Towards a CRISPR view of early human development:
applications, limitations and ethical concerns of genome editing in human embryos

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ABSTRACT

Developmental biologists have become increasingly aware that the wealth of knowledge generated through genetic studies of pre-implantation mouse development might not easily be translated to the human embryo. Comparative studies have been fueled by recent technological advances in single-cell analysis, allowing in-depth analysis of the human embryo. This field could shortly gain more momentum as novel genome editing technologies might, for the first time, also allow functional genetic studies in the human embryo. In this Spotlight article, we summarize the CRISPR-Cas9 genome editing system and discuss its potential applications and limitations in human pre-implantation embryos, and the ethical considerations thereof.

KEY WORDS: CRISPR, Embryo, Genome editing, Ethics

Introduction

Thirty years ago, the discovery of homologous recombination (HR) and its first application in modifying the genome of mice revolutionized the field of genetics (Thomas and Capecchi, 1986; Thomas et al., 1986). Owing to its low efficiency, however, HR-based gene targeting was confined to either simple organisms or required the selection of rare events in embryonic stem cells followed by blastocyst injection and time-consuming cross-breeding in order to obtain a homozygous mutant mice (Sargent et al., 1997). A breakthrough in this field was the discovery that enzymatically induced DNA double-strand breaks (DSBs) significantly increased the efficiency of HR-based targeting (Donoho et al., 1998; Rudin et al., 1989). DSBs in the genome trigger the inherent DNA repair machinery in the cell that repairs the break through two mechanisms: non-homologous end-joining (NHEJ) and homology-directed repair (HDR) (Sargent et al., 1997). In NHEJ, cleaved ends are joined back together, normally resulting in the addition or deletion of a few nucleotides that could induce a frame-shift mutation and lead to a loss of gene function. By contrast, in HDR, an external DNA fragment is used as a repair template, which can be exploited to introduce precise genetic modifications or exogenous sequences such as those of reporter genes (Donoho et al., 1998). Since then, numerous efforts have been made to develop sequence-specific nucleases (SSNs) that are capable of efficiently catalyzing genome editing in a large range of organisms through the introduction of DSBs.

The CRISPR-Cas9 system

To date, four different types of programmable SSNs have been developed: meganucleases, zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and CRISPR-associated (Cas) nucleases (Arnould et al., 2006; Cermak et al., 2011; Jinek et al., 2012; Kim et al., 1996; Urnov et al., 2005). Despite their recent discovery, Cas nucleases have quickly become the enzyme of choice for genome engineering. Among the most notable reasons is the fact that recognition of the desired target is not directed by a protein, which would require extensive protein engineering for specificity, but by a much more programmable RNA molecule. The most commonly used Cas enzyme, Cas9, is guided by a short RNA molecule known as single guide RNA (gRNA), which consists of two different components: the CRISPR RNA (crRNA), containing a targeting RNA sequence of about 20 nucleotides that can be redesigned to recognize any desired target in the genome; and a trans-activating CRISPR RNA (tracrRNA), which is an RNA sequence that base pairs with the crRNA that is essential for the Cas9 complex to bind to and cleave DNA. As a result of the efficiency of the technique and the ease with which single gRNAs can be generated, the CRISPR-Cas9 system is also amenable to multiplexing genome-editing experiments (Cong et al., 2013).

The current success and importance of the CRISPR-Cas9 system would never have been predicted by Francisco Mojica when he first described a curious structure consisting of an interspersed repeat array in the genome of an archaic microbe isolated from the marshes of Alicante in 1993 (Mojica et al., 1993, 1995). Those repeats, which he would later name clustered regularly interspaced short palindromic repeats, now known as CRISPR, were found to be part of an archaic adaptive immune system present in some microbes. This system is able to cleave the nucleic sequences of an invading virus and store a piece of it in a dedicated region of the prokaryote genome, the CRISPR locus, which serves as a memory reservoir of previous infections. In the event of a second infection, those stored sequences are expressed as short RNA molecules, called crRNA, and guide a Cas nuclease through sequence complementarity to the invading viral DNA, where it will generate DSBs and promote its degradation (Barrangou et al., 2007). Following the understanding that the system can be reduced to only three core components – the Cas9 nuclease, a crRNA and a tracrRNA (Sapranauskas et al., 2011) – it was further simplified by fusing the crRNA and the tracrRNA into a single molecule, the single gRNA (Jinek et al., 2012). When combined, this facilitated the successful genome editing in mammalian cells by the CRISPR-Cas9 systems (Cong et al., 2013; Mali et al., 2013). Owing to its simplicity, high efficiency and versatility, the CRISPR/Cas9 was quickly adopted by scientists around the world to perform genome editing in a large number of animals, ranging from mice to non-human primates (Niu et al., 2014; Wang et al., 2013).
Potential applications and limitations in fundamental biology

Understanding how normal human pre-implantation development is controlled could have implications for the treatment of infertility and stem cell-based regenerative medicine. Approximately one out of six couples experience infertility-related problems, so there is a significant need for a better understanding of how the embryo develops from a fertilized single cell zygote to a blastocyst that is ready to implant in the uterine wall. In the past 20 years we have established a deeper understanding of the genes and mechanisms that control pre-implantation development in the mouse, which has been due largely to well-developed gene-targeting methods. However, an increasing number of studies have identified striking differences between mouse and human pre-implantation development and pluripotency (Kuijk et al., 2012; Niakan and Eggan, 2013; Roode et al., 2012; van den Berg et al., 2011), highlighting the need for focused studies in human embryos. Thus far, functional studies in human embryos have been performed using pharmacological inhibition, which is generally restricted to signaling pathways and bears the risk of off-target effects. A recent pioneering paper undertook knockdown experiments using RNA interference (Durruthy-Durruthy et al., 2016). However, in mouse, RNA interference has repeatedly generated conflicting data that are not in line with subsequent genomic targeting (Blij et al., 2012). As such, the use of CRISPR-Cas9 to disrupt gene function is therefore an interesting alternative with which to dissect the very early regulatory events that shape embryonic development.

Besides being used in gene targeting through active genome modification, the Cas9 nuclease can also be exploited as a transcriptional repressor. If the catalytic domain of Cas9 is inactivated [termed dead-Cas9 (dCas9)], its binding to target DNA elements prevents further RNA polymerase-mediated transcriptional elongation. Alternatively, it can be fused to different effector domains that greatly expand the repertoire of CRISPR functions far beyond its use in loss-of-function experiments (Larson et al., 2013; Qi et al., 2013). Once fused to epigenetic modifiers, dCas9 can help in the study of chromatin modifications or act as a transcriptional repressor or activator, which might allow its use in gain-of-function experiments (Gilbert et al., 2013; Konermann et al., 2014). Furthermore, Cas9-null variants can also be fluorescently tagged and used to label distinct DNA loci in an attempt to elucidate the spatial organization of functional and structural elements in the genome, as well as to study the interactions between genes during live processes (Chen et al., 2013; Deng et al., 2015).

The potential to use CRISPR-Cas9 to disrupt gene function in human embryos is paralleled by technological breakthroughs that now allow us to measure global gene expression profiles at the single-cell level. Single-cell RNA sequencing has not only further identified differences between mouse and human, but has also provided a transcriptional atlas of human pre-implantation development that can now indicate which genes should be targeted for functional studies (Blakeley et al., 2015; Durruthy-Durruthy et al., 2016; Petropoulos et al., 2015; Tööönen et al., 2015; Xue et al., 2013; Yan et al., 2013). Furthermore, this technology will further facilitate analysis after gene targeting as it can both identify cells carrying modified mRNA and simultaneously show the functional result at the global transcriptional level in successfully targeted cells.

Despite its promise, there are still several potential limitations related to the use of CRISPR-Cas9 in human genome editing. The first one is mosaicism, which occurs when the cells divide before the genome editing takes place. This results in daughter cells that will either carry the CRISPR machinery and, eventually, display the mutation, or not carry it and therefore maintain an intact genome. In addition, targeting can also be mono-allelic or bi-allelic. Mosaicism and incomplete targeting are fairly common when using genome-editing techniques, as most of the CRISPR systems require cells that are actively dividing to enable the genome modification. Mosaicism may be more of a problem in the pre-implantation embryo where cell divisions occur fairly rapidly (Suzuki et al., 2014; Yen et al., 2014). In cell culture experiments, clones with correct targeting can be isolated. As such a strategy cannot be performed in the human embryo, the targeting strategy must either be efficient enough to result in a phenotype or the analysis methodology must also be able to identify the cells that have the correct and bi-allelic targeting. Single-cell RNA sequencing may be one such technology that could identify targeted cells through a changed mRNA sequence and relate that to potential transcriptional changes. The use of recombinant Cas9 protein and in vitro transcribed gRNAs (Cas9 RNP) would probably be preferential compared with traditional plasmid or mRNA delivery methods, as it has been shown to achieve lower rates of mosaicism in embryos (Blitz et al., 2013; Jao et al., 2013; Wang et al., 2013). This approach has also been used successfully to generate biallelic targeting in mice, zebrafish and frogs, which is needed in order to generate null phenotypes.

The second issue is off-target effects, which are the consequence of the nonspecific activity of the Cas nuclease in non-target locations of the genome (Cho et al., 2014; Pattanayak et al., 2013). Even though off-target events might be scarce, they should not be overlooked, as there is the possibility that another gene could be mutated, causing an effect or phenotype that could be confused with the one expected from the on-target mutation. As with mosaicism, off-target events pose less of a problem when working with cultured cells than with embryos, as it is feasible to pre-select cell lines that carry the desired genotype without unwanted off-target mutations (Wu et al., 2015). Several strategies are currently under investigation to reduce the risk of off-target effects, such as improved algorithms to design gRNAs and engineering Cas enzymes with higher fidelity and specificity (Kleinstiver et al., 2016; Slaymaker et al., 2015). In addition, this problem could be tackled by using multiple targeting strategies against the same gene to ensure that they produce the same phenotype.

The use of gene editing in human embryos also faces other considerations related to the availability of human embryos and the limited ways of analyzing the results. There are different categories of human embryos that can be used in research: embryos that are non-viable or not suitable for fertility treatment, viable ‘spare’ or supernumerary embryos following treatment, and embryos created specifically for research from donated oocytes and sperm. Non-viable or not suitable embryos would probably not be the first choice when trying to decipher normal development as they generally are of low quality and could display abnormal genomes, which requires taking great care when interpreting any results coming from them. Embryos created directly for research purposes would be beneficial from the perspective that the embryos can be genetically targeted at the zygote stage or even prior to fertilization, whereas supernumerary embryos are generally developed beyond the one-cell stage, which can make the targeting more challenging if all cells are to be injected. In addition, as assisted reproductive technology clinics generally transfer the best-quality embryos to patients and most of the available embryos have been in storage for many years, the quality of those ones available for research may not be the highest. Despite these constraints, several countries do not
allow the creation of embryos for the sole purpose of research, restricting the available material for research to supernumerary embryos.

Finally, there are limitations in the way the potential modified embryos can be analyzed. In contrast to animal studies in which the phenotype of a genetic modification can be analyzed following cross-breeding of genetically modified founders, the analysis after gene editing in human embryos must be performed in the primary targeted embryo during its subsequent in vitro culture. This means that the analysis is restricted to methods that are applicable to small samples ranging from a single to a few hundred cells. The time window for subsequent analysis is also restricted to the first week of development, as this is the period for which embryos have been robustly cultured. This does still allow for studies of several important processes, such as early gene regulation, zygotic genome activation, establishment of lineage and pluripotency, and X chromosome dose compensation. Recently, it was shown that human embryos can be supported to initiate peri-implantation development in vitro (Deglincerti et al., 2016; Shahbazi et al., 2016), which has now allowed researchers, for the first time, to access and study the second week of human development. It has been broadly agreed that human embryos may not be maintained in vitro for more than 14 days or after the appearance of the primitive streak, whichever comes first. This restriction was first introduced in the UK in 1984 through a report from the Committee on Human Fertilisation and Embryology known as the ‘Warnock Report’, which followed the world’s first IVF birth in 1978. Those 14 days mark the time when gastrulation begins, as well as the last developmental time point when the embryo can split to give rise to twins, therefore defining the moment an embryo becomes an ‘individual’. This limit has always been theoretical, but now that it has become a practical limit it may spark a renewed discussion.

Ethical considerations
When the potential of the CRISPR-Cas9 system was initially realized, ethical concerns were raised about the possibility of creating permanent and inheritable changes in the genome of human gametes and embryos. As a response to this development, a call for a moratorium was made for research involving genome modification of the human germline, or more specifically for the clinical applications of such technologies (Baltimore et al., 2015; Lanphier et al., 2015). Shortly after this call for a moratorium, two papers describing the use of CRISPR-Cas9 in human embryos were published (Kang et al., 2016; Liang et al., 2015). The two manuscripts aimed to evaluate the feasibility to edit the genome of human embryos for therapeutic goals, more specifically to edit the human β-globin (HBB) gene, which is normally mutated in β-thalassemia, or to edit the CCR5 locus, which is related to HIV resistance. Ethical approval was in place and additional measures were taken in both cases to meet potential ethical concerns. One such measure was to use non-viable (tri-pronuclear) embryos that were discarded during routine in vitro fertilization (IVF) procedures. However, both publications, especially Kang et al. ignited a firestorm of controversy, as they were the first attempt to perform genome editing in human embryos and they appeared shortly after the two articles suggesting a temporary moratorium. Despite the media attention, both groups clearly reported low overall gene correction efficiency using template-guided HDR, genetic mosaicism and a considerable number of off-target mutations. It is unclear whether this could be attributed to the use of embryos with three copies of the genome (triploids), to the use of one of the earliest versions of the gene editing technology (wildtype Cas9, known to have low HDR efficiency), or to both (Cong et al., 2013; Hsu et al., 2013; Mali et al., 2013; Ran et al., 2013). Although the frequency of gene correction could have possibly been higher if they were to use normal embryos and a more adequate version of the Cas9, such as the nickase Cas9, both studies still showed ~50% efficiency in generating indel mutations, which most probably would result in null mutations. This clearly shows that the CRISPR technology is currently not yet safe or efficient enough for therapeutic applications, but still supports its suitability for targeted disruption of individual genes, which can prove to be a powerful tool to gain fundamental knowledge of early human embryonic development.

Although CRISPR/Cas9-based germline editing is currently far from being efficient or safe enough to warrant clinical applications, we believe that the same technology could be extremely useful for basic research into early human development. Apart from the safety and efficiency aspects that are not as relevant for basic research, which other ethical issues could be considered? Arguments against the use of germline genome editing in basic research usually come in three forms. First, there is the argument that the legitimization of the technology for its use in basic research will inevitably lead to an increase in our knowledge on how to improve it and, therefore, will bring closer its potential translation into a clinical setting. However, such a gain of knowledge could also be viewed as beneficial, whether it qualifies the technology for clinical applications or not. Another argument is that the moral status of the embryo itself prevents germline modifications. This view is fully understandable but is not limited to germline modifications and would therefore prevent any type of experimentation on supernumerary embryos. A third argument is the ‘slippery slope’ argument, which suggest that embryo germline changes should not be performed as they open up the possibility of future misuse of the technology. In this case, one could argue that limiting fundamental in vitro research with donated human embryos to the first 14 days of development and not allowing the transfer of embryos used in research into a woman or into any other animal are reasonable regulations; indeed distinct restrictions along these lines are already in place so as to avoid any potential misuse. Such restrictions are in place in many countries, including the USA (although embryo research cannot be funded by the NIH), China and several European countries such as UK and Sweden (where researchers have recently obtained permission from their respective authorities to use CRISPR to genetically modify human embryos for basic-research purposes). Both the UK and Sweden have clear regulations against the reproductive uses of any gamete or embryo that has been subjected to any scientific research or treatment intended to produce inheritable genetic changes. They also have clear statements on the conditions that must be met by any research involving this type of human sample (Human Fertilisation and Embryology Act 2008, http://www.legislation.gov.uk/ukpga/2008/22/contents; Swedish Ministry of Health and Social Affairs, 2006). Moreover, in the case of the UK, there is a specialized organization, the Human Fertilisation and Embryology Authority (HFEA), dedicated to the licensing and oversight of this type of research, as well as any potential clinical application that involves the use of human eggs, sperm or embryos.

Conclusion
The application of CRISPR-Cas9 technology to human cells has evolved in parallel with increasingly powerful methods of cell culture and analysis. It is now possible to modify the genome of a human embryo in a highly efficient and specific way, to grow the
modified embryo in vitro for longer than ever before, and to analyze the regulatory consequences of the modification at the single cell level. With an increasing number of labs currently performing or about to embark on the gene editing of human embryos, we envision that the use of CRISPR-based genetics, together with these technological breakthroughs, will dramatically accelerate our efforts to decipher the mechanisms that control early human development. Although this in itself is a worthy pursuit, it is also hoped that such understanding will, ultimately, lead to improved infertility treatments and to the use of pluripotent stem cells in regenerative medicine.

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