PRIMER

Analyzing the effects of the insertion of an XIST Transgene in a primer explaining "Translating Dosage Compensation to Trisomy 21"

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ABSTRACT

Down Syndrome, also known as **Trisomy 21**, affects many worldwide. In fact, 1 in every 700 babies is born with it. This study, "Translating dosage compensation to trisomy 21," investigated what would happen to gene expression when **X-inactive specific transcript (XIST)** was inserted into Chromosome 21 (Jiang et al., 2013). Naturally, XIST is used in the body for the inactivation of one of the X chromosomes in females. It works by forming a Barr body to prevent overexpression of X-linked genes. The insertion of XIST demonstrated accurate addition to both Chromosome 19 and 21. When added to an extra Chromosome 21 in a cell line, XIST triggered **heterochromatin** modifications and DNA methylation to form a Barr body. A Barr body is a region that forms on a chromosome that stops gene expression. The insertion of XIST into Chromosome 21 also successfully silenced the expression from the XIST-carrying chromosome. This showed that the insertion of XIST could be used for future research on Down Syndrome and for research on other trisomy diseases to lessen potential effects of the diseases.

Key words: Down Syndrome, XIST, Barr body, Chromosome 21

Down Syndrome: disorder arising from an extra copy of chromosome 21

Trisomy 21: presence of all or part of a third copy of chromosome 21

XIST: X-inactive specific transcript, an RNA gene on the X-chromosome that acts in the natural process of X-inactivation

Heterochromatin: chromosome material that affects the activity of genes by modifying them and suppressing their activity

INTRODUCTION

Down Syndrome is a genetic disorder in humans caused by the presence of a third copy of Chromosome 21. It is the leading genetic cause of intellectual disabilities. In the United States, about half of the live births affected by trisomy are due to Down Syndrome. The three main types of Down Syndrome are normal, translocation, and mosaic. Normal Down Syndrome, which is what this primer focuses on, is when there is an extra copy of Chromosome 21, creating a trisomy. Translocation Down Syndrome is when a part of Chromosome 21 attaches to another chromosome during the formation of reproductive cells. Finally, Mosaic Down Syndrome is when the affected have an extra copy of Chromosome 21 in some of the body's cells, but not all.

Some of the babies affected with trisomy are stillborn or are spontaneously aborted early in the pregnancy. Others that survive until term commonly have birth defects. Most Down Syndrome babies are born with a low birth weight, a congenital heart defect, and physical abnormalities. People affected with Down Syndrome are also more susceptible to hearing loss, sleep apnea, ear infections, eye diseases, intestinal blockages, and anemia (Korenberg, et. al 1994). People with Down Syndrome may also have physical growth delays, characteristic facial features, a higher risk of developing respiratory problems, hearing difficulties, and leukemia.

As of now, there is no cure for Down Syndrome but there are treatments to lessen the associated health effects (Incerti et al., 2011). Treatments for Down Syndrome are based on the individual and their symptoms. Affected children are likely to receive care from a number of health professionals such as physicians, special educators, and social workers.

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For instance, most will participate in physical therapy to build motor skills and increase muscle strength, speech-language therapy to improve communication skills, occupational therapy to adjust their everyday tasks to fit their needs, and emotional or behavioral therapies such as coping with mental health issues and behaviors.

The paper by Jiang et al. (2013), which is the focus of this primer, tested whether **X-inactivation** can be used in a novel way to cure Down Syndrome. X-Chromosome inactivation is the silencing of one of the two X-chromosomes in female zygotes. This is done to decrease the dosage of the X-chromosome in the cells before most development begins. The process of **dosage compensation** naturally happens in all reproductive cells but if it malfunctions, **X-aneuploidy** occurs, which can cause genetic imbalances. In the process of inactivation, XIST silences the X-chromosome to establish facultative heterochromatin, also called a Barr body (Sahakyan, et. al 2018). The Barr body created is what disallows any expression from the second X-chromosome, stopping X-aneuploidies from occurring and adversely affecting the health of the individual.

The study uses transgenic XIST, which is a **transgene**, to silence the trisomy in Chromosome 21 in **induced pluripotent stem cells (iPSCs)** (Jiang et al., 2013). This transgene was modified to target the DYRK1A locus on Chromosome 21. It targeted this specific locus because it is the region that controls the phenotypic effects of Down Syndrome. The transgene contained XIST DNA and a **promotor** region that uses **doxycycline** (dox) which was included to induce the expression of the XIST. When XIST is inserted, it should become expressed once the cells are exposed to dox, therefore creating a Barr body and erasing the increased gene dosage of the extra chromosome, stopping any effects of Down Syndrome. To measure gene expression, a test was done across the XIST-targeted region. This test showed that the area where the Barr body was located, had no gene expression, illustrating an inactive Chromosome 21. This study, conducted by Jiang, et al., was concluded to be a success based on the fact that it showed that XIST can be used to study human chromosome silencing, and that trisomy silencing can be used to further study Down Syndrome, and therefore, should be brought into consideration for future gene therapy research (Jiang, et. al 2013).

X-inactivation: one X-chromosome is inactivated in an early embryonic cell

Dosage Compensation: mechanism by which species ensure that too much of one chromosome is not expressed

X-aneuploidy: presence of extra chromosomes in a cell

Transgene: a gene that is artificial or is from one organism inserted into a new organism.

Induced pluripotent stem cells (iPSCs): cells that can differentiate into any cell of the body

Promotor: sequence that sets where transcription starts

Doxycycline (Dox): broad-spectrum antibiotic that can control the tetracycline transactivator

METHODS

Cell Culture

Specific cells were needed to carry out this study. To model Down Syndrome, induced pluripotent stem cells (iPSCs) were used from a specific primary fibroblast line. iPSCs are mature differentiated cells that have been genetically altered so that they have the potential to develop into all types of cells in the body. The specific cells used had **pluripotency** markers and Trisomy 21. The cells were maintained by mouse irradiated embryonic fibroblasts, which are feeder cells that support iPSCs in culture. These feeder cells are present as a layer that provide extracellular secretions to help the iPSCs proliferate. The feeder cells are also irradiated so they cannot divide and were chosen for use because they are very common cells (Llames et al., 2015).

XIST Transgene

The transgene that was inserted into chromosome 21 consisted of DNA that contained the XIST coding sequence. The transgene contained a tetracycline controlled transactivator (rTA) that enabled the use of dox to activate XIST expression (Loew et al., 2010).

Insertion of XIST into trisomy

Zinc Finger Nucleases (ZFNs) are DNA cleavage enzymes that can be designed to target a specific part of the genome and cause a double stranded break (Carroll, 2011). In this case, Jiang et al. (2013) used the ZFNs to cause a double stranded break that would incorporate the XIST transgene as it was repaired.

First, the ZFNs were used to target the Adeno-Associated Virus Integration Site 1 (AAVS1) locus on Chromosome 19 and insert a XIST transgene. This approach was used because AAVS1 is easy to target for genetic engineering without affecting the growth of the cell, therefore working as a control for this experiment (Hendriks, et. al 2016). Once this was successful, ZFNs were made to target the DYRK1A locus on Chromosome 21, where they also inserted the XIST transgene. The DYRK1A locus was targeted because it is considered a Down Syndrome critical region that is responsible for phenotypic features that are pronounced in Down Syndrome (Bon et al., 2011).

The addition of XIST to Chromosome 21 triggered chromosome-wide transcriptional silencing. Inducible silencing of the trisomy *in vitro* was used to compare clones. The clones were descendants from a line of iPSCs that were engineered using ZFNs to carry the XIST transgene. The use of the clones *in vitro* allowed clearer differences to be seen due to variation in expression levels.

Fluorescent In Situ Hybridization (FISH): a laboratory technique that can be used to examine genes or chromosomes

Identifying a Barr body

To then identify if XIST RNA induced a Barr body in the pluripotent stem cells, several types of assay were carried out simultaneously. **Fluorescent in situ hybridization (FISH)** was used to identify the location of Chromosome 21 genes, immunofluorescence staining was used to detect proteins associated with heterochromatin, and an XIST probe was used to detect XIST RNA.

Disomic cells: cells containing two copies of a chromosome; normal cells

Trisomic cells: cells containing three copies of a chromosome; X-aneuploidy

Microarray analysis

Disomic and **trisomic** cells were treated with dox for 22 days to compare gene expression at the DYRK1A locus on Chromosome 21. After being treated, the RNA was extracted, processed, and hybridized to human gene expression arrays. This system allowed for comparison of the same cells grown in identical cultures, with or without XIST silencing. A 33% reduction in expression levels per cell was predicted when one Chromosome 21 was silenced compared to when all three chromosomes were fully expressed.

DNA methylation analysis

Another way epigenetic change was measured was through DNA methylation, because hypermethylation of CpG islands in gene promotors stabilizes gene inactivation by XIST. CpG sites are regions of DNA where a cytosine nucleotide has a guanine nucleotide after it. When this pattern occurs in high frequency, they are called CpG islands and can be methylated to change expression. To begin, the cell lines were grown with and without dox for 22 days. The DNA was then extracted and combined with bisulfite, which aids in sequencing the DNA and identifying methylated sites (Li and Tollefsbol, 2011). The bisulfite DNA was then amplified, fragmented, and hybridized following standard protocols.

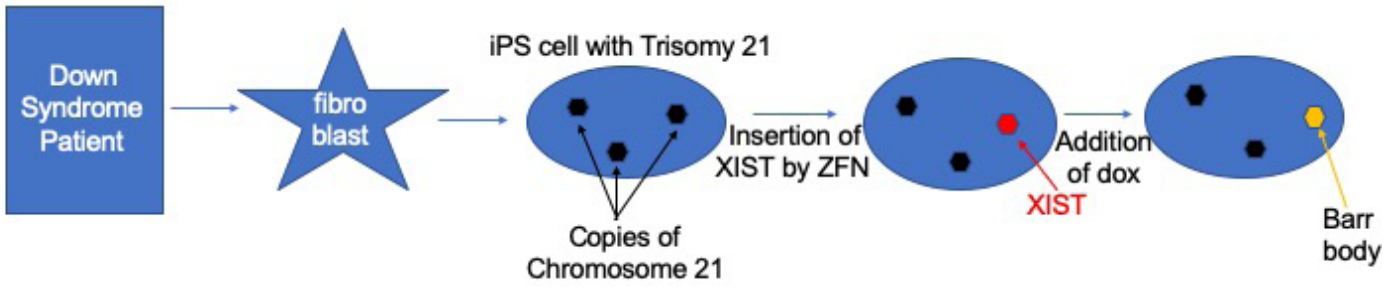

Figure 1. Process of the Insertion of the XIST transgene into iPSCs and activation of the transgene. Flow chart depicting the insertion of XIST into one of three copies of Chromosome 21. This results in the XIST-containing chromosome forming a Barr body. Figure based on Jiang, et al., 2013.

RESULTS

Summary of Main Findings

The overall purpose of this study was to transcriptionally silence one of the copies of the trisomy on Chromosome 21 by using a XIST transgene (Figure 1) (Jiang et al., 2013). Zinc Finger Nucleases were successfully used to insert a XIST transgene into the Chromosome 21 DYRK1A locus to induce the formation of a Barr body. The Barr body was formed by a series of heterochromatin modifications induced by XIST.

Transgene Components

pTRE3G Promotor: a promoter that is used to incorporate the tetracycline controlled activation of a transgene

The first objective of this study was to design a transgene that could induce expression of XIST. The transgene sequence consisted of 5' and 3' arms, pA region, the 14 kb XIST sequence, and **pTRE3G promotor** region (Figure 2). The locus was targeted by creating a double stranded break in the DNA where the transgene was then inserted. The XIST is inducible by dox due to the presence of the pTRE3G promotor which functions to turn on the expression of the XIST RNA in the transgene. In the absence of dox, the XIST gene will be turned off; in the presence of dox, the XIST RNA is expressed.

Insertion into Chromosome 19 and 21

The second objective of this study was to show that Zinc Finger Nucleases could be used to insert the XIST transgene into genomic DNA. First, they inserted a known transgene into the AAVS1 locus on Chromosome 19 as a test trial. The AAVS1 locus was used because the locus does not specifically affect the growth of the cell, thereby making it a good test site. With the help of ZFNs, the transgene demonstrated accurate addition.

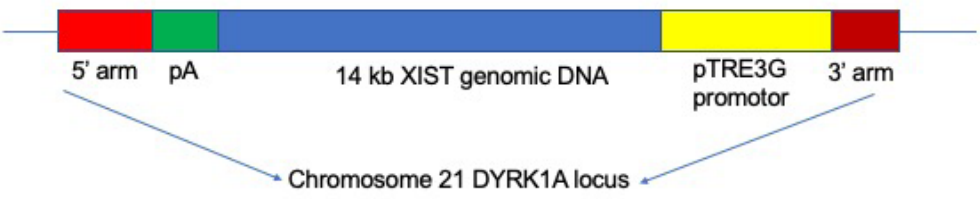

Figure 2. The transgene used in the XIST insertion. Illustration depicting the transgene that was inserted into Chromosome 21. Consists of a 5' arm, pA region, the XIST DNA encoding the XIST RNA needed to induce the formation of the Barr body, a promotor region containing a doxycycline response element which is used to turn the XIST gene on, and a 3' arm. Figure based on Jiang, et al., 2013.

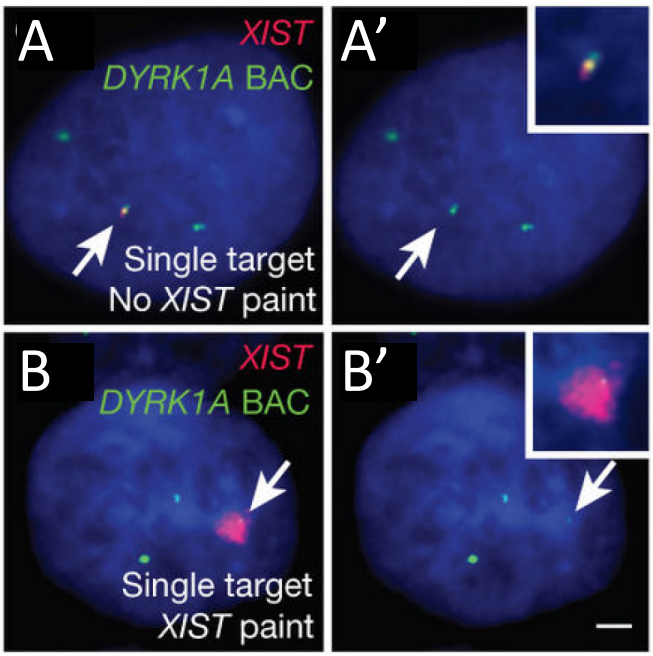

Figure 3. Image showing three genes targeted for XIST addition. A and B are the different clones with the same labeling patterns. **(A)** Shows three copies of the DYRK1A gene, two without without XIST (green) and one with an XIST insertion (red). **(A')** Higher magnification of a gene with XIST showing the overlap between the DYRK1A gene and XIST, producing a yellow color (red + green). **(B)** Shows three genes present with accurate addition of XIST to DYRK1A locus on one gene. **(B')** Up close visual of XIST addition. Scale bar: 2 μm. Reprinted by permission from Springer Naturre, Jiang et al., 2013.

Next, they modified the transgene to a sequence that was needed for their study, the XIST-containing transgene described above (Figure 2), which was triple the size of what had been used previously for other applications. This transgene also demonstrated accurate addition to the AAVS1 locus on Chromosome 19.

After both successful trials, they made several changes to their experimental data. First, they used a specific line of iPSCs with pluripotency markers and Trisomy 21. Pluripotency is the ability of the cell to differentiate into any cell type, so the pluripotency markers on the iPSCs illustrate this ability. Second, they used a ZFN that would target the DYRK1A locus on Chromosome 21. After these modifications, it was shown that the insertion of the transgene into the DYRK1A locus on Chromosome 21 demonstrated accurate addition (Figure 3).

Formation of Barr body

When the transgene containing XIST was inserted, Jiang et al. (2013) found evidence of Barr body formation. Five days after insertion, the Chromosome 21 carrying the XIST transgene became covered in heterochromatin marks, indicating a Barr body was present. To illustrate this, FISH was combined with immunofluorescence staining and used a probe to locate proteins, mRNA, and epigenetic marks which indicated the Barr body formation (Figure 4). This was tested in multiple clones with each trisomic Chromosome 21 containing the transgene. The clones each had condensed nuclei and histones that are specific for heterochromatin formation, further suggesting the formation of the Barr body.

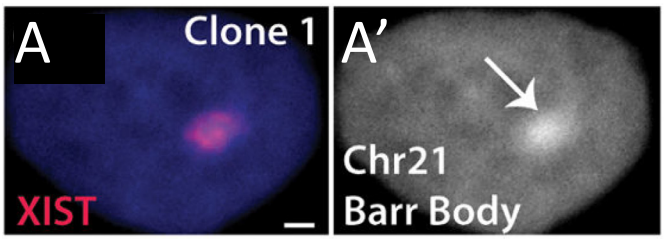

Figure 4. Barr body formation. (A) Formation of Barr body upon addition of XIST (red stain). **(A')** Shows localized area of XIST (white arrow) within the Barr body formed. Reprinted by permission from Springer Naturre, Jiang et al., 2013. Scale bar: 2 μm.

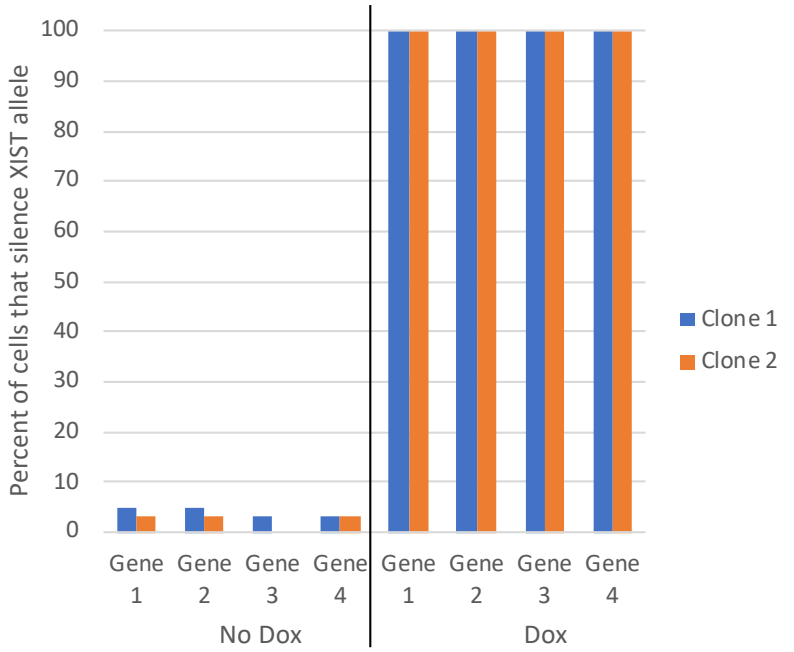

Figure 5. Effects of gene expression due to the presence of XIST. Graph depicting two clones and their percentage of genes (USP25, CXADR, ITSN1, COL18A1) that are silenced by XIST with and without doxycycline treatment. This was measured by FISH. Reprinted by permission from Springer Naturre, Jiang et al., 2013.

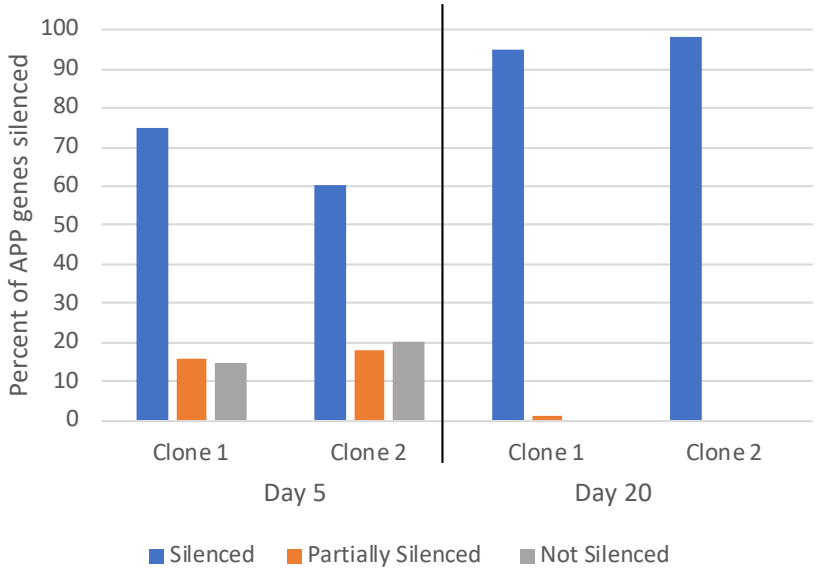

Figure 6. Effects on gene expression caused by XIST. Graph depicting two clones of iPSCs showing the percent of their descendent cells that had genes that were silenced by XIST. Reprinted by permission from Springer Naturre, Jiang et al., 2013.

A localized XIST-containing area was seen in over 85% of the cells examined. After five days, Barr body formation was seen in 90-100% of the cells examined (Figure 5). FISH was also used to identify the presence of transcription foci. With XIST expression, the foci are darker, illustrating that the allele became weaker or undetectable. After 20 days, complete silencing of the allele on Chromosome 21 was observed in all of the cells with accumulating XIST RNA.

Formation of a Barr Body induces the silencing of gene expression. Once the transgene was inserted and the presence of a Barr body was confirmed, the amount of gene expression was examined (Figure 6). This was done using **microarray analysis** and DNA methylation analysis. Microarray analysis was used to compare gene silencing between disomic and trisomic cells by treating the cells with dox. DNA methylation analysis was done on the cells grown with and without dox. The genomes of these cells were then sequenced to determine the presence of methylated CpG sites. More CpG sites indicated less expression of the trisomy on Chromosome 21.

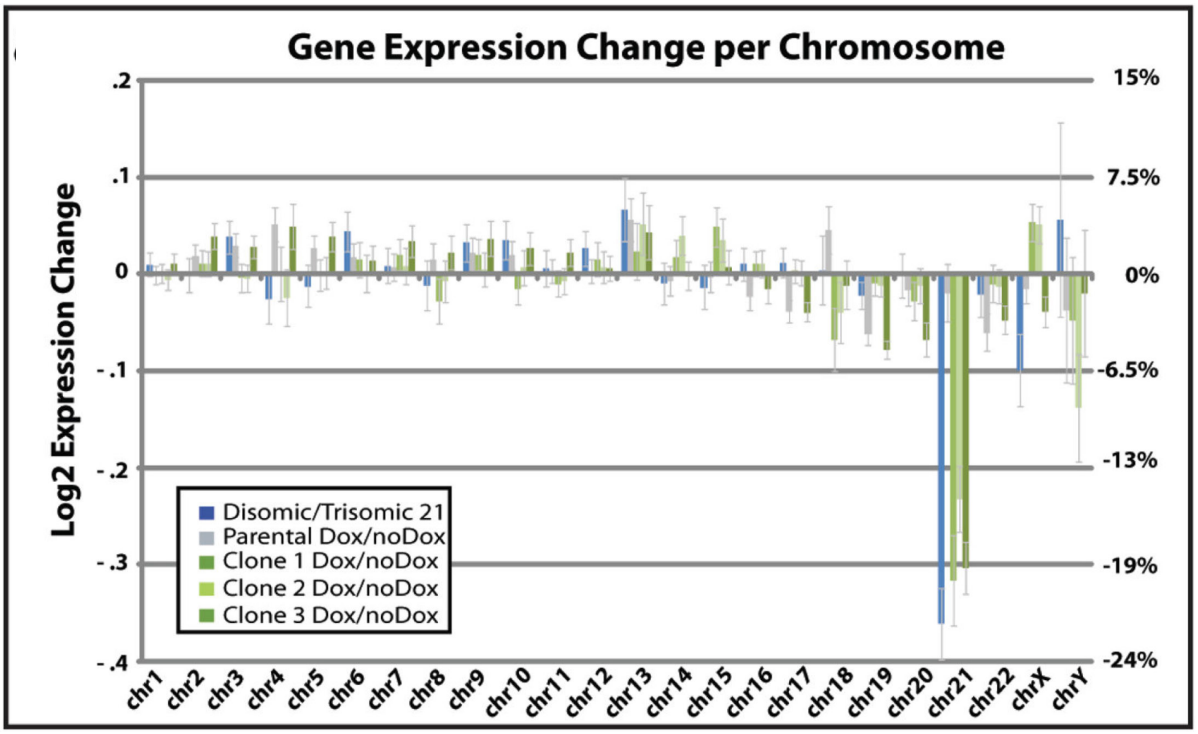

Figure 7. **Decline in gene expression levels dependent on the presence of dox.** Comparisons of gene expression in two clones with and without dox and between disomic and trisomic cells. Gene expression levels were measured using Microarray analysis. Reprinted by permission from Springer Naturre, Jiang et al., 2013.

Microarray Analysis: method that uses probes to detect the presence of genes

Microarray analysis was used to compare expression levels among cells. Use of this analysis showed that XIST-mediated dosage compensation corrected the expression of the extra Chromosome 21 to close to disomic levels. After XIST was inserted, expression of Chromosome 21 was reduced by about 20% in trisomic cells, similar to 22% in disomic cells without the trisomy. It also illustrated that more than 200 genes changed their expression levels upon addition of the transgene. As depicted in Figure 7, Chromosome 21 displayed the greatest change in expression due to the XIST inserted into the DYRK1A locus.

The insertion of XIST caused X-inactivation which was stabilized by the hypermethylation of CpG islands. Figure 8 shows the difference in methylation between Chromosome 21, which had the XIST transgene, Chromosome 22, and the X-chromosome,

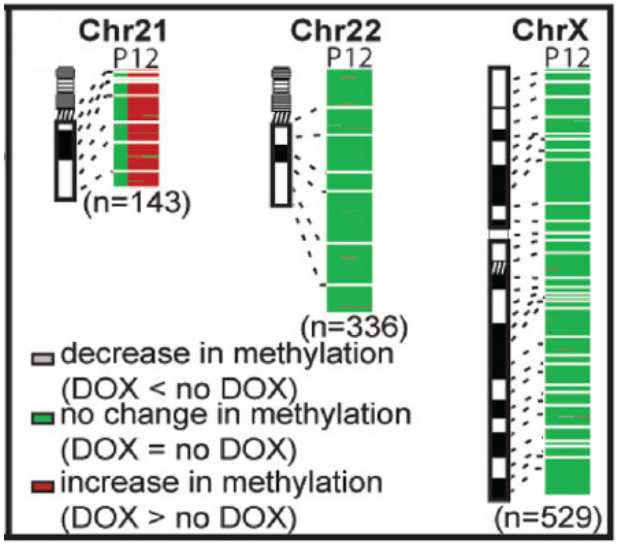

Figure 8. Image depicting the presence of methylated sites on each chromosome. Illustration showing the change in methylated CpG sites. The red coloring on Chromosome 21 indicates the increase in methylation compared to no change on Chromosome 22 and the X-chromosome. Reprinted by permission from Springer Naturre, Jiang et al., 2013.

which had not been modified. 97% of CpG island genes demonstrated an increase in DNA methylation across the entire Chromosome 21, indicating gene silencing of the Chromosome 21 trisomy.

DISCUSSION

This study, by Jiang et al., examined the effect of adding XIST to Chromosome 21 with the goal of silencing the trisomy plaguing Down Syndrome (Jiang et al., 2013). The researchers found that inserting XIST into Chromosome 21 caused decreased expression of genes near the DYRK1A locus where the transgene was inserted. This is important as genes in this region affect Down Syndrome facial phenotypes. Overall, the study created a system to study gene silencing through the insertion of a transgene, which has now paved the way for more research on Down Syndrome.

Using XIST to turn off an autosome

This study was the first to ever use the insertion of XIST for studying Down Syndrome and trisomy (Jiang et al., 2013). However, it built upon previous studies that uncovered the normal role that XIST has in X-inactivation. This occurs when XIST creates a condensed Barr body on the extra X-chromosome, therefore inhibiting transcription. For example, one study examined the triggering of chromosomal memory by using XIST to regulate histone methylation in X-inactivation (Kohlmaier et al., 2004).

Effect on Gene Expression

Previously, no research concerning the silencing of gene expression had been done using Chromosome 21. This is due to the fact that Down Syndrome and other trisomy diseases are hard to treat because of the number of genes they effect. For instance, Down Syndrome affects the DYRK1A locus which is connected to cranio-facial development, so research concerning this trisomy is hard to conduct. There has been research done on gene silencing in cancer by examining the promotor region dealing with hypermethylation (Herman and Baylin, 2003). There has also been research concerning CpG islands on Chromosome 21 with the knowledge that they can relate to X-chromosome inactivation, but the study never went any further (Takai and Jones, 2002).

Future Directions

The technique used in Jiang, et al. that dealt with inserting XIST into a chromosome to silence a trisomy can also be used in future studies. For example, this technique could be used to study the effects it would have on other trisomy diseases such as Trisomy 13 and 18. Patau Syndrome is when there is a trisomy on Chromosome 13. This causes severe intellectual disability and physical abnormalities. Because of the life-threatening medical problems associated with Trisomy 13, many infants die within their first days or weeks of life. Only about 10% of children with Trisomy 13 live past their first year (Taylor, 1968). Edwards Syndrome occurs when a trisomy forms with Chromosome 18. This trisomy is characterized by abnormalities in many parts of the body such as slow growth before birth, low birth rate, heart defects, and abnormalities of other organs. Due to life-threatening medical conditions associated with this disorder, most infants die before birth or within their first month of life (Taylor, 1968). The research discussed in this primer, using XIST to eventually silence the effects of the trisomy, could be a novel approach used to prevent the trisomy in these individuals, if pursued.

The methods used in this study could also potentially be used to study the effects of silencing the extra chromosome in Kleinfelter's Syndrome. Kleinfelter's Syndrome affects males and is caused by an extra X-chromosome. The extra X-chromosome affects production

of testosterone, reduces muscle mass, facial hair, and causes the enlargement of breast tissue (Lanfranco et al., 2004). To start research on diseases affecting gene expression, the strategy would have to be modified to specifically target the correct regions on the chromosomes being studied. For example, the transgene used in this study was modified to target the DYRK1A locus of Chromosome 21. Finding a similar locus on each of the other chromosomes affected by trisomy would also have to be examined to determine which would readily accept the transgene and demonstrate accurate addition. The technology to do this has greatly improved since 2003 with the development of the CRISPR/Cas9 genome editing system. Since CRISPR/Cas 9 recognizes a target site through simple base-pairing, it makes this type of experiment much more accessible (Ran et al., 2013).

There are many steps needed before the results of this study can be applied to human research. First, research using mammals with trisomy would have to be pursued. This would need a timeline developed. For example, the pregnancy would have to be known very early, as well as the Down Syndrome diagnosis, in order to insert the XIST before differentiation of cells would occur. If animal studies went well, then the next step could be research using human subjects. The first step in human research would be to try a natural pregnancy. If Down Syndrome developed in the baby, it would have to be known early to try the insertion of XIST. If this didn't work, the next technique pursued would be inserting XIST into zygotes produced *in vitro* via artificial implantation, meaning fertilization would occur manually in a lab with XIST insertion, and then the zygote would be implanted into the mother. If neither method of altering the embryo's genome worked, the last approach would be to develop drug therapies that would specifically target the pathways most impacted in Down Syndrome, which would at least lessen the effects on the body. Overall, the study by Jiang, et al. created a new platform for research on Down Syndrome that will likely be used in future studies in all areas of gene therapy.

ACKNOWLEDGEMENTS

I would like to thank Dr. Jennifer Liang, Hannah Campbell, Maddie Chalmers, and Emily Schroer for providing their undivided attention and continuous guidance throughout the development of this manuscript. Their knowledge and assistance have been tremendously helpful. I would also like to thank the Provost's Office of the University of MN and Center for Educational Innovation for allowing us to develop a high-quality publication through the Duluth Journal of Undergraduate Biology.

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