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CRISPR/Cas9 in Male Factor Infertility

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Abstract

Purpose of Review Male factor infertility is common and often multifactorial. A subset of these patients have underlying genetic etiologies. CRISPR/Cas9 is a simple and flexible gene editing tool with promising applications in this space. This review aims to summarize the advances that CRISPR/Cas9-based tools have brought to the field and propose future directions for study.

Recent Findings CRISPR/Cas9 has been applied successfully to spermatogonial stem cells (SSCs) and via pronuclear injection of zygotes to generate animal models of male factor infertility. These approaches have led to the high-throughput validation of candidate male fertility genes obtained either through genome-wide associated studies or testis-specific gene expression studies. One group has applied this further to SSCs in the correction of genetic infertility due to a mutation in the *Kit* gene.

Summary Application of CRISPR/Cas9 to the investigation and treatment of male infertility holds promise in identifying novel genetic causes of NOA. Gene editing in germ cells to treat genetic infertility is technically feasible, but has not been used in humans due to significant ethical concerns. Stringent regulations are imperative to ensure safe translation of this technology into human populations.

Keywords CRISPR · Cas9 · Genome editing · Infertility · Andrology · Spermatogonial stem cells · Germ cells · Germline

Introduction

Since its discovery, the CRISPR (cluster interspersed short palindromic repeats)/Cas9 system has emerged as the simplest and most flexible method for gene editing—namely, the precise addition, deletion, and alteration or an organism's genetic material [1]. It has become a seminal tool in biomedical research, as well as a hopeful candidate vehicle for therapeutic interventions and has been applied broadly to study different areas of biology. Male factor infertility is one such area. The underlying cause of male infertility is often multifactorial, but a subset of these, particularly in non-obstructive azoospermia (NOA), have underlying genetic etiologies. While some genetic causes of NOA have been identified, there are undoubtedly many causal genes remaining to be elucidated. CRISPR/Cas9 is a promising tool to expand our knowledge and therapeutic toolbox in this space. This review provides a background on CRISPR/Cas9 tools for mammalian gene editing, summarizes how CRISPR/Cas9 has been used to advance the study of male factor infertility, and proposes future directions as well as ethical considerations for the use of this technology in both studying and treating male factor infertility.

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CRISPR/Cas9 for Genome Editing

The CRISPR system was first described as a feature of the prokaryotic adaptive immune system that protects against pathogenic phages [2]. Bacteria or archaea that are subjected to a viral challenge capture short sequences of genetic material, termed protospacers, from the invading organism and incorporate these into their own genome at specific loci to form a CRISPR [2]. CRISPRs are named as such, because they are characterized by repeat sequences separated by protospacers. The CRISPRs are then transcribed into pre-CRISPR RNAs (crRNA) which bind by sequence complementarity to a trans-encoded RNA (tracrRNA) with complementarity to the pre-crRNA repeat region [3].

This complex is then loaded into a CRISPR-associated (Cas) endonuclease where the pre-crRNA is processed to a crRNA by bacterial RNase III. The loading of the tracr-

crRNA complex into the Cas endonuclease causes a conformational change in the protein leading to the formation of a channel which can accommodate target DNA sequences [4]. The resulting crRNA, tracrRNA, and Cas complex form an active nuclease complex that then can induce a double-strand DNA break at the target locus (Fig. 1).

Two classes of CRISPR systems have been identified. Class I systems include types I, III, and IV and are present in bacteria and archaea. The effector endonucleases of this class are composed of four to seven Cas protein subunits [5]. This complexity limits their applicability in the sphere of genome editing. Class II systems are less commonly occurring in nature and are composed of types II, V, and VI CRISPR systems. This class is characterized by a single multi-domain effector endonuclease [5]. While the particular of each type of CRISPR system is beyond the scope of this review, the best described of these is the type II Cas9 system. The Cas9 endonuclease targets 23 bp sequences composed of a 20 bp sequence that is complementary to the crRNA followed by an "NGG," where "N" designates any base [6]. The 3′ NGG sequence is called the protospacer adjacent motif (PAM) and is required for recognition by Cas9 which induces the dsDNA break 3 bases upstream of the PAM [7]. Further work showed that the tracrRNA and crRNA could be fused to form an artificial single-RNA guide (sgRNA) to direct Cas9 endonuclease activity [6].

Zinc-Finger Nucleases (ZFN) and transcription activator-like effector nucleases (TALENs) were the only two programmable nucleases available for targeted genome editing prior to the advent of CRISPR/Cas9 [8]. ZFNs are modular proteins composed of two domains: a nuclease domain based on the *FokI* restriction enzyme and a DNA-binding domain composed of zinc finger proteins (ZFPs) which determine the site specificity of the ZFN [9]. Sequence specificity is achieved by assembling multiple Cys2His2 zinc fingers with unique 3 bp DNA-binding sequences in tandem [10]. *FokI* nuclease domains must dimerize to cause a DNA double-strand break so ZFNs must be paired [11]. TALENs are structurally similar to ZFNs in that they are modular and contain a DNA cleavage domain and a DNA-binding domain. Like ZFNs, their DNA cleavage domain is based on the *FokI* restriction enzyme; however, their DNA recognition domain is based on concatenated transcriptional activator-like effectors (TALEs) which are derived from pathogenic plant bacteria of the *Xanthomonas* spp. [12]. Each TALE is composed of 33–35 AAs and recognizes a unique base in its major groove. Binding of individual bases is specified by the AAs located at position 12 and 13 which are termed the repeat variable diresidues (RVDs) [13]. The widespread use of both of these methods has however been hampered by the technical challenges associated with producing them and their lack of flexibility.

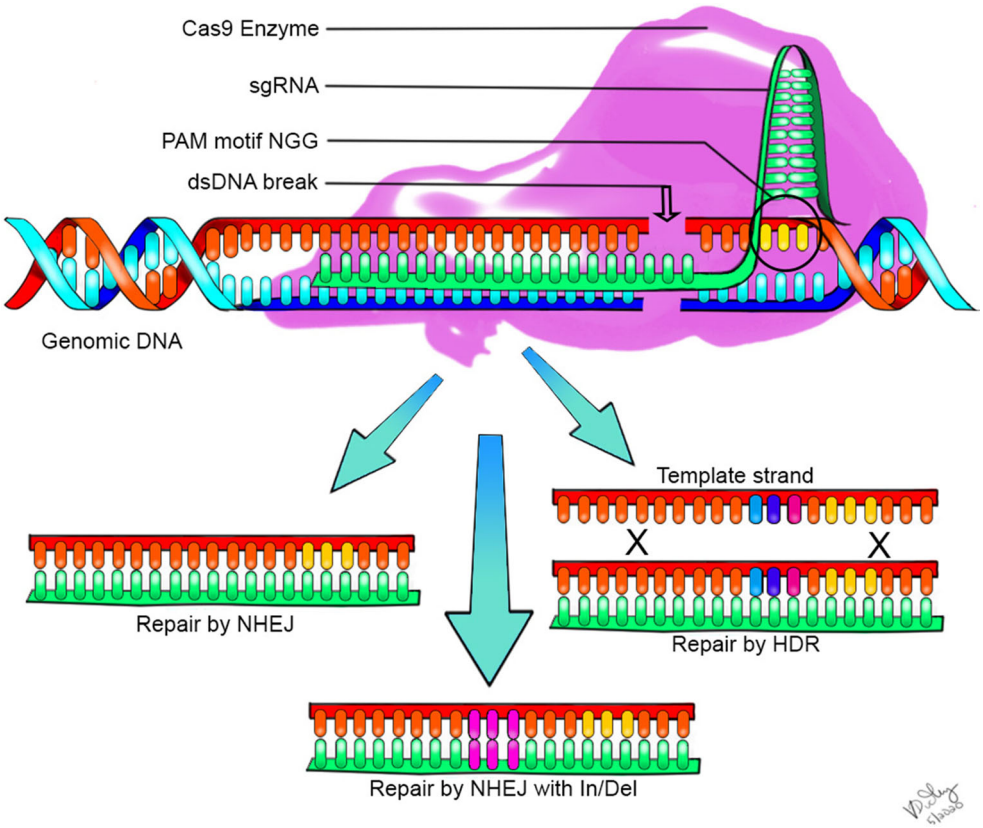

Fig. 1 The Cas9 endonuclease complex is guided by an sgRNA to a complementary 20 bp genomic DNA strand with adjacent PAM motif and induces a dsDNA break. The Cas9 enzyme induces a double-stranded DNA break 3 base-pairs from the "NGG" PAM sequence. The double-strand DNA break can be repaired by non-homologous end-joining (NHEJ), NHEJ with formation of insertion or deletion (in/del), or homology-directed repair (HDR)

In 2013, two groups showed that the CRISPR/Cas9 system could be used to induce double-strand breaks in the mammalian genome [14, 15]. This work highlighted several notable advantages that the mammalian optimized CRISPR/Cas9 system has over other genome-editing technologies. Beyond its ease of use, the Cas9 nuclease is not variable; therefore, by transfecting multiple sgRNAs into a single cell, multiple loci in the same cell can easily be targeted for multiplexed genome engineering. Several methods for delivering the Cas9 nuclease and sgRNAs to mammalian cells have also been devised. *S. pyogenes Cas9* is 4.2 kb, and can be delivered either by plasmid transfection or lentivirus, while the guides can be either transfected in the same plasmid as Cas9 under a U6 promoter, as a separate lentivirus [16, 17] with its own selection marker, or directly as PCR products [18]. In addition to the ease of design and delivery, CRISPR/Cas9 is also not as constrained as prior generations of genome-editing tools with regard to targetable loci in the genome. The "NGG" PAM requirement of Cas9 is not particularly limiting given that such a site occurs on average one in every eight base pairs [14].

The original application of this technology was to disrupt gene function by inducing DNA double-strand breaks with non-homologous end-joining (NHEJ) or to induce targeted gene modifications via DNA nicking and homology-directed repair (HDR) [14]. Since this original discovery however, the system has been re-engineered to perform other genomic functions. The creation of a catalytically inactive Cas9 protein (dCas9) combined with a sgRNA allowed for the targeting of specific genes and repression of gene expression [19, 20]. This dCas9 was subsequently fused to the transcriptional activator Vp64 which when targeted to promoter regions using sequence-specific sgRNAs induce the recruitment of the transcriptional machinery and subsequently target gene expression [21, 22]. Thus, the CRISPR/Cas9 system can be used to both downregulate and upregulate gene expression without changing coding DNA. Last, the system has also been repurposed to effect epigenetic changes at target genomic sites. A dCas9-Tet1 fusion protein was shown to specifically catalyze the demethylation of sgRNA-targeted promoters, while a dCas9-Dnmt3a fusion protein can catalyze methylation at specific targeted promoter sequences, thereby allowing the interrogation of site-specific epigenetic modification in vitro and in vivo [23, 24]. Targeted epigenetic activation has also been achieved using a dCas9-p300 which allows for acetylation of target promoters and robust gene activation [25].

While the CRISPR/Cas9 system has many advantages over traditional genome-editing tools, there are still technical barriers to its application in humans. One major concern is off-target effects which are related to both the sgRNA and the Cas9 endonuclease. First, there is a tradeoff between activity and specificity of sgRNAs, with shorter, less active sgRNAs conferring more specificity than longer more active guides [26]. Second, the sgRNA sequence is also important as the sgRNA-Cas9 complex can generally tolerate between 1 and 3 bp [15] and up to 5 bp mismatches. Third, high concentrations of either the guide or Cas9 can increase the likelihood of off-target effects [26]. Another concern is the immunogenicity of the DNA editing system itself. The Cas9 enzyme is found primarily in *Staphylococcus aureus* and *Streptococcus pyogenes*—two organisms which commonly infect humans. Indeed, pre-formed antibodies and T cells with activity specific to the Cas9 protein have been identified in humans [27, 28]. This is a technical challenge that will have to be overcome prior to planning human therapeutic interventions.

Male Factor Infertility

Infertility is described by the World Health Organization as the inability of a couple to conceive within 12 months of regular unprotected sex. It occurs among 15% of couples. Male factor infertility is present in 40–50% of couples presenting for infertility work-up [29]. Complete lack of sperm in ejaculate (azoospermia) is classified as being either obstructive or non-obstructive in nature. Obstructive causes are most commonly caused by infection, trauma, or congenital anomalies [30]. In this scenario, the testes remain capable of producing viable sperm.

Non-obstructive azoospermia (NOA) is a severe form of male factor infertility in which the ejaculate lacks sperm due to the inadequate or absent production of sperm from the testes. This can be from inadequate gonadotropin production or from intrinsic testicular impairment [31]. The treatments for this type of infertility can be invasive including microdissection testicular sperm extraction (mTESE) and intracytoplasmic semen injection (ICSI). Successful live births unfortunately only occur among 21.4% of couples pursuing this treatment [32]. An underlying etiology for NOA cannot be identified in roughly 80% of affected men; however, of those that are identified, genetic abnormalities represent the vast majority [31]. These include Y chromosome microdeletions, Klinefelter syndrome, KAL1 or FGFR1 defects in Kallmann syndrome, and even copy number variant deficits on the X chromosome [31]. Table 1 provides a detailed overview of our current understanding of causal genes in NOA. Thus, the application of genome-editing techniques such as CRISPR/Cas9 has significant potential in the investigation and treatment of infertile men.

Applying CRISPR/Cas9 Genome Editing to Treat Male Infertility

As our understanding of the genetic aberrancies underlying NOA and male infertility evolves, the potential to correct

Table 1 Genetic alterations associated with a NOA phenotype

Non-obstructive azoospermia		Genes/chromosomal abnormalities	References
Hypogonadotropic hypogonadism	Monogenic	KAL1, FGFR1, PROK2, PROKR2, CHD7, FGF8, GNRH, GNRHR, KISS1R, TAC3, TAC3R, DAX1, FSH, FSHR, LH	[33]
	Chromosomal translocations	46,XY/46,X,inv.(Y)(p11.2q11.2), mos46,XY,t(3;12)(p13;p13)/46,XY	
Klinefelter syndrome		47,XXY, mos47,XXY/46,XY	[34]
46,XX male		SRY, SOXA	[35]
Noonan syndrome		PTPN11, SOS1, KRAS, NRAS, RAF1, BRAF, SHOC2, MEK1, CBL	[36]
Mixed gonadal dysgenesis		Mos45,X/46,XY	[37]
X-linked infertility		AR, USP26, SOX3, TAF7, NXF2, TEX11	[36]
Yq11 chromosome microdeletions	AZFa AZFb	Candidate genes: USP9Y, DBY, UTY, TBY Candidate genes: CYorf15, RPS4Y2, EIF1AY, SMCY, XKRY, HSFY, PRY, RBMY	[38]
	AZFc	candidate genes: DAZ, CDY, BPY2, GOLGA2LY, CSPG4P1Y, TTY4	
Monogenic disorders		DMC1, DNAH6, MAGEB4, MCM8, MEIOB, MEI1, NPAS2, PSMC3IP, SPINK2, STX2, SYCE1, TAF4B, TDRD7, TDRD9, TEX14, TEX15, XRCC2, ZMYND15	[39]

these abnormalities and restore healthy spermatogenesis using gene editing techniques such as CRISPR/Cas9 exists. This technique could be applied to somatic cells, or spermatogonial stem cells in vitro, and then subsequently transplanted back into the patient's testis or differentiated into haploid germ cells in vitro. CRISPR/Cas9 is also well positioned for in vitro modeling and testing of gene expression and epigenetic regulation of germ cell or somatic cell functioning. This technique could be applied to both primarily derived testicular cells and human-induced pluripotent stem cells. Another potential application of such systems involves the correction of known paternally derived genetic abnormalities giving rise to disease in their offspring. These applications hold much promise in revolutionizing the directions of male infertility research; however, significant ethical considerations are imperative to consider given that the potential for undesired outcomes is vast.

CRISPR/Cas9 Genome Editing in Spermatogonial Stem Cells

Spermatogonial stem cell (SSC) transplantation has been proposed as a promising therapeutic intervention to restore fertility in men with NOA [40], or who are at risk of complete germ cell depletion [41], as it has been successfully accomplished in a number of pre-clinical animal models. This process was first described in mice by Brinster and Zimmermann in 1994 where they micro-injected SSC wild-type donor mice into the seminiferous tubules of azoospermic kit mutant mice or busulfan-treated mice resulting in the restoration of normal spermatogenesis [42]. This technique has been subsequently shown to work not only in rodents but also in zebrafish [43], pigs [44], cows [45], non-human primates [46, 47], and even across species [48]. This technique has not only been shown to generate viable sperm and healthy offspring in rodents [49–52] and large mammal models [53, 54] but also in non-human primates [47].

The use of CRISPR/Cas9 to target genes in SSCs was first reported in 2015. Early efforts were geared toward proving that CRISPR/Cas9-mediated gene editing in SSCs was not only technically feasible but could also yield offspring carrying the targeted mutation. Wu et al. report the generation of a mouse SSC cell line in which the *Crygc* gene was deleted using CRISPR/Cas9 and NHEJ-mediated in-del [55]. When injected into busulfan-treated mice, these mutant SSCs generated sperm and yielded offspring. These offspring phenocopied a previously described mouse model of nuclear cataracts caused by a 1 bp deletion in exon 3 of the *Crygc* gene resulting in a premature stop codon ($Crygc^{-/-}$). The authors then went on to use the same system with HDR to correct the genetic defect in SSCs from $Crygc^{-/-}$ mice to yield offspring with no cataracts. Importantly, the whole-genome bisulfite sequencing showed no difference in methylation pattern, and thus paternal imprinting status, between germline corrected *Crygc* mice and the $Crygc^{-/-}$ mice. The CRISPR/Cas9 system was also shown to be effective in evaluating fertility phenotypes in SSCs. As a proof of principle, mouse SSCs carrying a CRISPR/Cas9-mediated deletion in the *Stra8* gene which is responsible for regulating entry into meiosis were able to

populate *Kit* null mouse testes but did not generate sperm [56]. In rats, SSCs bearing mutations in *Epsti1*, a gene associated with alterations in sperm function and family size, were able to populate testes and generate mutant offspring at an expected frequency [57] suggesting that the gene is dispensable for fertility. The same study generated SSCs with homozygous deletions in the *Errb3* gene. *Errb3* encodes a receptor tyrosine kinase which is activated by Nrg1, a factor required for the clonal development of spermatogenic cells in vitro. *Errb3*-deleted SSCs were severely compromised in their ability to support development of spermatogenic colonies in vitro but displayed normal spermatogenic potential when transplanted into rats [57]. This approach has also been shown to work for studying fertility phenotypes related to non-coding genes. Micro-RNAs are of particular interest given their high level of expression and regulation at various stages of spermatogenesis. Chen et al. created SCCs with a doxycycline-inducible CRISPR/Cas9-mediated knock-out of micro-RNA 202 (miR-202) [58]. Using this approach, they showed that miR-202 is responsible for maintaining the stem cell pool by blocking premature differentiation both in vitro and in vivo.

While these studies showed the effectiveness of CRISPR/Cas9 for testing hypotheses in the field of male fertility, a major advance was the application of this technology for correcting genetic defects resulting in male factor infertility. Mice bearing spontaneous heterozygous mutations in the *Kit* gene ($Kit^{w/wv}$) have male factor infertility due to deficiency of spermatogonia [59]. Li et al. applied Cas9 and HDR to correct the Kit^{wv} mutation in SSCs which then resulted in restoration of fertility when re-transplanted into $Kit^{w/wv}$ mice [60]. Furthermore, these mice did not exhibit any defects in imprinting or off-target effects at predicted loci. These discoveries in mice suggest the future potential of applying such techniques to addressing genetic causes of male infertility in humans.

Of the men who suffer from NOA, a proportion lack sperm precursors all together. This is termed Sertoli cell-only phenotype. Others suffer from maturation arrest in which SSC fails to differentiate into sperm. This failure in differentiation may occur at various intermediary stages of spermatogenesis. Interestingly, despite the difference in phenotype, SSC can be isolated from a portion of patients with both subtypes of NOA and expanded in vitro [61]. In such men, the isolation of SSCs and the use of CRISPR/Cas9 could both accelerate the identification of and facilitate the correction of genetic defects resulting in either subtype of NOA. Thus, for men without current potential to produce sperm, this application has the potential to rescue a spermatogenic phenotype capable of producing sperm and subsequent injection for fertilization via intracytoplasmic sperm injection (ICSI). This approach however if performed in SSC's results in germline editing which is passed down to offspring and as such is currently the subject of ethical debate which is further discussed below. However, if particular genetic defects contributing to the NOA phenotype are localized to somatic cells in the testis, CRISPR/Cas9 editing may be used to rescue cell function to support spermatogenesis of primary germ cells. Application of such techniques for men with NOA will require a personalized medicine approach to identify the specific genetic abnormalities unique to each individual with NOA, confirm that the identified mutation is indeed disease causing, and correct the associated genetic abnormality with CRISPR/Cas9-based tools.

CRISPR/Cas9 for Modeling Human Infertility

While there are several well-described genetic causes of NOA, often a causative mutation is not identified, adding another layer of complexity in the application of genome editing to treat this condition. This being said, a number of candidate single-nucleotide polymorphisms (SNPs) associated with NOA have been identified either through genome-wide association studies [62–64] or targeted SNP interrogation [65–67]. While these studies use robust statistical methodology to show that certain gene variants segregate with the NOA phenotype, by their very nature, they lack functional genetic evidence that draws a causal link to the phenotype in question. Depending on the conservation of candidate genomic regions between mice and humans, CRISPR/Cas9 and HDR could be used to create SSCs or germline-edited mouse models that faithfully recapitulate these SNPs and test their effect on fertility.

Another area in which CRISPR/Cas9 technology has the potential to impact the field of infertility research and may offer therapeutic promise is the identification and treatment of genetic mutations resulting in infertility due to morphological abnormalities in sperm. Prior to the development of CRISPR/Cas9, genetic sequencing of consanguineous families and small cohorts of men with infertility allowed for the identification of several recessive genetic causes of oligo-/teratozoospermia (macrozoospermia, globozoospermia, and multiple morphological abnormalities of the flagella) including *AURKC*, *DPY19L2*, *CCDC39*, several of the dynein family genes, *DYX1C1*, *HYDIN*, and *LRRC6* [68]. The facility with which loss-of-function mutations in candidate genes could be validated in vivo, however, was hampered by the challenges associated with generating animal models.

The combination of increasingly available whole-exome sequencing in cohorts of affected men improved computational tools, and rapid generation of knock-out mouse models with CRISPR/Cas9 through pronuclear injection has allowed for high-throughput identification and validation of genes that result in sperm abnormalities. Indeed, since 2015, this approach has helped identify *CCDC36* [69], *SLC33A14* [70], *CABYR* [71], *CFAP43*, *CFAP44* [72], *CDC14A* [73], *RSPH6A* [74], *SLX/SLX1* [75], *ARMC2* [76], *TTC21A* [77], *CFAP65* [78], *QRICH2* [79], and *CFAP69* [80] as causative

mutations in humans and mice. This approach has also allowed for the rejection of many candidate genes with only anecdotal supportive evidence as potential causes of infertility. One recent study interrogated a list of 54 genes that were highly expressed in the testis and well conserved between mice and humans [81]. Pronuclear injection of CRISPR/Cas9 was used to efficiently generate knock-out mouse lines for 31 of these genes for which targeted alleles were not already available, all of which displayed normal fertility. As of yet, no study has used CRISPR/Cas9 with HDR in mice to model or correct specific human teratozoospermia causing gene mutations in vitro or in vivo.

Generally, this approach of generating germline gene-edited mouse models to model defective spermatogenesis is hampered by genes that result in an embryonic or perinatal lethal phenotype and by putative disease-causing genes that are not well conserved between humans and mice at the target locus. To circumvent this, one study has used the approach of creating mice with a humanized *CDK2* gene bearing a non-synonymous single-nucleotide polymorphisms (SNPs) expected to disrupt meiosis [82]. They showed that a homozygous Y15S substitution in the *CDK2* gene resulted in a Sertoli cell-only phenotype. Interestingly, the phenotype of these mice is different than $Cdk2^{-/-}$ mice highlighting the importance of such high-fidelity models when evaluating fertility phenotypes. Alternatively, CRISPR/Cas9-mediated genome editing could be applied to human SSC with or without testicular organoids [83]. Such in vitro approaches would be unhampered by the time and cost associated with developing animal models. Furthermore, the number of genes that can be simultaneously interrogated using this approach would be considerably higher. Indeed, several genome-scale CRISPR/Cas9 libraries have been generated and applied successfully to test essential genes in cancer cells [16, 17, 84, 85] and human pluripotent stem cells [86]. In addition to loss-of-function libraries, such genome-wide tools have been developed to interrogate the effects of transcriptional repression or activation [87, 88] as well as epigenetic modification [89]. These screening libraries have also been adapted for in vivo functional screens to evaluate how a population of CRISPR/Cas9-targeted cells behaves when an in vivo selection pressure is placed on it [90–92]. CRISPR/Cas9 screening can also be combined with other high-content methods such as single cell RNA sequencing to provide a comprehensive picture of gene function and regulation in complex systems such as immune cells and immune function [93]. While as of yet these high-throughput technologies have not been applied to the field of fertility, they offer exciting new prospects for advancing the field. Of course, any in vitro editing of human cells lines of the germline ontogeny will require careful consideration of the ethical pitfalls which arises should successful corrective genome-editing result in or have the potential to produce viable sperm and thus the potential for offspring with germline modifications.

Genome Engineering in the Human Germline: Advances and Ethical Considerations

While the prospect of curing genetic diseases using genome editing is exciting, the application of CRISPR/Cas9 in human SSCs, germ cells, or zygotes leads to the so-called germline editing in which genetic modifications are passed down from generation to generation. Given the heritable nature of such changes, the application of this technology in this sphere is subject to considerable ethical concern.

Shortly after CRISPR/Cas9 was identified as a promising genome engineering tool in mammalian cells, the system was applied through pronuclear injection in mouse zygotes to efficiently generate mice with germline modifications [94]. It was not however until 2015 that CRISPR/Cas9 was first applied to modify the human germline in non-viable tri-pronuclear zygotes [95]. This early study showcased some of the shortcomings associated with applying this technology to humans—namely, mosaicism and off-target mutations.

These early technical efforts were sufficient to raise ethical concerns and instigated the engagement of multiple stakeholders in the generation of a set of regulatory guidelines for the future of human genome engineering by the National Academies of Sciences, Engineering, and Medicine particularly in the setting of human clinical trials [96]. This report, while not recommending an outright ban on germline modification, proposes a stringent set of criteria and regulations that should govern and limit any human clinical applications of CRISPR/Cas9.

Since the generation of these guidelines, there have been ongoing technical improvements in using CRISPR/Cas9 for genome editing in humans. One group in particular was able to correct a pathogenic paternal *MYBPC3* mutation causing hypertrophic cardiomyopathy with no mosaicism or off-target effects [97]. Interestingly, this was achieved by modulating the cell-cycle phase at which genome editing occurs, and co-injecting the CRISPR/Cas9 system at the time of intracytoplasmic sperm injection, in-effect genome-editing sperm at the time of oocyte fertilization. These findings really highlight the imminence of applying such technologies to germline modification of male gametes using well-established techniques in fertility.

Indeed, more recently, the first germline-modified humans using CRISPR/Cas9 were reported in China [98]. These experiments prompted a public outcry from scientists and lay people alike and have led to the development of more stringent regulations against such practices. The practice is currently outlawed in over 30 countries including China where the experiments were conducted [99]. While the practice is not currently banned in the USA, stringent regulations are in place that de facto prohibit it [99].

Conclusion

Application of CRISPR/Cas9 to the investigation and treatment of male infertility holds significant promise to make breakthroughs in the field. Several groups have reported relevant successes with the technology to date. However, given the application to germ cells and subsequent germline mutations in humans, significant ethical considerations and the development of further consensus regulations are imperative to ensure safe conduct of pre-clinical and clinical trials necessary for the translation of this technology into human populations.

Compliance with Ethical Standards

Conflict of Interest Dr. Flannigan reports grants from American Society of Reproductive Medicine, grants from Canadian Urological Association Scholarship Foundation, grants from Canadian Institute for Health Research, grants from Vancouver Coastal Health Research Institute, and grants from New Frontiers Research Fund, outside the submitted work. Dr. Cina and Dr. Phillips have nothing to disclose.

Human and Animal Rights This article does not contain any studies with human or animal subjects performed by any of the authors.

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- • Of importance
- •• Of major importance

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