HIGH SCHOOL EDITION
Journal of Student Research
Volume 11 Issue 4 (2022)

Using CRISPR-Cas9 in Human Fetuses to Prevent Trisomy 16 and Trisomy 22 Induced Miscarriages

Emily Qin[1] and Nicole Guilz[2#]

[1]Newark Academy
[2]Columbia University
[#]Advisor

ABSTRACT

About 50% of abortions have been found to be caused by aneuploidy, roughly 60% of which are trisomy. The most common trisomy occurs on chromosome 16, 21, and 22. However, trisomy 21 (also called Down Syndrome) is viable in about 57% of cases, while trisomy 16 and 22 result in miscarriage in nearly every pregnancy. Thus, additional therapies and treatments must be explored, especially through quickly advancing techniques such as gene editing. Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) combined with the cleaving endonuclease CRISPR-associated protein (Cas9) harbors potential for targeted chromosome deletion, possibly increasing the chance of survival in fetuses with trisomy 16 or 22. However, the risks and safety benefits during genetic diagnosing and potential treatment of the fetus should also be considered. Successfully demonstrated approaches of editing or eliminating extraneous chromosomes in studies of both animal models and lab-cultured human embryos will be discussed. In particular, this paper will examine possible gene editing approaches such as elimination of entire chromosomes, large-scale deletions, and chromosomal truncations to target trisomy. In order to determine the efficacy of these approaches in trisomy, the use of CRISPR-Cas9 to specifically treat trisomy via autosomal deletion and counter-selection markers will be deliberated. Delivery methods for the in-utero therapy includes viral techniques such as retroviruses and adeno-associated vectors (AAVs) as well as non-viral techniques like electroporation and nanoparticles. This paper will propose hypothetical treatments for CRISPR-Cas9 in human embryos to target trisomy 16 and 22 while also examining the bioethical implications of doing so.

Introduction

Trisomy 16 and 22

Aneuploidy, the occurrence of an abnormal number of chromosomes, are responsible for about 50% of spontaneous abortions, and trisomy, three chromosomes, comprise roughly 60% of total aneuploidy (Čulić, V. et al., 2011). Most frequently, trisomy will appear on chromosomes 16, 21, and 22 (E. Littman. et al., 2014), though trisomy 21 (also known as Down Syndrome) is viable in about 57% of cases (Morris, J. K. et al., 1999). Chan-Wei et al. showed that out of 832 spontaneous abortions with 368 abnormal tests, 84.24% of the abnormalities was aneuploidy. Trisomy 16 had the highest rate, occurring 121 times out of the 310 aneuploidy cases, while trisomy 22 followed (Jia, Chan-Wei et al., 2015). Thus, trisomy 16 and 22, which miscarry in nearly every pregnancy, must be further researched for treatment development.

Typically, trisomy is caused by parental balanced translocations of chromosomes, which commonly occur during the Meiosis I or Meiosis II stage of parental germline cells (O'Connor, C, 2008). T. Hassold and P. Hunt demonstrated that this error would more frequently occur in maternal sex cells as opposed to paternal sex cells, although it is still possible for the error to occur in either parent (T. Hassold and P. Hunt, 2001). There is also higher risk

of trisomy when the fetus is conceived at older age (O'Connor, C, 2008). Autosomal trisomy has also been found to occasionally occur with partial monosomy, which is viable, though prognosis may differ depending on the type or severity of the monosomy (Chen, C et al., 2019).

If a fetus has mosaic trisomy, there is a likelihood of complications during birth and postnatally. Chen et al. studied 21 patients born with mosaic trisomy 22, in which about 60% faced developmental delay or other high rates of structural abnormalities (Chen, C et al., 2019). However, other forms of autosomal trisomy are rarely viable, and miscarriages nearly always occur in the first trimester of pregnancy (O'Connor, C et al., 2008). Thus, in utero intervention or germline gene therapy — gene editing in embryos — could potentially be used to target the source of the issue before fatal effects occur (Bergman, M.T., 2019).

In Utero Approaches to Medicine

Diagnosis

There are different ways to test a fetus for genetic disorders, each with distinct advantages and disadvantages. One approach is chorionic villus sampling (CVS), in which a needle is inserted into the womb and directed to the placenta using ultrasound to take a chorionic villi sample. This sample is then tested for genetic abnormalities (NHS, 2017). However, this testing can only be done between the tenth and twelfth weeks of pregnancy (Hopkins Medicine, 2019). Amniocentesis is another approach, in which a needle is inserted into the womb to take out amniotic fluid from within the placenta. This approach is performed later during pregnancy, between the fifteenth and twentieth weeks (Mayo Clinic, 2019). Unfortunately, all of these diagnosis approaches come with risks. CVS yields a miscarriage rate of about 1.39%, while amniocentesis miscarries in about 0.94% of pregnancies that undergo this testing (Saloman, L. et al., 2019).

Patients can also use one of several different types of fetal karyotyping. There are parental karyotypes, which can identify translocation carriers, and product of conception karyotypes (POC karyotypes), which are proficient in identifying fetal aneuploidy. Additionally, there are chromosomal microarrays (also called POC CMAs), which can identify aneuploidy in as early as 6 weeks into a pregnancy, and pre-implantation testing (PGT), which also possesses therapeutic potential (Papas, R. S., & Kutteh, W. H., 2021). Unfortunately, in general, fetal karyotyping is disadvantageous in terms of cost-efficiency and labor-intensity, and it usually has an undetermined prognosis (Papas, R. S., & Kutteh, W. H., 2021).

Fetal Intervention

There are a few types of in utero approaches to medicine, or fetal intervention. These include fetal surgery, fetoscopic intervention, stem cell transplants, and in utero gene therapy (IUGT). Fetal surgery and fetoscopic intervention are most commonly used to correct anatomic defects, so they are not suitable for curing genetic diseases. Stem cell transplants can be effective in targeting specific regions, though they do not possess high efficacy when treating genetic mutations in fetuses globally. The main approach that will be further discussed is IUGT, due to the ability to directly target and edit the additional chromosome that leads to trisomy (UCSF, 2022). The unique makeup of the CRISPR-Cas9 complex can lead to efficient targeting of autosomal trisomy that other gene editing techniques are not able to replicate.

CRISPR

Mechanisms

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) can be paired with Cas9, an endonuclease that cleaves DNA using guide RNA (gRNA) that is present in trans-activating crispr RNA (tracrRNA), in order to target and genetically edit a specific gene or DNA sequence. There is a sequence on the 5' side of the targeted DNA sequence

and another duplex RNA structure on the 3' side that binds to the Cas9 complex, referred to as the Protospacer Adjacent Motif (PAM). In general, CRISPR is relatively cost-effective and easy to use, especially in comparison to older gene editing techniques such as zinc fingers and TALENs. Using CRISPR, one can induce DNA deletions, insertions, replacements, modifications, labeling, or transcription modulation depending on the desired effect (Doudna, J. A., & Charpentier, E., 2014).

Using CRISPR for IUGT Delivery Methods

Generally, there are two types of delivery methods for in utero therapies: viral and non-viral. Viral methods include viral vectors that can encapsulate and deliver CRISPR mRNA to the patient's cells via injection, while non-viral methods utilize physical and biochemical strategies to deliver the therapy (Bulcha, J. T. et al., 2021).

Viral Methods: Viral methods of delivery include retroviruses, adenoviruses, and adeno-associated viruses. Retroviruses are single-stranded RNAs that can retrotranscribe their DNA through reverse transcriptase, and have low immunogenicity because of their low viral reproduction and are not likely to come into contact with the immune system (Vargas, J. E. et al., 2016). Lentiviruses are a subclass of retroviruses, and they can transduce dividing and non-dividing cells by encoding proteins that aid in nuclear localization of viral DNA. These viruses are also self-inactivating, which limits genotoxicity (Goswami, R. et al., 2019).

In addition to retroviruses, there are adenoviruses, which are non-enveloped viruses with double-stranded DNA genomes. They yield high immunogenicity rates, and thus, they are not suitable for IUGT and will not be discussed in this paper (Goswami, R. et al., 2019). Adeno-Associated Viruses (AAVs) are another viable method of IUGT delivery in animal models, which are single-stranded viruses that can be pseudotyped with 13 different serotypes (Davey, M. G et al., 2017). AAVs have the ability to transduce certain tissue or cell types, and they are less immunogenic than adenoviruses (Palanki, R. et al., 2021).

Non-Viral Methods: One non-viral delivery method includes naked nucleic acid injections, though its transfection efficacy is limited due to endonuclear degradation and mononuclear-phagocyte-system-induced apoptosis. An additional method is electroporation, which destabilizes the cell membranes using an electric field and delivers the nucleic acids through the pores of the cell. This approach is commonly used in vitro for embryonic cells, though there is possible risk of electric shock to the fetus when used in utero (Palanki, R. et al., 2021).

Another non-viral delivery approach is nanoparticles, which can be delivered to the fetus via injection into the mother. Nanoparticles can be inorganic, such as quantum dots (Murthy S.K., 2007), which have high biocompatibility and unique properties. However, there have not been any clinical trials performed with inorganic nanoparticles, and they are typically used for adult treatments in experiments both *in vivo* and *in vitro*. Nanoparticles can also be polymer-based, such as liposome-based nanoparticles (Murthy S.K., 2007), which is formed when polymer and co-polymer co-blocked with polyethylene glycol (PEG). Although polymer-based nanoparticles have high chemical diversity and are efficient to use to target specific cells, they are cytotoxic. Because this factor inhibits its effect in utero, they will not be discussed in this paper. A final type of nanoparticle is lipid-based nanoparticles, which are self-assembled nanostructures that have high carrying capacity. They have low risk of insertional mutagenesis, though they also lack transfection efficacy (Palanki, R. et al., 2021).

Viral approaches might be more appealing for in-utero CRISPR delivery, such as AAVs or retroviruses, as they may have more effective administration into the fetus through the mother. Nanoparticles may also be an effective option, though other non-viral delivery methods such as electroporation and microinjection can be difficult to administer or may have higher risk to the mother or fetus than viral delivery methods. Regardless, with consideration of all these approaches, a treatment utilizing CRISPR could be engineered to target trisomy and delivered in a variety of different ways during early stages of pregnancy.

Possible CRISPR Treatment Methods

This paper's approach for treating trisomy will be via the deletion of extraneous chromosomes 16 or 22 that occur as a result of an anomaly in germline cells. Some previous studies that have successfully deleted chromosomes in utero will be reviewed, and alternative methods will also be introduced, some of which do not utilize CRISPR. Finally, other studies that apply these chromosome deletion methods specifically to trisomy will be presented.

CRISPR Induced Chromosome Manipulation In Vitro

One approach to editing chromosomes in utero using CRISPR is through inter homolog recombination in meiosis to correct chromosome abnormalities in fetuses. During the in vitro fertilization process of the egg — specifically, during the meiosis II stage — sperm would be targeted with a CRISPR-Cas9 system to alter mutations of a specific gene. Alternatively, the CRISPR-Cas9 system may be delivered in a slightly later stage of pregnancy such as the two-cell stage or later. The approach of targeting a gene sequence on the affected chromosomes in a stage when trisomy first begins allows for the disease to be targeted and treated early on in the pregnancy (Zuccaro, M. V. et al., 2020). However, this editing method targets a specific gene on the chromosome that needs to be deleted, while an effective treatment for trisomy would require a full deletion of a chromosome. Thus, using this approach for a larger-scale deletion can possibly cause the loss of the chromosome to occur during cell proliferation (Muraki, K. et al., 2012).

A study done by Zuccaro, M. V. et al. in 2020 used this approach to target the Eyes Shut Homolog (EYS) in embryonic mice cells to treat blindness prenatally. Guide RNAs (gRNAs) were used to target the EYS gene in the paternal chromosomes, as the mutation occurred on the paternal 6q12 chromosome band. A double strand break (DSB) was induced at the target site, which caused a five Base Pair (BP) deletion in 63% of the total edited cells. Then, the edited cells repaired their reading frames successfully after undergoing the gene deletion. However, the study also found that deletion of the entire paternal allele occurred just as frequently as heterozygous indels in the embryos, meaning large-scale deletions could vary depending on the individual cell. Thus, even though this editing method holds potential to cause a large enough deletion that would lead to a chromosome knockout, it would still result in mosaicism in embryos (Zuccaro, M. V. et al., 2020). Thus, using larger target sites or multiplex editing could possibly yield effective results.

For example, targeting a long or large enough gene sequence on the extraneous autosome may cause the wipeout of the entire chromosome. In order to apply this to trisomy 16 or 22, one or multiple gene sequences on each chromosome would be targeted to achieve a full knockout (Qin, Y et al., 2021). Potential target gene sequences on chromosome 16 might be the eukaryotic translation initiation factor 3 subunit 8 (EIF3S8) gene, as it is fully duplicated nearly seven times on chromosome 16 and has a 98.33% homology rate, or similarity between each DNA sequence (Martin, J. et al., 2004). As a result, editing such a large number of sequences on a single chromosome may aid in a full chromosome knockout. A number of possible target sequences on chromosome 22 exist in the low-copy repeats (LCR22) that the 22nd chromosome is rich in. Examples of long-range duplications may be in the AL008723/AL021937 or AL031595/AL022339 sequences on the 22q11 band (Dunham, I. et al., 1999). Additional aspects of targeting these genes will be discussed in this review.

This approach was done by Qin et al. in 2021 to create sex-reversed female clonal mice by fully deleting the Y chromosome. Specifically, the study used gRNAs to target the *Rbmy1a1* gene sequences, which occur near the centrosome on the p arm of the chromosome. This approach was then utilized to genotype the XY cloned cells into cells with XO sex chromosomes. Electroporation was used to deliver a combination of green fluorescence protein (GFP) plasmids, CRISPR-Cas9, and the gRNAs into an embryonic mouse stem cell line. When applying an initial plasmid dose of 150 ng, Qin et al. found that there was an 87% successful chromosome deletion rate, and an optimal amount of 50% plasmid resulted in a 100% chromosome deletion rate in the twenty subcloned cells that were edited. The chromosome deletion was determined via DNA PCR analyses to check the loss of the *Uba1y* gene, which occurs

on the opposite end of the centromere on the p arm of the Y chromosome. Additionally, analyses were also checked for the deletion of *Ssty1* genes, which are a band of gene sequences present on the q arm of the Y chromosome. Cells with *Uba1y* gene deletion simultaneously had *Ssty1* gene deletion, confirming the total loss of the Y chromosome resulting from the deletion of the *Rbmy1a1* gene sequences (Qin, Y et al., 2021). Overall, Qin et al. had better results in deleting a full chromosome than Zuccaro et al. by aiming for a larger amount of gene sequences.

Similar to the previous methods that were discussed, the next approach utilizes DSBs in order to cut out a large portion of a chromosome. However, this method differs in that it uses two separate DSBs in different locations on a chromosome, completely taking out multiple gene sequences at once as opposed to smaller genes or frequent short breaks along the chromosome (Eleveld, T. F. et al. 2021). If this approach can be used to target the centrosome or large portions of the telomere, then the full chromosome may be deleted throughout cell proliferation. Alternatively, this approach might be able to be used to target locations at either arm of the chromosome for a full chromosomal elimination (Muraki, K et al., 2012).

Eleveld et al. completed a study using this approach in 2021, targeting DSBs at chromosome bands 11q13.4 and 11q25. The deleted portion spanned nearly the entire 11q telomere, as the study strived to eliminate terminal deletions of chromosome 11q in order to prevent neuroblastoma tumors. After a neuroblastoma cell line (SKNSH) was produced, a complex with Cas9 proteins and synthetic gRNAs were delivered into the cell line via electroporation. Additionally, a single-stranded DNA (ssDNA) virus was delivered to the cells with a 50 bp sequence that was homologous to the area that surrounded either DSB in order to properly facilitate the chromosome rejoining around the deleted section. The study yielded results of greater than 30% of the desired deletion in cloned cells, proving to be relatively effective in deletion, though improvements are necessary before applying this to trisomy 16 or 22 in utero. However, Sanger sequencing revealed that the ssDNA was possibly not used as a repair template, and further experimentation to delete the 6q chromosome — of which distal deletion is also commonly found in neuroblastoma patients — omitted the ssDNA. Out of 9 total cell clones, 4 did not undergo chromosomal deletion. However, a total rate of 5/9 of the cell clones yielded positive results for the deletion of the chromosome (Eleveld, T. F. et al., 2021).

As shown by Eleveld et al., this approach can potentially be successful with simply gRNAs targeting two separate locations on one chromosome. However, further studies must be done to improve cell-wide total chromosomal editing rate.

CRISPR Induced Chromosome Manipulation In Vivo

Although the previously mentioned approaches have not yet been performed in vivo, other studies have had success in chromosome manipulation in utero in animal models.

For example, Rossidis et al. aimed to use CRISPR in utero in mouse models to reduce cholesterol and coronary heart disease risk. Specifically, the study utilized a recently developed technology based on CRISPR called base editor, BE3, to target specific nucleotides on the PCSK9 gene without DSBs. Thus, BE3 was safer to use than conventional CRISPR in this particular study. An adenoviral vector was used to deliver BE3 along with a gRNA targeting codon W159 on the PCSK9 gene (Ad.BE3.Pcsk9), and was injected into the fetus of pregnant mice. The fetuses that were genetically edited showed consistent and high gene editing rates after 3 months, while mice that were injected postnatally at five weeks had decreased effects of gene editing at the three month time period. There was roughly a 2% indel rate in the genetically edited mice, which was comparable to the 40% indel rate in previous studies that used CRISPR to disrupt the PCSK9 gene in mice prenatally. Additionally, even after the injection, the organs of the dam showed no signs of gene editing, proving the localized effect of CRISPR in the mice fetuses. Thus, this study successfully demonstrated the efficiency, efficacy, and necessity of using gene editing to treat diseases prenatally (Rossidis, A. C. et al., 2018).

Additionally, Alapati et al. conducted a study in which CRISPR was utilized to genetically edit mice in utero to treat monogenic lung disease. In order to do so, an adenoviral vector was used to deliver Streptococcus pyogenes Cas9 (SpyCas9) along with a sgRNA targeting the loxP sites surrounding the mT/stop cassette. SpyCas9 was used

because of its efficiency as a Cas9 enzyme, and the Cas9 system was injected into the amniotic cavity of gestational day 16 mice fetuses. Using DNA Sanger sequencing, it was determined that indels were present in the loxP region three nucleotides away on the 5' end from the PAM site. Additional testing using PCR and immunohistochemistry (IHC) revealed no genetic editing in any other organs besides the lungs, proving success in pulmonary gene editing via intra-amniotic delivery with Ad vectors. Additional analysis was done using IHC and flow cytometry at embryonic day 19, as well as postnatally at 7 days, 30 days, and 6 months to determine persistence of gene editing after birth. The percentage of epithelial lung cells that were positive for chromosomal editing remained consistent over the course of testing up to the 6 month period, showing effective persistent results of in utero gene editing postnatally (Alapati, D., et al., 2019).

Neither study explored a full chromosome knockout in vivo, merely demonstrating positive results for fetal gene editing in vivo. However, Zuo et al. conducted a study in which the Y chromosome was eliminated in vivo in mouse fetuses. A construct containing a sgRNA targeting the Ssty2 gene was delivered into the fetus via electroporation along with enhanced green fluorescence protein (EGFP) into the mouse brains in utero to target the Y chromosome. DNA-FISH was used to determine the efficacy of the genetic editing in the mice. Results showed that 40% of the edited cells showed no Y chromosome signal, indicating an effective chromosome deletion in embryos in vivo (Zuo E et al., 2017).

Even though not many chromosome deletion techniques have been tested in vivo, the studies that have had success in doing so present potential for future studies to explore in vivo experiments to improve safety and efficacy of chromosome knockouts or large-scale deletions.

Applying CRISPR Chromosome Deletion to Trisomy

In addition to the chromosome deletion techniques mentioned, it is imperative to discuss the applications of these techniques specifically to trisomy. For example, other factors such as unwanted chromosome deletion must also be considered when creating a treatment for trisomy. Results from experiments that consider this factor can be researched in combination with the chromosome deletion methods previously mentioned for future in utero trisomy experimentation.

To further experiment on the possibilities of extraneous autonomous chromosome deletion, Zuo et al. tested the efficacy of autosome deletion in trisomy 21. Down syndrome iPSCs 21-A and 21-B were injected with two sgRNAs targeting sequence sites specific to hChr21 genes, 21-A consisting of 49 cleavage sites and 21-B consisting of 24 cleavage sites. Using DNA-FISH for analysis, results showed a 15% success rate of turning trisomy 21 into disomy. Off-target effects for autosomal deletion were identical to those of the Y-chromosome deletion, which demonstrated only 2 off-target sites in an XO mouse with 2,186 to 26,469 potential off-target sites for each sgRNA. Further testing using HIseq confirmed rare off-target sites for the CRISPR-targeted Kdm5d site (the control for the experiment), though multiple off-target sites did exist for the Ssty2 locus (the gene sequence that was targeted on the Y chromosome). Overall, results did not show high off-target mutation rates, though strong off-target sites should be contemplated when using CRISPR for a chromosomal knockout. However, the rate for additional chromosome deletion was random, and chances of a single chromosome being deleted during the experiments were relatively low. In fact, deletion of two chromosomes occurred most commonly in the experiment, leaving a single autosome after CRISPR editing (Zuo, E et al., 2017). Even though there are few off-target effects, further study must be conducted to limit CRISPR editing to a single chromosome as opposed to targeting all three and risking unwanted wipeout of multiple autosomes at once.

In addition, Abe et al. targeted trisomy in chromosome 2 using counter-selection markers in DT40 chicken cells. DT40 cells were then transfected at the OVA locus with puromycin and ganciclovir (GCV), which were used as counter-selection markers to target the puromycin resistant (puroR) and HSV-TK genes. Puromycin resistant clones were then used to create $OVA^{+/+/HSV-TK}$ cells, which were cultured in a solution containing GCV. Cells that lost the

HSV-TK gene on chromosome 2 were selected, and canonical Giemsa chromosome staining was then used to determine the efficacy of chromosome deletion in the selected cells. Out of 48 total cloned cells, there was a 100% success rate of reducing trisomy 21 into disomy. The experiment was performed again with E.coli guanine phosphoribosyltransferase (ECO-GPT) used as a negative-selection marker for the TOPBP1 locus occurring on chromosome 2, and results showed that a majority of the 25 total cloned cells showed two chromosome 2s. This study utilized artificial techniques, specifically selecting cells with antibiotics that would yield a productive experiment. Thus, this does not simulate or propose any specific solutions that would be effective in vivo, though the study still provides potential to create a successful trisomy treatment in vivo with further experimentation (Abe, T et al., 2021).

Even though neither study proposed a specific method that would be effective in a human trisomy treatment, both experiments demonstrated methods with potential for chromosome deletion to be applied specifically for trisomy.

In Utero Delivery Methods

The viral or physical methods to deliver CRISPR in vivo and in vitro can apply to in utero gene therapy delivery as well. Some of these techniques are more commonly used for delivery than others, as this review will discuss below.

Viral Vectors

Tran et al. used retroviruses for IUGT delivery in order to successfully transduce sheep fetal progenitor cells and ameliorate their target phenotype. The study aimed to induce prenatal tolerance to β-galactosidase, and sheep fetuses were directly injected with a MuLV-based retroviral supernatant. Over the 28 month period of the study, 12 of the 16 total sheep consistently showed proviral DNA and transgene expression, and 41 months after the injection, the total remaining animal models were able to produce hematopoietic progenitors while being in the presence of a G418 concentration of 2 mg/L. Such an environment would have been otherwise lethal, proving the efficacy of vector-encoded transgene expression in utero via retroviruses. Additional analysis on the day of immunization and 68 days post immunization using lacZ-specific PCR confirmed the presence of the lacZ gene in the sheep's PB mononuclear cells. Thus, it was concluded that there were no immune responses to the retroviral vectors or the transgenes that were injected prenatally (Tran, N.D. et al, 2001).

In addition to retroviruses, lentiviruses, a sub-type of retroviruses, have also been used to deliver IUGT in vivo. Shangaris P. et al used a lentiviral vector to normalize phenotypes in mouse lines with β-thalassemia in utero. Humanized mouse model fetuses of β-thalassemia were injected with a lentiviral vector expressing β-globin during the gestational period. The fetuses had nearly a 37% survival rate after the injections, though they had a significantly higher blood hemoglobin concentration than that of the untreated control mice. Thus, the study concluded that the increased hemoglobin expression was due to the increased transgene expression as well. However, off-target effects were present, as α-globin was also higher in the treated mice, though the IUGT did not have an effect on the γ-globin (Shangaris, P. et al., 2019).

Adeno-associated viruses are another viable approach to IUGT delivery. Davey et al. used AAVs to inject GFP into sheep fetuses in order to determine the immune tolerance. Sheep fetuses of 60 days were injected with either AAV6.2, AAV8, or AAV9 that expressed GFP, and after one month post transduction, serum assessments were used to determine the production of GFP antibodies. The fetuses that received AAV6.2.GFP or AAV8.GFP showed no antibody expression, though they still showed GFP presence. Fetuses that were injected with AAV9.GFP showed no GFP expression altogether, suggesting inefficiency in the GFP injection. However, the study was still able to achieve transgene expression after in-utero AAV delivery of GFP up to 6 months post natally (Davey, M. G. et al, 2017).

Non-Viral Vectors

Common physical methods for cell delivery should also be discussed for delivery into embryos, such as electroporation and microinjection. Electroporation is a method that creates nanometer-sized pores in the cellular membrane using a high-voltage electric current (Lino, C. A. et al, 2018). Cell type could affect the efficacy of electroporation, though it is a suitable method for delivery even in cells that are challenging to manipulate. However, due to the high amounts of shock that is needed for electroporation to occur, it is not typically used for in vivo delivery. In particular, mammalian cells are more sensitive to the high voltage shocks than bacterial cells are, making bacterial cells a more tolerant cell type to electroporation (Lino, C. A. et al, 2018).

Even though electroporation is currently not the most ideal method for in utero delivery, there are existing studies that have demonstrated successful results of using electroporation in vivo. For example, Shinmyo et al. performed an experiment in which CRISPR was used for gene knockout in the mouse brain via in utero electroporation. Initially, the experiment was performed in vitro to validate the effects of the plasmids before moving to in vivo experimentation. The experiment utilized pX330 plasmids containing humanized Cas9 and sgRNAs that targeted the Satb2 gene in HEK293T cells, which helped prove its necessary cell-autonomous presence in callosal axon projections. Specifically, pX330-Satb2-272, -524 and -2129 were used, while pCAG-EGxxFP target plasmid was used as a reporter plasmid for the results of the experiment. Additionally, pCAG-mCherry was used to mark transfected cells, and observations were made 48 hours post transfection. Transfected cells had a range of EGFP-positivity from 76%—89%. This method was also used for the in vivo experimentation, in which pX330-Satb2-272 was electroporated in utero into mice brains at embryonic day 15.5. Results were taken 2 days postnatally and showed cells that were successfully transduced with mCherry in the ventricular and subventricular zone of the mice brains. Although the birth rate was not noted, the experiment still demonstrated significant efficacy in gene editing when using electroporation in developing mouse brains (Shinmyo, Y et al, 2016).

Additionally, microinjection has had a significant impact in cell delivery with success rates that are near 100%. The process of microinjection consists of using a needle and microscope to directly pierce a cell membrane, and cargo can then be delivered into the cell via the injection. However, this approach is best suited for ex vivo and in vitro experiments, as direct injection makes in vivo work near impossible (Lino, C et al, 2018).

Regardless, Wang et al. was able to perform an experiment in which mouse embryos were microinjected with sgRNAs targeting the Tet1 gene. Fertilized eggs were injected with varying amounts of CRISPR-Cas9, and a RFLP assay was used to determine the frequency of altered alleles. The fertilized eggs were removed and the injection occurred ex vivo, though the injected eggs were then transferred into foster mothers. Using microinjection to deliver CRISPR-Cas9 into mouse embryos had low toxicity since a proportion of the blastocysts developed to birth regardless of injection material. Still, this approach would not be effective for in vivo delivery, though this study provided insight into the possibilities of microinjection in utero (Wang, H et al, 2013).

Nanoparticles can also be used for IUGT to encapsulate and deliver CRISPR effectively. Nanoparticles can be organic or inorganic, though organic nanoparticles are more favorable for in vivo experimentation due to its biodegradability and biocompatibility. Nanoparticles can be physically and chemically engineered in different ways, and their surfaces can be covered with various other molecules. Once nanoparticles are injected, they eventually break down and their cargo is released into the target sites (Pritchard, N et al, 2021).

Ricciardi et al. conducted an experiment in which polymeric nanoparticles were delivered to mice at gestational stages in vivo. The study aimed to improve β-thalassemia in mice postnatally after using in utero nanoparticle injections. Nanoparticles were coded with β-thalassemia-associated splice site mutations in intron 2 at position 654, as mice that were homozygous for the Hbb^{th-4} allele that is associated with β-thalassemia do not survive after birth. By turning homozygous Hbb^{th-4} mice into heterozygous ones, survival rate would increase. In the experiment, nanoparticles were injected into the vitelline vein in the mice at embryo day 15.5. Hemoglobin concentrations were observed postnatally at ages 6 and 10 weeks, and higher dosages of the nanoparticles resulted in greater improvement of hemo-

globin levels. Compared to the 69% birth rate in the untreated control mice, the mice that were injected with nanoparticles had a 100% birth rate, demonstrating extremely effective results of using nanoparticles for allele-specific editing (Ricciardi, A et al, 2018).

More specifically than nanoparticles, Lipid-Based Nanoparticles (LNPs) have also been used for cell delivery. They are commonly used for nucleic acid delivery, which allows for stable delivery of the nucleic acids into cells. Due to the fact that there are no viral components, LNPs have low immunogenicity and can be used in vivo, ex vivo, or in vitro for many different types of cells (Lino, C et al, 2018).

Riley et al. performed an experiment to test the suitability of lipid nanoparticles for mRNA delivery in utero. A library of 14 different LNPs was used, and polyamine-lipid cores were created from the reaction between polyamine molecules and alkyl tails after combining them. The ionizable lipids of LNPs allow for quick cellular uptake, and encapsulated mRNA is then delivered to the cytosol of the cell after endosomal escape. The mRNA used in the first part of this study was a luciferase mRNA, which was injected into mouse fetuses at gestational day 16. The LNPs were injected into the vitelline vein of the mice, and imaging analysis showed strong luciferase signals in the injected fetuses and no luciferase signals from the control. In order to perform further analysis, fetuses were surgically removed from the mothers and were each imaged using IVIS. After identifying two separate Regions of Interest (ROIs) onto different parts of the imaging, results showed that some fetuses received greater mRNA delivery than others depending on the LNP formulation. Thus, it was concluded that the ionizable lipids within the LNPs had a major effect on its capacity to deliver mRNA to the fetus. Additional analysis in the lungs, kidney, brain, intestines, and heart of the fetuses showed minimal luciferase signals, while the luciferase signal was strongest in the liver of the injected fetuses. Thus, further testing must be performed to determine more efficient locations or methods than injection into the vitelline vein to deliver LNPs to these organs. Birth rate of injected fetuses ranged from 72.4% to greater than 90% for different LNP formulations, and fetal liver toxicity was determined to be minimal based on the low amounts of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in the liver tissue as an immune response to the LNP injection. Mothers of the fetuses showed no difference in ALT or AST levels, suggesting no liver toxicity, liver damage, or inflammatory responses for the mothers of the injected fetuses (Riley, R et al., 2021).

Although experiments have yet to use any of these methods to deliver CRISPR in utero to treat trisomy, these studies, along with the ones mentioned previously for possible CRISPR treatments in utero, show potential to treat trisomy in early stages of pregnancy to eliminate the risk of miscarriage. Further studies must be conducted to determine safety and efficacy of such treatments, though there is great opportunity for the creation of this treatment in future years.

Discussion

Currently, there are no curative treatments for any kind of trisomy, whether they are viable after birth or not (O'Connor, C et al., 2008). The trisomy that make up the largest portion of spontaneous abortions, trisomy 16 and 22, are always fatal, and thus harbor the most urgency for a cure (Jia, Chan-Wei et al., 2015). Additionally, treatments that utilize genetic editing such as CRISPR-Cas9 appeal to trisomy as it can correct the genetic cause of the disease. Although using CRISPR in trisomy studies has not been not widely explored, there have still been many studies that propose methods that can one day be used in this area. Some cell delivery methods have already been applied for in utero delivery, though none of them have been used specifically in a trisomy treatment. With future research to create safer and more efficient methods, all of the studies discussed in this paper have potential to synthesize a final cure.

Bioethics

Ethics of Gene Editing

Alongside the growth of gene editing research exists concerns about the ethics of genetically editing humans, let alone human embryos. Although gene editing can provide many positive opportunities for the future, there are also many ethical factors that must be taken into consideration when changing the genetic makeup of a human being — or what has not even developed to be a human yet.

One particularly relevant case of genetic editing in vivo in a human embryo was performed by Jiankui He in 2018 at the Southern University of Science and Technology in Shenzhen, China. He aimed to make HIV-resistant babies by knocking out the CCR5 gene, which he did in twin female embryos. They were eventually born and named Lulu and Nana. In the study, the two embryos were implanted, Nana having a 1bp insertion and 4bp deletion of both her CCR5 alleles while Lulu only had a 15bp deletion of one of her alleles. Although frameshift mutations are widely known to have possible off-target effects on protein translation or expression, Jiankui did not perform any preliminary in vitro editing of cell clones to determine the possible consequences of such genetic editing. Thus, this researcher did not have a full understanding of the direct results that his editing would have made before performing the experiment in vivo. As a result, when cells were taken at the blastocyst stage for analysis (about 3-5 from each twin), Sanger chromatograms showed 3 different combinations of alleles in both twins. As an off-target effect, mosaicism had occurred. Specifically, in Nana's blastocyst, cells had either two normal copies of the allele, one normal copy and one with a 15bp deletion, or a normal copy and an allele with a large, unknown insertion. No tests were done in the study on the girls once they were born, so it was not determined if the girls' bodies were mosaic, though it would be reasonable to assume so. Another factor that Jiankui did not consider was other possible effects that editing the allele could have. As a side effect of editing the CCR5 allele, aside from possible HIV-resistance, Jiankui also increased the twins' chance of symptomatic infection and chance of death from West Nile virus, 71 influenza A, 71 , 72 and tick-borne encephalitis. From this study, many scientists determined that genetic editing should only be performed with the knowledge of as many risks as possible and all possible outcomes. However, at present, germline editing should not be done for humans. Jiankui's case seemed to be an outlier, and labs should take significantly more caution before administering such potentially life-altering and permanent changes to any human (Rothschild J, 2020).

Regardless, according to a 2018 survey, Americans still voted in favor of genetic editing to address disease and disability, though they were against genetically "enhancing" fetuses (NORC, 2018). Although Jiankui attempted to protect fetuses from a potentially fatal disease, he used unsafe, incautious, and unapproved methods of using gene editing in human embryos. Thus, though many agree that it is moral to genetically edit fetuses to prevent disease, it must be done in a deliberate manner.

Director of the Center for Bioethics at Harvard Medical School Robert Truog commented on the twins' gene editing case, saying that he believes germline gene editing is highly beneficial, but Jiankui He lacked a proper oversight of science (Bergman M.T., 2019). Genetic editing in human embryos to prevent otherwise fatal diseases such as trisomy 16 and 22 may raise some moral concerns in different religious or cultural contexts, though, as Truog mentions, is extremely beneficial from the medical point of view. However, Truog also notes the necessity of proper surveillance and definitively set limitations when performing such impactful experiments, which Jiankui He lacked in his experiment. In the future, when the proper definitions and restrictions are outlined for the scientific and medical community, novel research in areas such as genetic editing can still be made in morally acceptable manners. The morality does not lie in the ends of curing the fatal disease, though the means to justify the creation of such treatments without the proper regulations may be questioned.

Risk-Benefit Factor

Though a CRISPR treatment has the potential to cure a fetus' fatal condition, there are always possible off-target effects that may occur in the fetus. When thinking about off-target mutations, it is also imperative to consider the alternative of not using gene editing at all. For fatal diseases like trisomy 16 and 22 that always miscarry in the first

trimester, being able to bring a fetus to term at all would already be considered a success in the treatment. Of course, a completely curative treatment would be ideal, though using CRISPR for fatal conditions will nearly always yield improved results from the medical perspective. For example, Uddin et al. contemplated the risk-benefit factors of genetically editing in utero, using Jiankui He's experiment as a portion of their argument. They believe that instead of studying germline editing for a disease such as HIV that already has postnatal treatments and a low mortality rate, Jiankui should have experimented on a different disease with significantly higher potential of death (Uddin, F et al., 2020). To support this point, they mention an experiment that was performed in vitro with donor egg and sperm cells to genetically correct mutations in the MYBPC3 gene, which can cause Hypertrophic Cardiomyopathy (HCM). HCM is the most common cause for spontaneous cardiac deaths under the age of 30, so experiments for editing in this gene hold more merit than experiments for diseases that can be completely cured and prevented postnatally (Ma, H. et al., 2017).

There are additional factors to consider from financial, social, political, and other perspectives, but from a purely scientific standpoint, genetically editing a fetus with a terminal illness yields mostly positive results. The mother is not typically at risk as shown by studies mentioned in this paper, though further research must be done to ensure that CRISPR treatments remain localized in the fetus and do not affect the mother in any way.

Cost and Other Alternatives

Lives are priceless, and thus, curative treatments for fatal diseases can be extremely expensive. In some situations, parents may decide that losing the fetus would be more favorable — financially, socially, or other — than using gene editing approaches such as CRISPR to bring the fetus to term.

The cost of this CRISPR proposal to treat trisomy in utero will likely vary widely, as some components, such as a short RNA template, could be only $65, while more specialized or advanced CRISPR treatments could cost up to or more than $1 million. For example, Kim et al describes a treatment for Transthyretin Amyloidosis that cost over 1 million dollars and saw promising results (Kim, M., 2022). Additionally, a prenatal surgery to correct myelomeningocele costs upward of $20,000, depending on future complications, severity of the case, etc. (Werner E. F. et al., 2012). There are currently no existing in utero CRISPR therapies, though any combination of the previously mentioned prices may be potential costs of a future in vivo IUGT. Cost is thus an important decision point to make regarding in utero gene editing. The circumstances will always grant different solutions, and such decisions will vary on a case-by-case basis. However, in the case that future research can produce a treatment with guaranteed effects, nearly no price can be put on the life that was saved.

Social Implications

Presenting a cure for trisomy may encourage negative stigmas surrounding disabilities in society, such as in people with Down Syndrome. Disabilities advocates state that it is better to focus scientific advancements on improving the lives of those with disabilities rather than looking for them in genetic screenings prenatally (Leary, A, 2019). However, creating cures for trisomy 16 and 22 in particular could be thought of as no different than creating a treatment for any other disease with fatal prognoses. There are no negative stigmas being built around the possibility of treating conditions that result in spontaneous abortions. In fact, such research may have positive effects in society when parents learn that their fetus may have a chance at life with a disease that was previously not curable.

The same can be argued for any child potentially remedied with this treatment that might be considered "genetically enhanced." They would not be granted any unfairly additional characteristics such as heightened intelligence or physical ability, but rather given a chance at life with an otherwise incurable condition.

Future Directions

As with nearly all current CRISPR treatments, any kind of CRISPR-Cas9 system that will be used in utero should first be further researched to reduce off-target mutations and improve efficiency. It is especially necessary to do so in an IUGT, as off-target effects may affect the mother as well as the fetus. If CRISPR can have a more consistent on-target effect, there will be greater benefit than risk when utilizing it to treat fatal diseases.

Moreover, further experimentation with epigenetics may enable the silencing of an additional chromosome as opposed to a full deletion. This mechanism does not get rid of the entire chromosome, yet it could still mitigate the effect of an extra chromosome in an autosomal trisomy.

An additional factor that must be further researched for an effective trisomy 16 or 22 treatment is safer ways to deliver the treatment. Currently, both viral and non-viral methods of cell delivery have not been used for human in utero experimentation, which can be a potential focus of future research. If delivery methods can be made to be safer in addition to the treatment itself, cures for trisomy 16 and 22 could be manufactured and distributed much more cost- and time-efficiently.

Even though much more research must be conducted to make advancements in using CRISPR to treat trisomy 16 and 22, past studies have shown great potential in creating a future cure for spontaneous abortions.

References

1. Čulić, V., Lozic, B., Kuzmić-Prusac, I., Mijaljica, G., & Pavelić, J. (2011). Full trisomy 5 in a sample of spontaneous abortion and Arias Stella reaction. Medical science monitor: international medical journal of experimental and clinical research, 17(10), CS116–CS119. https://doi.org/10.12659/msm.881969
2. Littman, E., Phan, V., Harris, D., Severino, M., & La, A. (2014). The most frequent aneuploidies in human embryo are similar to those observed in the early pregnancy loss. Fertility And Sterility, 102(3), e344. doi: 10.1016/j.fertnstert.2014.07.1162
3. Morris, J. K., Wald, N. J., & Watt, H. C. (1999). Fetal loss in Down syndrome pregnancies. Prenatal diagnosis, 19(2), 142–145.
4. Jia, C. W., Wang, L., Lan, Y. L., Song, R., Zhou, L. Y., Yu, L., Yang, Y., Liang, Y., Li, Y., Ma, Y. M., & Wang, S. Y. (2015). Aneuploidy in Early Miscarriage and its Related Factors. Chinese medical journal, 128(20), 2772–2776. https://doi.org/10.4103/0366-6999.167352
5. O'Connor, C. (2008) Chromosomal abnormalities: Aneuploidies. Nature Education 1(1):172
6. Hassold, T., & Hunt, P. (2001). To err (meiotically) is human: the genesis of human aneuploidy. Nature reviews. Genetics, 2(4), 280–291. https://doi.org/10.1038/35066065
7. Chen, C. P., Huang, M. C., Chern, S. R., Wu, P. S., Chen, S. W., Chuang, T. Y., Town, D. D., & Wang, W. (2019). Mosaic trisomy 22 at amniocentesis: Prenatal diagnosis and literature review. Taiwanese journal of obstetrics & gynecology, 58(5), 692–697. https://doi.org/10.1016/j.tjog.2019.07.020
8. Bergman, M.T. (2019). Harvard researchers share views on future, ethics of gene editing. Retrieved 1 September 2022, from https://news.harvard.edu/gazette/story/2019/01/perspectives-on-gene-editing/
9. NHS. (2017). Chorionic villus sampling - What happens . Retrieved 16 August 2022, from https://www.nhs.uk/conditions/chorionic-villus-sampling-cvs/what-happens/
10. Hopkins Medicine. (2019). Chorionic Villus Sampling (CVS). Retrieved 14 September 2022, from https://www.hopkinsmedicine.org/health/treatment-tests-and-therapies/chorionic-villus-sampling-cvs#:~:text=CVS%20is%20usually%20done%20between,defects%2C%20such%20as%20spina%20bifida.
11. Amniocentesis - Mayo Clinic. (2022). Retrieved 16 August 2022, from https://www.mayoclinic.org/tests-procedures/amniocentesis/about/pac-20392914

12. Salomon, L. J., Sotiriadis, A., Wulff, C. B., Odibo, A., & Akolekar, R. (2019). Risk of miscarriage following amniocentesis or chorionic villus sampling: systematic review of literature and updated meta-analysis. Ultrasound in obstetrics & gynecology : the official journal of the International Society of Ultrasound in Obstetrics and Gynecology, 54(4), 442–451. https://doi.org/10.1002/uog.20353
13. Papas, R. S., & Kutteh, W. H. (2021). Genetic Testing for Aneuploidy in Patients Who Have Had Multiple Miscarriages: A Review of Current Literature. The application of clinical genetics, 14, 321–329. https://doi.org/10.2147/TACG.S320778
14. Techniques of fetal intervention. UCSF BCH Fetal Treatment Center - Techniques of Fetal Intervention. (n.d.). Retrieved August 3, 2022, from https://fetus.ucsf.edu/techniques-fetal-intervention/
15. Doudna, J. A., & Charpentier, E. (2014). Genome editing. The new frontier of genome engineering with CRISPR-Cas9. Science (New York, N.Y.), 346(6213), 1258096. https://doi.org/10.1126/science.1258096
16. Bulcha, J. T., Wang, Y., Ma, H., Tai, P., & Gao, G. (2021). Viral vector platforms within the gene therapy landscape. Signal transduction and targeted therapy, 6(1), 53. https://doi.org/10.1038/s41392-021-00487-6
17. Vargas, J. E., Chicaybam, L., Stein, R. T., Tanuri, A., Delgado-Cañedo, A., & Bonamino, M. H. (2016). Retroviral vectors and transposons for stable gene therapy: advances, current challenges and perspectives. Journal of translational medicine, 14(1), 288. https://doi.org/10.1186/s12967-016-1047-x
18. Goswami, R., Subramanian, G., Silayeva, L., Newkirk, I., Doctor, D., Chawla, K., Chattopadhyay, S., Chandra, D., Chilukuri, N., & Betapudi, V. (2019). Gene Therapy Leaves a Vicious Cycle. Frontiers in oncology, 9, 297. https://doi.org/10.3389/fonc.2019.00297
19. Palanki, R., Peranteau, W. H., & Mitchell, M. J. (2021). Delivery technologies for in utero gene therapy. Advanced drug delivery reviews, 169, 51–62. https://doi.org/10.1016/j.addr.2020.11.002
20. Murthy SK. Nanoparticles in modern medicine: state of the art and future challenges. Int J Nanomedicine. 2007;2(2):129-41. PMID: 17722542; PMCID: PMC2673971.
21. Zuccaro, M. V., Xu, J., Mitchell, C., Marin, D., Zimmerman, R., Rana, B., Weinstein, E., King, R. T., Palmerola, K. L., Smith, M. E., Tsang, S. H., Goland, R., Jasin, M., Lobo, R., Treff, N., & Egli, D. (2020). Allele-Specific Chromosome Removal after Cas9 Cleavage in Human Embryos. Cell, 183(6), 1650–1664.e15. https://doi.org/10.1016/j.cell.2020.10.025
22. Muraki, K., Nyhan, K., Han, L., & Murnane, J. P. (2012). Mechanisms of telomere loss and their consequences for chromosome instability. Frontiers in oncology, 2, 135. https://doi.org/10.3389/fonc.2012.00135
23. Qin, Y., Wong, B., Zhong, L., Geng, F., Parada, L. F., & Wen, D. (2021). Generation of Sex-Reversed Female Clonal Mice via CRISPR-Cas9-Mediated Y Chromosome Deletion in Male Embryonic Stem Cells. The CRISPR journal, 4(1), 147–154. https://doi.org/10.1089/crispr.2020.0074
24. Martin, J., Han, C., Gordon, L. A., Terry, A., Prabhakar, S., She, X., Xie, G., Hellsten, U., Chan, Y. M., Altherr, M., Couronne, O., Aerts, A., Bajorek, E., Black, S., Blumer, H., Branscomb, E., Brown, N. C., Bruno, W. J., Buckingham, J. M., Callen, D. F., … Pennacchio, L. A. (2004). The sequence and analysis of duplication-rich human chromosome 16. Nature, 432(7020), 988–994. https://doi.org/10.1038/nature03187
25. Dunham, I., Shimizu, N., Roe, B. A., Chissoe, S., Hunt, A. R., Collins, J. E., Bruskiewich, R., Beare, D. M., Clamp, M., Smink, L. J., Ainscough, R., Almeida, J. P., Babbage, A., Bagguley, C., Bailey, J., Barlow, K., Bates, K. N., Beasley, O., Bird, C. P., Blakey, S., … O'Brien, K. P. (1999). The DNA sequence of human chromosome 22. Nature, 402(6761), 489–495. https://doi.org/10.1038/990031
26. Eleveld, T. F., Bakali, C., Eijk, P. P., Stathi, P., Vriend, L. E., Poddighe, P. J., & Ylstra, B. (2021). Engineering large-scale chromosomal deletions by CRISPR-Cas9. Nucleic acids research, 49(21), 12007–12016. https://doi.org/10.1093/nar/gkab557 (chromosome deletion)
27. Li, L. B., Chang, K. H., Wang, P. R., Hirata, R. K., Papayannopoulou, T., & Russell, D. W. (2012). Trisomy correction in Down syndrome induced pluripotent stem cells. Cell stem cell, 11(5), 615–619. https://doi.org/10.1016/j.stem.2012.08.004

28. Chamberlain, J. R., Schwarze, U., Wang, P. R., Hirata, R. K., Hankenson, K. D., Pace, J. M., Underwood, R. A., Song, K. M., Sussman, M., Byers, P. H., & Russell, D. W. (2004). Gene targeting in stem cells from individuals with osteogenesis imperfecta. Science (New York, N.Y.), 303(5661), 1198–1201. https://doi.org/10.1126/science.1088757
29. Khan, I. F., Hirata, R. K., Wang, P. R., Li, Y., Kho, J., Nelson, A., Huo, Y., Zavaljevski, M., Ware, C., & Russell, D. W. (2010). Engineering of human pluripotent stem cells by AAV-mediated gene targeting. Molecular therapy : the journal of the American Society of Gene Therapy, 18(6), 1192–1199. https://doi.org/10.1038/mt.2010.55
30. Farr, C., Fantes, J., Goodfellow, P., & Cooke, H. (1991). Functional reintroduction of human telomeres into mammalian cells. Proceedings of the National Academy of Sciences of the United States of America, 88(16), 7006–7010. https://doi.org/10.1073/pnas.88.16.7006
31. Rossidis, A. C., Stratigis, J. D., Chadwick, A. C., Hartman, H. A., Ahn, N. J., Li, H., Singh, K., Coons, B. E., Li, L., Lv, W., Zoltick, P. W., Alapati, D., Zacharias, W., Jain, R., Morrisey, E. E., Musunuru, K., & Peranteau, W. H. (2018). In utero CRISPR-mediated therapeutic editing of metabolic genes. Nature medicine, 24(10), 1513–1518. https://doi.org/10.1038/s41591-018-0184-6
32. Alapati, D., Zacharias, W. J., Hartman, H. A., Rossidis, A. C., Stratigis, J. D., Ahn, N. J., Coons, B., Zhou, S., Li, H., Singh, K., Katzen, J., Tomer, Y., Chadwick, A. C., Musunuru, K., Beers, M. F., Morrisey, E. E., & Peranteau, W. H. (2019). In utero gene editing for monogenic lung disease. Science translational medicine, 11(488), eaav8375. https://doi.org/10.1126/scitranslmed.aav8375
33. Zuo, E., Huo, X., Yao, X., Hu, X., Sun, Y., Yin, J., He, B., Wang, X., Shi, L., Ping, J., Wei, Y., Ying, W., Wei, W., Liu, W., Tang, C., Li, Y., Hu, J., & Yang, H. (2017). CRISPR/Cas9-mediated targeted chromosome elimination. Genome biology, 18(1), 224. https://doi.org/10.1186/s13059-017-1354-4
34. Abe, T., Suzuki, Y., Ikeya, T., & Hirota, K. (2021). Targeting chromosome trisomy for chromosome editing. Scientific reports, 11(1), 18054. https://doi.org/10.1038/s41598-021-97580-1
35. Tran, N. D., Porada, C. D., Almeida-Porada, G., Glimp, H. A., Anderson, W. F., & Zanjani, E. D. (2001). Induction of stable prenatal tolerance to beta-galactosidase by in utero gene transfer into preimmune sheep fetuses. Blood, 97(11), 3417–3423. https://doi.org/10.1182/blood.v97.11.3417
36. Shangaris, P., Loukogeorgakis, S.P., Subramaniam, S. et al. In Utero Gene Therapy (IUGT) Using GLOBE Lentiviral Vector Phenotypically Corrects the Heterozygous Humanised Mouse Model and Its Progress Can Be Monitored Using MRI Techniques. Sci Rep 9, 11592 (2019). https://doi.org/10.1038/s41598-019-48078-4
37. Davey, M. G., Riley, J. S., Andrews, A., Tyminski, A., Limberis, M., Pogoriler, J. E., Partridge, E., Olive, A., Hedrick, H. L., Flake, A. W., & Peranteau, W. H. (2017). Induction of Immune Tolerance to Foreign Protein via Adeno-Associated Viral Vector Gene Transfer in Mid-Gestation Fetal Sheep. PloS one, 12(1), e0171132. https://doi.org/10.1371/journal.pone.0171132 (AAVs)
38. Lino, C. A., Harper, J. C., Carney, J. P., & Timlin, J. A. (2018). Delivering CRISPR: a review of the challenges and approaches. Drug delivery, 25(1), 1234–1257. https://doi.org/10.1080/10717544.2018.1474964
39. Wang, H., Yang, H., Shivalila, C. S., Dawlaty, M. M., Cheng, A. W., Zhang, F., & Jaenisch, R. (2013). One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. Cell, 153(4), 910–918. https://doi.org/10.1016/j.cell.2013.04.025
40. Pritchard, N., Kaitu'u-Lino, T., Harris, L., Tong, S., & Hannan, N. (2021). Nanoparticles in pregnancy: the next frontier in reproductive therapeutics. Human reproduction update, 27(2), 280–304. https://doi.org/10.1093/humupd/dmaa049
41. Ricciardi, A. S., Bahal, R., Farrelly, J. S., Quijano, E., Bianchi, A. H., Luks, V. L., Putman, R., López-Giráldez, F., Coşkun, S., Song, E., Liu, Y., Hsieh, W. C., Ly, D. H., Stitelman, D. H., Glazer, P. M., & Saltzman, W. M. (2018). In utero nanoparticle delivery for site-specific genome editing. Nature communications, 9(1), 2481. https://doi.org/10.1038/s41467-018-04894-2

42. Riley, R. S., Kashyap, M. V., Billingsley, M. M., White, B., Alameh, M. G., Bose, S. K., Zoltick, P. W., Li, H., Zhang, R., Cheng, A. Y., Weissman, D., Peranteau, W. H., & Mitchell, M. J. (2021). Ionizable lipid nanoparticles for in utero mRNA delivery. Science advances, 7(3), eaba1028. https://doi.org/10.1126/sciadv.aba1028 (lipid-based nanoparticles)
43. Rothschild J. (2020). Ethical considerations of gene editing and genetic selection. Journal of general and family medicine, 21(3), 37–47. https://doi.org/10.1002/jgf2.321
44. Human Genetic Engineering - AP-NORC. (2022). Retrieved 1 September 2022, from https://apnorc.org/projects/human-genetic-engineering/
45. Uddin, F., Rudin, C. M., & Sen, T. (2020). CRISPR Gene Therapy: Applications, Limitations, and Implications for the Future. Frontiers in oncology, 10, 1387. https://doi.org/10.3389/fonc.2020.01387
46. Ma, H., Marti-Gutierrez, N., Park, S. W., Wu, J., Lee, Y., Suzuki, K., Koski, A., Ji, D., Hayama, T., Ahmed, R., Darby, H., Van Dyken, C., Li, Y., Kang, E., Park, A. R., Kim, D., Kim, S. T., Gong, J., Gu, Y., Xu, X., … Mitalipov, S. (2017). Correction of a pathogenic gene mutation in human embryos. Nature, 548(7668), 413–419. https://doi.org/10.1038/nature23305
47. Schwartz, M. (2018). CRISPR is a gene-editing tool that's revolutionary, though not without risk. Retrieved 1 September 2022, from https://stanmed.stanford.edu/2018winter/CRISPR-for-gene-editing-is-revolutionary-but-it-comes-with-risks.html
48. Kim, M. (2022). With CRISPR gene editing, unique treatments begin to take off for rare diseases. The Washington Post. Retrieved September 1, 2022, from https://www.washingtonpost.com/health/2022/02/05/crispr-rare-diseases/
49. Werner, E. F., Han, C. S., Burd, I., Lipkind, H. S., Copel, J. A., Bahtiyar, M. O., & Thung, S. F. (2012). Evaluating the cost-effectiveness of prenatal surgery for myelomeningocele: a decision analysis. Ultrasound in obstetrics & gynecology: the official journal of the International Society of Ultrasound in Obstetrics and Gynecology, 40(2), 158–164. https://doi.org/10.1002/uog.11176
50. Leary, A. (2019, April 23). Why finding cures for genetic disabilities shouldn't be our main goal. Rooted in Rights. Retrieved September 1, 2022, from https://rootedinrights.org/why-finding-cures-for-genetic-disabilities-shouldnt-be-our-main-goal/