
Applications of CRISPR/Cas9 in Reproductive Biology

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Abstract

Genome editing is unravelling its benefits in wide areas of scientific development and understanding. The advances of genome editing from ZFNs and TALENs to CRISPRs defines its wide applicability. Reproduction is the fundamental process by which all organisms maintain their generations. CRISPR/Cas9, a new versatile genome editing tool has been recently tamed to correct several disease causing genetic mutations, spreading its arms to improve reproductive health. It not only edits harmful genetic mutations but is also applied to control the spread of parasitic diseases such as malaria by introducing selfish genetic elements, propagated through generations and population via reproduction. These applications led us to review the recent developments of CRISPRs use in reproductive biology.

Introduction

The blueprint of life and heredity is written in genome. The human body consists of trillions of cells which are divided in autosomal cells and gametes (Soh *et al.*, 2014). The autosomes consists of two genomes while gametes comprises a single genome. During the process of fertilization the two gametes, one from each partner, unite to form a zygote (Hughes and Page, 2015). The genome is built up of billions of DNA base pairs subdivided

into genes that interact with environment to develop a specific phenotype. Although extensive studies have been carried out but still the understanding of genes and its function is far from understanding because genes are differentially expressed. The present advances in genome editing technologies takes a leap towards endogenous genome modification, which gives rise to several opportunities to deal with diseases and undesired phenotypes (Khan *et al.*, 2016).

The continuous update to knowledge reservoir owes to molecular studies unfolding several important aspects of genes and their interactions with the environment enabling scientists to do forward and reverse genetics model cells and organisms to uncover the intricate mechanisms that may be taking place. Such studies also open avenues of its practical applications, especially in medicine. The genome manipulation was first made possible in the 1970s when *E. coli* was first manipulated for therapeutic protein production (Itakura *et al.*, 1977). The advent of gene silencing methods further increased the precise understanding of gene function that rely on homologous template. It is very precise but its application on a large scale remains inefficient. To overcome these limitations ZFNs and TALENs are developed which are proteins with DNA-binding domains (Dhanasekaran *et al.*, 2006; Reyon *et al.*, 2012) that are much more efficient than the gene silencing technologies; however, its

wider application is limited by unavailability of skilled technicians who can make a special targeting protein for each genomic target.

Clustered regularly interspaced short palindromic repeats (CRISPR/Cas) is the most recent and accurate tool to target nucleic acids of any nature to modify, edit or disrupt any gene. The CRISPR/Cas system can virtually be reprogrammed against any genome of interest to achieve required goals. This special immune response is developed by a unique set of CRISPR array which contains a protospacer from invading nucleic acid recognized by short protospacer adjacent motif (PAM) which helps the host resistant to its own immune response (Bolotin *et al.*, 2005; Barrangou *et al.*, 2007). The simple machinery of CRISPR/Cas system makes it a technology of choice in various biotechnological platforms including gene therapy, genome editing and therapeutic production. The CRISPR/Cas9 provides an efficient way to make targeted double-stranded breaks (DSBs) in DNA that can knock out any gene through non-homologous DNA repair pathway (NHEJ) and can stimulate template-based homologous repair through homology directed repair (HDR) (Khan *et al.*, 2016). The CRISPR/Cas9 system requires only the design of the new sgRNA against target, as compared to ZFNs and TALENs that needs special protein for each target.

Gene drives is a technique that can enhance the inheritance of a particular gene to increase its prevalence in the population (Windbichler *et al.*, 2011). In natural populations, genes sometimes gain a fitness bias that does not depend on the organism. The genes in a sexually reproducing organisms have a 50% chance of being inherited by offspring, but some genes somehow gain an evolutionary advantage such that they are passed on to more than 50% of offspring. This phenomenon is considered as natural 'gene drives' that initially is found in single organism and then is slowly transferred, over generations, to the whole population. Artificial gene drives can be a very useful way to control the vectors of several diseases, including malaria (Hammond *et al.*, 2015), dengue and zika transmitted by mosquitoes. The CRISPR/Cas9 system can be efficiently utilized to create artificial gene drives that can render mosquitoes sterile. Apart from disease control, CRISPR/Cas9 can be used to study several fertility-related genes in males, hence providing an important platform for molecular

genetic studies of fertility, development and control of disease-causing vectors. This review will focus on applications of the most versatile CRISPR/Cas9 in reproduction that is the fundamental process of species survival.

Gene drives and CRISPR/Cas9

The selfish genetic elements constitute gene drive systems that are biased towards their own inheritance in super-Mendelian fashion (Noble *et al.*, 2016; Champer *et al.*, 2016). These gene drives are considered as means to eradicate insect-borne diseases including malaria, zika and dengue (Hammond *et al.*, 2015). Naturally found gene drives are in the form of transposons, commonly known as jumping genes (Charlesworth *et al.*, 1989) or those causing segregation distortion (Tao *et al.*, 2001) and Medea elements (Chen *et al.*, 2007). The discovery of CRISPR/Cas9 genome editing technology has made it possible to create synthetic gene drives that can change the dynamics of natural populations (Champer *et al.*, 2016).

It is noteworthy that synthetic gene drives are widely studied and have potential to widely eradicate the protozoal, viral and helminthic diseases that causes a huge human disease burden (Zamanian and Andersen 2016). Synthetic gene drives to control vector-borne diseases have two principal objectives: population modification or population suppression (Burt, 2003, 2014). The eradication of the vector, for example mosquitos carrying the malaria parasite, is an example of population suppression. In contrast, if an allele important to disease resistance is introduced into a vector, or if an allele is transferred to a vector that makes it unable to transfer pathogen to host, this is a form of population modification gene drives. The concept of such synthetic gene drives is derived from natural gene drives but its progress was very slow until the use of the versatile CRISPR/Cas9 system (Zamanian and Andersen, 2016). The recent applications of CRISPR/Cas9 in *Drosophila* for mutagenic chain reaction (Gantz and Bier, 2015) and to achieve gene drives in malarial mosquito vectors that converts autocatalytic heterozygous locus to homozygous locus rendering mosquito unable to transmit malaria by spreading antimalarial effector genes through mutagenic chain reaction (Gantz *et al.*, 2015). Furthermore, this mutagenic chain

reaction was utilized recently against *Plasmodium falciparum* vector *Anopheles gambiae* to spread female sterility genes, hence targeting the reproduction capability of *Anopheles gambiae* (Hammond *et al.*, 2016). These studies open powerful avenues to control several other mosquito borne vector diseases including filarial nematodes by eradicating LF genes in wild populations that can be done by creating population suppression drives by spreading genes that compromise reproductive capacity and limit the mosquito populations transmitting *Wuchereria* and *Brugia* nematodes, the main aetiological agents of LF transfer in humans (Zamanian and Andersen, 2016).

The other kind of gene drive is sex-linked meiotic drive that occurs when certain alleles are biased at meiotic level and hence are transferred to gametes and ultimately to offspring. Several such kinds of meiotic gene drives are found in nature of which sex-linked genetic drives functions by preventing the gametes lacking meiotic drive to mature and help skew gender ratios (Helleu *et al.*, 2014; Champer *et al.*, 2016). The mechanisms by which the drives work is not fully understood, but mostly an X-shredder mechanism is widely accepted. If the X-shredder gene is on the Y chromosome it will quickly spread in a population and will suppress

the population. Often an endonuclease targeting X chromosome is designed for such drives in heterogametic X/Y species. A schematic illustrating endonuclease gene drives is shown in Fig. 8.1.

These approaches emphasizes the role of CRISPR/Cas9 genome editing technology beyond the study of fertility related genes and mechanistic pathways but provide a robust platform to spread desired gene drives efficiently in natural populations to control various vector-borne diseases of human health importance.

Genome engineering and reproductive biology

The gene modification in primates remains a challenge because of their long life cycles and hence make editing impractical. The gene introduction in primates is performed mostly with viral vectors that resulted in transgenic monkeys, however, the site of gene insertion and its number of copies are not controlled (Schubert, 2014). These limitations are overcome by CRISPR/Cas9 when Sha and colleagues established a technique where CRISPR system target specific DNA sequences and produce single-stranded DNA cuts at RNA-specified targets (Schubert, 2014). Three genes were targeted

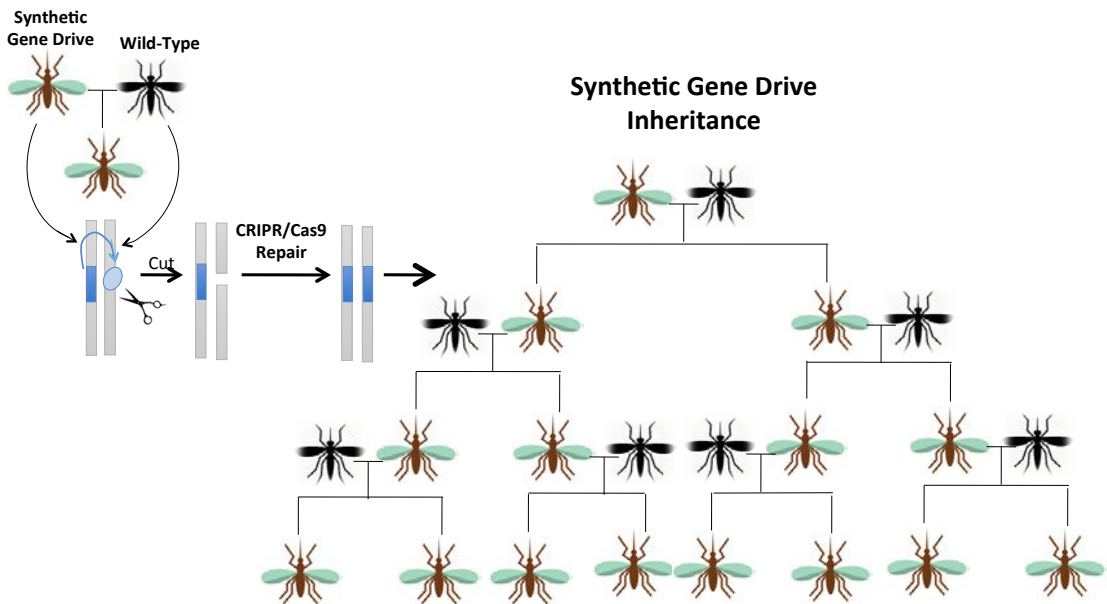


Figure 8.1 A schematic representing endonuclease gene drive using CRISPR/Cas9 system. A strategy to fight vector-borne diseases. Modified from Dicarlo *et al.*, 2015.

in monkey at one cell stage embryo in which two genes, *PRAG* and *RAG1*, were shown successfully knocked out in twins simultaneously (Niu *et al.*, 2014).

The recent years have witnessed gene editing in human embryos as the CRISPR/Cas9 come up with its applications in reproductive medicine with proof-of-principle to generate trippronuclear (3PN) zygotes (Liang *et al.*, 2015a). The results of Liang and colleagues show the use of CRISPR/Cas9 to efficiently cleave β -globin gene (*HBB*), although the homology directed repair (HDR) was found to be low, T7E1 assay and whole exome sequencing reveals its off-target effects that needs to be improved in future studies for its clinical applications. The reports that show correction of disease causing mutations for tyrosinaemia (Yin *et al.*, 2014), Duchenne muscular dystrophy (Khan *et al.*, 2016) by CRISPR systems open gates for its application to diseases causing infertility both in male and females.

A possible alternative to zygote editing is the editing of male and female germ cells at an immature stage by applying CRISPR/Cas9 system to get corrected mature sperms and oocytes which can be used in artificial assisted reproduction (Vasena *et al.*, 2016). The recent advances has made the genome editing more feasible in germlines, embryos, gametes and gamete precursors. To deal with the monogenic diseases, the mutations of which are often well characterized, and if can be corrected at sperm or at oocyte level, will produce disease-free individuals.

The 50% of genes related to fertility will not produce phenotype of fertility or infertility; hence it is a long tedious job to screen those phenotypes through generations. The fast-track CRISPR/Cas9 system can accomplish this task in months to determine whether a gene needs further study or not with its ability to precisely disrupt or induce multiple mutations to characterize genes, although redundancy of reproductive genes remains a challenge which can be overcome by properly designing the CRISPR/Cas9 system (Young *et al.*, 2015). Similarly, the effect of multiple genes on reproduction can be determined by utilizing the CRISPR/Cas9 system, as in the case of growth differentiation factor (*Gdf9*) and bone morphogenetic protein (*Bmp15*), two unrelated genes (Yan *et al.*, 2001).

CRISPR/Cas9 system will help develop knock-out models of mice and other animals for several genes as well as combined functioning of genes in pathways and its epigenetic interactions.

CRISPR/Cas system in male reproduction

The gene manipulation research in animal models has played an important role in assessment of reproduction processes. Reproduction is one of the best suited biological systems to which gene manipulation such as knock-out can be successfully applied. The spermatogenic or haploid germ cell genes that are important for fertility are more specific to the gonads and often comprise a single exon (Ikawa *et al.*, 2010). The breeding system involved in creating homozygous genetic manipulations in genes involved in gametogenesis, fertilization and conception. Whatever the reason, there are highly specific reproductively related genes of which the function is unveiled by making the use of gene disruption techniques to explore the vital factors of fertility. Gene disruption methods have long been advantageous to study male reproductive biology. However, because of the time and cost involved, this technology was not a practicable technique except in proficient laboratories. Genome editing tools such as CRISPR/Cas9 system has been extensively used to modify genes in organisms including human and animal cells, and hold a remarkable potential for clinical research applications. To date, an information gap remains in understanding of DNA repair mechanism in human embryos and in the efficiency and potential off-target effects using CRISPR/Cas9 technologies in human pre-implantation zygotes and early embryos (Liang *et al.*, 2015a). The introduction of the CRISPR/Cas9 system for gene disruption, has facilitated the modern era of genetic investigation. The application of CRISPR/Cas9 technology to generate gene disrupted mouse models to investigate the genetics of male fertility that look at the essential genetic component of male reproductive system (Young *et al.*, 2015). An important aspect to consider in the utilization of CRISPR/Cas9 system in reproduction is that 50% of fertility genes produce no distinct phenotype when disrupted (Okabe, 2014), fears no conclusion. With ease and precision of CRISPR/Cas9 system

this has revolved around, with worth perusing ability of producing fertility or infertility phenotype within few months, in addition to introduction of point mutation, ability to further investigate the molecular and epigenetic mechanisms of fertility related genes (Mashiko *et al.*, 2013). Currently the CRISPR/Cas9 systems are used to generate knockout mice to study the role of individual or multiple genes in reproduction where non-homologous end joining repair mechanism (NHEJ) is activated by DNA cleavage. The CRISPR/Cas9 systems are also used to introduce point mutations in mouse models of human conditions via homologous directed repair (HDR) mechanisms, as well as being used to analyse complex aspects of post-translational modifications (Young *et al.*, 2015). The use of CRISPR/Cas9 system circumvented these issue by allowing scientists to create multiple site mutations simultaneously which was previously impossible via conventional methods (Archambeault and Matzuk, 2014; Capecchi, 2005).

With the development of CRISPR/Cas9 system the knockout mouse models of testis specific genes are generated to study spermatogenesis process in male reproductive systems *in vivo*. The function of testis-specific component, *Slx2* in spermatogenesis was investigated by disrupting the *Slx2* gene with CRISPR/Cas9 system and knockout male mouse was obtained and is compared with the testis-specific genes of the wild type male mouse. Taken together, it was revealed that *slx2* itself does not play an essential role in spermatogenesis and the CRISPR/Cas9 technology can boost up the functional study of X-linked genes of testis *in vivo* (Li *et al.*, 2015).

CRISPR/Cas9 system could be used on growing sperm cells to produce gene-corrected mature sperm cells, which could be used for assisted reproduction. In male germline cells, it is not possible that mature post-meiotic sperms could be made subject to genetic manipulations, however, spermatogonial stem cells seem a better option for manipulation, particularly for the patients suffering from infertility having no sperm cells due to maturation arrest. Current progresses in *in vitro* spermatogonia stem cells culture seem to bring that prospect closer (Nickkholgh *et al.*, 2014). In a study, the *Crygc* gene in spermatogonial stem cells are mutated via CRISPR/Cas9 system. The mutated

spermatogonial stem cells undergo spermatogenesis after transplantation into seminiferous tubules of infertile mouse testes. Moreover, the disease causing mutations in *Crygc* gene in spermatogonial stem cells is repaired by non-homologous end joining or homology end repair mechanisms induced by CRISPR/Cas9 in mouse, resulting in spermatogonial stem cells carrying modified gene with no off-target mutations at entire genome sequencing and the offspring showed 100% efficient phenotype (Wu *et al.*, 2015). A better substitute for genetically modified spermatogonial stem cells seems to be the unprecedentedly simple CRISPR/Cas9 system (Chapman *et al.*, 2015; Sato *et al.*, 2015). Furthermore, the CRISPR/Cas9 system not only circumvents the nuclease engineering but also generates fewer off-target effects than ZFNs (Ul Ain *et al.*, 2015). According to recent studies, no obvious off-target genetic effects could be identified in SSCT experiments involving CRISPR/Cas9-mediated gene targeting transplantation of genetically modified SSC (Wu *et al.*, 2015).

Interestingly, CRISPR/Cas9 system has been shown to be successful in repairing the disease causing mutations in various species such as the *Crygc* gene mutation in mice which causes cataracts (Wu *et al.*, 2013), dystrophin gene which causes Duchenne muscular dystrophy (DMD) (Long *et al.*, 2014) and *Fah* mutation in hepatocytes are repaired by CRISPR/Cas9 system. As well as in humans, the CRISPR/Cas9 system has been used to correct β -thalassaemia, haemoglobin- β and dystrophin gene in pluripotent stem cells (Xie *et al.*, 2014). CRISPR/Cas9 catalysed the rat spermatogonial stem cells to regenerate spermatogenesis and showed long-term sperm-producing capability following transplantation in rat testes. Targeted mutations in germ line cells such as epithelial stromal interaction 1 (*Epsi1*) and receptor tyrosine protein kinase 3 (*Erb3*) were vertically transmitted from recipient to exclusive produce non-mosaic offspring. Monoclonal development of *Erb3* null germlines exposed recessive spermatogenesis defects and yielded modified offspring isogenic at targeted alleles. Therefore, spermatogonial gene modification with CRISPR/Cas9 provided a platform to produce targeted germ line modifications in rats, and to study spermatogenesis (Chapman *et al.*, 2015).

CRISPR/Cas system in female reproduction

CRISPR/Cas9 technology is especially useful in developmental studies in model organisms and the efficiency and specificity of CRISPR/Cas9 system mediated the genome editing in human embryonic cells. Recently, a group used CRISPR/Cas9 in human tripronuclear zygotes with one oocyte nucleus and two sperm nuclei to cleave the endogenous β -globin gene and repaired the β -globin gene through the non-crossover HDR mechanism (Liang *et al.*, 2015a). The embryos are edited directly through cytoplasmic pronuclei microinjection of zygote with no off-target genetic modifications. Although the efficiency of genome editing in embryos is low, but still several studies have been done in different animals such as rat, sheep, cattle, pigs and dogs to demonstrate the feasibility and efficiency of genome editing in animals (Shao *et al.*, 2014; Zou *et al.*, 2015; Jeong *et al.*, 2016). This technique can prevent the chances of genetic disorders, for example a study on cataract development demonstrated successful 1 bp deletion in a specific gene in mouse offspring (Wu *et al.*, 2013).

Microinjection of Cas9 or TALENs in non-human primates led to the birth of modified offspring (Niu *et al.*, 2014). The efficiency of genetic modifications by Cas9 or TALENs in mammalian zygote ranges from 0.5% to 40.9% per injected zygote (Araki and Ishii, 2014). Recently, CRISPR/Cas9 editing technique was performed in human zygote to validate its specificity and fidelity (Liang *et al.*, 2015b). This group injected 86 3PN zygotes along with other DNA molecules designated as new DNA with CRISPR/Cas9 and 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). Furthermore, a few number of off-target mutations were found, which were supposed to have been introduced by CRISPR/Cas9 complex acting in another part of genome. Notably, a specific portion of genome known as exome was verified for off-target mutations and such mutations could be higher due to mosaic nature of gene correction in edited embryos (Vassena *et al.*, 2016). As a possible alternative to the

zygote approach, genome modifications could also be applied during gametogenesis. In this aspect, the CRISPR/Cas9 system could be applied on growing oocytes to generate genetically modified oocytes, which would be subsequently useful for assisted reproductive technology, thus the off-target mutations could be overcome in the following generation (Vassena *et al.*, 2016). In female germ line cells, the oocyte is easily accessible for genetic modifications. However, *in vitro* maturation of oocytes at germinal vesicle stage is applied for necessary manipulation, though efficiency and accuracy of CRISPR/Cas9 should be investigated during meiosis (Vassena *et al.*, 2016). The schematic illustrating the CRISPR/Cas9 editing is shown in Fig. 8.2. The mutations leading to monogenic diseases such as cystic fibrosis are well characterized in patients and these mutations can be corrected in germ line cells and patients can produce oocytes which are free from mutations. Therefore, they produce healthy embryos and offspring that are not carrier for these disease causing mutations in the population. (Vassena *et al.*, 2016). Besides this, recently a group used CRISPR/Cas9 technology to modify the pig genome and produced genetically modified pigs by introducing CRISPR/Cas9 mutated CD1D or CD163 into somatic cells. The CRISPR/Cas9 was introduced *in vitro* produced porcine zygote and the system was effective in generating mutations in CD1D, CD163 and eGFP with 100% targeting accuracy in embryos at blastocyst stage. Direct inoculation of CRISPR/Cas9 targeting CD1D or CD163 into zygotes resulted in offspring which have mutations on both alleles with only one CD1D pig having a mosaic genotype. It was concluded that the CRISPR/Cas9 system can be used by two methods. The CRISPR/Cas9 can be used to modify somatic cells followed by somatic cell nuclear transfer as well as the CRISPR/Cas9 components can also be used in *in vitro*-produced zygotes to generate pigs with specific genetic modifications (Whitworth *et al.*, 2014).

In addition to its application in genome editing, the feasibility and specificity of CRISPR/Cas9 technology offers unique opportunities to expedite the control of insect vector diseases through the development of gene drive system (Esvelt *et al.*, 2014). A CRISPR/Cas9 based translation for the control of human malaria insect vector involves the development of endonuclease based gene drive

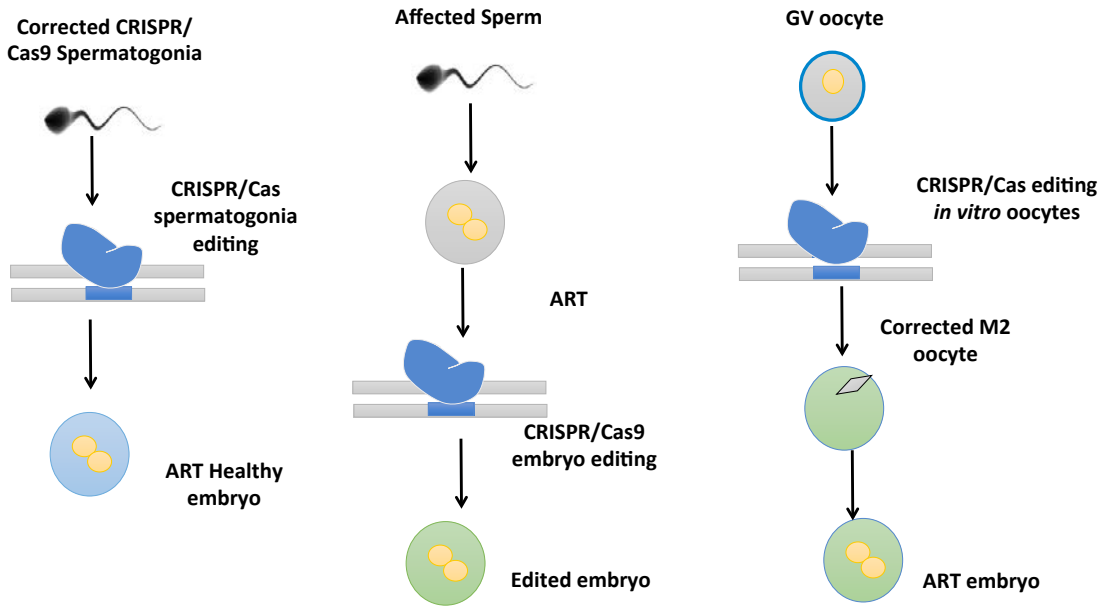


Figure 8.2 A schematic illustrating the use of CRISPR/Cas9 in sperm, oocyte and embryo genome editing. The corrected sperms and oocytes can be used in artificial reproduction techniques to produce healthy embryos.

systems that interfere with disease transmitting ability of *Anopheles gambiae* mosquitoes, that could be attained by blocking or reducing the reproductive ability of parasite or insect vector. A CRISPR/Cas based gene drive system was developed in both male and female sexes of human malaria vector *A. gambiae* to yield recessive mutation in female fertility genes before or during the gamete formation process (Deredec *et al.*, 2008; Hammond *et al.*, 2016).

CRISPR/Cas technology has opened up opportunities for the correction of human mitochondrial diseases by editing mitochondrial DNA in mouse oocyte incorporated into patient cells. This riveting progress may inspire the development of unique remedy for human maternally inherited diseases. The highly efficient CRISPR/Cas-targeted mutant mitochondrial DNA in both human and animal model cells revealed in future healthy babies born from oocytes of patients where mutant mtDNA is cleaned and the residues of mutant DNA is reduced below the level required for disease manifestation. Regardless of all potential benefits narrated, the authors also instructed a risk that embryos might be unable to implant in uterus when mtDNA copy number in the 'amended' embryos was below a specific threshold (Wang *et al.*, 2015).

In comparison with former techniques, current genome editing technologies particularly CRISPR/Cas9 is not only precise but also inexpensive and very efficient. Further, there is increased acceptance and use of techniques for assisted reproduction, which are likely to be required for genome editing in human embryos.

Conclusion

The CRISPR/Cas system is revolutionizing the speed of genome editing of disease-causing mutations and is also capable of producing corrected mature sperms and oocytes that can be used to generate disease-free individuals. It has also been efficiently applied to produce synthetic gene drives in populations to control earlier and emerging vector-borne diseases. The safe applications of CRISPRs in reproduction can guarantee huge reduction in genetic as well as vector-borne parasitic diseases.

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