The technical risks of human gene editing

Benjamin Davies

Welcome Centre for Human Genetics, University of Oxford, Oxford OX3 7BN, UK

Email: ben.davies@well.ox.ac.uk

Running title: The technical risks of human gene editing

Abstract

A recent report from Dr. He Jiankui concerning the birth of twin girls harbouring mutations engineered by CRISPR/Cas nucleases has been met with international condemnation. Beside the serious ethical concerns, there are known technical risks associated with CRISPR/Cas gene editing which further raise questions about how these events could have been allowed to occur. Numerous studies have reported unexpected genomic mutation and mosaicism following the use of CRISPR/Cas nucleases, and it is currently unclear how prevalent these disadvantageous events are and how robust and sensitive the strategies to detect these unwanted events may be. Although Dr. Jiankui's study appears to have involved certain checks to ascertain these risks, the decision to implant the manipulated embryos, given these unknowns, must nonetheless be considered reckless. Here I review the technical concerns surrounding genome editing and consider the available data from Dr. Jiankui in this context. Although the data remains unpublished, preventing a thorough assessment of what was performed, it seems clear that the rationale behind the undertaking was seriously flawed; the procedures involved substantial technical risks which, when added to the serious ethical concerns, fully justify the widespread criticism that the events have received.

Key words: gene editing, CRISPR, nuclease, mutagenesis, Cas9.

23

Introduction

The development of site-specific nucleases over the last decade now make it possible to introduce precise changes into the DNA sequence of our cells (Carroll, 2017). In particular, the RNA-guided, CRISPR/Cas family of nucleases are very easy to design against specific genomic target sequences and high efficiencies of mutagenesis can be achieved (Sander, et al., 2014). These qualities are making the therapeutic application of CRISPR/Cas nucleases to tackle genetic disease feasible and there has been a diverse range of success stories published in preclinical models (Porteus, 2019). For example, CRISPR/Cas9 nucleases have been designed to ablate the mutation responsible for muscular dystrophy, restoring normal gene expression (Long, et al., 2014). In both small (Nelson, et al., 2016) and large (Amoasii, et al., 2018) animal models, viral delivery of these nucleases into the diseased muscle was shown to restore muscle condition and strength. Such studies typify the exciting new field of therapeutic gene editing and highlight its potential in tackling genetic disease.

The target cell for therapeutic gene editing needs to be carefully considered to maximise the therapeutic potential and its longevity. Clearly, editing a stem cell population would have clear advantages for a prolonged therapeutic effect. Indeed, CRISPR/Cas nucleases have been introduced into haematopoietic stem cells to correct the underlying mutations responsible for sickle-cell anaemia (Vakulskas, et al., 2018) and there is thus considerable interest in this approach for long-lasting treatments for genetic diseases of the blood.

The application of gene editing tools in somatic stem cell therapies has raised the possibility that they could be applied in the ultimate stem cell, the 1-cell embryo, allowing the genetic correction to be permanent and thus present in all cells of the resulting individual. Furthermore, a successful genetic manipulation of the 1-cell embryo would lead to the

presence of the experimentally-induced mutation in the germ cells of the manipulated individual, and thus the inheritance of these genome edits would be achieved. Early work introducing CRISPR/Cas nucleases into the 1-cell mouse embryo by microinjection demonstrated that genetic modification of the whole organism could be achieved efficiently and confirmed the inheritance of the genetic change in subsequent generations (Wang, et al., 2013).

CRISPR/Cas nucleases in human embryos

Reports soon emerged that CRISPR/Cas could indeed be used to manipulate the human 1-cell embryo. The first published studies, used discarded tripronuclear zygotes and achieved mutagenesis efficiencies of up to 50%, with specific gene editing (i.e. the incorporation of information from a co-injected repair template) occurring at around 15% (Kang, et al., 2016; Liang, et al., 2015). This was quickly followed by a further report of efficient mutagenesis in healthy 2-pronuclear embryos (Tang, et al., 2017). Two more substantial articles followed, both published in Nature (Fogarty, et al., 2017; Ma, et al., 2017). The first demonstrated efficient correction of a dominant pathogenic mutation at the *MYBPC3* gene, encoding a cardiac myosin-binding protein, in heterozygous human embryos, and proposed a mechanism of inter-homologue repair using the wild-type allele as a repair template (Ma, et al., 2017). The second study focussed on the use of the technology to explore the role of genes involved in pre-implantation human development and achieved targeted mutation of the gene encoding the transcription factor *OCT4* (*POU5F1*) in 71% of manipulated embryos (Fogarty, et al., 2017). More recent studies have successfully achieved gene editing using exogenous repair templates to repair pathogenic mutations (Tang, et al., 2018) or to introduce specific reporter sequences (Yao, et al., 2018).

An alternative strategy for achieving site-specific change within the genome has been reported: base editing, which relies upon the fusion of enzymatic domains to the CRISPR/Cas machinery, capable of chemically converting one nucleotide base to another (Rees, et al., 2018). Base editing has also been successfully applied within the human 1-cell embryo (Li, et al., 2017; Liang, et al., 2018) and pathogenic mutations have been successfully corrected using this technology (Liang, et al., 2017; Zeng, et al., 2018).

These studies demonstrate that it is now feasible to achieve mutations and edits in human embryos at manageable frequencies and suggest that the tools for therapeutic germline editing are now available. There are numerous ethical concerns surrounding this technology which have been widely discussed and reviewed elsewhere (van Dijke, et al., 2018), but a major requirement before germline editing can be considered is to assess the safety of the manipulations. Investigations of CRISPR/Cas9 mutagenesis both in cell culture experiments and in embryos have highlighted a number of disadvantageous consequences, where research is needed to assess and mitigate the risk and optimise methods permitting the reliable detection of such events. There thus remains an appreciable amount of technology development and optimisation to be done before therapeutic editing can be considered.

Off-target mutagenesis

Soon after the demonstration that CRISPR/Cas9 could be used for targeted manipulation of the mammalian genome, reports emerged that its use carries a risk of unintended mutagenesis at closely matched genomic sequences (Fu, et al., 2013). This so-called off-target mutagenesis is also more pronounced than initially expected as the commonly used Cas9 enzyme can tolerate certain mismatches within its targeting sequence (Figure 1A). Many of the studies addressing off-target mutagenesis have been performed in cell culture

93 experiments where the CRISPR/Cas enzymes are transfected into millions of cells, the
94 genomic DNA of which is then deep-sequenced to ascertain levels of accuracy. These types of
95 experiment may overestimate the risk of off-target mutagenesis occurring when the
96 CRISPR/Cas nucleases are applied in a single cell, i.e. the 1-cell embryo. Indeed, one carefully
97 controlled study in mouse used whole genome sequencing on a trio (sequencing both
98 parental and offspring DNA) to address off-target mutagenesis resulting from a 1-cell embryo
99 microinjection experiment and were unable to detect any events in the founder mice
100 analysed (Iyer, et al., 2018). In contrast, a larger study investigated founder rodent lines
101 generated with multiple CRISPR/Cas9 enzymes addressing a number of different target
102 sequences and found that almost 30% of the mutant lines harboured putative off-target
103 mutations (Anderson, et al., 2018). Interestingly, base editors designed to convert cytidine to
104 thymidine residues were also found to have substantial off-target effects when applied within
105 the mouse 1-cell embryo (Zuo, et al., 2019). Some of the human studies have also analysed
106 the resulting embryos for off-target effects. Candidate off-target sites, localised using
107 bioinformatic approaches, have been analysed by either Sanger or next generation
108 sequencing. One of the studies confirmed an off-target mutation in two of the resulting
109 embryos (Liang, et al., 2015). All of the other studies found no evidence for significant levels
110 of off-target mutation (Fogarty, et al., 2017; Kang, et al., 2016; Ma, et al., 2017; Tang, et al.,
111 2017). In contrast with the results from the cytidine to thymidine base editors in mouse (Zuo,
112 et al., 2019), only low or entirely absent levels of off-target mutagenesis was detected when
113 these reagents were applied in human embryos (Li, et al., 2017; Liang, et al., 2017; Zeng, et
114 al., 2018).

115 The risk of off-target mutagenesis is thus clearly dependent upon the target sequence and
116 can be reduced by designing CRISPR/Cas nucleases against truly unique genomic sequences;

a number of online algorithms are available to facilitate this improved design (Haeussler, et al., 2016; Hodgkins, et al., 2015). These bioinformatic assessments of off-target risk can be somewhat flawed however, as many of the available tools do not take into consideration human genetic sequence variation: a true off-target profile, and thus risk assessment for a selected CRISPR/Cas nuclease can only really be accomplished by establishing a personalised genome. Indeed, studies have suggested that naturally occurring human SNPs can alter the off-target landscape of site-specific nucleases substantially (Lessard, et al., 2017).

It has also been shown that the concentration and persistence of the nuclease can increase the chance of off-target cleavage (Kim, et al., 2014; Zuris, et al., 2015), and subsequently a number of approaches aimed at limiting the activity of the nuclease have been shown to reduce the level of off-target mutation (Chen, et al., 2016; Shen, et al., 2018). Structural investigations and molecular evolution of the Cas9 nuclease have enabled the design of variant sequences which show increased levels of accuracy (Kleinstiver, et al., 2016; Slaymaker, et al., 2016) and reduced risk of off-target mutagenesis in rodent models (Anderson, et al., 2018). Furthermore, orthologues of Cas nucleases from alternative bacterial species have been shown to have increased levels of accuracy (Kim, et al., 2016; Teng, et al., 2018). A recent study also improved the accuracy of CRISPR/Cas effectors by altering the structure of its cofactor guide-RNA (Kocak, et al., 2019), highlighting a different approach to addressing this problem.

Taken together, there is a measurable risk of off-target mutagenesis when applying CRISPR/Cas nucleases in cells and embryos, but there has been significant technology refinement and bioinformatics tool development to reduce these risks substantially. Nonetheless, more research is needed in this area to assess the risk, improve the accuracy of

the enzymes and to explore methods for detecting these off-target events, before these gene editing tools can be applied therapeutically.

Mosaicism

The microinjection of CRISPR/Cas nucleases into mouse zygotes soon revealed that the CRISPR/Cas nucleases frequently retain activity after the first embryo cleavage event. Consequently, there is an appreciable risk that individual cells of the 2-cell or even 4-cell embryo harbour different combinations of wild-type and mutant alleles. The resulting organism is thus frequently a genetic mosaic, with different combinations of mutations in different parts of the animal (Figure 1B). This phenomenon is easily demonstrated by microinjection experiments in mouse embryos which use CRISPR/Cas nucleases designed against coat colour genes. Addressing the Tyrosinase gene, loss of function of which leads to the albino phenotype, clear somatic mosaicism was evident as the majority of founder offspring show a speckled, patchy coat colour, rather than complete albinism (Yen, et al., 2014). Clear evidence of mosaicism was also found in the human studies (Fogarty, et al., 2017; Ma, et al., 2017; Tang, et al., 2017; Yao, et al., 2018).

Mosaicism may be tackled by altering the timing of the nuclease activity within the 1-cell embryo, effectively restricting its activity to the 1-cell stage. Reducing the half-life of the nuclease through the use of a destabilised version of Cas9 was shown to reduce mosaicism whilst editing non-human primate embryos (Tu, et al., 2017). Another improvement was achieved by delivering the nuclease to in-vitro fertilised embryos by electroporation, permitting the delivery of the CRISPR/Cas machinery at a very early developmental stage, even before pronuclei have formed (Hashimoto, et al., 2016). One study in human embryos was able to effectively eliminate mosaicism by introducing the CRISPR/Cas reagents at the

same time as performing the fertilisation by intracytoplasmic sperm injection (Ma, et al., 2017).

Large deletions and rearrangements

The mutagenesis occurring following the application of CRISPR/Cas nucleases relies upon the innate DNA repair machinery of the target cells. Most frequently, the induced double-strand-break (DSB) is repaired by non-homologous end joining, which can lead to the introduction of small deletions and insertions. The mutations are frequently small in size, the most common being a single nucleotide insertion or deletion (Chakrabarti, et al., 2018; Taheri-Ghahfarokhi, et al., 2018). Nonetheless, larger deletions do occur and there is evidence to suggest that, on occasion, the repair event can result in large kilobase-scale deletions. In one in vivo study, introducing CRISPR/Cas nucleases as a virus to correct a mutation in the *Otc* gene, an appreciable rate (6.5%) of disruptive large deletions was found (Yang, et al., 2016). More recently, various studies have observed large deletions occurring at significant levels following the use of CRISPR/Cas9 in vitro (Kosicki, et al., 2018) and when applied within the 1-cell embryo (Parikh, et al., 2015; Shin, et al., 2017). These unexpectedly large deletions have the capacity to delete whole genes or cause misregulation of nearby expressed sequences (Figure 1C).

In addition to large deletions, a mouse study has revealed that complex rearrangements can occur following the application of nucleases in the 1-cell embryo (Boroviak, et al., 2017). These events seem particularly prevalent when using multiple CRISPR/Cas nucleases which cleave in cis. The repair of the resulting two or more DSBs can result in the deletion of the intervening sequence (which is often the aim of the experiment) but also the inversion of the sequences can occur, as well as many surprising duplications and insertion events.

Interestingly, the prevalence of these deletions and rearrangements may have been underestimated since these complex events are often invisible to the molecular assays used to genotype the resulting mutations. Simple PCR-based genotyping strategies can be compromised by the deletion encompassing the primer binding sites. Short-read next generation sequencing technologies are not well equipped to detect and assess genomic inversions and duplications. Genomic technologies based on long-reads or more traditional assessment of target locus integrity by Southern blotting or fluorescent in-situ hybridisation analysis may help detection, but these methods are difficult to apply and may not very applicable for genotyping embryo biopsy material.

The difficulty in detecting large deletions has fuelled controversy surrounding the inter-homologue repair mechanism proposed when CRISPR/Cas9 nucleases are used to selectively ablate a pathogenic mutation present heterozygously (Ma, et al., 2017). It has been suggested that the inability to detect a rearranged or damaged mutant allele could lead to the misinterpretation that the allele has been repaired from the intact wild-type allele (Adikusuma, et al., 2018; Egli, et al., 2018), although follow-up analysis of the original study provided evidence arguing against this explanation (Ma, et al., 2018). At the moment we know too little about the dominant DNA repair machinery active within the early preimplantation embryos and these discussions highlight the requirement for further research to fully establish what repair events are likely, how they can be harnessed for therapeutic effect and how disadvantageous large deletions and rearrangements of the target locus can be detected.

On-site damage and biallelic modification

The high efficiency of CRISPR/Cas nucleases frequently leads to the mutagenesis of both autosomal copies of a target gene. Where a complete loss-of-function is therapeutic, this is,

of course, advantageous; however, there are frequently situations where only one copy of a gene needs to be addressed, in particular when trying to correct or ablate dominant heterozygous mutations. Although CRISPR/Cas nucleases can be designed against the mutated copy of a gene, the tolerance of Cas9 for small mismatches may make it challenging to design nucleases that can discriminate between a mutant copy and a normal copy of a gene (Figure 1D). Interestingly, in the human study which successfully applied CRISPR/Cas nucleases to correct a dominant heterozygous mutation in the *MYBPC3* gene (Ma, et al., 2017), the mutation chosen for this proof-of-concept study was a 4-bp deletion. This relatively large mutation allowed the nuclease to be designed specifically against this mutant allele, thus eliminating the risk of mutating the wild-type allele. The majority of disease-associated mutations, however, are single nucleotide changes where discrimination may be challenging, and mutagenesis of the normal copy of the gene or even reprocessing and subsequent mutagenesis of the correctly repaired mutation would be expected to occur at appreciable frequencies. Development of enzymes with a higher level of discrimination may help the selective correction of the mutant alleles. The developed Cas enzymes and orthologues with reported higher accuracy may be very useful in this context (Kim, et al., 2016; Kleinstiver, et al., 2016; Slaymaker, et al., 2016; Teng, et al., 2018).

Another approach to help improve the predictability of gene editing outcomes is emerging from the analysis of large numbers of mutagenesis events. It has become clear that, for a specific target sequence, certain mutational outcomes can be quite common. Part of the explanation for this lies in regions of micro-homology that lie upstream and downstream of the target site (Bae, et al., 2014), but recent studies have identified other key attributes of the underlying primary sequence that can be used to predict the predominant pattern of repair (Allen, et al., 2018; Chakrabarti, et al., 2018; Shen, et al., 2018; Taheri-Ghahfarokhi, et

al., 2018). These studies demonstrate the power of using machine learning tools and large data set analysis to help make CRISPR/Cas9 mutagenesis more predictive. With the help of these new tools, it could be possible to achieve the desired repair of a mutation simply by design considerations alone. Indeed, one of the recent papers in this area has already confirmed the feasibility of this approach by using the predictive outcome of DSB processing to repair a pathogenic mutation (Shen, et al., 2018).

The reported birth of gene edited twins

Despite technical improvements addressing the shortcomings, there remains uncertainty about the prevalence, extent and detection of genomic damage and mosaicism. Considerable research and technical development is needed to quantify and address the issues before therapeutic gene editing can be considered. Given these unresolved safety concerns, it was alarming to hear the reports emerging late last year from the Southern University of Science and Technology, Shenzhen, China, which suggested that human embryos had been manipulated by CRISPR/Cas9, reimplanted into the mother and carried to term. The principal scientist involved in this study, Dr. He Jiankui, reported his results at the Second International Summit on Human Genome Editing in Hong Kong (National Academies of Sciences, et al., 2019), and described the birth of twin girls, Lula and Nana, who both carried CRISPR/Cas9 engineered mutations. Dr. Jiankui attempted to introduce loss-of-function mutations into the gene encoding the CCR5 chemokine receptor, a co-receptor for certain subtypes of HIV virus.

A naturally occurring *CCR5* variant involving a deletion of 32 bp, introduces a premature STOP codon into the gene, resulting in the expression of a truncated protein which is not able to act as an HIV co-receptor. Subsequently, individuals homozygous for this so called Δ32 mutation are resistant to infection by certain HIV subtypes. There has been wide interest in

this mutation, since an HIV-infected patient has been effectively cured of viral infection by an allogeneic stem-cell transplantation with haematopoietic stem cells from a donor homozygous for this Δ32 CCR5 variant (Hutter, et al., 2009). The aim of the Dr. Jiankui's study was to engineer loss-of-function mutations within the *CCR5* gene in human embryos generated by IVF from parents where the father was infected with HIV. In doing so, the goal was to protect the resulting embryos from HIV infection.

Flawed scientific rationale and experimental design

The scientific rationale behind the study is questionable for a number of reasons. Firstly, there are established protocols involving semen washing which can be used to reduce the risk of infection when using HIV infected semen in assisted reproductive therapy (Zafer, et al., 2016). There appears no need to invoke genome editing for this purpose. Secondly, CCR5 is a co-receptor for one subtype of HIV; a different chemokine receptor, CXCR4, can also act as a co-receptor for different classes of HIV. In patients with *CCR5* mutations, CXCR4-tropic HIV subtypes can, albeit inefficiently, enter the cells through this alternative receptor which would continue to be expressed (Agrawal, et al., 2004). Potentially the edited offspring would thus still be susceptible to HIV infection, despite engineered mutations in their *CCR5* receptor gene.

With respect to the experimental design, the genome engineering strategy adopted did not involve the incorporation of the naturally occurring Δ32 mutation, despite the fact that a previous study in human embryos showed successfully that the Δ32 mutation could be incorporated at the *CCR5* gene with a repair template (Kang, et al., 2016). Instead, a random mutagenesis approach was adopted, albeit at the same position within the gene, which would be expected to lead to the incorporation of de-novo mutations within the gene. The biological

consequences of these novel mutations are impossible to predict and could lead to a global *CCR5* knock-out by affecting mRNA or protein stability.

Importantly, it has been established that stable expression of the *CCR5* Δ32 variant is essential to convey HIV resistance (Agrawal, et al., 2007), thus mutations that cause a global knock-out might not lead to the immunity which was the primary goal of the study. More worrying, results from *Ccr5* knock-out mouse models are revealing that there may be other consequences of CCR5 loss-of-function, besides HIV entry. *Ccr5* loss-of-function led to an increased severity following infection of influenza virus (Falcon, et al., 2015) and West Nile virus (Durrant, et al., 2015) and have implicated *CCR5* function in neuronal plasticity (Zhou, et al., 2016) and recovery after brain injury (Joy, et al., 2019). Another recent study explored human UK Biobank data to assess the impact of the *CCR5* Δ32 variant on longevity and reported an estimated 21% increase in mortality for individuals homozygous for this mutation (Wei, et al., 2019). Mutations at *CCR5*, especially those with uncharacterised consequences on protein stability and expression, might thus be expected to have unpredictable and disadvantageous consequences.

Genotyping data from Dr. Jiankui's study

The results of Dr. Jiankui's study remain unpublished and thus the primary data has not been peer reviewed, making it difficult to assess the study. However, from his presentation at the Second International Summit on Human Genome Editing, we can learn some of the genotyping approaches he adopted in an attempt to mitigate the known problems of CRISPR/Cas nucleases outlined above.

Following CRISPR/Cas9 injection, the embryos were cultured to the blastocyst stage in vitro and the *CCR5* genotype was then assessed by pre-implantation genetic diagnosis (PGD) using

whole genome sequencing on trophectoderm biopsies. Two embryos were selected for transplantation: the first of which (Lulu) harboured +1 bp and -4 bp frameshift alleles, predicted to encode truncation mutations that are similar but different from the natural occurring Δ32 mutation. As discussed above, the biological activity of these mutations is completely unclear.

The second embryo (Nana) harboured a 15 bp deletion on one allele, while the other allele remained unedited. This 15 bp deletion results in an in-frame deletion that would effectively remove 5 amino-acids from the mature CCR5 peptide chain. Dr. Jiankui hypothesised that this allele might destabilise the CCR5 structure near to the HIV binding site, but no experimental validation of this assumption was presented. The in-frame deletion would almost certainly result in a mature protein of completely unknown function; one could envisage a dominant effect which might compromise the function of organ systems in which CCR5 is known to play a role.

The fact that an embryo with an in-frame deletion and with a remaining intact copy of the *CCR5* gene were selected for implantation is highly questionable. The unedited *CCR5* allele would still be expressed and thus a normal version of this chemokine receptor would remain on the surface of T-cells, thus rendering the cells infectable by HIV, at odds with the primary goal of the study.

Dr. Jiankui highlighted in his presentation that the parents made the decision concerning whether this embryo should be implanted, although it is entirely unclear how the parents were advised or counselled. Indeed, in the question and answer session that followed his presentation, it became clear that the scientists directly involved in the study may have

performed the genetic counselling of the parents, rather than correctly qualified and trained genetic counsellors.

Known problems of CRISPR/Cas mutagenesis were addressed

Concerning off-target site mutagenesis, theoretical off-target sites were bioinformatically assessed using the parental genomes to allow the consequence of SNP variation to be considered. As mentioned above, naturally occurring genome variation has the potential to alter the off-target landscape substantially (Lessard, et al., 2017) and it is interesting that this study did indeed ascertain a personalised genome for this purpose. These theoretical off-targets were combined with experimentally reported off-target sites from published in-vitro experiments which used the same target site to establish a panel of risk sites within the genome. PGD using whole genome sequencing on biopsied cells from the embryos revealed the presence of a single intergenic off-target site within Lulu's genome. A decision was thus made to knowingly implant an embryo harbouring a CRISPR/Cas induced mutation at an off-target site; although localised to an intergenic region of the genome, the functional consequences of this off-target mutation are unclear. Intriguingly, this off-target was no longer detected in fetal DNA analysis obtained from maternal blood during gestation and in the cord and placental samples obtained at birth, indicating that the initial PGD result might have been an artefact of whole genome amplification or reflect a low-level mosaicism in the trophectoderm cells biopsied.

Concerning mosaicism, the CRISPR/Cas9 reagents were applied during the ICSI fertilisation procedure using the same approach as adopted previously (Ma, et al., 2017). Similarly, despite what appears to be mosaic sequence traces in the PGD Sanger sequencing, the results of the

whole genome sequences revealed equal proportions of two alleles in each of the implanted embryos, suggesting that the embryos were not genetic mosaics.

Concerning large deletion analysis, the presence of large deletions was investigated by searching for chimeric sequencing reads arising from two regions of the genome in cis. Interestingly, in one edited embryo, not selected for implantation, a 6 kb deletion at the *CCR5* target site was indeed found, confirming the prevalence of this kind of repair outcome. The embryos chosen for implantation revealed no evidence of large deletions.

Despite these relatively thorough sequencing experiments which, to a degree, seek to mitigate the known problems of CRISPR/Cas mutagenesis outlined above, it is unclear how thorough and complete the analysis was. Without an in-depth assessment of the primary data, it is impossible to know whether the investigations were sufficient to completely rule out non-specific mutagenesis events, large deletions or genomic rearrangements.

Conclusions

Given the above technical and scientific issues, combined with the grave ethical concerns (Krimsky, 2019), it is not surprisingly that Dr. Jiankui's study has been widely condemned as being a reckless and premature use of the technology. As a direct result, experts in the field have called for a moratorium on germline editing (Lander, et al., 2019), which has been widely supported by the scientific community. It is important to recognise however that this moratorium concerns the implantation of edited embryos. Indeed, many scientists, clinicians, patient groups and ethicists support that research is needed to understand and address the risks involved. There is thus an understanding that this research may necessitate the use of human embryos, and the argument has been made that intentionally refraining from engaging in life-saving research is not morally defensible (Savulescu, et al., 2015). However,

at this point in time, at the very beginning of this emerging field with many unknowns, the implantation of edited embryos cannot be justified.

Research is needed to fully understand the repair outcomes occurring following the action of CRISPR/Cas9 nucleases within the early embryo, especially those involving repair templates. Further improvements in the accuracy of nucleases by either mutagenesis or molecular evolution, or by mining the bacterial and archeal kingdoms for alternative more accurate enzymes, would be advantageous. Improving methodologies for the detection of off-target mutation and large deletion and rearrangement events is also an area where continued research would be beneficial. Furthermore, a more thorough examination of the effects of base editing technology would also be of considerable interest.

PGD provides one alternative strategy for combatting genetic disease. However, there are concerns that, with reproductive age increasing in the Western world and given that PGD is known to impact reproductive success (Steffann, et al., 2018), the number of viable embryos from which healthy individuals can be selected may frequently be too low for PGD selection to provide an effective solution. There are, of course, also situations where PGD cannot provide a solution, for example, where one patient is homozygous for a mutation. It is thus not too far-fetched to imagine a growing necessity to consider human germline editing in the future. It is thus clear that, in parallel with research addressing the safety concerns, the debate exploring ethical aspects of human germline editing must continue.

Funding

The author is supported by a Wellcome Trust Core Award Grant (203141/Z/16/Z)

Conflict of interest

The author has nothing to declare.

References

Adikusuma F, Piltz S, Corbett MA, Turvey M, McColl SR, Helbig KJ, Beard MR, Hughes J, Pomerantz RT, Thomas PQ. Large deletions induced by Cas9 cleavage. *Nature* 2018; **560**: E8-E9.

Agrawal L, Jin Q, Altenburg J, Meyer L, Tubiana R, Theodorou I, Alkhatib G. CCR5Delta32 protein expression and stability are critical for resistance to human immunodeficiency virus type 1 in vivo. *J Virol* 2007; **81**: 8041-8049.

Agrawal L, Lu X, Qingwen J, VanHorn-Ali Z, Nicolescu IV, McDermott DH, Murphy PM, Alkhatib G. Role for CCR5Delta32 protein in resistance to R5, R5X4, and X4 human immunodeficiency virus type 1 in primary CD4+ cells. *J Virol* 2004; **78**: 2277-2287.

Allen F, Crepaldi L, Alsinet C, Strong AJ, Kleshchevnikov V, De Angeli P, Palenikova P, Khodak A, Kiselev V, Kosicki M *et al.* Predicting the mutations generated by repair of Cas9-induced double-strand breaks. *Nature biotechnology* 2018.

Amoasii L, Hildyard JCW, Li H, Sanchez-Ortiz E, Mireault A, Caballero D, Harron R, Stathopoulou TR, Massey C, Shelton JM *et al.* Gene editing restores dystrophin expression in a canine model of Duchenne muscular dystrophy. *Science* 2018; **362**: 86-91.

Anderson KR, Haeussler M, Watanabe C, Janakiraman V, Lund J, Modrusan Z, Stinson J, Bei Q, Buechler A, Yu C *et al.* CRISPR off-target analysis in genetically engineered rats and mice. *Nat Methods* 2018; **15**: 512-514.

Bae S, Kweon J, Kim HS, Kim JS. Microhomology-based choice of Cas9 nuclease target sites. *Nat Methods* 2014; **11**: 705-706.

Boroviak K, Fu B, Yang F, Doe B, Bradley A. Revealing hidden complexities of genomic rearrangements generated with Cas9. *Scientific reports* 2017; **7**: 12867.

Carroll D. Genome Editing: Past, Present, and Future. *The Yale journal of biology and medicine* 2017; **90**: 653-659.

Chakrabarti AM, Henser-Brownhill T, Monserrat J, Poetsch AR, Luscombe NM, Scaffidi P. Target-Specific Precision of CRISPR-Mediated Genome Editing. *Mol Cell* 2018.

Chen Y, Liu X, Zhang Y, Wang H, Ying H, Liu M, Li D, Lui KO, Ding Q. A self-restricted CRISPR system to reduce off-target effects. *Molecular Therapy* 2016; **24**: 1508-1510.

Durrant DM, Daniels BP, Pasieka T, Dorsey D, Klein RS. CCR5 limits cortical viral loads during West Nile virus infection of the central nervous system. *Journal of neuroinflammation* 2015; **12**: 233.

426 Egli D, Zuccaro MV, Kosicki M, Church GM, Bradley A, Jasin M. Inter-homologue repair in
427 fertilized human eggs? *Nature* 2018; **560**: E5-E7.

428 Falcon A, Cuevas MT, Rodriguez-Frandsen A, Reyes N, Pozo F, Moreno S, Ledesma J,
429 Martinez-Alarcon J, Nieto A, Casas I. CCR5 deficiency predisposes to fatal outcome in
430 influenza virus infection. *The Journal of general virology* 2015; **96**: 2074-2078.

431 Fogarty NME, McCarthy A, Snijders KE, Powell BE, Kubikova N, Blakeley P, Lea R, Elder K,
432 Wamaitha SE, Kim D *et al.* Genome editing reveals a role for OCT4 in human embryogenesis.
433 *Nature* 2017; **550**: 67-73.

434 Fu Y, Foden JA, Khayter C, Maeder ML, Reyon D, Joung JK. High-frequency off-target
435 mutagenesis induced by CRISPR-Cas nucleases in human cells. *Nature biotechnology* 2013;
436 **31**.

437 Haeussler M, Schonig K, Eckert H, Eschstruth A, Mianne J, Renaud JB, Schneider-Maunoury
438 S, Shkumatava A, Teboul L, Kent J *et al.* Evaluation of off-target and on-target scoring
439 algorithms and integration into the guide RNA selection tool CRISPOR. *Genome Biol* 2016;
440 **17**: 148.

441 Hashimoto M, Yamashita Y, Takemoto T. Electroporation of Cas9 protein/sgRNA into early
442 pronuclear zygotes generates non-mosaic mutants in the mouse. *Dev Biol* 2016; **418**: 1-9.

443 Hodgkins A, Farne A, Perera S, Grego T, Parry-Smith DJ, Skarnes WC, Iyer V. WGE: a CRISPR
444 database for genome engineering. *Bioinformatics* 2015; **31**: 3078-3080.

445 Hutter G, Nowak D, Mossner M, Ganepola S, Mussig A, Allers K, Schneider T, Hofmann J,
446 Kucherer C, Blau O *et al.* Long-term control of HIV by CCR5 Delta32/Delta32 stem-cell
447 transplantation. *N Engl J Med* 2009; **360**: 692-698.

448 Iyer V, Boroviak K, Thomas M, Doe B, Riva L, Ryder E, Adams DJ. No unexpected CRISPR-Cas9
449 off-target activity revealed by trio sequencing of gene-edited mice. *PLoS genetics* 2018; **14**:
450 e1007503.

451 Joy MT, Ben Assayag E, Shabashov-Stone D, Liraz-Zaltsman S, Mazzitelli J, Arenas M,
452 Abduljawad N, Kliper E, Korczyn AD, Thareja NS *et al.* CCR5 Is a Therapeutic Target for
453 Recovery after Stroke and Traumatic Brain Injury. *Cell* 2019; **176**: 1143-1157.e1113.

454 Kang X, He W, Huang Y, Yu Q, Chen Y, Gao X, Sun X, Fan Y. Introducing precise genetic
455 modifications into human 3PN embryos by CRISPR/Cas-mediated genome editing. *Journal of*
456 *assisted reproduction and genetics* 2016; **33**: 581-588.

457 Kim D, Kim J, Hur JK, Been KW, Yoon SH, Kim JS. Genome-wide analysis reveals specificities
458 of Cpf1 endonucleases in human cells. *Nature biotechnology* 2016; **34**: 863-868.

459 Kim S, Kim D, Cho SW, Kim J, Kim JS. Highly efficient RNA-guided genome editing in human
460 cells via delivery of purified Cas9 ribonucleoproteins. *Genome Res* 2014; **24**: 1012-1019.

461 Kleinstiver BP, Pattanayak V, Prew MS, Tsai SQ, Nguyen NT, Zheng Z, Joung JK. High-fidelity
462 CRISPR–Cas9 nucleases with no detectable genome-wide off-target effects. *Nature* 2016;
463 **529**: 490-495.

464 Kocak DD, Josephs EA, Bhandarkar V, Adkar SS, Kwon JB, Gersbach CA. Increasing the
465 specificity of CRISPR systems with engineered RNA secondary structures. *Nature*
466 *biotechnology* 2019.

Kosicki M, Tomberg K, Bradley A. Repair of double-strand breaks induced by CRISPR-Cas9 leads to large deletions and complex rearrangements. *Nature biotechnology* 2018; **36**: 765-771.

Krimsky S. Ten ways in which He Jiankui violated ethics. *Nature biotechnology* 2019; **37**: 19-20.

Lander ES, Baylis F, Zhang F, Charpentier E, Berg P, Bourgain C, Friedrich B, Joung JK, Li J, Liu D *et al.* Adopt a moratorium on heritable genome editing. *Nature* 2019; **567**: 165-168.

Lessard S, Francioli L, Alfoldi J, Tardif JC, Ellinor PT, MacArthur DG, Lettre G, Orkin SH, Canver MC. Human genetic variation alters CRISPR-Cas9 on- and off-targeting specificity at therapeutically implicated loci. *Proc Natl Acad Sci U S A* 2017; **114**: E11257-e11266.

Li G, Liu Y, Zeng Y, Li J, Wang L, Yang G, Chen D, Shang X, Chen J, Huang X *et al.* Highly efficient and precise base editing in discarded human tripronuclear embryos. *Protein & cell* 2017; **8**: 776-779.

Liang P, Ding C, Sun H, Xie X, Xu Y, Zhang X, Sun Y, Xiong Y, Ma W, Liu Y *et al.* Correction of beta-thalassemia mutant by base editor in human embryos. *Protein & cell* 2017; **8**: 811-822.

Liang P, Sun H, Zhang X, Xie X, Zhang J, Bai Y, Ouyang X, Zhi S, Xiong Y, Ma W *et al.* Effective and precise adenine base editing in mouse zygotes. *Protein & cell* 2018; **9**: 808-813.

Liang P, Xu Y, Zhang X, Ding C, Huang R, Zhang Z, Lv J, Xie X, Chen Y, Li Y *et al.* CRISPR/Cas9-mediated gene editing in human tripronuclear zygotes. *Protein & cell* 2015; **6**: 363-372.

Long C, McAnally JR, Shelton JM, Mireault AA, Bassel-Duby R, Olson EN. Prevention of muscular dystrophy in mice by CRISPR/Cas9-mediated editing of germline DNA. *Science* 2014; **345**: 1184-1188.

Ma H, Marti-Gutierrez N, Park S-W, Wu J, Hayama T, Darby H, Van Dyken C, Li Y, Koski A, Liang D *et al.* Ma et al. reply. *Nature* 2018; **560**: E10-E23.

Ma H, Marti-Gutierrez N, Park SW, Wu J, Lee Y, Suzuki K, Koski A, Ji D, Hayama T, Ahmed R *et al.* Correction of a pathogenic gene mutation in human embryos. *Nature* 2017; **548**: 413-419.

National Academies of Sciences E, Medicine, Policy, Global A. The National Academies Collection: Reports funded by National Institutes of Health *Second International Summit on Human Genome Editing: Continuing the Global Discussion: Proceedings of a Workshop-in Brief*. 2019. National Academies Press (US)

Nelson CE, Hakim CH, Ousterout DG, Thakore PI, Moreb EA, Castellanos Rivera RM, Madhavan S, Pan X, Ran FA, Yan WX *et al.* In vivo genome editing improves muscle function in a mouse model of Duchenne muscular dystrophy. *Science* 2016; **351**: 403-407.

Parikh BA, Beckman DL, Patel SJ, White JM, Yokoyama WM. Detailed phenotypic and molecular analyses of genetically modified mice generated by CRISPR-Cas9-mediated editing. *PLoS One* 2015; **10**: e0116484.

Porteus MH. A New Class of Medicines through DNA Editing. *N Engl J Med* 2019; **380**: 947-959.

Rees HA, Liu DR. Base editing: precision chemistry on the genome and transcriptome of living cells. *Nat Rev Genet* 2018; **19**: 770-788.

508 Sander JD, Joung JK. CRISPR-Cas systems for editing, regulating and targeting genomes.
509 *Nature biotechnology* 2014; **32**: 347-355.

510 Savulescu J, Pugh J, Douglas T, Gyngell C. The moral imperative to continue gene editing
511 research on human embryos. *Protein & cell* 2015; **6**: 476-479.

512 Shen C-C, Hsu M-N, Chang C-W, Lin M-W, Hwu J-R, Tu Y, Hu Y-C. Synthetic switch to
513 minimize CRISPR off-target effects by self-restricting Cas9 transcription and translation.
514 *Nucleic acids research* 2018.

515 Shen MW, Arbab M, Hsu JY, Worstell D, Culbertson SJ, Krabbe O, Cassa CA, Liu DR, Gifford
516 DK, Sherwood RI. Predictable and precise template-free CRISPR editing of pathogenic
517 variants. *Nature* 2018; **563**: 646-651.

518 Shen MW, Arbab M, Hsu JY, Worstell D, Culbertson SJ, Krabbe O, Cassa CA, Liu DR, Gifford
519 DK, Sherwood RI. Predictable and precise template-free CRISPR editing of pathogenic
520 variants. *Nature* 2018.

521 Shin HY, Wang C, Lee HK, Yoo KH, Zeng X, Kuhns T, Yang CM, Mohr T, Liu C, Hennighausen L.
522 CRISPR/Cas9 targeting events cause complex deletions and insertions at 17 sites in the
523 mouse genome. *Nature communications* 2017; **8**: 15464.

524 Slaymaker IM, Gao L, Zetsche B, Scott DA, Yan WX, Zhang F. Rationally engineered Cas9
525 nucleases with improved specificity. Science 2016; 351: 84-88.

526 Steffann J, Jouannet P, Bonnefont JP, Chneiweiss H, Frydman N. Could Failure in
527 Preimplantation Genetic Diagnosis Justify Editing the Human Embryo Genome? Cell stem
528 cell 2018; 22: 481-482.

529 Taheri-Ghahfarokhi A, Taylor BJM, Nitsch R, Lundin A, Cavallo AL, Madeyski-Bengtson K,
530 Karlsson F, Clausen M, Hicks R, Mayr LM et al. Decoding non-random mutational signatures
531 at Cas9 targeted sites. Nucleic Acids Res 2018; 46: 8417-8434.

532 Tang L, Zeng Y, Du H, Gong M, Peng J, Zhang B, Lei M, Zhao F, Wang W, Li X et al.
533 CRISPR/Cas9-mediated gene editing in human zygotes using Cas9 protein. Molecular
534 genetics and genomics : MGG 2017; 292: 525-533.

535 Tang L, Zeng Y, Zhou X, Du H, Li C, Liu J, Zhang P. Highly efficient ssODN-mediated homology-
536 directed repair of DSBs generated by CRISPR/Cas9 in human 3PN zygotes. Mol Reprod Dev
537 2018; 85: 461-463.

538 Teng F, Cui T, Feng G, Guo L, Xu K, Gao Q, Li T, Li J, Zhou Q, Li W. Repurposing CRISPR-Cas12b
539 for mammalian genome engineering. Cell discovery 2018; 4: 63.

540 Tu Z, Yang W, Yan S, Yin A, Gao J, Liu X, Zheng Y, Zheng J, Li Z, Yang S et al. Promoting Cas9
541 degradation reduces mosaic mutations in non-human primate embryos. Scientific reports
542 2017; 7: 42081.

543 Vakulskas CA, Dever DP, Rettig GR, Turk R, Jacobi AM, Collingwood MA, Bode NM, McNeill
544 MS, Yan S, Camarena J et al. A high-fidelity Cas9 mutant delivered as a ribonucleoprotein
545 complex enables efficient gene editing in human hematopoietic stem and progenitor cells.
546 Nat Med 2018; 24: 1216-1224.

547 van Dijke I, Bosch L, Bredenoord AL, Cornel M, Repping S, Hendriks S. The ethics of clinical
548 applications of germline genome modification: a systematic review of reasons. Human
549 reproduction (Oxford, England) 2018; 33: 1777-1796.

550 Wang H, Yang H, Shivalila CS, Dawlaty MM, Cheng AW, Zhang F, Jaenisch R. One-step
551 generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome
552 engineering. Cell 2013; 153: 910-918.

553 Wei X, Nielsen R. CCR5-Δ32 is deleterious in the homozygous state in humans. Nature
554 Medicine 2019; 25: 909-910.

555 Yang Y, Wang L, Bell P, McMenamin D, He Z, White J, Yu H, Xu C, Morizono H, Musunuru K et
556 al. A dual AAV system enables the Cas9-mediated correction of a metabolic liver disease in
557 newborn mice. Nature biotechnology 2016; 34: 334-338.

558 Yao X, Zhang M, Wang X, Ying W, Hu X, Dai P, Meng F, Shi L, Sun Y, Yao N et al. Tild-CRISPR
559 Allows for Efficient and Precise Gene Knockin in Mouse and Human Cells. Developmental
560 cell 2018; 45: 526-536.e525.

561 Yen S-T, Zhang M, Deng JM, Usman SJ, Smith CN, Parker-Thornburg J, Swinton PG, Martin JF,
562 Behringer RR. Somatic mosaicism and allele complexity induced by CRISPR/Cas9 RNA
563 injections in mouse zygotes. Developmental Biology 2014; 393: 3-9.

564 Zafer M, Horvath H, Mmeje O, van der Poel S, Semprini AE, Rutherford G, Brown J.
565 Effectiveness of semen washing to prevent human immunodeficiency virus (HIV)
566 transmission and assist pregnancy in HIV-discordant couples: a systematic review and meta-
567 analysis. Fertility and sterility 2016; 105: 645-655.e642.

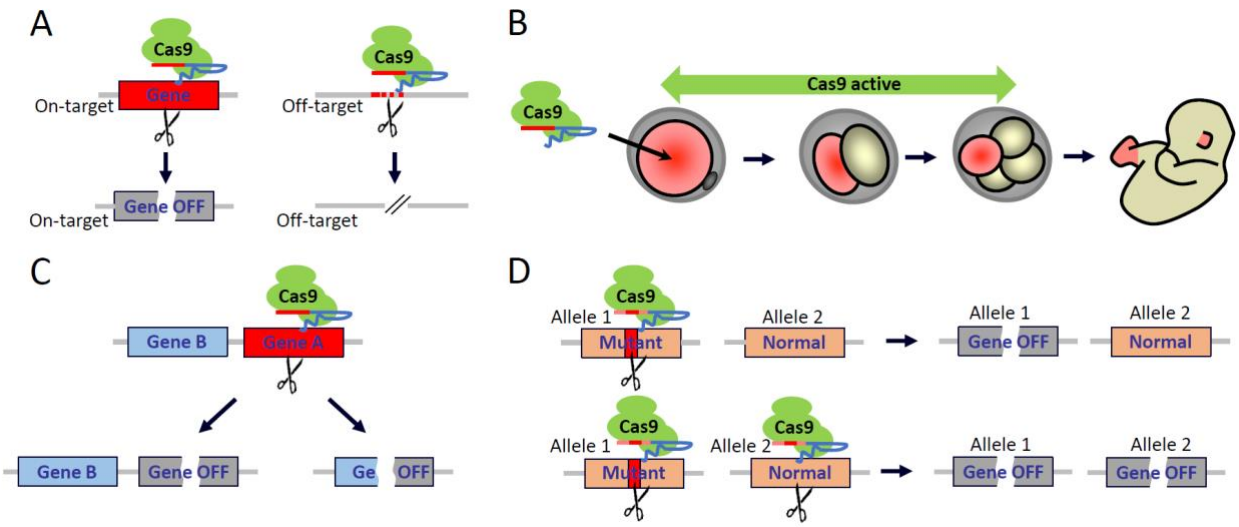
568 Zeng Y, Li J, Li G, Huang S, Yu W, Zhang Y, Chen D, Chen J, Liu J, Huang X. Correction of the
569 Marfan Syndrome Pathogenic FBN1 Mutation by Base Editing in Human Cells and
570 Heterozygous Embryos. Mol Ther 2018; 26: 2631-2637.

571 Zhou M, Greenhill S, Huang S, Silva TK, Sano Y, Wu S, Cai Y, Nagaoka Y, Sehgal M, Cai DJ et al.
572 CCR5 is a suppressor for cortical plasticity and hippocampal learning and memory. Elife
573 2016; 5.

574 Zuo E, Sun Y, Wei W, Yuan T, Ying W, Sun H, Yuan L, Steinmetz LM, Li Y, Yang H. Cytosine
575 base editor generates substantial off-target single-nucleotide variants in mouse embryos.
576 Science 2019; 364: 289-292.

577 Zuris JA, Thompson DB, Shu Y, Guilinger JP, Bessen JL, Hu JH, Maeder ML, Joung JK, Chen Z-
578 Y, Liu DR. Cationic lipid-mediated delivery of proteins enables efficient protein-based
579 genome editing in vitro and in vivo. Nature biotechnology 2015; 33: 73.

580



581

582 **Figure Legends**

583 **Figure 1 - Disadvantageous outcomes of CRISPR/Cas mutagenesis within the 1-cell**

584 **embryo.**

585 A) Off-target mutagenesis. In addition to the correct mutagenesis event, similar sequences

586 elsewhere in the genome are also mutated leading to unpredictable effects. B) Mosaicism.

587 Prolonged activity of the nuclease within the developing embryo can lead to different

588 mutations in different parts of the resulting individual. C) Large deletions. The left panel

589 shows the intended mutagenesis event where a target gene is inactivated. The right panel

590 shows a potential consequence of a large deletion event, where a neighbouring gene is also

591 inactivated. D) On-site damage. The top panel shows the intended mutagenesis event, with

592 a CRISPR/Cas nuclease specifically recognising only the mutant allele, leading to its

593 inactivation. The bottom panel shows the biallelic mutation that could occur if the

594 CRISPR/Cas nuclease is not able to discriminate the mutant sequence from the normal

595 sequence.

596