Published in final edited form as:
Expert Rev Mol Med. ; 12: e15. doi:10.1017/S1462399410001468.

Local Signaling Environments and Human Male Infertility: What Can Be Learned from Mouse Models

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Abstract

Infertility is one of the most prevalent public health problems facing young adult males in today's society. A clear, treatable cause of infertility cannot be determined in a large number of these patients, and a growing body of evidence suggests that infertility in many of these men may be due to genetic causes. Studies utilizing animal models, and most importantly, mouse knockout technology, have been integral not only for the study of normal spermatogenesis but also for identifying proteins essential for this process, which in turn are candidate genes for causing human male infertility. Successful spermatogenesis depends on a delicate balance of local signaling factors, and this review focuses specifically on the genes that encode these factors. Normal functioning of all testicular cell types is not only essential for normal fertility but, as recently hypothesized, may also be crucial to prevent germ cell oncogenesis. Analysis of these processes using mouse models *in vivo* has provided investigators with an invaluable tool to effectively translate basic science research to the research of human disease and infertility.

Keywords

male infertility; Sertoli cell; spermatogenesis; knockout mouse; testis signaling

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Further reading, resources, and contacts

American Society for Reproductive Medicine http://www.asrm.org/
Society for Assisted Reproductive Technology http://www.sart.org/
European Society of Human Reproduction and Embryology http://www.eshre.com/page.aspx/3
OMIM: Testicular tumors http://www.ncbi.nlm.nih.gov/entrez/dispomim.cgi?id=273300/
International Male Contraception Coalition http://www.malecontraceptives.org/
Mammalian Reproductive Genetics http://mrg.genetics.washington.edu/
Papers with testicular expression profiling:

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Mouse Models of Spermatogenesis

Animal models have been indispensable to the study of a number of biological processes, and fertility research is no exception. Histological analysis in 1871 allowed Viktor von Ebner to first describe the cycle of spermatogenesis in the rat (Ref. 1,2). In 1878, the studies of Enrico Sertoli provided detailed evidence of the origin of the cell types in the testis, correctly distinguishing the types of maturing germ cells – spermatogonia, spermatocytes, and spermatids – and also correctly identifying their support cell, which von Ebner later termed the "Sertoli cell" (Ref. 1,2) (See Figure 1 for an overview of spermatogenesis (Ref. 3)). For many years thereafter, understanding of the mechanisms of spermatogenic regulation was limited, and research was heavily focused on proteins that were affected by a few naturally occurring genetic mutations. However, knowledge of the molecular basis of infertility exponentially increased with the advent of mouse gene knockout technology. Since up to 30% of male infertility cases are likely due to genetic causes, mouse models of infertility may be instrumental in directing treatment of these patients (Ref. 4). This review will focus on spermatogenesis with an emphasis on how the local signaling environment regulates germ cells and how mouse models of infertility have enriched our knowledge of testis development which may directly relate to the genetic causes of human male infertility.

Spermatogonial Regulation

Spermatogonia are the progenitor population of male germ cells. Unlike the ovary, which has a fixed pool of oocytes for the lifetime of an organism, testicular germ line stem cells continually proliferate. Similar to other stem cell types, the most important function of spermatogonial stem cells is to balance self-renewal with differentiation. If there is not adequate differentiation, sperm counts will suffer. On the other hand, if there is not adequate self-renewal, the pool of germ cells will progressively become exhausted. This balance is dependent on both extrinsic and intrinsic factors. The extrinsic factors are derived from the spermatogonial stem cell niche, an environment that is propagated by signaling factors from the vasculature (Ref. 5) and also those originating more locally in adjacent peritubular myoid cells, Leydig cells, and, most importantly, Sertoli cells (Ref. 6). A number of factors are important for the niche including glial cell line-derived neurotrophic factor (GDNF), kit ligand (KITL), activin A, and bone morphogenic protein 4 (BMP4), to name a few (Ref. 7). *In vitro* and *in vivo* studies have shown the impact of these signaling factors on intrinsic spermatogonial stem cell factors and spermatogonial self-renewal versus differentiation (Figure 2).

Glial Cell Line-Derived Neurotrophic Factor—GDNF, which was first discovered as a pro-survival factor for midbrain dopaminergic neurons, is a distant member of the transforming growth family β (TGFβ) superfamily that is expressed and secreted by Sertoli cells (Ref. 8,9,10). A subset of spermatogonia express the major GDNF receptors, RET (ret proto-oncogene) and GFRA1 (GDNF family receptor alpha 1) (Ref. 8,10,11), and this led to interest in investigating the roles of GDNF signaling in spermatogonial stem cells *in vitro*. It was found that not only did GDNF enhance stem cell maintenance (Ref. 12), but it was also found to be essential for the maintenance of stem cell activity in long term cultures (Ref. 13,14). These results led to interest in GDNF receptors as markers of "stemness" in spermatogonial stem cell cultures. However, studies utilizing GFRA1 antibodies in magnetic activated cell sorting procedures to enrich for $GFRA1^+$ cells found that although a subset of spermatogonial stem cells did express this GDNF receptor, the overall stem cell pool was heterogeneous with respect to receptor expression (Ref. 15,16). In contrast to those findings, siRNA-mediated knockdown of *Gfra1* in mouse type A spermatogonial cultures led to suppression of proliferation and induction of markers of spermatogonial differentiation (Ref. 10). In addition, phosphorylation of tyrosine 1062 of RET, a known binding site for many of

its downstream signaling components, was reduced in the absence of GFRA1 (Ref. 10) suggesting that RET may also play a pivotal role in the prevention of differentiation and the maintenance of self-renewal of this cell type.

Transgenic mouse models were critical in the discovery of the functional importance of GDNF to the spermatogonial stem cell niche and the translation of the *in vitro* findings to a live organism. *Gdnf*-heterozygous mutant mice were fertile but contained a number of seminiferous tubules with progressive depletion of spermatogonial pools, and conversely, transgenic mice that overexpressed GDNF accumulated masses of undifferentiated spermatogonia postnatally (Ref. 8). To circumvent the neonatal lethality of *Gdnf*-, *Gfra1*-, and *Ret knockout* mice, testes from these mice were transplanted into the back of castrated nude mice (Ref. 11). Consistent with signaling of GDNF through a receptor complex that incudes RET and GFRA1, the null testes phenocopied each other and exhibited impaired spermatogonial stem cell proliferation, decreased markers of spermatogonia stem cells, and increased markers of differentiation in the spermatogonial population (Ref. 11). Knockin mice have also been utilized to replace tyrosine 1062 of RET with a phenylalanine codon, rendering it incapable of phosphorylation. The testes from these mice showed marked atrophy, and RET-expressing spermatogonia became almost undetectable by 3 weeks of age, revealing *in vivo* that Y1062 of RET is essential for the self-renewal of spermatogonial stem cells (Ref. 17).

Kit ligand—Spontaneous mutations in the mouse *Steel* (*Sl*) locus encoding kit ligand (official gene symbol *kitl*, also known as stem cell factor) were the first clues in the identification of the importance of KITL in spermatogenesis (Ref. 18,19). KITL is a signaling factor synthesized by Sertoli cells in the postnatal testis (Ref. 20) that binds and activates the KIT receptor (official gene symbol *kit*, also known as *c-kit*) located on differentiating spermatogonia and early spermatocytes (Ref. 21). Similar to KITL, the importance of KIT in spermatogenesis was well known due to spontaneous mutations in the *white spotting* (*W*) locus that encodes it (Ref. 18,19). Early work utilizing one such mutant (W^v) led to the discovery that W^v-heterozygous mice that were made cryptorchid to eliminate all differentiating germs cells and leave only undifferentiated spermatogonia were never able to progress to differentiating spermatogonia after surgical reversal (Ref. 22). Injection of the ACK2 blocking antibody that prevents KIT activation led to reduced mitosis of differentiating spermatogonia but had no effect on the mitosis of undifferentiated spermatogonia or the meiosis of spermatocytes (Ref. 21). The effect on undifferentiated spermatogonia was further corroborated by later studies revealing that SSCs do not express KIT mRNA or protein (Ref. 23,24). When wild-type spermatogonial stem cells were transplanted into KITL mutant mice, undifferentiated spermatogonia were able to proliferate, but the cells were never able to differentiate into more mature spermatogonia (Ref. 25). However, these same cells when re-transplanted to KIT mutant testes were able to differentiate and reestablish complete spermatogenesis (Ref. 25). Further proof of the importance of Sertoli cell expression of KITL and germ cell expression of KIT came when KITL mutant germ cells were transplanted into KIT mutant testes and were shown to successfully rescue the infertility in those mice (Ref. 26).

Mouse models have also been critical in discovering the relationship between intrinsic stem cell factors and KIT/KITL signaling. One such intrinsic protein is zinc finger and BTB domain containing 16 (ZBTB16 also known as Plzf or Zfp145), a DNA sequence-specific transcriptional factor. ZBTB16 is expressed in undifferentiated spermatogonia, and mice with mutations in the *Zbtb16* locus have progressive infertility due to depletion of the spermatogonial stem cell pool (Ref. 27,28). ZBTB16 directly represses the transcription of *Kit*, and undifferentiated spermatogonia from *Zbtb16*-null mice have a relative enrichment of KIT-positive cells, leading to the hypothesis that ZBTB16-mediated repression of *Kit* is at

least partially responsible for its function in maintaining the undifferentiated state in spermatogonia (Ref. 29).

The previous studies emphasized the relationship between KIT/KITL signaling and spermatogonial differentiation. However, studies in *Kit* mutant mice indicated that KIT/KITL signaling is not necessary for the initial differentiation of juvenile undifferentiated spermatogonia into KIT-positive cells but is necessary for the maintenance and proliferation of those cells (Ref. 30). Adding to this finding is a recent study that discovered that KIT mutant testes contain spermatogonial stem cells that could be enriched in culture and reintroduced into recipient KIT mutant testes to successfully reestablish all stages of spermatogenesis (Ref. 31). This result casts doubt on the dogma that KIT/KITL signaling is an absolute requirement for spermatogonial differentiation. In another interesting study, it was shown that GDNF and fibroblast growth factor 2 can reprogram KIT-positive spermatogonia to dedifferentiate into spermatogonial stem cells, raising the possibility that "stemness" can be acquired by differentiating progenitor cells in the testis (Ref. 32). Although it may be quite complicated, the studies in the GDNF and KITL pathways support the hypothesis that there is a balance between self-renewal and differentiation of spermatogonial stem cells, and studies in humans support the hypothesis that alterations in this balance can lead to infertility and/or cancer. This topic will be discussed in greater length in a later section.

Meiotic Initiation

After undifferentiated spermatogonia commit to entering a differentiation pathway, these cells undergo a couple of mitotic divisions and then differentiate into primary spermatocytes that subsequently undergo meiotic division. Initiation of meiosis is dependent upon intrinsic as well as extrinsic signals, similar to what has been discussed about spermatogonial maintenance in the previous section. Also, similar to their role in maintaining the spermatogonial stem cell niche, Sertoli cells are critical in controlling the extrinsic signaling environment that influences meiotic initiation. Much of the work in this field has focused on the role of retinoic acid signaling and the regulation of the permissive meiotic environment (Ref. 33).

Extrinsic Retinoic Acid Signaling—Historically, it has been observed that vitamin A deficiency in rodents causes spermatogenic defects and complete loss of meiotic germ cells (Ref. 43). This was the first evidence that retinoids, the metabolic derivatives of vitamin A, are important to spermatogenesis. During embryogenesis, the mesonephros expresses a major enzyme of retinoic acid synthesis (ALDH1A2) and, consequently, is the main source of retinoic acid in the embryonic gonad (Ref. 37). Postnatally, Sertoli cell expression of ALDH1A1 may play a more prominent role in retinoic acid (RA) production as *Aldh1a1*-null testes have reduced expression of RA-sensitive genes at postnatal day 5 (Ref. 35,44). Although there appears to be abundant levels of RA present throughout gonadal development, there are also prominent sex-specific differences in RA availability to the germ cells, the discovery of which helped to clarify the role of RA in meiotic initiation.

Meiosis begins embryonically in females and postnatally in males. A major retinoid-degrading enzyme – cytochrome P450, family 26, subfamily b, polypeptide 1 (CYP26B1) – is expressed sex-specifically in Sertoli cells of the male gonads (Ref. 37). Deletion of *Cyp26b1* causes male germ cells to enter meiosis precociously at the same embryonic time point that female germ cells normally initiate meiosis (E13.5) (Ref. 37,38), and CYP26B1-null embryonic testes have elevated RA levels (Ref. 38). Addition of a P450 antagonist to E12.5 gonadal cultures reduces the expression of spermatogonial stem cell markers and increases the expression of meiotic markers (Ref. 37), and meiosis is induced in gonadal

cultures if they are treated with a synthetic retinoid that is resistant to CYP26B1-mediated metabolism (Ref. 38). Further cementing the importance of the role of RA in meiotic initiation is the observation that meiotic markers are suppressed in female gonadal cultures that have been treated with a synthetic antagonist of RA (Ref. 37).

Intrinsic Competency Factors—Stimulated by retinoic acid gene 8 (*Stra8*) is an RA-responsive gene that is expressed in premeiotic germ cells in both the male and female gonads (Ref. 41,45,46). RA treatment of cultured gonocytes, spermatogonia, or whole neonatal testes strongly induces *Stra8* expression (Ref. 45). *Stra8* knockout males show a block in spermatogenesis immediately preceding meiotic prophase and show no signs of meiotic recombination or chromosomal synapsis (Ref. 41). So, these results beg the question – since RA is quite abundant in many tissue types in development, why are germ cells the only cell type to respond to RA by initiating meiosis? Meiotic competence must not only depend on an extrinsic signal but also an intrinsically permissive environment.

Deleted in azoospermia-like (*Dazl*) is a germ cell-specific gene that is expressed in the embryonic male and female gonads (Ref. 47,48). *Dazl* knockout female gonads have defective meiotic chromosome condensation and synapsis and severely suppressed endogenous *Stra8* mRNA levels (Ref. 49). In contrast to fetal ovaries, wild-type fetal testes do not normally undergo meiotic initiation, but when cultured in the presence of RA, they respond by upregulating *Stra8* mRNA (Ref. 49). However, culturing of *Dazl*-null male gonads with RA does not result in *Stra8* mRNA upregulation (Ref. 49). Interestingly, germ cells in *Stra8*-null testes express an important protein for meiotic synapsis, synaptonemal complex protein 3 (SYCP3), but it is not loaded onto chromosomes as it normally is during proper meiosis (Ref. 41). DAZL directly binds to the 3'-untranslated region of *Sycp3* mRNA and enhances its translation (Ref. 50), and *Sycp3* is severely suppressed in *Dazl*-null germ cells (Ref. 49). Taken together, these results suggest that DAZL enables meiotic competence of germ cells by promoting SYCP3 expression and that RA-induced STRA8 promotes meiotic progression by enabling the proper localization of SYCP3.

Spermatozoan Maturation

As germ cells progress through the meiotic divisions of spermatogenesis, they migrate from the basal layer of the seminiferous tubule towards the adluminal compartment and are eventually released into the tubular lumen as mature spermatozoa. During this maturation process, Sertoli cells are responsible for a number of important functions including: 1) re-forming the blood-testis barrier as the germ cells transit from the basal to adluminal compartments, 2) providing proper signaling and nutrients, 3) secreting tubular fluid to maintain the seminiferous tubular lumen, and 4) remodeling the constantly changing junctions between Sertoli cells and maturing germ cells (Ref. 2,3). Improper Sertoli cell functioning during spermatogenesis could cause a myriad of spermatozoan defects including misshaping of the sperm head, improper formation of the sperm tail leading to reduced fertility, or decreased sperm counts, just to name a few. Androgen signaling through the androgen receptor is a major signaling pathway that is crucial for proper Sertoli cell regulation and support of spermatogenesis (Ref. 51).

Androgen Receptor—In 1970, Lyon and Hawkes isolated the testicular feminization (*Tfm*) mouse mutant, an X-linked mutation that caused XY mice to develop external female genitalia, cryptorchidism, and a spermatogenic block at the spermatocyte to round spermatid transition (Ref. 52). Mapping of the *Tfm* mutation revealed a single base deletion in the amino-terminus of the gene encoding the androgen receptor (*Ar*) that results in a frameshift mutation and premature termination of AR at amino acid 412 (Ref. 53). Later studies showed that *Tfm* mutant mice had reduced Sertoli cell numbers at birth, but by puberty, *Tfm*

mutant testes were no different from wild-type testes that were made cryptorchid surgically as shown by their similar histology and gene expression levels (Ref. 54). Knockout mice that were engineered to have a null allele of *Ar* phenocopy the *Tfm* mutant mice, but researchers were still left wondering if the spermatogenic defects in these mice were due to defects in androgen signaling or cryptorchidism (Ref. 51,55).

Conditional knockout mice were developed using Cre-loxP technology to knock out the androgen receptor in specific cells or tissues. A number of different laboratories made Sertoli cell-specific knockouts of *Ar* (Ref. 56,57,58). None of these conditional knockout mice had cryptorchidism but did vary in the severity of the spermatogenic block, with some experiencing a block between the spermatocyte and spermatid stages (Ref. 56,57) and another showing a block between the round spermatid and elongating spermatid stages (Ref. 58). Despite these differences, all of these Sertoli cell-specific knockouts of *Ar* were shown to have defects in both the permissive and instructive environment driving the later stages of spermatogenesis. The permissive environment of late spermatogenesis refers to the adluminal compartment that is protected by the blood-testis barrier. The integrity of the blood-testis barrier is essential to shield the developing germ cells from being influenced by signaling from any source besides Sertoli cells or other germ cells and also functions as an immunological barrier. Transcript levels of a variety of genes involved in tubular restructuring and blood-testis barrier maintenance, including proteases and protease inhibitors, cell adhesion molecules, and tight junction components, were affected in all of the aforementioned Sertoli cell-specific *Ar* knockout mouse models (Ref. 59,60,61). One functional assay indeed showed that Sertoli cell-specific *Ar* knockout mice experienced disruption of blood-testis barrier integrity (Ref. 62). The instructive environment was also affected as mRNAs for proteins involved in transport and metabolism were misregulated (Ref. 59,61). Interestingly, one of the genes that was upregulated in the knockout was alcohol dehydrogenase 1, the rate limiting enzyme of retinol to retinoic acid conversion, which raised the possibility of cross talk between AR and RA pathways (Ref. 59).

Clinical Implications/Applications

Research using mouse models has produced a wealth of knowledge about the signaling pathways and intrinsic factors that are essential to spermatogenesis. Applying this knowledge to human disease is vitally important, and some steps have been taken in that direction (reviewed in (Ref. 4)). There are a number of chromosomal abnormalities and gene mutations, including Klinefelter's syndrome and cystic fibrosis transmembrane conductance regulator mutations, respectively, that have been identified as related to male infertility, but the following section will focus instead on the pathways that we have discussed in previous sections. We will also discuss the relationship of these molecular pathways in the pathogenesis of testicular cancer and the relationship between male infertility and cancer.

Genetics of Male Infertility

It is estimated that 15% of male infertility patients suffer from chromosomal alterations and single gene mutations that may be the cause of disease (reviewed in (Ref. 64,65)). One common chromosomal aberration found in patients with severe infertility is microdeletions in the long arm of the Y chromosome, which is estimated to cause 10–15% of azoospermia (absence of sperm in the ejaculate) and 5–10% of severe oligozoospermia (low sperm counts) (Ref. 64). This commonly deleted portion of the Y chromosome has been divided into 3 "azoospermia factor" regions: AZFa, AZFb, and AZFc. Deletions of AZFc are frequently found in patients with severe disease (3–7%), and this locus contains *DAZ1–4*, the Y chromosome paralogs of the autosomal gene, *DAZL* (Ref. 64). In one study that covered a decade of treating infertility, 71.4% of patients with an AZFc deletion had sperm in their testes that could be retrieved, and two-thirds of those cases resulted in pregnancy via

assisted reproductive techniques (Ref. 66). In a new twist, new AZFc haplotypes termed "gr/gr" or b2/b3, which both result in loss of *DAZ3* and *DAZ4*, have been identified as linked to infertility but have also been found in normozoospermic males as well (Ref. 64,65). As suggested from knockout of *Dazl* in mice, which causes loss of germ cells and complete absence of gamete production (Ref. 39), *DAZL* in humans has been examined as a candidate for male infertility. One study identified 4 novel missense mutations in *DAZL* with one homozygous *DAZL*-null male patient presenting as infertile (Ref. 67). This group hypothesized that *DAZL* mutations may account for the variations in infertility found in patients with partial deletions in AZFc; however, they were not able to discern a link between autosomal *DAZL* function and severity of male infertility in the population they examined (Ref. 68).

Idiopathic infertility that is not related to chromosomal alterations may be due to single gene mutations or polymorphisms. One of the most widely appreciated and best-characterized genes found mutated in infertile men is the androgen receptor, estimated to be associated with infertility in 2–3% of all cases of azoospermia/oligozoospermia (Ref. 64). The *AR* gene can be mutated in a number of ways resulting in mild to complete androgen insensitivity. The cases of complete androgen insensitivity phenocopy the *Tfm* mice, in which the 46,XY patients present as phenotypic females with undeveloped gonads, whereas the mild forms present as phenotypic males with low sperm counts. In the phenotypic males with low sperm counts, studies have found that there is a significant relationship between *AR* mutations and infertility; however, no one has been able to match a specific *AR* polymorphism to disease severity (Ref. 69,70). Polymorphisms that have been examined in the *AR* gene relate to two sites in exon 1 that exhibit variable stretches of CAG or GGC repeats (Ref. 64). In a mouse model of Kennedy disease (a neurodegenerative disorders caused by CAG expansion in the *AR* gene), 113 glutamine codons were knocked into exon 1 of *Ar* (Ref. 71). These mice experienced progressive infertility not as a result of loss of AR function but instead due to insoluble AR fractions created in Sertoli cell nuclei that led to cytoskeletal abnormalities and decreased support of germ cells (Ref. 71). When examining CAG polymorphisms in groups of infertile patients, different groups had different results, but overall, many studies found a relationship between CAG expansion and infertility (Ref. 72). Unlike in the mouse, it is unclear whether this low level of CAG expansion affects AR transactivation.

Germ Cell Tumors and Testicular Dysgenesis Syndrome

Type II testicular germ cell tumors (TGCTs), including seminomas and non-seminomas, arise from malignant primordial germ cells or gonocytes. They are the most prevalent type of male germ cell tumor and account for 1% of all cancers in male Caucasians and up to 60% of malignancies in those between 20 and 40 years-old (Ref. 73). Multiple studies have linked TGCT occurrence to subfertility, cryptorchidism, and genitourinary tract malformations (Ref. 73). This knowledge has led to the testicular dysgenesis syndrome (TDS) hypothesis (Ref. 74). According to the TDS hypothesis, TGCTs form embryonically when a localized disturbance in niche environment impairs the differentiation of fetal gonocytes. These gonocytes do not mature and remain in the testis as carcinoma *in situ*. Carcinoma *in situ* cells proliferate during the peripubertal period and, with the help of abnormal signaling, acquire genetic changes that progress to TGCT in the adult. Environmental factors and genetic mutations and polymorphisms in the pathways that were discussed in previous sections are all hypothesized to be vital to the development of TGCTs as they all affect the gonocyte niche.

Hormonal Disruption—A rat model of the early pathogenesis of TDS gave clues towards defining pathways that may be affected in human disease (Ref. 75). Dibutyl phthalate administration to gravid mothers induced cryptorchidism, hypospadias, infertility, and testis

abnormalities in their male offspring, similar to those seen in human TDS (Ref. 75). Cryptorchid testes often showed areas of focal dysgenesis containing partially formed testicular cords with mislocalized Sertoli cells, Leydig cells, and gonocytes, and this focal dysgenesis might have been caused by suppressed testosterone *in utero* affecting peritubular myoid cell function (Ref. 75). Dysgenic and "Sertoli cell only" tubules contained Sertoli cells expressing markers of immaturity (Ref. 75). Since this model of early pathogenesis was very similar to human disease (Ref. 76), gene expression analysis in this system could lead to the discovery of important pathways in human disease. One such study found that phthalate esters, including dibutyl phthalate, affected a number of systems including genes necessary for proper steroid hormone synthesis (i.e., testosterone) and genes important for germ cell-Sertoli cell junctions and signaling (including KIT) (Ref. 77). In humans, there has not been a definitive study linking phthalate ester exposure to TDS, but one study found that high exposure to prenatal phthalates corresponded to decreased anogenital distance, penile width, and testicular descent in male infants – all signs of low prenatal testosterone (Ref. 78).

Non-Hormonal Factors—One major finding from the study assessing gene expression changes in the rat model of TDS was the decreased expression of KIT receptor in the dysgenic testes (Ref. 77). Conversely, *Kitl*-heterozygous mice have increased incidence of TGCTs (Ref. 79). Is it possible that KIT activating mutations are selected for in the *Kitl*-heterozygous mice and that the downregulation of KIT receptor may be the major reason why rat models of TDS using phthalate esters do not recapitulate the TGCTs seen in human disease? Activating mutations of *KIT* are often seen in TGCTs (Ref. 80,81) and have been associated with bilateral tumor development (Ref. 82,83). The use of KITL immunostaining has also been proposed as novel diagnostic marker for early malignant germ cells (Ref. 84). In a mouse model, mutation of the tyrosine codon of KIT that is necessary for phosphatidylinositol (PI) 3'-kinase activation led to infertility (Ref. 85). This was in direct contrast to one human study that found a *KIT* mutation that was associated with TGCT pathogenesis that also constitutively activated PI3 kinase *in vitro* (Ref. 81). Another *in vitro* study indicated that KIT activation of PI3 kinase upregulates cyclin D3 and promotes cell cycle progression, which would explain its dichotomous role in the aforementioned studies (Ref. 86).

One cohort study and one retrospective literature review found that even when excluding cryptorchidism as a confounding factor, there is still a significant positive association between subfertility and increased risk of testicular cancer (Ref. 87,88). All of these studies that have determined genetic link for infertility and cancer raise the concern that better screening for genetic causes of infertility may be vital so that these patients can be referred for genetic counseling and possibly preimplantation genetic screening if they are attempting to conceive using assisted reproductive technologies (ART).

Research in Progress and Outstanding Research Questions

Although there has been a lot of outstanding research in both the clinical and basic science fields, more needs to be done to translate "wet lab" benchwork into bedside patient therapies. Testicular dysgenesis syndrome and embryonic stem (ES) cell technology are two fields that have both benefited from extensive basic science research.

Testicular Cancer

We previously outlined a couple of areas that have seen major progress with regards to translational research, but we also would like to highlight some areas that may need more attention. The first genetic study trying to link AR mutations/trinucleotide repeat expansions to testicular cancer saw an increased incidence of null mutations of AR and certain

trinucleotide repeat polymorphisms in TGCT patients (Ref. 89), but not much has been done on this topic since that study. Another important deletion in infertility, the gr/gr haplotype (*DAZ3*/*DAZ4*), has been associated with an increased risk of developing TGCTs in one report (Ref. 90). Studying the functions of AR and DAZ orthologs in humans will be vital to determining the causes of TDS.

Although we have compelling mouse models for the role of KIT/KITL in infertility and compelling human data for the role of this signaling system in TGCTs, very little is known about KIT or KITL mutations in human infertility. We were only able to find two studies that looked at KIT in infertility (Ref. 86,91), with only one of them finding a positive association (Ref. 86). A mouse model in which GDNF was overexpressed demonstrated an early phenotype of infertility but at later time points developed seminomatous tumors (Ref. 92). Although this model does not accurately recapitulate human disease, it is unclear if the GDNF pathway is important to human TDS, as we were not able to find any human studies examining this ligand or its receptors. Again, better understanding of the genetics of human male infertility with regards to the KITL and GDNF pathways may lead to better understanding of TDS. Research in this field is moving in the right direction with the establishment of the International Testicular Cancer Linkage Consortium (Ref. 93).

Promise of ES cell technology/SSC culturing

New studies using *DAZ* and its homologs are revolutionizing the field of ES cell technology and highlighting some of the differences between mouse and human ES cells. Overexpression of DAZL in mouse ES cells caused cells to differentiate into phenotypic male and female germ cells without any extrinsic signaling (Ref. 94). In a human ES cell study, on the other hand, researchers had to overexpress all DAZ homologs (DAZ1–4, DAZL, and BOULE) and supplemented the differentiation medium with multiple BMP ligands (BMP4, 7, and 8b) to make the human cells meiotically competent (Ref. 95). Interestingly, both culture systems seemed to also result in the appearance of Sertoli-like cells which raises the possibility that germ cells may also be influencing the development of Sertoli cells and not just the other way around or, alternatively, that the crosstalk between both cell types is necessary for their coordinated development. The surprisingly different requirements of mouse and human ES cells to achieve meiotic competence may just be a reflection of the innate differences between mouse and human ES cells, as mouse ES cells have many strong germ cell markers and may represent a germ cell-like lineage, unlike human ES cells that may be more like cells from the epiblast (Ref. 96). These studies also support the theories that DAZ homologs are the causative factor in infertility in patients with Y-chromosome microdeletions and that DAZ homologs are intrinsic meiotic competence factors that control germ cell identity.

Future Directions

Of the approximately 15% of all U.S. couples that experience infertility, one to two-thirds of these cases are due, at least in part, to male factor infertility (Ref. 97,98). Scientific breakthroughs in ART have revolutionized the field of reproductive endocrinology and allowed many of these couples to conceive biological offspring. However, there are still many uncertainties regarding the long-term consequences of ART, especially in light of the genetic issues that we have outlined throughout. Treatments of severe male factor infertility are limited (Ref. 99,100), and the need for more treatments is enormous especially for couples experiencing infertility due to male factor alone. Although the idea of gene therapy for the treatment of male infertility seems far-fetched (Ref. 101) and genetic treatment of male factor infertility may or may not reduce the monetary cost of ART, treatments of male infertility would mitigate the medical cost placed on the female patient who must endure hormonal injections ridden with side effects and improve the health of the fetus, since many

of the long term consequences of reproductive technologies are still unknown (Ref. 102,103,104,105).

Acknowledgments

Acknowledgements and funding

Research in the Matzuk laboratory on male reproduction and testicular cancer has been supported by National Institutes of Health (U01HD060496, R01HD057880, P01HD036289, and R01CA60651). R.L.N. is a student in the Medical Scientist Training Program at Baylor College of Medicine and has been supported in part by the Edward J. and Josephine G. Hudson Scholar Fund. We would also like to thank our peer reviewers for their help with improving this review.

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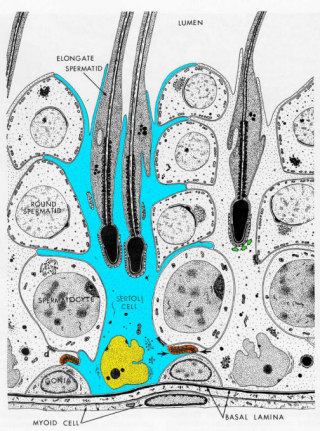

Figure 1.

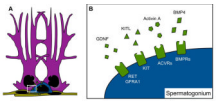

Figure 2.

Table 1

Mouse knockouts of select meiotic genes

Gene Symbol	Gene Name	Reproductive Phenotype	Fertility Status	Age of phenotype	Ref.
Aldh1a1	aldehyde dehydrogenase family 1, subfamily A1	Testes have reduced expression of retinoic acid-sensitive genes (such as *Stra8*)	Fertile	P5	(Ref. 34,35)
Aldh1a2	aldehyde dehydrogenase family 1, subfamily A2	Knockout mice show severe morphological defects by embryonic day 9.5	Lethal	N/A	(Ref. 36)
Cyp26b1	cytochrome P450, family 26, subfamily b, polypeptide 1	Precocious meiotic entry due to increased embryonic levels of gonadal retinoic acid; subsequent pachytene arrest and apoptosis	Infertile	E13.5	(Ref. 37,38)
Dazl	deleted in azoospermia-like	Reduced embryonic development and survival of XY germ cells, reduced expression of germ cell markers, increased germ cell apoptosis	Infertile	E14.5-15.5	(Ref. 39,40)
Stra8	stimulated by retinoic acid gene 8	Failure of meiotic prophase with loss of meiotic chromosome cohesion, synapsis, and recombination	Infertile	P10	(Ref. 41)
Sycp3	synaptonemal complex protein 3	Defects in chromosome synapsis during meiosis, increased germ cell apoptosis at the zygotene stage	Infertile	P12	(Ref. 42)

N/A, not applicable; E, embryonic day; P, postnatal day

Expert Rev Mol Med. Author manuscript; available in PMC 2011 March 14.

Table 2

Select mouse mutants with defects in androgen receptor expression and/or function

Mutation	Cre Promoter	Cell type affected	Reproductive Phenotype	Fertility Status	Ref.
Spontaneous (*Tfm*)	Not applicable	All	Feminized external genitalia, hypogonadal, cryptorchidism with a spermatogenesis block	Infertile	(Ref. 52,53)
Flox (deleted exon 2)	β-Actin	All	Female-like appearance, hypogonadal, low serum testosterone, spermatogenic arrest at pachytene stage	Infertile	(Ref. 55)
Flox (deleted exon 2)	Anti-Müllerian Hormone (Guillou)	Sertoli cells	Normal testis descent and male urogenital tract, hypogonadal, block in meiotic progression with severely decreased spermatids	Infertile	(Ref. 56)
Flox (deleted exon 2)	Anti-Müllerian Hormone (Guillou)	Sertoli cells	Normal testis descent and male urogenital tract, spermatogenic arrest at the diplotene premeiotic stage, low serum testosterone	Infertile	(Ref. 57)
Flox (inverted exon 1)	Anti-Müllerian Hormone (Braun)	Sertoli Cells	Normal testis descent and male urogenital tract, unaffected meiotic progression, spermatogenic block from the late-round to elongating spermatid stage	Infertile	(Ref. 58)
Flox (deleted exon 3)	Anti-Müllerian Hormone (Guillou)	Sertoli cells	Normal testis descent and male urogenital tract, block in meiotic progression with severely decreased spermatids, normal serum testosterone	Infertile	(Ref. 63)

Inverted exon 1, loss of start codon and deletion of entire protein; deleted exon 2, frameshift deletion of first zinc finger of the AR DNA binding domain and premature termination of the protein; deleted exon 3, in frame deletion of second zinc finger domain of the AR DNA binding domain