

## The power of mouse genetics to study spermatogenesis

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## Abstract

Approximately 80 million people suffer from infertility worldwide, and nearly half of all infertility cases are attributed to a male factor (Matzuk and Lamb, 2002). Therefore, progress in reproductive genetics becomes crucial for future diagnosis and treatment of infertility. In recent years, enormous progress has been made in this field. More than 400 mutant mouse models with specific reproductive abnormalities have been produced, and numerous human association studies have been discovered. However, the translation of basic science findings to clinical practice remains protracted, with only modest progress in the application of novel findings to clinical genetic testing and cures. To date, the most significant findings in male infertility remain numerical and structural chromosomal abnormalities and Y-chromosome microdeletions in infertile men. Thus, we anticipate that future genetic investigations will focus on infertile men with a normal somatic karyotype, but with various spermatozoal defects like insufficient production of spermatozoa (oligozoospermia), inadequate motility (asthenozoospermia), abnormal morphology (teratozoospermia), or combinations of these defects. Ultimately, basic advances in mammalian non-human reproduction will translate to clinical advances in human reproduction and testing infertile humans, thereby helping to improve diagnostics and health care for infertile patients.

**Keywords:** *reproduction, spermatogenesis, mouse model, human male infertility*

## Introduction

Over the last 18 years, the Matzuk laboratory has been focused on deciphering the processes of normal and abnormal reproduction in mammals. Because fertility cannot be evaluated in a test tube, our laboratory has been using knockout and knockin transgenic mouse models to dissect mammalian TGF $\beta$  superfamily signaling pathways, to study ovarian and testicular cancers, to define the functions of known and novel germ cell-expressed genes, and to identify unique germ cell targets for contraception. To understand the role of specific proteins *in vivo*, we have produced over 70 transgenic mouse models, including mice lacking TGF $\beta$  superfamily ligands, binding proteins, and receptors, downstream signaling proteins, and their nuclear targets [e.g., growth differentiation factor 9 (GDF9), bone morphogenic protein 15 (BMP15), inhibins (INHA), activins (INHBA), and activin receptor type 2A (ACVR2A), SMADs, and pentraxin 3]. Our studies identified inhibin as the first secreted tumor suppressor factor that inhibits testicular and ovarian tumor development (Matzuk, et al., 1992). Our research also showed that ACVR2A is a key regulator of follicle stimulating hormone (FSH) synthesis and secretion from the anterior pituitary gland, and in the absence of ACVR2A, FSH levels are

suppressed, resulting in a hypogonadal phenotype (Matzuk, et al., 1995). Many mouse models have also been used to study female reproduction and/or genes function in both females and males (reviewed in Matzuk and Lamb, 2002; Matzuk and Lamb, 2008).

### **Genes important in the male germline**

To study the genes and mechanisms that direct male reproduction, we have generated mutant mice that lack key proteins at almost every step of spermatogenesis (Table 1). To identify such critical proteins, we utilized functional genomic approaches, including analysis of evolutionary conservation, protein domain homology, and genomic synteny between mouse and human genomes. Our work effectively led to the discovery of essential genes at key stages of spermatogenesis. We identified several genes that show defects prior to as well as after meiotic division. Our studies with Dr. Debra Wolgemuth's group demonstrated that mutant mice, homozygous for a deletion of cyclin A1 gene, show spermatogenesis arrest prior to the first meiotic division (Liu, et al., 1998). Mutant *Ccn1* null males present with increased germ cell apoptosis, desynapsis abnormalities, and reduction of Cdc2 kinase activation at the end of meiotic prophase, leading to meiotic arrest. Therefore, we conclude that cyclin A1 is essential for the mitosis –meiosis transition during spermatogenesis.

Other collaborative studies with Drs. Norman Hecht and Richard Schultz led to the discovery of a key role of the germ-cell-specific protein from the Y-box family of DNA-/RNA-binding proteins, namely MSY2, also known as YBX2 (Yang, et al., 2005). MSY2 was proposed to be a coactivator of transcription in postmeiotic spermatids by stabilizing the storage of parental mRNAs in the cytoplasm. Mice lacking *Msy2* demonstrate disrupted spermatogenesis in postmeiotic germ cells with many misshapen and multinucleated spermatids, resulting in azoospermia. While increased apoptosis was observed, the major defect was due to large reductions of the mRNA levels in postmeiotic male germ-cell transcripts. Interestingly, defects in *Msy2* null female mice were observed at later stages of oogenesis in adults and include increased oocyte loss, anovulation, and multiple morphological defects of oocytes and follicles. Thus, *Msy2* represents one of a small number of germ-cell-specific genes whose deletion leads to the disruption of both spermatogenesis and oogenesis.

Considerable progress has been made in investigation of several genes involved in the later stages of spermatogenesis. To study proteins responsible for normal morphology of the flagella and acrosome in mature spermatozoa, we have created a number of mutant mice, including knockout models of tektin 3 (TEKT3), tektin 4 (TEKT4), and zona pellucida binding proteins 1 (ZPBP1) and 2 (ZPBP2) (Table 1). We demonstrated that homozygous deletions of

these genes causes ultrastructural defects in the spermatozoal flagella and sperm motility defects that in turn leads to subfertility and infertility (Roy, et al., 2007; Roy, et al., 2009). Sperm that are null for *Tekt4* exhibit severely reduced forward progression and uncoordinated movement of the flagellum. Transmission electron microscopy revealed that spermatozoal flagellar ultrastructure is mainly unaltered. However, the ineffective flagellar strokes in *Tekt4*-null sperm lead to nearly 10-fold higher consumption of intracellular ATP, resulting in rapid loss of progressive motility. Although male mice lacking TEKT3 are fertile, they similarly produce sperm with reduced motility and forward progression, and increased flagellar structural bending defects. Furthermore, male mice null for both TEKT3 and TEKT4 show subfertility, suggesting partial nonredundant roles for these two proteins in sperm physiology. Thus, tektins are necessary for the proper coordinated beating of the sperm flagellum and forward movement and are potential candidate genes for nonsyndromic asthenozoospermia in humans.

To understand the final stages of spermatogenesis and fertilization, we constructed mouse models with a knockout of two novel proteins expressed in the acrosome of mature spermatozoa, zona pellucida binding protein 1 and 2 (ZPBP1 and ZPBP2, respectively) (Lin, et al., 2007). Mutant male mice lacking ZPBP1, ZPBP2, or both demonstrate abnormal acrosome formation due to various morphological defects of the sperm head. Male mice null for *Zpbp1* were sterile and present with characteristic abnormal round-headed sperm morphology (similar to globozoospermia) and no forward sperm motility. The abnormal phenotype of null *Zpbp1* is probably caused by improper acrosome compaction, ultrastructural acrosome fragmentation, and disruption of the Sertoli-spermatid junctions, resulting in the inability of oocyte fertilization. Males missing ZPBP2 were subfertile, and produced dysmorphic sperm with a reduced ability to penetrate the zona pellucida. Further studies of mutant *Zpbp2* mice demonstrated that aberrant acrosomal membrane invaginations resulted in abnormal morphology. While double mutant mice heterozygous for the *Zpbp1* mutation and homozygous for the mutant *Zpbp2* allele have more severe morphological defects and reduced fecundity, mice double homozygous mutant were sterile, similar to the *Zpbp1* null mice. These results suggested that the two paralogous genes play important structural roles during spermiogenesis and likely cooperate in sperm-egg interaction.

### **The immortal male germline.**

Spermatogenesis is a continuous process originating from spermatogonial stem cells (SSCs). Because SSCs can self-renew and maintain in an undifferentiated state, sperm are produced for the lifetime of an adult male. Self-renewal of SSCs is strictly regulated by extrinsic stimuli as well as intrinsic signal pathways and transcriptional mechanisms. Extrinsic factors that

regulate self-renewal and differentiation of SSCs have been analyzed. GDNF (glial cell line derived neurotrophic factor) is an essential growth factor for SSCs to self-renew and is produced by Sertoli cells. GDNF stimulates SSCs to self-renew by signaling through a heterodimer of the tyrosine kinase, RET, and GFR $\alpha$ 1 (Figure 1) (GDNF receptor family alpha 1) (Jing, et al., 1996; Treanor, et al., 1996). In testes heterozygous for GDNF mutation, spermatogenesis is impaired by defects of self-renewal of SSCs. In contrast, there is an abnormal increase of SSCs in testes with overexpression of GDNF (Meng, et al., 2000). When normal SSCs are transplanted into GDNF-deficient testes, SSCs cannot be maintained in an undifferentiated state and disappear from the transplanted testes (Naughton, et al., 2006). Basic fibroblast growth factor (bFGF) can enhance self-renewal of SSCs together with GDNF *in vitro*, although SSCs cannot self-renew with bFGF alone (Kubota, et al., 2004). Recently, colony stimulating factor 1 (CSF1) was shown to enhance self-renewal activity of SSCs in the presence of GDNF and bFGF *in vitro* (Oatley, et al., 2009). On the other hand, SCF (stem cell factor, also known as Kit ligand; KITL) is an essential cytokine for spermatogonia to differentiate. In testes of mice mutant for KITL, undifferentiated spermatogonia accumulate due to a failure in differentiation (Ohta, et al., 2000). Transplantation experiments reveal that the *Kit* tyrosine kinase receptor, the receptor of KITL, is absent in SSCs and present in differentiated spermatogonia (Shinohara, et al., 2000).

Extrinsic signals stimulate downstream signal transduction pathways as well as transcription machineries. The most important transcriptional regulator of SSCs is PLZF (promyelocytic leukemia zinc finger; also known as ZFP145 and ZBTB16) (Figure 1) (Buaas, et al., 2004; Costoya, et al., 2004). PLZF is a POK (POZ/BTB and Kruppel) type transcription suppressor and regulates transcription by negative cooperation with the co-repressors and HDAC (Barna, et al., 2002). The expression of PLZF is restricted to spermatogonial stem cells and early spermatogonia. Mutations in the *Plzf* locus lead to male infertility by progressive loss of germ cells. In PLZF mutant testes, spermatogonia exist at birth and spermatozoa are produced in the first few rounds of spermatogenesis, but ultimately all germ cells disappear. PLZF mutant SSCs fail to colonize when transplanted in germ cell depleted testes. However, environmental somatic cells in PLZF null testes can support self-renewal of wild type SSCs. PLZF may regulate genes related to differentiation through epigenetic regulations because PLZF interacts with polycomb group proteins and HDAC (Barna, et al., 2002).

SSCs express OCT4 (Octomer-4, also known as POU domain class 5 transcription factor 1, Pou5f1), which is essential for cell pluripotency and cell survival of embryonic stem (ES) cells and primordial germ cells (PGCs). OCT4 is a transcription factor specifically expressed in ES cells, preimplantation embryo, and germ cells (Pesce, et al., 1998). In testes, OCT4 localizes to

spermatogonia and is used as a marker of SSCs. Recently, OCT4 was shown to be important for SSC maintenance by knockdown experiments (Dann, et al., 2008). OCT4 knockdown SSCs can not colonize in both culture and transplanted testes. Because PLZF is not affected by knockdown of OCT4, OCT4 and PLZF function in different pathways to maintain the SSCs in an undifferentiated state.

A basic helix loop helix (bHLH) transcription factor, neurogenin 3 (NGN3), localizes to only early spermatogonia (between A<sub>s</sub> and A<sub>al</sub>) in testes (Yoshida, et al., 2004). NGN3 is a useful marker of SSCs, because NGN3 starts to express in spermatogonia after birth, but is not expressed in PGC. At present, the role of NGN3 in SSCs remains unclear. Germ-cell specific bHLH transcription factor SOHLH1 (testis and ovary-specific bHLH transcriptional factor 1) and SOHLH2 are expressed in type A spermatogonia (Ballow, et al., 2006a; Ballow, et al., 2006b). In testes lacking SOHLH1 or SOHLH2, differentiation of spermatogonia is inhibited and only type A spermatogonia remain (Ballow, et al., 2006a; Hao, et al., 2008; Toyoda, et al., 2009). SOHLH1 and SOHLH2 can form heterodimers and control transcription in spermatogonia. Interestingly, expression of NGN3 is decreased in SOHLH1 null testes, which are enriched in undifferentiated spermatogonia, suggesting that SOHLH1 might regulate transcription of NGN3 (Ballow, et al., 2006a).

Oatley et al., identified genes regulated by GDNF using an *in vitro* culture system. Among these genes, the gene that showed the most dramatic change in expression is BCL6B (B cell CLL/lymphoma 6; also known as BAZF) (Oatley, et al., 2006). BCL6B, like PLZF, is a POK family transcriptional repressor. SSCs, in which BCL6b was down-regulated by siRNA, were decreased in both number and size of colonies. Furthermore, colony formation was decreased when BCL6B knockdown SSCs were transplanted into recipient testes. Although BCL6B null mice are fertile, about 24% of seminiferous tubules degenerated and/or showed defects in spermatogenesis. BCL6B is suggested to be an important molecule in self-renewal of SSCs and is a gene that connects the GDNF pathway and SSC self-renewal. ETV5 (Ets variant 5, also known as ERM) and LHX1 (Lim homeobox 1) were other transcription factors regulated by GDNF and shown to be important for SSC maintenance *in vitro* (Oatley, et al., 2007).

Whereas PLZF and BCL6B are essential for SSC self-renewal and regulate transcription negatively, TAF4B (TATA box-binding protein (TBP) associated factor 4b), was shown to be an important protein in SSC self-renewal that regulates transcription positively (Figure 1) (Falender, et al., 2005). TAF4B is a germ cell-specific component of the TFIID transcription complex. TAF4B null mice are fertile at a young age but become sterile after 11 weeks of age (Freiman, et al., 2001). TAF4B localizes to spermatogonia and spermatids but is not expressed in meiotic cells

or somatic cells in testis. Although there were no defects in neonatal TAF4B null testes, proliferation defects and decreased expression of SSC marker genes began to appear at 3 days of age. Degeneration of spermatogenesis became more severe by 8 weeks of age, and almost all tubules lacked germ cells by 8 months of age. Because spermatogenesis was recovered by transplantation of normal SSCs into TAF4B null testes, the surrounding environment could support spermatogenesis and mutant SSCs lacked self-renewal activity. Thus, TAF4B likely activates SSC genes related to self-renewal, proliferation, and differentiation.

Recently, somatic cells were shown to be converted into pluripotent state by induction of four transcription factors only (Park, et al., 2008b; Takahashi, et al., 2007; Takahashi and Yamanaka, 2006; Wernig, et al., 2007; Yu, et al., 2007). Progress with induced pluripotent stem (iPS) cells is a major topic in regenerative medicine because iPS cells allow us to exclude various conflicts about moral issue and immunotolerance (Park, et al., 2008a). On the other hand, SSCs can be converted into a pluripotent state in culture *in vitro* (Conrad, et al., 2008; Guan, et al., 2006; Kanatsu-Shinohara, et al., 2004). Interestingly, SSCs express all of the genes that are required for induction of pluripotency, *i.e.* *Oct4*, *Sox2*, *Klf4*, *Myc*, *Lin28* except for *Nanog* (Kanatsu-Shinohara, et al., 2005; Oatley, et al., 2006). Constitutive expression of NANOG enables autonomous self-renewal of ES cells, whereas lack of NANOG leads to early embryonic lethality (Chambers, et al., 2003; Mitsui, et al., 2003). In addition, NANOG is important for PGC maturation during embryonic development (Chambers, et al., 2007).

In summary, maturation of SSC from PGC is associated with inactivation of *Nanog* and induction of pluripotency in SSC requires reactivation of *Nanog*. There are many similarities between SSCs and pluripotent stem cells. However, despite accumulating information about the transcriptional network in SSC self-renewal after development of transplantation technology and generation of germ cell specific knockout mice, complete knowledge is still limited. The understanding of regulatory mechanisms of SSC self-renewal may provide unique insights into not only self-renewal of other stem cells, but also nuclear reprogramming, because SSCs share many transcription factors with ES cells and is the only stem cell which can transfer genetic information to next generation.

### **Discovery of the first germ cell intercellular bridge protein (TEX14)**

Cytokinesis, which separates a mother cell into two daughter cells, occurs in all multicellular organisms. In somatic cells, abscission of the midbody is the last step of cytokinesis and separates completely the two daughter cells. In contrast to somatic cells, abscission is blocked in differentiating germ cells and results in germ cells interconnected by a stable structure called

the intercellular bridge (Dym and Fawcett, 1971; Huckins, 1978). In 1955, Fawcett used electron microscopy of cat spermatids to convincingly define the intercellular bridge as a stable cytoplasmic channel connecting the germ cells, and within a short period, intercellular bridges were identified to be conserved in a wide variety of species including man (Fawcett, et al., 1959). The conservation among various species and the interconnection of as many as 650 germ cells in a syncytium suggests that intercellular bridges have key roles in multiple stages of spermatogenesis in mammals. The reason why intercellular bridges connect mammalian germ cells is still unknown, although intercellular bridges are believed to function in synchronization of germ cells as well as permitting the passage of organelles and molecules between germ cells (especially important post-meiotically in haploid spermatids) (Braun, et al., 1989; Ventela, et al., 2003).

We identified a testis expressed 14 (*Tex14*) as a germ cell-specific gene by using *in silico* subtraction (Wu, et al., 2003a). In parallel, Wang *et al* identified a portion of TEX14 as a spermatogonial-enriched gene protein (Wang, et al., 2001). We showed that full length TEX14 is a 162.5 kD protein with three ankyrin repeats, a protein kinase-like domain, and a leucine zipper dimerization motif (Wu, et al., 2003a). TEX14 localizes to male and female germ cell intercellular bridges (Greenbaum, et al., 2007) (Figure 2A). Knockout of *Tex14* disrupts intercellular bridges in male and female mice, but only arrests meiosis in spermatocytes. As a result, null *Tex14* mice present with disrupted spermatogenesis and sterility only in males (Greenbaum, et al., 2006). Our studies indicate that TEX14 is the first essential protein of the germ cell intercellular bridge and demonstrate that intercellular bridges are essential for fertility and spermatogenesis.

In testes, the A type single ( $A_s$ ) spermatogonium is the stem cell for spermatogenesis and is the only post- natal male germ cell without intercellular bridges (Figure 2B). Intercellular bridges are typically observed in all differentiating spermatogonia after the  $A_s$  cell differentiates to form A paired ( $A_{pr}$ ) spermatogonia and through the haploid germ cell stage. TEX14-positive intercellular bridges interconnect  $A_{pr}$  spermatogonia cells and continue to interconnect male germ cells up through formation of mature spermatozoa (Greenbaum, et al., 2006). Thus, the intercellular bridges and TEX14 are specific markers for the differentiation process and initiation of the spermatogenesis pathway.

We developed a biochemical method to enrich intercellular bridges from mouse testes, using TEX14 as a marker protein (Greenbaum, et al., 2007), and performed proteomic analysis to identify 19 cytokinesis proteins, including Mitotic Kinesin-Like Protein 1 (*MKLPI*) and Male germ cell Rac GTPase-activating protein 1 (*MgcRacGap1*) that are necessary for cytokinesis and

also components of the intercellular bridge (Matuliene and Kuriyama, 2004; Van de Putte, et al., 2001). TEX14 converts these midbody proteins into stable intercellular bridge components (Greenbaum, et al., 2007). The mechanisms by which these proteins come together to prevent the completion of cytokinesis and form a stable intercellular bridge and the precise functions of the intercellular bridge throughout spermatogenesis remain elusive. We are studying the interactions among these bridges and cytokinesis proteins, and how a stable intercellular bridge forms. We believe that TEX14 and other intercellular bridge proteins are unique targets for male contraception, and small molecule contraceptives that disrupt the intercellular bridge have important clinical applications. Furthermore, defining how intercellular bridges function in germ cell biology and spermatogenesis is the long-range objective of our basic research program.

### **Translation to practice**

Although findings in knockout mouse models are very important for the dissection of mammalian reproductive pathways, the discovery of genetic defects in humans, translation of these findings to clinical diagnoses, and providing potential targets for therapeutics is an ultimate goal. While the investigation of mutant animal models have been extremely useful in studying simple diseases, study of genetic defects causing human infertility is complicated by the enormous number of genes involved and the phenotypic heterogeneity of infertility (Matzuk and Lamb, 2008).

One noteworthy example of the translational research in the Matzuk laboratory is the study of human KLHL10 in infertile oligozoospermic patients. Our earlier mouse knockout studies have shown that the germ cell-specific *kelch-like 10* (KLHL10) protein is involved in protein ubiquitination, is critical for maturation process of mouse spermatozoa, and is one of the essential proteins for survival of postmeiotic spermatozoa (Wang, et al., 2006; Yan, et al., 2004). Male mice heterozygous for the *Klh10* gene deletion demonstrate a failure of spermatozoal maturation, resulting in oligozoospermia. These results suggested to us that a mutated *KLHL10* gene in human males might have dominant effect as well. Consequently, we devised a strategy to determine whether mutations in *KLHL10* might be associated with human oligozoospermia and male infertility.

Although transcription is repressed in mature sperm, many postmeiotically-expressed mRNAs and microRNAs persist in human ejaculate spermatozoa and are even delivered to the oocyte (reviewed by (Krawetz, 2005). Accordingly, detection of specific RNAs would provide an imprint of testicular gene expression. Therefore, we developed a non-invasive, high-throughput

screening technique that effectively utilizes reverse transcription–polymerase chain reaction (RT–PCR) of germline mRNAs obtained from human ejaculate samples of oligozoospermic and normozoospermic infertile men (Yatsenko, et al., 2006). We employed this approach to study *KLHL10* mutations in oligozoospermic infertile patients and identified 7 of 550 patients (1.2%) with missense and/or splicing mutations in *KLHL10* (Figure 3)(Yatsenko, et al., 2006). Importantly, we confirmed the pathogenic effect of identified *KLHL10* mutations. We demonstrated abnormal gene splicing via RT-PCR, and using an *in vitro* yeast 2-hybrid functional assay, showed abnormal protein dimerization of mutant proteins with the various missense mutations. These results support our initial hypothesis that mutations in human *KLHL10* have a dominant effect on human spermatogenesis.

Recently, more encouraging discoveries toward nonsyndromic male infertility have been reported. Dr. Ray’s group reported that a novel testis-expressed gene, aurora kinase C (*AURKC*), previously shown to play a role in meiotic division in mice (Tang, et al., 2006), is responsible for male infertility (Dieterich, et al., 2007). Male patients harboring homozygous single-nucleotide deletion in the *AURKC* gene presented with large-headed multiflagellar polyploid spermatozoa that showed nearly 100% morphologically abnormal spermatozoa with oversized irregular heads, abnormal midpiece and acrosome, and up to six flagella. In another report, the spermatogenesis-specific gene *SPATA16* was shown to be responsible for globozoospermia in 3 related male infertile patients (Dam, et al., 2007). This study described three affected brothers from a consanguineous family, in which each brother is homozygous for mutation in the *SPATA16*. However, a follow-up study of unrelated patients with complete or partial globozoospermia failed to identify *SPATA16* mutation, highlighting the common difficulty in identifying genetic mutations in such a complex etiology as male infertility.

### **Implications for the future**

Current discoveries of crucial genetic and molecular aspects of mammalian reproductive pathways is a fundamental step toward clinical management as well as effective therapy. Therefore, more human genetic studies of male infertility and novel effective non-invasive screening detection methods in the germline are needed for future progress. Ultimately, recognized genetic alterations associated with human spermatogenesis defects should be applied for various diagnostic applications in the fertility clinic.

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## Figure Legends

### Figure 1. Essential molecules regulating self-renewal of spermatogonial stem cell (SSC).

Glial cell line-derived neurotrophic factor (GDNF) stimulates SSC self-renewal, and basic fibroblast growth factor (bFGF) and colony stimulating factor 1 (CSF1) enhances this activity. Transcriptional repressor, encoded by *Bcl6b* (B cell CLL/lymphoma 6, member b) and the transcription factors, *Etv5* (Ets variant 5) and *Lhx1* (LIM homeobox 1), are activated by the GDNF stimuli. Independently from GDNF signaling, the transcriptional repressor PLZF (promyelocytic leukemia zinc finger; also known as ZBTB16 and ZFP145) and transcription factor TAF4b (TATA box binding protein (TBP)-associated factor) regulate SSC self-renewal.

**Figure 2. A. The localization of TEX14 in the testis.** Immunofluorescence using anti- TEX14 antibody shows that TEX14 localizes to intercellular bridge. (Red, TEX14; Blue, DAPI) The tissue is a six-weeks-old male mouse testis. **B. A summary of intercellular bridges.** In testes, the A type single ( $A_s$ ) spermatogonium is the stem cell. The daughter cells of  $A_s$  spermatogonia are self-renewed  $A_s$  spermatogonia without intercellular bridges and  $A_{pr}$  spermatogonia interconnecting by intercellular bridges. All differentiating germ cells, which are present after the  $A_s$  cell differentiates to form an A paired ( $A_{pr}$ ) spermatogonia and through the haploid germ cell stages, are connected by intercellular bridges. TEX14 localizes initially to the midbody during cytokinesis and its presence results in stable intercellular bridges in differentiating germ cells. TEX14 is a specific marker for intercellular bridges, the differentiation process, and the initiation of the spermatogenesis pathway. Details of the model are as follows: Black arrow, dividing cell; Orange arrow, maturing cell; Red ring, midbody and TEX14; Purple bar, intercellular bridge; Turquoise blue and Bright green circles, cytoplasm of stem cell and differentiating germ cell; Blue, maturing nucleus during spermiogenesis.

**Figure 3.** Distribution of identified *KLHL10* mutations among categories of sperm concentration in infertile men. Three major categories are shown in upper part of the figure, severe (red) and moderate (pink) oligozoospermia, and normozoospermia (green). The lower part shows all of the *KLHL10* identified mutations in respect to their corresponding position on the KLHL10 protein. Mutations present above the protein are only present in oligozoospermic patients, whereas those depicted below to the protein are also observed in normozoospermic patients (believed to be rare polymorphisms). Splicing\* - denotes splicing *KLHL10* mutation, c.1302+121\_124del4bp. Known functional domains of the KLHL10 protein are shown by different colors and named after respective domains, as BTB, BACK, and K1- K6 (Kelch motives 1-6).

**Table 1.** Mouse models with single gene mutations and reproductive defects. Mutations were generated by using embryonic stem cell knockout technology and were created and/or phenotypically characterized by the Matzuk laboratory. Only the first reference is cited.

	<i>Mouse Gene - target</i>	<i>Sex affected</i>	<i>Reproductive defect</i>	<i>Mouse Phenotype</i>	<i>Reference</i>	<i>Chr</i>
1.	Activin receptor-type IIA ( <i>Acvr2a</i> )	Both	Antral follicle block (F); small testes, delayed initiation of copulation, reduced mount, and intromission frequencies, and increased mount, intromission, and ejaculation latencies (M)	Infertile (F) Subfertile (M)	(Ma, et al., 2005; Matzuk, et al., 1995)	2
2.	Anti-Müllerian hormone receptor ( <i>Amhr2</i> )	Male	Uteri development causes obstruction	Secondary infertility	(Mishina, et al., 1996)	15
3.	Bone morphogenetic protein 15 ( <i>Bmp15</i> )	Female	Defects in cumulus-oocyte complex (COC) formation and ovulation	Subfertile	(Yan, et al., 2001)	X
4.	Cyclin A1 ( <i>Ccna1</i> )	Male	Block in spermatogenesis before the first meiotic division; haploinsufficiency observed in 129S6/SvEv inbred background	Infertile	(Liu, et al., 1998)	3
5.	Folliculogenesis specific basic helix-loop-helix ( <i>Figla</i> )	Female	No primordial follicles develop at birth and oocytes die	Infertile	(Soyal, et al., 2000)	6
6.	Follistatin ( <i>Fst</i> )	Female	Presence of male-specific coelomic vessel in ovary and loss of oocytes	Postnatal lethal	(Yao, et al., 2004)	13
7.	Follicle stimulating hormone $\beta$ ( <i>Fshb</i> )	Both	Pre-antral block in folliculogenesis (F); decreased testis size (M)	Infertile (F)	(Kumar, et al., 1997)	2
8.	Germ cell protein w Ankyrin repeats, Sterile and leucine Zipper motifs ( <i>Gasz</i> )	Male	Disrupted structure of intermitochondrial cement, zygotene-pachytene spermatocytes block	Infertile	(Ma, et al., 2009)	6
9.	Growth differentiation factor-9 ( <i>Gdf9</i> )	Female	Folliculogenesis arrest at the one-layer follicle stage	Infertile	(Dong, et al., 1996; Elvin, et al., 1999)	11
10.	Inhibin $\alpha$ ( <i>Inha</i> )	Both	Granulosa/Sertoli tumors, gonadotropin hormone-dependent	Infertile (F) Secondary infertility (M)	(Matzuk, et al., 1992)	1
11.	Inhibin/activin $\beta$ B ( <i>Inh<math>\beta</math>b</i> )	Female	Delivery and nursing defects	Subfertile	(Vassalli, et al., 1994)	1
12.	Kelch-like 10 ( <i>Klhl10</i> )	Male	Dysmorphic spermatozoa and impaired motility	Infertile	(Yan, et al., 2004)	11

(Hetero)						
13.	Luteinizing hormone $\beta$ ( <i>Lhb</i> )	Both	Failure of spermatogenesis (M) and folliculogenesis (F)	Infertile	(Ma, et al., 2004)	7
14.	LIM homeobox protein 8 ( <i>Lhx8</i> )	Female	Primordial to primary follicle block and oocyte loss	Infertile	(Pangas, et al., 2006)	3
15.	NOBOX oogenesis homeobox ( <i>Nobox</i> )	Female	Primordial to primary follicle block and oocyte loss	Infertile	(Rajkovic, et al., 2004)	6
16.	Nucleophosmin/nucleoplasmin 2 ( <i>Npm2</i> )	Female	Maternal effect gene; partial block at 1 cell to 2 cell embryo stage	Subfertile	(Burns, et al., 2003)	14
17.	2'-5' Oligoadenylate synthetase 1D ( <i>Oas1d</i> )	Female	Defects in folliculogenesis and ovulatory efficiency	Subfertile	(Yan, et al., 2005)	5
18.	Oxytocin ( <i>Oxt</i> )	Both	Maternal nursing defects (F); social memory defect and increased aggression (M)	Fertile	(Nishimori, et al., 1996)	19
19.	Oxytocin receptor ( <i>Oxtr</i> )	Both	Maternal nursing defects (F); social memory defect and increased aggression (M)	Fertile	(Takayanagi, et al., 2005)	6
20.	Platelet activating factor acetylhydrolase, isoform 1b, beta1 subunit ( <i>Pafah1b1; Lis1</i> )	Male	Gene trap insertion; spermiogenic block, spermatids fail to form correct acrosomes and nuclei are distorted in size and shape	Infertile	(Yan, et al., 2003)	11
21.	Sma/MAD homolog 5 ( <i>Smad5</i> )	Both	Developing embryos lose primordial germ cells	Lethal	(Chang and Matzuk, 2001)	13
22.	Superoxide dismutase 1 ( <i>Sod1</i> )	Female	Folliculogenesis defect, failure to maintain pregnancy	Subfertile	(Ho, et al., 1998; Matzuk, et al., 1998)	16
23.	Spermatogenesis and oogenesis-specific basic helix-loop-helix 1 ( <i>Sohlh1</i> )	Both	Spermatogenesis block at meiotic entry (M); primordial to primary follicle block and oocyte loss (F)	Infertile	(Pangas, et al., 2006)	2
24.	Tektin 3 ( <i>Tekt3</i> )	Male	Sperm motility defects	Fertile	(Roy, et al., 2009)	11
25.	Tektin 4 ( <i>Tekt4</i> )	Male	Sperm motility defects and ultrastructural defects in flagellum	Subfertile	(Roy, et al., 2007)	17
26.	Testis expressed gene 14 ( <i>Tex14</i> )	Male	Absence of germ cell intercellular bridges, meiotic defects and block at pachytene stage	Infertile	(Greenbaum, et al., 2006)	11
27.	Y box protein 2 ( <i>Ybx2; Msy2</i> )	Both	Elongating spermatid arrest (M); follicular atresia, oocyte loss, anovulation (F)	Infertile	(Yang, et al., 2005)	11

28.	Zygote arrest 1 ( <i>Zar1</i> )	Female	Maternal effect gene, block at 1 cell to 2 cell embryo stage	Infertile	(Wu, et al., 2003b)	5
29.	Zona pellucida binding protein ( <i>Zpbp</i> )	Male	Abnormal acrosome and globozoospermia	Infertile	(Lin, et al., 2007)	11
30.	Zona pellucida binding protein 2 ( <i>Zpbp2</i> )	Male	Abnormal acrosome formation and sperm head defects	Subfertile	(Lin, et al., 2007)	11

**Abbreviations:** Chr, mouse chromosome location of the gene; M, male; F, female; Hetero, haploinsufficiency phenotype observed in heterozygous mice. Definitions: asthenozoospermia - reduced sperm motility; azoospermia - no sperm; globozoospermia – rounded sperm head; oligozoospermia - low sperm counts; teratozoospermia - abnormal sperm head morphology.

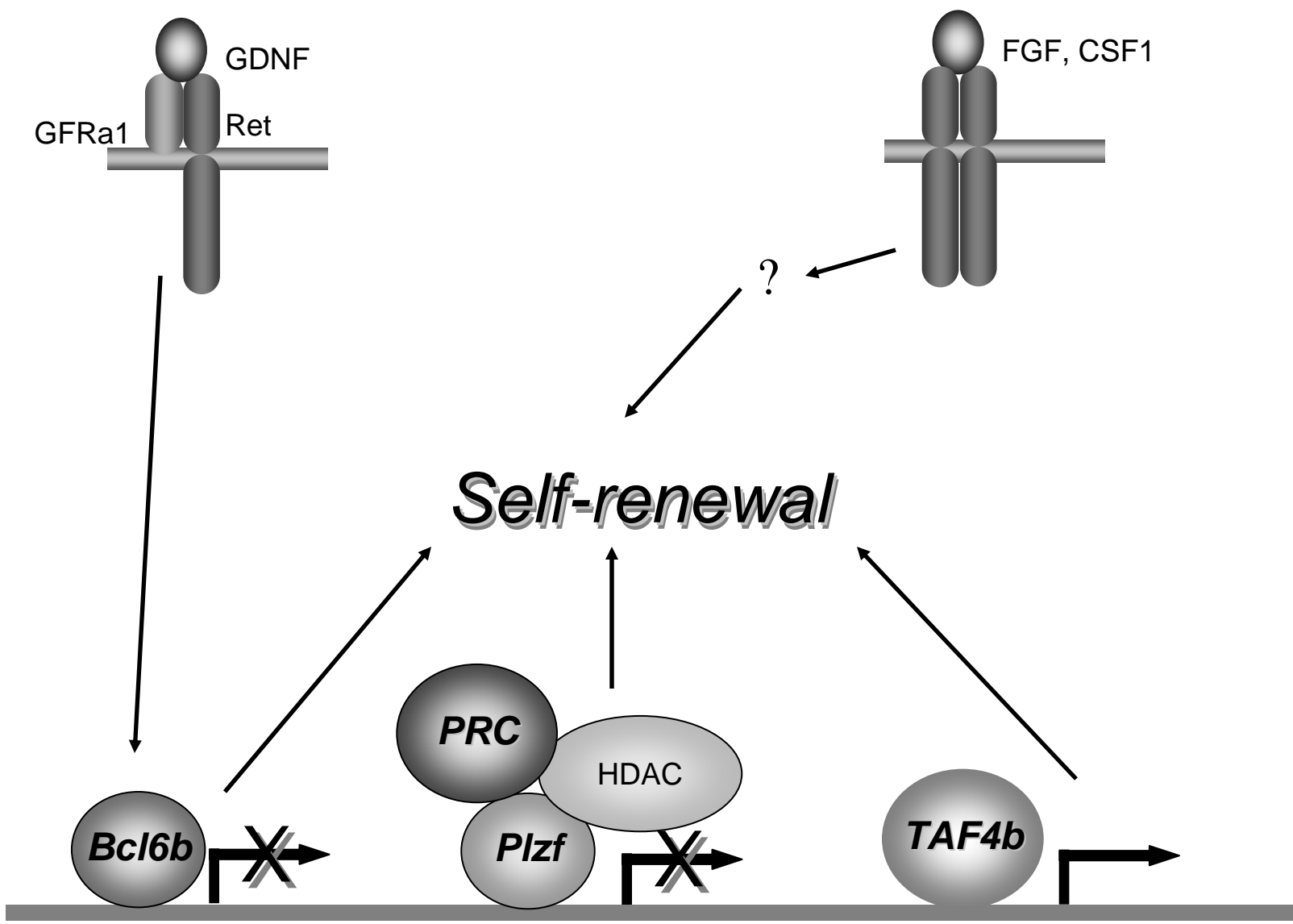
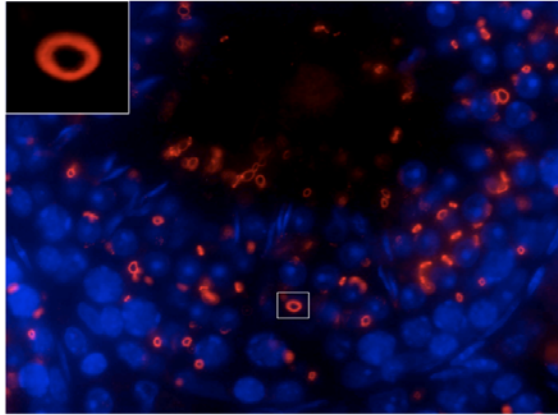
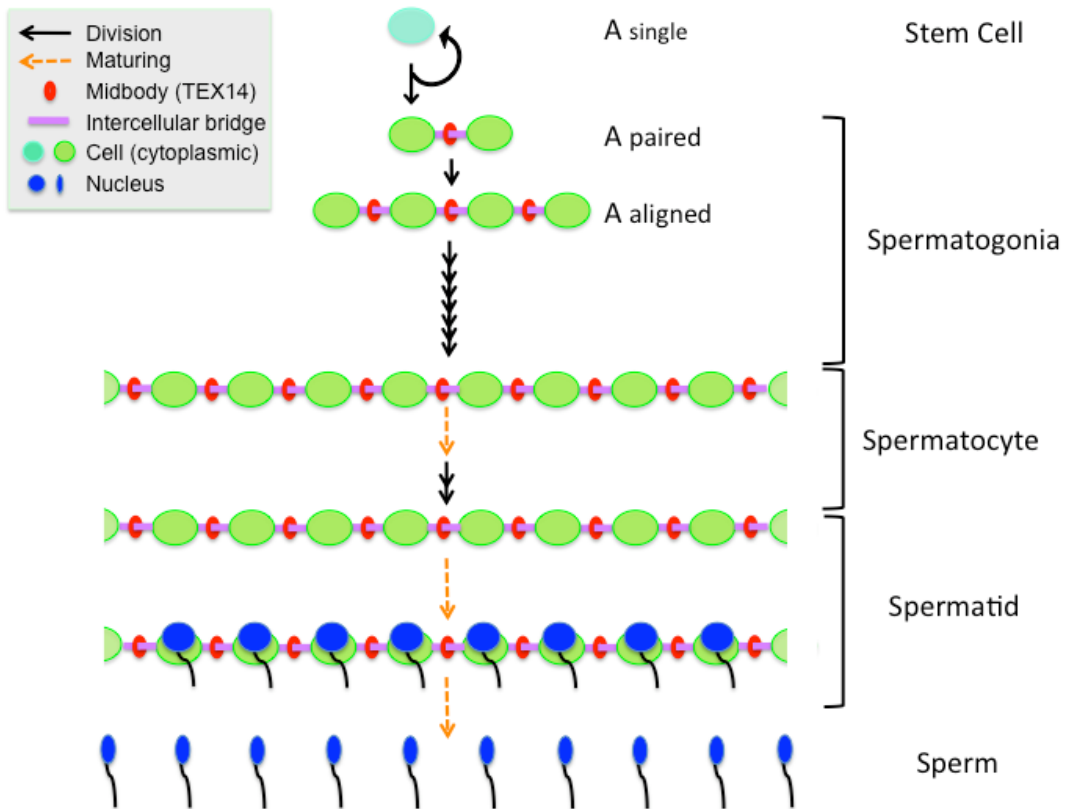


Figure 2

A



B



**Figure 3**

