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CRISPR/CAS9-Mediated Gene Editing In Human Gametes: A Review

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Gene editing exploits endogenous DNA repair pathways to introduce precise modifications into the genome. The CRISPR system was first identified in Escherichia coli (1987) and, based on Cas protein architecture, is divided into Class I (types I, III, IV) and Class II (types II, V, VI). The widely adopted CRISPR/Cas9 system comprises a guide RNA (gRNA) and the Cas9 endonuclease, which orchestrate genome editing through a tripartite mechanism: target sequence recognition via gRNA binding to a complementary DNA site adjacent to a protospacer adjacent motif (PAM), double-stranded DNA cleavage by two nuclease domains (RuvC cleaving the non-target strand and HNH the target strand), and repair of the resulting break by cellular pathways. This approach has been applied to in vitro fertilization-derived embryos and meiotically developing oocytes to disrupt or correct genes, offering potential for eliminating heritable diseases. However, concerns remain regarding off-target effects that introduces unwanted genetic mutations, necessitating improved specificity and ethical scrutiny.

Keywords: CRISPR/Cas9; DNA; Gametes; Gene editing; Infertility.

Gene editing employs endogenous DNA repair mechanisms to introduce precise, targeted modifications into the human genome, significantly advancing genetic research. Gene editing is a process that make use of the biochemical processes that naturally repair DNA damage. Various nuclease mechanisms are able to introduce DNA breaks at specified sites in the genome. The Cas9–sgRNA gene editing platform, which is based on the bacterial adaptive CRISPR immune system, has gained attention in gene editing recently and is now a standard procedure in many research laboratories across the globe due to the ease of use.[1] Recombinant DNA technology is a set of techniques used to recombine (join) DNA Segments of two or more distinct DNA molecules which are put together to produce a recombinant DNA molecule. A recombinant DNA molecule is able to enter a cell and replicate there under specific conditions, either by itself or by following chromosome integration.[2] Any species provide DNA sequences that are used to generate recombinant DNA molecules. In some cases, bacterial and plant DNA are put together, or fungus and human DNA are mixed.

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The chemically-mediated creation of DNA also produces DNA sequences that are not found in nature and incorporate them into recombinant molecules. It is possible to create and introduce any DNA sequence into a wide range of living organisms through the use of synthetic DNA and recombinant DNA technology.[3]

Infertility

A reproductive disorder known as infertility that affects both sexes and is marked by infertility following a period of regular, unprotected sexual activity lasting twelve months or longer.[4] It is classified into two types: primary infertility, which occurs when there has never been a successful pregnancy, and secondary infertility, which occurs when there has been at least one successful pregnancy but still no baby.

Male infertility

Male infertility is typically characterized by issues with sperm ejection, low sperm count, or poor morphology and motility.[5] Spermatogenesis comprises a sequence of cellular processes, such as mitosis-induced self-renewal of spermatogonial stem cells and spermatogonia, spermatocyte transformation and differentiation, meiosis I/II-induced haploid spermatid generation, and spermiogenesis-induced final morphological maturation of spermatids to become spermatozoa. Thus, these processes entail a variety of cellular activities in the testis and are intricately regulated by signalling and hormonal axes.[6-10]

Female infertility

Infertility in the female reproductive system originate from problems in the ovaries, uterus, fallopian tubes, and endocrine system, among other factors[5]. In the female embryo, a number of intraovarian and extraovarian components regulate the highly complex process of creating gametes, leading to a progeny organism.[11] The process through which the oocyte, is formed is known as oogenesis. There are many interactions between the developing oocyte and the granulosa and cumulus cells that surround it in this multi-step process. When oogonia are formed from primordial germ cells (PGC), about the 12th week of a woman's pregnancy, oogenesis starts in the foetal ovaries as soon as the embryo's development expands.[12-15]

Evolution and milestones of CRISPR technology

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) were initially identified in the genomic DNA of Escherichia coli by Ishino et al. (1987) at Osaka University, Japan.[18] Subsequently, in 1995, Francisco Mojica, a Spanish microbiologist, significantly advanced the understanding of CRISPR loci by identifying analogous sequences in the archaeal genome of Haloferax mediterranei, thereby suggesting a conserved function across prokaryotic domains.[19] The first experimental evidence elucidating the functional mechanism of the CRISPR-Cas system emerged in 2007 through studies conducted on Streptococcus thermophilus, a bacterium used in yogurt fermentation. This research was led by Rodolphe Barrangou and Philippe Horvath under the auspices of the Danish biotechnology firm Danisco.[20] Since the 1980s, Danisco had developed an extensive repository of bacterial strains, enabling detailed analyses of bacteriophage-host interactions. These investigations revealed the adaptive nature of the CRISPR system, wherein the incorporation of novel spacer sequences into the CRISPR locus correlated with acquired immunity against corresponding bacteriophages. Building on these findings, the researchers pioneered a CRISPR-based strategy for bacterial immunization, culminating in one of the earliest patents in this domain, filed in 2005.[21,22] The CRISPR-Cas9 system relies on a small RNA molecule that is processed and transcribed from the CRISPR locus. This molecule directs Cas proteins to external nucleic acid sequences that contain identical genetic information. A study group headed by John van der Oost at Wageningen University in the Netherlands was the first to identify these RNA components, which are called CRISPR RNAs (crRNAs).[23] Essential for in vitro reconstituting of the CRISPR-Cas9 system, an extra short RNA molecule involved in crRNA maturation was found in 2011 by Emmanuelle Charpentier's lab. The activation of Cas9 nuclease activity was demonstrated to require this RNA, which is known as trans-activating CRISPR RNA (tracrRNA). A significant step towards the programmable and practical use of the CRISPR-Cas9 system for genome editing was the conceptual advancement that crRNA and tracrRNA are created into a single chimeric RNA, called single-guide RNA (sgRNA).[24,25]

Components of CRISPR

CRISPR/Cas systems are broadly classified into two major classes based on the structural organization and functional characteristics of their associated Cas proteins: Class I, comprising types I, III, and IV, and Class II, comprising types II, V, and VI.[27] Class I systems utilize multi-subunit protein complexes to mediate interference, whereas Class II systems rely on a single, multifunctional Cas protein to perform the same function. Two fundamental elements are required for the CRISPR/Cas9 system to function: the Cas9 endonuclease and the guide RNA (gRNA). Genome editing initially made use of the Cas9 protein, which originated from Streptococcus pyogenes (SpCas9). The capacity to produce specific double-stranded DNA breaks at specific places has earned this big, multidomain protein, which contains 1,368 amino acids, the nickname "molecular scissor".[28] The two primary structural domains of Cas9 are the recognition (REC) and nuclease (NUC) domains. The REC lobe, which is made up of the REC1 and REC2 domains, helps with target recognition by binding the guide RNA. A number of domains are located in the NUC lobe. One of these is the RuvC nuclease domain, which cleaves the non-target DNA strand. The other is the protospacer adjacent motif (PAM)-interacting domain, which binds to the target DNA's PAM sequence and guarantees sequence specificity.[29] An 18–20 nucleotide sequence complementary to the target DNA is found in the CRISPR RNA (crRNA) component of the guide RNA, while a scaffold of stem-loop structures required for Cas9 binding and activation is formed by the trans-activating CRISPR RNA (tracrRNA).[28] These RNAs work in tandem to enable site-specific DNA cleavage by directing the Cas9 protein to particular genomic locations.

CRISPR–CAS System mechanism

The three main steps of the CRISPR/Cas9 system for editing genomes are identifying targets, cleaving DNA, and repairing damaged DNA.[30] Synthetic single-guide RNAs (sgRNAs) contain CRISPR RNA (crRNA) components that base-pair with complementary sequences in the target gene, directing the Cas9 endonuclease to the target DNA. When sgRNA is not present, Cas9 stays in an inactive state. Cas9 will insert a double-strand break (DSB) at a position three nucleotides before the protospacer adjacent motif (PAM)[31] when base pairing is successful. Although the precise sequence and length of the PAM differ among bacterial species, it is usually a brief, conserved DNA sequence located just downstream of the target site and usually ranging from 2 to 5 base pairs in length. The most popular version used for genome editing, SpCas9, which is derived from Streptococcus pyogenes, identifies the PAM sequence 5^{1}-NGG-3^{1}. Despite our limited understanding of the exact molecular mechanism, Cas9 is able to unwind local DNA strands and generate an RNA-DNA heteroduplex by recognising the PAM site. The catalytic domains of Cas9 are activated by this conformational shift. The DNA strand that is complementary to the sgRNA is cleaved by the HNH domain, and the DSBs that are caused by the RuvC domain are mainly blunt-ended. The endogenous DNA repair pathways of the host cell then fix these breaks, finishing the genome editing process.[28,32]

Some DNA strands experience double-strand breaks (DSBs). The crRNA directs Cas9. TracrRNA stabilizes a structure before Cas9 enabling to break the target DNA. sgRNA is responsible for target gene recognition [sgRNA (teal)].[34] These domains (RuvC, HNH) then interact with the Cas proteins to effectively modify the genomes of different animals by introducing DSBs in the DNA at specific locations. The HNH (histidine–asparagine–histidine) nuclease domain of Cas9 cleaves the DNA strand complementary to the guide RNA, whereas the RuvC-like (resolvase C-like) nuclease domain cleaves the non-complementary strand.[35] In both type I and type II CRISPR/Cas systems, the target of interference is foreign DNA containing a protospacer adjacent motif (PAM), a short, conserved sequence essential for target recognition and cleavage. The Cas9 protein breaks the target DNA on both strands using its RuvC and (HNH) domains. For Cas9 to break DNA, it primarily requires the PAM sequence. A 20-base stretch offers selectivity for binding in sgRNA. There are two methods for repairing DNA DSBs: HDR, which requires the presence of a template which results in NHEJ, a loose but permanent knockout of a gene, or knock-in or gene replacement.[36] In type I and type II CRISPR/Cas systems, only foreign DNA sequences containing a protospacer adjacent motif (PAM)—a short,

conserved nucleotide sequence—are specifically recognized and targeted for interference.[37]

The basic mechanism by which all living things sustain their generations is reproduction. Recently, a novel, adaptable genome editing technique called CRISPR/Cas9 was developed to fix genetic abnormalities that cause a number of diseases, expanding its ability to enhance reproductive health.[39] Using CRISPR and ARTs together has made it easier to modify the genomes of embryos created using IVF and other similar procedures. CRISPR/Cas9 is especially useful when it comes to IVF. IVF-produced embryos have the potential to have particular genes disrupted or edited by CRISPR, which could improve particular features or prevent hereditary diseases.[40] In general, CRISPR technology has the capacity to improve IVF results and encouraging the development of new uses for the reproductive systems of both men and women.[41]

Application of CRISPR in reproductive biology

Sperm

Genome editing technologies have the ability to revolutionize spermatogenesis research

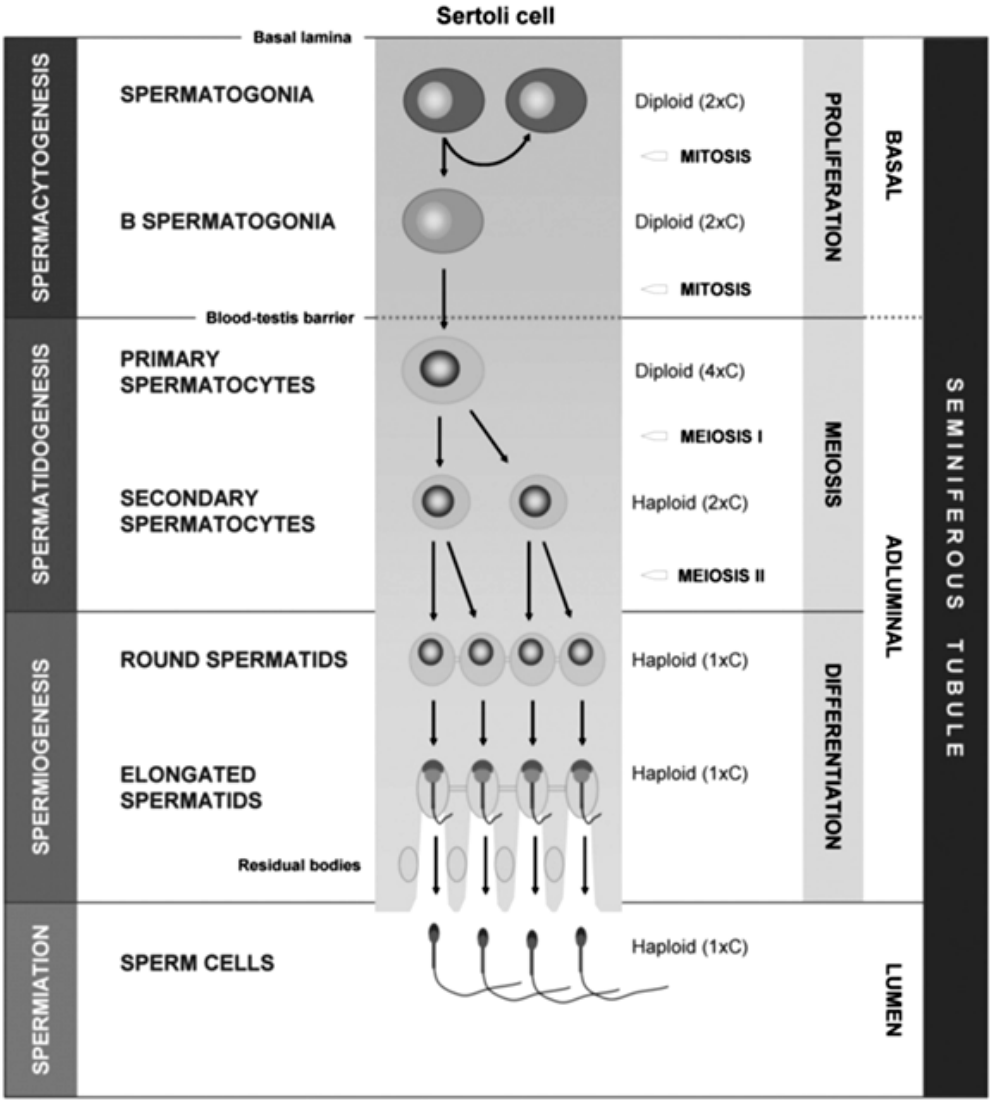

Fig. 1. Flow chart representing stages of spermatogenesis, germ cell characteristics in each stage and compartments of seminiferous tubules.[16]

and shed light on the molecular mechanisms behind male infertility disorders. This was demonstrated by the successful application of CRISPR/Cas9-mediated gene editing in mouse spermatogonial stem cells (SSCs).[42] An effective and easily accessible in vivo method for studying gene function during spermatogenesis is the CRISPR/Cas9-based spermatogenic cell-specific knockdown system.[43] A proof of concept was carried out in rat SSCs using targeted gene editing at the Epsti1 locus. This locus is involved in epithelial-stromal interactions, and the results showed that the genome could be effectively modified. Epsti1 mutations in humans have been

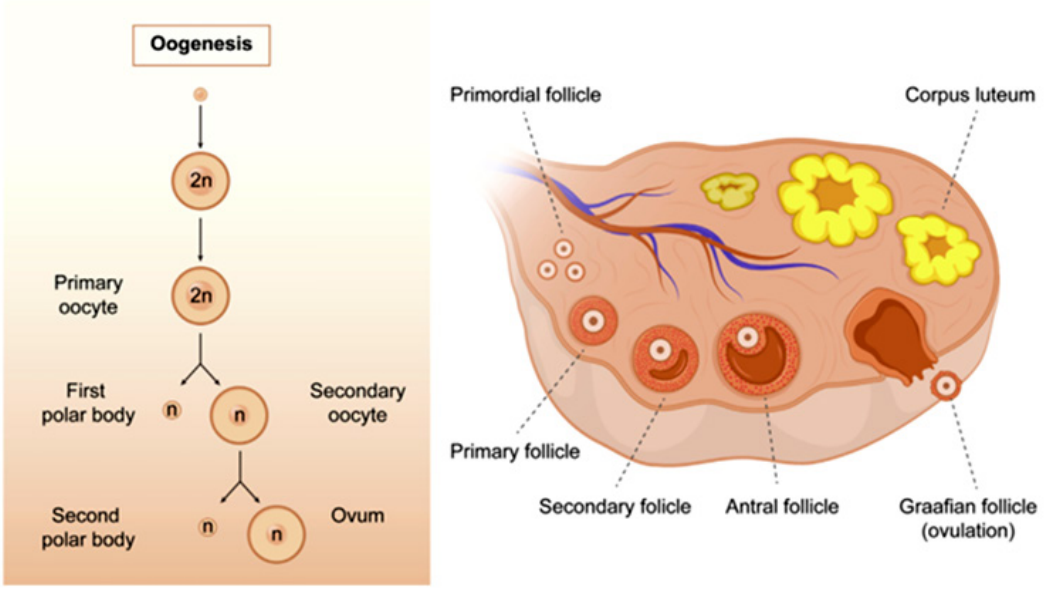

Fig. 2. This diagram illustrates the process of oogenesis which begins with transformation of the oogonia into mature oocyte.[17]

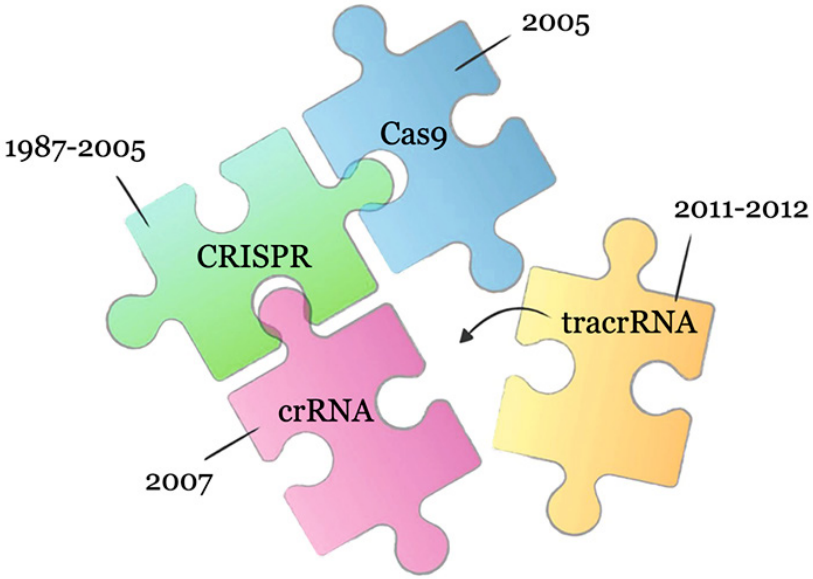

Fig. 3. The historical chronology of the CRISPR-Cas9 system's component findings.[26]

linked to changes in sperm function, which is worth noting.[44] With SSCs readily available, a potential platform for homology-directed repair (HDR) mediated by CRISPR/Cas9 to address harmful mutations has emerged. This method has been proven effective in a model of cataract in mice by restoring normal gene function after an ex vivo correction of a disease-causing mutation in SSCs using CRISPR-Cas9 and HDR.[45-47] These results raise the possibility that recovering spermatogenesis in individuals with genetically-caused non-obstructive azoospermia (NOA) could be possible through targeted gene editing in SSCs.[48]

Oocyte

Generating genetically modified female germ cells for use in assisted reproductive technologies (ARTs) has been made possible by the CRISPR/Cas9 system, which has shown great promise in genetically modifying developing oocytes. These changes could be useful in preventing off-target mutations from being passed down across generations. Oocytes are a good candidate for germline modification because they are easily accessible. To test the efficacy and accuracy of CRISPR/Cas9-mediated gene editing, oocytes must first reach the germinal vesicle (GV) stage of in vitro maturation, and then the following meiotic development is required.[39]

Numerous animal models, including pigs and mice, have demonstrated the successful use of CRISPR/Cas9 to mammalian oocytes and embryos. It is possible to treat hereditary diseases by directly modifying the genome at these early phases of development. To further our understanding of the molecular pathways essential for embryogenesis, genome editing techniques in oocytes and embryos enable in-depth mechanistic research of early developmental processes.[49]

The dCas9-DNMT and dCas9-TET complexes are epigenome editing tools that have been used to alter DNA methylation patterns in embryos and oocytes from mammals. To fix aberrant methylation linked to familial Angelman syndrome, for instance, microinjected dCas9-TET-based systems have been used in mice oocytes.[50] Furthermore, a CRISPR-based approach integrating sgRNA and dCas9-DNMT3a has been used to successfully edit seven separate

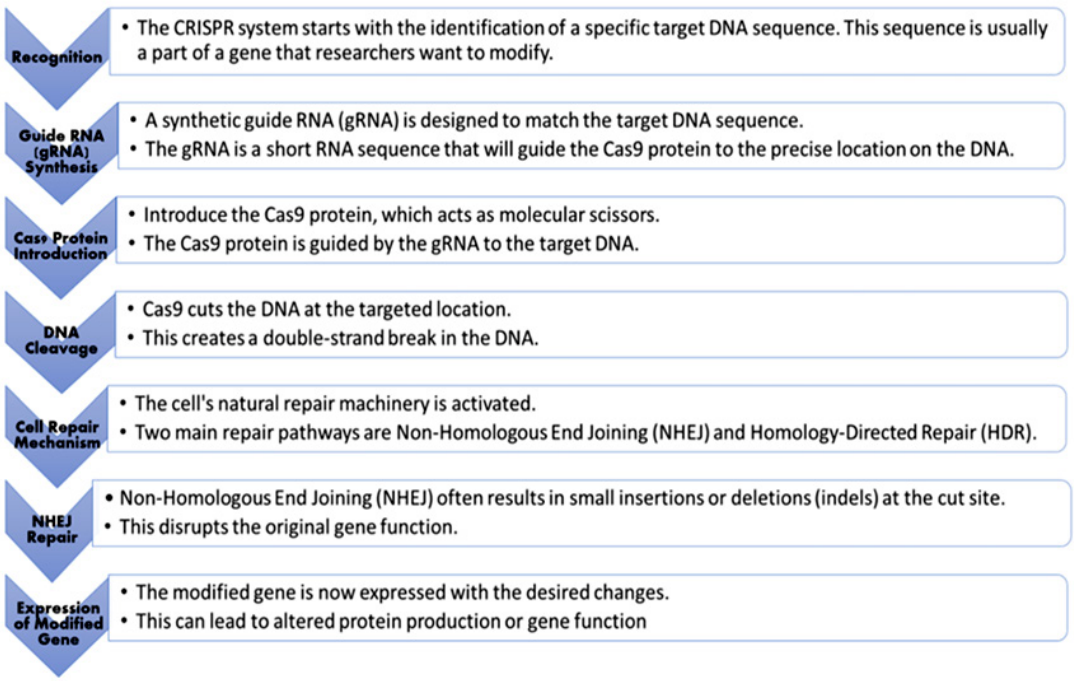

Fig. 4. This represents successive steps of CRISPR/Cas9 system which includes target identification and modification of gene.[33]

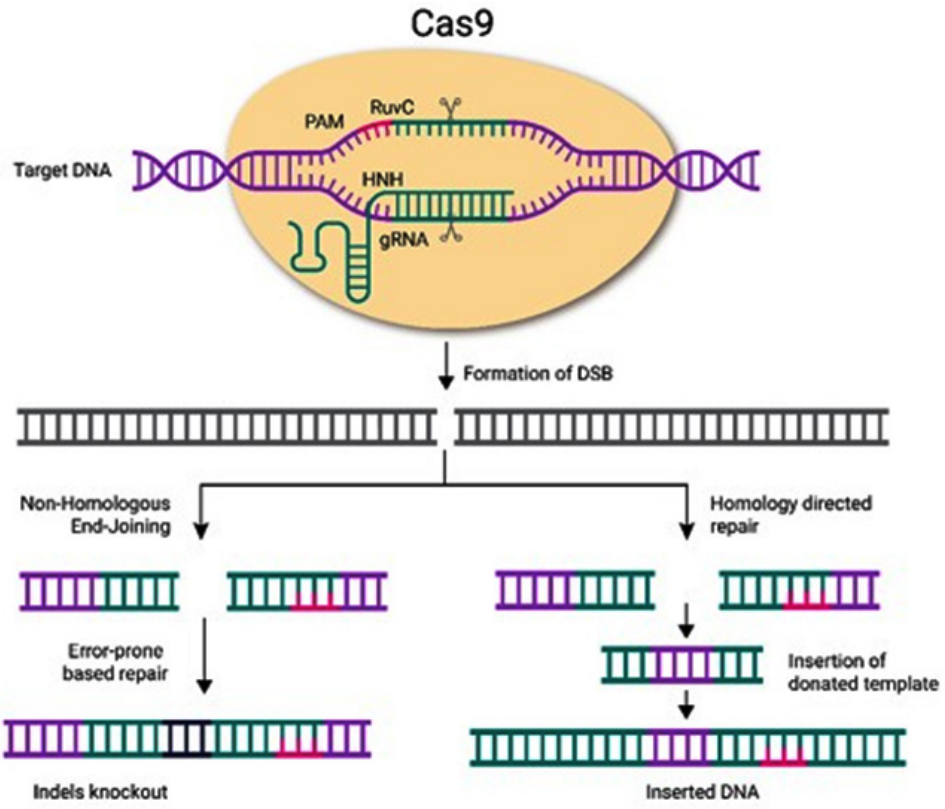

Fig. 5. CRISPR and the Cas9 nuclease mechanism are represented in symbolic terms.[38]

genomic imprinting loci in single unfertilised oocytes, leading to the production of genetically changed children after fertilisation.[51] In light of these developments, studies investigating oocyte methylation have gained popularity as a possible strategy for the treatment of non-genetic maternal hereditary disorders.[52]

Researchers have shown that injecting Cas9 cRNA into metaphase II (mII) oocytes before sperm and guide RNA (gRNA) has the capacity to increase editing efficiency, prolong Cas9 expression, and improve the results of genome modification. Edited embryos and healthy offspring could be produced using this co-injection technique.[53] To further emphasise the significance of timing genome editing with DNA synthesis and particular cell cycle stages for optimal efficiency, it was found that inserting CRISPR/Cas9 components into M-phase oocytes successfully removed mosaicism in cleaving embryos.[54]

Even in mature oocytes with condensed chromatin, CRISPR/Cas9 was able to elicit high-efficiency alterations, according to the experimental findings. Gene editing is not the best approach for GV-stage oocytes, but they are made more mutable by blocking nuclear export and raising nuclear Cas9 levels. There were no negative impacts on meiotic progression or early embryonic development observed in pig models when CRISPR components were microinjected into immature oocytes.[55-59] Genome editing in oocytes going through meiosis confirmed their eligibility as targets for CRISPR/Cas9-based therapies, as it led to a noticeably increased mutation efficiency.[60,61]

Embryo

To correct pathogenic mutations in the germline, an optimized CRISPR/Cas9-based strategy has been developed that leverages the endogenous DNA repair mechanisms active in early embryos. This approach enabled the precise,

efficient, and accurate correction of a heterozygous mutation in the MYBPC3 gene—implicated in hypertrophic cardiomyopathy (HCM)—in human preimplantation embryos. This was achieved by co-injecting sperm, Cas9 protein, guide RNA (gRNA), and single-stranded oligodeoxynucleotides (ssODNs) into metaphase II (MII) oocytes, without inducing significant off-target effects or large deletions.[62-64]

When it comes to in vitro fertilization and preimplantation genetic diagnosis, the CRISPR/Cas9 method could be used to increase the quantity of embryos available for transfer.[65] The use of CRISPR on human embryos has the potential for removing all genetic defects from the genome.[66.]

Advantages of CRISPR

Mutations are induced on several locations at once with the use of CRISPR/Cas9. Previously, this couldn't be accomplished in a single round using traditional methods.[67,68] It was necessary to create several mutant mouse lines using these traditional techniques, which necessitated repeated crossbreeding to produce mice with various mutations for study. As established by Wang et al., (2013) who simultaneously targeted the Tet1 and Tet2 genes, CRISPR/Cas9 may target multiple locations.[67] Because CRISPR/Cas9 has the potential to create homozygous mice in the founding generation, analysis completed much more quickly and researchers are capable to generate more data than ever before by expanding their list of target genes.[69]

In the field of reproductive biology, the development of CRISPR/Cas9 technology is very encouraging. It is possible to thoroughly study the functional roles of potential genes implicated in spermatogenesis by using CRISPR/Cas9-mediated transcriptional suppression. This method allows for the assessment of gene-specific roles in sperm maturation. Furthermore, by fusing CRISPR/Cas9 systems with fluorescent tags, it is possible to establish the chromosomal localisation of genes specific to sperm. By making it easier to see and map spermatogenesis-related gene loci, these methods provide light on important regulatory mechanisms supporting male reproductive function.[70]

Limitations

One of the major challenges in applying CRISPR/Cas9 for gene therapy is the high frequency of off-target effects (OTEs), which have been reported at rates of e™50% in some studies.[71] Additional limitations of the CRISPR/Cas9 system include unintended on-target effects, suboptimal homology-directed repair (HDR) efficiency, and the persistent difficulty of precisely controlling genome edits.[72–74] These issues raise concerns about the genomic integrity of edited embryos, as unanticipated mutations carry the risk of having deleterious consequences for progeny. As CRISPR technology continues to evolve, previously unrecognized editing errors continue to emerge, highlighting the need for comprehensive assessment and refinement prior to clinical application.[75,76]

Furthermore, embryos exhibit a distinct response to CRISPR-Cas9-induced DNA damage compared to somatic cells, a phenomenon not yet fully understood. Editing outcomes in embryos remain highly variable and unpredictable, frequently resulting in diverse forms of genomic damage. Notably, approximately 50% of edited embryonic cells display detectable abnormalities, indicating a high burden of unintended effects. These findings suggest that genome editing protocols optimized for somatic cells are not directly transferable to embryos. The induction of double-strand breaks (DSBs) by Cas9 in embryonic genomes occasionally leads to undetected or inaccurately repaired lesions due to incomplete knowledge of embryonic DNA repair pathways. Collectively, these challenges underscore the current unsuitability of CRISPR-Cas9 for clinical germline genome editing and emphasize the need for further investigation and ethical deliberation before its application in mammalian eggs and embryos.[49,77]

Bioethics

An important ethical consideration with CRISPR/Cas9 is the possibility of off-target consequences leading to alterations that were not intended. If such changes occur in germline cells, they could be passed on to future generations, potentially leading to unknown biological consequences. CRISPR/Cas9 safety needs to be improved by increasing its specificity and carefully identifying both desirable edits and off-target modifications. Another issue is mosaicism, in which certain cells are not altered, resulting in mutant cells that still cause illness

and decreasing the efficacy of treatment.[78] As mentioned earlier, the existing guidelines indicate that CRISPR technology should not be utilized for genetic editing in germ cells or embryos intended for implantation due to the uncertain and serious possible repercussions of off-target editing, the implications of modifying the intended target itself, the emergence of mosaicisms (the existence of two distinct cell lines derived from the same zygote), and the potential creation of new diseases along with the risk of impacting an entire generation of humans with these conditions.[79]

CONCLUSION

CRISPR/Cas9 technology holds transformative potential in reproductive biology, particularly in the genetic editing of human gametes. Because of its accuracy, effectiveness, and capacity to target certain DNA sequences, it is a useful tool for repairing genetic abnormalities at the gamete or zygote stage. Pre-fertilization gene editing has shown encouraging results in applications in spermatogonial stem cells (SSCs) and metaphase II oocytes, setting the stage for preventing monogenic hereditary diseases and enhancing embryo quality. Furthermore, improving embryo selection and combining CRISPR with methods such as preimplantation genetic diagnosis (PGD) are expected to greatly increase the success rates of assisted reproductive technologies (ART).

Although it offers many advantages, significant ethical and technical barriers still prevent CRISPR from being used clinically in reproductive medicine. There is considerable safety issues related to embryonic mosaicism, off-target effects, and unusual genomic rearrangements. Long-term follow-up research and strict regulatory frameworks are also necessary due to the possibility of irreversible germline changes. It is also necessary to carefully consider the ethical concerns of heredity, consent for future generations, and institutional concerns. Future research efforts should prioritize the optimization of target specificity, the minimization of associated risks, and the facilitation of transparent ethical discourse to guide the safe and responsible clinical implementation of CRISPR/Cas9 in reproductive health.

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This research did not involve human participants, animal subjects, or any material that requires ethical approval.

Informed consent statement

This study did not involve human participants, animal subjects, or any material that requires ethical approval.

Clinical trial registration

This research does not involve any clinical trials.

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Not Applicable.

Authors contribution

Esha Kumari: Conceptualization, Methodology, Writing- Original Draft; Neha Banu: Data Collection, Analysis, Writing- Review and Editing; Katrina Marbaniang: Analysis, Editing and Supervision; Faridha Jane R.M. Momin: Analysis, Editing and Supervision; Barry Cooper Hynniewta: Visualization, Supervision, Project Administration.

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