Review

Selected Genetic Aspects of Male Infertility – What Animal Models Tell Us

(male infertility / spermatogenesis / animal models / genetic control of male fertility / pathogenesis of male infertility / mutations causing male infertility)

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Abstract. Many advances have been recently made in understanding the genetic control of fertility in model systems. This review concentrates on genetic causes of male factor infertility in mammalian models. More than 150 genes proved to be important for the male fertility in mammals and the list is continuously growing. Most of those genes were discovered using gene targeting in the mouse. Here, several interesting male infertility mutations are described with regard to the pathogenesis of reproduction failure. A detailed table comprising most of the genes causing male infertility is presented as supplementary Table 1, at http://www.img.cas.cz/fb/v49no4_table1.html, including the corresponding references.

Infertility is a major health problem: approximately 15% of couples worldwide suffer from infertility, and the male factor accounts for about half of these cases (for review see Feng, 2003). A large proportion of these men fail to conceive because of lack of sperm (azoospermia) or too little sperm (oligospermia). Other phenotypic manifestations include abnormal sperm morphology (teratospermia) and insufficient sperm motility (asthenospermia). Although the cause of these defects of spermatogenesis is often unclear in humans, recent efforts point out the importance of the environmental as well as genetic factors in the development of male infertility. The explosive growth of assisted reproduction (*in vitro* fertilization techniques, IVF) should focus our attention to the genetic causes as, of course, unrestricted use of these techniques could lead to transmission of the responsible genetic defects to successive generations and their unwanted accumulation in the population. Our knowledge of underlying genetic defects could, however, promote appropriate genetic counselling (prevention) or even gene therapy. Because of the small family size and the lack of transmission from affected males (with the exception of IVF), humans are not ideal for the experimental dissection of infertility "genes". Animal models are thus a very important tool for infertility research. There are both the classical genetic approaches, that is studying spontaneous or ethylnitrosurea (ENU)-induced mutations as well as the modern "reverse genetics" of gene targeting and transgenic animals. Overall, over 150 male infertile or subfertile mouse models have been described, not counting the appropriate models in other species. In humans, on the contrary, most of the genetic cases of male infertility must escape detection, as long as karyotype analysis, mutation screening of *CFTR* and Y chromosome deletion analysis are the only genetic tests commonly offered to the infertile patients. Therefore, any estimation of the true incidence of genetic defects in infertile men is, as I believe, highly speculative, although several authors conclude that a genetic cause may be responsible for about 30% (e. g. McLachlan et al., 1998) mainly nonobstructive cases. In case of male subfertility, the incidence of genetic aetiology may be even higher (60% according to Lilford et al., 1994). However, there are also genetically determined obstructive azoospermias, as is the case in patients with cystic fibrosis.

The wide variety of different genes expressed in the germ cells, somatic cells in the testis (especially Sertoli and Leydig cells), epididymis and other parts of the male genital tract is critical for the development of fully functional sperm. In other words, disruption of many different cellular networks and pathways may lead to

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Abbreviations: CBAVD – congenital bilateral absence of vas deferens, CFTR - cystic fibrosis transmembrane conductance regulator, CSL – cranial suspensory ligament, ENU – ethylnitrosourea, IVF – *in vitro* fertilization, PGC – primordial germ cells. Further abbreviations concerning the genes and their products can be found in supplementary Table 1.

the male infertility. There is also evidence that up to 1500 genes contribute to male fertility in Drosophila (Hackstein et al., 2000), suggesting that the genetic control of mammalian male fertility could be of at least comparable complexity. It seems to agree well with the high incidence of spermatogenesis defects in mammalian models. That is especially true of the mouse knockout experiments, where the occurrence of male infertility is frequently unforeseen, but quite common. The variety of genes described to affect spermatogenesis range from cell cycle control (e.g. *Tp53*, see Yin et al., 1998; Fujisawa et al., 2001) to neurohormonal regulation (e.g. *Ace*, see Hagaman et al., 1998), thus revealing the complex nature of spermatogenesis control.

It would be almost impossible to describe here all mutations resulting in male infertility in mammals. Therefore, an attempt was made to select the most interesting or surprising cases and those with specific (non-syndromic) disruption of male gametogenesis. However, neurohormonal regulation of spermatogenesis will not be discussed and the main focus will be on intrinsic defects of genes expressed in testis and epididymis. An overview of the spermatogenic cycle is given in Fig. 1.

Sex determination

Through the cytogenetic studies of Turner syndrome (X0 females) and Klinefelter syndrome (XXY males) it was concluded that Y is the male determining chromosome (Welshons and Russell, 1959). Later on, through analysis of human and mouse sex reversal syndromes (XX males and XY females) it was shown that a single gene *Sry* (sex-determining region Y) is responsible for the initiation of male development (Berta et al., 1990; Jäger et al., 1990). The Sry protein is a transcription factor of the high mobility group (HMG) family. It is expressed in the supporting cells of the indifferent gonad and turns their fate to become Sertoli cells, which then drive the primordial germ cells (PGC) to become prospermatogonia. So it is not the PGC sex, but the supporting cell lineage sex that is responsible for the sex determination of the whole gonad. Indeed, XY PGC can develop as oocytes in female embryos as well as XX PGC can develop as prospermatogonia in male embryos. The direct downstream target of Sry is Sox9, a transcription factor also belonging to the HMG family. Mutations in this gene cause campomelic dysplasia (in these patients sex reversal is combined with skeletal dysplasia (Foster et al., 1994; Wagner et al., 1994). What is the nature of the differentiation signal transmitted to PGC (and the role of PGC themselves) is not yet clear enough, but prostaglandin D2 is one of the candidates (Adams and McLaren, 2002). A more active role of the supporting cells is illustrated by mouse *kitl* (protooncogene kit ligand, identical to stem cell factor - SCF) and c-*kit* (protooncogene, SCF receptor) mutations blocking PGC migration and resulting in infertility without altering sex determination (e.g. Manova et al., 1990; Matsui et al., 1990; Guerif et al., 2002).

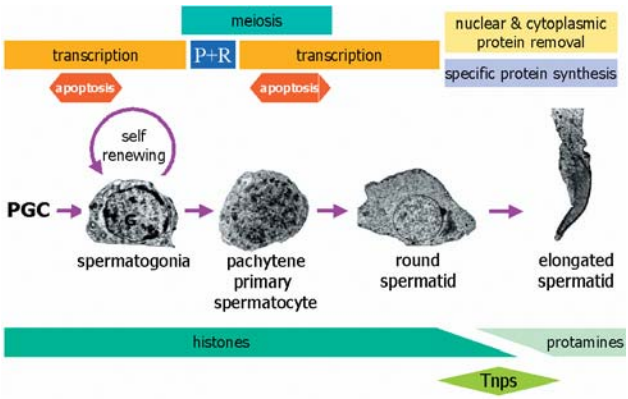

Fig. 1. Overview of mammalian spermatogenesis as a continuous cyclic developmental process involving differentiation of primordial germ cells (PGC) into spermatogonia, spermatogonia proliferation, meiosis and postmeiotic differentiation of a round spermatid into a mature spermatozoon. The underlying molecular processes are shown: **upper part:** transcriptional phases, meiosis with homologous chromosome pairing and recombination (P+R) and apoptosis checkpoints, **middle:** disctinct phases of rat spermatogenesis in transmission electron microscope (photographs courtesy of Z. Jirsová), **lower part:** histone to protamine transition.

Descent of the testis

Cryptorchidism is the most common disorder of sexual development in the newborn (about 3%, at 1 year less than 1%, see e.g. Cortes, 1998). Complications of impaired testicular descent include not only infertility, but also increased risk for testicular malignant tumours (for review see Hutson et al., 1997).

Mutations of the mouse genes *Insl3*, *Great* (G-protein coupled receptor that affects testicular descent) and *Hoxa10* result in male infertility secondary to cryptorchidism. The sexual dimorphic position of the gonads is dependent on the dimorphic development of the two parts of genital mesentery, the cranial suspensory ligament (CSL) and gubernaculum (caudal genital ligament). In males, the transabdominal phase of testicular descent is characterized by movement of the testis into the inguinal region, as a result of gubernaculum development and CSL regression. On the other hand, in females, CSL and not gubernaculum develops, keeping thus the ovary adjacent to kidney. During the second, inguinoscrotal phase, descent of the testis to the scrotum is governed by gubernacular regression. CSL regression is thought to be controlled by androgens produced by Leydig cells. Gubernacular outgrowth is stimulated by Insl3 (insulin-like hormone 3), another product of Leydig cells (Nef and Parada, 1999; Zimmermann et al., 1999). The nature of hypothetical

Insl3 receptor in gubernaculum is not yet clear. It is thus interesting that a receptor with an unknown ligand, Great (G-protein coupled receptor affecting testis descent), is expressed in the gubernaculum and its mutation caused cryptorchidism both in mice and in a clinical case (Gorlov et al. 2002). Another example of genetically determined cryptorchidism is targeted mutation in *Hoxa10*. However, this is not likely to be the cause of human idiopathic cryptorchidism, because the mutation also severely affects female reproduction and, moreover, lumbal vertebrae show anterior homeotic transformation (Satokata et al., 1995).

Testicular temperature is lower than the core body temperature. Prolonged exposure of the undescended testis to the increased temperature has been for a long time (Crew 1922) believed to be the cause of compromised spermatogenesis in cryptorchidism (Nishimune et al., 1978). Early surgical reposition (orchiopexy) leads to normal testis development, including fertility. On the other hand, examination of rodent testis exposed to a single heat stress or experimental cryptorchidism revealed apoptosis of pachytene spermatocytes as a mechanism of germ cell degeneration (Yin et al., 1998; Lue et al., 1999). Very similar pathology was found in testes of transgenic mice expressing human Hsf1 (heat shock transcription factor 1), a transcription regulator of heat shock proteins. One can hypothesize that Hsf1 functions in testes as a temperature sensor, which drives spermatocytes, in contrast to other cell types, to death in case of elevated temperature to prevent genetically damaged germ cells to complete spermatogenesis (Nakai et al., 2000).

Problems with sperm transit from epididymis

The sympathetic nervous system plays a key role in vas deferens contraction, which propels sperm into the ejaculate. ATP is an important neurotransmitter mediating the sympathetic action, because the deletion of its receptor, P2X1, reduced vas deferens motoric response to sympathetic nerve stimulation by 60% and also reduced male fertility by 90% (Mulryan et al., 2000).

Obstruction in the male genital tract is a common cause of infertility in men; however, most cases are acquired. One well-studied example of inherited obstructive azoospermia is congenital bilateral absence of vas deferens (CBAVD) in *CFTR* (cystic fibrosis transmembrane conductance regulator) mutations. The mutations in *CFTR* are either associated with classical cystic fibrosis or with CBAVD, as a monosymptomatic atypical form accountable, however, for increased risk of cystic fibrosis in the offspring in case of *in vitro* fertilization (Stuhrmann and Dörk, 2000).

Sertoli cell dysfunction

Dhh (desert hedgehog) is one of the earliest signalling molecules expressed by Sertoli cells. Its receptor Patched is expressed on Leydig cells and peritubular cells. In *Dhh*-null male mice Leydig cells are absent (consequently, there is a lack of testosterone production and feminization resembling androgene insensitivity syndrome). The spatial organization of tubules is severely affected – there are defects in basal lamina causing anastomotic tubules, extracordal gonocytes and apolar Sertoli cells (Bitgood et al., 1996; Clark et al., 2000; Pierucci-Alves et al., 2001).

In RXRβ (retinoid X receptor beta)-null mice infertility results from oligoasthenoteratozoospermia. In older males seminiferous tubules progressively degenerate. RXRβ is expressed exclusively by Sertoli cells, which are in the mutant filled progressively by unsaturated triglycerides. Sertoli cells are thus solely responsible for this defect (Kastner et al., 1996). Other molecules involved in retinoic acid signalling are also functionally relevant to spermatogenesis, although their effects are exerted not only by Sertoli cells. Retinoic acid itself plays a major role in spermatogenesis, as males deficient in vitamin A after weaning exhibit gradual germ cell loss and vacuolization of the seminiferous epithelium (e.g. Thompson et al., 1961). A very similar pattern of affliction is also seen in RARα (retinoic acid receptor alpha) deficient mice (Lufkin et al., 1993), as well as in a gene trap mutation in a retinoic acid-induced gene *E-MAP-115* (*Mtap7*, coding for epithelial microtubule-associated protein of 115 kDa). The latter was shown to reduce the development of Sertoli cell typical microtubule bundles as a hypothetical substrate of Sertoli cell dysfunction and germ cell loss. Furthermore, there was an abnormality in the microtubule manchette of the elongating spermatid (Komada et al., 2000).

Sertoli cells are believed to be responsible for the formation of microenviroment for germ cells, including the secretion of the fluid transporting spermatozoa to the epididymis. The luminal fluid is rich in K^+ ion, similar to endolymph of the inner ear (Tuck et al., 1970). $Na^+K^+2Cl^-$ contransporter 1 (Nkcc1, gene *Slc12a2*) is involved in transport of salts across epithelial tissues. The null allele as well as deletion mutant of Nkcc1 results in deafness, vestibular defect and male infertility caused by germ cell degeneration (Pace et al., 2000).

Contacts in the germinal epithelium

Infertility can result from disruption of Sertoli cell-germ cell adhesion. In case of targeted mutation of *Man2a2* (alfa mannosidase X, MX), impaired synthesis of GlcNAc-terminated N-glycans on the surface of spermatogenic cells leads to failure of germ cell-Sertoli cell adhesion. Consequently, the number of spermatogenic cells is strongly reduced due to premature release. Epididymis then contains immature cells and only very few fertilization-competent spermatozoa (Akama et al., 2002). Targeted mutations of *claudin 11* (neurological deficit and infertility, see Gow et al., 1999) and *connexin 43* demonstrated the key role of tight and gap junctions,

respectively, during the process of spermatogenesis. The latter has a more profound functional impact, causing neonatal mortality due to heart abnormality (Juneja et al., 1999).

Germ cell proliferation

Germ cell proliferation begins in embryogenesis and with the exception of a short prenatal-prepubertal period, spermatogonial stem cells proliferate (at the highest rate in the body) throughout life. This process is well regulated, as might be expected, by the genes involved in growth and apoptosis. Spermatogonial growth factors include e.g. SCF (stem cell factor, see above), M-CSF (macrophage colony stimulating factor, Cohen et al., 1997), GM-CSF (granulocyte-macrophage colony stimulating factor, Robertson et al., 1999), Gdnf (glial cell neurotrophic factor, Meng et al., 2000) and bone morphogenetic proteins Bmp7, Bmp8a and Bmp8b (Zhao et al., 1996, 1998, 2001). From downstream effectors let us mention PI3K (phosphatidylinositol 3-kinase), recognized as a second messenger in SCF signalling (Blume-Jensen et al., 2000). The rate of apoptosis in spermatogonia is very high, in rats about 75%. A balance of anti-apoptotic members of the Bcl2 family (Bcl6, BclX, Bclw) and pro-apoptotic Bax protein is extremely important for germ cell survival in both sexes. In males, the absence of either Bax (Knudson et al., 1995), BclX (Rucker et al., 2000) or Bclw (Ross et al., 1998, 2001) causes male infertility and the absence of Bcl6 causes subfertility (Kojima et al., 2001). However, other members of apoptotic pathways are not negligible, e.g. the absence of Apaf1 (apoptotic protease activating factor 1) leads to spermatogonial degeneration and male infertility, although only in 5% mice which survive into adulthood, 95% die from complications of defective neural development (Honarpour et al., 2000).

Meiosis-recombination-DNA repair-apoptosis

Meiosis, a process of cell division unique to germ cells, is necessary for production of haploid gametes and extremely important, from the evolutionary point of view, for both the integrity and diversity of the genome. Recombination of homologous chromosomes occurs during prophase of the first meiotic division. Recombination increases genetic variation by reassorting linkage groups and plays a mechanical role in chromosome segregation. Reciprocal exchange between homologous, nonsister chromatids provides a physical connection between the maternal and paternal chromosomes that allows them to orient properly on the meiotic spindle and to segregate accurately at the first division (Baudat et al., 2000). Errors during the recombination process damage genome integrity, so appropriate DNA repair is absolutely necessary. In fact, homologous recombination in meiosis and homologous recombination as a mechanism of double-strand DNA break repair are functionally very similar. If the repair of the DNA damage is unsuccessful, spermatocytes are removed by apoptosis. There exists a low threshold for apoptosis of male gametes with errors in their genome, because it would be evolutionarily unprofitable if any damaged spermatozoon could fertilize an oocyte. Although the basis of meiosis is the same in males and females, details are quite different and so the function of many factors is requisite only for the male meiosis (Hunt and Hassold, 2002).

About 20 genes are known to disrupt the meiotic division in male mice. The particular stage of meiotic failure associated with these genes is indicated in Fig. 2.

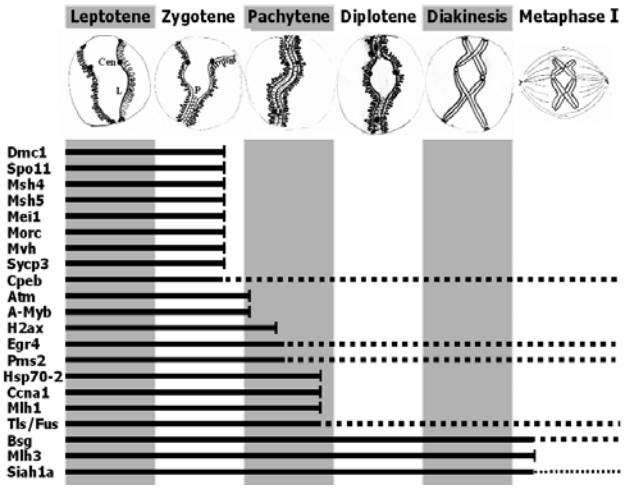

Fig. 2. The timing of meiotic defects of spermatogenesis - comparison of mutations in different genes involved. **Upper part**: schematic representations of the prophase of meiosis I and metaphase I, **Cen** – centromere, **L** – lateral elements of the synaptonemal complex, **P** – protein axes (central element) of the synaptonemal complex, **r** – recombination nodule. **Lower part**: meiotic arrest phenotypes. Short vertical bar - complete meiotic arrest, dashed line - incomplete arrest. For detailed description of each mutation see the text and the supplementary Table 1.

Failure of spermatogenesis occurred in mice double homozygous for *Brca1* and *p53* mutation due to a block in early prophase of the first meiotic division (Cressman et al, 1999). Cyclin A1 is expressed exclusively in germ cells and promotes transition at G2/M phase. Male mice deficient in cyclin A1 are infertile and their testes demonstrate a premeiotic spermatogenesis block (Liu et al., 1998). Zinc finger transcription factor Egr4 is required for male meiosis. In mice deficient in Egr4, spermatocytes are arrested in the pachytene stage and undergo massive apoptosis. Although the arrest is incomplete, the few produced spermatozoa are morphologically abnormal (Tourtellotte et al., 1999). TLS/FUS is an RNA-binding protein that contributes to the N-terminal half of fusion oncoproteins implicated in the development of human liposarcomas and leukaemias. Male mice homozygous for an induced mutation in *TLS*

are sterile with a marked increase in the number of unpaired and mispaired chromosomal axes in pre-meiotic spermatocytes. The role of TLS in chromosome pairing is hypothesized to involve binding of TLS to the nascent transcript rather than to DNA (Kuroda et al., 2000). Targeted disruption of the gene coding for heat shock protein Hsp70-2 abolished the first meiotic division of spermatocytes, as well as drove them to apoptosis, although female meiosis did not show any abnormalities. Hsp70-2 was shown to be associated with synaptonemal complexes. Synaptonemal complexes in the spermatocytes of *Hsp70-2*$^{-/-}$ mice assembled, but became abnormal by late prophase (Dix et al., 1996). Histone H2ax is essential for assembly of DNA repair complexes on radiation-damaged DNA, as shown by gene targeting. Its function is also indispensable for male meiosis, because *H2ax*$^{-/-}$ mice display pachytene arrest of spermatogenesis (Celeste et al., 2002). Morc (microrchidia) is a mutation caused by transgene insertion, deleting part of the *morc* gene. The function of this gene is not known, but it is essential for progression of spermatocytes past the zygotene stage (completion of synapsis, Watson et al., 1998). Protooncogene *A-myb* (alternative symbol *Mybl1*) is essential for male meiosis and for breast development in females, its deletion causes pachytene arrest of spermatogenesis (Toscani et al., 1997). Deficiency of synaptonemal complex protein Scp3 disrupts male meiosis, leading to infertility (Yuan et al., 2000). However, female meiosis proceeds, although the oocytes are aneuploid, causing embryonal death (Yuan et al. 2002). Murine Vasa homolog (Mvh) is an ATP-dependent RNA helicase indispensable for germ cell proliferation and meiosis. In knockout male mouse, zygotene spermatocytes undergo massive apoptosis (Tanaka et al., 2000). Eventually, many genes directly involved in DNA repair are necessary for completion of meiosis, e.g. *Pms2*, *Mlh1* (Edelmann et al., 1996), *Mlh3* (Lipkin et al., 2002), *Msh4* (Kneitz et al., 2000) and *Msh5* (de Vries et al., 1999, Edelmann et al., 1999). In case of *Pms2*, the meiosis defect is male-specific (Baker et al., 1995).

Sperm differentiation (spermiogenesis)

Spermiogenesis is the differentiation of spermatozoa from haploid spermatids. This process of extensive cellular remodelling with accompanying chromatin condensation presents many unique features and requires many male-specific gene products. The nucleus of the mature spermatozoon is the most compacted from all mammalian cell types, enabling it thus to swim towards the egg without any extra weight. Moreover, the maturing spermatozoa get rid of the extra cytoplasm in a process called cytoplasmic extrusion, leaving a "cytoplasmic droplet" (Cortadellas and Durfort, 1994). A diagram of a mature mammalian spermatozoon is shown in Fig. 3.

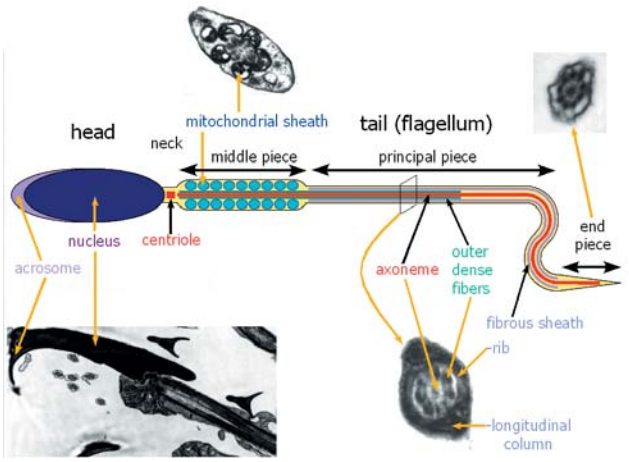

Fig. 3. Morphology of the mammalian spermatozoon. **Middle:** schematic drawing. **Upper part and lower part**: illustrative photographs of the Norway rat (*Rattus norvegicus*) spermatozoa taken by a transmission electron microscope (photographs courtesy of Z. Jirsová). **Top left:** transversal section through the midpiece of the sperm flagellum. **Top right:** transversal section of the endpiece of the sperm flagellum. **Bottom left:** longitudinal section of the sperm head and proximal part of the tail (midpiece). **Bottom right:** transversal section through the principal piece of the tail.

Chromatin is remodelled by replacement of the ubiquitous and testis-specific histones by transitional nuclear proteins and then by protamines. After histones dissociate from DNA, supercoiling is removed from DNA by inducing single-strand breaks by a yet unrecognized enzyme, DNA is then stabilized by transitional nuclear proteins (Tnp1 and Tnp2) to allow for single-strand break repair (Caron et al., 2001) until an eventually stable DNA-protamine (Prm1 and Prm2) complex forms (for review see e.g. Wouters-Tyrou et al., 1998.). Inactivation of *Tnp1* or *Tnp2* leads to subfertility, although the pathogenesis of the defect is slightly different. Deletion of *Tnp1* causes compensatory rise in Prm2 and Tnp2 levels, abnormal rod-shaped chromatin condensation, in some spermatozoa then blunted headtips and poor motility. There is a decrease in the number of litters and in the litter size by 70% (Yu et al., 2000). The absence of *Tnp2* prevents the completion of chromatin condensation. In spite of that, fertility is only slightly compromised (decrease in the litter size, Zhao et al., 2001). *Tnp2* is not conserved among mammals. In humans Tnp2 mRNA is expressed at a very low level and only 85% of histones are replaced by protamines. This resembles the mouse knockout, but it is a normal state for men (Schlüter et al., 1993). In contrast to Tnps, protamine deficiency has a much more profound impact on male infertility as haploinsuficiency (mutation of only one allele) of either *Prm1* or *Prm2* disrupts nuclear condensation and processing of Prm2 (which is synthetised as a precursor). Consequently, sperm function is impaired and further genetic transmission of both

mutant and normal protamine alleles is stopped (Cho et al., 2001, 2003). This is possible due to the fact that normally spermatids develop not as isolated cells, but all the spermatids originated in a single spermatogonial cell form syncytium. The normal function of the syncytia is sharing RNAs and proteins and it can compensate for haploinsufficiency of many alleles on autosomes (Braun et al., 1989).

Chromatin remodelling has one important consequence - genes in DNA free of histones cannot be transcribed and normal transcriptional regulation of gene activity is impossible. The beginning of spermiogenesis is characterized by a massive wave of transcriptional activity, giving rise to all necessary proteins for differentiation. The master switch of haploid-specific genes is believed to be CREMτ (cyclic AMP responsive element modulator) transcription factor. It is expressed specifically in round spermatids and acts through the CRE elements, which are present in many testicular haploid-specific genes including protamines and *Tnps*. Indeed, knockout of the *CREM* gene results in a complete block of spermiogenesis with resulting azoospermia (Blendy et al., 1996; Nantel et al., 1996). To activate trancription, CREM interacts with the central general transcription factor TFIID, in particular with TFIID subunit TBP (TATA-binding protein). TBP is expressed ubiquitously, but *TBP* mRNA is upregulated in pachytene spermatocytes and round spermatids (Schmidt and Schibler, 1995; Persengiev et al., 1996). In testis, however, there is an additional partner of CREM - TLF (TBP-like factor). TLF was recently reported to activate transcription from TATA-less promoters (Ohbayashi et al., 2003). The impact of an induced mutation of *TLF* on spermiogenesis is very similar to the mutation in *CREM* – a complete block in spermatid differentiation accompanied with spermatid apoptosis (Martianov et al. 2001, 2002; Zhang et al., 2001).

Nature solved the lack of transcriptional regulation in developing spermatids by simply shifting regulation toward translation - a stock of all mRNAs needed for later sperm differentiation and function made during the postmeiotic period is then sequestered as mRNP (messenger ribonucleoprotein particles) to chromatoid bodies. Multiple RNA-binding proteins interact with specific sequences at the 3'UTR or with the polyA tail and both repress translation and prevent mRNA degradation. These translationally repressed mRNAs must be again released at a specific time to fulfil their function (Braun, 2000; Steger, 2001). Phosphorylation of RNA-binding proteins appears to play a role in this process as was shown for some testicular RNA-binding proteins, e.g. Ybx2 (Msy2), TB-RBP and TLS/FUS. Phosphorylation is a relevant posttranslational modification of protamine 2, as proved by mutation of *Camk4* (coding for Ca^{2+}/calmodulin-dependent protein kinase IV), with impairment of spermatogenesis at the stage of elongating spermatids (Wu et al., 2000). Similar features were also presented by mutation in *Csnk2a2* (coding for casein kinase 2, α´ subunit), although with a specific defect in anterior head and acrosome development, expressing phenotypically as globozoospermia (Xu et al., 1999). The fine tuning of regulation of spermiogenesis by phosphorylation is demonstrated by the null mutation in *Styx* (coding for phosphoserine, -threonine and -tyrosine interaction protein) causing severe oligospermia due to disruption of spermiogenesis. Styx was implicated in the regulation of putative RNA-binding protein Crhsp-24 (Wishart and Dixon, 2002). The function of 3'UTR of translationally repressed RNAs is illustrated by the example of human Tnp2: the incomplete condensation of chromatin with low levels of Tnp2 (see above) can be explained by the absence of a conserved 8 nt motif in 3'UTR, leading to insufficient mRNA storage (Schlüter et al., 1993; Steger, 2001). However, in this case there is no functional consequence. On the other hand, mutations in RNA-binding proteins are frequently associated with male infertility. For example, in mice, targeted mutation of Protamine 1-binding protein (PRBP, gene *Tarbp2*) caused severe oligospermia. It was shown, however, that PRBP participates in Prm1 translation activation rather than repression (Zhong et al., 1999). Mutation of Miwi (a homologue of Drosophila *piwi, piwi* = P-element induced wimpy testis), a cytoplasmic RNA-binding protein, caused arrest at the round spermatid stage, thus resembling CREM knockout. Miwi was shown to bind several spermatid mRNAs, including Tnp1 and Ace (Deng and Lin, 2002).

RNA-binding proteins also back the aetiology of Y chromosome-associated infertility. More than 10% of azoospermic men have mutations (mostly microdeletions) of the long arm of the Y chromosome, of the so-called azoospermia factor locus (AZF). It is divided in three subloci (AZFa, AZFb and AZFc, for review, see Foresta et al., 2001). In case of AZFc, the infertility is associated with loss of function of *DAZ* (deleted in azoospermia), several-copy gene coding for an RNA-binding protein (Reijo et al., 1995; Saxena et al., 1996). Its autosomal homologues DAZL (Teng et al., 2002, for humans; Ruggiu et al., 1997, for mice) and Boule (Xu et al., 2001) were also implicated in the regulation of spermatogenesis. Although many RNA-binding proteins function postmeiotically, Dazl is expressed in spermatogonia and during the meiotic prophase and, furthermore, its mutation leads to the block of spermatogonia A to B transition (Schrans-Stassen et al., 2001). AZFb candidate is another multicopy gene *RBM* (RNA-binding motif protein, Elliott, 2000).

Extensive cellular remodelling also requires extensive protein breakdown. Degradation of various cellular proteins can serve not only to get rid of those proteins, which are simply no more useful, but can also help downregulate signalling pathways. The ubiquitin sys-

tem can fulfil both these functions. Various components of the ubiquitin system were indeed found mutated in both mouse models and humans with male infertility. In humans, mutations were revealed in *USP9Y* (coding for ubiquitin-specific protease 9, Y chromosome, in the AZFa region) in azoospermic men (Sun et al., 1999). Inactivation of ubiquitin-conjugating DNA repair enzyme Ube2b in mice causes (nonsyndromic) male infertility, with abnormal postmeiotic chromatin condensation (Roest et al., 1996; Baarends et al., 2003). But the ubiquitin system is not functionally confined to spermiogenesis, as inactivation of another ubiquitin-like DNA repair gene *mHR23B* causes (among other abnormalities) Sertoli cell-only syndrome (Ng et al., 2002). As well the ubiquitin ligase component Siah1a is required for meiosis in males - its inactivation causes block in metaphase I (Dickins et al., 2002).

Sperm motility

Sperm motility is required for normal fertilization. Therefore, it is no surprise that defects in flagellar structures lead to male infertility. A well-studied example represent mutations in axonemal dyneins, which cause primary ciliary dyskinesis or Kartagener syndrome with bronchiectasis, sinusitis, male infertility (due to immotile sperm) and in case of Kartagener syndome also *situs viscerum inversus* (e.g. Neesen et al., 2001; Ibanez-Tallon et al., 2002). Insertional inactivation of the murine *kisimo* locus is an example of defective flagellum assembly. Kisimo complexes with chaperonin-containing t-complex polypeptide 1e and this complex helps in assembly of cytoskeletal proteins. If there is a mutation, elongated spermatids have missing or abnormal flagella with disorganized microtubuli (Yanaka et al., 2000).

In mice bearing the targeted disruption of *CatSperm*, deficiency of cAMP-mediated Ca^{2+} influx to the principal portion of the tail also leads to the male infertility with severely decreased sperm motility, although the *CatSperm*$^{-/-}$ spermatozoa are able *in vitro* to fertilize eggs with removed zona pellucida (Ren et al., 2001).

Movement of a spermatozoon is a high energy-demanding process. ATP is produced by oxidative phosphorylation in the mitochondrial sheath. Deletion of Smcp (sperm mitochondria-associated cysteine-rich protein, synonym mitochondrial capsule selenoprotein, Mcsp), a structural protein associated with the keratinous capsules of sperm mitochondria, seriously affects sperm motility, despite normal sperm morphology (Nayernia et al., 2002). Another example illustrating the role of ATP in sperm motility is the targeted mutation of Vdac3 (mitochondrial voltage-dependent anion channel 3), a mitochondrial outer membrane protein involved in transport of ATP and other anions. Loss of Vdac3 not only disrupts sperm motility, but also leads to abnormalities of the flagellum, arising during the epididymal transit (Sampson et al., 2001).

Akap4 (A-kinase anchoring protein 4) is the most abundant protein in the fibrous sheath, a cytoskeletal structure present in the principal piece of the sperm flagellum. Akap4 act as a scaffold for protein complexes (including Protein kinase A) involved in regulation of flagellar function and its mutation results in flagellum distortion and loss of sperm motility (Miki et al., 2002).

Motility defects were implicated in the aetiology of t-haplotype-associated sterility and hybrid sterility. However, discussion of this rather complex phenomenon is beyond the scope of this review, a curious reader can consult several reviews, e.g. Olds-Clarke (1997) or Forejt (1996).

Acrosome formation

Acrosome is a cytoplasmic vesicle containing many enzymes that function during the penetration of zona pellucida. The major component is acrosin, a serine protease. However, acrosin mutation revealed that acrosin is dispensable for fertilization (Baba et al., 1994; Adham et al., 1997). On the other hand, some genes involved in acrosome formation did indeed influence fertility.

One example was the aforementioned casein kinase 2 (Csnk2a2), whose defect showed globozoospermia. Hrb (HIV-1 Rev-binding protein) is essential for formation of acrosome from proacrosomic vesicles. In wild-type spermatids, Hrb is associated with the cytosolic surface of the proacrosomic vesicles. Despite that proacrosomic vesicles form in *Hrb*-deficient mice, they cannot fuse together (Kang-Decker et al., 2001). The round shape of Hrb-deficient spermatozoa is similar to that observed in the casein kinase-deficient mice. Both conditions are not compatible with fertility.

The spermatozoa from mice deficient in GOPC (Golgi-associated PDZ- and coiled-coil motif-containing protein) showed complete lack of mature acrosomes and globozoospermia, because of defective fusion of the Golgi-derived transport vesicles to the acrosomal cap (Yao et al., 2002).

Deficiency of Dpl (Doppel, homologue of PrP^c prion protein, a GPI-anchored protein) causes subtotal male infertility. There are defects in development of elongating spermatids and defective acrosomal reaction - spermatozoa bind to zona pellucida, but are unable to penetrate (Behrens et al., 2002).

Sperm maturation and fertilization

Maturation of sperm in the epididymis is a necessary prerequisite for many sperm functions, including motility and sperm-egg interaction. Sperm maturation appears to be a multistep process. Several players were identified: fertilin β is a sperm membrane glycoprotein that mediates sperm-egg membrane binding (as a

heterodimer with fertilin α attaching to oolemma integrins). It is present on sperm as a precursor and proteolytically cleaved to its mature form in the corpus epididymis. Indeed, mice deficient in fertilin β are infertile due to a defect in sperm adhesion to zona pellucida (Cho et al., 1998). Another membrane protein cyritestin was also shown to be necessary to sperm-egg interaction in mice (Shamsadin et al., 1999). Both fertilin and cyritestin belong to the same family ADAM (a disintegrin and metalloproteinase domain). Thus, it is quite surprising that both cyritestin and fertilin α genes are nonfunctional in humans (Jury et al., 1997; Grzmil et al., 2001). Hypothetically, sperm ADAM proteins are functionally redundant in humans, but not in mice. Nevertheless, it was shown recently that loss of fertilin β or cyritestin in mice leads to loss of expression of multiple sperm proteins by an unknown mechanism. Conceivably, these proteins can be fairly more important for the sperm-egg interaction than fertilin or cyritestin (Nishimura et al., 2001).

Two genes acting genetically upstream of fertilin, during its posttranslational processing, were associated with infertility: mutation of calmegin, a membrane-bound chaperone of the endoplasmic reticulum, was shown to disrupt fertilin α/β heterodimerization with the infertility phenotype resembling fertilin β deficiency (Ikawa et al., 1997, 2001). Targeted mutation in Inpp5b (type II inositol polyphosphate 5-phosphatase) associates a defect in inositolpolyphosphate signalling in epididymal epithelium with defective fertilin β processing and male infertility (Hellsten et al., 2001). The effector protease cleaving fertilin is unknown, one hypothetical candidate could be PC4, testis-specific proprotein convertase, the importance of which was proved by gene targeting (Mbikay et al., 1997).

Deep insight into one aspect of sperm maturation was provided by targeted disruption of the protooncogene *c-ros*, an orphan membrane receptor with an intracellular tyrosine kinase domain, expressed in the caput epididymidis. Mice lacking *c-ros* lack prepubertal differentiation of the epididymal initial segment. The spermatozoa show flagellar angulation, which compromises motility, and the spermatozoa are unable to enter the oviduct. This defect is due to the impaired development of the volume regulatory mechanisms of spermatozoa, normally acquired in the initial segment, as the same flagellar angulation can be induced in normal caput epididymidis spermatozoa by incubation in media or in normal mature spermatozoa from cauda epididymidis by volume-sensitive ion channel blocker quinine. Moreover, the angulation can be released by demembranation (Yeung et al., 1999, 2000, 2002).

The *Ace* (angiotensin-converting enzyme) gene was found to code for a somatic protein (sACE) and a testicular specific protein (tACE). tACE is essential for mouse fertility, although sperm of mice homozygous for *Ace* mutation have normal sperm counts, motility and morphology. However, those spermatozoa were defective in transport into the oviduct and in binding zona pellucida, thus suggesting a capacitation and fertilization defect (Krege et al., 1995; Hagaman et al., 1998). Haploinsufficiency of *ApoB* (apolipoprotein B), another gene associated rather with cardiovascular phenotypes, also obviates binding of spermatozoa to zona pellucida, in addition of decreased sperm count and motility (Huang et al., 1996).

Conclusion

Although a large amount of genetic and biochemical data have accumulated, the picture of male genital system development and spermatogenesis is far from completion. Gene targeting proved recently to be the most fruitful method to associate genes with functions. However, different approaches can still show their potential for our understanding of this exceptionally complex biological network. Especially large-scale mutagenesis projects can lead to new findings, considering the advantage of mammalian draft genomes, which make it possible to link the phenotype to the gene in a more straightforward way. In addition, we can now more easily turn back and dissect many classical mutations – not only in mice but also in other mammals (e.g. the Norway rat has been recently sequenced and other organisms will follow).

It is clear that spermatogenesis is especially sensitive to the balance of many cellular processes, and mutations in many genes can cause infertility. This has major drawbacks for genetic counselling in humans. Testing for mutations in all genes known to affect fertility using current approaches is in principle technically possible, but financially unsupportable. Moreover, there are little data about the role of particular genes in human infertility (in fact, for most above-mentioned genes we know nothing). Therefore, it will be a major challenge to transfer data from animal models to man and thus to improve the diagnostics and therapy of infertile men.

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