromosome uses epigenome-wide mechanisms (Hall and Lawrence 2010). The ability to insert a single *XIST* transgene in any locus provides a more powerful tool to study *XIST* function, and our effort also almost triples the size of transgenes that can be thus targeted for a host of other applications.

From a translational perspective, trisomy silencing has immediate impact as a new means to define the poorly understood cellular pathways deregulated in Down's syndrome, and creates the opportunity to derive and study various patient-compatible cell types potentially relevant to Down's syndrome therapeutics. Inducible trisomy silencing *in vitro* compares otherwise identical cultures, allowing greater discrimination of differences directly due to chromosome 21 overexpression distinct from genetic and epigenetic variation between transgenic sub-clones, or potentially even rare disomic sub-clones isolated from a trisomic population ((Lavon et al. 2008; Li et al. 2012) and this study). *XIST* expression triggers not only chromosome 21 repression, but a defined effect on the genomic expression profile, and reverses deficits in cell proliferation and neural progenitors, which has implications for hypocellularity in the Down's syndrome brain (Guidi et al. 2011; Haydar and Reeves 2012). This new approach can illuminate the cohort of genes and cognate pathways most consistently impacted in Down's syndrome, to inform the search for drugs that may rebalance those pathways and cell pathologies.

This general strategy could be extended to study other chromosomal disorders, such as trisomy 13 and 18, often fatal in the first 1–2 years.

Finally, the more forward-looking implication of this work is to bring Down's syndrome into the realm of consideration for future gene therapy research. Although development of any clinical gene therapy is a multi-step process, any prospect requires that the first step, functional correction of the underlying genetic defect in living cells, is achievable. We have demonstrated that this step is no longer insurmountable for chromosomal imbalance in Down's syndrome. Our hope is that for individuals and families living with Down's syndrome, the proof-of-principle demonstrated here initiates multiple new avenues of translational relevance for the 50 years of advances in basic X-chromosome biology.

Materials and methods

Cell culture

HT1080 TetR cells (Invitrogen) and female Down's syndrome human primary fibroblast line (Coriell) (AG13902) were cultured as recommended by the supplier. Down's syndrome iPS cell parental line (DS1-iPS4) was provided by G. Q. Daley (Children's Hospital Boston)(Park et al. 2008) and maintained on irradiated mouse embryonic fibroblasts (iMEFs) (R&D Systems, PSC001) in hiPSC medium containing DMEM/F12 supplemented with 20% knockout serum replacement (Invitrogen), 1 mM glutamine (Invitrogen), 100 μM non-essential amino acids (Invitrogen), 100 μM β-mercaptoethanol (Sigma) and 10 ng ml^{-1} FGF-β (Invitrogen, PHG0024). Cultures were passaged every 5–7 days with 1 mg ml^{-1} of collagenase type IV (Invitrogen).

ZFN design

ZFNs against the human *AAVS1* locus on chromosome 19 have been previously described (DeKelver et al. 2010). ZFNs against the *DYRK1A* locus were designed using an archive of pre-validated zinc finger modules (Urnov et al. 2010; Doyon et al. 2011), and validated for genome editing activity by transfection into K562 cells and Surveyor endonuclease-based measurement of endogenous locus disruption ('Cel1' (Miller et al. 2007; Guschin et al. 2010)) exactly as described (Doyon et al. 2011). Southern blotting for targeted gene addition was performed exactly as described (Urnov et al. 2005; Moehle et al. 2007) on SphI-digested genomic DNA probed with a fragment corresponding to positions Chr21:38825803+38826056 (hg19).

XIST and rtTA/puro plasmid construction

Fourteen-kilobase human *XIST* cDNA, a splicing isoform of full-length *XIST* cDNA, was subcloned into pTRE3G (Clontech, catalogue no. 631167). Two homologous arms (left arm, 690 bp; right arm, 508 bp) of *DYRK1A* gene on chromosome 21 were amplified by PCR from primary Down's syndrome fibroblasts (AG13902) (Coriell) and cloned into the pTRE3G vector (human chromosome 21 *DYRK1A* left arm primers: forward 5′-GCCGTATACCATTAACTCTTTACTGTTC-3′, reverse 5′-TCTGTATACGTAAACTGGCAAAGGGGTGG-3′; human chromosome 21 *DYRK1A*right arm primers: forward 5′-ATTTCGCGAACGGGTGATGAGCAGGCTGT-3′, reverse 5′-CCGTCGCGAAAACCAGAAAGTATTCTCAG-3′). The pEF1α-3G rtTA-pA cassette from pEF1α-Tet3G vector (Clontech) was subcloned into a plasmid for targeted gene addition to the *PPP1R12C*/*AAVS1* locus (DeKelver, 2010), which contains a unique HindIII site flanked by two 800-bp stretches of homology to the ZFN-specified position in the genome.

Dual-targeted addition of human Down's syndrome iPS cells and generation of stable targeted clones

The Down's syndrome iPS cell line was cultured in 10 μM of Rho-associated protein kinases (ROCK) inhibitor (Calbiochem; Y27632) 24 h before electroporation. Single cells (1×10^7) were collected using TryPLE select (Invitrogen), re-suspended in 1× PBS and electroporated with a total of 55 μg DNA including five plasmids (*XIST*, *DYRK1A* ZFN1, *DYRK1A* ZFN2, rtTA/puro and *AAVS1* ZFN) with both 3:1 and

5:1 ratios of *XIST*:rtTA/puro. The electroporation conditions were 220 V and 750 μF (BioRad Gene Pulser II System). Cells were subsequently plated on puromycin-resistant DR4 MEF feeders (Open Biosystems, catalogue no. MES3948) in hiPSC medium supplemented with ROCK inhibitor for the first 24 h. Over 300 colonies remained after 12 days of 0.4 μg ml^{-1} puromycin selection and 245 randomly chosen individual colonies across 36 pooled wells were examined by interphase DNA/RNA FISH for the presence and expression of *XIST*, correct targeting and retention of trisomy (because some subclones lacked *XIST* or showed just two *DYRK1A* DNA signals). Over 100 individual clones were isolated and characterized, and those of interest, containing targeted *XIST* on one of three *DYRK1A* loci, were frozen. Six single target clones with good pluripotent morphology, OCT4 positive staining, correct targeting to one trisomic chromosome, and good *XIST* RNA paint were expanded for further characterization. One double and one triple target line, two non-target clones, and one disomic clone were also isolated and frozen. Targeting and correct chromosome number (47) was confirmed by interphase and metaphase FISH and genome integrity was confirmed by high-resolution G-band karyotype and CGH array.

Chromosome preparation

iPS cells were treated with 100 ng ml^{-1} KaryoMAX colcemid (Invitrogen) for 2–4 h at 37 °C in a 5% CO_2 incubator. Cells were trypsinized, treated with hypotonic solution, and fixed with methanol:acetic acid (3:1). Metaphases were spread on microscope slides, and at least 20 analysed per clone. Karyotype analysis was done on pro-metaphase chromosomes using Standard Giemsa-trypsin G band methods.

CGH array

CGH was performed in the Cytogenetics Laboratory at University of Massachusetts Medical School. Genomic Microarray analysis using University of Massachusetts Genomic Microarray platform (Human Genome Build hg19) was performed with 1 µg of DNA. The array contains approximately 180,000 oligonucleotides (60-mers) that represent coding and non-coding human sequences and high-density coverage for clinically relevant deletion/duplication syndromes and the telomeric and pericentromeric regions of the genome. Data were analysed by BlueFuse Multi, v3.1 (BlueGnome, Ltd).

DNA/RNA FISH and immunostaining

DNA and RNA FISH were carried out as previously described(Clemson et al. 1996; Hall et al. 2002a; Hall et al. 2002b; Byron et al. 2013). The *XIST* probe is a cloned 14-kb *XIST* cDNA (the same sequence as the *XIST* transgene) in pGEM-7Zf(+) (Promega). Six chromosome 21 gene probes are BACs from BACPAC Resources (*DYRK1A*, Rp11-105O24; *APP*, RP11-910G8; *USP25*, RP11-840D8; *CXADR*, RP11-1150I14; *ITSN1*, RP11-1033C16; *COL18A1*, RP11-867O18). DNA probes were labelled by nick translation with either biotin-11-dUTP or digoxigenin-16-dUTP (Roche). In simultaneous DNA/RNA FISH (interphase targeting assay), cellular DNA was denatured and hybridization performed without eliminating RNA and also treated with 2 U μl^{-1} of RNasin Plus RNase inhibitor (Promega). For immunostaining with RNA FISH, cells were immunostained first with RNasin Plus and fixed in 4% paraformaldehyde before RNA FISH. Antibodies were as follows: H3K27me3 (Millipore, 07-449), UbH2A (Cell

Signaling, 8240), H4K20me (Abcam, ab9051), macroH2A (Millipore, 07-219), OCT4 (Santa Cruz, sc-9081), PAX6 (Stemgent, 09-0075), SOX1 (R&D Systems, AF3369).

Allele-specific SNP analysis

Primers were designed to amplify 3′ untranslated regions of chromosome 21 genes reported to contain SNPs (Table 2.1). Total cDNA was used from three transgenic clones with and without *XIST* induction for 22 days. RT–PCR products were sequenced by GENEWIZ. Of ~10 genes examined, four were heterozygous and informative in the patient Down's syndrome iPS cell line used here.

Microarray analysis

Three independently targeted subclones plus the parental chromosome 21 trisomic (non-targeted) iPS cell line were grown with or without doxycycline (2 μg ml^{-1}) for 22 d. Normal male iPS cell and disomic isogenic lines were also cultured for 22 d and total RNA was extracted with a High Pure RNA extraction kit (Roche) in triplicate for each, processed with a Gene Chip 3′ IVT express kit (Affymetrix), and hybridized to Affymetrix human gene expression PrimeView arrays. Array normalization was performed with Affymetrix Expression Console Software with Robust Multichip Analysis (RMA)(Irizarry et al. 2003). Probe sets with the top 60% of signal values were considered present and 'expressed' and were used for all further analysis. Data in Fig. 4 has no other threshold applied. When designated, a gene expression change significance threshold was applied using a two-tailed *t*-test comparing samples with or without doxycycline in triplicate ($n = 3$) (Table 2.1, $P < 0.01$). For the ~200 genes found to significantly change in all three clones (in text), a *t*-test with $P < 0.001$ was applied.

Table 2.2 Primers for Chr21 gene amplification (allele-specific SNP silencing analysis)

genes	Forward primer	reverse primer
ADAMTS1	5' - TCTCTGAAACCATAGCAGCCA -3'	5' - CTTGTGCAGACCATCCCTGC -3'
ETS2	5' - GCCTTTTGCAACCAGGAACAGC -3'	5' - ATCACACAGAAGAACGTGGAGC -3'
SPA13	5' - AACTCTGCTCCAAATGCCGA -3'	5' - CCTGTACATCATTCTCTGCTTGG -3'
TIAM1	5' - TGGGGTGATTTGCTTTCCAGTGC -3'	5' - GTGCAGTGTCTGCCCCAAGC -3'

Microarray data interpretation

Using extraction-based methods, changes on just one of three alleles (from the *XIST*-bearing chromosome) will be diluted by the other two. If all three chromosomes are fully expressed, this would predict a 33% reduction in chromosome 21 expression levels per cell when one chromosome 21 is fully silenced. However, 33% would apply only if chromosome 21 genes are fully overexpressed to start, and previous evidence and results in this study show this is not the case for many genes. Previous microarray studies have analysed expression levels of chromosome 21 in Down's syndrome patient cells, although such analyses are hampered by the extensive genetic and epigenetic differences between any two individuals (Prandini et al. 2007). The fraction of chromosome 21 genes detected as overexpressed varies with the study and tissue, but generally is in the 19–36% range(Prandini et al. 2007; Biancotti et al. 2010), with individual gene increases often in the ~1.2–1.4 range (less than the theoretical 1.5). For example, one study of Down's syndrome embryoid bodies showed that only 6–15% of genes appeared significantly upregulated, but this was comparing non-isogenic samples of different ES cell isolates (Biancotti et al. 2010).

Our trisomy correction system allows direct comparison of the same cells grown in identical parallel cultures, with and without *XIST*-mediated chromosome silencing. Our data show a ~20% reduction in chromosome 21 expression overall; importantly, this level of reduction is seen either when the third chromosome is silenced in trisomic cells, or when disomic and trisomic cells are compared. This 20% reduction represents an average

per cell for all three chromosomes, but corresponds to a 60% reduction in expression for just one chromosome 21 (the one silenced by *XIST* RNA, as shown here).

Apart from our goal here of trisomy dosage compensation, these results add significantly to understanding the extent of chromosome 21 overexpression in Down's syndrome, by providing a more comprehensive analysis that shows that expression of most genes is increased, but less than the theoretical 1.5-fold.

qRT–PCR

qRT–PCR was performed for eight downregulated chromosome 21 genes determined by microarray on a Bio-Rad MyiQ real-time PCR detection system in triplicate for clone 3 with/without doxycycline treatment for 22 d. The β-actin gene was used as an internal standard for calculation of expression levels. Primers for eight chromosome 21 genes and β-actin were described in Table 2.3.

DNA methylation analysis

The parental line and two independent targeted lines were grown with and without doxycycline for 22 d, in duplicate cultures. Genomic DNA was extracted using PureLink Genomic DNA mini kit (Invitrogen) and 750 ng bisulphite modified with the Alternative Incubation Conditions from the EZ DNA methylation kit (Zymo Research). 160 ng of bisulphite DNA was amplified, fragmented and hybridized to Illumina Infinium HumanMethylation450 array following the standard protocol as outlined in the user guide. CpG islands were defined as high and intermediate CpG densities using the CpG

density classifications based on those used previously (Weber et al. 2007). The program CpGIE was used to locate HC and IC islands on the X chromosome and chromosomes 21 and 22. When multiple probes in CpG islands were associated with the same TSS, an average genic methylation value was calculated. These average genic values were compared before and after doxycycline induction using the Mann–Whitney *U*-test. Analysis was based on CpG islands within promoters of 143 chromosome 21 genes (Figure 2.7c).

The average methylation value was 6% on chromosome 21 before *XIST* induction, and increased to 20–21% in both subclones after induction. Because any methylation increase on the transgenic chromosome would be diluted by the presence of three chromosome 21 copies, this suggests the range of 60% methylation on the one *XIST*-coated chromosome, which is within the range seen for the inactive X chromosome (Weber et al. 2007).

Cell proliferation analysis

Eight different iPS cell lines (parental line, one non-targeted subclone, and six independent targeted subclones) were passaged onto 6-well plates at equal cell densities per well of each line and grown with or without doxycycline for 7 d. At least four replicates of each line were analysed in two independent experiments. Rigorous measures were taken to minimize and control for any minor variations in seeding densities of iPS cells, which cannot be plated as single cell suspensions. First, the analysis was done twice for six different transgenic clones, in each case comparing triplicate plates of corrected versus not corrected (doxycycline versus no doxycycline). To avoid differences in plating

efficiencies of doxycycline and no doxycycline cells, we performed the experiments over a time course that did not require passage. For each of the six transgenic clones, the parental line and one negative control (non-targeted) subclone, a single well of Down's syndrome iPS cells (without doxycycline) was used to generate a cell suspension (cells and small disaggregated clumps). Next, equal aliquots of the cell suspension were plated into each of six wells four times (not relying on one measurement but the average of four for seeding each well). After plating, doxycycline was added to three of the six wells, and the cultures were maintained for 7 d. For images, plates were fixed, stained with 1 mg ml^{-1} crystal violet (Sigma) in 70% ethanol for 30 min and scanned to generate TIFF images. For cell counts, single cells were collected by TryPLE select and counted using Beckman Coulter Z1 Particle Counter.

Differentiation of neural progenitors and irreversibility in cortical neurons

For differentiation, independent *XIST*-transgenic iPS cell clones and the parental Down's syndrome iPS cell line were dissociated with Accutase (Innovative Cell Technologies) and 4×10^5single cells were plated on Matrigel-coated 6-well plates in mTeSR1 medium (Stemcell technologies). Once the cell culture reached 90–100% confluence, neural induction was initiated by changing the culture medium to neural induction medium, a 1:1 mixture of N2- and B27-containing media supplemented with 500 ng ml^{-1} noggin (R&D Systems), 10 µM SB431542 (Tocris Bioscience), and 1 µM retinoic acid (Sigma, catalogue no. R2625), with/without treatment of doxycycline for the specified times. The neural rosettes were counted and their diameter measured for at least 300 rosettes (sampled in random areas from triplicate dishes). At day 14, the doxycycline-induced

culture had an average rosette diameter of 142 μm ± 0.55 μm in clone 1 and 141 μm ± 3.49 μm in clone 3. Rosettes could not be measured at the same time point in the uncorrected culture, as they had not formed. At day 17, the uncorrected culture had neural rosettes of similar number and size for both clones 1 (140 μm ± 0.87 μm) and 3 (140 μm ± 1.09 μm). The corrected culture could not be accurately compared for day 17 because the rosettes had become so mature and often had merged. After 17 d, neural rosettes were collected by dissociation with dispase and replated on poly-ornithine and laminin-coated plastic dishes in N2- and B27-containing media including 20 ng ml^{-1} FGF2. After a further 2 d, FGF2 was withdrawn to promote differentiation of cortical neurons. To test for the irreversibility of silencing, two independent clones were differentiated to cortical neurons in the presence of doxycycline for 70 days to initiate silencing. They were then split into parallel cultures grown with and without doxycycline for another 30 days, and and *XIST* and *APP* expression analysed by RNA FISH.

Chapter III: Trisomy Silencing by XIST normalizes known Down Syndrome cell pathogenesis as demonstrated for hematopoietic defects *in vitro*

Preface

Work described in this chapter was submitted to Nature Communications (as shown below) and is currently under revision. I essentially did all the experiments except the neural differentiation which was done by Jun Jiang.

Jen-Chieh Chiang, Jun Jiang, Peter E. Newburger, and Jeanne B. Lawrence, **Dosage compensating trisomy 21 in DS iPS cells mitigates abnormalities associated with hematopoietic differentiation. Submitted to** *Nature Communications.*

Introduction

Down syndrome (DS), caused by trisomy 21, is the most common human chromosomal disorder, occurring in about one in every 750 newborns in the United States, and bringing enormous medical and social costs to millions worldwide. Children with DS consistently have mild to moderate cognitive disability which often progresses in adulthood, and it is now recognized that 80% develop early-onset Alzheimer Disease. Trisomy 21 also confers high risk of congenital heart disease, metabolic changes, and hematopoietic abnormalities. This study focuses on the common hematopoietic abnormalities seen in DS neonates which confer high-risk of transient myeloproliferative disorder (TMD) and 500-fold greater incidence of AMKL, in addition to a 20-fold greater risk for ALL. Many or most DS individuals, even as adults, also show less acute hematological abnormalities, including immune system defects and extreme susceptibility to viral infections, a prominent cause of morbidity and death in this population (Kusters et al. 2009; Megarbane et al. 2009; Gardiner 2010; Ram and Chinen 2011; Bruwier and Chantrain 2012; Sullivan et al. 2016).

Children with Down Syndrome are typically sociable, happy and valued members of families, but biomedical research for DS has lagged that of less common monogenetic disorders, and better experimental and therapeutic strategies are needed. Work here tests the feasibility that a novel approach involving chromosome silencing with an XIST transgene can normalize an established DS cell pathogenesis *in vitro*, a question that has not been previously addressed for any system. We test the phenotypic effects of induced chromosome repression in an *in vitro* model of human fetal hematopoiesis, the system for which cellular phenotypes of Trisomy 21 are best established from clinical studies, and

studies of fetal liver cells (Chou et al. 2008; Tunstall-Pedoe et al. 2008; Roy et al. 2012). Several aspects of DS hematopathogenesis have been shown recapitulated in studies comparing human trisomic and disomic iPS cells, particularly those using protocols that mirror fetal hematopoiesis (Maclean et al. 2012; Banno et al. 2016), as we utilize here. Our first priority was to address the extent to which expression of a single gene, XIST, can normalize hematopoietic phenotypes associated with Trisomy 21, including whether chromosome silencing impacts particular steps of hematopoiesis consistent with predictions of other studies (Lancrin et al. 2012; Maclean et al. 2012; Banno et al. 2016). In addition, the tightly-controlled inducible experimental system used allowed us to not only corroborate but extend certain important points regarding the specific effects of trisomy 21 on hematopoiesis, notably the unconfirmed hypothesis that overactive IGF signaling is present and important in trisomy 21-associated myeloid disorders (Klusmann et al. 2010a; Bhatnagar et al. 2016).

For single-gene disorders it is more straightforward to identify a gene mutation and determine the specific cells and pathways that underlie a phenotype, as is essential for the development of targeted drug therapies. For most major systems impacted in Down syndrome, however, it is not even clear what specific cell-types underlie various phenotypes, nor is it known how many of the ~300 genes on chromosome 21 have any effect when expressed just 50% more. Inbred mouse models of DS have been valuable and a number of candidate genes identified (reviewed in (Herault et al. 2017)), but, with the exception of the known role of APP in Alzheimer Disease, genes and pathways that underlie major DS phenotypes have yet to be established. In fact, alternative concepts of DS hold that much of the pathology is not due to specific chromosome 21 genes but to

the physical presence of an extra chromosome causing general stress or mitotic/cell-cycle defects that negatively impact cell function and stability (Sheltzer et al. 2017). Studies in yeast and cultured mouse cells show that an additional copy of any chromosome causes a proliferative disadvantage, due to the proteomic stress of collective low level over-expression of many genes, rather than a few specific "dosage-sensitive" genes (Oromendia et al. 2012; Blank et al. 2015).

Understanding pathogenesis is needed for traditional drug therapeutics, but identification of specific gene targets is required for gene-therapy strategies which have been progressing rapidly for mono-genic disorders, bolstered by an ongoing revolution in development of powerful *in vivo* gene editing and delivery technologies (Naldini 2015). Such hopeful progress, however, has not even been a consideration for chromosomal imbalances, given the insurmountable obstacle of correcting many genes across a chromosome. However, gene-based therapy for DS could become more thinkable, even without identification of one or a few target genes, if insertion of a single "epigenetic switch" to suppress chromosome-wide transcription can effectively mitigate cell pathogenesis and normalize phenotypic outcome.

Our laboratory has demonstrated an innovated approach for targeted regulation of the epigenome by insertion of a single gene, XIST, into one of three chromosome 21s in iPS cells derived from a DS patient (Jiang et al. 2013). The X-linked *XIST* naturally controls X-chromosome inactivation in human female cells, producing a long non-coding RNA that coats the X-chromosome *in cis* to induce a series of chromatin modifications that stably silence transcription across that X chromosome (Brown et al. 1992; Clemson et al. 1996). Insertion of XIST into a trisomic autosome allowed Jiang et al. to

demonstrate that in absence of selection against silencing (of a disomic autosome), *XIST* has a remarkably comprehensive capacity to epigenetically silence genes across the autosome(Jiang et al. 2013). The focus of this prior study was to demonstrate that a targeted *XIST* transgene could indeed induce chromosome-wide transcriptional silencing of a trisomic chromosome, which was shown in undifferentiated iPS cells by eight different methods, including CpG promoter methylation and reduction of chromosome 21 transcriptional output to near normal disomic levels.

Here we address for the first time the critical next question: can "trisomy silencing" effectively normalize or mitigate defects in cell function and pathogenesis which underlie DS phenotypes? A priori, it cannot be assumed that *XIST*-mediated transcriptional repression would be sufficiently robust to correct cell pathogenesis, even in cells that still carry the physical presence of the extra chromosome 21. Hence, direct determination of this is critical to any future prospect of "chromosome therapy", and for the utility of this experimental approach to investigate trisomy 21 effects in different cell systems. Inducible expression of *XIST*, as used here, provides a powerful test system and was applied to the hematopoietic system as our priority for several reasons. First, hematopoietic cell pathologies are the most clearly defined and established, allowing a rigorous test of this key point. Moreover, hematopoietic abnormalities are important clinically, most acutely for 20-30% of infants that develop TMD, a pre-cursor to leukemia. However, less severe hematopoietic abnormalities are widely present in DS patients that have broader impacts on health, ranging from high susceptibility to infections to potentially inflammation that may even impact cognition or Alzheimer pathology (Roizen and Amarose 1993; David et al. 1996; de Hingh et al. 2005; Bloemers

et al. 2010). There is also greater accessibility of hematopoietic cells by established bone marrow or cord blood procedures.

Finally, because DS hematopoiesis involves *over-production* of certain cell types, this system allows us to address a key question: whether silencing a trisomic chromosome in any cultured cells might enhance cell proliferation/fitness in a non-specific manner due to relief of "aneuploidy stress", not necessarily by correcting specific defects in a developmental program. *A priori*, silencing trisomy 21 in the hematopoietic system might actually *increase* the over-proliferation of the trisomic hematopoietic cell-types. Alternatively, silencing trisomy may normalize over-production of these blood cell types, indicating successful correction of a specific defect in hematopoiesis.

Therefore, in this study, we determined the effects of induced XIST expression during differentiation along the hematopoietic pathway, to mimic DS fetal liver hematopoiesis which other evidence indicates is the pathogenic source of TMD and AMKL in DS children. Mutations of *GATA1* are consistently present in both TMD and AMKL leukemic blasts (Ahmed et al. 2004; Ge et al. 2006), however excessive production of erythroid and megakaryocytic cells can be observed as early as in second trimester DS fetal liver. This and other evidence (Chou et al. 2008; Tunstall-Pedoe et al. 2008) indicates that trisomy 21 generally causes overproduction of hematopoietic cells in fetal hematopoiesis, which is a precursor in many infants to acquisition of GATA1s mutation, further promoting the clinical morbidity or mortality of TMD and AMKL.

Results

Trisomy 21 silencing mitigates excessive production of megakaryocytes and erythrocytes

Figure 3.1a summarizes the experimental design in which a dox-inducible full-length *XIST* cDNA was inserted into one of three chromosome 21s in iPS cells derived from a male DS patient, as previously described (Jiang et al. 2013). This prior study focused on establishing that the RNA properly localized to one chromosome 21 and comprehensively silenced genes across that chromosome *in cis*, using numerous methods to demonstrate chromosome-wide "trisomy silencing" in undifferentiated cells. Here we investigate the ability of trisomy silencing to normalize the well-established hematopoietic cell pathologies using a previously characterized all-isogenic panel of DS iPS cell subclones, including four independent XIST-transgenic clones as well as the non-transgenic parental trisomic cells and an isogenic disomic subclone (from a cell which spontaneously lost one chromosome 21). A strength of this system is the ability to induce silencing of one chromosome 21 in parallel cultures of otherwise identical cell populations, thus minimizing other sources of variation that can arise between even isogenic iPSC clones (Prandini et al. 2007; Hall et al. 2008; Nazor et al. 2012).

For perspective in investigating effects of induced-XIST expression on hematopoiesis, it is instructive to consider that expression of transgenic XIST for just a few days was shown to strongly enhance proliferation of undifferentiated iPS cells (Jiang et al. 2013). Furthermore, in cells differentiated down the neural lineage, proliferation of cells and formation of neural rosettes (neural progenitors) was enhanced by XIST-induced silencing, as illustrated in Figure 3.1b and previously reported (Jiang et al. 2013).

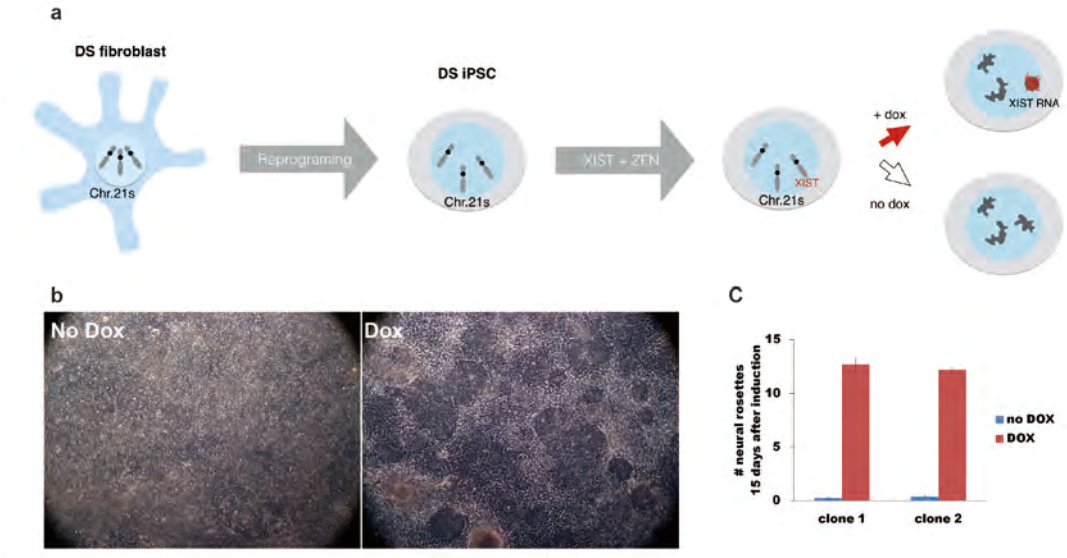


Figure 3.1 System to study the effect of trisomy 21 expression on DS related pathologies.

(a) Schematic of the inducible *XIST* RNA-mediated silencing system in male Down Syndrome iPSCs. (b) Neural stem cell formation after 15 days neural differentiation. The samples treated with dox have significant more neural stem cells. (c) Quantification of the number of rosettes at day 15 for 2 of the isogenic subclones.

These findings affirm that expression of XIST RNA is not toxic, but rather has beneficial effects on cell proliferation and viability, as shown for undifferentiated and early neural cells. Comparing effects of XIST-induced trisomy silencing on production of neural and hematopoietic cells is informative because the clinical impact on these two systems suggests contrasting effects on hematopoiesis versus neurogenesis. Although gross brain structure and size are similar in DS, there appears to be hypocellularity with fewer neurons in some brain regions (Lott 2012). The enhanced kinetics of cell proliferation and neural stem cell formation upon chromosome 21 silencing suggests that chromosome 21 over-expression may cause developmental delay in an early step of neural development, however a deficit in formation of neural stem cells is not an established phenotype of DS, and was not apparent in comparison of non-isogenic trisomic and disomic iPS cells (which showed general variability between lines) (Shi et al. 2012). Therefore, it remained possible that trisomy silencing would enhance proliferation and production of any or most cells, especially if this relieves a general "aneuploidy stress" as reported in cultured cells (see Introduction). Hence, it was of interest to determine if *XIST* expression would accentuate, have no-effect, or actually decrease (correct) over-production of hematopoietic cell-types seen in DS children.

The elevated risk of developing TMD and AMKL in DS is characterized by markedly increased proliferation of megakaryocytes, usually accompanied by increased erythrocytes. This property was affirmed in studies of DS fetal liver cells *in vitro* (Chou et al. 2008; Tunstall-Pedoe et al. 2008; Roy et al. 2012) and in two DS iPS cell studies that mirror fetal hematopoiesis (Maclean et al. 2012; Banno et al. 2016), but not another that reflected primitive hematopoiesis, an earlier stage of hematopoiesis (Chou et al.

2012). Therefore, we adopted a protocol that mimics fetal hematopoiesis from iPS cells differentiated through embryoid bodies with a cytokine cocktail, as shown by Maclean et al. (2012), and in Figure 3.2a.

Our prior study extensively demonstrated XIST induces chromosome silencing that is largely complete within 3-5 days of induction (Jiang et al. 2013), but we confirmed chromosome silencing in our hematopoietic differentiation system, by examining XIST RNA for proper localization to the chromosome, H3K27me3, an epigenetic hallmark of heterochromatin silencing, and gene silencing based on loss of one of three *APP* transcriptional foci, specifically from the *XIST* coated chromosome. After 9 days of hematopoietic differentiation, more than 90% of XIST-positive cells exhibited a well localized XIST RNA "paint", H3K27me3 spread across the chromosome (with *XIST* RNA) (Figure 3.2b) and transcriptional silencing of the *APP* locus on the targeted chromosome (Figure 3.2c).

These experiments were examined using parallel cultures (with and without dox-induced XIST) for four independent transgenic subclones (termed clones 1, 3, 4, and 5), as well as the non-transgenic trisomic parental line and the disomic subclone (also plus/minus doxycycline). Our first goal was to address the central question of whether induction of the XIST transgene can rebalance over-production of hematopoietic cell-types examined for the differentiation "end state", in colony forming assays. EBs at day 14, which contain hematopoietic progenitor cells, were dissociated and equal amounts of cells for each sample were plated for colony forming assay or examined by FACS analysis (Figure 3.2a). The experiment was done at least three times, and for some comparisons four times. The morphology of colonies and the quantitative results from

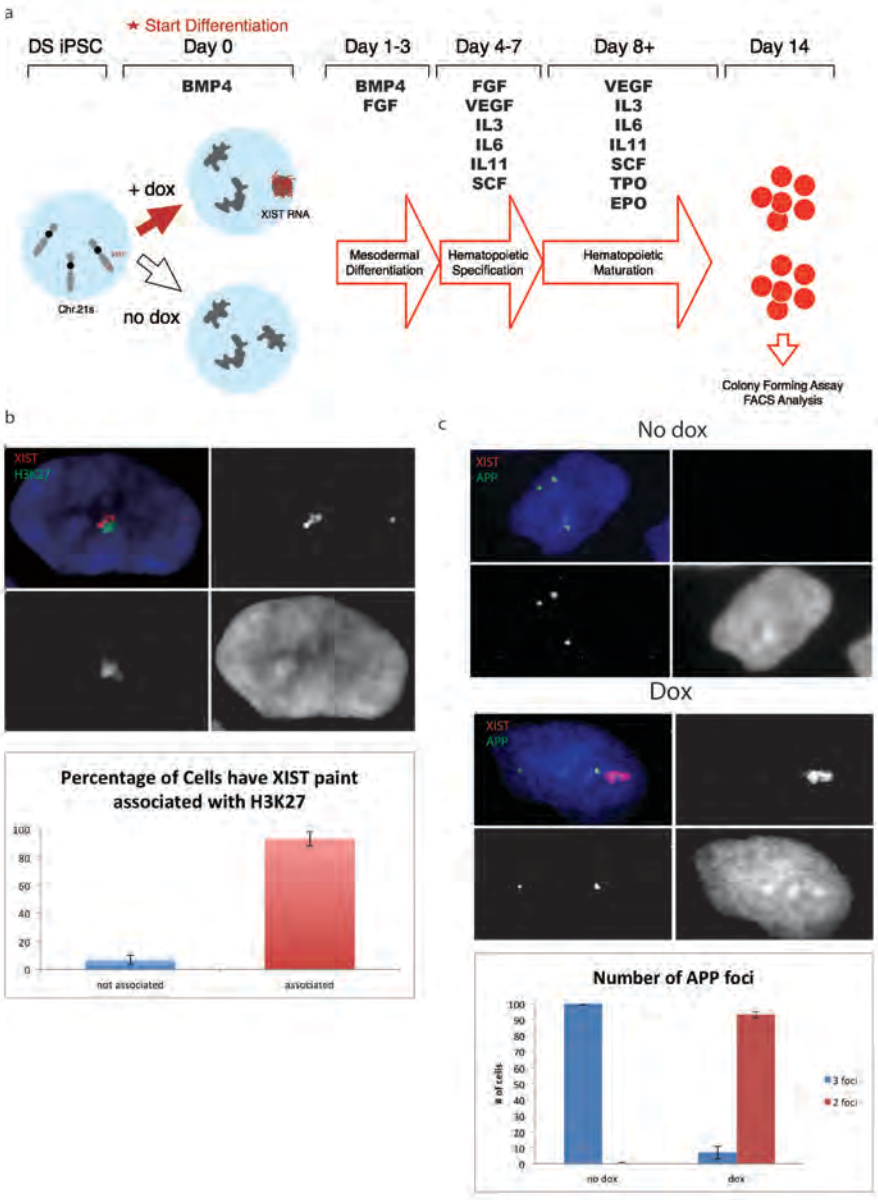


Figure 3.2 Trisomy silencing during hematopoietic differentiation.

(a) Schematic of hematopoietic differentiation from DS iPSCs. (b) Association of H3K27me3 heterochromatic mark with XIST RNA in day 9 differentiated cells treated with dox. Quantification shows over 90% of cells have H3K27me3 marker associated with XIST paints. (c) RNA FISH of *APP* and *XIST* on day 9 differentiating cells. *APP* RNA is transcribed from all three foci in untreated cells (top). In treated cells (bottom), only two *APP* transcription foci are detected, indicating transcriptional silencing induced by *XIST* expression. Quantification shows over 90% of *XIST* expressing cells have only two *APP* transcription loci (which are not from *XIST* expressing chromosome). Error bars for (b, c) represent SEM from three independent scorings.

multiple experiments are shown and summarized in Figure 3.3 and Figure 3.4, respectively.

The first point to note is that doxycycline treatment of the non-transgenic parental or disomic lines had no significant effect on the production of the four different types of hematopoietic cell colonies, affirming that the effect of dox on the transgenic clones is due to *XIST* expression to induce chromosome silencing. Comparison of the trisomic parental and disomic subclone is included for reference, although this is based on just one comparison of separate subclones, our overall findings are compared and consistent with other studies showing that DS trisomic iPS cells generate more megakaryocyte and erythrocyte colonies than euploid cells (Figure 3.4a,b) (Maclean et al. 2012; Roy et al. 2012; Banno et al. 2016). Most importantly, data in Figure 3.4 shows that a marked, statistically significant reduction in megakaryocyte and erythrocyte colonies is consistently seen with doxycycline treatment to induce XIST RNA, in all four transgenic clones in multiple experiments. Of the four colony types examined, megakaryocytic colonies (CFU-Mk) and erythroid colonies (CFU-E) were by far the most abundant, and they dropped by 50% or more when comparing parallel samples with and without *XIST*-RNA and chromosome silencing (Figure 3.4a, b). The observations for CFU-MK and CFU-E are consistent with laboratory observations that DS newborns overproduce erythrocytes and megakaryocytes, and therefore demonstrate that these phenotypes are normalized or greatly mitigated by *XIST* RNA mediated chromosome silencing.

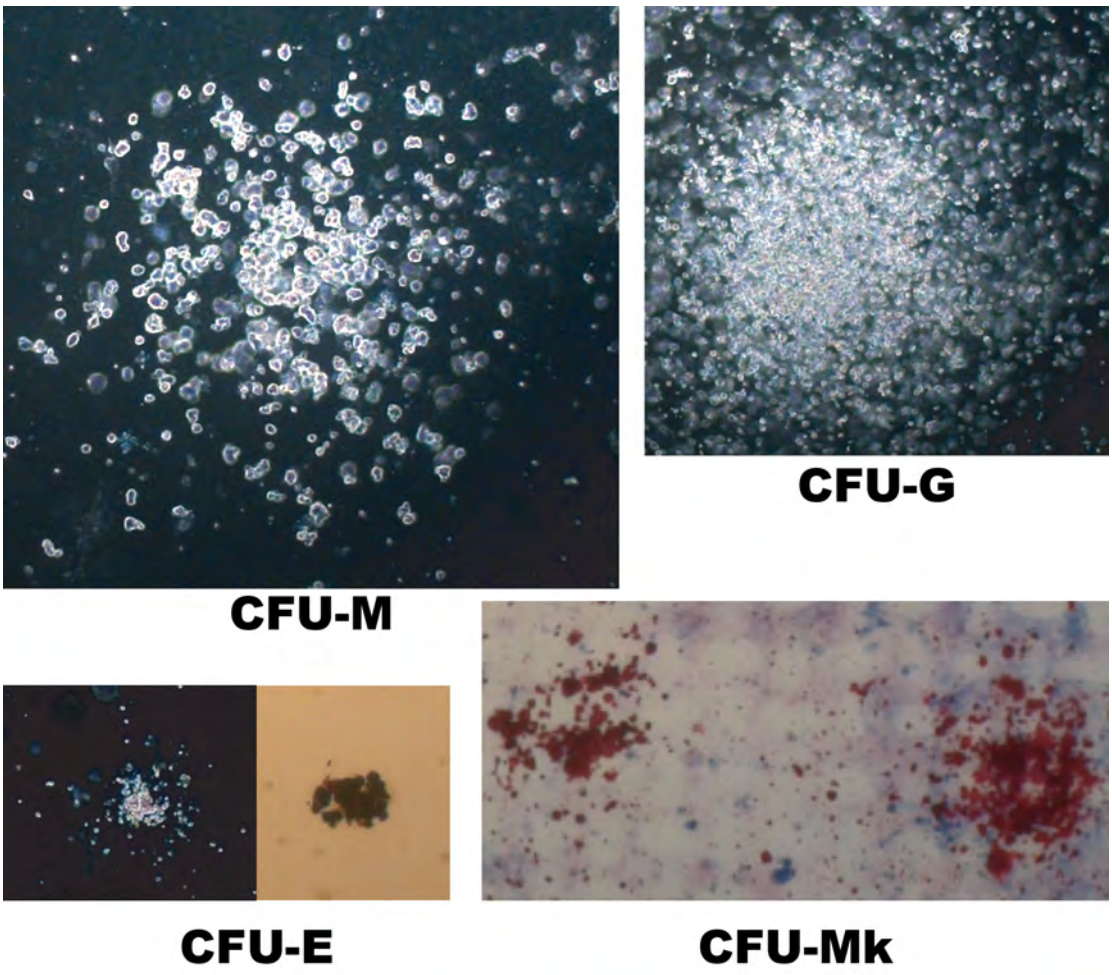


Figure 3.3 Morphology of hematopoietic colonies.

Morphologies of CFU-M, CFU-G, CFU-E, and CFU-Mk is shown here. For CFU-E, the left and right parts of the diagram are before and after DAB staining, respectively. CFU-M: Monocyte; CFU-G: Granulocytes; CFU-E: Erythrocytes; CFU-Mk: Megakaryocytes.

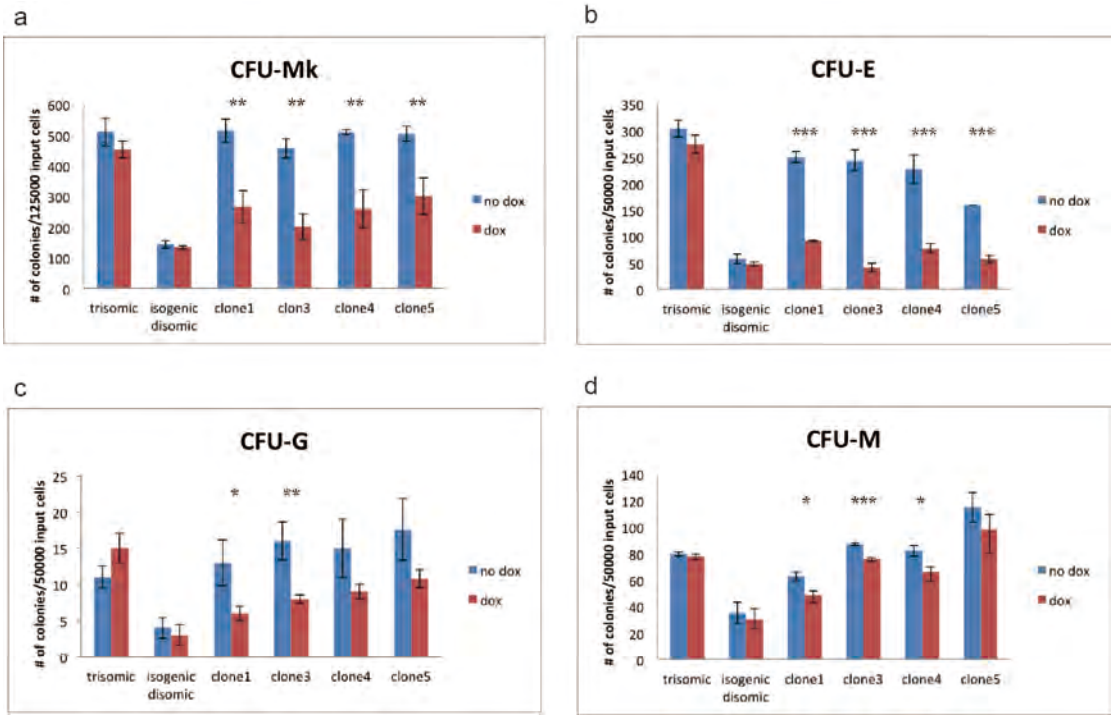


Figure 3.4 Colony-forming potential of DS iPSCs with and without *XIST*-induced chromosome 21 silencing.

Colony forming potential of multiple isogenic transgenic DS iPS lines with and without *XIST*-induced chromosome 21 silencing for (a) megakaryocytes, (b) erythrocytes, (c) granulocytes, and (d) monocytes. Error bars represent SEM from three independent experiments and p values were calculated by Student t test; *P < 0.05, **P < 0.01, ***P < 0.001.

The effect of trisomy 21 on CFU-MK and CFU-E is the greatest and has been firmly established, however it is much less clear, from both clinical and experimental observations, whether trisomy 21 affects the production of granulocytes and monocytes (Chou et al. 2012; Maclean et al. 2012; Roy et al. 2012; Banno et al. 2016). Our cell panel provides an opportunity to test this question as this all-isogenic and inducible system may detect more subtle differences. As shown in Figure 3.4c, results suggest there is a modest but reproducible decrease in the reductions of granulocyte colonies (CFU-G) as a function of chromosome 21 silencing. While the low numbers of CFU-G (in all samples) makes this comparison more difficult, it was statistically significant in some pair-wise comparisons, and some reduction was seen consistently in all four transgenic clones (and the disomic subclone compared to parental). Therefore, these results strongly suggest some impact on granulocyte production of trisomy 21 over-expression. Monocyte colonies (CFU-M) showed a very small effect, statistically significant for some comparisons. Again, while smaller less significant differences were seen for comparisons of each individual transgenic clone, when analyzed collectively, results indicate treated samples (and disomic cells) showed significant reduction for both colony types.

Therefore, these results of colony forming assays demonstrate that applying *XIST*-mediated silencing of one chromosome 21 can normalize the abnormal over-production of differentiated hematopoietic cells in colony forming assays, *in vitro*.

Trisomy silencing identifies endothelial-to-hematopoietic transition (EHT) as key to the overproduction of hematopoietic progenitors

The above results demonstrate that chromosome 21 silencing successfully normalized known defects in the ultimate production of differentiated hematopoietic cell types, by markedly reducing the over-production of megakaryocyte and erythrocyte colonies. This alone is a major milestone demonstrating feasibility to correct imbalance in the end-product of hematopoiesis. However, this also supports that this system provides a means to study the basic steps in pathogenesis that are impacted by dosage effects of trisomy 21. Thus we next investigated: at what step(s) during development of hematopoietic cells does trisomy silencing have an effect, and does that coincide with what is known from other studies, or advance what is currently known? Hematopoiesis is a complex, multi-step process that involves formation and differentiation of many different types of progenitors. Studies using human iPSC/ESC cell systems suggest that *in vitro* differentiation mimics most developmental events in embryonic hematopoiesis and thus can be valuable to model early human hematopoiesis (Vodyanik et al. 2006; Kennedy et al. 2007; Nostro et al. 2008; Choi et al. 2009; Grigoriadis et al. 2010; Choi et al. 2012; Kennedy et al. 2012; Elcheva et al. 2014; Sturgeon et al. 2014; Ditadi et al. 2015). Several major stages in the complex process of hematopoiesis are outlined in Figure 3.5a, and the markers used for flow sorting to isolate and study distinct cell populations are indicated. During embryogenesis in different model organisms, hematopoietic stem cells (HSC) arise from a specialized endothelium population called hemogenic endothelium (HE). HE cells are present only transiently during development and difficult to identify due to dual expression of markers for hematopoietic and

endothelial cells (Figure 3.5a) (Swiers et al. 2013). The formation of hematopoietic stem cells from HE is known as the endothelial-to-hematopoietic transition (EHT). HSCs are capable of self-renewal and have potential to differentiate into progenitor cells of all hematopoietic lineages. Prior work comparing normal and DS fetal liver found increased numbers of hematopoietic progenitor cells (Chou et al. 2008; Tunstall-Pedoe et al. 2008), and studies using iPS cell systems have also reported increased production of hematopoietic progenitor cells indicating that trisomy 21 has its effect at an early step in hematopoietic differentiation (Chou et al. 2012; Maclean et al. 2012; Banno et al. 2016).

Hematopoietic differentiation through embryoid bodies was induced from XIST-transgenic DS iPS cells with and without dox-induced trisomy silencing, and cells were examined at different times in the process and for different cell populations, as indicated in Figure 3.5a. CD34 has been widely used to broadly identify cell populations containing hematopoietic stem and progenitor cells; however, the $CD34^+$ population is very heterogeneous and another marker, CD43, has been shown the earliest expressed after hematopoietic commitment during *in vitro* hematopoietic differentiation from pluripotent cells (Vodyanik et al. 2006). Therefore, we examined both CD34 and CD43 simultaneously to maximize coverage of early hematopoietic precursors. As shown in Figure 3.5b, based on expression of these two markers, three populations ($CD34^+/CD43^-$, $CD34^+/CD43^+$, $CD34^-/CD43^+$) are detected at day 11 and day 14 EBs. As CD43 is the earliest marker of full hematopoietic commitment following EHT, the two $CD43^+$ populations ($CD34^+CD43^+$ and $CD34^-CD43^+$) most closely represent committed hematopoietic progenitors, within the differentiating embryoid bodies.

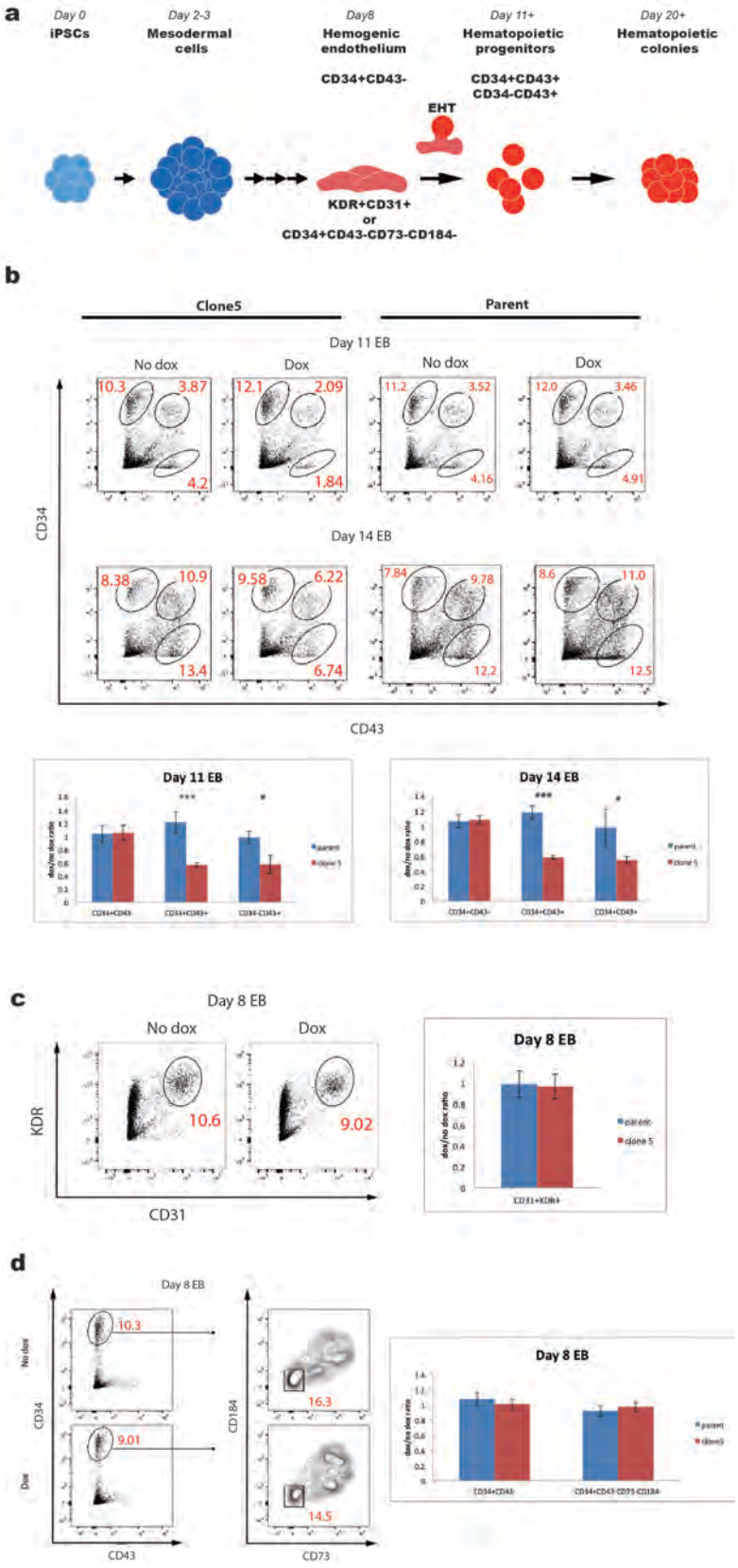

a
Day 0
iPSCs
Day 2-3
Mesodermal cells
Day8
Hemogenic endothelium
CD34+CD43-
Day 11+
Hematopoietic progenitors
CD34+CD43+
CD34-CD43+
Day 20+
Hematopoietic colonies
EHT
KDR+CD31+
or
CD34+CD43-CD73-CD184-
b
Clone5
Parent
Day 11 EB
No dox
Dox
No dox
Dox
10.3
3.87
4.2
12.1
2.09
1.84
11.2
3.52
4.16
12.0
3.46
4.91
Day 14 EB
8.38
10.9
13.4
9.58
6.22
6.74
7.84
9.78
12.2
8.6
11.0
12.5
CD34
CD43
Day 11 EB
Day 14 EB
c
Day 8 EB
No dox
Dox
10.6
9.02
KDR
CD31
Day 8 EB
d
Day 8 EB
10.3
9.01
16.3
14.5
No dox
Dox
CD34
CD43
CD184
CD73
Day 8 EB

Figure 3.5 Impact of chromosome 21 silencing on hematopoietic populations during hematopoietic differentiation from DS iPSCs

(a) A simplified schematic of the hematopoietic differentiation process. EHT: Endothelial to hematopoietic transition. (b) Early hematopoietic progenitor populations detected at day 11 and 14 differentiation for clone 5. Quantifications are represented as the ratio of doxcycycline-treated cells to untreated cells. Transgenic cells which *XIST* is induced have less $CD43^{+}$ early hematopoietic progenitor cells at both day 11 and 14 of differentiation (c,d) Hemogenic-like populations identified during hematopoietic differentiation for clone 5. Quantifications are represented as the ratio of treated cells to untreated cells. Chromosome 21 silencing does not affect the formation of hemogenic endothelium-like populations at day 8 of differentiation, using either of the two sets of markers to define this population. Error bars represent SEM from 5 independent experiments and p values were calculated by Student t test; *P < 0.05, **P < 0.01, ***P < 0.001.

Consistent with results from other studies, induced chromosome silencing successfully resulted in significant reduction in $CD43^+$ hematopoietic progenitor cells, for both the $CD34^-$ and $CD34^+$ subpopulations. Importantly, there is only marginal if any effect of trisomy 21expression on the formation of the $CD34^+CD43^-$ population, which lacks the CD43+ marker indicative of full hematopoietic commitment. Notably, as differentiation continues from day 11 to day 14, the two $CD43^+$ populations are expanding while the $CD34^+CD43^-$ population shrinks, consistent with $CD34^+CD43^-$ being the earlier precursor of the two $CD43^+$ populations.

These findings support prior evidence that the over-production of differentiated hematopoietic colonies is preceded developmentally by an over-production of hematopoietic progenitor cells. However, it is not known at what earlier step the excess progenitors arise. The ability of the inducible chromosome 21 silencing system to recapitulate hematopoietic abnormalities seen in clinic and laboratory drove our interest to use this inducible system (isogenic and iso-epigenetic) to further define when during hematopoietic differentiation defects arise due to trisomy 21. Given that trisomy 21 status strongly impacted the $CD43^+$ committed hematopoietic progenitors (but not the likely earlier $CD34^+/CD43^-$ cells), we wanted to ask if the earlier population of the bipotential hemogenic endothelium (HE) is also over-produced in trisomic cells, indicating an even earlier progenitor is impacted. Alternatively, if the HE population numbers were unaffected, this would indicate a defect involving excess EHT, the process known as endothelial to hematopoietic transition, which produces cells fully committed as hematopoietic progenitors (from bipotential HE cells). KDR^+CD31^+ cell populations at day 8 of *in vitro* differentiation have been reported to contain some HE-like cells

(Kennedy et al. 2007). Consistent with earlier observations, we found no significant difference in the formation of $KDR^{+}CD31^{+}$ cells (Figure 3.5c), however this is not clearly established to reflect the HE (Maclean et al. 2012; Banno et al. 2016). Therefore, we adapted recent improvements in identification of HE using other markers to more closely examine and identify the HE-like cell population. A more recent study showed that HE can be further enriched by using four markers, in the $CD34^{+}CD43^{-}CD73^{-}CD184^{-}$ population (Ditadi et al. 2015). Hence, we analyze this population at day 8 post-differentiation, with and without chromosome silencing. As shown in Figure 3.5d, results further support that there was no significant difference in formation of the HE-enriched population. Results using analysis of two different sets of markers for the HE-enriched population indicate trisomy 21 does not affect earlier steps leading to HE formation, but rather enhances EHT and the over-production of $CD43^{+}$ hematopoietic progenitors.

In sum, results show that trisomy silencing reduces the over-production of undifferentiated hematopoietic progenitors reported in other studies, but the analysis here goes further to indicate that overproduction of hematopoietic progenitor cells is not evidenced in the HE population, but arises due to an overactive EHT process.

Trisomic $CD43^{+}$ hematopoietic progenitors have increased colony forming potential for megakaryocyte and erythrocyte

Based on the results of our own and others (Maclean et al. 2012; Banno et al. 2016), the increased production of hematopoietic colonies is due to increased representation of hematopoietic progenitors plated into the colony forming assay. However, studies on DS fetal liver cells demonstrated more numerous hematopoietic

colonies generated from the same number of starting progenitor cells (Chou et al. 2008; Tunstall-Pedoe et al. 2008; Roy et al. 2012), suggesting increased colony forming potential in trisomic hematopoietic progenitor cells. To further examine and confirm this in the *in vitro* hematopoietic differentiation system, which hasn't been done, we investigated whether trisomy 21 also affects formation of various colony types from $CD43^+$ early hematopoietic progenitors within EBs. Therefore, we purified $CD43^+$ cells from day 14 EBs and then plated the same number of $CD43^+$ cells from each sample in colony forming assays. As shown in Figure 3.6, we observed marked decreases in CFU-Mk and CFU-E, a marginally significant difference in CFU-G, and slight reduction in CFU-M formation in silenced samples of transgenic subclones. In addition, the CFU-Mk colonies in treated samples were usually smaller than those in untreated samples, which is reflective of enhanced megakaryocyte proliferation in DS. To summarize, by utilizing our inducible trisomy silencing system, we affirm that trisomic hematopoietic progenitor cells generated in vitro show increased colony forming potential (shown previously for DS fetal liver studies); trisomy silencing of $CD43^+$ progenitors reduces the number of colonies generated, especially for megakaryocytes and erythrocytes. Therefore, our results indicate that trisomy 21 not only promotes excess production of $CD43^+$ hematopoietic progenitors, but also increases these progenitors' colony forming potential for megakaryocytic and erythroid colonies, likely through excess production of megakaryocyte-erythroid progenitors (MEP) within $CD43^+$ hematopoietic progenitors.

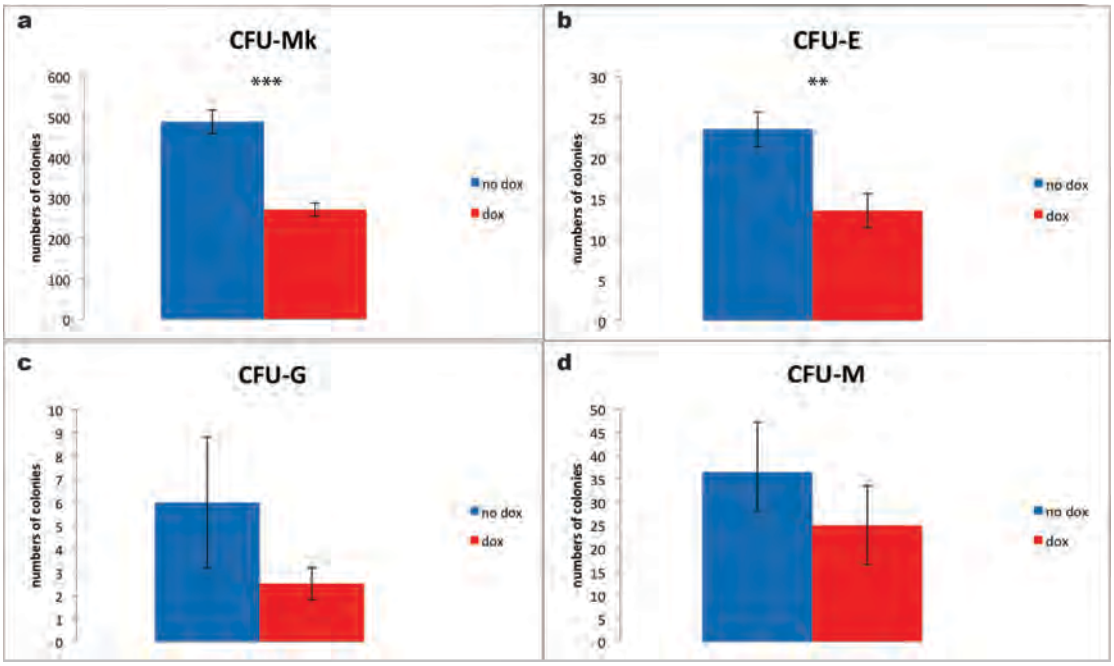


Figure 3.6 Colony-forming potential of purified day 14 CD43$^+$ hematopoietic progenitors with and without *XIST*-induced chromosome 21 silencing.

Colony forming potential from equal numbers of purified day 14 differentiated CD43$^+$ hematopoietic progenitor cells for (a) megakaryocytes, (b) erythrocytes, (c) granulocytes, and (d) monocytes. Error bars represent SEM from three independent experiments and p values were calculated by Student t test; *P < 0.05, **P < 0.01, ***P < 0.001.

Chromosome silencing indicates Trisomy 21 causes overactive IGF signaling that promotes over-production of CD43+ hematopoietic progenitors

Finally, having demonstrated that trisomy silencing corrects developmental defects in Trisomy 21 hematopoiesis, we investigated the utility of this inducible experimental system to examine gene expression changes as a function of silencing the third chromosome 21. The heterogeneous nature of hematopoietic cell populations (even as defined by specific markers) and the rapidly changing levels of hematopoietic regulators during hematopoiesis likely contribute to the observations in other studies that consistent changes in gene expression due to trisomy 21, even for known hematopoietic regulators encoded on chromosome 21, have alluded identification (Maclean et al. 2012; Roy et al. 2012). Since some of the expression variability between samples will also be due to comparison of different stem cell clones, we tested whether inducible chromosome silencing might be able to discern consistent changes, focusing on a small panel of genes of interest, as previously examined by Maclean et al (2012).

As referenced by Maclean et al. (2012) we were particularly interested in an important mechanistic hypothesis that excessive insulin growth factor (*IGF*) signaling in fetal hematopoiesis drives over-production of myeloid cells in TMD and AMKL. The IGF pathway is well-established to broadly impact cell proliferation, has been linked to a number of other types of cancers (Denduluri et al. 2015), and was implicated by one study as specifically elevated in fetal hematopoiesis and DS-associated TMD and DS-AMKL. Klusmann et al. (2010) found that genes in the *IGF* signaling pathway were substantially over-expressed specifically in AMKL associated with trisomy 21. They showed that fetal liver but not adult hematopoiesis depends upon IGF signaling,

suggesting that differences in IGF signaling may explain why DS-related leukemia arises specifically from fetal liver hematopoiesis in neonates and infants, but not in adults. Elevated mRNA levels for several IGF signaling genes were observed in DS-AMKL cancer lines but not in non-DS AMKL lines, and DS blasts were especially sensitive to inhibition of IGF signaling. While they showed GATA1s mutation contributes to this (through effects on E2F target genes), they also speculated that Trisomy 21 itself may cause overactive IGF signaling. However, this was not tested in this study focused on TMD and AMKL cells (which have a GATA1s mutation). Maclean et al. (2012) did not detect consistent differences in any genes examined, including IGF signaling genes, comparing hematopoiesis of trisomic and euploid iPS cells (without GATA1s). However, as stated by Roberts and colleagues in a recent review, the mechanism whereby Trisomy 21 itself leads to over-production of megakaryocytes and erythrocytes remains an important unknown question, but "*differences in the expression or responsiveness to the developmentally regulated IGF signaling pathway remain an attractive candidate*"(Bhatnagar et al. 2016).

We performed RT-qPCR on RNA from purified day 14 $CD43^+$ cells, examined in three independent experiments comparing parallel cultures with and without XIST-induced chromosome silencing. As shown in Figure 3.7a and b, for many genes, most of which are highly dynamic and cell-type specific hematopoietic regulators, no significant patterns were evident, similar to the findings of Maclean et al. (2010). In contrast to Maclean et al., however, we did detect consistent, statistically significant differences in five of these same genes, all five of which have been discussed as potentially involved as contributing to trisomy 21 hematopathology (Figure 3.7a). For chromosome 21 genes

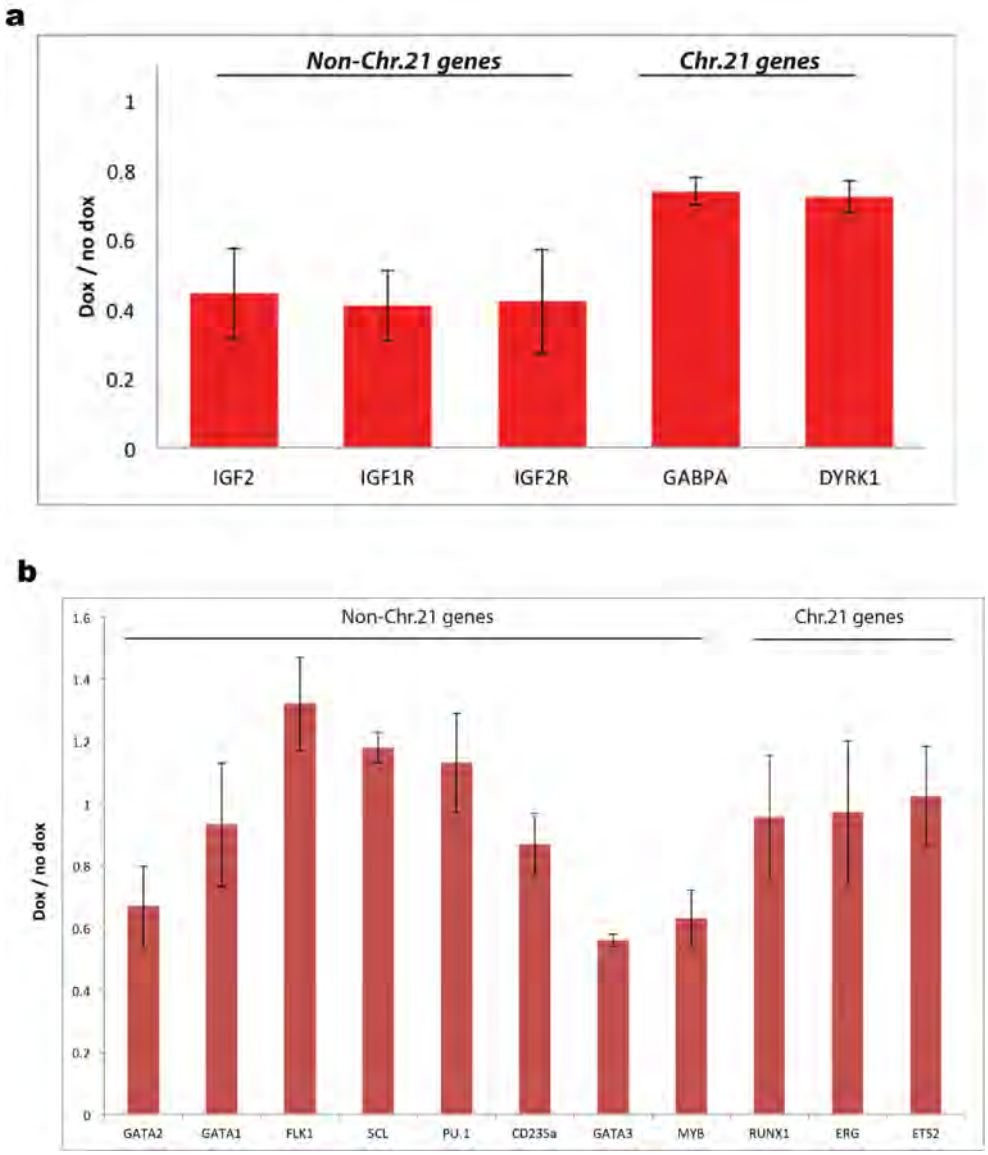


Figure 3.7 Effects of trisomy 21 silencing on gene expression **in CD43$^+$ early hematopoietic progenitor cells.**

Gene expression analysis on (a) three IGF signaling related genes and two chromosome 21 genes in purified day 14 CD43$^+$ hematopoietic progenitors, and on (b) a panel of hematopoietic regulators, which are dynamically changing in different hematopoietic sub-populations. Expression levels were normalized to GAPDH and represented as the ratio of treated cells to untreated cells. Error bars represent SEM from three independent experiments.

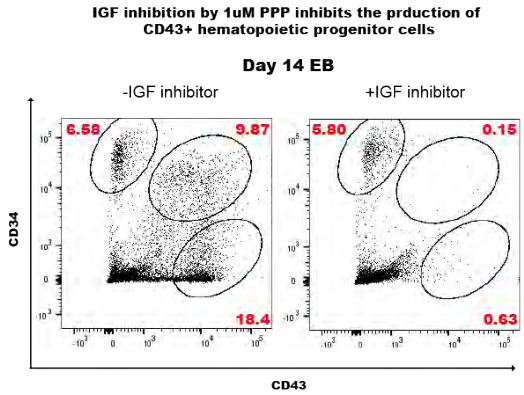
DYRK1A and *GABPA*, a reduction in mRNA levels approaching the 1/3 level expected (due to silencing one of three chromosome 21s) was evident. The sensitivity of our inducible system to detect such modest changes likely also reflects wide expression of these two broad regulators in much of the heterogeneous $CD43^+$ hematopoietic cell population. We note that *GABPA* and *DYRK1A* are expressed early even in undifferentiated iPS cells (Jiang et al. 2013 and unpublished). Most importantly, results in Figure 3.7a demonstrate that indeed there is an increase in expression of IGF signaling genes (not on chromosome 21) in the trisomic versus disomic state. All three *IGF* signaling genes examined, *IGF2*, *IGF1R*, and *IGF2R*, were each markedly down-regulated by about 60% in trisomic $CD43^+$ cells when one chromosome 21 is silenced by induced *XIST* expression. These genes changed with greater magnitude and significance than did genes encoded on chromosome 21. As shown in Figure 3.7a, GABPA and DYRK1A mRNA levels were reduced to about 70%, consistent with their upregulation in trisomy silencing one of three alleles. Since none of the IGF-related genes examined here are on chromosome 21, results indicate they are mis-regulated by trisomic expression of one or more chromosome 21 genes. We note that *GABPA* is known to be involved in cell cycle progression (Yang et al. 2007) and that regulatory elements of *IGF2* and *IGF2R* have binding sites recognized by *GABPA*.

Klusman et al. (2010) also showed that fetal but not embryonic or adult hematopoiesis was dependent on IGF signaling and sensitive to IGF signaling inhibition, and that AMKL and TMD cells from DS patients (which carry both trisomy 21 and a GATA1s mutation) were more sensitive than non-DS AMKL at a given concentration. Since changes in IGF signaling have not yet been corroborated, it would be important

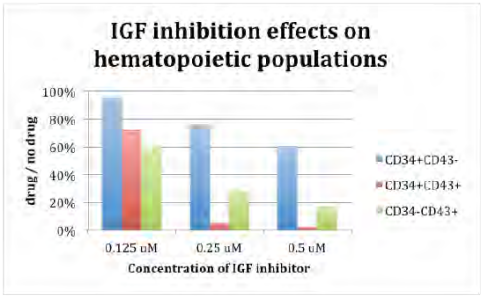
first to corroborate that in any system, but also to address whether and when this sensitivity is present in steps prior to acquisition of the GATA1s mutation. Using the same inhibitor (PPP) as Klusman et al. (2010), we examined effects on the same three flow-sorted hematopoietic populations studied above, as summarized in Figure 3.8 ($CD34^+/CD43^-$, $CD34^-/CD43^+$ and $CD34^-/CD43^-$). Treatment of trisomic cells began on Day 8 and then cells were evaluated on Day 14 of differentiation. The first experiment used the same PPP concentration as Klusman et al. on trisomic cells and served to reveal that production of $CD43^+$ hematopoietic progenitor cells specifically was almost completely eliminated by inhibition of IGF signaling (Figure 3.8a). In contrast, there was very little effect on the earliest hematopoietic population of $CD34^+/CD43^-$ cells (corresponding to hemogenic endothelium-enriched population prior to HET), affirming that the drug was not generally toxic to cell proliferation and that IGF impacted a specific point in hematopoiesis (Figure 3.8a).

Since the evidence indicates that IGF signaling would be required for fetal hematopoiesis for normal or trisomy 21 samples, any greater effect of IGF signaling on trisomy 21 cells would only be evident within a certain concentration window of IGF inhibitor, as indicated by Klusman et al. (2010). Therefore, we next tested three different inhibitor concentrations on both the untreated trisomic cultures, and the parallel culture with XIST-induced chromosome silencing. Once again results affirm marked effects on the production of $CD34^+/CD43^+$ and $CD34^-/CD43^+$ cells, much more than the earliest $CD34^+$ population (Figure 3.8b). Notably, these results coincide nicely with the cell populations and developmental step (involving the hemogenic-to-endothelial transition) identified above to be most affected by silencing trisomy 21. Further, a very

a


IGF inhibition by 1uM PPP inhibits the prduction of CD43+ hematopoietic progenitor cells
Day 14 EB
-IGF inhibitor
+IGF inhibitor
6.58
9.87
18.4
5.80
0.15
0.63
CD34
CD43

b


IGF inhibition effects on hematopoietic populations
drug / no drug
100%
80%
60%
40%
20%
0%
0.125 uM
0.25 uM
0.5 uM
Concentration of IGF inhibitor
CD34+CD43-
CD34+CD43+
CD34-CD43+

c

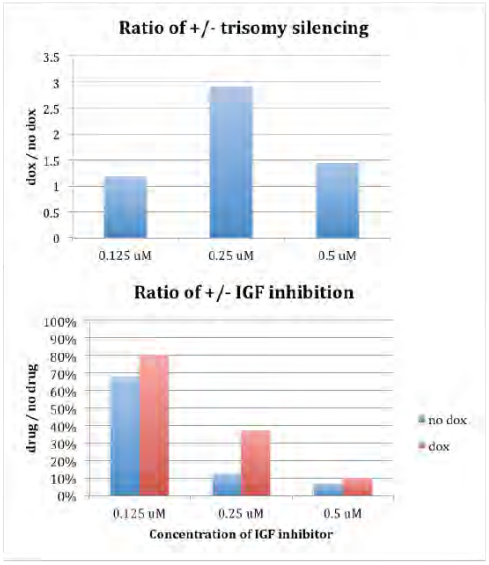

Effect of trisomy silencing on sensitivity to IGF inhibition for CD43+ cells
Ratio of +/- trisomy silencing
dox / no dox
3.5
3
2.5
2
1.5
1
0.5
0
0.125 uM
0.25 uM
0.5 uM
Ratio of +/- IGF inhibition
drug / no drug
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
0.125 uM
0.25 uM
0.5 uM
Concentration of IGF inhibitor
no dox
dox

Figure 3.8 Trisomy 21 contributes to increased sensitivity of $CD43^+$ hematopoietic progenitor production **to IGF inhibition.**

(a) Inhibition of IGF signaling by 1 uM PPP, an IGF inhibitor, has distinct effects on the $CD34^+$ and $CD43^+$ populations. Production of $CD43^+$ early hematopoietic progenitor populations are more sensitive to IGF inhibition whereas $CD34^+CD43^-$ hemogenic endothelium enriched population is only slightly affected. (b) Analysis of the effects of IGF inhibition at three lower concentrations identifies the window of sensitivity for the $CD43^+$ cells. (c) Trisomy silencing reduces the sensitivity of $CD43^+$ cell production to IGF signaling inhibition, suggesting greater reliance of trisomic $CD43^+$ progenitors on IGF signaling.

close link between IGF signaling and over-expression of chromosome 21 is further evidenced by the finding that silencing of one chromosome 21 substantially reduced the sensitivity to IGF inhibition of the $CD43^+$ population in parallel cultures induced to silence one chromosome 21 (Figure 3.8c).

Therefore, both gene expression analysis and the IGF inhibitor studies support the conclusion that trisomy 21 itself (prior to GATA1s mutation) increases *IGF* signaling, which in turn promotes excessive production of $CD43^+$ hematopoietic progenitor cells in Down Syndrome (Figure 3.9). Results further demonstrate the potential of that expression of one gene, XIST, can sufficiently rebalance chromosome 21 gene expression levels to reduce excess *IGF* signaling and normalize the pathological over-production of specific hematopoietic cell populations that underlie Down Syndrome-associated myeloid defects. Figure 3.9 provides a summary of findings and model regarding the effect of trisomy 21 on distinct steps in the hematopoietic process.

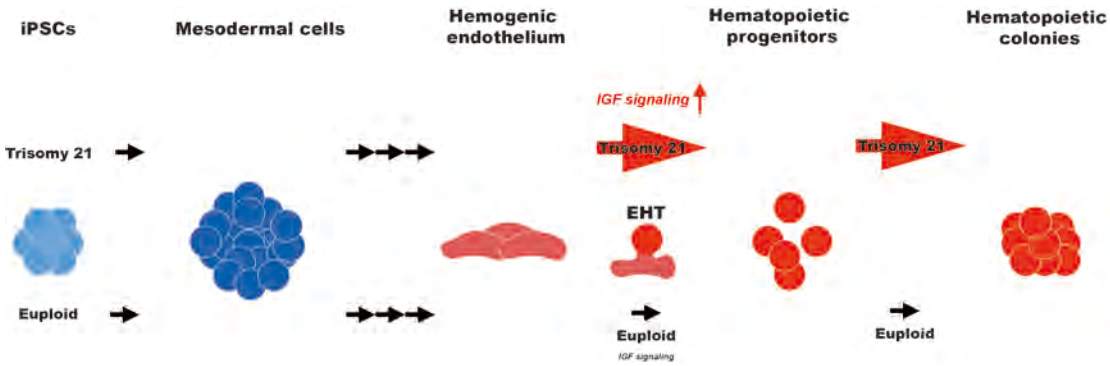


Figure 3.9 Schematic of how trisomy 21 affects hematopoietic differentiation.

Trisomy 21 increases the production of early hematopoietic progenitor cells by enhancing the endothelial-to-hematopoietic transition, accompanied by increased IGF signaling. Trisomy 21 also increases the colony forming potential of these hematopoietic progenitor cells.

Discussion

Previously, we demonstrated that an inducible XIST cDNA could spread heterochromatin and transcriptionally repress most genes across one chromosome 21 in DS iPS cells. However, it was the first priority of this study to address the critical next question: is *XIST*-mediated "trisomy silencing" sufficiently effective to normalize cell physiology and mitigate pathogenesis of known DS-cellular phenotype(s). Here we address whether this approach involving expression of transgenic *XIST* could correct the complex regulatory mechanisms that give rise to hematopoietic cell pathologies in DS. In-depth analysis of well-established hematopoietic cell phenotypes consistently shows that trisomy silencing indeed effectively corrects cell pathogenesis and phenotypes. Following *XIST*-expression to induce heterochromatin formation across one chromosome 21, the over-proliferation of megakaryocyte and erythrocyte colonies is sharply reduced, in all four transgenic iPS clones in multiple experiments. Similarly, this also corrects an excess production of earlier hematopoietic progenitors. This reduction in cell proliferation is not due to negative effects of doxycycline or *XIST* since no such effect was seen in doxycycline-treated control cells. Importantly, we highlight that *XIST* expression has opposite effects on proliferation of hematopoietic versus neural cells; if these same trisomic iPS cells are directed down the neural lineage they proliferate more rapidly with *XIST* expression, as do undifferentiated iPS cells (Jiang et al. 2013). These findings are consistent with differences in the clinical features of trisomy 21 for the neural and hematopoietic systems in DS patients. This cell-type specificity, and the specificity of effects on distinct hematopoietic precursor populations, supports the conclusion that silencing one chromosome 21 does not just relieve general "aneuploidy

stress" but it corrects specific changes in developmental programs impacted by dosage-sensitive genes on chromosome 21.

The clear demonstration that XIST expression alone can correct cell development and function to normalize a major DS cellular phenotype, in cells which still carry the extra chromosome, is itself novel and significant. However, the results go further to demonstrate the utility of the inducible XIST strategy to investigate steps in cell pathogenesis, as further discussed below.

Inducible chromosome silencing as a strategy to illuminate steps in cell pathogenesis due to trisomy 21

The implications of our results for longer-term prospects of "chromosome therapy" will be discussed below, but we first consider the extent to which these results confirm and extend understanding the underlying biology of hematopoietic pathogenesis in DS. The consistency between our results and findings from others that compared hematopoiesis for trisomic and disomic cells (Chou et al. 2012; Maclean et al. 2012; Roy et al. 2012; Banno et al. 2016) validates the trisomy silencing approach as a means to analyze the underlying biology by which trisomy 21 contributes to hematopoietic abnormalities. In fact, use of this experimental approach allowed us to advance what is known by providing evidence for certain important hypotheses. By making the chromosome 21 silencing event inducible, we exclude inter-clonal variations that commonly exist among different iPS lines. Our results affirm prior evidence that hematopoietic progenitors (as well as differentiated cells) are produced in excess, but further found no increase in the formation of the hemogenic endothelium(HE)-enriched population with and without trisomy silencing, by using multiple markers. $CD43^{+}$

committed early hematopoietic progenitor cells were consistently higher with trisomy 21 expression; since these cells derive from the bi-potential HE by the developmental step know as endothelial to hematopoietic transition (EHT), our study points to a defect in the HET as a key to the overproduction of hematopoietic cells in DS. Based on our results, *RUNX1,* an essential chromosome 21 gene involved in differentiation of committed hematopoietic progenitor cells (Chen et al. 2009), is highly likely involved in enhancing EHT in the context of trisomy 21. Banno et al. (2016) recently reported that an extra copy of *RUNX1* was indispensable for excessive production of hematopoietic progenitor cells during the differentiation of trisomic iPS cells. However, there are multiple hematopoietic regulators, including miRNAs, on chromosome 21 that may also contribute, and genes encoded on other chromosomes and impacted by even non-hematopoietic chromosome 21 genes may be important as well, as further suggested in our results.

IGF signaling as a major contributor to Trisomy 21 Pathogenesis

Our findings show that specific non-chromosome 21 genes involved in IGF signaling are reproducibly impacted by trisomy 21 expression and support the hypothesis that this is a major driver of excessive hematopoietic cell proliferation. We show for the first time that cells expressing three chromosome 21s have consistently higher expression of three IGF genes examined relative to the same cells with one chromosome 21 silenced. Importantly, this impact on IGF signaling is evident in trisomic cells prior to any leukemic (or pre-leukemic) state and without GATA1s mutation. Our results provide important corroboration for the hypothesis of Klusman et al. (2010) that IGF signaling is important in fetal megakaryopoiesis and contributes to leukemogenesis in DS. While

their results indicated the mutant GATA1s no longer sufficiently inhibits IGF signaling, our findings show that increased IGF signaling (and sensitivity of growth to IGF inhibition) can be observed in trisomic $CD43^{+}$ early hematopoietic progenitors. However, our results do not counter the conclusion of Klusman et al. that GATA1s mutation impacts IGF signaling, as these authors also speculate that trisomy 21 itself may also impact IGF signaling. If the combined action of trisomy 21 and GATA1s cooperates to enhance IGF signaling to more critical levels, this would explain why neither trisomy 21 nor GATA1s alone leads to TMD or leukemia. Hence, we suggest these two types of mutation operate together during fetal hematopoiesis to push the same proliferation-promoting pathway to dangerous levels.

In sum, use of the tightly-controlled inducible system to study effects of trisomy 21 allowed us to not only confirm but substantially extend evidence that overactive *IGF* signaling is a key component of trisomy 21 that promotes development of hematopoietic abnormalities. This system will also be useful to further investigate gene expression changes at different stages of hematopoietic differentiation as a function of trisomy, and we currently have RNA sequencing on multiple time points of *in vitro* hematopoiesis in progress. This may better define changes in dynamic regulators, such as *RUNX1*, but will also determine if there are basal changes in non-hematopoietic genes. For example, it will be interesting to determine if certain chromosome 21 genes, such as *DYRK1A* and *GABPA*, are expressed (at one-third increased levels) prior to elevation of IGF signaling, since both *GABPA* and *DYRK1A* have been suggested to have a role in DS myeloid defects (Chou et al. 2012; Malinge et al. 2012). Certain chromosome 21 genes that are widely expressed could have wide impacts on basic cellular pathways, such as IGF

signaling. In fact, it is interesting to speculate that changes in the insulin-based signaling pathways might be widely present in Down Syndrome patient cells which could underlie the almost universal metabolic changes that make individuals with DS prone to obesity and more frequent diabetes. Additionally, potential linkage between *RUNX1* overexpression and increased IGF signaling in the context of trisomy 21 has not been shown, but since IGF signaling is essential for supporting the growth of fetal live HSCs (Zhang and Lodish 2004), it is interesting to consider whether *RUNX1* regulates IGF signaling or vice versa, in both euploid and trisomic 21 conditions.

Implications for longer-term prospects of "chromosome therapy"

The first step in development of any gene therapy approach is to demonstrate that the genetic abnormality can be corrected *in vitro*. Since *XIST*-mediated chromosome silencing is not a traditional "correction" of a genetic mutation, it was essential to show that this "translational epigenetics" strategy could improve cellular or molecular phenotypes. This study demonstrates that autosomal expression of a single gene, *XIST*, can mitigate DS hematopoietic abnormalities *in vitro*. *Importantly, this correction can be achieved even without knowing which or how many specific genes are involved in controlling these abnormalities.* Given that many research groups are making progress on developing human HSCs from iPS cells for therapeutic purposes (Wahlster and Daley 2016), findings here have direct applicability for such approaches. Though challenges remain, it has been shown that *Xist* can initiate chromosome silencing in hematopoietic progenitor cells (Savarese et al. 2006), and bone marrow transplantation of genetically modified HSC has also been tested on patients with hematopoietic diseases in several clinical trials. Therefore, the present study provides a proof-of-principle for the potential

future development of *ex vivo* gene therapy for certain circumstances for DS children. Children with trisomy 21 who develop TMD are at particularly high risk to acquire DS-AMKL, which could lead to harsh treatments and severe outcomes. Although children with DS-AMKL generally have higher survival rates after chemotherapy when compared to non-DS children, the toxicity is also often even greater for DS children and the disease is still life-threatening (Bhatnagar et al. 2016). Since *GATA1s* mutation alone does not lead to leukemia, it is now possible to consider that dosage-compensating chromosome 21 expression in DS-TMD children might eventually become a viable alternative to preempt the development of DS-AMKL in the substantial subset of DS children who have TMD.

Finally, it is important to note that the experimental strategy demonstrated here, and phenotypic benefits of chromosome silencing in the hematopoietic system, are potentially relevant to all the bodily systems and functions impacted by changes to blood due to trisomy 21. For example, these results have relevance to hematopoietic cells of the lymphoid system, which are impacted by trisomy 21 to increase the risk for ALL, a more commonly seen leukemia for which the success of chemotherapy is substantially less than for DS-AMKL. Since the hematopoietic progenitor cells studied here (and corrected by trisomy silencing) could also give rise to cells of the lymphoid system more broadly, and this might mitigate immune defects that impact many people with DS (Kusters et al. 2009; Ram and Chinen 2011; Sullivan et al. 2016). For example, high susceptibility to infections is one of the most significant health problems for individuals with DS. Furthermore, many recent studies suggest a link between immune system and progression of neurodegenerative diseases. Therefore, being able to correct DS

hematopoietic abnormalities could bring broader potential benefits beyond leukemia to immune or even cognitive functions, or possibly the early-onset Alzheimer disease so prevalent in DS.

To summarize, this study demonstrates the feasibility of mitigating a major pathology in DS by induced silencing of the third chromosome 21 through ectopic *XIST* expression. In addition, the inducible system for chromosome silencing provides a new tool to study DS hematopoiesis, as well as other aspects of DS pathologies. Lastly, these results further highlight the longer-term clinical prospects for development of this innovative approach, which should be further explored for hematological and other aspects of the Down Syndrome.

Materials and Methods

iPS Cell culture

iPS lines were maintained as previously described(Jiang et al. 2013). Briefly, iPS cells were maintained on irradiated mouse embryonic fibroblasts (R&D Systems) in iPSC medium containing KnockOut-DMEM/F12 supplemented with 20% KnockOut serum replacement (ThermoFisher), 1X GlutaMax (ThermoFisher), 100 μM non-essential amino acids (ThermoFisher), 100 μM β-mercaptoethanol (Sigma) and 10 ng ml−1 FGF-β (ThermoFisher). Cultures were passaged with 1 mg/ml collagenase type IV (ThermoFisher) every week.

Neural differentiation

Differentiation of neural stem cell was carried out as previously described (Jiang et al. 2013) and chapter II.

RNA fluorescence in situ hybrization and immunostaining

Immunostaining for H3K27me3 and RNA **fluorescence in situ hybrization** for XIST and APP were performed as described previously(Byron et al. 2013; Jiang et al. 2013).

Hematopoietic differentiation

iPS cells were differentiated as previously described(Maclean et al. 2012). Briefly, iPSCs were passaged from feeders to growth factor reduced matrigel (Corning). After 24 – 48 hours, iPSCs were lifted by collagenase B (Roche) and cultured in suspension in low attachment plate (Corning) to form embryoid bodies (EB). This is considered as day 0 of

differentiation and *XIST* expression was turned on by doxycycline treatment (500 ng/ml). Cytokines were added according to protocol shown by Maclean et al(Maclean et al. 2012). After 14 days of differentiation, EBs were dissociated and single cells were plated in MegaCult medium for generating megakaryocytic colonies and MethoCult medium for erythroid, monocyte, and granulocyte colonies (Stem Cell Technologies). After 10 days, colonies were fixed and scored according to the manufacturer's instructions. All cytokines were purchased from Pepro Tech except Erythropoietin (R&D Systems).

Flow cytometry

Cells from dissociated embryoid bodies were filtered through 50 μm filter (Partec) before stained for FACS analysis. All antibodies were purchased from BD Bioscience, including anti-CD34-APC, anti-CD43-FITC anti-CD31-PE, anti-CD31-FITC, anti-CD73-PE, anti-CD184- PE-Cy7, and anti-CD309- Alexa Flour 647. Cells were stained for 30 minutes at 4 degree in the dark then washed twice with PBS supplemented with 2% FBS before flow cytometry. 4',6-diamidino-2-phenylindole (DAPI) was used for selection of live cells.

qRT-PCR

RNA was isolated from purified $CD43^{+}$ hematopoietic progenitor cells using RNeasy kit (Qiagen). cDNA was generated by iScript cDNA kit (BioRad). IQ SYBR Green supermix (BioRad) was used for qPCR reactions. All reactions were done in triplicate and the expression levels were normalized by expression of GAPDH.

Statistical analysis

All experiments were done at least in triplicate and repeated independently. For cell counting, at least three random regions on the slides were scored for 100 cells for each experiment. For colony forming assays, three independent plates (for MethoCult) and three independent slides (for MegaCult) were scored for the number of colonies of each type. For flow cytometry analysis, five independent experiments were performed. For qPCR, reactions were performed in triplicate in three independent experiments. One-tailed Student t test was used to determine the significant level of differences between treated and untreated samples. Differences were considered to be significant when $P < 0.05$. Error bars represent standard error of the mean.

Chapter IV: Initial investigation of effect of trisomy 21 on angiogenesis

Introduction

Individuals with Down syndrome (DS) have elevated risk for various defects and abnormalities associated with multiple compartments of the body, including leukemia, as described in previous chapters. Additionally, DS patients have features that are traditionally thought to be cancer-prone. For example, increased levels of reactive oxygen species (ROS) are detected in various cell types from DS patients and mouse models, including neurons, fibroblasts, and lymphocytes (Busciglio and Yankner 1995; Komatsu et al. 2006; Zana et al. 2006). High levels of ROS may partly result from an additional copy of chromosome 21 genes involved in oxidative metabolism, such as *SOD1* and *CBS*. This often leads to higher rate of DNA damage and mitochondria dysfunction (Del Bo et al. 2001; Coskun et al. 2010), both are highly correlated with cancer progression (Boland et al. 2013; Hsu et al. 2016). In addition, defective DNA repair mechanisms have also been reported to be a feature of DS cells (Morawiec et al. 2008), which may contribute to the GATA1s mutations consistently seen in DS-TMD and DS-AMKL cells, as described in previous chapters (Cabelof et al. 2009). Despite these changes that might be expected to increase susceptibility to cancer, multiple studies have shown a decreased incidence of most tumor types in DS individuals compared to age matched euploid individuals, with the exception for leukemia and germ cell cancers (Hasle et al. 2000; Nizetic and Groet 2012). This observation presents a paradox for what researchers would have predicted based on multiple cancer-prone cellular conditions caused by trisomy 21. Interestingly, the malignant cells for the two types of cancer for which DS individuals are more prone, childhood leukemia and germ cell cancers, are restricted to cell types of fetal origin and

the increased risk does not extend to other types of cancer of typically adult onset (Hasle et al. 2000; Yang et al. 2002). Therefore, how trisomy 21 protects DS individuals from most types of solid tumors is of interest broadly for the field of cancer research.

Some properties of DS cells may potentially be less favorable for cancer progression. For instance, fibroblasts from DS individuals and mouse models have been reported to exhibit reduced ability for proliferation and migration (Kimura et al. 2005; Delom et al. 2009). Hence, this suggests that certain genes on chromosome 21 may have inhibitory effects on cell cycle and mobility. Notably, our result in chapter II that showed increased proliferation rate in iPSCs after trisomy silencing further supports this hypothesis. Additionally, certain genes on chromosome 21 are suggested to be tumor suppressive. For example, *ETS2* was implicated protective from colon cancer in a mouse model of DS (Sussan et al. 2008) and the biological functions of DYRK1A also thought to be cancer suppressive. (Canzonetta et al. 2008; Laguna et al. 2008; Park et al. 2010; Litovchick et al. 2011). However, the tumor suppressive properties of these genes may be cell context dependent, as they may also be oncogenic in other cell types (Xu et al. 2008; Birger and Izraeli 2012; Malinge et al. 2012).

Another plausible hypothesis for solid tumor protection in DS individuals is the reduced angiogenesis theory. Although not involved in tumor initiation, angiogenesis is crucial for tumor progression, as rapid tumor growth requires sufficient vasculature to support nutrient supply and circulation. High levels of serum endostatin, an endogenous inhibitor of angiogenesis produced from cleavage of collagen XVIII, encoded by the *COL18A1* gene on chromosome 21, are seen in DS individuals (Zorick et al. 2001). Notably, clinical trials using endostatin as a potent anti-angiogenic drug have been

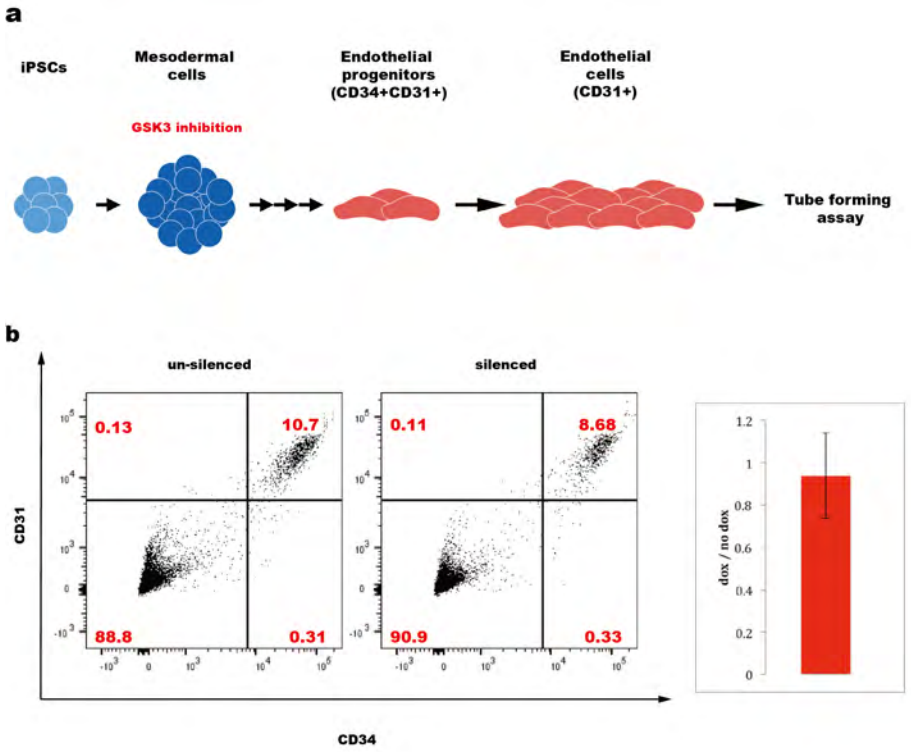
conducted in non-DS patients (Herbst et al. 2002; Thomas et al. 2003). Additionally, two mouse models of DS, Ts65Dn and Tc1, showed reduced support for growth of transplanted melanoma cells and these inhibitory effects on tumor growth were attribute to the dosage of the *Rcan1* gene (Baek et al. 2009; Reynolds et al. 2010). A possible mechanism is that the combined effect of increased *DYRK1A* and *RCAN1* dosage (on chromosome 21) causes dysregulation of NFAT signaling, which reduces responsiveness to VGEF, an important angiogenic signal (Arron et al. 2006). However, a contradictory result (also shown in Ts65Dn mice) was that the increased survival rate for aggressive tumors is not related to attenuated angiogenesis (Yang and Reeves 2011). Therefore, more studies are needed to examine whether there is indeed an effect of trisomy 21 on angiogenesis.

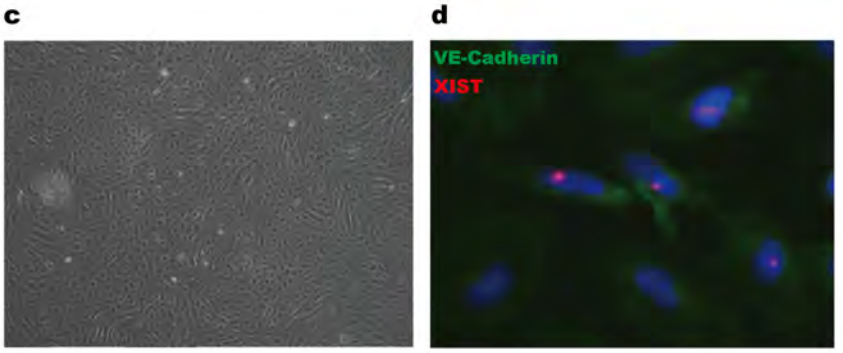
In this chapter, we extend the use of our system for investigating hematopoiesis to examining the capacity of trisomic cells for angiogenesis, as a function of trisomy silencing. Preliminary results of multiple experiments investigating the effect of trisomy 21 on angiogneisis will be described.

Preliminary results

To address the question of whether or not trisomy 21 reduces angiogenesis, an advantageous approach is to compare the aniogenic capacity of two endothelial populations that differ only in the number of active chromosome 21s (3 versus 2). Therefore, I worked to adopt a differentiation protocol for differentiation of iPSCs to endothelial progenitors (Lian et al. 2014). Differentiation was triggered by inhibition of GSK3 in a specialized medium that promotes the generation of $CD31^{+}CD34^{+}$ endothelial progenitor cells from iPSCs. After 5 days of differentiation, I was able to acquire $CD31^{+}CD34^{+}$ endothelial progenitor cells that constitute approximately 10% of the differentiation culture (Figure 4.1a, b). Isolation of these endothelial progenitor cells by positive selection of $CD34^{+}$ cells was performed through FACS sorting. Isolated $CD34^{+}$ cells were then expanded in culture condition favoring growth of endothelial cells and these cells were positive for VE-Cadherin, an endothelial marker (Figure 4.1c, d). Therefore, I was able to effectively differentiate endothelial cells from DS iPSCs.

To assess the effects of trisomy 21 on the first process of endothelial differentiation then subsequently on the angiogenic capacity of iPSC-derived endothelial cells, I conducted differentiation experiments using our transgenic DS iPSCs with and without silencing the targeted chromosome 21 to generate endothelial cells. As shown in figure 4.1, in three independent experiments there was no significant difference in the amount of $CD34^{+}CD31^{+}$ endothelial progenitor cells generated from cells with and without trisomy silencing, suggesting trisomy silencing does not affect the differentiation efficiency of CD34+CD31+ endothelial progenitor cells, at least not at the level that can be detected in my approach. In addition, culturing these isolated endothelial progenitor


a
iPSCs
Mesodermal cells
GSK3 inhibition
Endothelial progenitors (CD34+CD31+)
Endothelial cells (CD31+)
Tube forming assay
b
un-silenced
silenced
0.13
10.7
88.8
0.31
0.11
8.68
90.9
0.33
CD31
CD34
dox / no dox
1.2
1
0.8
0.6
0.4
0.2
0


c
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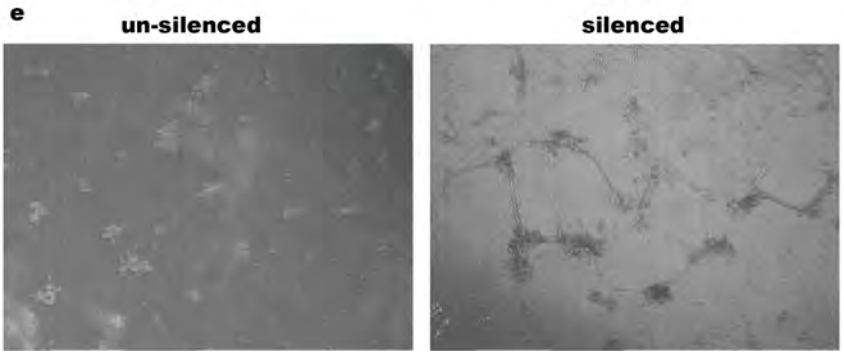
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Figure 4.1 Endothelial differentiation from *XIST* targeted DS iPSCs

(a) Simple schematic of endothelial differentiation process. iPSCs were cultured with GSK3 inhibition to induce formation of $CD34^+CD31^+$ endothelial progenitor cells. Endothelial progenitor cells were isolated and cultured in conditions promotes growth of $CD31^+$ endothelial. After expansion, equal amounts of cells from the endothelial cultures of trisomy silenced and non-silenced samples were plated for tube forming assay. (b) The formation of $CD34^+CD31^+$ endothelial progenitor cells in trisomy silenced and non-silenced samples. The quantification was calculated as the percentage of $CD34^+CD31^+$ endothelial progenitor cells from trisomy silenced sample divided by the percentage of $CD34^+CD31^+$ endothelial progenitor cells from non-silenced sample. There is no significant difference in the formation of $CD34^+CD31^+$ endothelial progenitor cells after trisomy silencing. (c) Morphology of cultured $CD31^+$ endothelial cells. (d) Trisomy silenced $CD31^+$ cells were stained positive for VE-Cadherin, an endothelial marker, and *XIST* RNA paint. (e) The result of the first tube-forming assay performed. Trisomy silenced endothelial cells generated significantly more tube-like structures than non-silenced cells. However, two subsequent experiments were not able to reproduce this result.

cells did not show observable differences in proliferation rate between trisomy corrected and non-corrected populations. Notably, consistent with results in previous chapters showing the effects of trisomy 21 correction are cell type specific (increased and decreased the formation of neural stem cells and hematopoietic progenitor cells, respectively), endothelial progenitor cells proved to be a cell type whose differentiation is not affected by trisomy 21 correction. Hence, we conclude that trisomy 21 does not affect the formation of endothelial progenitor cells, at least not as discernible in these differentiation experiments *in vitro*.

To examine if trisomy 21 affects angiogenesis, a tube forming assay was performed to access the ability of trisomy corrected and non-corrected endothelial cells to form tube-like structures in response to VEGF, an angiogenic cytokine. This process is thought of as an *in vitro* mimic of capillary formation under angiogenic stimulus *in vivo*. The first experiment showed significant differences in the number of tubes formed, with strikingly more tubes generated from endothelial cells in which *XIST*-mediated trisomy correction was induced (Figure 4.1e). However, two subsequent experiments did not reproduce this result, since no difference in the amount of tubes formed was observed. However, in the latter two experiments (where no differences in tube formation were seen) are unreliable in that neither cell population (with or without trisomy silencing) formed tubes well.

We conclude from these experiments that trisomy 21 does not cause defective formation of the endothelial progenitor cells, since all three experiments were consistent on that part. However, to determine if there is a difference in angiogenic competence of these endothelial cells will require more experiments with optimal conditions for

performing tube-forming assay. Whether or not trisomy 21 can reduce angiogenesis, remains an interesting and important biological question related to the unexpected reduction in risk for solid tumor development in DS individuals.

Discussion and future directions

Despite the promising result showed in my first tube-forming assay experiment, the inconsistent and questionable results shown in subsequent experiments prevent us from drawing a conclusion on the effect of trisomy 21 on angiogenesis. Since these experiments were not performed in the context of the cancer environment, if an inhibitory effect on angiogenesis is truly present, the results would indicate intrinsic defects of endothelial cells in tube formation due to trisomy 21. However, our results do provide solid evidence that endothelial cells form at the same rate, irrespective of trisomy 21.

It should be considered that the differentiation protocol we conducted here produced endothelial progenitor cells of fetal origin. However, cancer protection in DS individuals only occurs in adulthood and cells of adult type may behave differently from cells with fetal orgin. For example, fetal liver hematopoietic stem cells (HSCs) and adult bone marrow HSCs differ in their capacity for self-renewal and differentiation. Similarly, fetal and adult types of red blood cells express different sets of hemoglobins with different affinities for oxygen. Therefore, the endothelial cells we differentiated from iPSCs do not fully resemble adult type endothelial cells, which may have reduced angiogenic capacity in the presence of trisomy 21. Additionally, tumor angiogenesis is a complex mechanism with many angiogenic factors involved. In our experiment, the only angiogenic factor we used to induce tube formation is VEGF and it is possible that a difference in angiogenesis may reflect the differences in responsiveness to other types of angiogenic factors (not present in this system).

Interestingly, the pathogenesis of Alzheimer's disease (AD) has also been linked to dys-regulated angiogenesis. However, in contrast to the reduced angiogenesis theory

for tumor protection in DS individuals, increasing evidence suggested that the pathogenesis of AD involves hyperactive angiogenesis in response to neural inflammation in the brain (Vagnucci and Li 2003; Desai et al. 2009). Defective blood brain barrier function was identified as a prominent clinical feature of AD patients (Farrall and Wardlaw 2009) and findings from AD mouse models suggested that overproduction of amyloid plaques, a key feature of AD and DS brains, causes extensive angiogenesis that disrupts the permeability of blood brain barrier, which contribute significantly to the disease progression (Biron et al. 2011). Notably, in the cancer protection hypothesis, reduced angiogenesis is likely due to an intrinsic characteristic of trisomic endothelial cells, but in AD brains, hyperactive angiogenesis is hypothesized to result from excessive production of extrinsic factors from the trisoimc environment, such as amyloid plaques, that directly or indirectly promote angiogenesis. Endothelial cells that form the blood brain barrier have properties distinct from endothelial cells in other parts of the body. Although a differentiation protocol for producing endothelial cells with blood brain barrier properties has been described (Lippmann et al. 2012), modeling and testing this hypothesis involve multiple cell types in the brain and therefore in this case, an *in vivo* model might be preferable.

In sum, given the links between angiogenesis and DS phenotypes, elucidating properties of angiogenesis in DS is an important task in the field and the research findings may not only benefit DS individuals but also AD and cancer patients.

Materials and methods

iPSC culture

XIST targeted DS iPSCs were maintained on vitronectin-coated plate in Essential 8 (E8) medium (Thermo Fisher Scientific). 6-well plates were pre-coated with 5ug/ml vitronectin (1 ml/well) for 1 hour at room temperature before plating iPSCs. To passage, iPSCs were treated with 0.5 mM EDTA (in PBS) at room temperature for 5 minutes. After removing EDTA, E8 medium were then added and iPSCs were broken into small clumps before distributed into new wells. Medium were changed every day and cells were passaged every 3 to 4 days.

Endothelial differentiation

As noted in the main text, endothelial differentiation protocol was based on a published method (Lian et al. 2014). Briefly, iPSCs were dissociated into single cells with Accutase (Thermo Fisher Scientific) then seeded into 6 well plate pre-coated with vitronectin with concentration 40000 cells/well in E8 medium supplemented with 5 uM ROCK inhibitor Y-27632 (Selleckchem) for 24 hours (day -3). At day 0, cells were treated with 6 mM CHIR99021 (Selleckchem) for 2 days in LaSR basal medium, which consists of Advanced DMEM/F12 (Thermo Fisher Scientific) supplemented with 2.5 mM GlutaMAX (Thermo Fisher Scientific) and 60 mg/ml ascorbic acid (Sigma, A8960). At day 2, cells were treated with fresh LaSR medium (without CHIR99021) for 3 to 4 days, followed by FACS analysis and isolation for $CD34^{+}$ cells.

Endothelial cell culture

Day 5 differentiation cultures was isolated for $CD34^+$ endothelial progenitor cells using CD34 MicroBead Kit (Miltenyi Biotec). Plates were pre-coated with 0.1% Fibronectin (Sigma) before seeded with isolated $CD34^+$ cells. Cells were cultured in Human Endothelial-SFM supplemented with bFGF (20 ng/ml) and EGF (10 ng/ml). Cytokines were purchased from PeproTech.

Flow cytometry

All antibodies were purchased from BD Bioscience, including anti-CD34-APC, anti-CD43-FITC and anti-CD31-PE. $CD34^+$ cells isolated from day 5 differentiation were filtered through 50 μm filter (Partec) before stained for FACS analysis. Cells were stained for 30 minutes at 4 degree in the dark then washed twice with PBS supplemented with 2% FBS before flow cytometry. 4',6-diamidino-2-phenylindole (DAPI) was used for selection of live cells.

Tube forming assay

Endothelial cells were seeded into one well of 24 well plate (pre-coated with 250 ml Matrigel from BD Bioscience) in 0.4 ml EGM-2 medium (Lonza) (~100,000 cells/well). 50 ng/ml VEGF (PeproTech) was added to induce vesicular tube formation. Tube formation was observed by light microscopy after 16 to 24 hours of incubation.

in situ hybridization and immunostaining

Methods for *in situ* hybridization and immunostaining are described in chapter II. Rabbit anti-human CD144 (VE-Cadherin) was purchased from eBioscience.

Chapter V: Final Summary and Discussions

In its broadest sense, this thesis work demonstrated the notion of "translational epigenetics". Instead of trying to directly correct specific genes on chromosome 21 in DS, which is not feasible due to the number of genes involved, we took an alternative approach to epigenetically rebalance the transcriptome of DS cells. By inducible inactivation of one chromosome 21 via *XIST* mediated chromosome inactivation, we showed chromosome wide transcriptional silencing of the targeted chromosome. Further, we show in cells that still carry the extra chromosome that this rebalancing of the transcriptome effectively normalized cellular defects associated with DS, demonstrated for the hematopoietic system for which cell phenotypes are best known. This demonstration of the effectiveness for DS phenotypes at the cellular level makes more conceivable potential development of a novel therapeutic strategy of "chromosome therapy" for DS. However, it also immediately provides a well-controlled human cellular model system for studying the basic biology of trisomy 21 pathogenesis in cell, which could also advance development of traditional therapies. The implications of these two aspects of DS will be the focus of the discussion below, but first I will briefly summarize the importance of this work for basic research in chromosome regulation.

XIST RNA has a remarkable capacity to silence an autosomal chromosome

While the ability of XIST RNA to initiate X chromosome silencing *in cis* has been known for decades, scientists have been focusing on studying the mechanism behind this biological process. In this thesis work, we instead tested the feasibility of silencing a problematic extra autosome that impacts many children and adults. Results

showed a very comprehensive and efficient ability of *XIST* RNA to silence an autosome, in trisomic cells where there is no selection against silencing this chromosome. Hence, XIST mediated chromosome silencing is not specific to X chromosome sequences, but rather to the chromosome structure into which *XIST* is integrated. Several hypotheses have been proposed for how XIST RNA localizes and silences the X chromosome and our results clearly establish that XIST localization and silencing shows little if any specificity to the genomic sequence or topological structure of X chromosome. While not our focus here, we note that the robust ability of *XIST* to inactivate an autosome indicates that the strategy developed here would be applicable to other trisomies, such as trisomy 13 and 18, which lead to death in early years of life.

XIST-engineered DS iPSCs provides a needed model system to study initiation of *human* chromosome silencing by *XIST*

Given that X chromosome inactivation (XCI) normally occurs early in embryogenesis and human embryos are not accessible, current knowledge of the X chromosome inactivation process largely depends on experimental results from mouse models. Even with the invention of techniques for ESC isolation and iPSC reprogramming, *in vitro* human female PSCs tend to already have one X chromosome silenced precociously or have other epigenetic anomalies (Hall et al. 2008), thus a human cell model for studying the process of XCI from its initiation is still lacking. Although our trisomy silencing system is for chromosome 21, characteristics of XCI are shared with chromosome 21 silencing, such as *XIST* RNA coating, recruitment of various heterochromatic hallmarks, DNA methylation, and formation a DNA-dense of Barr body.

Therefore, besides being a valuable tool for DS research, our XIST-engineered DS iPSCs provide the field with the first human cell model for studying the process of XIST mediated chromosome silencing.

Inducible chromosome silencing provides an isogenic and iso-epigenetic system to model human Down Syndrome cell pathologies

One of the biggest challenges for researchers to identify dosage sensitive genes or pathways responsible for DS symptoms is the genetic variations among DS individuals. Although trisomy 21 is the consistent chromosomal abnormality of DS, the clinical symptoms vary significantly among DS individuals. While some DS individuals are able to speak well and function close to more normal levels, others need lifelong help even for performing daily tasks. Notably, a significant proportion of DS pregnancies cannot even survive and end in miscarriage or stillbirth. Thus, trisomy 21 has both consistent and variable impacts on different DS individuals, but investigating this is thwarted by the broad genetic variation that exists between all individuals. This natural variation, and the genetic complexity of DS has made it difficult to clearly determine the direct effects of trisomy 21 on cells and different systems of the body.

An obvious approach to identify pathogenic genes or pathways is to compare cell phenotypes and transcriptome datasets from normal and disease individuals. However, due to the variation in symptoms seen between DS individuals and the genetic variability among all people (control and disease populations), it is difficult to establish which pathology is caused by specific trisomy 21 genes, or even the particular cell types and pathways impacted. Although mouse models of DS have been created to compare mice

with identical genetic backgrounds (except the presence of an extra chromosome), the species differences between human and mouse and certain weaknesses of available mouse models still make it difficult to extrapolate experimental findings, and some phenotypes are seen only in human or only in mice with DS.

Since the invention of iPSC reprogramming techniques, these pluripotent cells became a popular choice for modeling development of human diseases at the cellular level, allowing study of how defects arise in patients. However, significant genetic variations exist among iPS lines made from different individuals. Therefore, comparing iPS lines derived from different individuals is less useful than comparison of “isogenic” iPS lines derived from the same individual. However, even with this improvement, there are often still significant variations between isogenic iPS cell clones. Epigenetic variation can come from heterogeneity of the cell population reprogrammed, differences in reprograming of each cell clone, or epigenetic “drift” that is common during stem cell culture. Additionally, the process of reprogramming can be mutagenic and thus introduce genetic variations between iPSC lines from different reprogramming events or methods. Furthermore, the genome and epigenome of iPSCs is known to be unstable and prolonged culture of iPSCs may select for cells harboring alterations that promote faster growth. Therefore, direct comparisons between two different iPSC lines, even from the same individual, are often less informative, necessitating comparison of many distinct iPSC lines/clones before drawing strong conclusions.

For this work we generated an isogenic disomic subclone by isolating DS iPSCs that randomly lost the extra chromosome 21 during the process of culturing. This provides an isogenic disomic (normal euploid) line that is genetically identical except for

one chromosome 21 being lost. While this comparison is useful to include and can be informative, separately cloned and cultured isogenic iPSC sub-clones can accumulate epigenetic changes that influence their behaviors or phenotypes. In contrast, in our inducible chromosome silencing system, we compare essentially the same cell populations in parallel with the only difference being expression of two versus three active chromosome 21s. While there is a need to correct for any potential effect of doxycycline itself, this design minimizes other potential sources of variation which likely made it plausible for our results to reveal certain effects of trisomy 21, such as enhanced IGF gene expression and signaling in early hematopoietic progenitor cells (chapter III).

Other regulators on chromosome 21 could play important roles in hematopoietic abnormalities

While transcriptional regulators are heavily involved in the control of the hematopoietic system, there is growing evidence to indicate that posttranslational regulation by miRNAs is also very important. Five miRNAs have been identified on chromosome 21, including miR-let-7c, miR-99a, miR-125b, miR-155, and miR-802. While several hematopoietic regulators on chromosome 21, including *RUNX1, ERG, ETS2*, are potential contributors for development of DS-AMKL, they are not highly expressed in DS-TMD or DS-AMKL cells. In contrast, Klusmann et al. showed increased expression of miR-125b in DS-TMD and DS-AMKL cells and inhibition of miR-125b expression inhibits their proliferation (Klusmann et al. 2010b). They also demonstrated that overexpression of miR-125b increases proliferation and self-renewal of MEPs, the precursor cells for megakaryocytes and erythrocytes. This is inline with our results

showing the overproduction of these two types of hematopoietic colonies occurs due to trisomy 21 (without GATA1s or other mutations), consistent with this as the initial driving event for DS-AMKL. Their finding suggests an oncogenic role of miR-125b in DS-AMKL. Additionally, miR99a, miR125b, and miR155 have been shown enriched in HSCs and, moreover, enforced expression of miR125b and miR155 in HSCs causes myeloproliferative disorder which progresses to acute myeloid leukemia (O'Connell et al. 2010). All of these findings suggest a possible role of chromosome 21 encoded miRNAs in the pathogenesis of DS associated hematopoietic abnormalies. This is therefore an exciting area which could be further investigated in our well-controlled experimental system to study hematopoietic pathogenesis associated with DS.

Altered chromatin states may contribute to hematopoietic abnormalities associated with DS individuals

The process of hematopoietic differentiation is very complicated and tightly controlled by transcriptional programs that activate lineage specific and repress non-lineage specific genes. As discussed in the introduction (chapter I), hematopoietic transcription regulators encoded on chromosome 21, such as *RUNX1*, *ERG*, and *ETS2*, are likely to contribute to hematopoietic abnormalities in trisomy 21. In particular, our findings implicating EHT as key to trisomy 21 effects fits well with other evidence that *RUNX1* dosage impacts the production of hematopoietic progenitors (Banno et al. 2016) and that *RUNX1* is involved in regulation of EHT. However, for lineage specification, to ensure appropriate maintenance of the transcription program, precise coordination between hematopoietic transcription factors and chromatin states is also required. Two

chromosome 21 genes, *USP16* and *HMGN1*, known to be broadly involved in chromatin modifications and remodeling, have also been suggested to participate in pathogenesis of other common hematopoietic deficits associated with DS (although not necessarily the DS-TMD and DS-AMKL).

USP16 encodes a deubiquitinase that counteracts the ubiquitination function of Polycomb repressive complex (PRC1), which is essential for self-renewal of multiple types of stem cells. A 1.5-fold increase in expression of *Usp16* in HSCs isolated from Ts65Dn mice was reported to significantly reduce the global level of H2AK119 ubiquitination and cause defective self-renewal capacity, suggested by failed bone marrow engraftment in secondary transplantation (Adorno et al. 2013). Notably, this defective self-renewal of HSCs due to *Usp16* overexpression has been linked to aging (Souroullas and Sharpless 2013). Interestingly, certain phenotypes of aged HSCs from normal individuals mimic other types of hematopoietic abnormalities broadly implicated in DS, such as reduced self-renewal, myeloid-biased differentiation, and impaired lymphoid differentiation that may contribute to defective immune functions.

Another recent study using Ts1Rhr mice, a mouse model of DS with triplication of 31 genes orthologous to human chromosome 21 genes, implicated chromatin changes due to the *HMGN1* gene in etiology of DS-ALL. Lane et al. reported defective differentiation of progenitor B cells in the bone marrow and these cells exhibited increased self-renewal capacity *in vitro*, and upon introduction of common mutations found in DS-ALL these progenitor B cells promoted development of ALL (Lane et al. 2014). Transcriptional profiling of these progenitor B cells revealed global reduction of H3K27me3 level on genes whose promoter is marked with both H3K27me3 and

H3K4me3, suggesting overexpression of these genes in Ts1Rhr progenitor B cells (Lane et al. 2014). Moreover, pharmacological restoration of H3K27me3 rescued the phenotypes observed in Ts1Rhr progenitor B cells, suggesting the involvement of these overexpressed genes in the pathogenesis of DS-ALL (Lane et al. 2014). Among the 31 genes triplicated in Ts1Rhr mice, *HMGN1* encodes nucleosome-binding proteins capable of promoting chromatin decompaction and modulating gene transcription. Overexpression of *Hmgn1* in mouse B progenitor cells recapitulates transcription profiling and many phenotypes of progenitor B cells in Ts1Rhr mice (Lane et al. 2014). Therefore, *HMGN1* triplication may underlie the development of DS-ALL. However, whether *HMGN1* dosage effects may contribute to development of DS-AMKL is unknown.

In sum, overexpression of certain genes on chromosome 21, such as *USP16* and *HMGN1,* may have broader effects on the global chromatin states, which could influence many genes and pathways that contribute to development of leukemia.

The long-term prospects that XIST-mediated chromosome silencing can potentially be developed as a treatment for DS children associated with leukemia

In this thesis work we introduced a novel approach to epigenetically correct the chromosomal imbalance in DS cells “on demand”, and demonstrates for the first time that insertion of one gene can normalize a DS developmental defect at the cellular level, which we show the utility of this as an experimental strategy to examine DS pathogenesis and a potential strategy of developing “chromosome therapy” for DS patients in the future.

The accessibility of the cell type of interest is one key to the potential feasibility for chromosome therapy for the various medical challenges DS patients encounter. Currently, the hematopoietic stem cells (HSCs) are the only adult stem cell type in routine clinical use because they are easier to acquire (through isolating bone marrow cells, which are enriched for hematopoietic stem and progenitor cells), and they are able to reconstitute the damaged hematopoietic system after transplantation. Consistent with other findings (Maclean et al. 2012), our results clearly show that trisomy 21 increases production of hematopoietic progenitors, and thus trisomy 21 alone can cause this initial step towards the acquisition of DS-TMD and DS-AMKL. It is known that DS-AMKL is a multistep disease and DS children with TMD can have severe clinical effects, and are at high risk to acquire DS-AMKL before they reach the age of 4 (whether the DS-TMD appears clinically to be resolved or not) (Bhatnagar et al. 2016). Hence, theoretically it would be possible to correct bone marrow cells isolated from DS-TMD patients by XIST-mediated trisomy silencing and then transplant back into the patient. This can theoretically prevent DS-TMD children from further acquiring DS-AMKL, which is more severe and requires chemotherapy. Although DS-AMKL patients have higher 5-year survival of cytarabine-based chemotherapy when compared to non-DS-AMKL patients, they suffer with harsh side effects and toxicity, and the overall mortality rate is around 20% (Bhatnagar et al. 2016). While DS-TMD blasts already harbor the GATA1s mutations essential for further leukemia progression, it has been established that the GATA1s mutation alone (without trisomy 21) is not sufficient to drive the development DS-AMKL. In addition, this autologous transplantation would prevent the adverse effects due to immunocompatiability issues (of allogenic transplantations) and would avoid the

need for the time-consuming process to acquire a matched bone marrow donor. Moreover, although we did not examine the effect of trisomy silencing on the lymphoid compartment of the hematopoietic system, lymphoid cells can be derived from the hematopoietic progenitor cells studied here. Hence, this approach can potentially have applicability to DS-ALL as well, since DS-ALL patients have significantly worse prognosis and lowered overall survival rate in response to chemotherapy. Since cord blood is also enriched for Hematopoietic stem and progenitor cells (HSPCs) and could be an alternative source for stem cells and chromosome therapy for the greater hematopoietic risks for DS children, cord blood should be more routinely saved for DS newborns.

Despite these prospects, there are still several obstacles to any therapeutic application remain to be overcome. Genetic manipulation has been thought difficult in primary cells, especially for HSCs. HSCs are characterized by self-renewal capacity and multipotency, however, an ideal condition for culturing HSCs has been lacking and *in vitro* culture of HSCs often results in loss of these two critical properties. This has significantly limited the process of gene editing in hematopoietic stem cells *in vitro*. Initiation of XIST mediated chromosome silencing has been shown in mouse HSCs (Savarese et al. 2006), although not directly demonstrated in human HSCs. This can be tested in our system by turning on *XIST* expression at the stage where HSCs emerge. An alternative strategy to avoid any uncertainties about genetic engineering in HSCs is to produce them from patient derived iPSCs. This strategy has been sought for many blood disorders but was not demonstrated as feasible, because *in vitro* generated HSCs failed to engraft in bone marrow. However, a very recent study now describes the first

breakthrough, successful production of engraftable HSCs from human pluripotent stem cells *in vitro* (Sugimura et al. 2017). Although the efficiency for engraftment is low, this demonstrates the promise to further develop therapeutic strategies for DS associated hematopoietic disorders. Therefore, despite the challenges ahead, the possibility for XIST-based chromosome therapy merits further testing and development for select DS patients.

Correction of DS hematopoietic abnormalities by XIST mediated trisomy silencing has implications beyond the hematopoietic system

Although this thesis work demonstrated the correction of cellular phenotypes mainly in the myeloid compartment of the hematopoietic system, it has implications beyond DS associated leukemia. One example is the defective immune system. Detailed mechanisms for the defective immune response and increased rate of respiratory infections in most DS individuals are still largely unknown and not extensively studied. However immune cells, such as B and T cells, belong to the hematopoietic system, making correction of hematopoietic abnormalities a potential path to improve the immunological system. Future work could investigate this by comparing the behavior of immune cells, using the inducible trisomy silencing shown here. One example, defective chemotaxis of neutrophils seen in DS could be tested using this system.

Another intriguing implication is that “normalizing” hematopoiesis may even have potential to improve some aspects of neurological deficits in DS individuals. Increasing evidence point to the interplay between the immune and neural systems and the involvement of immune and inflammatory responses in the development of

complicated neurodegenerative disorders, including Alzheimer's Disease. Brain tissues are constantly under immunosurveillence to prevent damage, and several myeloid cells have been recognized to play essential roles in both health and diseases. For example, microglia are macrophages (member of the hematopoietic system) which reside in the central nervous system (CNS), participating in the development of neural circuits, maintenance of synapses, and neurogenesis (Gomez Perdiguero et al. 2015). Microglia are also responsible for defending the CNS against various types of pathogenic factors, and produce various inflammatory cytokines that may compromise the function and survival of neurons. Notably, emerging evidence suggests neuroinflammation is an important component of Alzheimer's disease pathologies, a hallmark of which is accumulation of Aβ peptides in brain tissues, Aβ produced by cleavage of APP. In normal brain tissues, microglia recognize Aβ peptides and participate in the clearance of Aβ peptides through phagocytosis. One hypothesis for Alzheimer's disease pathogenesis is the impairment of microglial clearance of Aβ peptides, and the production of inflammatory cytokines that may damage brain tissues. Whether microglia, a type of hematopoietic cell, are defective in DS brain tissues has not been established, however this serves as one example of how correcting components of the hematopoietic system could have unexplored broader benefits on the health and well-being of individuals with DS.

Concluding remarks and future directions

Collective results presented in this thesis work demonstrate a novel approach to dosage compensate chromosome 21 genes in DS cells without complete elimination of

the extra chromosome 21, and establish that this improves several phenotypes commonly seen in DS hematopoietic cells. While an alternative concept holds that much of the DS pathologies are due to the physical presence of an extra chromosome that impacts cell function and fitness but not to the expression of genes on that chromosome (Sheltzer et al. 2017), our results suggested the improved phenotypes were not caused by the physical presence of an extra chromosome 21, but by the specific developmental program controlled by genes on chromosome 21. Because trisomy silencing affected different DS cell types in opposite ways (increased proliferation on iPSCs, increased formation of neural stem cells, and decreased formation of hematopoietic progenitor cells), this ruled out the possibility that decreased hematopoietic differentiation might be due to an adverse effect of expressing a large non-coding RNA in the cells. The consistency between our results (comparing trisomy silenced cells to non-silenced cells) and results from other studies (comparing disomic to trisomic cells) provides a proof-of-principle for "chromosome therapy" for DS patients and validates the usefulness of our system for modeling and studying DS pathogenesis. Further, the iso-epigenetic feature of our system allows us to compare cells in a more controlled manner, with less variability, and identify pathogenic genes or pathways that might otherwise be obscured or difficult to detect. An example is the increased IGF signaling in trisomic hematopoietic progenitor cells (chapter III), which was not found by comparing disomic to trisomic cells (Maclean et al. 2012).

As described in previous sections, to better understand the pathogenesis of DS hematopoietic defects, the transcriptome profiling analysis on hemogenic endothelium–like populations will help identify more dysregulated gene networks associated with the

defects. One candidate gene to look for is *RUNX1*, as its involvement in both normal and DS hematopoiesis has been reported (Chen et al. 2009; Banno et al. 2016). Additionally, miRNAs and chromatin modifiers are also of interest based on their broader effects on the whole genome and their dysregulations in DS leukemic cells (Klusmann et al. 2010b; Adorno et al. 2013; Lane et al. 2014).

A key to successful chromosome therapy is the effectiveness of *XIST*-mediated chromosome silencing in the pathogenic cell types. Although iPSCs can theoretically differentiate into all cell types in the body, the process can be difficult and inefficient. Therefore, determining whether *XIST* RNA can silence a chromosome in differentiated cells (by inducing *XIST* expression later in the differentiation process) can significantly increase the potential of applying chromosome therapy clinically, especially for easily accessible cell types such as hematopoietic cells. Additionally, DS mouse models have well-characterized phenotypes throughout the body that can be tested for correction after targeted expression of *Xist* RNA from the extra chromosome. This will allow a more complete examination of *XIST*-mediated chromosome therapy on a living organism, which will be the key to the future development of chromosome therapy for DS patients.

Reference

Adamo L, Garcia-Cardena G. 2012. The vascular origin of hematopoietic cells. *Developmental biology* **362**: 1-10.

Adorno M, Sikandar S, Mitra SS, Kuo A, Nicolis Di Robilant B, Haro-Acosta V, Ouadah Y, Quarta M, Rodriguez J, Qian D et al. 2013. Usp16 contributes to somatic stem-cell defects in Down's syndrome. *Nature* **501**: 380-384.

Ahmed M, Sternberg A, Hall G, Thomas A, Smith O, O'Marcaigh A, Wynn R, Stevens R, Addison M, King D et al. 2004. Natural history of GATA1 mutations in Down syndrome. *Blood* **103**: 2480-2489.

Ait Yahya-Graison E, Aubert J, Dauphinot L, Rivals I, Prieur M, Golfier G, Rossier J, Personnaz L, Creau N, Blehaut H et al. 2007. Classification of human chromosome 21 gene-expression variations in Down syndrome: impact on disease phenotypes. *Am J Hum Genet* **81**: 475-491.

Al-Kasim F, Doyle JJ, Massey GV, Weinstein HJ, Zipursky A, Pediatric Oncology G. 2002. Incidence and treatment of potentially lethal diseases in transient leukemia of Down syndrome: Pediatric Oncology Group Study. *J Pediatr Hematol Oncol* **24**: 9-13.

Antonarakis SE. 2017. Down syndrome and the complexity of genome dosage imbalance. *Nat Rev Genet* **18**: 147-163.

Arron JR, Winslow MM, Polleri A, Chang CP, Wu H, Gao X, Neilson JR, Chen L, Heit JJ, Kim SK et al. 2006. NFAT dysregulation by increased dosage of DSCR1 and DYRK1A on chromosome 21. *Nature* **441**: 595-600.

Augui S, Filion GJ, Huart S, Nora E, Guggiari M, Maresca M, Stewart AF, Heard E. 2007. Sensing X chromosome pairs before X inactivation via a novel X-pairing region of the Xic. *Science* **318**: 1632-1636.

Baek KH, Zaslavsky A, Lynch RC, Britt C, Okada Y, Siarey RJ, Lensch MW, Park IH, Yoon SS, Minami T et al. 2009. Down's syndrome suppression of tumour growth and the role of the calcineurin inhibitor DSCR1. *Nature* **459**: 1126-1130.

Baialardo EM, Felice MS, Rossi J, Barreiro C, Gallego MS. 1996. Tandem triplication and quadruplication of chromosome 21 in childhood acute lymphoblastic leukemia. *Cancer Genet Cytogenet* **92**: 43-45.

Banno K, Omori S, Hirata K, Nawa N, Nakagawa N, Nishimura K, Ohtaka M, Nakanishi M, Sakuma T, Yamamoto T et al. 2016. Systematic Cellular Disease Models Reveal Synergistic Interaction of Trisomy 21 and GATA1 Mutations in Hematopoietic Abnormalities. *Cell Rep* **15**: 1228-1241.

Barakat TS, Gunhanlar N, Pardo CG, Achame EM, Ghazvini M, Boers R, Kenter A, Rentmeester E, Grootegoed JA, Gribnau J. 2011. RNF12 activates Xist and is essential for X chromosome inactivation. *PLoS Genet* **7**: e1002001.

Barlow GM, Chen XN, Shi ZY, Lyons GE, Kurnit DM, Celle L, Spinner NB, Zackai E, Pettenati MJ, Van Riper AJ et al. 2001. Down syndrome congenital heart disease: a narrowed region and a candidate gene. *Genet Med* **3**: 91-101.

Barr ML, Bertram EG. 1949. A morphological distinction between neurones of the male and female, and the behaviour of the nucleolar satellite during accelerated nucleoprotein synthesis. *Nature* **163**: 676.

Bertrand JY, Chi NC, Santoso B, Teng S, Stainier DY, Traver D. 2010. Haematopoietic stem cells derive directly from aortic endothelium during development. *Nature* **464**: 108-111.

Bertrand JY, Kim AD, Violette EP, Stachura DL, Cisson JL, Traver D. 2007. Definitive hematopoiesis initiates through a committed erythromyeloid progenitor in the zebrafish embryo. *Development (Cambridge, England)* **134**: 4147-4156.

Bhatnagar N, Nizery L, Tunstall O, Vyas P, Roberts I. 2016. Transient Abnormal Myelopoiesis and AML in Down Syndrome: an Update. *Curr Hematol Malig Rep* **11**: 333-341.

Biancotti JC, Narwani K, Buehler N, Mandefro B, Golan-Lev T, Yanuka O, Clark A, Hill D, Benvenisty N, Lavon N. 2010. Human embryonic stem cells as models for aneuploid chromosomal syndromes. *Stem cells (Dayton, Ohio)* **28**: 1530-1540.

Birger Y, Izraeli S. 2012. DYRK1A in Down syndrome: an oncogene or tumor suppressor? *J Clin Invest* **122**: 807-810.

Biron KE, Dickstein DL, Gopaul R, Jefferies WA. 2011. Amyloid triggers extensive cerebral angiogenesis causing blood brain barrier permeability and hypervascularity in Alzheimer's disease. *PloS one* **6**: e23789.

Blank HM, Sheltzer JM, Meehl CM, Amon A. 2015. Mitotic entry in the presence of DNA damage is a widespread property of aneuploidy in yeast. *Molecular biology of the cell* **26**: 1440-1451.

Bloemers BL, van Bleek GM, Kimpen JL, Bont L. 2010. Distinct abnormalities in the innate immune system of children with Down syndrome. *J Pediatr* **156**: 804-809, 809 e801-809 e805.

Boisset JC, van Cappellen W, Andrieu-Soler C, Galjart N, Dzierzak E, Robin C. 2010. In vivo imaging of haematopoietic cells emerging from the mouse aortic endothelium. *Nature* **464**: 116-120.

Boland ML, Chourasia AH, Macleod KF. 2013. Mitochondrial dysfunction in cancer. *Front Oncol* **3**: 292.

Brockdorff N, Ashworth A, Kay GF, McCabe VM, Norris DP, Cooper PJ, Swift S, Rastan S. 1992. The product of the mouse Xist gene is a 15 kb inactive X-specific transcript containing no conserved ORF and located in the nucleus. *Cell* **71**: 515-526.

Brown CJ, Ballabio A, Rupert JL, Lafreniere RG, Grompe M, Tonlorenzi R, Willard HF. 1991a. A gene from the region of the human X inactivation centre is expressed exclusively from the inactive X chromosome. *Nature* **349**: 38-44.

Brown CJ, Hendrich BD, Rupert JL, Lafreniere RG, Xing Y, Lawrence J, Willard HF. 1992. The human XIST gene: analysis of a 17 kb inactive X-specific RNA that contains conserved repeats and is highly localized within the nucleus. *Cell* **71**: 527-542.

Brown CJ, Lafreniere RG, Powers VE, Sebastio G, Ballabio A, Pettigrew AL, Ledbetter DH, Levy E, Craig IW, Willard HF. 1991b. Localization of the X inactivation centre on the human X chromosome in Xq13. *Nature* **349**: 82-84.

Bruwier A, Chantrain CF. 2012. Hematological disorders and leukemia in children with Down syndrome. *Eur J Pediatr* **171**: 1301-1307.

Busciglio J, Yankner BA. 1995. Apoptosis and increased generation of reactive oxygen species in Down's syndrome neurons in vitro. *Nature* **378**: 776-779.

Byron M, Hall LL, Lawrence JB. 2013. A multifaceted FISH approach to study endogenous RNAs and DNAs in native nuclear and cell structures. *Curr Protoc Hum Genet* **Chapter 4**: Unit 4 15.

Cabelof DC, Patel HV, Chen Q, van Remmen H, Matherly LH, Ge Y, Taub JW. 2009. Mutational spectrum at GATA1 provides insights into mutagenesis and leukemogenesis in Down syndrome. *Blood* **114**: 2753-2763.

Cantor AB, Orkin SH. 2002. Transcriptional regulation of erythropoiesis: an affair involving multiple partners. *Oncogene* **21**: 3368-3376.

Canzonetta C, Mulligan C, Deutsch S, Ruf S, O'Doherty A, Lyle R, Borel C, Lin-Marq N, Delom F, Groet J et al. 2008. DYRK1A-dosage imbalance perturbs NRSF/REST levels, deregulating pluripotency and embryonic stem cell fate in Down syndrome. *Am J Hum Genet* **83**: 388-400.

Carrel L, Willard HF. 2005. X-inactivation profile reveals extensive variability in X-linked gene expression in females. *Nature* **434**: 400-404.

Chen MJ, Yokomizo T, Zeigler BM, Dzierzak E, Speck NA. 2009. Runx1 is required for the endothelial to haematopoietic cell transition but not thereafter. *Nature* **457**: 887-891.

Choi KD, Vodyanik MA, Togarrati PP, Suknuntha K, Kumar A, Samarjeet F, Probasco MD, Tian S, Stewart R, Thomson JA et al. 2012. Identification of the hemogenic endothelial progenitor and its direct precursor in human pluripotent stem cell differentiation cultures. *Cell Rep* **2**: 553-567.

Choi KD, Yu J, Smuga-Otto K, Salvagiotto G, Rehrauer W, Vodyanik M, Thomson J, Slukvin I. 2009. Hematopoietic and endothelial differentiation of human induced pluripotent stem cells. *Stem cells (Dayton, Ohio)* **27**: 559-567.

Chou ST, Byrska-Bishop M, Tober JM, Yao Y, Vandorn D, Opalinska JB, Mills JA, Choi JK, Speck NA, Gadue P et al. 2012. Trisomy 21-associated defects in human primitive hematopoiesis revealed through induced pluripotent stem cells. *Proceedings of the National Academy of Sciences of the United States of America* **109**: 17573-17578.

Chou ST, Opalinska JB, Yao Y, Fernandes MA, Kalota A, Brooks JS, Choi JK, Gewirtz AM, Danet-Desnoyers GA, Nemiroff RL et al. 2008. Trisomy 21 enhances human fetal erythro-megakaryocytic development. *Blood* **112**: 4503-4506.

Chow JC, Ciaudo C, Fazzari MJ, Mise N, Servant N, Glass JL, Attreed M, Avner P, Wutz A, Barillot E et al. 2010. LINE-1 activity in facultative heterochromatin formation during X chromosome inactivation. *Cell* **141**: 956-969.

Chu C, Zhang QC, da Rocha ST, Flynn RA, Bharadwaj M, Calabrese JM, Magnuson T, Heard E, Chang HY. 2015. Systematic discovery of Xist RNA binding proteins. *Cell* **161**: 404-416.

Clarke RL, Yzaguirre AD, Yashiro-Ohtani Y, Bondue A, Blanpain C, Pear WS, Speck NA, Keller G. 2013. The expression of Sox17 identifies and regulates haemogenic endothelium. *Nature cell biology* **15**: 502-510.

Clemson CM, McNeil JA, Willard HF, Lawrence JB. 1996. XIST RNA paints the inactive X chromosome at interphase: evidence for a novel RNA involved in nuclear/chromosome structure. *The Journal of cell biology* **132**: 259-275.

Coskun PE, Wyrembak J, Derbereva O, Melkonian G, Doran E, Lott IT, Head E, Cotman CW, Wallace DC. 2010. Systemic mitochondrial dysfunction and the etiology of

Alzheimer's disease and down syndrome dementia. *J Alzheimers Dis* **20 Suppl 2**: S293-310.

Cossec JC, Lavaur J, Berman DE, Rivals I, Hoischen A, Stora S, Ripoll C, Mircher C, Grattau Y, Olivomarin JC et al. 2012. Trisomy for synaptojanin1 in Down syndrome is functionally linked to the enlargement of early endosomes. *Hum Mol Genet* **21**: 3156-3172.

Cotton AM, Lam L, Affleck JG, Wilson IM, Penaherrera MS, McFadden DE, Kobor MS, Lam WL, Robinson WP, Brown CJ. 2011. Chromosome-wide DNA methylation analysis predicts human tissue-specific X inactivation. *Hum Genet* **130**: 187-201.

Couzens D, Cuskelly M, Haynes M. 2011. Cognitive development and Down syndrome: age-related change on the Stanford-Binet test (fourth edition). *Am J Intellect Dev Disabil* **116**: 181-204.

Couzens D, Haynes M, Cuskelly M. 2012. Individual and environmental characteristics associated with cognitive development in Down syndrome: a longitudinal study. *J Appl Res Intellect Disabil* **25**: 396-413.

Csankovszki G, Nagy A, Jaenisch R. 2001. Synergism of Xist RNA, DNA methylation, and histone hypoacetylation in maintaining X chromosome inactivation. *The Journal of cell biology* **153**: 773-784.

Cumano A, Godin I. 2007. Ontogeny of the hematopoietic system. *Annu Rev Immunol* **25**: 745-785.

David O, Fiorucci GC, Tosi MT, Altare F, Valori A, Saracco P, Asinardi P, Ramenghi U, Gabutti V. 1996. Hematological studies in children with Down syndrome. *Pediatr Hematol Oncol* **13**: 271-275.

Davisson MT, Schmidt C, Akeson EC. 1990. Segmental trisomy of murine chromosome 16: a new model system for studying Down syndrome. *Prog Clin Biol Res* **360**: 263-280.

Davisson MT, Schmidt C, Reeves RH, Irving NG, Akeson EC, Harris BS, Bronson RT. 1993. Segmental trisomy as a mouse model for Down syndrome. *Prog Clin Biol Res* **384**: 117-133.

de Hingh YC, van der Vossen PW, Gemen EF, Mulder AB, Hop WC, Brus F, de Vries E. 2005. Intrinsic abnormalities of lymphocyte counts in children with down syndrome. *J Pediatr* **147**: 744-747.

DeKelver RC, Choi VM, Moehle EA, Paschon DE, Hockemeyer D, Meijsing SH, Sancak Y, Cui X, Steine EJ, Miller JC et al. 2010. Functional genomics, proteomics, and regulatory DNA analysis in isogenic settings using zinc finger nuclease-driven transgenesis into a safe harbor locus in the human genome. *Genome research* **20**: 1133-1142.

Del Bo R, Comi GP, Perini MP, Strazzer S, Bresolin N, Scarlato G. 2001. Down's syndrome fibroblasts anticipate the accumulation of specific ageing-related mtDNA mutations. *Ann Neurol* **49**: 137-138.

Delom F, Burt E, Hoischen A, Veltman J, Groet J, Cotter FE, Nizetic D. 2009. Transchromosomic cell model of Down syndrome shows aberrant migration, adhesion and proteome response to extracellular matrix. *Proteome Sci* **7**: 31.

Denduluri SK, Idowu O, Wang Z, Liao Z, Yan Z, Mohammed MK, Ye J, Wei Q, Wang J, Zhao L et al. 2015. Insulin-like growth factor (IGF) signaling in tumorigenesis and the development of cancer drug resistance. *Genes Dis* **2**: 13-25.

Desai BS, Schneider JA, Li JL, Carvey PM, Hendey B. 2009. Evidence of angiogenic vessels in Alzheimer's disease. *J Neural Transm (Vienna)* **116**: 587-597.

Ditadi A, Sturgeon CM, Tober J, Awong G, Kennedy M, Yzaguirre AD, Azzola L, Ng ES, Stanley EG, French DL et al. 2015. Human definitive haemogenic endothelium and arterial vascular endothelium represent distinct lineages. *Nature cell biology* **17**: 580-591.

Doetschman TC, Eistetter H, Katz M, Schmidt W, Kemler R. 1985. The in vitro development of blastocyst-derived embryonic stem cell lines: formation of visceral yolk sac, blood islands and myocardium. *J Embryol Exp Morphol* **87**: 27-45.

Down JL. 1995. Observations on an ethnic classification of idiots. 1866. *Ment Retard* **33**: 54-56.

Doyon JB, Zeitler B, Cheng J, Cheng AT, Cherone JM, Santiago Y, Lee AH, Vo TD, Doyon Y, Miller JC et al. 2011. Rapid and efficient clathrin-mediated endocytosis revealed in genome-edited mammalian cells. *Nature cell biology* **13**: 331-337.

Driscoll DA, Gross S. 2009. Clinical practice. Prenatal screening for aneuploidy. *N Engl J Med* **360**: 2556-2562.

Eiges R, Urbach A, Malcov M, Frumkin T, Schwartz T, Amit A, Yaron Y, Eden A, Yanuka O, Benvenisty N et al. 2007. Developmental study of fragile X syndrome using human embryonic stem cells derived from preimplantation genetically diagnosed embryos. *Cell stem cell* **1**: 568-577.

Eilken HM, Nishikawa S, Schroeder T. 2009. Continuous single-cell imaging of blood generation from haemogenic endothelium. *Nature* **457**: 896-900.

Elcheva I, Brok-Volchanskaya V, Kumar A, Liu P, Lee JH, Tong L, Vodyanik M, Swanson S, Stewart R, Kyba M et al. 2014. Direct induction of haematoendothelial programs in human pluripotent stem cells by transcriptional regulators. *Nat Commun* **5**: 4372.

Farrall AJ, Wardlaw JM. 2009. Blood-brain barrier: ageing and microvascular disease--systematic review and meta-analysis. *Neurobiol Aging* **30**: 337-352.

Ferencz C, Neill CA, Boughman JA, Rubin JD, Brenner JI, Perry LW. 1989. Congenital cardiovascular malformations associated with chromosome abnormalities: an epidemiologic study. *J Pediatr* **114**: 79-86.

Gardiner KJ. 2010. Molecular basis of pharmacotherapies for cognition in Down syndrome. *Trends Pharmacol Sci* **31**: 66-73.

Ge Y, Dombkowski AA, LaFiura KM, Tatman D, Yedidi RS, Stout ML, Buck SA, Massey G, Becton DL, Weinstein HJ et al. 2006. Differential gene expression, GATA1 target genes, and the chemotherapy sensitivity of Down syndrome megakaryocytic leukemia. *Blood* **107**: 1570-1581.

Gomez Perdiguero E, Klapproth K, Schulz C, Busch K, Azzoni E, Crozet L, Garner H, Trouillet C, de Bruijn MF, Geissmann F et al. 2015. Tissue-resident macrophages originate from yolk-sac-derived erythro-myeloid progenitors. *Nature* **518**: 547-551.

Grigoriadis AE, Kennedy M, Bozec A, Brunton F, Stenbeck G, Park IH, Wagner EF, Keller GM. 2010. Directed differentiation of hematopoietic precursors and functional osteoclasts from human ES and iPS cells. *Blood* **115**: 2769-2776.

Gropp A, Giers D, Kolbus U. 1974. Trisomy in the fetal backcross progeny of male and female metacentric heterozygotes of the mouse. i. *Cytogenet Cell Genet* **13**: 511-535.

Grossman TR, Gamliel A, Wessells RJ, Taghli-Lamallem O, Jepsen K, Ocorr K, Korenberg JR, Peterson KL, Rosenfeld MG, Bodmer R et al. 2011. Over-expression of DSCAM and COL6A2 cooperatively generates congenital heart defects. *PLoS Genet* **7**: e1002344.

Guidi S, Ciani E, Bonasoni P, Santini D, Bartesaghi R. 2011. Widespread proliferation impairment and hypocellularity in the cerebellum of fetuses with down syndrome. *Brain Pathol* **21**: 361-373.

Guralnick MJ. 2010. Early Intervention Approaches to Enhance the Peer-Related Social Competence of Young Children With Developmental Delays: A Historical Perspective. *Infants Young Child* **23**: 73-83.

Guralnick MJ. 2011. Why Early Intervention Works: A Systems Perspective. *Infants Young Child* **24**: 6-28.

Guschin DY, Waite AJ, Katibah GE, Miller JC, Holmes MC, Rebar EJ. 2010. A rapid and general assay for monitoring endogenous gene modification. *Methods Mol Biol* **649**: 247-256.

Hall LL, Byron M, Butler J, Becker KA, Nelson A, Amit M, Itskovitz-Eldor J, Stein J, Stein G, Ware C et al. 2008. X-inactivation reveals epigenetic anomalies in most hESC but identifies sublines that initiate as expected. *J Cell Physiol* **216**: 445-452.

Hall LL, Byron M, Sakai K, Carrel L, Willard HF, Lawrence JB. 2002a. An ectopic human XIST gene can induce chromosome inactivation in postdifferentiation human HT-1080 cells. *Proceedings of the National Academy of Sciences of the United States of America* **99**: 8677-8682.

Hall LL, Clemson CM, Byron M, Wydner K, Lawrence JB. 2002b. Unbalanced X;autosome translocations provide evidence for sequence specificity in the association of XIST RNA with chromatin. *Hum Mol Genet* **11**: 3157-3165.

Hall LL, Lawrence JB. 2010. XIST RNA and architecture of the inactive X chromosome: implications for the repeat genome. *Cold Spring Harb Symp Quant Biol* **75**: 345-356.

Harewood L, Robinson H, Harris R, Al-Obaidi MJ, Jalali GR, Martineau M, Moorman AV, Sumption N, Richards S, Mitchell C et al. 2003. Amplification of AML1 on a duplicated chromosome 21 in acute lymphoblastic leukemia: a study of 20 cases. *Leukemia* **17**: 547-553.

Hasle H, Clemmensen IH, Mikkelsen M. 2000. Risks of leukaemia and solid tumours in individuals with Down's syndrome. *Lancet* **355**: 165-169.

Hattori M, Fujiyama A, Taylor TD, Watanabe H, Yada T, Park HS, Toyoda A, Ishii K, Totoki Y, Choi DK et al. 2000. The DNA sequence of human chromosome 21. *Nature* **405**: 311-319.

Haydar TF, Reeves RH. 2012. Trisomy 21 and early brain development. *Trends Neurosci* **35**: 81-91.

Heard E. 2005. Delving into the diversity of facultative heterochromatin: the epigenetics of the inactive X chromosome. *Curr Opin Genet Dev* **15**: 482-489.

Heerema NA, Raimondi SC, Anderson JR, Biegel J, Camitta BM, Cooley LD, Gaynon PS, Hirsch B, Magenis RE, McGavran L et al. 2007. Specific extra chromosomes

occur in a modal number dependent pattern in pediatric acute lymphoblastic leukemia. *Genes Chromosomes Cancer* **46**: 684-693.

Herault Y, Delabar JM, Fisher EMC, Tybulewicz VLJ, Yu E, Brault V. 2017. Rodent models in Down syndrome research: impact and future opportunities. *Dis Model Mech* **10**: 1165-1186.

Herbst RS, Hess KR, Tran HT, Tseng JE, Mullani NA, Charnsangavej C, Madden T, Davis DW, McConkey DJ, O'Reilly MS et al. 2002. Phase I study of recombinant human endostatin in patients with advanced solid tumors. *J Clin Oncol* **20**: 3792-3803.

Hernandez D, Mee PJ, Martin JE, Tybulewicz VL, Fisher EM. 1999. Transchromosomal mouse embryonic stem cell lines and chimeric mice that contain freely segregating segments of human chromosome 21. *Hum Mol Genet* **8**: 923-933.

Hertzberg L, Vendramini E, Ganmore I, Cazzaniga G, Schmitz M, Chalker J, Shiloh R, Iacobucci I, Shochat C, Zeligson S et al. 2010. Down syndrome acute lymphoblastic leukemia, a highly heterogeneous disease in which aberrant expression of CRLF2 is associated with mutated JAK2: a report from the International BFM Study Group. *Blood* **115**: 1006-1017.

Hsu CC, Tseng LM, Lee HC. 2016. Role of mitochondrial dysfunction in cancer progression. *Exp Biol Med (Maywood)* **241**: 1281-1295.

Iacovino M, Chong D, Szatmari I, Hartweck L, Rux D, Caprioli A, Cleaver O, Kyba M. 2011. HoxA3 is an apical regulator of haemogenic endothelium. *Nature cell biology* **13**: 72-78.

Irizarry RA, Bolstad BM, Collin F, Cope LM, Hobbs B, Speed TP. 2003. Summaries of Affymetrix GeneChip probe level data. *Nucleic acids research* **31**: e15.

Jaffredo T, Gautier R, Brajeul V, Dieterlen-Lievre F. 2000. Tracing the progeny of the aortic hemangioblast in the avian embryo. *Developmental biology* **224**: 204-214.

Jaffredo T, Gautier R, Eichmann A, Dieterlen-Lievre F. 1998. Intraaortic hemopoietic cells are derived from endothelial cells during ontogeny. *Development (Cambridge, England)* **125**: 4575-4583.

Jiang J, Jing Y, Cost GJ, Chiang JC, Kolpa HJ, Cotton AM, Carone DM, Carone BR, Shivak DA, Guschin DY et al. 2013. Translating dosage compensation to trisomy 21. *Nature* doi:10.1038/nature12394.

Jonkers I, Barakat TS, Achame EM, Monkhorst K, Kenter A, Rentmeester E, Grosveld F, Grootegoed JA, Gribnau J. 2009. RNF12 is an X-Encoded dose-dependent activator of X chromosome inactivation. *Cell* **139**: 999-1011.

Kennedy M, Awong G, Sturgeon CM, Ditadi A, LaMotte-Mohs R, Zuniga-Pflucker JC, Keller G. 2012. T lymphocyte potential marks the emergence of definitive hematopoietic progenitors in human pluripotent stem cell differentiation cultures. *Cell Rep* **2**: 1722-1735.

Kennedy M, D'Souza SL, Lynch-Kattman M, Schwantz S, Keller G. 2007. Development of the hemangioblast defines the onset of hematopoiesis in human ES cell differentiation cultures. *Blood* **109**: 2679-2687.

Khan AJ, Evans HE, Glass L, Skin YH, Almonte D. 1975. Defective neutrophil chemotaxis in patients with Down syndrome. *J Pediatr* **87**: 87-89.

Kim H, Kim JS. 2014. A guide to genome engineering with programmable nucleases. *Nat Rev Genet* **15**: 321-334.

Kimura M, Cao X, Skurnick J, Cody M, Soteropoulos P, Aviv A. 2005. Proliferation dynamics in cultured skin fibroblasts from Down syndrome subjects. *Free Radic Biol Med* **39**: 374-380.

Kissa K, Herbomel P. 2010. Blood stem cells emerge from aortic endothelium by a novel type of cell transition. *Nature* **464**: 112-115.

Klusmann JH, Godinho FJ, Heitmann K, Maroz A, Koch ML, Reinhardt D, Orkin SH, Li Z. 2010a. Developmental stage-specific interplay of GATA1 and IGF signaling in fetal megakaryopoiesis and leukemogenesis. *Genes & development* **24**: 1659-1672.

Klusmann JH, Li Z, Bohmer K, Maroz A, Koch ML, Emmrich S, Godinho FJ, Orkin SH, Reinhardt D. 2010b. miR-125b-2 is a potential oncomiR on human chromosome 21 in megakaryoblastic leukemia. *Genes & development* **24**: 478-490.

Komatsu T, Lee MC, Miyagi A, Shoji H, Yoshino F, Maehata Y, Maetani T, Kawamura Y, Ikeda M, Kubota E. 2006. Reactive oxygen species generation in gingival fibroblasts of Down syndrome patients detected by electron spin resonance spectroscopy. *Redox Rep* **11**: 71-77.

Korbel JO, Tirosh-Wagner T, Urban AE, Chen XN, Kasowski M, Dai L, Grubert F, Erdman C, Gao MC, Lange K et al. 2009. The genetic architecture of Down syndrome phenotypes revealed by high-resolution analysis of human segmental trisomies. *Proceedings of the National Academy of Sciences of the United States of America* **106**: 12031-12036.

Kuhl C, Atzberger A, Iborra F, Nieswandt B, Porcher C, Vyas P. 2005. GATA1-mediated megakaryocyte differentiation and growth control can be uncoupled and mapped to different domains in GATA1. *Molecular and cellular biology* **25**: 8592-8606.

Kusters MA, Verstegen RH, Gemen EF, de Vries E. 2009. Intrinsic defect of the immune system in children with Down syndrome: a review. *Clinical and experimental immunology* **156**: 189-193.

Laguna A, Aranda S, Barallobre MJ, Barhoum R, Fernandez E, Fotaki V, Delabar JM, de la Luna S, de la Villa P, Arbones ML. 2008. The protein kinase DYRK1A regulates caspase-9-mediated apoptosis during retina development. *Developmental cell* **15**: 841-853.

Lancrin C, Mazan M, Stefanska M, Patel R, Lichtinger M, Costa G, Vargel O, Wilson NK, Moroy T, Bonifer C et al. 2012. GFI1 and GFI1B control the loss of endothelial identity of hemogenic endothelium during hematopoietic commitment. *Blood* **120**: 314-322.

Lancrin C, Sroczynska P, Stephenson C, Allen T, Kouskoff V, Lacaud G. 2009. The haemangioblast generates haematopoietic cells through a haemogenic endothelium stage. *Nature* **457**: 892-895.

Lane AA, Chapuy B, Lin CY, Tivey T, Li H, Townsend EC, van Bodegom D, Day TA, Wu SC, Liu H et al. 2014. Triplication of a 21q22 region contributes to B cell transformation through HMGN1 overexpression and loss of histone H3 Lys27 trimethylation. *Nature genetics* **46**: 618-623.

Lavon N, Narwani K, Golan-Lev T, Buehler N, Hill D, Benvenisty N. 2008. Derivation of euploid human embryonic stem cells from aneuploid embryos. *Stem cells (Dayton, Ohio)* **26**: 1874-1882.

Lee B, Davidson BL. 2011. Gene therapy grows into young adulthood: special review issue. *Hum Mol Genet* **20**: R1.

Lee JT, Lu N. 1999. Targeted mutagenesis of Tsix leads to nonrandom X inactivation. *Cell* **99**: 47-57.

Lee JT, Strauss WM, Dausman JA, Jaenisch R. 1996. A 450 kb transgene displays properties of the mammalian X-inactivation center. *Cell* **86**: 83-94.

Lengerke C, Grauer M, Niebuhr NI, Riedt T, Kanz L, Park IH, Daley GQ. 2009. Hematopoietic development from human induced pluripotent stem cells. *Ann N Y Acad Sci* **1176**: 219-227.

Li LB, Chang KH, Wang PR, Hirata RK, Papayannopoulou T, Russell DW. 2012. Trisomy correction in Down syndrome induced pluripotent stem cells. *Cell stem cell* **11**: 615-619.

Lian X, Bao X, Al-Ahmad A, Liu J, Wu Y, Dong W, Dunn KK, Shusta EV, Palecek SP. 2014. Efficient differentiation of human pluripotent stem cells to endothelial progenitors via small-molecule activation of WNT signaling. *Stem Cell Reports* **3**: 804-816.

Liewluck T, Miravalle A. 2015. Immune-Mediated Neurological Disorders. *Curr Neurol Neurosci Rep* **15**: 61.

Lippmann ES, Azarin SM, Kay JE, Nessler RA, Wilson HK, Al-Ahmad A, Palecek SP, Shusta EV. 2012. Derivation of blood-brain barrier endothelial cells from human pluripotent stem cells. *Nature biotechnology* **30**: 783-791.

Litovchick L, Florens LA, Swanson SK, Washburn MP, DeCaprio JA. 2011. DYRK1A protein kinase promotes quiescence and senescence through DREAM complex assembly. *Genes & development* **25**: 801-813.

Lott IT. 2012. Neurological phenotypes for Down syndrome across the life span. *Prog Brain Res* **197**: 101-121.

Lu Z, Zhang QC, Lee B, Flynn RA, Smith MA, Robinson JT, Davidovich C, Gooding AR, Goodrich KJ, Mattick JS et al. 2016. RNA Duplex Map in Living Cells Reveals Higher-Order Transcriptome Structure. *Cell* **165**: 1267-1279.

Lyon MF. 1961. Gene action in the X-chromosome of the mouse (Mus musculus L.). *Nature* **190**: 372-373.

Maclean GA, Menne TF, Guo G, Sanchez DJ, Park IH, Daley GQ, Orkin SH. 2012. Altered hematopoiesis in trisomy 21 as revealed through in vitro differentiation of isogenic human pluripotent cells. *Proceedings of the National Academy of Sciences of the United States of America* **109**: 17567-17572.

Malinge S, Bliss-Moreau M, Kirsammer G, Diebold L, Chlon T, Gurbuxani S, Crispino JD. 2012. Increased dosage of the chromosome 21 ortholog Dyrk1a promotes megakaryoblastic leukemia in a murine model of Down syndrome. *J Clin Invest* **122**: 948-962.

Mateizel I, De Temmerman N, Ullmann U, Cauffman G, Sermon K, Van de Velde H, De Rycke M, Degreef E, Devroey P, Liebaers I et al. 2006. Derivation of human embryonic stem cell lines from embryos obtained after IVF and after PGD for monogenic disorders. *Hum Reprod* **21**: 503-511.

McGrath KE, Frame JM, Fromm GJ, Koniski AD, Kingsley PD, Little J, Bulger M, Palis J. 2011. A transient definitive erythroid lineage with unique regulation of the beta-globin locus in the mammalian embryo. *Blood* **117**: 4600-4608.

McHugh CA, Chen CK, Chow A, Surka CF, Tran C, McDonel P, Pandya-Jones A, Blanco M, Burghard C, Moradian A et al. 2015. The Xist lncRNA interacts directly with SHARP to silence transcription through HDAC3. *Nature* **521**: 232-236.

Megarbane A, Ravel A, Mircher C, Sturtz F, Grattau Y, Rethore MO, Delabar JM, Mobley WC. 2009. The 50th anniversary of the discovery of trisomy 21: the past, present, and future of research and treatment of Down syndrome. *Genet Med* **11**: 611-616.

Miller JC, Holmes MC, Wang J, Guschin DY, Lee YL, Rupniewski I, Beausejour CM, Waite AJ, Wang NS, Kim KA et al. 2007. An improved zinc-finger nuclease architecture for highly specific genome editing. *Nature biotechnology* **25**: 778-785.

Miyabara S, Gropp A, Winking H. 1982. Trisomy 16 in the mouse fetus associated with generalized edema and cardiovascular and urinary tract anomalies. *Teratology* **25**: 369-380.

Moehle EA, Rock JM, Lee YL, Jouvenot Y, DeKelver RC, Gregory PD, Urnov FD, Holmes MC. 2007. Targeted gene addition into a specified location in the human genome using designed zinc finger nucleases. *Proceedings of the National Academy of Sciences of the United States of America* **104**: 3055-3060.

Moindrot B, Cerase A, Coker H, Masui O, Grijzenhout A, Pintacuda G, Schermelleh L, Nesterova TB, Brockdorff N. 2015. A Pooled shRNA Screen Identifies Rbm15, Spen, and Wtap as Factors Required for Xist RNA-Mediated Silencing. *Cell Rep* **12**: 562-572.

Monfort A, Di Minin G, Postlmayr A, Freimann R, Arieti F, Thore S, Wutz A. 2015. Identification of Spen as a Crucial Factor for Xist Function through Forward Genetic Screening in Haploid Embryonic Stem Cells. *Cell Rep* **12**: 554-561.

Morawiec Z, Janik K, Kowalski M, Stetkiewicz T, Szaflik J, Morawiec-Bajda A, Sobczuk A, Blasiak J. 2008. DNA damage and repair in children with Down's syndrome. *Mutat Res* **637**: 118-123.

Mullighan CG, Collins-Underwood JR, Phillips LA, Loudin MG, Liu W, Zhang J, Ma J, Coustan-Smith E, Harvey RC, Willman CL et al. 2009. Rearrangement of CRLF2 in B-progenitor- and Down syndrome-associated acute lymphoblastic leukemia. *Nature genetics* **41**: 1243-1246.

Nakajima-Takagi Y, Osawa M, Oshima M, Takagi H, Miyagi S, Endoh M, Endo TA, Takayama N, Eto K, Toyoda T et al. 2013. Role of SOX17 in hematopoietic development from human embryonic stem cells. *Blood* **121**: 447-458.

Naldini L. 2015. Gene therapy returns to centre stage. *Nature* **526**: 351-360.

Nazor KL, Altun G, Lynch C, Tran H, Harness JV, Slavin I, Garitaonandia I, Muller FJ, Wang YC, Boscolo FS et al. 2012. Recurrent variations in DNA methylation in human pluripotent stem cells and their differentiated derivatives. *Cell stem cell* **10**: 620-634.

Nikolaev SI, Garieri M, Santoni F, Falconnet E, Ribaux P, Guipponi M, Murray A, Groet J, Giarin E, Basso G et al. 2014. Frequent cases of RAS-mutated Down syndrome acute lymphoblastic leukaemia lack JAK2 mutations. *Nat Commun* **5**: 4654.

Nikolaev SI, Santoni F, Vannier A, Falconnet E, Giarin E, Basso G, Hoischen A, Veltman JA, Groet J, Nizetic D et al. 2013. Exome sequencing identifies putative

drivers of progression of transient myeloproliferative disorder to AMKL in infants with Down syndrome. *Blood* **122**: 554-561.

Nizetic D, Groet J. 2012. Tumorigenesis in Down's syndrome: big lessons from a small chromosome. *Nature reviews Cancer* **12**: 721-732.

North T, Gu TL, Stacy T, Wang Q, Howard L, Binder M, Marin-Padilla M, Speck NA. 1999. Cbfa2 is required for the formation of intra-aortic hematopoietic clusters. *Development (Cambridge, England)* **126**: 2563-2575.

Nostro MC, Cheng X, Keller GM, Gadue P. 2008. Wnt, activin, and BMP signaling regulate distinct stages in the developmental pathway from embryonic stem cells to blood. *Cell stem cell* **2**: 60-71.

O'Connell RM, Chaudhuri AA, Rao DS, Gibson WS, Balazs AB, Baltimore D. 2010. MicroRNAs enriched in hematopoietic stem cells differentially regulate long-term hematopoietic output. *Proceedings of the National Academy of Sciences of the United States of America* **107**: 14235-14240.

O'Doherty A, Ruf S, Mulligan C, Hildreth V, Errington ML, Cooke S, Sesay A, Modino S, Vanes L, Hernandez D et al. 2005. An aneuploid mouse strain carrying human chromosome 21 with Down syndrome phenotypes. *Science* **309**: 2033-2037.

Ohno S, Kaplan WD, Kinosita R. 1959. Formation of the sex chromatin by a single X-chromosome in liver cells of Rattus norvegicus. *Experimental cell research* **18**: 415-418.

Okuda T, van Deursen J, Hiebert SW, Grosveld G, Downing JR. 1996. AML1, the target of multiple chromosomal translocations in human leukemia, is essential for normal fetal liver hematopoiesis. *Cell* **84**: 321-330.

Orkin SH, Zon LI. 2008. Hematopoiesis: an evolving paradigm for stem cell biology. *Cell* **132**: 631-644.

Oromendia AB, Dodgson SE, Amon A. 2012. Aneuploidy causes proteotoxic stress in yeast. *Genes & development* **26**: 2696-2708.

Ottersbach K, Dzierzak E. 2005. The murine placenta contains hematopoietic stem cells within the vascular labyrinth region. *Developmental cell* **8**: 377-387.

Paik EJ, Zon LI. 2010. Hematopoietic development in the zebrafish. *Int J Dev Biol* **54**: 1127-1137.

Palis J, Yoder MC. 2001. Yolk-sac hematopoiesis: the first blood cells of mouse and man. *Experimental hematology* **29**: 927-936.

Park IH, Arora N, Huo H, Maherali N, Ahfeldt T, Shimamura A, Lensch MW, Cowan C, Hochedlinger K, Daley GQ. 2008. Disease-specific induced pluripotent stem cells. *Cell* **134**: 877-886.

Park J, Oh Y, Yoo L, Jung MS, Song WJ, Lee SH, Seo H, Chung KC. 2010. Dyrk1A phosphorylates p53 and inhibits proliferation of embryonic neuronal cells. *J Biol Chem* **285**: 31895-31906.

Patil DP, Chen CK, Pickering BF, Chow A, Jackson C, Guttman M, Jaffrey SR. 2016. m(6)A RNA methylation promotes XIST-mediated transcriptional repression. *Nature* **537**: 369-373.

Polani PE, Adinolfi M. 1980. Chromosome 21 of man, 22 of the great apes and 16 of the mouse. *Dev Med Child Neurol* **22**: 223-225.

Prada N, Nasi M, Troiano L, Roat E, Pinti M, Nemes E, Lugli E, Ferraresi R, Ciacci L, Bertoni D et al. 2005. Direct analysis of thymic function in children with Down's syndrome. *Immun Ageing* **2**: 4.

Prandini P, Deutsch S, Lyle R, Gagnebin M, Delucinge Vivier C, Delorenzi M, Gehrig C, Descombes P, Sherman S, Dagna Bricarelli F et al. 2007. Natural gene-expression variation in Down syndrome modulates the outcome of gene-dosage imbalance. *Am J Hum Genet* **81**: 252-263.

Ram G, Chinen J. 2011. Infections and immunodeficiency in Down syndrome. *Clinical and experimental immunology* **164**: 9-16.

Reynolds LE, Watson AR, Baker M, Jones TA, D'Amico G, Robinson SD, Joffre C, Garrido-Urbani S, Rodriguez-Manzaneque JC, Martino-Echarri E et al. 2010. Tumour angiogenesis is reduced in the Tc1 mouse model of Down's syndrome. *Nature* **465**: 813-817.

Rhodes J, Hagen A, Hsu K, Deng M, Liu TX, Look AT, Kanki JP. 2005. Interplay of pu.1 and gata1 determines myelo-erythroid progenitor cell fate in zebrafish. *Developmental cell* **8**: 97-108.

Rhodes KE, Gekas C, Wang Y, Lux CT, Francis CS, Chan DN, Conway S, Orkin SH, Yoder MC, Mikkola HK. 2008. The emergence of hematopoietic stem cells is initiated in the placental vasculature in the absence of circulation. *Cell stem cell* **2**: 252-263.

Roberts I, Alford K, Hall G, Juban G, Richmond H, Norton A, Vallance G, Perkins K, Marchi E, McGowan S et al. 2013. GATA1-mutant clones are frequent and often unsuspected in babies with Down syndrome: identification of a population at risk of leukemia. *Blood* **122**: 3908-3917.

Roizen NJ, Amarose AP. 1993. Hematologic abnormalities in children with Down syndrome. *Am J Med Genet* **46**: 510-512.

Roy A, Cowan G, Mead AJ, Filippi S, Bohn G, Chaidos A, Tunstall O, Chan JK, Choolani M, Bennett P et al. 2012. Perturbation of fetal liver hematopoietic stem and progenitor cell development by trisomy 21. *Proceedings of the National Academy of Sciences of the United States of America* **109**: 17579-17584.

Roy A, Roberts I, Norton A, Vyas P. 2009. Acute megakaryoblastic leukaemia (AMKL) and transient myeloproliferative disorder (TMD) in Down syndrome: a multi-step model of myeloid leukaemogenesis. *British journal of haematology* **147**: 3-12.

Sailani MR, Makrythanasis P, Valsesia A, Santoni FA, Deutsch S, Popadin K, Borel C, Migliavacca E, Sharp AJ, Duriaux Sail G et al. 2013. The complex SNP and CNV genetic architecture of the increased risk of congenital heart defects in Down syndrome. *Genome research* **23**: 1410-1421.

Salehi A, Delcroix JD, Belichenko PV, Zhan K, Wu C, Valletta JS, Takimoto-Kimura R, Kleschevnikov AM, Sambamurti K, Chung PP et al. 2006. Increased App expression in a mouse model of Down's syndrome disrupts NGF transport and causes cholinergic neuron degeneration. *Neuron* **51**: 29-42.

Sasaki K, Yagi H, Bronson RT, Tominaga K, Matsunashi T, Deguchi K, Tani Y, Kishimoto T, Komori T. 1996. Absence of fetal liver hematopoiesis in mice deficient in transcriptional coactivator core binding factor beta. *Proceedings of the National Academy of Sciences of the United States of America* **93**: 12359-12363.

Savarese F, Flahndorfer K, Jaenisch R, Busslinger M, Wutz A. 2006. Hematopoietic precursor cells transiently reestablish permissiveness for X inactivation. *Molecular and cellular biology* **26**: 7167-7177.

Savva GM, Morris JK, Mutton DE, Alberman E. 2006. Maternal age-specific fetal loss rates in Down syndrome pregnancies. *Prenatal diagnosis* **26**: 499-504.

Scott EW, Simon MC, Anastasi J, Singh H. 1994. Requirement of transcription factor PU.1 in the development of multiple hematopoietic lineages. *Science* **265**: 1573-1577.

Sheltzer JM, Ko JH, Replogle JM, Habibe Burgos NC, Chung ES, Meehl CM, Sayles NM, Passerini V, Storchova Z, Amon A. 2017. Single-chromosome Gains Commonly Function as Tumor Suppressors. *Cancer Cell* **31**: 240-255.

Shi Y, Downes M, Xie W, Kao HY, Ordentlich P, Tsai CC, Hon M, Evans RM. 2001. Sharp, an inducible cofactor that integrates nuclear receptor repression and activation. *Genes & development* **15**: 1140-1151.

Shi Y, Kirwan P, Smith J, MacLean G, Orkin SH, Livesey FJ. 2012. A human stem cell model of early Alzheimer's disease pathology in Down syndrome. *Sci Transl Med* **4**: 124ra129.

Sleegers K, Brouwers N, Gijselinck I, Theuns J, Goossens D, Wauters J, Del-Favero J, Cruts M, van Duijn CM, Van Broeckhoven C. 2006. APP duplication is sufficient to cause early onset Alzheimer's dementia with cerebral amyloid angiopathy. *Brain* **129**: 2977-2983.

Souroullas GP, Sharpless NE. 2013. Stem cells: Down's syndrome link to ageing. *Nature* **501**: 325-326.

Sturgeon CM, Ditadi A, Awong G, Kennedy M, Keller G. 2014. Wnt signaling controls the specification of definitive and primitive hematopoiesis from human pluripotent stem cells. *Nature biotechnology* **32**: 554-561.

Sugimura R, Jha DK, Han A, Soria-Valles C, da Rocha EL, Lu YF, Goettel JA, Serrao E, Rowe RG, Malleshaiah M et al. 2017. Haematopoietic stem and progenitor cells from human pluripotent stem cells. *Nature* **545**: 432-438.

Sullivan KD, Lewis HC, Hill AA, Pandey A, Jackson LP, Cabral JM, Smith KP, Liggett LA, Gomez EB, Galbraith MD et al. 2016. Trisomy 21 consistently activates the interferon response. *Elife* **5**.

Sussan TE, Yang A, Li F, Ostrowski MC, Reeves RH. 2008. Trisomy represses Apc(Min)-mediated tumours in mouse models of Down's syndrome. *Nature* **451**: 73-75.

Swiers G, Rode C, Azzoni E, de Bruijn MF. 2013. A short history of hemogenic endothelium. *Blood cells, molecules & diseases* **51**: 206-212.

Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S. 2007. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* **131**: 861-872.

Thomas JP, Arzoomanian RZ, Alberti D, Marnocha R, Lee F, Friedl A, Tutsch K, Dresen A, Geiger P, Pluda J et al. 2003. Phase I pharmacokinetic and pharmacodynamic study of recombinant human endostatin in patients with advanced solid tumors. *J Clin Oncol* **21**: 223-231.

Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM. 1998. Embryonic stem cell lines derived from human blastocysts. *Science* **282**: 1145-1147.

Tian D, Sun S, Lee JT. 2010. The long noncoding RNA, Jpx, is a molecular switch for X chromosome inactivation. *Cell* **143**: 390-403.

Tunstall-Pedoe O, Roy A, Karadimitris A, de la Fuente J, Fisk NM, Bennett P, Norton A, Vyas P, Roberts I. 2008. Abnormalities in the myeloid progenitor compartment in Down syndrome fetal liver precede acquisition of GATA1 mutations. *Blood* **112**: 4507-4511.

Urbach A, Benvenisty N. 2009. Studying early lethality of 45,XO (Turner's syndrome) embryos using human embryonic stem cells. *PloS one* **4**: e4175.

Urbach A, Schuldiner M, Benvenisty N. 2004. Modeling for Lesch-Nyhan disease by gene targeting in human embryonic stem cells. *Stem cells (Dayton, Ohio)* **22**: 635-641.

Urnov FD, Miller JC, Lee YL, Beausejour CM, Rock JM, Augustus S, Jamieson AC, Porteus MH, Gregory PD, Holmes MC. 2005. Highly efficient endogenous human gene correction using designed zinc-finger nucleases. *Nature* **435**: 646-651.

Urnov FD, Rebar EJ, Holmes MC, Zhang HS, Gregory PD. 2010. Genome editing with engineered zinc finger nucleases. *Nat Rev Genet* **11**: 636-646.

Vagnucci AH, Jr., Li WW. 2003. Alzheimer's disease and angiogenesis. *Lancet* **361**: 605-608.

Vodyanik MA, Thomson JA, Slukvin, II. 2006. Leukosialin (CD43) defines hematopoietic progenitors in human embryonic stem cell differentiation cultures. *Blood* **108**: 2095-2105.

Wahlster L, Daley GQ. 2016. Progress towards generation of human haematopoietic stem cells. *Nature cell biology* **18**: 1111-1117.

Wang Q, Stacy T, Binder M, Marin-Padilla M, Sharpe AH, Speck NA. 1996. Disruption of the Cbfa2 gene causes necrosis and hemorrhaging in the central nervous system and blocks definitive hematopoiesis. *Proceedings of the National Academy of Sciences of the United States of America* **93**: 3444-3449.

Weber M, Hellmann I, Stadler MB, Ramos L, Paabo S, Rebhan M, Schubeler D. 2007. Distribution, silencing potential and evolutionary impact of promoter DNA methylation in the human genome. *Nature genetics* **39**: 457-466.

Wechsler J, Greene M, McDevitt MA, Anastasi J, Karp JE, Le Beau MM, Crispino JD. 2002. Acquired mutations in GATA1 in the megakaryoblastic leukemia of Down syndrome. *Nature genetics* **32**: 148-152.

Welshons WJ, Russell LB. 1959. The Y-Chromosome as the Bearer of Male Determining Factors in the Mouse. *Proceedings of the National Academy of Sciences of the United States of America* **45**: 560-566.

Wiseman FK, Al-Janabi T, Hardy J, Karmiloff-Smith A, Nizetic D, Tybulewicz VL, Fisher EM, Strydom A. 2015. A genetic cause of Alzheimer disease: mechanistic insights from Down syndrome. *Nat Rev Neurosci* **16**: 564-574.

Wutz A, Rasmussen TP, Jaenisch R. 2002. Chromosomal silencing and localization are mediated by different domains of Xist RNA. *Nature genetics* **30**: 167-174.

Xu D, Dwyer J, Li H, Duan W, Liu JP. 2008. Ets2 maintains hTERT gene expression and breast cancer cell proliferation by interacting with c-Myc. *J Biol Chem* **283**: 23567-23580.

Xu W, Weissmiller AM, White JA, 2nd, Fang F, Wang X, Wu Y, Pearn ML, Zhao X, Sawa M, Chen S et al. 2016. Amyloid precursor protein-mediated endocytic pathway disruption induces axonal dysfunction and neurodegeneration. *J Clin Invest* **126**: 1815-1833.

Yang A, Reeves RH. 2011. Increased survival following tumorigenesis in Ts65Dn mice that model Down syndrome. *Cancer research* **71**: 3573-3581.

Yang Q, Rasmussen SA, Friedman JM. 2002. Mortality associated with Down's syndrome in the USA from 1983 to 1997: a population-based study. *Lancet* **359**: 1019-1025.

Yang ZF, Mott S, Rosmarin AG. 2007. The Ets transcription factor GABP is required for cell-cycle progression. *Nature cell biology* **9**: 339-346.

Yoshida K, Toki T, Okuno Y, Kanezaki R, Shiraishi Y, Sato-Otsubo A, Sanada M, Park MJ, Terui K, Suzuki H et al. 2013. The landscape of somatic mutations in Down syndrome-related myeloid disorders. *Nature genetics* **45**: 1293-1299.

You SH, Lim HW, Sun Z, Broache M, Won KJ, Lazar MA. 2013. Nuclear receptor co-repressors are required for the histone-deacetylase activity of HDAC3 in vivo. *Nat Struct Mol Biol* **20**: 182-187.

Yu T, Li Z, Jia Z, Clapcote SJ, Liu C, Li S, Asrar S, Pao A, Chen R, Fan N et al. 2010. A mouse model of Down syndrome trisomic for all human chromosome 21 syntenic regions. *Hum Mol Genet* **19**: 2780-2791.

Zana M, Szecsenyi A, Czibula A, Bjelik A, Juhasz A, Rimanoczy A, Szabo K, Vetro A, Szucs P, Varkonyi A et al. 2006. Age-dependent oxidative stress-induced DNA damage in Down's lymphocytes. *Biochemical and biophysical research communications* **345**: 726-733.

Zhang CC, Lodish HF. 2004. Insulin-like growth factor 2 expressed in a novel fetal liver cell population is a growth factor for hematopoietic stem cells. *Blood* **103**: 2513-2521.

Zhu L, Zhong M, Zhao J, Rhee H, Caesar I, Knight EM, Volpicelli-Daley L, Bustos V, Netzer W, Liu L et al. 2013. Reduction of synaptojanin 1 accelerates Abeta clearance and attenuates cognitive deterioration in an Alzheimer mouse model. *J Biol Chem* **288**: 32050-32063.

Zorick TS, Mustacchi Z, Bando SY, Zatz M, Moreira-Filho CA, Olsen B, Passos-Bueno MR. 2001. High serum endostatin levels in Down syndrome: implications for improved treatment and prevention of solid tumours. *Eur J Hum Genet* **9**: 811-814.

Zovein AC, Hofmann JJ, Lynch M, French WJ, Turlo KA, Yang Y, Becker MS, Zanetta L, Dejana E, Gasson JC et al. 2008. Fate tracing reveals the endothelial origin of hematopoietic stem cells. *Cell stem cell* **3**: 625-636.