Review Article on Genetic Causes and Management of Male Infertility

Defining new genetic etiologies of male infertility: progress and future prospects

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Abstract: Male infertility is a common and complex disease, manifesting as a wide range of phenotypes, ranging from apparently normal semen parameters with an inexplicable inability to conceive, to the complete absence of sperm production. The diversity of male infertility phenotypes, coupled with the extreme complexity of spermatogenesis has significantly confounded the identification of the underlying genetic causes for these conditions, though incremental progress has been made, particularly in the past decade. In this review, we discuss the progress that has been made to date, tools and resources that have proven effective in accelerating discovery of novel genetic markers for male infertility, and areas in which we see the greatest potential for advancing the field in the coming years. These include the development and use of robust phenotyping tools, the continued development of *in vitro* and animal models for variant validation, increased utilization and refinement of whole genome approaches for discovery, and further expansion of consortia that assemble groups of clinicians and basic researchers with the unified goal of disentangling the complex genetic architecture of male infertility. As these resources mature, and funding agencies increasingly recognize the importance of these efforts for improving human health, the discovery of novel genetic markers for male infertility will certainly continue to accelerate.

Keywords: Male infertility; genetic marker; genetics; mutation; genome; sequencing; epigenetics

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Introduction

Approximately 15% of couples trying to conceive are infertile, and male factors are responsible for 40–50% of these cases (1,2). The diagnosis of male fertility is usually performed through the observation of sperm number, motility, and morphology under the microscope (2). Nevertheless, the clinical value of semen analysis (or seminogram) has been called into question (3,4). Therefore, the identification of new, predictive and effective male infertility markers is a must, and several multicenter projects and international consortia are currently actively searching for new male infertility markers in the context of genetics and epigenetics.

The aim of this review is to highlight the most important advances in the field of genetics of male infertility, to describe the main methodologies and techniques for identification and validation of novel infertility markers, to review current efforts being made to improve the diagnosis and treatment of male infertility, and to discuss how recent and future findings will advance clinical practice in the near future.

Established genetic components of male infertility

Disease-associated genetic variants can be broadly categorized

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as highly penetrant monogenic and associated variants. Highly penetrant monogenic variants are those which disrupt function of a single gene and result in a consistent phenotype, while associated variants may be present in affected or unaffected individuals, but the variant frequency is significantly associated with the disease state, and the variant may not directly impact gene function.

In the context of male infertility, several genetic causes of spermatogenic impairment are well established and already in clinical use, while others have been described more recently and vary in current clinical evidence (*Table 1*). The vast majority of assisted reproductive technology (ART) centers and reproductive urologists routinely utilize genetic screening in the diagnostic workup of infertile men, particularly those displaying azoospermia or severe oligozoospermia.

Highly penetrant monogenic variants

For example, established guidelines (16) indicate that infertile patients with non-obstructive azoospermia (NOA) and/or severe oligozoospermia should undergo cytogenetic testing (karyotype) and Y chromosome deletion testing due to the high prevalence of Klinefelter (47,XXY) syndrome [5–15% of NOA and severe oligozoospermic men (17)] and Y chromosome microdeletions or *AZF* deletions [5–10% of NOA and severe oligozoospermic men; 0.5% of infertile patients (17)]. Less frequent causes of male infertility include 46,XX male (1:20,000 new born; 0.9% of NOA men) or the Robertsonian translocations, inversions, and reciprocal translocations, ten-fold more frequent in men with oligozoospermia than in men with normozoospermia (5,6).

In the context of obstructive azoospermia, *CFTR* mutations are the main cause for agenesis of the vas deferens or obstruction. Subjects with *CFTR* mutations are excellent candidates for ICSI, using sperm retrieved from testis or epididymis because spermatogenesis in these patients is normal. The prevalence of *CFTR* mutations is about 5% in infertile patients and 50–60% in obstructive azoospermic patients (5).

In addition to Klinefelter's syndrome, *AZF* deletions and *CFTR* mutations, only a small number of gene variants have reached the level of high clinical validity in the field of Andrology, however that number is growing as genome-wide approaches are applied increasingly in male infertility research.

Several genes that harbor variants with very strong evidence for a highly penetrant monogenic role in male infertility include *ANOS1*, *AR*, *CFTR*, *CHD7*, *CYP11B1*, *FANCM*, *SRY*, *STAG2*, and *TEX11*, in NOA; *DPY19L2* and *SPATA16* associated with globozoospermia; *AMH*, *AMHR2*, *CYP11A1*, *CYP17A1*, *CYP19A1*, *CYP21A2*, *FGFR1*, *GNRHR*, *KISS1R*, *LHB*, *LHCGR*, *NR0B1*, *NR5A1*, *PROKR2*, *SRD5A2*, *TACR3*, and *WT1* associated with low or very low sperm count; and *AURKC*, *DNAH1*, *CFAP43* and *CFAP44*, *SUN5*, *WDR66* associated with multiple morphological abnormalities of the flagellum (MMAF) (8).

In current clinical practice, the screening for *AR* (androgen receptor) mutations has been introduced in some selected instances of quantitative sperm disorders due to the prevalence in infertility patients. Androgens and androgen receptors are essential for normal male sexual development before birth and during puberty and are crucial for the maintenance of the male phenotype and spermatogenesis. A mutation in *AR* [situated on the X chromosome (Xq11-12)], can cause androgen insensitivity syndrome. More than 1,000 mutations in *AR* gene have been described and the prevalence of clinically relevant AR mutations in azoospermic and oligozoospermic men is about 2–3% (5).

The list of highly penetrant monogenic variants with strong evidence for a role in male infertility has grown rapidly in the past few years and will continue to grow at an accelerated pace, largely driven by whole genome and exome sequencing approaches coupled with larger sample sets and carefully selected familial cases of infertility (6,8).

Associated variants

Moreover, some gene polymorphisms are considered potential risk factors for spermatogenic failure, or they may display association with male infertility without directly impacting gene function. The list of well validated variants includes variants in exon 1 of *AR* (9), *CYP1A1* (10), *DAZL* (11), *ESR1* and *2* (12), *ER* (13), *FSHR* (14), and *MTHFR* (15) polymorphisms among others, due to the existence of meta-analysis certifying the positive associations with male infertility (5,18). Gr/gr deletions likewise have been shown to be significantly associated with male infertility based on several large studies and meta-analyses. Smaller than *AZF* deletions, gr/gr deletions remove only a small part of the *AZFc* region. The prevalence of these microdeletions is estimated to be 6.8% in infertile men (4.3% in oligozoospermic men, 6.5% in men with severe oligozoospermia and 8.6% in azoospermic men) and 3.9% in controls (7), though frequencies vary by ethnicity and Y haplogroup (19).

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Table 1 Summary of male infertility genetic markers, highly penetrant monogenic variants and associated variants (gene polymorphisms) where the association with male infertility was confirmed by meta-analytic genetic association studies (high confidence association)

Genetic marker	Genetic test preference	Prevalence/primary phenotype	Primary reviews and meta-analysis
Chromosomal anomalies: aneuploidies			
47,XXY (Klinefelter syndrome)	Karyotype analysis	5–10% in azoospermia and 2–5% in severe oligospermia	(5)
Male 46,XX (la Chapelle syndrome)	Karyotype analysis	0.9% in azoospermia and oligozoospermia	(5)
Robertsonian and reciprocal translocations	Cytogenetics	0.5–1.0% in azoospermia and oligozoospermia	(5)
Chromosomal structural anomalies: Y microdeletions			
Y microdeletions (AZFa, AZFb and AZFc)	Array CGH, MLPA, PCR	5–10% in azoospermia and oligozoospermia (AZFa =0.5–1.0%, AZFb =0.5–1.0%, AZFc =3–7%)	(5)
Y microdeletions gr/gr	PCR-related techniques/sequencing	6.8% of infertile men (4.3% in oligozoospermia, 6.5% in severe oligozoospermia and 8.6% in azoospermia)	(6,7)
Genes in which highly penetrant monogenic variants have been identified			
ADGRG2	PCR-related techniques/sequencing	Azoospermia	(8)
AMH	PCR-related techniques/sequencing	Abnormal reproductive organ development	(8)
AMHR2	PCR-related techniques/sequencing	Abnormal reproductive organ development	(8)
ANOS1	PCR-related techniques/sequencing	Abnormal hypothalamic function	(8)
AR	PCR-related techniques/sequencing	2–3% in azoospermia and oligozoospermia; Abnormal reproductive organ development	(5,8)
AURKC	PCR-related techniques/sequencing	Teratozoospermia	(8)
CCDC39	PCR-related techniques/sequencing	Oligoasthenozoospermia	(8)
CCDC40	PCR-related techniques/sequencing	Asthenozoospermia	(8)
CFAP43	PCR-related techniques/sequencing	Teratozoospermia	(8)
CFAP44	PCR-related techniques/sequencing	Teratozoospermia	(8)
CFAP69	PCR-related techniques/sequencing	Teratozoospermia	(8)
CFTR	PCR-related techniques/sequencing	5% in infertile men, 50–60% in obstructive azoospermia; absence of vas deferens	(5,8)
CHD7	PCR-related techniques/sequencing	Abnormal hypothalamic function	(8)
CYP11A1	PCR-related techniques/sequencing	Adrenal gland dysfunction	(8)
CYP11B1	PCR-related techniques/sequencing	Adrenal gland dysfunction	(8)
CYP17A1	PCR-related techniques/sequencing	Adrenal gland dysfunction	(8)
CYP19A1	PCR-related techniques/sequencing	Adrenal gland dysfunction	(8)
DNAH1	PCR-related techniques/sequencing	Teratozoospermia	(8)
DPY19L2	PCR-related techniques/sequencing	Teratozoospermia	(8)
FANCM	PCR-related techniques/sequencing	Azoospermia	(8)
FGF8	PCR-related techniques/sequencing	Abnormal hypothalamic function	(8)

Table 1 (*continued*)

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Table 1 (*continued*)

Genetic marker	Genetic test preference	Prevalence/primary phenotype	Primary reviews and meta-analysis
FGFR1	PCR-related techniques/sequencing	Abnormal hypothalamic function	(8)
GNRHR	PCR-related techniques/sequencing	Pituitary dysfunction	(8)
HSD3B2	PCR-related techniques/sequencing	Adrenal gland dysfunction	(8)
KISS1R	PCR-related techniques/sequencing	Abnormal hypothalamic function	(8)
LHB	PCR-related techniques/sequencing	Pituitary dysfunction	(8)
LHCGR	PCR-related techniques/sequencing	Leydig cell dysfunction	(8)
LRRC6	PCR-related techniques/sequencing	Asthenozoospermia	(8)
MAMLD1	PCR-related techniques/sequencing	Abnormal reproductive organ development	(8)
NR0B1	PCR-related techniques/sequencing	Adrenal gland dysfunction	(8)
NR5A1	PCR-related techniques/sequencing	Abnormal reproductive organ development	(8)
PIH1D3	PCR-related techniques/sequencing	Asthenozoospermia	(8)
PKD1	PCR-related techniques/sequencing	Asthenozoospermia	(8)
PLXNA1	PCR-related techniques/sequencing	Abnormal hypothalamic function	(8)
PMFBP1	PCR-related techniques/sequencing	Teratozoospermia	(8)
PROK2	PCR-related techniques/sequencing	Abnormal hypothalamic function	(8)
PROKR2	PCR-related techniques/sequencing	Abnormal hypothalamic function	(8)
SOX10	PCR-related techniques/sequencing	Abnormal hypothalamic function	(8)
SOX2	PCR-related techniques/sequencing	Abnormal hypothalamic function	(8)
SOX3	PCR-related techniques/sequencing	Abnormal reproductive organ development	(8)
SRD5A2	PCR-related techniques/sequencing	Abnormal reproductive organ development	(8)
SRY	PCR-related techniques/sequencing	Abnormal reproductive organ development	(8)
SUN5	PCR-related techniques/sequencing	Teratozoospermia	(8)
TEX11	PCR-related techniques/sequencing	Azoospermia	(8)
TEX15	PCR-related techniques/sequencing	Azoospermia	(8)
TACR3	PCR-related techniques/sequencing	Abnormal hypothalamic function	(8)
WDR11	PCR-related techniques/sequencing	Abnormal hypothalamic function	(8)
WDR66	PCR-related techniques/sequencing	Teratozoospermia	(8)
WT1	PCR-related techniques/sequencing	Abnormal reproductive organ development	(8)
Variants with robust associations (polymorphisms)			
AR gene exon 1	PCR-related techniques/sequencing	Idiopathic male infertility	(9)
CYP1A1	PCR-related techniques/sequencing	Idiopathic male infertility	(10)
DAZL	PCR-related techniques/sequencing	Idiopathic male infertility	(11)
ESR1 and 2	PCR-related techniques/sequencing	Idiopathic male infertility	(12)
ER	PCR-related techniques/sequencing	Idiopathic male infertility	(13)
FSHR	PCR-related techniques/sequencing	Idiopathic male infertility	(14)
MTHFR	PCR-related techniques/sequencing	Idiopathic male infertility	(15)

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The efficiency with which new disease-highly penetrant monogenic or associated variants will be discovered in the context of male infertility largely depends upon a handful of factors. These factors include the use of genome-wide approaches that enable the discovery of rare variants, careful phenotyping for precise classification of infertility phenotypes and large-scale collaborative studies to leverage the cumulative resources (patient samples along with assay and analytical resources) of multiple groups with a common goal.

Tools for genomic discovery

There is a long and growing list of genomic tools available for the discovery of novel genetic variants associated with male infertility. These include PCR- and array-based approaches as well as more recently refined whole exome and whole genome sequencing (WGS) approaches.

Conventional techniques

CGH arrays

The comparative genomic hybridization array (aCGH), is a well-stablished molecular cytogenetic method for the analysis of copy number variations (CNVs), which include submicroscopic insertions and deletions in the genome that can disrupt gene function. aCGH is used in some ART clinics to detect aneuploidies, well-characterized microdeletion/microduplication syndromes and sub-telomeric or other unbalanced chromosomal rearrangements (20). However, this technique is not able to identify balanced chromosomal alterations such as translocations and inversions. Using aCGH, the risk variant *TEX11* was described only 5 years ago as cause of meiotic arrest and azoospermia in infertile men (21).

In aCGH, genomic DNA of the patient and control are differentially labeled with fluorescent probes such as Cyanine 3 (Cy3) and Cy5, following which equal amounts of labeled genomic DNA from a test and a reference sample are co-hybridized to an array containing the DNA targets, and the slides are scanned into image files using a microarray scanner. The spot intensities are measured, and the ratio of the fluorescence intensities is proportional to the ratio of the numbers of copies of specific DNA sequences. If there is an altered Cy3:Cy5 ratio, this indicates a loss or a gain of patient DNA at that specific genomic region.

MLPA technique

Genomic imbalances detected by aCGH are usually validated with other cytogenetic and molecular methods that can include customized multiplex ligation-dependent probe amplification (MLPA) assays (22). These analyses are based on multiplexed size-separation of the amplification products of a maximum of 40–50 target sequences in parallel in a single PCR reaction. Nowadays, more than 300 probe sets are commercially available and are specific for a very large range of common and rare genetic disorders. The main advantage of MLPA is the low cost of the technique (23). In the case of MLPA technique, one study allowed the description of some mutations in *DMRT1* gene as the associate variant (24).

SNP arrays

Single nucleotide polymorphism (SNP) arrays are routinely used to detect common polymorphisms within a population. SNP arrays enable the assessment of hundreds of thousands to millions of polymorphisms across the genome to determine whether specific SNPs occur more frequently in a disease cohort compared with controls. They are most frequently used for genome-wide association studies of common polymorphisms but can also be used to detect CNVs based on probe intensities. In the past decade SNP arrays have been relatively widely employed in the study of male infertility, which has been important in characterizing the genetic architecture of the disease (see *Table 1*).

Next generation sequencing approaches

With significant advances in next generation sequencing (NGS) technologies and rapidly increased adoption of NGS, sequencing costs have decreased rapidly. This has resulted in significant shifts in the tools employed for disease variant discovery (25). While there is still some utility in characterizing frequencies of common polymorphisms and CNVs, WGS and whole exome sequencing (WES) have largely supplanted array-based technologies for gene discovery. WGS, as the name suggests, attempts to sequence the entire genome, however, technically the procedures cover about 95–96% of the genome due to the difficulty to sequence and assemble some regions (e.g., high GC content, large repeat regions, centromeres or telomeres). On the other hand, WES focuses on sequencing only the protein coding sequences. The primary advantages of WES are significantly reduced sequencing costs since less than

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2% of the genome comprises exonic regions, the lower data storage costs, and more straightforward analyses (26). Whether it's WES or WGS, data analysis remains the largest bottleneck in genomic studies, however the repertoire of available tools for sequence analysis is growing rapidly (27,28). Due to the error rates associated with the various NGS platforms, variants identified by NGS should be confirmed by Sanger sequencing technology (29,30).

Study design considerations

In addition to the importance of using the appropriate genomic tools for discovery of novel disease-causing variants, proper patient selection and phenotyping are an absolutely necessary component of study design. Team science approaches have proven critical in the study of numerous complex diseases and are increasingly emerging in the field of reproductive medicine.

Patient phenotyping

Careful phenotypic classification of infertile cases is of critical importance in the successful execution of genomic discovery studies. The identification of discrete and specific phenotypes has proven to yield early successes in the identification of novel genetic variants underlying male infertility. Two such examples are globozoospermia and MMAF. Globozoospermia is a specific defect of the sperm head, characterized by the absence of a sperm acrosome. Early genomic studies of both familial and sporadic cases successfully identified causal mutations in *DPY19L2* and *SPATA16* that cumulatively account for a large percentage of cases (31,32). Likewise, similar genomic analyses in men displaying MMAF have identified mutations in a number of genes including *DNAH1*, *CFAP43*, and *CFAP44*, among others (33,34). These cases illustrate the value of patient selection and phenotyping for genomic studies (8,35).

These examples are in contrast to the relatively slower-paced success in identifying genetic variants associated with spermatogenic impairment including oligozoospermia and NOA. While the formation of an acrosome or a competent flagellum during the late stages of spermiogenesis requires the concerted function of a relatively small number of genes, and disruption of either process yields a very specific phenotype, the process of spermatogenesis involves the concerted function of thousands of genes, and the disruption of any gene required for spermatogenesis can theoretically result in spermatogenic impairment.

This reality is reflected in the fact that, to date only a few high confidence variants have been discovered to explain severe spermatogenic impairment. Notably, more specific phenotypic characterization of NOA phenotypes based on testis histology data has proven to be important in variant discovery. Specifically, the genetic evaluation of patients with complete meiotic arrest, a relatively less common form of NOA, has produced a higher diagnostic rate than evaluation of the NOA population as a whole. More refined tools for molecular phenotyping of different categories of spermatogenic impairment or other classes of male infertility, such as single cell RNA sequencing will certainly improve patient classification and the diagnostic yield of genomic studies.

Family-based studies

The genomic analysis of families with two or more infertile siblings has proven to be an efficient and cost-effective tool for discovering high-confidence causal variants. In particular, families displaying increased consanguinity harbor a larger portion of the genome that is homozygous by descent, and thus are more likely to harbor homozygous recessive mutations. There are multiple examples in the literature of male infertility-causing mutations (29,30,36). Likewise, genomic analyses of non-consanguineous in which multiple siblings display the same infertility phenotype has proven successful. Screening both affected and unaffected siblings as well as both parents can reduce the number of potential variants to investigate many-fold. The continued identification of these families will be critical in identifying novel highly penetrant monogenic or associated variants.

Collaborative approaches

As medical research has evolved to integrate big data, population-scale approaches, team science has become absolutely imperative. This evolution has given rise to a large number of consortia focused on specific diseases and conditions. In recent years, the value of large-scale collaborations has become increasingly appreciated in the field of reproductive medicine as well. Two international genomics consortia [Genetics of Male Infertility Initiative (GEMINI; https://gemini.conradlab.org/)], and the International Male Infertility Genomics Consortium (IMIGC; http://www.imigc.org/)] have been organized in the past several years with the specific aim of organizing clinicians and basic researchers to enable the investigation

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of the genetic basis for various etiologies of male infertility. The fruits of these efforts are just beginning to emerge with several recent publications and numerous others in preparation or under review (30,37,38). In addition, the Male Reproductive Health Initiative (MRHI) has been organized to increase global awareness of male reproductive health and advocate to funding agencies the need for greater investment in research (39), and ReproUnion (https://reprounion.eu/) is organized to address big questions in reproductive medicine through international collaborations. Lastly, the Andrology Research Consortium (ARC) was founded to standardize data collection, clinical information and therapeutic approaches for the treatment of male infertility (40). As these and other efforts emerge and further mature, they will continue to accelerate the advancement of research in reproductive medicine.

Functional validation

While the identification of novel variants with plausible associations with male infertility is important, the functional validation of these variants is necessary to confirm variant function and to characterize the underlying mechanisms for infertility associated with specific variants. This is a huge undertaking considering the number of variants that are expected to underlie male infertility, however continuing improvements in genome editing tools, the emergence of novel research models and progress in the development of *in vitro* systems for the study of spermatogenesis will expand our ability to perform functional validation.

Animal models

The use of animal models for scientific purposes is both a longstanding practice in biological research, and a frequent matter of societal debate. However, this is an indisputably essential step to validate the results of a genetic study as well as to definitively establish a genetic marker, even though not all results obtained on animals can be directly translated to humans.

In reproduction, three different animal models are widely used to validate genomic discoveries: fruit fly, zebrafish and mice, due to their cost efficacy, easy maintenance, genetic homology and high fertility rates.

The fruit fly (*Drosophila melanogaster*) has been extensively studied for over a century as a model organism for genetic investigations because, on the molecular level, many similar features and pathways with humans were described. The main advantages are: short life cycle, ease of culture and maintenance, low number of chromosomes, small genome size (in terms of base pairs) and polytene chromosomes that permits a high level of gene expression. The main utility in reproductive studies is that Drosophila has a relatively short life span of 60–80 days, which makes it attractive for life span studies (41).

In the last two decades, zebrafish (*Danio rerio*) has emerged as a powerful model to study vertebrate development and disease because it has a short generation time, simplifying genetic manipulation and analysis, has a small size, has a high fecundity, and embryos are transparent. This last characteristic facilitates live imaging of developmental processes and make this animal model ideal to study infertility and reproduction (42).

Finally, mice (*Mus musculus*) are one of the most used animal models because of their phylogenetic relatedness and physiological similarity to humans and the ease of maintaining and breeding them in the laboratory. In addition, the availability of many inbred strains makes it ideal for conducting controlled genetic studies. Because mice are one of the most studied mammalian models and display many reproductive similarities with humans, mouse models have been described as "*invaluable in dissecting the molecular mechanisms underlying a number of complex diseases like infertility*" (43). Specifically, in rodent models it is worth recognizing that factors affecting male fertility may not adversely affect spermatogenesis, due to the highly efficient spermatogenesis seen in rodents, even though they may have a clinically important role in human male fertility (44).

Genetic manipulation

The generation of gene mutants in animal models, is necessary for demonstrating evidence for the role of a variant in male infertility. There are many tools available for the generation of gene mutants in animals. Here we describe a few including: N-ethyl-N-nitrosourea (ENU), morpholino oligonucleotides (MO), zinc finger nucleases (ZFN), Transcription activator-like effector nucleases (TALEN) and Clustered regularly interspaced short palindromic repeats (CRISPR/Cas) (*Table 2*).

The ENU mutagenesis protocol is a very easy way to produce a mutant. ENU is an alkylating agent that transfers its ethyl group to nitrogen or oxygen radicals in DNA, resulting in base mispairing that causes base pair substitution. ENU is principally used when there is a need for highly efficient induction of point mutations randomly distributed throughout the germline (56). Therefore, the

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Table 2 Characteristics of the primary mutagenesis techniques. In order of discovery: N-ethyl-N-nitrosourea (ENU), morpholino oligonucleotides (MO), zinc finger nucleases (ZFN), transcription activator-like effector nucleases (TALEN) and clustered regularly interspaced short palindromic repeats (CRISPR/Cas)

Technique	Main characteristic	Permanent mutations?	Cost	Ease of design	Off-target effects	Primary references
ENU	Mutagen. Produces 1 new mutation in every 700 loci approx.	Mutations made are permanent and heritable	Very inexpensive: $50–200	Easy design, difficult screening	N/A	(45,46)
MO	~25 base antisense oligomers	Temporal knockdown (up to five days later approx.)	Inexpensive: $400–2,000	Easy	Low	(47,48)
ZFN	Restriction enzyme. Double-strand break induced by FokI	Mutations made are permanent and heritable	Expensive (non-validated): $7,000–10,000	Difficult	High	(49,50)
TALEN	Restriction enzyme. Double-strand break induced by FokI	Mutations made are permanent and heritable	Less expensive than ZFN (non-validated): $3,000–5,000	Moderate	Low	(51,52)
CRISPR/Cas	In conjunction with Cas (restriction enzyme). Single- or double-strand break induced by Cas. Recognize PAM sequences. Produces precise base modifications	Mutations made are permanent and heritable	Inexpensive: $500–1,000	Easy	Variable (usually low)	(53-55)

most interesting ENU application is the identification of novel alleles important in embryonic organogenesis. However, a genome-wide mutagenesis screen is mandatory to map the mutations generated by ENU (57).

On the other hand, MO is a type of antisense oligomer molecule designed to modify gene activity by blocking RNA translation or splicing (47). MOs are chemically synthesized, are similar to small interfering RNAs (siRNAs) and are typically injected into embryos at the 1-cell stage (58). MOs are typically 25-base oligomers with high specificity when designed appropriately. However, because the mechanism of action is based in gene expression modification instead of DNA modification, the duration of the effect is short-lived (typically 5–6 days), allowing the measure of the effects in early embryo development, but not later developmental stages. It is important to recognize that MO-induced phenotypes are often more severe than those of the corresponding mutants (59).

ZFN are the first generation of genome manipulation elements based on restriction enzymes (typically the restriction endonuclease FokI) (49). Zinc fingers are the most common DNA binding domain found in eukaryotes and are comprised of ~30 amino acids that interact with nucleotide triplets (each ZNF typically recognizes 3–6 nucleotide triplets). Zinc finger domains (usually 3 to 6) can be designed to target specific DNA sequences, and this enables zinc-finger nucleases to produce a double-strand brake (DSB) that promotes the nonhomologous DNA end joining (NHEJ) pathway activation. This technology can serve to induce random insertion-deletions (indels) when the NHEJ machinery works off-target or, using a supplied DNA fragment as a template for allele editing (50). Similarly, TALENs are based on restriction enzymes, however TALENs typically work with greater specificity and efficiency than ZFN. The main problem of ZFN and TALEN is that they can lead to toxicity or lethality due to binding at off-target sites resulting in the induction of undesired DNA cleavage (60). Even though TALEN design is generally more straightforward than ZNFs, to clone the large TALEN modules in series is challenging (51).

The latest gene editing technology is the CRISPR-Cas system discovered in the late 1980's (53) but largely applied in gene editing only in the last 20 years (54,55). CRISPR systems are RNA-based bacterial defense mechanisms, primarily discovered in archaea organism, designed to recognize and eliminate foreign DNA from invading bacteriophage and plasmids. The system consists of a specific endonuclease, usually called Cas, that is directed to cleave a target sequence by a guide RNA (gRNA). Again, similar to the ZNF and TALEN systems, the CRISPR-Cas

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system can be used to either introduce specific mutations or insertions by co-injecting an engineered DNA construct or to introduce random mutations at the site of DNA cleavage by NHEJ machinery. In contrast to ZNF and TALEN, the design is easy, and the cost of this technology is relatively inexpensive (60).

In vitro systems

While animal models play a critical role in the validation of candidate mutations, there are limitations to the use of animal models. These include the cost of animal experiments, the time required for the development and completion of animal experiments, and the inherent genomic and reproductive differences between model organisms and humans. In many cases, genome sequences are not sufficiently conserved among species to accurately model specific mutations.

The culture of testicular tissues or cells *in vitro* to recapitulate spermatogenesis overcomes many of the shortcomings associated with model organism research, however *in vitro* culture of spermatogonial stem cells and *in vitro* spermatogenesis have not been achieved in humans in spite of success in mice (61,62) and tremendous efforts in humans (63). As a more complete understanding of human testis physiology and signaling cascades is gained, and as tools for three-dimensional culture of complex tissues continue to improve, *in vitro* spermatogenesis in humans will certainly be achieved. These advances coupled with CRISPR/Cas gene editing will enable the precise assessment of the impact of many variants on spermatogenesis. In addition, it will pave the way for the ability to restore spermatogenic capacity *in vitro*.

Beyond genetic markers for male infertility

Epigenetic modifications

Unlike the genome, the epigenome is highly variable between cells and is dynamic and can be influenced more readily by environmental and lifestyle factors. In the past two decades several studies have demonstrated that the sperm epigenome influences sperm function and fertilization. The basic epigenetic factors that exist in spermatozoa are histone and chromatin modifications, DNA methylation, and non-coding RNAs (ncRNAs) (64). Some authors suggest that the last two can be useful as epigenetic markers of male infertility.

DNA methylation

Numerous articles have described an association between male infertility and methylation abnormalities at particular imprinted loci in spermatozoa (65-68). Generally, these studies suggest that aberrations in the sperm DNA methylation epigenome can lead to defects throughout spermatogenesis, thereby impairing spermatogenesis or fertilization. However, the suggested link between sperm epigenetic changes and male sub-fertility is still a matter of debate, because the published studies are typically case-control analyses where the clinical relevance of the associations are uncertain.

In order to evaluate the clinical validity of some methylation variants, a recent meta-analytic analysis has been published and demonstrated that male infertility is associated with altered sperm DNA methylation at *H19*, *MEST*, and *SNRPN* imprinted genes, suggesting these as good candidate biomarkers of male infertility (69). *H19* is one of the most studied imprinted gene for male infertility, encodes for a 2.3-kb non-coding mRNA and is strongly expressed during embryogenesis. This gene is located in chromosome 11 near the insulin-like growth factor 2 (*IGF2*) gene and is only expressed from the maternally inherited chromosome. This gene includes five exons and four introns and has different patterns of activation depending on the cell type and age (different between fetal life and adult life) suggesting that is involved in tissue differentiation (70). On the other hand, *MEST* encodes a member of the alpha/beta hydrolase superfamily and is located in chromosome 7. This gene is imprinted, and unlike *H19*, is monoallelically expressed from the paternal allele in fetal tissues (71). Finally, *SNRPN* is located within the Prader-Willi Syndrome critical region on chromosome 15 and is likewise expressed from the paternal allele. It encodes a component of the small nuclear ribonucleoprotein complex and may contribute to tissue-specific alternative splicing. This could be an important marker because some authors propose that ICSI predisposes to abnormal imprinting although there is not general consensus in this regard (72). More studies with these three epigenetic biomarkers are required to corroborate the associations suggested.

ncRNAs

Traditionally it was believed that the role of the male gamete was limited to the narrow window of fertilization as a simple vehicle for male DNA delivery to the embryo. However, several studies have demonstrated that this is not the case,

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and the male contribution, far from being confined to DNA, has been extended to a wide variety of molecules including coding and ncRNAs (73,74). Some studies have proposed that these transcripts are not just random remnants from early spermatogenesis stages but constitute a stable population that has been selectively retained, suggesting an important role in early zygotic development and postulating them as important infertility biomarkers (75).

Among ncRNAs, microRNAs (miRNAs) have been suggested as useful biomarkers of male infertility. Some authors suggested a panel of five well-known miRNAs in sperm (hsa-miR-34b*, hsa-miR-34b, hsa-miR-34c-5p, hsa-miR-429, and hsa-miR-122) as a novel noninvasive biomarker to diagnose patients with subfertility (76). However, in a recent study other authors suggested that pairs of miRNAs could be a more effective way to apply these epigenetic biomarkers, based on specificity and sensitivity values. Salas-Huetos and collaborators described the presence of 48 stable pairs of miRNAs in sperm of a fertile population of men (73) and suggested this group of miRNA pairs including miR-942-5p/miR-1208 and miR-34b-3p/miR-93-3p with a potential role in predicting fertility in a validation study (77).

Conclusions/future directions

Characterization of the underlying genetic basis for male infertility has long been a research focus for many groups. Early efforts focused on single gene screens by Sanger sequencing. These efforts successfully identified a handful of highly penetrant monogenic risk variants, and a large number of spurious associations. As genomic technologies have advanced, and whole genome approaches have been increasingly employed in the field, significant progress in understanding the genetic architecture of male infertility, as well as identifying novel highly penetrant monogenic risk variants, has been made.

To ensure continued progress in the field, future efforts should focus on the continued development of whole genome screens, improvements in analytical tools, novel phenotyping approaches, expanded sample sets, and advancements in *in vitro* and *in vivo* variant validation methods. In addition, the continued investigation of other factors such as epigenetics, lifestyle factors and exposures will improve our understanding of the etiologies of male infertility. Appropriately powered studies that will make significant strides in characterizing the genetic basis of male infertility can only be made through the combined efforts of scientists and clinicians collaborating across a broad spectrum of expertise. As these principles are increasingly employed, our understanding of the genetic basis for male infertility will likely expand rapidly in the coming years.

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