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Genome engineering through CRISPR/Cas9 technology in the human germline and pluripotent stem cells

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BACKGROUND: With the recent development of CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 genome editing technology, the possibility to genetically manipulate the human germline (gametes and embryos) has become a distinct technical possibility. Although many technical challenges still need to be overcome in order to achieve adequate efficiency and precision of the technology in human embryos, the path leading to genome editing has never been simpler, more affordable, and widespread.

OBJECTIVE AND RATIONALE: In this narrative review we seek to understand the possible impact of CRISR/Cas9 technology on human reproduction from the technical and ethical point of view, and suggest a course of action for the scientific community.

SEARCH METHODS: This non-systematic review was carried out using Medline articles in English, as well as technical documents from the Human Fertilisation and Embryology Authority and reports in the media. The technical possibilities of the CRISPR/Cas9 technology with regard to human reproduction are analysed based on results obtained in model systems such as large animals and laboratory rodents. Further, the possibility of CRISPR/Cas9 use in the context of human reproduction, to modify embryos, germline cells, and pluripotent stem cells is reviewed based on the authors' expert opinion. Finally, the possible uses and consequences of CRISPR/cas9 gene editing in reproduction are analysed from the ethical point of view.

OUTCOMES: We identify critical technical and ethical issues that should deter from employing CRISPR/Cas9 based technologies in human reproduction until they are clarified.

WIDER IMPLICATIONS: Overcoming the numerous technical limitations currently associated with CRISPR/Cas9 mediated editing of the human germline will depend on intensive research that needs to be transparent and widely disseminated. Rather than a call to a generalized moratorium, or banning, of this type of research, efforts should be placed on establishing an open, international, collaborative and regulated research framework. Equally important, a societal discussion on the risks, benefits, and preferred applications of the new technology, including all relevant stakeholders, is urgently needed and should be promoted, and ultimately guide research priorities in this area.

Key words: human embryo / CRISPR/Cas9 / genome editing / germline modification / stem cells / oocyte / sperm

Introduction

The possibility of editing the genome of cells in a targeted manner has its basis in the experimental observation that DNA constructs harbouring stretches of homology are able to interact and eventually integrate at the target site in the genome, assisted by the endogenous homologous recombination machinery of the cells (Thomas and Capecchi, 1986; Thomas *et al.*, 1986). While broadly successful in several cell types, until recently, DNA editing has been virtually unviable when targeting germline and human embryonic cells. However, the development of novel and highly efficient DNA editing tools such as CRISPR/Cas9 systems allow for fast, inexpensive, and precise gene editing. The possibility of creating permanent changes in the DNA of gametes and embryos, which will be inherited through the generations, have been met by the scientific community with differing attitudes, ranging from a call for a ban on human germline modification to cautious approval of further research (Hinxton group statement: statement on genome editing technologies and human germline genetic modification, www.hinxtongroup.org/hinxton2015_statement.pd). Meanwhile, the first report of an attempt of gene editing in a human embryo has been published, igniting further debate (Liang *et al.*, 2015a, b).

In this review, we analyse the issue of germline and embryo modification by reviewing the available literature on applications and potential uses of genome editing using CRISPR/Cas9, and report on the possible use of this technology in the reproductive field, as well as on the ethical implications it entails. The use of CRISPR/Cas9 technology for gene therapy on adult stem cells, which represents another important area of medicine where CRISPR/Cas9 editing will be of great value, and as recently demonstrated in haematopoietic stem cells (Mandal *et al.*, 2014), falls outside the scope of this paper and is not discussed.

Methods

The literature search for the preparation of this non-systematic review was carried out using Medline articles written in English language, as well as technical documents from the Human Fertilisation and Embryology Authority (HFEA) and reports in the media.

Technical aspects of gene editing

Historically, the use of gene targeting as a strategy to repair pathogenic mutations has been repeatedly proposed. For example, haemoglobin genes in haematopoietic cells were originally selected for gene targeting repair on the perspective that patients with thalassaemia and sickle cell anaemia could theoretically be treated with genome-edited repaired cells (Smithies *et al.*, 1985). The main hurdle arising from the early attempts at genome editing was the very poor targeting efficiency of the available techniques, which was highly dependent on the cell line and the specific locus to be targeted. The initial attempts to improve targeting efficiencies focused on the development of negative selectable markers and the identification of proteins of the endogenous homologous recombination machinery (Mansour *et al.*, 1988; Meyn, 1993). However, a significant breakthrough was achieved when researchers found that a dramatic increase in targeting efficiency could be obtained when double strand breaks (DSBs) adjacent to the integration sites were generated (Donoho *et al.*, 1998). Inspired by those results, sequence-specific nucleases (SSNs) such as engineered zinc finger nucleases, TALENS (transcription activator-like effector nucleases) and CRISPR (clustered regularly interspaced short palindromic repeats) were developed (Urnov *et al.*, 2005; Miller *et al.*, 2011). SSNs opened the possibility of achieving genome edition in more therapeutic contexts, such as *in vivo* genome editing of hepatocytes in living animals (Li *et al.*, 2011) or muscle of dystrophin knockout mice (Jinek *et al.*, 2012; Long *et al.*, 2016; Tabebordbar *et al.*, 2016). The application of SSNs, in particular CRISPR/Cas9, results in easy, inexpensive, precise, and efficient targeting, and the impressive development since their discovery has eventually allowed the manipulation of human embryos (Liang *et al.*, 2015a, b). This was proof of principle that genetic alteration of germline by direct manipulation of human embryos is feasible.

CRISPR/Cas9 endonuclease belongs to a prokaryotic adaptive immune system able to cleave infecting DNA and store short foreign sequences of incoming viruses and plasmids in the host genome of invaded bacteria. Such stored sequences when expressed as RNA molecules, called short crRNAs, interact with CRISPR/Cas9 endonucleases to search and locate complementary sequences and eventually generate DSBs sequence specifically as a first step towards target degradation (Mojica *et al.*, 2005; Barrangou *et al.*, 2007). A third element is necessary for the cleavage to occur, called tracrRNA. A fusion of the crRNA and tracRNA in a single RNA molecule, called guiding RNA (gRNA) is routinely used during genetic engineering tasks. The fourth requirement for CRISPR/Cas9 endonuclease to work is a so-called, PAM (protospacer adjacent motif) composed of the sequence NGG (Jinek *et al.*, 2012). The ability to guide an endonuclease to any given sequence using a short molecule of RNA makes the CRISPR/Cas9 system particularly suited to generate DSBs at will in virtually any target DNA. Once generated, DSBs activate and recruit non-homologous end joining (NHEJ) and homologous recombination enzymatic machineries to repair DNA injuries (Symington and Gautier, 2011). Upon mutation of one of the catalytic sites, CRISPR/Cas9 generates nicks instead of DSBs: since nicked DNA preferentially stimulates the endogenous recombination enzymatic activity over the NHEJ

pathway, wild type CRISPR/Cas9 has been predominantly used to generate mutations, as damaged DNA repaired through NHEJ occurs with higher mutagenicity (Cong *et al.*, 2013; Mali *et al.*, 2013b), while CRISPR/Cas9 single mutants (CRISPR/Cas9 nickases) are more suitable for gene targeting applications where a donor plasmid or oligonucleotide is cotransfected (Chen *et al.*, 2015). CRISPR/Cas9 null variants devoid of nuclease activity are routinely used for interference silencing of transcriptional activity when directed towards exons, or to deliver CRISPR/Cas9 fusion proteins to genomic promoters with specific functions such as transcriptional activation or repression (Bikard *et al.*, 2013; Qi *et al.*, 2013). CRISPR/Cas9 null variants can also be used as a probe to label sequence-specific genomic loci fluorescently without global DNA denaturation (Deng *et al.*, 2015) as well as for targeted epigenetic regulation (Thakore *et al.*, 2015). Other Cas endonucleases have been also demonstrated to be effective tools for DNA engineering tasks. In particular, Cas9 endonuclease from *Staphylococcus aureus* (SaCas9), whose open reading frame is considerably smaller, is a powerful tool when adeno-associated vectors (AAV) are used as delivery method since the cargo capacity of AAVs cannot accommodate the longer CRISPR/Cas9 cDNA originally isolated from *Streptococcus pyogenes* (Ran *et al.*, 2015). In this context it is worth mentioning the recently identified Cpf1 endonuclease, which introduces staggered DNA double-stranded breaks and does not need tracrRNA (Zetsche *et al.*, 2015).

A matter of concern when working with SSNs are so called off-targets effects, or unspecific activity towards other locations in the genome that share sequence homology with the target (Miller *et al.*, 2011). Bioinformatics resources can help decreasing the likelihood of selecting a highly repetitive sequence and thus the chance of unwanted off-target activity (Park *et al.*, 2015). Importantly, CRISPR/Cas9 modified versions exist that keep similar target efficiencies as the wildtype CRISPR/Cas9 while reducing off-target activity (Mali *et al.*, 2013a, b; Ran *et al.*, 2013; Slaymaker *et al.*, 2016). In the context of germline manipulation, it is expected that nickases or CRISPR/Cas9 protein modified versions, although somewhat less active than wild type CRISPR/Cas9, would be preferred in order to minimize undesired off-target effects.

Donor templates used in combination with SSNs require much shorter stretches of homology (1–2 kb) compared with classic targeting construct configurations (5–8 kb), thus facilitating construction (Byrne *et al.*, 2015). Also, correction can be pursued through homology-dependent repair using exogenously supplied oligonucleotides, avoiding the need to clone donor plasmids (Chen *et al.*, 2015). Thus, SSNs facilitate the generation of targeting constructs to the point that oligonucleotides can be used to repair mutations. Importantly, simple generation of a pair of distant DSBs also simplifies more elaborated genome editing experiments such as chromosomal translocations or inversions (Torres *et al.*, 2014), which previously required more sophisticated loxP-Cre based strategies (van der Weyden *et al.*, 2009).

CRISPR/Cas9 and the guiding RNAs can be provided as DNA plasmids, AAV viruses (Ran *et al.*, 2015), chemically synthesized and *in vitro* transcribed RNA (Cong *et al.*, 2013; Mali *et al.*, 2013a, b), chemically modified RNA (Hendel *et al.*, 2015), proteins (Liang *et al.*, 2015a, b), or ribonucleoprotein complexes (Schumann *et al.*, 2015), and they can be delivered by simple injection, electroporation or infection into stem cells (Cong *et al.*, 2013; Mali *et al.*, 2013a, b), embryos (Qin *et al.*, 2015), spermatogonial stem cells (Wu *et al.*, 2015), oocytes (Sato *et al.*, 2015), or androgenetic haploid embryonic stem cells (Zhong *et al.*, 2015). Since residual retrotranscriptase activity can be detected in stem cells (Macia *et al.*, 2011), proteins or ribonucleoproteins complexes should be, at least theoretically, the safest strategy to deliver CRISPR/Cas9 and guiding RNAs when editing the germline.

As efficiency will be a central issue, it should be stressed that a number of chemical molecules (Yu *et al.*, 2015) and culturing strategies (Hatada *et al.*, 2015) have been reported to increase the efficiency of genome editing. However, before using any of those, it would be essential to test possible detrimental effects on cells or embryos exposed to such chemicals or experimental conditions. Also bioinformatics tools exist that predict CRISPR efficiency towards targets (Moreno-Mateos *et al.*, 2015) and should improve outcomes by decreasing the mosaicism rate.

Targeting the germline for genome editing

Gene editing in preimplantation embryos

Specific considerations must be taken into account depending on the target population in which germline gene editing is to be performed: embryos, gametes, or stem cells.

For embryos, the editing system is directly microinjected into the cytoplasm or pronuclei of zygotes, and some type of screening is then necessary to select embryos with a correctly edited genome and no off-target genetic modifications (Fig. 1). The efficiency of genomic editing in embryos is low, the main problem being the generation of mosaic embryos as a result of inefficient nuclease cutting and/or inaccurate DNA repair before the embryo undergoes cleavage. Still several studies in different animal models such as rat, cattle, sheep, dogs and pigs have demonstrated the feasibility of gene editing in animals (Shao *et al.*, 2014; Yoshimi *et al.*, 2014; Heo *et al.*, 2015; Zou *et al.*, 2015). That the technique can prevent the onset of a genetic disorder, for example cataract development, was demonstrated by the study of (Wu *et al.*, 2013) who successfully corrected a 1 bp deletion in a specific gene in mouse offspring. In non-human primates, microinjection of Cas9 or TALENs into zygotes led to the birth of modified offspring (Liu *et al.*, 2014; Niu *et al.*, 2014). The efficiency of genomic modifications into mammalian zygotes ranges from 0.5 to 40.9% per injected zygotes, by TALENs or Cas9 (Araki and Ishii, 2014). Very recently, the technique of CRISPR/Cas9 editing was also performed in human zygotes to verify its specificity and fidelity (Liang *et al.*, 2015a, b). This group injected 86 donated 3PN zygotes with CRISPR/Cas9, along with other molecules designed to insert new DNA. Of the 71 embryos that survived injection, 54 DNA samples were successfully amplified and genetically tested. This revealed that 28 genome-edited zygotes were successfully spliced, and that only four (5.6% of the total) contained the correct genetic material inserted through homologous recombination. The edited embryos were mosaic, with results similar to findings in other model systems (Yen *et al.*, 2014). In addition, a substantial number of 'off-target' mutations were identified, which were presumed to have been introduced by the CRISPR/Cas9 complex acting in other parts of the genome, intrinsic abnormalities of the embryos originating from the 3PN zygotes, or a combination of both. It has to be taken into account that only a proportion of the genome, known as the exome, was verified for off-target mutations, so the number of off-target mutations could be higher. Importantly, given the mosaic nature of the gene corrections in the edited embryos, it would be highly inaccurate to predict gene-editing outcomes through preimplantation genetic diagnosis (PGD).

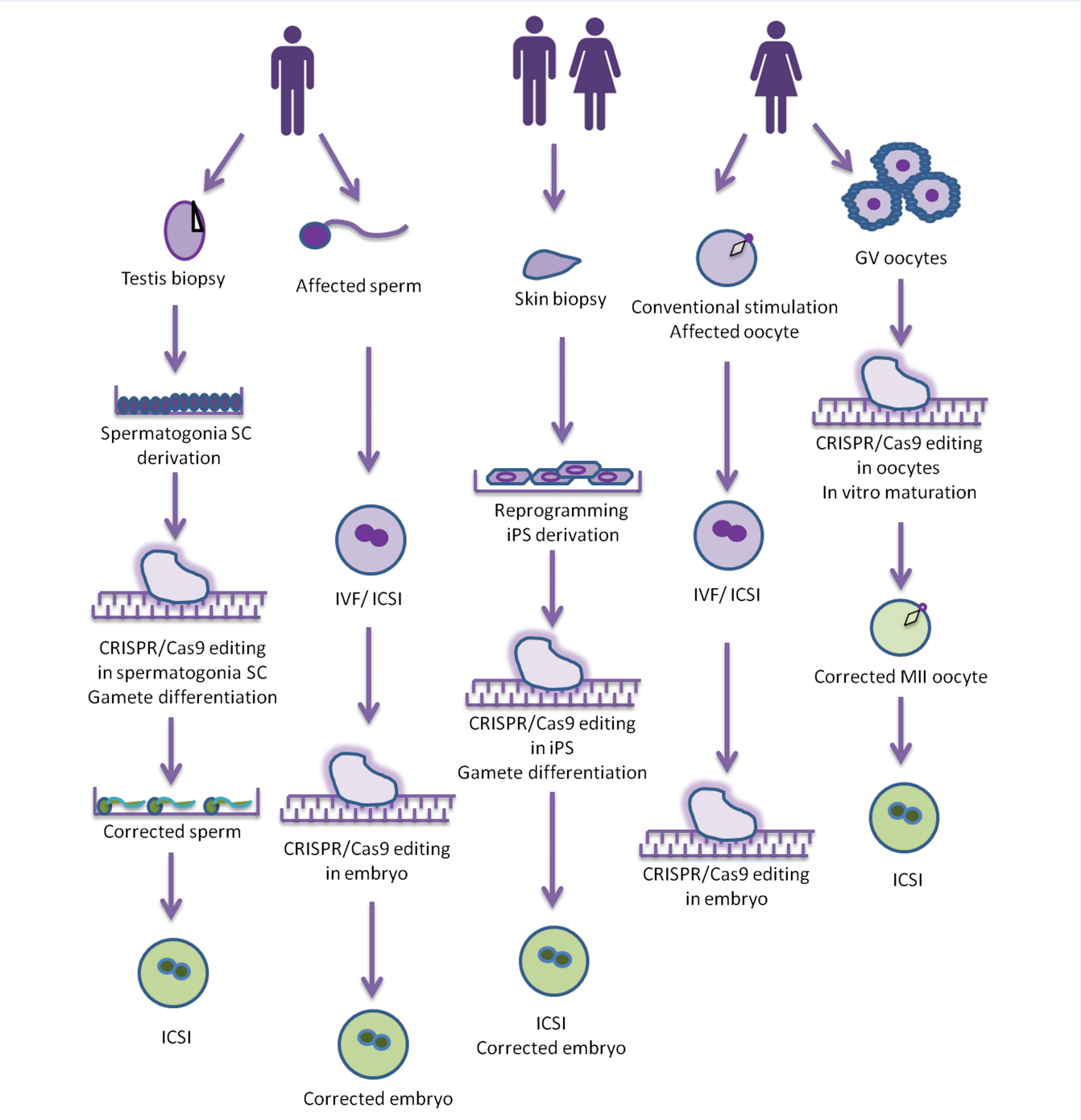

Figure 1 Summary of possible routes to editing of the germline genome in men and women. SC, stem cell; MII, metaphase II; GV, germinal vesicle; iPS, induced pluripotent stem cell; CRISPR, clustered regularly interspaced short palindromic repeats.

Gene editing of male and female germ cells

As a possible alternative to the zygote approach, gene modifications could also be applied during gametogenesis. In this manner, the CRISPR/Cas9 system could be used on growing immature oocytes or sperm to generate gene-corrected mature sperm or oocytes, which could subsequently be used for assisted reproductive technology (ART; Fig. 1). In this way, genetic conditions could be overcome in the following generation. In the male germ cell line, it is not easily conceivable that mature postmeiotic sperm could be subject to genetic modifications. Spermatogonial stem cells seem to be a better target, especially if the patient suffers from infertility due to maturation arrest and no mature sperm cells are present. Recent advances in *in vitro* spermatogonia stem cells (SSC) culture seem to bring that prospect closer (Nickkholgh *et al.*, 2014).

In the female germ line, the oocyte is of course much more easily accessible for genetic manipulation. However, the low number of mature oocytes collected per patient and the low efficiency of the technique represent a major difficulty for CRISPR application. *In vitro* maturation, applied to oocytes at the germinal vesicle stage could offer a time window for performing the necessary manipulations, although the efficiency and safety of the CRISPR/Cas9 system during meiosis should be investigated.

Pluripotent cells editing and differentiation

Finally, the last theoretical option of genomic editing to prevent a genetic disorder in the progeny is genomic editing to correct the disorder in pluripotent stem cells (induced Pluripotent Stem cells—iPSC—or Somatic Cell Nuclear Transfer-human Embryonic Stem Cells—SCNT-hESC) obtained from a diseased patient. Unlike embryos, pluripotent stem cells can be grown easily in bulk amounts, and can also sustain single cell passaging which makes them an ideal source for gene editing experiments with CRISPR/Cas9 system. Lastly, these pluripotent stem cells would need to be differentiated towards oocytes or sperm containing the genetically corrected information, and could thus be used in ART. The possibility of creating stem cell-derived gametes was shown by two landmark papers in mice (Hayashi *et al.*, 2011, 2012) and research is nowadays being performed to attain similar progression in human to overcome certain types of infertility (Vassena *et al.*, 2015). Evidently, much more experimental work in animal models is needed to investigate the safety and efficiency of this last method of germline correction through genome editing.

Possible uses of genome editing in reproduction

Germline modifications for genetic disease correction

The correction of mutations leading to monogenic diseases such as cystic fibrosis or sickle cell disease, is conceptually straightforward, as the disease-causing mutation is usually well characterized in the patients. Correction of the mutations in the germ line could allow the patients to produce sperm or oocytes that are free from the mutation, and therefore produce embryos and offspring that are not only healthy, but also not carriers, therefore changing the relative frequency of a mutation in the population. However, there are very few situations in which the application of CRISPR/Cas9 technology could be of benefit for individuals at risk of conceiving children with monogenic diseases. In classical families at risk for autosomal recessive diseases, for instance, two carrier-parents have a one-in-four risk of affected offspring. In case they are aware of this, they can be treated by PGD, a well-established technique that offers high risk couples the possibility of the birth of healthy children, or even prenatal diagnosis, without incurring the added risks of genetic manipulation. The same reasoning can be applied to couples where one partner is affected with an autosomal dominant disease, and have a one-in-two chance of disease transmission.

The use of CRISPR/Cas9 in more exceptional circumstances can be put forward, such as the case of both members of a couple affected with the same monogenic disease who wish to have a healthy child with their own genetic material. As an example, the life expectancy of CF patients has increased tremendously in the past decades, with intensive and early symptomatic treatment, with possible transplantation of the lungs and heart, and most recently with drugs that open CFTR channels for ion transport that have also been made available. Healthy pregnancies have been described in women with CF, and ICSI with testicular biopsy has long since been introduced and used for males with congenital absence of the vas deferens, many of whom harbour CF mutations (omim.org/entry/602421). In those cases, it would suffice to correct the affected genes in the germ line of one of the prospective parents, and all children of this couple would be healthy carriers.

Another example of possible candidates for germline editing is constituted by patients who are homozygous for an autosomal dominant disease such as Huntington's disease. In the case of Huntington's disease, patients who carry two affected alleles are more severely affected than those who carry only one allele, but appear to have the same age of onset. Autosomal dominant polycystic kidney disease also displays childhood or earlier onset when one of both alleles is milder, and the second one leads to more severe disease. Homozygotes for two alleles leading to severe disease are probably lost *in utero*. Many other autosomal dominant diseases, such as achondroplasia and Marfan syndrome (http://www.omim.org/entry/154700), are severe or lethal in the homozygous form. Myotonic dystrophy type 1 (DM1) or Steinert's disease seems to be the exception to this bleak list; however, only 13 patients with homozygous DM1 have been described to date (Carroll *et al.*, 2013).

CRISPR/Cas9 technology could also be envisaged as a mean to correct chromosomal aberrations. The Robertsonian translocation 21;21 is, for instance, a classic example of a chromosomal aberration present in healthy carriers, but that leads to trisomy 21 at every conception. Robertsonian translocations occur when two acrocentric chromosomes (chromosomes with very short p-arms that consist exclusively of repetitive sequences) fuse together at the centromeres with loss of the short p-arms. These translocations can occur between acrocentric chromosomes such as 13, 14, 15, 21 and 22. The Robertsonian translocation between the two chromosomes 21, however, inevitably leads to a trisomy 21. A possible approach would be to use CRISPR/Cas9 to separate the two chromosomes and to restore both the centromere and the missing p-arms. However, even when taking into consideration the extremely rapid evolution of the CRISPR/Cas9 toolkit, this particular application may be quite far from being applied.

Other candidates for correction would be genes related to infertility. Only a handful of genes are known to cause infertility, but the most common and best described genetic causes of infertility are chromosomal in nature: 45,X (Turner syndrome), 47,XXY (Klinefelter syndrome) and Y chromosome deletions. Modern technology such as whole genome sequencing will help to identify new genes involved in infertility, although the main hurdle here is the identification of patients. In classical genetics, genes are identified through different individuals in one family, which by definition will not be the case in genetic causes of infertility. Even if we will identify genes or other genetic variants causing infertility, we will then be faced with the limited choice of germ cells to manipulate.

Finally, it could be envisaged to correct mutations in mitochondrial DNA (mtDNA) that are present in the oocyte. Mitochondrial diseases are caused by a mutation in a proportion of the mtDNA molecules present in the patient's cells, a phenomenon called heteroplasmy. Mutations in the mtDNA are transmitted exclusively via the oocyte, which can carry between 10 000 and 100 000 mtDNA copies. Reddy *et al.* (2015) demonstrated in a mouse model that TALEN could be used to selectively

eliminate mtDNA molecules with a specific polymorphism from mouse zygotes. It could be envisaged that a similar strategy, using CRISPR/Cas9 instead of TALEN could eliminate mutated mtDNA molecules from the oocyte or the zygote (Wang *et al.*, 2015).

Correction of non-medical conditions

The germline 'selection' of non-medical conditions would be undoubtedly technically much more challenging. Changing the germ line to achieve more desirable traits such as certain physical characteristics, eliminate traits perceived as detrimental (such as bad eyesight or short stature), or improve other traits such as intelligence or talents for sports or art would be of a very high complexity, because these traits are often polygenic and the phenotype is often the result of the interaction between several genes and the environment. Moreover, because of their complexity, simple selection such as used in PGD would not be an option. Even for simple traits such as the colour of hair or skin we are only starting to unravel the underlying genetics. Our skin or hair colour is not simply a matter of how much melanin our cells produce, and with the knowledge acquired from whole genome sequencing, it turns out that many other regulatory sequences, that is, sequences that do not code for proteins but regulate such genes, are of great influence. For instance, blond hair is partly a result of a single nucleotide polymorphism (SNP) in a regulatory region upstream of *KITLG*, a gene with an essential role in the development, migration and differentiation of cell types such as melanocytes, blood cells and germ cells (Guenther *et al.*, 2014). The SNP located in an enhancer region prevents binding of a transcription factor (LEF1) causing a lower expression of KITLG and therefore a lower pigmentation specifically in hair follicles. This example may seem trivial, but it illustrates very clearly how little we know of how physical traits—and by extension other traits such as musical or mathematical talents for example—are determined at a molecular basis. It is clear now that our environment has a direct influence on gene expression, and therefore on phenotype. It is assumed that much of this influence is exerted through epigenetic regulation of gene expression, but it will take many more years before a clear understanding of epigenetics is achieved. Trying to influence these complex traits through germ line modification before the whole picture is clear, is doomed to fail at best, and dangerous at worst.

Fundamental research and technology development

Apart from its potential future clinical use, genome editing by CRISPR/Cas9 has a tremendous value as a tool to address fundamental questions of human and animal developmental biology. The following topics of basic research involving genome editing technology can be envisaged: (i) research to understand and improve the technique of genome editing itself on the different types of target cells which can later be used to modify the germline; (ii) genome editing used as a tool to address fundamental questions of developmental biology: altering developmental genes with CRISPR/Cas9 could help to reveal their functions.

Ethical considerations

Limiting ourselves to applications in the field of reproduction, three categories of benefits may be distinguished: increased knowledge and understanding of developmental processes and gene functioning that may help to develop or improve medical technologies, correction of defects that cause infertility in would-be parents, and correction of diseases in future children. As such, gene editing may increase the reproductive autonomy of people (Sugarman, 2015).

The main argument against gene editing in embryos and gametes is that it implies germ line gene modifications. In many countries and in a many international conventions, germ line modification is forbidden (Araki and Ishii, 2014). Although one frequently refers to an international consensus, there is in fact no such consensus. The clearest deviation from this rule is the acceptance of mitochondrial, or rather spindle or pronuclear, replacement in the United Kingdom. The UK parliament eventually decided that this objection was insufficient to block the application of mitochondrial replacement that constitutes a germline modification. Connected to the objection of germ line modification are a number of other aspects of lesser importance such, for instance, the argument that these modifications are made without the consent of future generations (Collins, 2015). However, this is true for any intervention that affects future generations, including conception itself. No person consented to being created and no person consented to having the particular gene set that he or she has.

Of greater importance is the concern for the safety of future children. There seems to be unanimity among the scientific community that application of gene editing to alter the germ line in germ cells or embryos intended for reproduction is premature and unacceptable at present. The issues of responsible innovation and the introduction of new techniques have special relevance in the context of ART (Dondorp and de Wert, 2011). A comparison with the applications in ART can be useful to reveal the specificities of similarities with experimenting in reproduction. The American Society for Gene and Cell Therapy and the Japan Society of Gene Therapy concluded that germ line modification in humans is unacceptable because 'the results of such experiments are not susceptible to long-term evaluation in a scientifically reasonable time scale' (Friedmann *et al.*, 2015). This statement implies that almost all techniques in ART (including IVF, ICSI, cryopreservation, etc.) are ethically unacceptable experiments since they all may have intergenerational effects that cannot be determined at the moment of application. Although this is a serious concern, it does not need to result in a prohibition. Animal studies, early human embryo research and long-term follow-up might be the best we can do (Haites and Lovell-Badge, 2011; Bioethics, 2012).

Apart from the germ line modification itself, the matter of research on embryos has also raised numerous questions. The Liang study (Liang *et al.*, 2015a, b) avoided this matter to a certain extent through the use of non-viable (tripronucleated) embryos. Meanwhile, it could be argued that it is too early to start research on good quality human embryos because of the (too) limited knowledge of the technique at present (Kaiser and Normile, 2015). Given the relative scarcity of good quality embryos for research, respectful use implies that there must be a reasonable chance that the research will lead to valuable findings. It can be argued that the Liang paper did only confirm what we knew from animal research and added no new knowledge. Still, research on normal embryos will be needed in the future if introduction to clinical practice is considered. Moreover, for a number of experiments, creation of embryos for research will also be necessary. Most supernumerary embryos in IVF are beyond the one-cell stage and the gene editing techniques may then be more prone to lead to mosaicism (Hinxton Group, 2015).

A last argument that is often brought forward is that gene editing will result in the modification of the human genome. It is far from clear what is

meant by this argument. The first meaning seems to be based on the underlying idea that there is a catalogue of human genes that is fixed and transmitted from generation to generation. First, any mutation results in a modification of the human gene pool. Second, when gene editing is used to repair or reinsert a gene that exists in the human gene pool, there would be no modification in the relevant sense. A second possible meaning would be that we would be changing the human species. However, this would require large scale applications. Although this seems unlikely, it is not impossible. Still, as long as the techniques are used to correct defects and diseases, their effects would be similar to preimplantation and prenatal screening applied at the moment. A closely related argument against genome editing regards the threat to human dignity and the concern for a renewal of eugenics (UNESCO International Bioethics Committee, 2015). However, the current genetic testing and screening procedures certainly have a much greater eugenic effect and this concern is not strong enough to prohibit their application. Again, as long as the editing technology is used to correct and prevent diseases, there seems to be no good reason to stop the introduction.

The final evaluation of gene editing techniques to avoid diseases in future offspring will depend at least partially on the comparison with other techniques such as gamete donation and preimplantation genetic diagnosis. According to some, there are few compelling medical applications justifying gene editing of embryos, precisely because of the existence of alternatives (Collins, 2015; Lanphier *et al.*, 2015). Gene editing will almost certainly increase the risk for the health of the future offspring compared with the existing alternatives. So gene editing would be mainly an option when PGD is not possible but these cases are likely to be very rare, as we argued above (Lander, 2015). How much added risk for the future child can be accepted for a couple to have a genetically related child? A crucial question then will be the value attributed to genetic parenthood.

More specific new questions will be generated in the context of ART. Should gene editing be seen as an alternative to PGD? Could it be applied for couples who have few healthy embryos after IVF or should it also be considered to avoid discarding affected embryos? Although the decisions are presented as pure cost-benefit analyses, the balance will be partially based on moral considerations regarding the status of the embryo and on views about the disposition of (supernumerary) embryos. People who object to discarding embryos may prefer the solution of the modification of germ cells. The different options will have to be balanced on morally relevant criteria such as safety, efficacy and accessibility.

Matters of social justice may also arise. If these techniques have proven to be safe and efficient, should society guarantee equitable access by all those in need? (Newson and Wrigley, 2015). Given the general trend in this domain, the issue of patenting may become crucial, not only in the further development of the technology but also in the accessibility of possible therapies (Hurlbut *et al.*, 2015). As part of the latter question, priorities will have to be determined on the basis of seriousness of the disease, incidence, alternative approaches, etc. (Hinxton Group, 2015). Similar to the context of PGD, each society will have to decide what kinds of interventions to accept. Based on the concerns mentioned above, we propose that all applications of genome editing techniques that may affect future offspring (whether or not they involve germline modifications) should be evaluated by a committee with the necessary scientific expertise. Countries that allow embryo research normally have such a committee in place. If a country has no such committee, it should be installed before applications are considered. A good example of this mechanism is the recent review and approval from the HFEA for a research group at the Francis Crick Institute in the UK to conduct experiments involving CRISPR/Cas9 editing of human preimplantation embryos (http://guide.hfea.gov.uk/guide/ShowPDF.aspx?ID=5966).

Conclusions

Newly developed sequence-specific nucleases based technologies have resulted in higher targeting efficiency of the genome, easier construction of donor vectors, the possibility of genome editing with oligonucleotides, direct genome editing by electroporation of embryos and the possibility of multiplexed genome editing. This technical breakthrough makes the possibility of editing the human germline, either through gametes, gamete precursors, embryo manipulation or through pluripotent stem cells, more feasible than in the past. However, before CRISPR/Cas9 technology could be translated to the clinic, some problems will need to be resolved; the main issue is mosaicism, together with off-target effects and unwanted random genome integration of oligonucleotides and constructs. Overcoming such limitations will depend on intensive research that needs to be transparent and widely disseminated. Calls for a moratorium on, or outright banning of, this type of research is not the best course of action. Nonetheless, once technical issues will be completely resolved, the editing of the human germline to prevent the birth of an affected individual should only be considered when already established methods that do not entail manipulation of the genome, such as PGD, are not available.

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Conflict of interest

None declared.

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