

Impaired male fertility and atrophy of seminiferous tubules caused by haploinsufficiency for *Foxa3*

Rüdiger Behr, Sara D. Sackett, Irina M. Bochkis, Phillip Phuc Le, Klaus H. Kaestner*

Department of Genetics, University of Pennsylvania Medical School, 560 CRB, 415 Curie Blvd., Philadelphia, PA 19104-6145, USA

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Abstract

Foxa1, 2 and 3 (formerly HNF-3 α , - β and - γ) constitute a sub-family of winged helix transcription factors with multiple roles in mammalian organ development. While all three *Foxa* mRNAs are present in endoderm derivatives including liver and pancreas, only *Foxa3* is expressed in the testis. Here we demonstrate by genetic lineage tracing that *Foxa3* is expressed in postmeiotic germ and interstitial Leydig cells. The germinal epithelium of *Foxa3*-deficient testes is characterized by a loss of germ cells secondary to an increase in germ cell apoptosis that ultimately leads to a Sertoli cell-only syndrome. Remarkably, not only the *Foxa3*^{-/-} mice but also *Foxa3*^{+/-} mice exhibited loss of germ cells. This cellular phenotype caused significantly reduced fertility and testis weight of both *Foxa3*^{-/-} and *Foxa3*^{+/-} mice. Using microarray analysis, we found a dramatic downregulation of the zinc finger protein 93 and the testicular tumor-associated paraneoplastic Ma antigen (PNMA) and increased expression of a number of genes including zinc finger protein 94 and several kallikrein 1-related peptidases which could account for at least part of the observed phenotype. In summary, we have identified *Foxa3* as a transcriptional regulator with a dominant phenotype in germ cell maintenance and suggest *FOXA3* as a potential candidate gene for subfertility in man.

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Introduction

Subfertility and infertility in men are widespread yet poorly understood health problems. Approximately 15% of all couples of reproductive age are infertile, and the causes of infertility are roughly equally distributed between genders (Nieschlag, 2001). Germ cell production in the mammalian testis depends on intimate biochemical and physical interactions between somatic Sertoli cells and germ cells (Mruk and Cheng, 2004; Sharpe et al., 2003). In addition, normal Leydig cell function is also critical for proper sperm production in rodents and men (Andersson et al., 2004; Qian et al., 2001; Rich et al., 1979). Hence, it is well established that there is a functional network between the somatic cell types, i.e. the Sertoli and the Leydig cells, and the germ cells (Gnessi et al., 1997, 2000; Huleihel and

Lunenfeld, 2004; Jegou et al., 1984; O'Donnell et al., 2001; Weinbauer and Wessels, 1999).

Spermatogenesis can be divided into three phases: phase 1 consists of up to eleven mitotic divisions of the germinal stem cells (i.e. the spermatogonia), phase 2, which is meiosis of the spermatogonia, and phase 3, which comprises the postmeiotic differentiation of the haploid spermatids. Thus, theoretically each germinal stem cell division can result in the production of several thousand sperm cells (de Rooij, 2001). Due to the exponential nature of the process even a small decrease in the efficiency of spermatogonial proliferation or survival, caused for instance by abnormal Sertoli or Leydig cell function, can result in a dramatic reduction in sperm count and fertility.

While gene targeting studies in mice have identified many factors as being important in spermatogenesis (Cooke and Saunders, 2002; de Rooij and de Boer, 2003; Escalier, 2001; Matzuk and Lamb, 2002; Venables and Cooke, 2000), none of the *Fox* (*Forkhead box*) or winged helix DNA binding proteins

* Corresponding author. Fax: +1 215 573 5892.

E-mail address: kaestner@mail.med.upenn.edu (K.H. Kaestner).

have been shown to play a role in this process thus far. Here we demonstrate using genetic lineage tracing analysis that the transcription factor *Foxa3*, previously known as hepatocyte nuclear factor 3 γ or HNF-3 γ , is expressed in Leydig cells and in spermatids. Mice either homozygous or heterozygous for a *Foxa3* null allele exhibit reduced male fertility secondary to increased germ cell apoptosis. We identify several candidate genes possibly linking the lack of *Foxa3* with the observed phenotype and conclude that *Foxa3* plays an important role in the maintenance of a healthy germinal epithelium. We suggest that mutations in the human *FOXA3* gene might contribute to subfertility in man.

Results and discussion

Foxa3 is expressed in Leydig cells and in spermatids

Foxa3 is the only member of the *Foxa* subfamily of transcription factors expressed in testes, and, as such, a candidate gene for a regulator of spermatogenesis (Kaestner et al., 1994; Lai et al., 1991). *Foxa3* mRNA was expressed from day 6 to day 70 during postnatal testicular development in the mouse (Fig. 1a), suggesting a role for *Foxa3* in testicular development as well as the maintenance of the adult status. Because none of the available antibodies produced consistent staining patterns that were absent from *Foxa3* null tissue (data not shown), we decided to localize *Foxa3* expression further by genetic lineage tracing. In genetic lineage tracing (see scheme in Fig. 1b), the control elements of the gene of interest, here *Foxa3*, are used to drive the site-specific DNA recombinase Cre. In all cells where Cre is expressed, a loxP flanked stop sequence is removed, allowing for the expression of a marker gene, in our case β -galactosidase. Because the Cre activity produces a permanent mark in the genome, all descendants of the first cell that expressed Cre will be labeled as well. An essential requirement for this technique is a Cre transgene that accurately mimics the expression of the endogenous gene. Therefore, we employed a 170 kb yeast artificial chromosome (YAC) encompassing the entire *Foxa3* locus to direct expression of Cre, as this transgene has been shown to faithfully recapitulate the expression of the endogenous *Foxa3* gene (Hiemisch et al., 1997; Lee et al., 2005). As shown in Figs. 1d and e, both Leydig cells and spermatids were β -galactosidase positive in *Foxa3Cre/Rosa26* reporter mice. No staining was observed in *Rosa26* reporter mice alone (Fig. 1c). Not all Leydig cells were positive for lacZ in *Foxa3Cre/Rosa26* reporter mice, which most likely reflects the heterogeneous physiological state of Leydig cells. For instance, it is well established that Leydig cells also differ in the expression of the androgen receptor (AR), with a subpopulation of Leydig cells expressing no AR (Vornberger et al., 1994). *Foxa3Cre* expression in spermatids as evidenced by β -galactosidase staining starts shortly before nuclear condensation of spermatids. However, as judged by the phenotype of *Foxa3*-deficient mice described below, *Foxa3* expression in spermatids is of minor physiological importance compared to its expression in Leydig cells.

Foxa3 mutant mice show abnormal testicular development

Next we investigated testicular development in *Foxa3* mutant mice. In contrast to normal mice, aging *Foxa3*-deficient testes could not support spermatogenesis throughout the entire germinal epithelium (Fig. 2). At the postnatal age of 40 days, when the testis produces the first sperm cells, no histological defects were seen in *Foxa3* mutant mice (data not shown). In *Foxa3*-deficient testes of 3-month-old mice a few abnormal, but no completely degenerated tubules were visible (Figs. 2a and b). During the next months, the phenotype became more pronounced and in 8-month-old mice a significant portion of tubules was degenerated (Figs. 2c and d). In mice older than 1 year about a third of all tubular cross sections exhibited severe impairment of spermatogenesis. While the mutant testes at any age also contained tubules with apparently normal spermatogenesis, directly adjacent tubules were often degenerating (Figs. 2d and e) or completely devoid of germ cells (Fig. 2f). Degenerating tubules were randomly distributed over the testes. Remarkably, the last remaining germ cells in degenerating tubules were elongated spermatids and mature sperm cells (Fig. 2e). This indicates that the observed phenotype is likely caused by impairment of early germ cell proliferation or survival, and not by a specific block during sperm maturation, even though *Foxa3* is expressed during spermiogenesis. After passing an early critical checkpoint, the developing germ cells can complete meiosis and spermiogenesis, even if the following germ cell generations are completely missing and the germinal epithelium is almost atrophied (Fig. 2e). Hence, histologically, the lack of *Foxa3* in spermatids does not affect spermiogenesis. At the late stages of tubular degeneration as seen in Fig. 2f, some of the Sertoli cell nuclei have lost the pyramidal shape normally found in Sertoli cells supporting ongoing spermatogenesis.

Foxa3 mutant testes exhibit increased apoptosis in the germinal epithelium

The maintenance of a normally composed germinal epithelium depends on a finely tuned balance between proliferation, differentiation, and apoptosis of the germ cells (de Rooij, 2001). We analyzed germ cell proliferation using BrdU incorporation as a marker for DNA synthesis in replicating cells. At 2 h after injection mitotically dividing spermatogonia and primary spermatocytes were labeled. There was no difference in the number of BrdU-positive cells between wild type and mutant testes (Fig. 3a), indicating that the defect in the *Foxa3*^{-/-} mice was not caused by decreased proliferation of germ cells. Even in strongly degenerated tubules BrdU-positive germ cells were seen. In order to quantify germ cell apoptosis we carried out TUNEL assays on testicular tissue sections (Figs. 3b and c). We found a significantly increased number of TUNEL-positive cells per TUNEL-positive tubular cross-section in the *Foxa3*-mutant as well as in the heterozygous testes compared to controls ($p < 0.02$ wt vs. heterozygous and homozygous mutants) (Fig. 3d). Furthermore, the percentage of tubules containing TUNEL-positive cells was slightly increased

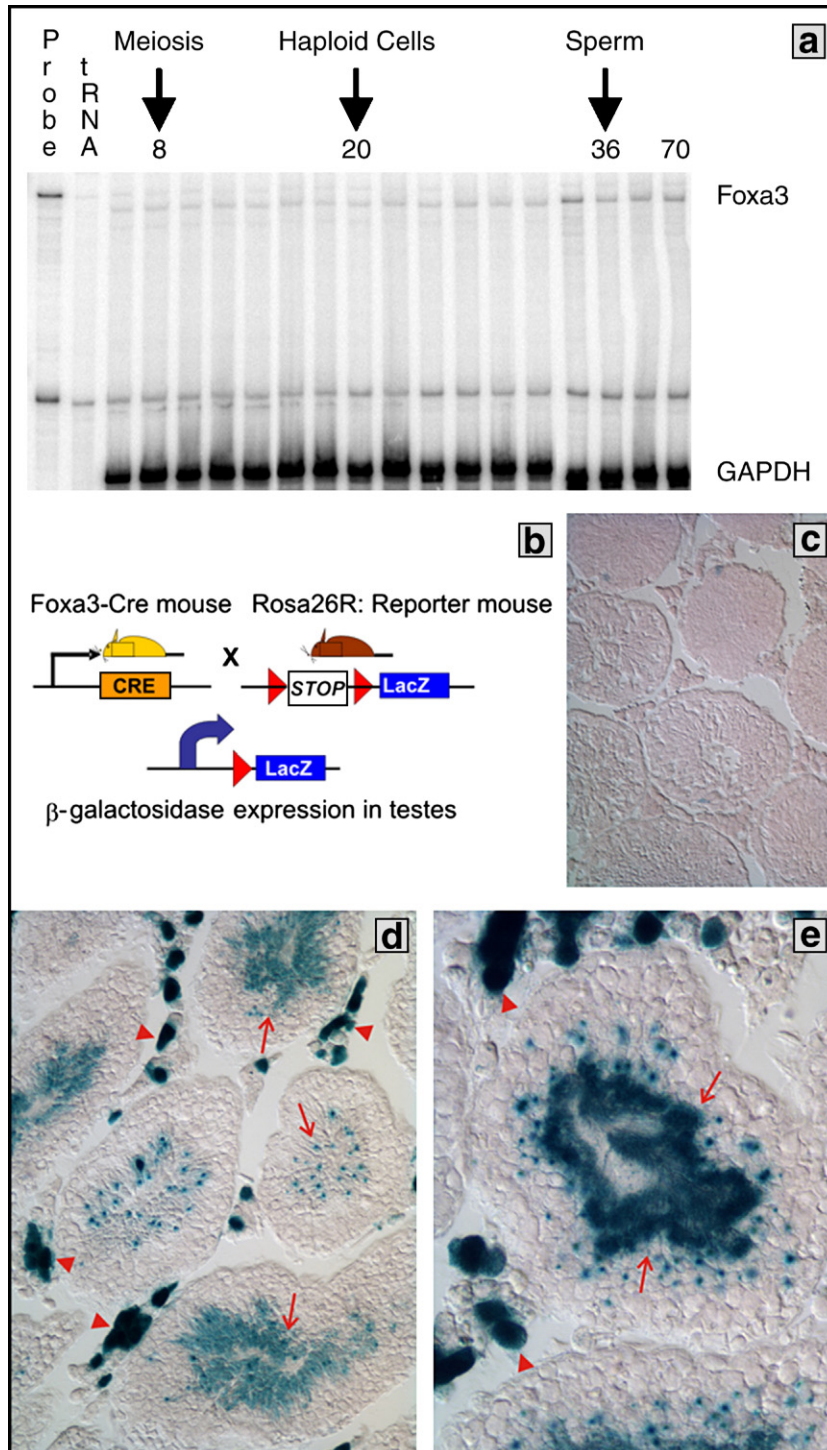


Fig. 1. Testicular expression of *Foxa3*. (a) Messenger RNA expression analysis by RNase protection assay. *Foxa3* mRNA is detectable in the mouse testis throughout postnatal development. The mRNA abundance is increased approximately twofold in the adult testis when compared to the 6-day-old testis (normalized to GAPDH mRNA). RNA was prepared from testis of postnatal days 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 36, 40, and 70, respectively. (b) Scheme of the genetic lineage tracing employed to track *Foxa3* expression in the testis. Beta-galactosidase expression occurs only in those cells that express *Foxa3* or are the descendants of *Foxa3*-expressing cells. (c) The control mouse (Rosa26R, no Cre), shows no β -galactosidase expression in the testes. (d, e) Mice carrying both the β -galactosidase reporter and the *Foxa3*-Cre transgene (Rosa26R; *Foxa3*Cre mice) display robust staining in Leydig cells (red arrowheads) and in spermatids (arrows).

(wt: 15.1%, het: 17.1%, mut: 18.9% of TUNEL-positive tubules). Altogether, the number of apoptotic germ cells in the *Foxa3*-deficient testes was increased 1.77-fold. This difference

becomes even more relevant taking in consideration that mostly spermatogonia are affected by apoptosis as judged from the position of the labeled cells in the germinal epithelium. The

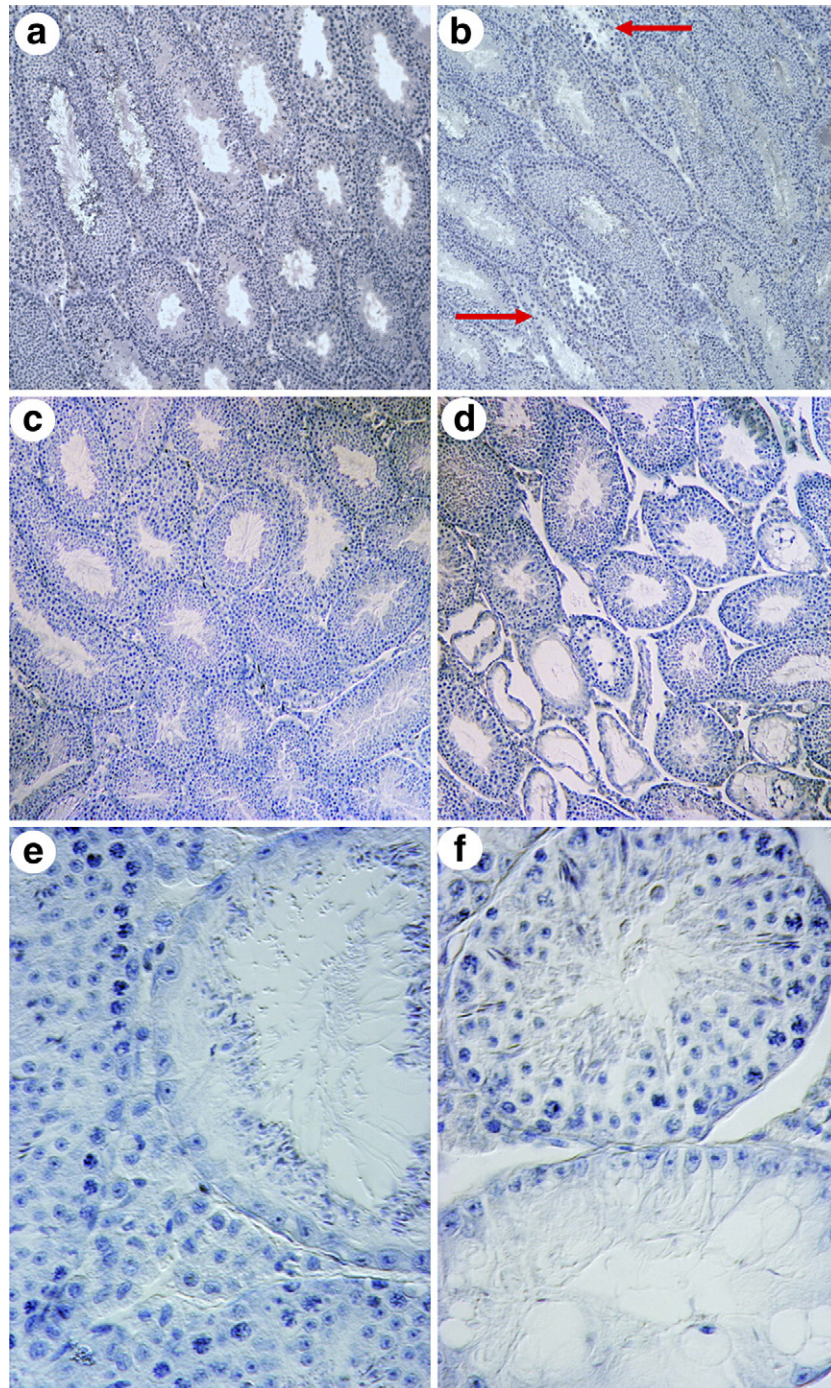


Fig. 2. Testicular degeneration in *Foxa3*-deficient mouse testes. (a) Histological section of a wild type mouse testis at the age of 3 months. All seminiferous tubules contain somatic Sertoli cells as well as germ cells and exhibit ongoing germ cell production. (b) Mutant testis at the age of 3 months. Sporadic tubulus with early signs of degeneration, i.e. thinning of the germinal epithelium, can be seen (arrow). (c) Histological section of a wild type mouse testis at the age of 8 months showing normal spermatogenesis in all tubules. (d–f) Histological sections of *Foxa3*-deficient mouse testes at 8 months of age. The testis contains both tubules with normal appearance and ongoing spermatogenesis as well as severely degenerated tubules. The characteristic feature of the defective tubules is a selective loss of germ cells. The Sertoli cells remained within the tubule resulting in a focal Sertoli cell-only syndrome. (e) An almost completely atrophied seminiferous tubule. The only germ cells present are elongated spermatids. All spermatogonia, spermatocytes, and round spermatids are missing. The elongated spermatids are arrayed on the adluminal surface of the Sertoli cells as they usually are shortly before sperm release from the germinal epithelium (spermiation). (f) A Sertoli cell only tubule (bottom) adjacent to a tubule with complete spermatogenesis (top). The Sertoli cell nuclei characterized by their triangular shape and their prominent nucleoli have aligned with the basal lamina indicating the absence of any germ cells. Heterozygous testes show histological pictures similar to the homozygous mutant testes. Magnification of a–d=100 \times ; e and f=600 \times .

extinction of even one A_{single} spermatogonium, the stem cell for germ cell production, will result in the loss of the entire germ cell population within a region of the germinal epithelium. Thus

increased germ cell apoptosis is expected to result in seminiferous tubules devoid of germ cells as seen in the *Foxa3*^{-/-} mice (Fig. 2).

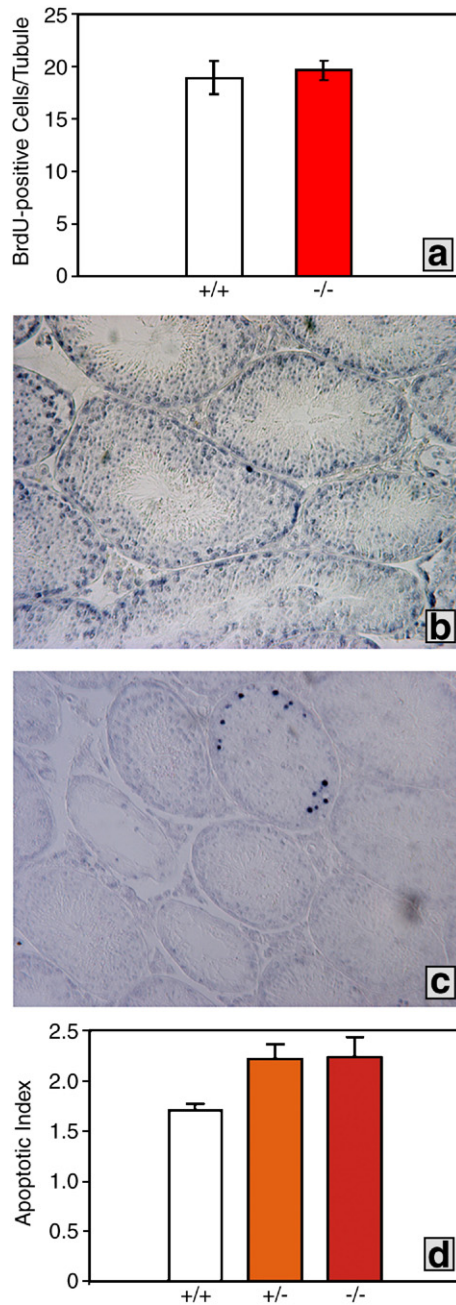


Fig. 3. Testicular proliferation and apoptosis in *Foxa3*-deficient mice. (a) Proliferation rates, as determined by BrdU labeling of cells in S-phase during spermatogenic stages VII and VIII, are not different between wild type and *Foxa3*-deficient mice ($p > 0.35$). (b) Apoptosis in a wild type mouse testis as revealed by the TUNEL-technique. Only a minor proportion of all tubules contains one or more TUNEL-positive cells. All other tubules are devoid of TUNEL-positive cells. As judged from the appearance and the location of the cells in the seminiferous tubule the apoptotic cells are almost exclusively spermatogonia and spermatocytes. (c) *Foxa3*-deficient mice lose their germ cell population by apoptosis. The tubule undergoing degeneration exhibits a high number of apoptotic germ cells, again mainly spermatogonia and spermatocytes. Note the already degenerated tubules which are devoid of TUNEL-positive cells. (d) Apoptotic index expressed as TUNEL-positive cells per tubule containing TUNEL-positive cells. The mutant as well as the heterozygous testes show a significant increase in the number of apoptotic cells ($p < 0.05$).

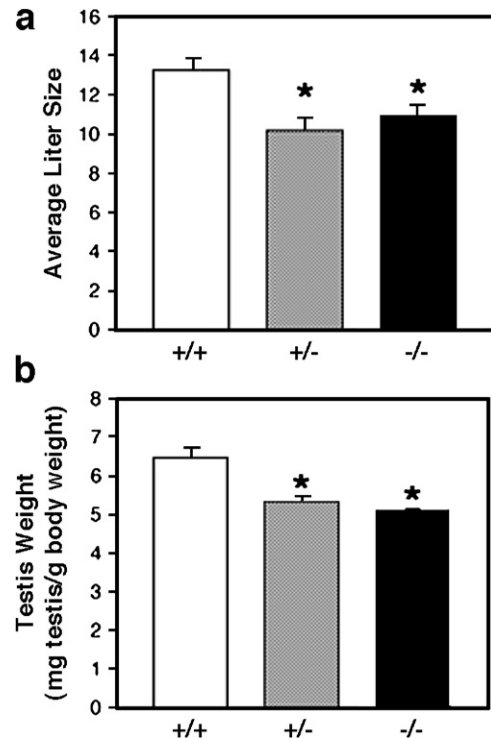


Fig. 4. Reduced male fertility and testis weight in *Foxa3*-deficient mice. (a) The testicular defect in *Foxa3*-deficient mice results in reduced fertility. Male age-matched mice of each genotype were housed together with a female CD1 wt mouse. The average litter size in the mutant ($p < 0.05$) and in the heterozygous group ($p < 0.02$) was significantly lower than in the wt group. (b) The *Foxa3*-mutant ($p = 0.003$) as well as the heterozygous ($p = 0.007$) mice show a significantly reduced testis weight compared to the wild type controls. Data are presented as paired testis weight [mg] per body weight [g].

Foxa3 deficiency reduces male fertility

To test whether the morphological defects described above had an impact on the reproductive capability of the *Foxa3* mutant mice we mated 3-month-old *Foxa3*^{+/+}, *Foxa3*^{+/-}, and *Foxa3*^{-/-} males ($n = 8$ in each group) for 5 months to wild type females. Interestingly, the average litter size of the homozygous mutant as well as of the heterozygous group was significantly smaller than in the wild type group ($p < 0.05$) (Fig. 4a). The litter sizes of the *Foxa3*^{+/-} and *Foxa3*^{-/-} groups did not differ significantly. The biological relevance of the defect in spermatogenesis is emphasized by the fact that not all males were fertile in the mutant and in the heterozygous group.

The mating data correlate well with the testicular weight in relation to body weight at 8 months of age. While this coefficient is 6.48 for the wt group (mg paired testis weight per g bodyweight), the coefficient for the *Foxa3*^{+/-} and the *Foxa3*^{-/-} group is 5.43 and 5.10, respectively ($P \leq 0.0001$) (Fig. 4b). The bodyweight did not differ significantly among the three experimental groups ($P \geq 0.2$).

Hormonal analysis

Qualitatively and quantitatively normal spermatogenesis is dependent on several hormones. We analyzed circulating FSH,

mechanism of these regulatory relationships will need to be established experimentally in the future.

The changes in gene expression, especially of the kallikrein genes, provide a possible explanation of the phenotypic changes described above. The kallikreins are a subgroup of the serine protease enzyme family (Clements et al., 2004; Diamandis and Yousef, 2002; Lundwall et al., 2006). Kallikreins are involved in propeptide processing (Blaber et al., 1987; Lundwall et al., 2006; Thomas et al., 1981) and have clinical relevance as prognostic factors for several types of endocrine-related tumors such as breast, ovarian and prostate cancer (Clements et al., 2004; Diamandis and Yousef, 2002). Moreover, kallikreins have been associated with such diverse biological processes as skin desquamation, tooth development, neural plasticity, and Alzhei-

mer's disease (Clements et al., 2004). Matsui and colleagues (Matsui et al., 2000, 2005; Matsui and Takahashi, 2001) have isolated three cDNAs from the mouse testis, Klk1b21, Klk1b24, and Klk1b27 (formerly mGK-21 or mKlk21, mGK-24 or mKlk24, and mGK-27 or mKlk27, respectively; Lundwall et al., 2006), which are related to human kallikrein-1 and are expressed in the testis exclusively in Leydig cells. We found all three kallikreins expressed in mouse testes significantly upregulated in the absence of *Foxa3*. Therefore, it is conceivable that massively deregulated kallikrein expression disturbs testicular function in *Foxa3*-deficient mice by paracrine mechanisms. In fact, the kallikrein–kinin system has been suggested previously to play an important role in paracrine regulation of the seminiferous epithelium (Monsees et al., 1997).

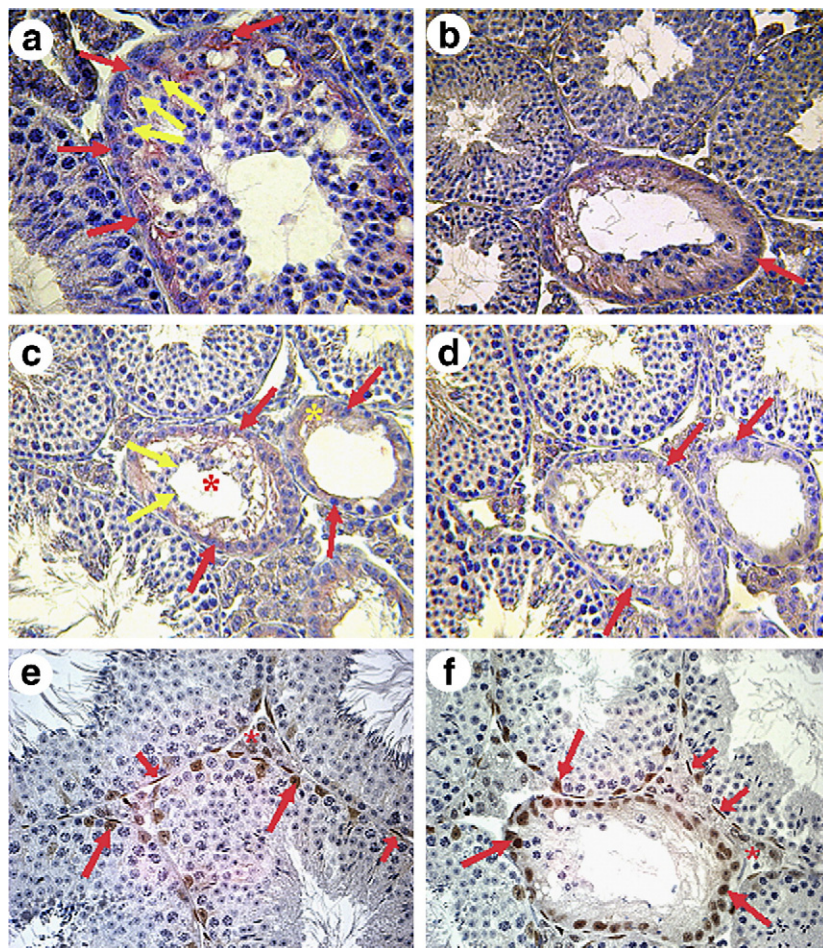


Fig. 6. Degenerating seminiferous tubules in *Foxa3*-deficient testes re-express anti-Müllerian hormone (AMH) as revealed by immunohistochemistry (a–d). (a) A seminiferous tubule in the early phase of degeneration. The thickness of the germinal epithelium appears to be normal, although vacuolization of the epithelium has already occurred. Germ cells present in this tubule are mostly round spermatids as judged from nuclear morphology. Earlier germ cell stages (spermatogonia and spermatocytes) are rather rare. Germ cells are devoid of any pink stain (yellow arrows) but the surrounding cytoplasm of the Sertoli cells is clearly stained (red arrows). Tubules with normal appearance do not exhibit any staining (lower left part). (b) A degenerating tubule in a more advanced stage than that shown in panel a. One generation of spermatocytes (presumably maturing to spermiation) is still present in this tubule. Sertoli cell nuclei have aligned along the tubular wall and the cytoplasm is clearly stained by the AMH antibody (red arrow). (c) A tubule during degeneration (red asterisk) still containing some germ cells (yellow arrows) and a tubule at the final stage of degeneration (yellow asterisk), which exhibits a Sertoli cell-only phenotype characterized by the complete loss of germ cells. At this stage, the Sertoli cells have flattened and extend little beyond their nuclei. Again, AMH immunoreactivity is widespread. Red arrows point out AMH positive Sertoli cells. (d) Negative control omitting the AMH antibody from the diluent. Also, the adult wild type control mice exhibited no staining while Sertoli cells in young testes up to p10 showed strong cytoplasmic Sertoli cell staining (data not shown). (e) Expression of the androgen receptor (AR; brown stain) in a wt testis as revealed by immunohistochemistry. Sertoli cells (long red arrows), peritubular myoid cells (short red arrows) and Leydig cells (red asterisk) are AR positive. (f) AR expression in an 8-month-old mutant testis showing staining in the same cell types as the age-matched wild type control. Remarkably, also Sertoli cells in almost completely degenerated tubules still express the AR.

The relatively minor changes in the testicular transcriptome of the mutant testes accompanied by the significant histological impairments may be explained by the fact that the effects of increased kallikrein activity in the testis affect primarily the proteome.

Zinc finger proteins (Zfp) 93, 94, 95, and 96 are closely related DNA binding proteins with predominant or exclusive expression in testis (Shannon et al., 1996; Shannon and Stubbs, 1998; Weissig et al., 2003). Zfp94 steady state mRNA levels were increased approximately fourfold in *Foxa3*-mutant mice compared to controls. In contrast, Zfp93 is strongly down-regulated in the mutant testis. The Riken cDNA 0710005i19, which was down-regulated fourfold in the *Foxa3*-deficient testes, encodes the mouse homolog of the human MA2 antigen. Expression of MA2 in healthy tissues in humans is restricted to the brain. However, in patients with testicular cancer exhibiting concomitant neurological disorders (paraneoplastic limbic and/or brain-stem encephalitis), MA2 antigen was also present in testicular tumors, suggesting a role for MA2 during testicular tumorigenesis (Voltz et al., 1999). Whether reduced MA antigen mRNA expression in *Foxa3* mutant mice contributes to testicular germ cell loss remains to be resolved.

Sertoli cells in degenerating tubules re-express anti-Müllerian hormone

Although anti-Müllerian hormone (AMH) itself is not essential for Sertoli cell differentiation (Behringer et al., 1994), it is switched on very early in fetal Sertoli cells and is a very reliable marker for the maturation state of this cell type (Sharpe et al., 2003). In normal testis, AMH is specifically expressed in immature Sertoli cells until puberty. With the appearance of meiotic germ cells and androgen sensitivity of Sertoli cells, AMH expression is strongly down-regulated in the normal testis and mature Sertoli cells are devoid of AMH (Sharpe et al., 2003). Interestingly, the Sertoli cells within the Sertoli cell-only tubules in the *Foxa3* mutant testes expressed AMH protein as revealed by immunohistochemistry (Figs. 6a–d). Moreover, AMH protein expression appeared to be induced even where meiotic and postmeiotic germ cells were still present (Figs. 6a and b), indicating that the loss of germ cells was not the primary cause of AMH protein accumulation. Likewise, transcriptional reactivation of the AMH locus is not a likely explanation for the reappearance of AMH protein, as mRNA levels were not altered in the mutants (data not shown). The molecular mechanisms underlying the reappearance of the AMH antigenicity in Sertoli cells remain to be investigated, but it is tempting to speculate that increased kallikrein activity, which alters the local crosstalk between Leydig and Sertoli cells, may contribute to this effect.

Usually, AMH is down-regulated when Sertoli cells start expressing the androgen receptor as a sign of maturity (Sharpe et al., 2003). Interestingly, in *Foxa3*-deficient mice the Sertoli cells in degenerated tubules still express the AR (Figs. 6e and f) indicating an intermediate state of the Sertoli cells between maturity and dedifferentiation.

Summary and model of the progression of the testicular lesion

Foxa3 is expressed in spermatids and in Leydig cells. According to our current view the lack of *Foxa3* in Leydig cells is causative for the phenotype described above. Since spermiogenesis proceeds normal even in the absence of *Foxa3* in spermatids, this transcription factor appears to be dispensable in germ cells for completed spermatogenesis. However, lack of *Foxa3* in Leydig cells causes significantly increased Kallikrein mRNA levels. We hypothesize that increased Kallikrein activity in the mutant testes severely disturbs paracrine communication between testicular cells. As a consequence, Sertoli cells may fail to maintain their fully differentiated state, as evidenced by the presence of AMH antigenicity, and lose their ability to support and regulate the early steps of spermatogenesis, which leads to an increase in germ cell apoptosis and eventually to degeneration of seminiferous tubules.

Conclusions

In conclusion, we have shown that *Foxa3* is necessary for the maintenance of the testicular germ cell population and male fertility in mice and provide evidence that the germinal epithelium in testes lacking *Foxa3* is not able to maintain its differentiated phenotype. Surprisingly, *Foxa3* is haploinsufficient in the testes, an unusual finding for transcription factors in this organ. We have identified several differentially expressed genes which may contribute to the phenotypic findings. Strikingly, the mutant testes express increased levels of all testicular kallikrein proteases, which may disturb the local communication between the Leydig cells and the cells of the seminiferous tubules. Approximately 8% of all men are infertile and in a large subset of these patients the underlying cause is unknown (Nieschlag, 2001). The phenotype of *Foxa3*-deficient mice resembles a human spermatogenic disorder called mixed atrophy, which is characterized by a variable degree of degeneration of the seminiferous tubules. The molecular mechanisms causing this disorder are unknown. However, it is tempting to speculate that mixed atrophy in a subset of patients may be caused by mutations in the *FOXA3* locus.

Materials and methods

Mice

The derivation of the *Foxa3* null allele has been described (Kaestner et al., 1998). Mice were genotyped by PCR on tail DNA as described previously. The *Foxa3* null allele was backcrossed to C57BL6 inbred mice for 11 generations to obtain an incipient congenic strain (>99% C57BL6 contribution) prior to the onset of this study. Use of an inbred background reduces the variability of the phenotype, as all potential modifier loci are homozygous in all animals studied. For genetic lineage tracing experiments, *Foxa3*-Cre mice (Lee et al., 2005) were bred to Rosa26 lacZ reporter mice (Soriano, 1999). Testis from 3-month-old mice were prepared for β -galactosidase staining as described (Hiemisch et al., 1997).

Mating experiments

Age-matched males ($n=8$ in each group; +/+, +/-, -/-) from our *Foxa3* breeding colony were housed together with age-matched CD1 females (Charles

River). At the outset of the study male mice were 3 months of age, i.e. when the phenotype became histologically evident. The study was continued for 5 months. Three times a week all cages were inspected and pups counted. At 2 to 3 weeks after birth the offspring were removed from the cages. At the termination of the study the body weight and the paired testis weight of the male mice was determined.

Hormone analysis

At termination of the mating experiments serum was collected from age-matched males ($n=6$ per group) and levels of FSH, testosterone, and estradiol were determined by AniLytics, Inc. (Gaithersburg, MD).

Histology and immunohistochemistry

After sacrifice of the mice the testes were removed and cut into halves. Two halves were fixed in Bouin's solution for 4 h. One-half testis was fixed overnight in 4% PFA, while the remaining half of was frozen at -80°C . The fixed tissue was paraffin-embedded and used for histological evaluation, detection of apoptotic cells, and BrdU-localization. Immunohistochemistry was performed on Bouin-fixed tissue essentially as described (Behr and Weinbauer, 1999) using the primary antibody (sc-6886; Santa Cruz Biotechnology Inc.; CA, USA) to detect anti-Müllerian hormone. As positive control we used newborn mouse testis, where we obtained specific and exclusive staining of the Sertoli cells. In negative controls the primary antibody was omitted. Androgen receptor was detected similarly using the AR (N-20) antibody (sc-816; Santa Cruz Biotechnology Inc.).

Proliferation assay

Proliferation of cells was detected by the incorporation of BrdU into genomic DNA. A dose of 10 mg BrdU (Zymed)/kg bodyweight was intraperitoneally injected 2 h before sacrifice. The tissue was fixed in 4% PFA and paraffin-embedded. After re-hydration and antigen retrieval by cooking in a microwave oven in 10 mM citric acid buffer, the BrdU antigen was detected using an alkaline phosphatase-conjugated BrdU-specific antibody (Roche Molecular Biochemicals). Histologically normal seminiferous tubules in spermatogenic stages VII and VIII were evaluated. During these stages all tubules exhibit BrdU-positive cells. Labeled cells were counted in at least 20 round tubular cross sections per animal ($n=6$ per group).

Apoptosis assay

Apoptotic cells were detected on Bouin-fixed tissue sections using the TUNEL technique (Wijsman et al., 1993). Paraffin-embedded sections were dewaxed and microwaved in 10 mM citric acid buffer for 8 min. For visualization of DNA double strand breaks the in situ cell death detection kit (Roche Molecular Biochemicals) was used according to the manufacturer's protocol.

Microarray and RNA analysis

Four wild type and three *Foxa3*^{-/-} testes samples, each containing 1 μg of total RNA, were hybridized after a single round of amplification to Affymetrix MOE430v2 oligo chips. Data was provided as absent/present calls and intensity values. We normalized the data using the GeneChip Robust Multi-Array Analysis (GCRMA) algorithm (Wu et al., 2003), implemented in a R package (<http://www.bioconductor.org/repository/devel/vignette/gcrma.pdf>). Fold-changes were calculated as the ratio of the geometric mean between wild type and *Foxa3*^{-/-} intensities.

Statistical analysis of the microarray data was completed using the significance of microarrays (SAM) (Tusher et al., 2001) package with a false discovery rate (FDR) of 10% and fold change cutoff of absolute value of 2.0. Annotation for each 'spot' was downloaded from the Affymetrix web site. The data have been deposited into ArrayExpress. Individual steady state transcript levels were determined by real-time PCR (QPCR) using the SYBR Green Master Mix from Stratagene on a Stratagene MX3000 QPCR instrument. RNase

protection analysis was basically carried out as described previously (Kaestner et al., 1994) using the RPAII kit from Ambion (Austin, TX, USA). The protected RNA fragments were separated on 8 M urea denaturing 6% acrylamide gels and the radioactive bands visualized using a PhosphorImager (Molecular Dynamics). Primer sequences used can be obtained from Kaestner Lab website (<http://www.med.upenn.edu/kaestnerlab/reagents.shtml>).

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