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REVIEW

Genetic causes of spermatogenic failure

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Approximately 10%–15% of couples are infertile, and a male factor is involved in almost half of these cases. This observation is due in part to defects in spermatogenesis, and the underlying causes, including genetic abnormalities, remain largely unknown. Until recently, the only genetic tests used in the diagnosis of male infertility were aimed at detecting the presence of microdeletions of the long arm of the Y chromosome and/or chromosomal abnormalities. Various other single-gene or polygenic defects have been proposed to be involved in male fertility. However, their causative effects often remain unproven. The recent evolution in the development of whole-genome-based techniques and the large-scale analysis of mouse models might help in this process. Through knockout mouse models, at least 388 genes have been shown to be associated with spermatogenesis in mice. However, problems often arise when translating this information from mice to humans.
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INTRODUCTION

Infertility, defined as the inability to conceive after at least 1 year of regular and unprotected intercourse, affects approximately 10%–15% of couples.[1,2] It is estimated that a male factor is partially responsible for the fertility problems in approximately half of the couples. In this review, we will focus on those cases where spermatogenesis is deficient. Problems during spermatogenesis are reflected in a lower or absent production of spermatozoa and are described by routine semen analysis using terms such as 'azoospermia', 'oligozoospermia', 'teratozoospermia' or 'asthenozoospermia', or a combination of the last three ('oligoasthenoteratozoospermia'). Because the main objective of this paper is to discuss 'spermatogenic failure', we focus here on non-obstructive causes of male infertility and not on patients in whom sperm cells are produced but fail to reach their destination, i.e., obstructive azoospermia.

The underlying cause of these abnormalities in sperm production can either be acquired, congenital, or both. Currently, it is estimated that in approximately 40% of men, the diagnosis remains to be elucidated.[3] In view of assisted reproductive techniques, it is especially important to gain information about the genetic causes of male infertility, as these defects can be transmitted across generation(s).

ROUTINE TESTS

Currently, routine genetic analyses in the clinical diagnosis of non-obstructive azoospermia or oligozoospermia are limited to the investigation of the presence microdeletions of long arm of the Y chromosome (Yq) and/or chromosomal abnormalities. One of the first genetic tests to be performed in patients with severe idiopathic male infertility is karyotype analysis. Karyotype abnormalities are detected in ~5% of patients with fertility problems, and this prevalence increases to >13% when only considering men with azoospermia.[4–6] Most of the chromosomal abnormalities involve the sex chromosomes, with Klinefelter syndrome (47,XXY) being the most commonly detected karyotype abnormality in infertile men.[7] The vast majority of patients with the non-mosaic form of Klinefelter syndrome are azoospermic. Yet, a recent review showed that mature spermatozoa can be detected in ~44% of these patients.[8] It is suggested that some foci with residual spermatogenesis might be present and that these foci are derived from normal 46,XY spermatogonia.[9,10] Multiple studies have also shown that the majority of sperm cells have a normal haploid chromosomal content.[10,11]

Besides numerical abnormalities, structural defects are also detected 5–10 times more frequently in infertile men.[4,12] The formation of normal bivalents during meiosis is disrupted in patients with structural abnormalities (mainly with respect to translocations), leading to the expectation of impaired meiosis and a maturation arrest of spermatogenesis. However, in most of the patients with structural changes in the chromosome structure, oligozoospermia is observed. Therefore, it is also not surprising that the frequency of Robertsonian translocations, reciprocal translocations and inversions is higher in men with oligozoospermia compared with azoospermic men and men in the general population.[12]

It is also well known that Yq microdeletions are associated with male infertility. In 1992, Ma *et al.*[13] reported the first Yq microdeletions. Since then, over 90 papers have been published describing the frequency of Yq microdeletions in different patients and population groups. A re-evaluation of the literature, including >13 000 infertile men, showed that the prevalence of Yq microdeletions is ~7.4%. In an azoospermic population, the prevalence is higher (9.7%), while in oligozoospermic men, the prevalence is 6.0% (**Table 1**).

The Yq contains three 'azoospermia factor (AZF)' regions: AZFa, AZFb and AZFc. Deletions of the complete AZFc region are most

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Table 1 Frequency of Yq microdeletions in patients with azoospermia or oligozoospermia. The group total also includes patients with undefined or unclassified semen parameters

	Total	*Deletions*	%
Azoospermia (n)	3157	305	9.7
Oligozoospermia (n)	3473	209	6
Total (n)	13 097	969	7.4

Abbreviation: Yq, long arm of the Y chromosome.

frequently detected (69%), followed by deletions of the AZFb region (14%) and deletions of the AZFa region (6%) (**Figure 1**). However, some papers report aberrant deletion patterns that were not confirmed. Consequently, the actual frequencies of AZF deletions in different patient groups might be slightly smaller, compared to the numbers deducted from all published papers. Furthermore, at least 12 AZFa+b deletions were reported. These deletions cannot be explained from the repeat structures present on the Yq, and their relevance remains doubtful. However, not all of the Yq microdeletions can be explained by non-allelic homologous recombination.[14]

The current guidelines for the detection of Yq microdeletions recommend the use of two markers in each AZF region in two multiplex PCR reactions. Each PCR reaction also has to include a marker for sex-determining region Y (*SRY*), located on the short arm of the Y chromosome, and a marker for *ZFX/ZFY*, a gene located on the X and Y chromosomes.[15] Furthermore, for each test, positive (normal male), negative (normal female) and no template (water) controls should be included.

Deletions encompassing the complete AZFa or AZFb region are always associated with the complete absence of mature spermatozoa upon testicular biopsies. At the testicular level, the majority of the patients with an AZFa deletion have a Sertoli cell-only syndrome, while the most common phenotype among patients with an AZFb deletion is a maturation arrest of spermatogenesis.[16] For both patient groups, no sperm cells are left in their testis. Consequently, the diagnosis of an AZFa or AZFb deletion has important consequences for adequate counselling of the patients; a testicular biopsy is unnecessary because of the absence of sperm cells for intracytoplasmic sperm injection (ICSI). One rare exception has been described in which the complete AZFb region was absent in a severe oligozoospermic man.[17] However, it is interesting to note that testicular sperm extraction in this man was unsuccessful in retrieving spermatozoa, further underlying the negative predictive value of the complete AZFb deletion for testicular sperm retrieval in azoospermic men.

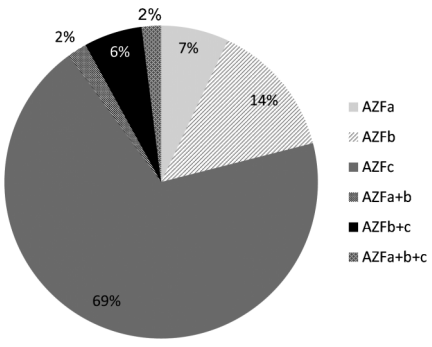

Figure 1 Distribution of Yq microdeletions among the three AZF regions. AZF, azoospermia factor; Yq, long arm of the Y chromosome.

The complete absence of the AZFc region, in contrast, causes a more heterogeneous phenotype, ranging from azoospermia to severe oligozoospermia (<5 million spermatozoa per ml). We estimated that spermatozoa could be found in approximately 70% of patients with an AZFc deletion.[18] Consequently, for these patients, ICSI remains possible. Because sons conceived after ICSI have a high chance of having impaired spermatogenesis, appropriate genetic counselling is necessary to explain the consequences of ICSI and to inform these men of the possible alternatives or additional treatments, such as preimplantation genetic diagnosis to select female embryos.

Screening for the presence of gr/gr deletions, which are partial deletions of the AZFc regions, is not performed in most of the routine genetic testing laboratories. Other reports, including one from our group, have shown an increased incidence of gr/gr deletions in men with fertility problems.[19–21] However, these gr/gr deletions are also detected in men with normal semen parameters and should therefore be considered more as a risk factor for male infertility rather than a causative factor. Besides these gr/gr deletions, which are associated with decreased sperm parameters, other partial deletions can be detected on the Y chromosome.[22] These include b1/b3 deletions and b2/b3 deletions, which are presumably neutral changes. Furthermore, duplications and other structural changes are observed in the AZFc region of the Y chromosome.[22] From several publications, it is obvious that the distribution of these alterations is not equal among different populations,[21,22] which makes the interpretation of the consequences of these changes a challenge.

SINGLE-GENE DEFECTS VERSUS POLYGENIC CAUSES

Until recently, single-gene defects were the focus of most of the published studies. However, it is obvious that in some of the patients, a combination of mutations or polymorphisms might cause fertility problems. Potentially, a combination of congenital/genetic and environmental factors might eventually be recognized as the cause of fertility problems. Yet, the number of patients affected by a single-gene defect remains unclear. **Table 2** gives an overview of genes that have been tested by one or more research groups. However, the majority of these studies fail to identify a mutation that is associated with the examined phenotype.

Single-gene defects

We believe that, especially in men with 'well-defined' and specific defects during sperm production, mutations in a single-gene might be responsible for the observed phenotype.

In this respect, rare cases with a well-defined sperm abnormality, such as globozoospermia or macrocephalic sperm cells, are interesting subjects for study. Indeed, the mutations in these patient groups have already been reported. In two families with multiple infertile men caused by globozoospermia, Dam *et al.*[23] and Liu *et al.*[24] detected mutations in the *SPATA16* (spermatogenesis associated protein 16) and *PICK1* (protein interacting with c kinase 1) genes, respectively. Dam *et al.*[23] detected a homozygous mutation in the *SPATA16* gene in three brothers of an Ashkenazi Jewish family. This mutation consists of an amino-acid substitution and confers the removal of a splice site. The subsequent screening for mutations in the *SPATA16* gene in 29 patients with globozoospermia failed to identify other changes in this gene. The *SPATA16* gene is presumably involved in the formation of the acrosome. It was observed that this protein translocates from the Golgi to the acrosome during spermiogenesis.[25] In the second study by Liu *et al.*,[24] a potential homozygous mutation was detected in the *PICK1* gene of a single patient with globozoospermia from

Table 2 Genes tested with consideration of human non-syndromic spermatogenesis or sperm defects, with special emphasis on genes tested at the DNA level

Patients	*Phenotype*	*One study*	*Multiple studies*	*References*
Azoospermia	Maturation arrest of spermatogenesis	*DNMT3L, FKBP6, FKBPL, MEI1, MSH4, STRA8, TAF7L*	*RBMY*[a], *SYCP3*	36–39, 41, 45, 74, 75
	Sertoli cell-only syndrome		*BPY2*[a], *DBY*[a], *USP9Y*[a]	76–83
	Not defined	*ART3, PRDM9, SOHLH1, TAF7L, ZNF230*		42,85–87
Abnormal semen parameters	Teratozoospermia	*CSNK2A2, GOPC, HRB, PICK1, SPATA16, eNOS*	*AURKc, DPY19L2*	23, 24, 27–30, 85
	Asthenozoospermia	*ADCY10, CATSPER3/4, DNAI1, DNAH5, DNAH11, eNOS, HFE, PLA2G6, SPAG16, TNFalpha A, TNFR1, TNFR2*	*AKAP3/4, CATSPER1/2*	33–37, 89–96
	OAT or not defined	*DNMT3b, eNOS, HIWI2/3, OAZ3, PON1/2, SCA1*	*CDY1*[a], *CYP1A1, DAZ*[a], *DAZL, ESR1/2, GSTM1, GSTT1, GSTP1, HSFY*[a], *KLHL10, POLG, PRM1/2, TNP1/2, TSPY*[a]	19, 97–118
Infertile men (undefined or mixed)		*FKBPL, GAMT, H1FNT, H2BFWT, HFE, HSP90, MS, MTR, MTRR, NANOS2, NANOS3, NR5A1, NRIP1, PUM2, NALP14, SLC6A8, TSSK2, TSSK6, UTP14C*	*APOB, AR, BOULE, c-KIT, KITLG CYP19A1, CREM, DDX25, FAS, FASLG, FKBP6, FSH, FSHR, LH, LHCGR, MTHFR, SHBG, UBE2B, USP26, YBX2 (=MSY2)*	19, 102, 119–151

Abbreviation: OAT, oligoasthenoteratozoospermia.
[a] These genes are located in the AZF regions on the Y chromosome. For some of the *Yq* genes, gene-specific deletion and/or mutation screening has been performed (*USP9Y*, *DBY*). For other genes, this method of screening was impossible because of the multicopy nature of the genes; for some of these genes, the copy number has been determined in infertile men.[152]

consanguineous parents. This change was absent in 100 normozoospermic Chinese controls.[24] Moreover, *PICK1* is also presumably involved in the formation of the acrosome.[26] Although the gene showed a ubiquitous expression pattern, Xiao *et al.*[26] showed that the major abnormality in Pick1$^{-/-}$ mice was infertility. In two publications concerning patients with globozoospermia, a homozygous deletion was detected on chromosome 12 encompassing the *DPY19L2* gene.[27,28] One paper described a 200-kb deletion in a consanguineous Jordanian family and three unrelated patients,[28] while the second research group detected the homozygous deletion in 15 out of 20 globozoospermic men that were tested using single-nucleotide polymorphism (SNP) arrays.[27] Additionally, in patients with large-headed polyploid multiflagellar sperm cells, a mutation was detected in the *AURKc* (aurora kinase c) gene, which is involved in chromosome segregation and cytokinesis. The typical phenotype of large headed sperm cells is especially detected in North African men, where the carrier frequency of the mutation is estimated to be 1/50.[29,30]

Visser *et al.*[31] analysed 30 patients with isolated asthenozoospermia for the presence of mutations in nine genes that were selected on the basis of the phenotype observed in knockout mouse models. They identified four *CATSPER* genes, which form the ion channel essential for the calcium influx during sperm capacitation. The genes *GAPDHS*, *PLA2G6* and *ADCY10* code for enzymes specifically expressed in sperm, and *SLC9A10* is a sodium hydrogen exchanger.[31] A total of 10 potential mutations were detected in seven of these genes (*ADCY10*, *AKAP4*, *CATSPER1*, *CATSPER2*, *CATSPER3*, *CATSPER4* and *PLA2G6*), yet all of the changes were heterozygous alterations. However, three patients had multiple changes in the investigated genes. Previous studies reported a man with partial deletions in the *AKAP3* and *AKAP4* genes that caused isolated asthenozoospermia.[32] In addition, mutations in the *CATSPER1* gene and deletion of the *CATSPER2* gene had been previously associated with asthenozoospermia.[33–35] However, in most of the patients, a reduced sperm number and an increased number of morphological abnormal spermatozoa were also detected.

Another interesting patient group is men with a maturation arrest of spermatogenesis. Spermatogenesis can arrest at different stages, although primarily, an arrest during meiosis is observed. Therefore, abnormalities in genes essential for meiosis are possible candidates for the defect in spermatogenesis. Yet, as suggested above, chromosomal abnormalities can also be the underlying cause of the failure to complete meiosis. This idea emphasizes the need to perform karyotype analysis before or in parallel with testing for the presence of gene mutations. Different groups have investigated the involvement of the *SYCP3* (synaptonemal complex gene 3) gene in male infertility.[36–38] Miyamoto *et al.*[36] detected a single change in two patients, which was predicted to alter the function of the protein. Two studies have investigated the *SYCP3* gene for the presence of mutations in association with recurrent miscarriages.[39,40] Three patients (two women and a man) were described with changes in the *SYCP3* gene that were potentially linked to their problems, i.e., maintaining a pregnancy, which might be due to an abnormal chromosomal constitution of the foetus.[39,40] The *TAF7L* gene has also been studied in relation to the maturation arrest of spermatogenesis or azoospermia.[41,42] In the first study, four non-synonymous changes were detected with equal frequencies in the patient and control groups.[41] The second study identified three of these four changes in their patient population and concluded that one of the changes present in exon 13 could be linked with azoospermia. The X-linked transcription factor *TAF7L* translocates from the cytoplasm to the nucleus during meiosis,[43] suggesting a function during meiosis. Yet, subsequent studies in mice showed that sperm cells were still produced, although at a lower rate, with abnormal morphology and motility.[44] This result indicated that patients with oligoasthenoteratozoospermia would have been a more appropriate group to screen.

In another study, Sato *et al.*[45] looked at the presence of mutations in the meiosis defective 1 (*MEI1*) gene. This gene was selected based on knockout mouse models that showed a meiotic arrest due to impaired chromosome synapsis.[46] Two synonymous SNPs were potentially associated with maturation arrest of spermatogenesis in Americans of European origin but not in Israelis. One SNP, resulting in a single amino-acid change, was detected in one patient and not in the controls. However, due to the low number of patients and controls analysed, the physiological meaning of this amino acid change could not be proven, because it failed to reach statistical significance.

These studies in patients with maturation arrest of spermatogenesis illustrate some of the pitfalls and obstacles that should be considered when investigating genetic causes of spermatogenesis defects or when drawing conclusions from the published studies.

1. The number of patients analysed is often too low to draw solid conclusions. The observation is often intrinsic to patient groups under investigation; it is hard to find large numbers of patients with a specific phenotype.
2. The ethnicity of patients and controls should match. Some SNPs are common in certain population groups, but rare or absent in other groups. However, sometimes it is hard to exclude that either the patient or the control has 'foreign' ancestors.
3. Often, no functional studies have been performed. Therefore, it is hard to predict the consequences of the observed changes, especially considering amino acid substitutions.
4. When analysing data, one should also consider the consequence of heterozygous versus homozygous changes. Even when functional analyses show that the function of a mutant protein is altered, a second 'normal' protein might compensate for the loss. Compensation has been observed in mouse studies where heterozygous mice are often fertile. Only a homozygous knockout of a gene completely disrupts the function of the gene product.
5. One should also consider that the function of genes might be different when comparing the mice and humans.
6. Furthermore, in knockout mice, often a large part of the gene has been removed. Thus, the consequences of a small in-frame deletion or amino acid substitution might be less severe than that predicted from the mouse study. This phenomenon was observed in studies investigating changes in the *SYCP3* gene, where mutations were compatible with fertility (but associated with miscarriages).[39,40] Knockout male mice were completely sterile, but in these mice, an important fraction of the gene was missing.[47]
7. When no knockout mouse studies are available, the phenotype caused by mutations might be predicted based on the expression pattern of the gene of interest. Yet again, caution should be taken. As shown with the *TAF7L* gene, the observed phenotype could be less severe than that predicted from the expression pattern.

From these 'pitfalls', it is obvious that even 'specific' phenotypes should be handled with care, and even for these groups, multiple factors might be involved in the aetiology of the disease. When analysing unselected groups of patients, it is even more important to consider the aforementioned difficulties. The number of papers describing mutations in genes that are clearly associated with the observed fertility problems in patients remains severely limited.

Polygenic causes

As mentioned above, single-gene defects are especially expected in patients with a 'specific' phenotype. Yet, the majority of patients visiting fertility clinics for male factor infertility suffer from poor semen parameters. For men with unexplained oligozoospermia, it is difficult to predict whether a defect in a single gene causes the fertility problems. Indeed, the cause might be multifactorial and include defects in one or more genes and potentially be combined with environmental factors. Each factor on its own can be considered as a 'risk factor'. In extremes, Sertoli cell-only syndrome (the complete absence of germ cells in the testicular tissue) could also be caused by an accumulation of risk factors. Yet in these patients, also single-gene defects can be expected, for instance, in genes essential to maintain the stem cell pool of spermatogonia.

Two well-studied risk factors are the gr/gr deletions and *MTHFR* gene polymorphisms. The gr/gr deletions have already been discussed in a previous section. We believe that the impact of gr/gr deletions is dependent on the genetic background and is potentially under the influence of environmental factors. Consequently, the patients will still have normal sperm counts or be classified as oligozoospermic. Therefore, it is essential to gain more insight into these genetic factors that should be considered as risk factors because the presence of a single, isolated risk factor might have only a small influence on spermatogenesis. Consequently, when analysing the controls, one might (incorrectly) conclude that this factor/polymorphism has no influence on male infertility. It will be an ongoing challenge to map genetic risk factors that might have an impact on the efficiency of sperm production. Again, we should consider the same interpretation errors that are encountered with the identification of single-gene defects. In particular, differences in ethnicity should be considered. As with the C677T SNP, the background in which the *MTHFR* gene is expressed might be important for the consequences of the SNP. The *MTHFR* gene is essential for folate metabolism. It is suggested that in countries with a low dietary intake of folates, the homozygous C677T polymorphism might be associated with male infertility, as folates are essential for DNA methylation.[48] Tüttelmann *et al.*[19] performed a meta-analysis of eight published studies that showed a clear association between homozygous change and decreased spermatogenesis. Alternatively, some SNPs might be more common in ethnic subpopulations without affecting infertility. In the case of gr/gr deletions, it was observed that these deletions are fixed on the Y haplogroups Q1 and D2b, which are present in high frequencies in China and Japan, respectively.[49–51] It is supposed that protective mechanisms are present on these Y chromosomes that counteract with the gr/gr deletions.

The development of whole-genome approaches, as described in the next paragraph, will enable the identification of changes in multiple genes simultaneously and will thus facilitate the identification of polygenic causes. Yet, the interpretation of the data will be the most difficult part of these studies.

IMPLEMENTATION OF NEW TECHNIQUES

The implementation of whole-genome approaches, such as SNP arrays, array comparative genomic hybridisation analysis and whole-genome or exome analysis through next generation sequencing, will enable researchers to analyse multiple genes in parallel. These studies will be useful in identifying polygenic causes and single-gene defects. This approach also has the advantage of avoiding the selection bias of genes to be included in studies on (in)fertility. The current studies are primarily based on what is already known about genes from mouse studies.

SNP arrays have already been used in studying familial cases of male infertility. Dam *et al.*,[23] for instance, were able to identify a mutation in the *SPATA16* gene after minimizing the region of interest through linkage analysis by SNP arrays. Nevertheless, large families with multiple fertile and infertile men are difficult to find.

Until now, a single pilot study has been published in which the authors performed a 'genome-wide SNP association study' to identify SNPs that were linked to male infertility.[52] A follow-up study showed that some of the SNPs might be associated with azoospermia or oligozoospermia.[53] Yet, this study failed to identify 'real causes' of male infertility, but rather, identified factors that were only present in infertile males, and not in the controls. These SNPs could be considered as potential risk factors.

Through array comparative genomic hybridisation, deletions or increased copy numbers can be detected in the whole genome. The main limitation of array comparative genomic hybridisation is the resolution of the platform used, meaning that small rearrangements might be missed. Moreover, mutations or translocations cannot be detected. One study described the involvement of copy number variations in patients with disorders in sexual development.[54] Although the majority of these patients also face fertility problems, spermatogenesis failure is not the only phenotypic abnormality in these patients.

To our knowledge, whole-exome or whole-genome analysis through next generation sequencing has not been described in relation to the study of male infertility. Again, the interpretation of the data will be difficult. Therefore, it is important to select well-defined and extremely specific patient groups in which single-gene defects are more likely.

Whole-genome approaches have the advantage that defects can be detected in genes with an unknown function, thereby avoiding the manual selection of genes based on their known expression pattern or described phenotype in knockout mouse studies. Whole-genome sequencing techniques also represent a potentially well-suited approach to characterize complex spermatogenic impairment phenotypes resulting from disturbances in multiple genes. Furthermore, novel insights into epigenetic mechanisms regulating spermatogenesis might be acquired. Epigenetic deviations have been shown to be potentially responsible for male infertility; examples are an abnormal protamine 1/protamine 2 ratio and aberrant methylation patterns in *DAZL* and *CREM*.[55,56]

First, more insight needs to be gained into the function of the genes that are involved in spermatogenesis. However, during spermatogenesis, numerous genes are expressed under the influence of hormones, but also of autocrine, paracrine and juxtacrine factors between the various testicular compartments, making it impossible to model this process completely *in vitro*.[57] Therefore, many models for studying the role of genes in spermatogenesis have been used. The mouse is the model organism of choice for this purpose, mainly because mouse spermatogenesis is comparable to that in humans. Furthermore, mice have a short reproductive cycle with large litter sizes, are not expensive to accommodate and their embryos are easy to manipulate at the genetic level.[58]

MOUSE MODELS

The technique primarily used to study a gene function *in vivo* is the generation of knockout mouse models, where a gene is inactivated or 'knocked out' by replacing or disrupting it (**Figure 2**). Consequently, the role of the defective gene(s) can be determined. In the Mouse Genome Informatics database (http://www.informatics.jax.org/), over 388 knockout mouse models with impaired spermatogenesis are currently described. The technique to generate knockout mouse models is based on the reverse genetic approach; the function of a gene can be predicted by alterations of the expression of a specific gene, followed by the evaluation of the phenotypic outcome. However, the ablation of a critical gene can result in unexpected embryonic death, making the analysis of the role of this gene in spermatogenesis impossible. Conditional and inducible knockout models can be made to prevent this. In conditional knockouts, the gene is inactivated only in specific tissues, using Cre–LoxP or Flp–FRT site-specific recombination systems. In inducible knockout models, the gene of interest is fused with an antibiotic sensitive gene such that it will become disrupted when the antibiotic is administered.[59] A recent example using conditional knockout mice was applied to determine the testicular function of the transcription factor *Gata4* in adult mice.[60] *Gata4* knockout mice died from defects in ventral morphogenesis and heart development at embryonic day 9.5.[61] Therefore, Cre–LoxP recombination in conjunction with Amhr2–Cre was used to delete the *GATA4* gene only in the Sertoli cells, and consequently, the function could be studied at a later stage.[61] At

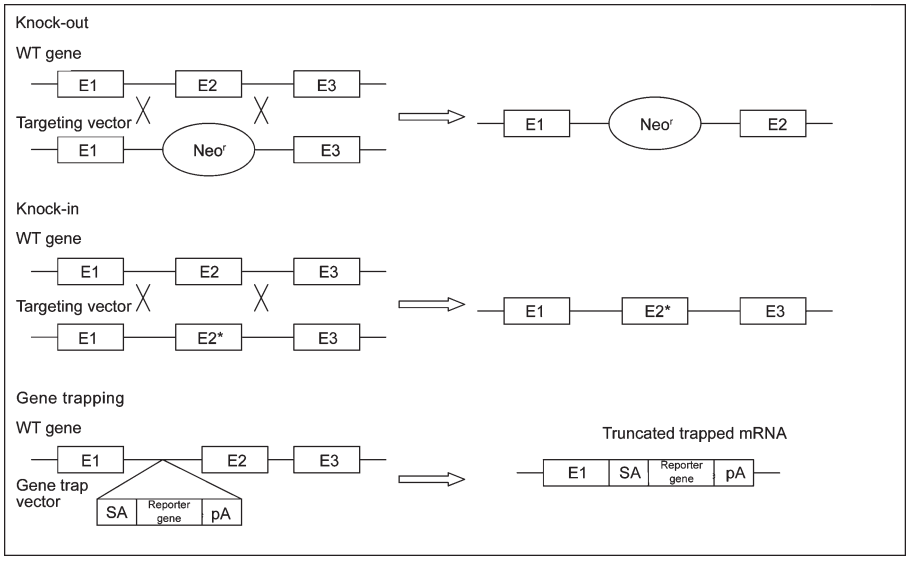

Figure 2 Scheme of knockout, knockin and gene trapping methodologies. pA, plasminogen activator; SA, splice acceptor; WT, wild type; E, exon.

6 months, these knockout mice showed decreased sperm counts and sperm motility, resulting in testicular atrophy and loss of fertility.[61]

A variant of the knockout approach is the creation of knockin models in which mutations are introduced in the genome by replacing the original gene by its mutant version using homologous recombination (**Figure 2**).

In the International Knockout Mouse Consortium, different groups collaborate to mutate all of the protein-encoding genes in the mouse using a combination of gene trapping and gene targeting in C57BL/6 mouse embryonic stem cells. Gene trapping is a high-throughput method in which gene trap cassettes are inserted either randomly across the genome or at a specific site, resulting in gene ablation[62,63] (**Figure 2**). The International Knockout Mouse Consortium includes the following programs: the Knockout Mouse Project (USA), the European Conditional Mouse Mutagenesis Program (Europe), the North American Conditional Mouse Mutagenesis Project (Canada) and the Texas A&M Institute for Genomic Medicine (USA) (http://www.knockoutmouse.org/).

A disadvantage of the reverse genetic approach is that prior knowledge of the gene's function is needed, and therefore, only genes with an expected role in spermatogenesis will be detected. This is not the case in the forward genetic or phenotypic-driven approach, which starts with the selection of a model with a phenotype of interest, and subsequently determines the underlying genetic cause. As described above, gene trapping disrupts genes at random. Another forward genetic approach is whole-genome mutagenesis in which high rates of point mutations are randomly introduced throughout the whole genome. This approach is primarily performed using the alkylating agent *N*-ethyl-*N*-nitrosourea (ENU), which causes mutations in all cells, particularly in premeiotic spermatogonial stem cells. After the selection of mice with the desired phenotype, the causal mutation can be identified through linkage analysis, followed by sequencing of the candidate genes or the currently preferred method of whole-genome sequencing. Instead of null alleles, single base-pair substitutions are generated, which adequately reflect the disease-causing mutations that are predicted in human and can also help in determining critical domains for protein function. The first large-scale ENU mouse mutagenesis programmes were implemented at the end of 1996 in Germany and the United Kingdom.[64,65] In 2002, the Reproductive Genomics Program was set up at the Jackson Laboratory to develop mouse models of infertility using ENU mutagenesis (http://reproductivegenomics.jax.org). Currently, 38 models expressing male infertility have been generated in this programme, and the chromosomal location is known for 30 of them.[66] Through this program and in subsequent individual studies aiming to characterize the underlying genetic defect of the observed phenotypes, several novel genes were identified that cause male infertility. These genes include *Brwd1*, which is necessary for the completion of gametogenesis;[67] *Capza3*, which is involved in the removal of excess cytoplasm during spermiation;[68] and *eIF4G3*, a translation initiation factor.[69] Furthermore, mutations in *Nsun7* result in a rigid flagellar midpiece of the sperm cells that causes decreased progressive motility[70,71] and mutations in *Hei10* impair alignment of the chromosomes at the metaphase plate in both spermatocytes and oocytes.[72]

These mouse studies will provide useful information about the function of proteins involved in spermatogenesis. Furthermore, we might obtain information concerning the consequences of the mutation or deletion of the corresponding genes. However, as mentioned above, caution should be taken in translating the results found in mice to humans. Some biological processes such as the process of the sperm–egg interaction can be different between mice and humans.[73] Furthermore, similar genes could have different functions. Whereas the knockout of a certain gene results in infertility in mice, the function of one gene could compensate for another in humans.

CONCLUSIONS

Despite substantial efforts over the last decade, the genetic causes of spermatogenetic failure still remain largely unknown. It has been estimated that more than 2300 genes play a role in spermatogenesis.[59] Mutations in each of these genes could theoretically cause male infertility. Only a few of these genes have been investigated in humans, and most of the detected alterations could not be demonstrated to cause infertility. Through the use of knockout mouse models, 388 genes have already been shown to be involved in spermatogenesis, but translating these results to humans should be done with care. One reason for this caution is that a large part of male infertility in humans is not caused by monogenic homozygotic mutations except for well-defined cases such as globozoospermia. Considering that thousands of genes are involved in male fertility, it could be possible that innumerable combinations of heterozygous base pair changes or risk factors could cause male infertility. Thus, the molecular diagnosis of infertility would be difficult with the current available technologies. The recent evolution in the development of whole genome-based techniques and the large-scale analysis of mouse models will hopefully help to identify more infertility-related mutations and risk factors. In addition, epigenetics has created a promising avenue in the field of male infertility. The development of an adequate in vitro human model for spermatogenesis would also be helpful.

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The authors declare no competing financial interets.

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