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Commentary

Reproductive medicine involving genome editing: clinical uncertainties and embryological needs

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A B S T R A C T

Genome editing based on site-directed nucleases facilitated efficient and versatile genetic modifications in human cells. However, recent reports, demonstrating CRISPR/Cas9-mediated genome editing in human embryos have raised profound concerns worldwide. This commentary explores the clinical justification and feasibility of reproductive medicine using germline genome editing. Despite the perceived utility of reproductive medicine for treating intractable infertility, it is difficult to justify germline genome editing from the perspective of the prospective child. As suggested by the UK legalization regarding mitochondrial donation, the prevention of genetic disease in offspring by genome editing might be acceptable in limited cases of serious or life-threatening conditions, where no alternative medicine is available. Nonetheless, the mosaicism underlying human embryos as well as the off-target effect by artificial nucleases will likely hamper preimplantation genetic diagnosis prior to embryo transfer. Such considerations suggest that this type of reproductive medicine should not be developed toward a clinical application. However, the clinical uncertainties underscore the need for embryology that can address fundamental questions regarding germline aneuploidy and mosaicism using genome editing.

Introduction

The genetic modification of germ cells or zygotes (germline) can impact the entire body of the progeny as well as subsequent generations via modified germ cells. For this reason, germline genetic modification has been considered to be effective against some genetic diseases. Transferring donor oocyte-derived cytoplasm (containing mitochondrial DNA (mtDNA)) to putatively non-viable oocytes or zygotes was practised in cases of unexplained infertility from the late 1990s to the early 2000s. However, such cytoplasmic transfers resulted in pregnancies affected with Turner syndrome (Barritt et al., 2001b), fetal deaths (Zhang, 2003) and the onset of pervasive developmental sisorder in progeny (Barritt et al., 2001b). Conversely, the UK has recently become the first country to allow the clinical use of karyoplast transfer to an enucleated donor oocyte or zygote (so-called mitochondrial donation) in order to prevent the inheritance of pathogenic mtDNA mutations in offspring (HFEA, 2015).

Genome editing tools, such as zinc-finger nucleases (ZFN), transcription activator-like effector nucleases (TALEN) and the clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9, have facilitated the insertion of an exogenous gene, correcting a gene mutation (or copying of a variant) and disrupting an endogenous gene in human cells. The artificial, site-directed nucleases can unintentionally break DNA double strands at non-target sites (Ishii, 2015b; Kim and Kim, 2014), although a recent clinical trial concluded that the infusion of T cells modified by ZFN is safe in HIV-positive patients, despite no investigation of off-target mutations in the infused cells (Tebas et al., 2014). With regard to germline genome editing, two groups recently reported that the microinjection of CRISPR/Cas9 into tripronuclear zygotes can produce human embryos with an intentional genetic modification, but also indicated three technical problems: low efficiency of on-target gene modification, off-target mutations and the mosaicism of genetic modification in the embryos (Kang et al., 2016; Liang et al., 2015). More recently, a non-human primate (NHP) study demonstrated that the microinjection of optimized ZFN/TALEN into zygotes can avoid the mosaicism of genetic modification in resultant monkeys, causing them to display immune-deficiency similar to human patients (Sato et al., 2016). These reports suggest that reproductive medicine involving genome editing is theoretically feasible although there are still concerns regarding the safety and efficacy related to its clinical use. However, the two human embryo editing studies raised serious concerns over its

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medical use and non-medical (social) use worldwide, prompting several global discussions such as the International Summit on Human Gene Editing (NASEM, 2015).

The present commentary discusses the two objectives of reproductive medicine involving germline genome editing: infertility treatment and disease prevention. Then, the clinical feasibility of such reproductive medicine is examined in terms of risk assessment. In addition, the wider implications of the findings are discussed in scientific contexts.

Infertility treatment

According to the latest report on the treatments involving assisted reproductive technology by the European Society of Human Reproduction and Embryology, pregnancy rates in 2011, while the overall number of assisted reproductive technology cycles has continued to increase, decreased slightly to those reported in 2010. For all IVF cycles, the clinical pregnancy rates per aspiration and per transfer were stable with 29.1 and 33.2%, respectively. Moreover, for intracytoplasmic sperm injection (ICSI), the corresponding rates were stable with 27.9 and 31.8%, respectively (Calhaz-Jorge et al., 2016). To enhance the assisted reproductive technology success rate, personalization is one of future directions (Simon, 2013). Since half of idiopathic infertility cases are considered to have a genetic basis (Singh and Schimenti, 2015), there is a tremendous need for personalized reproductive medicine, which may be achieved by correcting a mutation responsible for infertility through genome editing (Ishii, 2015a). For instance, human oocytes with a missense mutation in the *TUBB8* undergo developmental arrest after fertilization (Feng et al., 2016b). Currently, two relevant reports are available. In 2016, the first case report identified seven *TUBB8* mutations that were responsible for oocyte meiosis I arrest in seven of the 24 families, using exome sequencing (Feng et al., 2016a). The second report discovered nine new *TUBB8* mutations in 10 patients from nine families, displaying phenotypic variability (Feng et al., 2016b). Among them, oocytes having any of three missense mutations (I210V, T238M and N348S) are of particular note. Such oocytes could extrude the first polar body and could be fertilized, despite subsequent developmental arrest. Genome editing-mediated *TUBB8* correction in premature oocytes, such as GV stage oocytes, could recover their developmental potential, although the remaining transcripts from mutated *TUBB8* could disturb the formation of microtubule via de-novo synthesis of the functional protein. It should also be noted that genome editing-mediated gene correction has not been demonstrated in mammalian oocytes.

Moreover, *TEX11* mutations cause meiotic arrest and azoospermia in infertile males (Yatsenko et al., 2015). If spermatogonial stem cells (SSC) can be retrieved from the patient's testis, viable spermatozoa could be generated from genetically corrected SSC *in vitro* in the near future (Ishii, 2015a; Ishii and Pera, 2016). Of note, a recent report demonstrated that rat offspring were born using spermatozoa regenerated following the transplantation of CRISPR/Cas9-modified SSC (Chapman et al., 2015), although SSC transplantation is still experimental in humans.

However, its use is currently unjustifiable. First, genetic modification in humans is still in the early stages. Although at least 2356 clinical trials of somatic gene therapy have been conducted worldwide, fewer than 10 products have gone on to be approved (JGM, 2016). Second, very few cases of the clinical use of human germline genetic modification have been reported. Only ooplasmic transfer and pronuclear transfer were practised for treating intractable infertility by transferring donor oocyte-cytoplasm to a patient's oocyte, or by transferring karyoplast including pronuclei to an enucleated donor zygote (Barritt et al., 2001a; Ishii, 2015b; Zhang, 2003). Third, such cytoplasmic transfers are suspected to have imposed congenital anomalies upon resultant children in some cases. Ooplasmic transfer resulted in pregnancies affected with Turner syndrome, and the onset of Pervasive Developmental Disorder after birth, pronuclear transfer led to fetal deaths (Barritt et al., 2001b; Zhang, 2003). With regard to ooplasmic transfer, the Food and Drug Administration discussions in 2002 suggested that the cytoplasmic transfer caused inappropriate mitochondrial distribution in oocytes that led to such congenital anomalies. Given that germline genome editing can potentially affect progeny with the substantial risk of off-target effects, its development for infertility treatment, which will likely promote its widespread use, should be avoided from the perspective of the prospective child's welfare.

Disease prevention

Assisted reproductive techniques are practised with prior consent by parents. However, widely accepted assisted reproductive techniques such as IVF involve no intentional genetic intervention. Therefore, under what conditions does parental consent justify the germline genetic intervention from the viewpoint of a child's welfare?

Consider the UK regulatory framework on mitochondrial transfer (HFEA, 2015). Such intervention is deemed legal provided the germline modification focuses mtDNA (not nuclear DNA) and intends to prevent the maternal transmission of 'serious' mitochondrial disease to offspring. This is employed when a mother carries the risk of transmitting the disease to the child. Its practice is limited to serious conditions among various forms of mitochondrial disease. Similarly, disease prevention via germline genome editing might be accepted in some countries. Notably, in the International Summit on Human Gene Editing, mitochondrial disease was addressed as a candidate for germline genome editing (NASEM, 2015). Interestingly, the elimination of pathogenic mtDNA using mito TALEN (mitochondria-targeted nucleases to selectively reduce mtDNA haplotypes) requires no oocyte donation, which is indispensable for mitochondrial donation in the UK (Reddy et al., 2015). Aside from cases of serious mitochondrial disease, the use of germline genome editing seems compelling in cases of definite inheritance of a serious or life-threating autosomal genetic disorder where preimplantation genetic diagnosis (PGD) is inapplicable, such as autosomal dominant diseases in which one or both parents is homozygous (e.g. Huntington disease) or autosomal recessive diseases where both parents are homozygous (e.g. cystic fibrosis) (Ishii, 2015b). At least four reports have demonstrated that genome editing-mediated gene correction and the recovery of phenotypes are feasible in mouse and rat experiments (Ishii, 2015a).

In such rare cases, the benefits of genome editing for a prospective child is likely to exceed the risks, such as the failure of disease prevention and off-target mutations (Ishii, 2015a, 2015b). Therefore, parental consent for germline genome editing might be justifiable.

Clinical potential

Genome editing frequently results in precise genetic modifications at target sites in the genome, by introducing site-directed

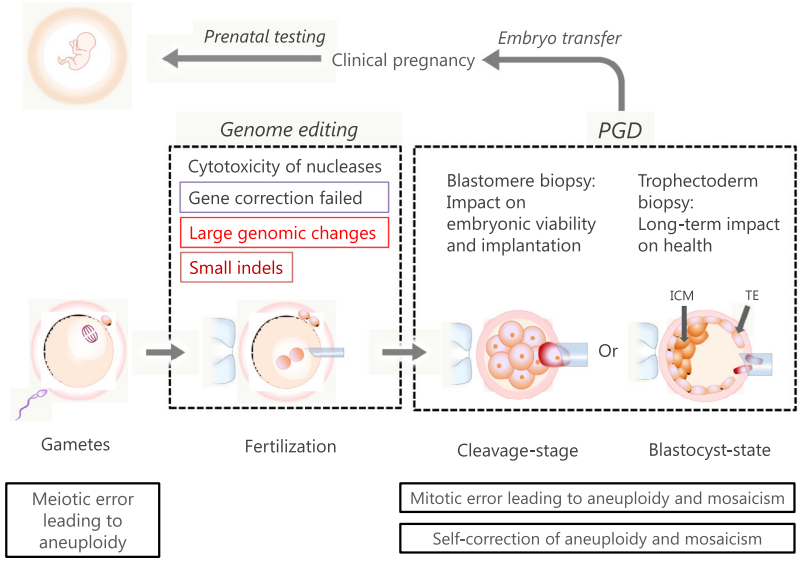

Figure 1 – The clinical uncertainties in reproductive medicine involving genome editing. This medicine is initiated by microinjecting artificial nucleases into human embryos. Subsequently, preimplantation genetic diagnosis (PGD) is carried out to investigate on-target modifications and no significant off-target effects (large genomic alternations and small indels). In dotted-line boxes, potential risks in each procedure are indicated. Bold-line boxes show the factors underlying the human germline, which will impact genome editing as well as PGD. ICM = inner cell mass; TE = trophectoderm.

nucleases into human cells. However, it is important to contemplate whether preventive medicine involving genome editing is truly feasible in clinical settings (**Figure 1**).

The introduction of nucleases manifests cytotoxicity against human embryos, potentially reducing the number of viable embryos (Kang et al., 2016; Liang et al., 2015). Applying mito TALEN to oocytes may fail to eliminate aberrant mtDNA under the threshold level of disease onset. In cases of autosomal dominant diseases, the microinjection of nucleases into zygotes may fail to correct bi-allelic mutations at the one-cell stage. Furthermore, the microinjection may also lead to mosaicism of genetic modification in the resultant embryos if gene correction initiates at the two-cell or later stages (Kang et al., 2016; Liang et al., 2015). Furthermore, the artificial nucleases could break DNA double strands at off-target sites for reasons such as the improper selection of a target sequence. Subsequently, off-target double-strand breaks could induce large-scale genomic alternations such as translocations, inversions and large deletions in addition to small insertions or deletions (indels) of various lengths, including point mutations (Kim and Kim, 2014; Liang et al., 2015). Although the above-mentioned concerns have been frequently addressed in somatic cell genome editing, these are also the case in the germline.

As the NHP experiment demonstrated, such risks are expected to be reduced by the careful design of the guiding molecule (zinc-finger domains in ZFN, TALE in TALEN and guide RNA in CRISPR/Cas9) and prior profiling of genome-wide off-target effect (Sato et al., 2016). However, the risks with genome editing will be substantial in clinical settings because the subjects are of human origin, not laboratory animals (Kang et al., 2016; Liang et al., 2015). Therefore, PGD following genome editing is mandatory prior to embryo transfer (Ishii, 2015a). PGD using a single blastomere biopsied from cleavage-stage embryos is common in assisted reproductive technology centres. However, this diagnosis is performed using the low amount of initial starting DNA, which might lead to a misdiagnosis for reasons such as allele drop-out. Instead, PGD should be performed using several cells biopsied from the trophectoderm at the blastocyst-stage (SenGupta et al., 2016). This type of PGD can more reliably detect bi-allelic modifications without large-scale genomic changes.

Nevertheless, clinical uncertainties may occur in the diagnosis. First, it would be difficult to detect small indels, including point mutations, arising from off-target effects in the embryonic genome. The use of next-generation sequencing (NGS), such as whole-genome or whole-exome sequencing, following whole genome amplification (WGA) is likely to be considered, because the indels created in the promotors and terminators as well as protein-coding regions may impact the gene expression and health of resultant children. However, the WGA introduces artefacts into the sample (SenGupta et al., 2016). Additionally, it is likely that NGS can hardly distinguish small indels from a single nucleotide polymorphism (SNP) or spontaneous mutations (Liang et al., 2015). Furthermore, chromosome instability is common even in embryos derived from young fertile women. In addition to mosaicism for whole chromosome aneuploidies and uniparental disomies, segmental chromosomal imbalances frequently occur in cleavage-stage embryos (Vanneste et al., 2009). The microinjection of artificial nucleases into human embryos with chromosomal instability may also increase the rate of chromosomal breakage and aneuploidy via off-target effects. The aberrant genomic constitutions and mosaicism are likely to produce misleading PGD results in blastocyst-stage embryos. Even if PGD can confirm that the biopsied trophectoderm cells have corrected genes and no significant chromosomal abnormalities, the genetic condition in the inner cell mass (ICM) may be different from that of the biopsied cells (Taylor et al., 2014).

Such considerations suggest the difficulties in risk assessment following germline genome editing in clinics. Nonetheless, one might assert that clinical management using amniotic diagnosis or chorionic villus sampling can be performed after embryo transfer and subsequent pregnancy since the benefits of genome editing will be likely to exceed the risks. However, the reproductive medicine may

fail to prevent the onset of certain diseases in offspring due to chromosomal mosaicism in the ICM. As the two reports on the CRISPR/Cas9 treatment of tripronuclear zygotes (Kang et al., 2016; Liang et al., 2015), genome editing may result in low efficiency of gene correction in the polysomic cells, which may lead to the onset of an autosomal dominant disease in offspring. Additionally, the resultant offspring may still be affected, or develop another disease after birth, in childhood or adulthood. Indeed, recent mouse studies have shown that offspring who underwent embryo biopsy are at a high risk of late-onset neurodevelopmental and metabolic diseases (Sampino et al., 2014; Yu et al., 2009).

Scientific implications

The aforementioned analysis also identified the lack of fundamental knowledge regarding mosaicism, which has been suggested to be as high as 70% and 90% in cleavage- and blastocyst-stage embryos derived from IVF, respectively (Taylor et al., 2014). Whole chromosome imbalances may arise due to the meiotic error. After fertilization, mitotic error can also cause chromosomal breakage fusion-bridge cycles, resulting in complex patterns of segmental aneuploidy at the cleavage stage (Vanneste et al., 2009). Simultaneously, such aneuploidies might be in part self-corrected, resulting in the mosaic embryo (Barbash-Hazan et al., 2009; Munne et al., 2005). Meanwhile, mitotic error at the cleavage stage can by itself lead to general or confined mosaicism at the blastocyst stage (Taylor et al., 2014).

It is scientifically and clinically essential to address questions about when, where and how aneuploidy, its self-correction and mosaicism arise in the human germline. Genome editing is useful in such embryological studies. For instance, EGFP-dead Cas9 (inactive form of enzyme) fusion can be used to map hot spots of chromosomal breakage through the visualization of loci harbouring repetitive sequences, such as telomeres (Chen et al., 2013). With regard to self-correction, previous studies have suggested that the actual fetus only derives from three cells of the ICM, whereas chromosomally abnormal cells can be forced away from the inner cell mass (Taylor et al., 2014). How then is the cell fate determined in human embryogenesis? In mouse embryos at the 8-cell stage, the polarized cells have actomyosin and an apical domain enriched with 'certain proteins' (Plusa and Hadjantonakis, 2016). As cells divide asymmetrically, one daughter cell with the apical domain becomes low contractile, whereas the other cell abundant in actomyosin becomes highly contractile. A gene-expression activated by Yap guides the low contractile cell to trophectoderm at the 32-cell stage. Highly contractile cells become the ICM. Certain proteins at the apical domain may be found using the SLENDER (single-cell labeling of endogenous proteins by CRISPRCas9-mediated homology-directed repair) method, which label proteins via CRISPR-Cas9-mediated homology-directed repair in embryonic cells (Mikuni et al., 2016). Previous research suggests that certain culture conditions and/or hormonal stimulation protocols may impact aneuploidy and subsequent mosaicism (Munne et al., 1997). In-vitro research using human embryos donated from patients or the NHP embryos, is expected to provide in-depth insight into aneuploidy and mosaicism by modifying culture conditions and utilizing CRISPR/Cas9-based platforms. Thus, versatile genome editing is expected to enhance our knowledge regarding the mosaicism in human early embryos.

Conclusions

Although genome editing is a robust genetic engineering tool, its straightforward use is, at present, unfeasible in clinical settings because our knowledge is still lacking regarding human genetic modifications as well as the genetics of the human germline. However, genome editing can be used to obtain fundamental knowledge regarding aneuploidy and mosaicisms, which will enhance genetic counselling to help infertile patients choose among reproductive options, contribute to clinical management and enhance the assisted reproductive technology success rate.

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