Rex1 (Zfp42) null mice show impaired testicular function, abnormal testis morphology, and aberrant gene expression

Naira C. Rezende a,b,1, Mi-Young Lee a,1, Sébastien Monette c, Willie Mark d, Ailan Lu a,2, Lorraine J. Gudas a,b,*

a Department of Pharmacology, Weill Cornell Medical College of Cornell University, 1300 York Avenue, New York, NY 10065, United States
b BCMB Graduate Program, Weill Cornell Medical College of Cornell University, 1300 York Avenue, New York, NY 10065, United States
c Center of Comparative Medicine and Pathology, Weill Cornell Medical College of Cornell University, 1300 York Avenue, New York, NY 10065, United States
d Developmental Biology Program, Sloan-Kettering Institute, 1275 York Avenue, New York, NY 10065, United States

Abstract

Rex1 (Zfp42), GeneID 132625, is a gene whose expression is closely associated with pluripotency/multipotency in both mouse and human embryonic stem cells. To study the function of the murine Rex1 gene in vivo, we have used cre/lox technology to create Rex1(fllox) mice and mice deficient in Rex1 gene function. Rex1−/− males are characterized by an age-associated decrease in sperm counts, abnormal sperm morphology, and mild testicular atrophy. We characterized global patterns of gene expression in primary germ cells by microarray and identified the growth hormone responsive gene, GRP11, as a transcript present at a 4.5 fold higher level in wild type (WT) compared to Rex1−/− mice. We analyzed immature germ cell (Dazl), proliferating (PCNA), and Sertoli cell populations, and quantitated levels of apoptosis in Rex1−/− as compared to WT testes. We evaluated the expression of proteins previously reported to correlate with Rex1 expression, such as STAT3, phospho-STAT3, p38, and phospho-p38 in the tests. We report a distinct cellular localization of total STAT3 protein in Rex1−/− affected testes. Our data suggest that loss of Rex1 leads to impaired testicular function.

© 2011 Elsevier Inc. All rights reserved.

Introduction

Understanding the cell signaling networks commonly used in defining the pluripotent state is critical to understanding stem cell identity and differentiation. However, more must be learned about the functional role of a gene whose expression is associated with pluripotency, namely Rex1, in the context of adult and embryonic stem cell biology. The Rex1 (Zfp42) gene (GeneIDs: mouse, 22702; human, 132625), a member of the zinc finger protein family that also includes YY1 (Kim et al., 2007), was identified by our laboratory (Hosler et al., 1989) and is widely used as one of seven markers of human and murine embryonic stem (ES) cells (Brivanlou et al., 2003).

We first identified Rex1 as an abundant transcript expressed in F9 teratocarcinoma stem cells; the transcription of Rex1 rapidly decreased upon addition of all-trans retinoic acid (RA), an active form of vitamin A (retinol) (Hosler et al., 1989, 1993). Subsequently, we characterized Rex1 expression during mouse development and showed that Rex1 mRNA is primarily detected in trophoblast derived tissues and in the inner cell mass of the blastocyst (ICM), the source of embryonic stem cells in culture (Rogers et al., 1991). In adult mice, Rex1 mRNA is expressed in a subset of male germ cells, spermatocytes actively undergoing meiosis (Rogers et al., 1991). Rex1 (mRNA and protein) is selectively expressed in dividing cells of the human testes and ovaries (Kristensen et al., 2008), suggesting a conserved functional role. Rex1 transcripts are also detected in a variety of pluripotent cells, normal primary human keratinocytes (NHK), human prostate epithelial cells (PrEC), renal parenchymal tissue, and some human neoplastic cell lines, such as MDA-MB-468 mammary carcinoma cells, SCC-15 head and neck squamous carcinoma cells, and NTERA2 teratocarcinoma cells (Kristensen et al., 2010; Mongan et al., 2006; Mongan and Gudas, 2007; Raman et al., 2006). Interestingly, we also showed that Rex1 expression increases in mouse digits during tissue regeneration/wound healing (Agrawal et al., 2010; Wang et al., 2010).

Most of what is functionally known about Rex1 comes from results obtained in cell differentiation studies, and/or analysis of the genes regulating its expression. First, our laboratory created Rex1 knockout (Rex1−/−) F9 teratocarcinoma stem cell lines by homologous recombination, and demonstrated that F9 Rex1−/− cells fail to differentiate completely into visceral endoderm (VE) in aggregate...
cells are primarily responsible for testosterone production (Hogarth et al., 1993; Rosfjord and Rizzino, 1994; Shi et al., 2006). Not surprisingly, Rex1 is one of a few select genes shown to be re-expressed in induced pluripotent stem cells (iPS cells) (Chan et al., 2009). iPS cells hold enormous potential not only for regenerative medicine, but also for studies in developmental biology, drug development, and the derivation of patient specific stem cell lines. Analysis of the promoter of the human Rex1 gene by our laboratory showed that human prostate cancer cells exhibited lower Rex1 promoter activity than normal prostate epithelial cells in culture (Lee et al., 2010).

Spermatogenesis, the proliferation and maturation of germ cells leading to formation of spermatocytes, occurs from puberty to death in male mammals. The testis is composed of germ cells as well as somatic cells, such as Sertoli cells and Leydig cells (Meng et al., 2000; O'Shaughnessy et al., 2009; Yoshinaga et al., 1991). Testicular germ cells consist of spermatogonial stem cells which can undergo self-renewal as well as differentiate into spermatocytes, spermatids, and finally into spermatocytes, the mature form of sperm. Sertoli cells produce growth factors, such as glial cell line-derived neurotrophic factor (GDNF), that maintain germ cell functions, and endocrine hormones such as inhibin, which acts on the hypothalamic–pituitary–gonadal axis (Hogarth and Griswold, 2010; Meng et al., 2000). Leydig cells are primarily responsible for testosterone production (Hogarth and Griswold, 2010). We wanted to understand the functions of Rex1 in stem cells in vivo and thus generated Rex1–/− mice by using a conditional knockout strategy (Cre–LoxP). We show here that Rex1−/− mice are viable, fertile, and grossly similar to WT littermates. Rex1 null mice were also generated by a conventional gene targeting method (Meng et al., 2000). Upon further characterization we discovered that Rex1−/− males showed an age associated decrease in sperm counts, abnormal sperm morphology, and mild testicular atrophy, phenotypes that could reflect a defect in the stem cell pool. Our data, collectively, indicate that Rex1 plays an important role in germ cell differentiation. Here we report experiments characterizing the germ cell compartment of Rex1 null mice in detail.

Materials and methods

Reagents

Unless stated, all reagents were purchased from Sigma Aldrich (St. Louis, MO, USA).

Construction of the Rex1 targeting vector and generation of Rex1−/− mice

We previously generated a phage library with genomic DNA for the Rex1 gene (Hosler et al., 1993). To generate a targeted deletion of the protein coding exon, Rex1 exon 4, we first subcloned the LoxP sequence from the pBS64 plasmid (Baubonis and Sauer, 1993) into the BglII sites of plasmid pgRex1-1.38 (3′ of Rex1 exon 4) (Fig. 1A). We then ligated vectors pgRex1-1.37 and pgRex1-1.38 at their HindIII restriction sites (Fig. 1A). Subsequently, we subcloned the PGK-neo/MCI-TK cassette (pNENOKLXO plasmid) into the NsiI site within intron 3′ of Rex1. This strategy created a targeting vector of approximately 12.7 kb (Fig. 1A); the 5′ region of this targeting vector contains 2.49 kb homologous to Rex1 intron 3, 3.86 kb of selection markers flanked by LoxP sites (PGK-neo, MCI-TK), 2.28 kb of the Rex1 gene (including all of exon 4), a LoxP site, and a 3′ arm consisting of a 4.07 kb genomic region 3′ of Rex1 exon 4.

This construct was linearized and introduced into the C57 mouse ES cell line (originally derived from 129S1/SvImj) by electroporation using a Bio-Rad electroporator at a voltage of 230 mV and a capacitance of 500 μF. Drug resistant clones were isolated after G418 selection (300 μg/ml active G418), and a total of 116 cell clones were screened by Southern blot analysis. Eight positive clones from cells that had undergone homologous recombination were obtained. One clone, # 66, was injected into C57BL/6 blastocysts to generate chimeric mice. Germ-line transmission was confirmed by mating chimeras to wild type C57BL/6 mice. Seven Rex1+/lox−/− female mice were chosen to mate with CAG-cre male mice, which are on the C57BL/6 background, to excise exon 4 of the Rex1 gene and/or the PKG-neo-MCI-TK cassette. The Cre gene in these mice is under the control of the cytomegalovirus immediate early enhancer linked to the chicken beta-actin promoter so that the Cre gene is expressed early and ubiquitously (Sakai and Miyazaki, 1997). Recombination was detected by PCR-based genotyping (the primers used for this genotyping are in Supplemental Table 2), and heterozygous mice (Rex1+/−) were inter-crossed to generate the Rex1−/− null mice. To determine the genotypes by PCR, two primer sets, P1F and P2R, and P1F and P4R were used. To confirm the genotype by Southern blot, genomic DNA was first digested with BglII. A 0.9 kb probe, located in genomic DNA 3′ of the targeting construct, was obtained by digesting pgRex1-1.46 plasmid DNA with HindIII/BglII, and hybridized to BglII digested genomic DNA. To prove homologous recombination we generated a second probe targeting the genomic DNA 5′ of the targeting construct. This second probe (0.3 kb) was obtained by digesting pgRex1-1.51 plasmid DNA with HindIII/BglII, and hybridized to EcoRII digested genomic DNA. The locations of the primer sets are depicted in Fig. 1A. For these studies, mice were partially backcrossed into the C57BL/6J background, and thus they represent the N3 generation. Mice are now at the N12 generation with respect to the Rex1 knockout in the C57BL/6J background.

Sperm counts, morphology evaluation, and testes weight measurements

Either 6–7 week or 4–6 month old male mice, both Rex1+/+ and Rex1−/−, were sacrificed by cervical dislocation. For sperm counts, our protocol was adapted from Yu et al. (2006). Briefly, the cauda epididymis was removed, minced in 3 ml of PBS (pH 7.4), and incubated for 5 min at room temperature by gently rocking. The samples were transferred to 15 ml tubes and centrifuged at 500 × g for 5 min. Then, the supernatants were used to count sperm with a hemocytometer. Three independent counts were performed for each mouse and the number of sperm was averaged. Briefly, sperm from 23 WT and 17 Rex1−/− mice were counted for the 6 week age group, and sperm from 10 WT and 20 Rex1−/− mice were counted for the four to six month age group. For sperm morphology evaluations, samples were prepared according to Ribeiro et al. (1987). Briefly, 0.9 ml of the supernatant, prepared as above, was transferred to 0.1 ml of 1% Eosin Y in a 1.5 ml tube and incubated for 30 min at 22 °C. The solution (10–15 μl) was used to prepare a smear on a slide, and this was air-dried. Mounting medium was added and sperm morphology was analyzed under the microscope. For testes weight measurements, both testes from each animal were removed, weighed, and the numbers were averaged.
Fig. 1. (A) Strategy and structure of targeting vector and strategy for generating Rex1+/− mice. (B, C) Southern blot strategy verifying the accuracy of the targeting event. (B) 3′ Southern: genomic DNA from two WT, three heterozygous, and two knockout mice was digested with BglII, and the probe (0.9 kb) was prepared by digesting pgRex1-1.46, a genomic fragment outside of the targeting construct, was prepared by digesting pgRex1-1.46 with HindIII/BglII. WT mice yield a band of 4.97 kb, heterozygous mice yield a 4.97 kb band and a 6.6 kb band, and knock out animals yield a 6.6 kb band. (C) 5′ Southern: genomic DNA from two WT, three heterozygous, and two knockout mice was digested with EcoRI, and the probe (0.3 kb) was prepared by digesting pgRex1-1.51, a genomic fragment outside of the targeting construct, with BglII. WT mice yield a band of 8.12 kb, heterozygous mice yield a 8.12 kb band and a 5.6 kb band, and knock out animals yield only a 5.6 kb band. (D) Genotype by PCR: genomic DNA from mouse tails is routinely genotyped by semi-quantitative PCR. We detect the recombination event by using a three primer PCR reaction: (1) specific forward primer homologous to an intragenic genomic region (P1F), in combination with two unique reverse primers: (2) a primer complementary to the Rex1 first exon (P2R); (3) and a primer complementary to the exogenous LoxP site (P4R). WT mice yield one band of approximately 350 bp, homozygous null animals yield a band of 470 bp, and heterozygous animals yield both bands. Primer sequences are listed in Supplemental Table 2. (D, E) RT-PCR: total RNA was isolated from testis and 2 μg of RNA was used to make cDNA using reverse transcriptase (Superscript, Invitrogen). 2 μl of 1:5 diluted cDNA was used to amplify mRex1 (D), and 36B4 (E) was used as a loading control. Rex1 primer pairs used for this are called Rex1F and Rex1R and result in a band of 670 bp; this band identity was verified by sequencing. 36B4 primers are called 36B4F and 36B4R and result in a 450 bp band. All primer sequences are listed in Supplemental Table 2.
Testicular cell preparation and cell cycle analyses

Testicular cells were prepared for cell cycle analysis following a protocol adapted from Malkov et al. (1998) and Browning. Briefly, testes were removed and decapsulated by making a small incision in the testis. The contents of the testes were collected through the incision into a 15 ml tube containing 5 ml ice-cold M199-BSA media (9.5 g tissue culture medium M199 (Invitrogen, Carlsbad, CA), 2.2 g NaHCO₃, 1 g bovine serum albumin in 1 l distilled water, pH 7.3–7.4). 1–2 ml of 2 mg/ml collagenase was added to this tube, and the contents were incubated for 40 min at 37 °C with vigorous shaking. Then, the tubes were placed on ice and incubated to allow the seminiferous tubules to settle. The supernatants were discarded and the seminiferous tubules were washed twice in 10 ml of M199-BSA medium. 5 ml of M199-BSA medium containing 2.5 μg/ml trypsin and 1 U/ml DNase I was added to seminiferous tubules, and they were incubated for 20 min at 37 °C. The reactions were stopped by transferring the tubes to ice and the cords were disaggregated by pipetting up and down with a Pasteur pipette several times. The resulting suspensions were filtered through a 50 μm nylon mesh and washed twice with M199-BSA medium and the cells were counted. A total of 2 × 10⁶ cells were used for each cell cycle analysis. Cell cycle analyses were performed following the manufacturer’s guide (Becton Dickinson Immunocytometry System, San Jose, CA). Cells (2 × 10⁶) were resuspended in 875 μl of cold PBS containing 0.1% sodium azide and fixed by adding 125 μl of cold 2% paraformaldehyde in PBS and incubating for 1 h at 4 °C. Cells were centrifuged for 5 min at 250 g at 4 °C and the supernatant was aspirated. Cells were then permeabilized by incubating them in 1 ml of PBS containing 0.05% Tween 20 for 15 min at 37 °C. After washing the cells with 1 ml of PBS containing 0.1% sodium azide, cells were stained with propidium iodide (PI) staining solution (50 μl of 1 mg/ml of PI, 1 μl of 100 mg/ml RNase A in 1 ml PBS), and cell cycle analysis was performed on a Becton-Dickinson FACs Calibur in the Weill Cornell FACS Core.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

The TUNEL assay was performed following the manufacturer’s guide (TUNEL enzyme, Ref: 11767305001, TUNEL label Ref: 11767291910, Roche, Basel, Switzerland); paraffin sections of testis (5 μm-thickness) were deparaffinized for 5 min in Histo-Clear (National Diagnostics, Atlanta, GA) twice, and then rehydrated in a series of serially diluted ethanol solutions (100%, 95%, 70%, and then H₂O). After washing with PBS, the tissue sections were incubated with primary antibodies: GRTP1 (1:500, Sigma, PRS4589), Sox9 (1:200, H-90, sc-20095, Santa-Cruz Biotechnology, Santa Cruz, CA), DazL (1:400, ab34139, Abcam, Cambridge, MA), PCNA (1:100, M0879, Dako, Carpinteria, CA) overnight at 4 °C for Sox9, or 1 h at 22 °C for GRTP1, DazL and PCNA. Following three washes with PBS the sections were incubated with secondary antibodies, either anti-rabbit HRP for Sox9 and DazL (superpicture HRP polymer conjugated rabbit primary, 87-9263, Invitrogen, Carlsbad, CA) or anti-mouse HRP for PCNA (M.O.M kit, PK-2200, Vector Labs, Burlingame, CA) for 10 min or 30 min at 22 °C, respectively. DAB signals were generated and sections were counterstained with Hematoxylin. We provided total Stat3 (Cell Signaling Technology, 9132), phospho-Stat3 (Cell Signaling Technology, 9145), p38 MAPK (Cell Signaling Technology, 9212, lot 16), and phospho-p38 MAPK (Cell Signaling Technology, 4631, lot 6) antibodies for automated staining at the Memorial Sloan Kettering Cell Biology Core Facility.

Germ cell (GC) RNA isolation, labeling and microarray hybridization

Germ cell RNA was isolated from whole testis following an adaptation of a well established protocol (Malkov et al.). Briefly, testes were decapsulated and digested with 0.1 mg/ml of collagenase type II (Invitrogen) for 40 min. After collagenase digestion, the dissociated seminiferous tubules were isolated by gravity sedimentation, and germ cells were further disconnected from tissue by digestion with 2.5 μg/ml trypsin (Lonza) supplemented with 1 U/ml DNase (Invitrogen). Single cells were filtered through a 50 μm nylon mesh, washed twice in separation medium (1) for 5 min at 300 g, and counted. Total germ cell RNA (GC RNA) was extracted using TRIZOL reagent (Invitrogen). GC RNA from two WT mice and three Rex₁⁻/⁻ mice were labeled and hybridized to oligonucleotide microarrays (Gene-Chip Mouse Gene 1.0 ST (Affymetrix)), according to standard protocols at the Weill Cornell Medical College Microarray Core Facility (the GC RNA of one wt mouse was analyzed twice).

Microarray data analysis

Raw data CEL files were normalized using the Robust Multi-Array (RMA-16) algorithm in GeneSpring GX 11.0.2 software (Agilent). Triplicate samples were grouped as wild type (wt) and homozygous null (Rex₁⁻/-) and statistical analysis was calculated with unpaired t-tests applied to log-transformed data.

Results

Rex1 null animals are characterized by premature age-associated testicular germ cell depletion, decreased sperm count, and abnormal sperm morphology

To investigate the functions of Rex1 in germ cell biology we have generated a mouse with a targeted deletion of the Rex1 gene (Fig. 1A). Initial attempts by our laboratory to create Rex1 null mice failed so we used Cre/Lox recombination to produce a conditional Rex1 knockout mouse. A LoxP site was inserted into the ‘3’ end of the Rex1 exon 4 while the selectable marker genes, the Neo-TK cassette, were flanked by LoxP sites as well (Fig. 1A). After germline transmission of the
targeted allele, Rex1fox/wt mice were mated with a transgenic mouse line carrying the chicken β-actin promoter (Niwa et al., 1991) directing Cre recombinase expression in all tissues (Sakai and Miyazaki, 1997). Rex1+/− mice were then identified, indicating excision of both the Neo-TK gene and exon 4 of the Rex1 gene (Figs. 1B, C, D). We obtained WT, heterozygous, and homozygous mutant Rex1 mice. Rex1+/− mice are viable and fertile and there appear to be no sex preferences among the progeny obtained (Table 1). As expected, Rex1 null mice failed to express Rex1 message by semi-quantitative PCR (Figs. 1E, F). We tested two commercially available antibodies against murine Rex1 (Millipore MAB4316, Abcam ab28141), and two custom generated Rex1 antibodies (Alpha Diagnostics), but failed to detect endogenous Rex1 protein by Western blot analysis of WT mouse testicular tissue, although the antibodies could detect mouse Rex1 over-expressed in Cos cells (data not shown).

To explore the physiological consequences of the loss of Rex1, the structure and function of the testes from Rex1 null mice were examined in detail. First, testis histology of six week (WT, n = 23, KO, n = 17) and 4-6 month old (WT, n = 10, KO, n = 20) mice was evaluated by H&E staining (Figs. 2B, D, C, E). We found that six of the Rex1+/− mutant testes showed a mild to moderate decrease in the number of germ cells in the seminiferous tubules (KO six week, n = 5, KO 4-6 month old, n = 1). None of the WT, either 6 week or 4-6 month old, showed this phenotype. This phenotype was seen most predominantly in spermatids (both early and late), but also occasionally in spermatocytes. In affected testes depleted seminiferous tubules were observed multifocally, admixed with normal tubules. We sacrificed two 1 year old animals for testis histology, at 13 months old (Supplemental Figs. 1A, B). Germ cell depletion is known to naturally occur with increased age, and we observed mild germ cell depletion, admixed with physiologically normal tubules in both WT and Rex1+/− 1 year old male mice (Supplemental Fig. 1). To investigate further the germ cell defect in Rex1+/− males, we performed sperm counts in animals of various ages. The same mice were evaluated for sperm counts (Fig. 2H) and testes weight (Fig. 2I). Both testes were removed from each mouse, weighed, and averaged. No significant difference was seen between six week old WT and Rex1+/− mice, though a decline in the number of spermatocytes was observed in Rex1+/− animals between four to six months of age (Fig. 2H). The average sperm count of WT mice in the four to six month age group was 502 ±104 ± 103/male/epididymis for WT mice, and it was 301 ±104 ± 50/male/epididymis in Rex1+/− mice (p = 0.03). We also determined testis weights of six-week old WT mice and Rex1+/− mice. The tissue weights were 80 ±4 mg and 77 ±7 mg, respectively (Fig. 2G). No statistically significant differences were observed in testis weights between 6 week old WT vs. Rex1+/− mice (p = 0.324), and the same is true for 4-6 month old mice.

We studied the sperm in 9 Rex1+/− and 10 WT mice between 4 and 6 months old, and noted the morphology to be different (Figs. 2F, G). In many Rex1+/− mice most sperm had bent tails just distal to the head (2G, white arrows). In addition, some sperm heads lacked cytoplasmic content, indicated by weaker staining (2G, blue arrows). The magnitude of this phenotype varied, suggesting incomplete penetrance. We conclude that Rex1 null animals are characterized by mild germ cell depletion, and a premature age-associated decrease in sperm counts.

Rex1+/− ovaries have similar numbers of follicles, corpora lutea, and ceroid pigment as compared to WT ovaries

In mammals the development of female germ cells entails a complex and tightly regulated process. At birth, the murine female ovaries contain primary oocytes arrested at stage I of mitosis (Pepling, 2006). Following puberty, a number of oocytes will periodically respond to environmental hormonal clues, resume mitosis, and mature further (Pepling, 2006). We previously failed to detect Rex1 transcript in murine ovaries (Rogers et al., 1991). Nevertheless Kristensen et al. (2008) reported human REX-1 staining specifically in the cycling oocytes of gestation week 40. To investigate a role for Rex1 in female germ cell development we undertook a comparison of the number of follicles, corpora lutea and ceroid pigment in a group of mice across three distinct age groups, 21 days old, 6 weeks old, and 1 year old. We reasoned that if transient Rex1 expression in cycling cells affects the number of female germ cells we would be able to see a quantitative difference in the number of follicles and corpora lutea between WT and Rex1+/− mice. In general, ovary morphology was grossly similar between WT and Rex1+/− mice. At 21 days old the average number of follicles in the WT mice was 52.6 as compared to 75.5 in Rex1+/− mice (p = 0.06) (Figs. 3A, B, Table 2). At 6 weeks old the average number of follicles in the WT mice was 37.6 as compared to 24.6 in Rex1+/− mice (p = 0.4), and there was an average of one corpus luteum per WT mice and 1.3 per Rex1+/− mice (Figs. 3C, D, Table 2). At 1 year old, the average number of follicles in the WT mice was 8.5 as compared to 7.8 in Rex1+/− mice (p = 0.8), and there was an average of 7.25 corpus lutea per WT mice and 4.3 corpus lutea per Rex1+/− mice (p = 0.09) (Figs. 3E, F, Table 2). We note mild lymphocytic infiltration in the ovarian bursa, which was observed in both WT and Rex1+/− mice (Table 2). Overall, we conclude that Rex1 expression does not affect the number or the morphology of follicles and corpus luteum in Rex1+/− mice.

Depletion of the germ cell compartment in Rex1+/− males cannot be explained by defects in cell cycle distribution

Previously, our laboratory utilized microarray approaches to identify transcripts that were differentially expressed between wild type and Rex1+/− mouse ES cells (Scotland et al., 2009). One of the differentially expressed transcripts was cyclin D2, which showed a six fold decrease in Rex1+/− mES cells. To determine if alterations in cell cycle distribution, potentially indicating a block in cell cycle progression, could explain the decrease in sperm counts in Rex1+/− mice, we measured the DNA content of cells in the seminiferous tubules of 4-6 month old mice (Fig. 3). Comparing WT (mean ± S.E) (Figs. 4A, C), and Rex1+/− mice (mean ± S.E) (Figs. 4B, C), we observed no significant difference in the distribution of cells with 4N DNA content (19.2 ± 1.6% vs 16.9 ± 0.8%) and 2N DNA content (9.7 ± 0.8% vs 8.5 ± 0.4%). A small difference in the spermatid and spermatocyte populations was observed between WT and mutant mice; WT exhibited 68.9 ± 2% of cells with 1N DNA content and Rex1 mutants 72.9 ± 0.8% (p = 0.03). These results indicate that defects in cell cycle distribution/progression cannot explain the reduction in sperm count in older Rex1+/− males.

Microarray analysis of WT and Rex1+/− primary germ cells reveals effects on the transcriptome

To investigate if Rex1 expression influences the transcriptome in testicular cells we performed microarray analyses on isolated primary germ cells from the seminiferous tubules of six week old WT and Rex−/− mice. We were not able to detect Rex1 mRNA by RT-PCR in

Table 1

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT (Rex1+/+)</td>
<td>60 (0.32)</td>
<td>50 (0.3)</td>
</tr>
<tr>
<td>HT (Rex1+/+)</td>
<td>78 (0.41)</td>
<td>68 (0.4)</td>
</tr>
<tr>
<td>KO (Rex1−/−)</td>
<td>52 (0.27)</td>
<td>51 (0.3)</td>
</tr>
<tr>
<td>Total</td>
<td>190</td>
<td>169</td>
</tr>
</tbody>
</table>
isolated primary germ cells from Rex1−/− mice using primers homologous to exons three and four (Fig. 5B). Three genes showed at least a four fold decrease in expression in Rex1 null mice. Two are unknown genes (Fig. 5A, and Supplemental Table 1), whereas the third is the Growth Hormone Regulated TBC Protein-1, GRTP1, which shows decreased mRNA expression in germ cells of Rex1−/− mice (~4.5 fold, Fig. 5B) (Lu et al., 2001). Although primary germ cells of Rex1 null mouse express less GRTP1 mRNA than germ cells from WT mice.
Fig. 3. Histological evaluation of Follicles, Corporea Lutea, and Ceroid Pigment in WT vs Rex1−/− mice. Ovary histology of twenty one day, six week, and 1 year old mice was evaluated by H&E staining (WT A, C, E; Rex1−/− B, D, F). In panels A thru D black arrows highlight examples of follicles; in panels E and F black arrows highlight examples of corpus luteum. We analyzed ovaries across a range of different ages (WT, n=10, Rex1−/−, n=13 animals). Scale: Black bars denote 100 μm.

Characterization of apoptotic cells, STAT3, phospho-STAT3(Tyr705), p38 and phospho-p38 positive cells in the testes of Rex1−/− mice

Bhandari et al. (2010) have recently shown that Rex1 acts as an inhibitor of the pro-apoptotic p38 mitogen-activated protein kinase (p38-MAPK) in human mesenchymal stem cells. The abnormal morphology of many seminiferous tubules and the lower sperm counts seen in Rex1−/− mutants may reflect an increase in apoptosis. To test this, we performed TUNEL assays to assess the level of apoptosis in the testicular cells of Rex1−/− mice. We defined a 100× magnification section of comparable slides as a field, and evaluated the TUNEL signal in the testes from six week old mice (WT, n=21, KO, n=19). The average number of apoptotic cells observed per field in WT testes was 39±4 (Fig. 6A, C), while the average number of apoptotic cells in Rex1−/− testes was 56±13 (Fig. 6B, C). We observed a large variance in the number of cells undergoing apoptosis within the testes of different WT and Rex1−/− mice, but with the exception of an outlier (Fig. 6B) most animals displayed comparable levels of apoptosis. The apoptotic cells were mostly immature germ cells located in the outer layers of the seminiferous tubule, and we found no correlation between the number/location of immature germ cells and germ cell depletion.

The p38 mitogen activated protein kinases are a well-known class of proteins involved in responses to physiological stress (Han et al., 1994). Since human mesenchymal stem cells in culture show a direct relationship between Rex1 expression and activation of p38 by phosphorylation, we assessed primary germ cells from WT and Rex1−/− mice for phospho-p38 levels. We stained testes for phospho-p38 (P-p38) (WT, n=20, KO, n=19 animals) (Supplemental Figs. 2A, B), and for total p38 levels (WT, n=21, KO, n=19) (Supplemental Figs. 2D, E). There was a high variation in staining within both WT and Rex1−/− samples. Ten Rex1−/− mice displayed phospho-p38 expression as compared to seven WT mice. In most cases phospho-p38 was detected in Sertoli cells (Supplemental Fig. 2B, black arrows), and occasionally in germ cells (Supplemental Fig. 2B, white arrows). There was no statistically significant
difference between the numbers of seminiferous tubules expressing phospho-p38 between WT and Rex1−/− mice (Supplemental Fig. 2C). Comparable total p38 levels were observed in the cytoplasm of both Sertoli and a range of immature germ cells in WT and Rex1−/− (Supplemental Figs. 2D, E). In mature spermatids, p38 staining was detected in the nucleus of unaffected seminiferous tubules (Fig. 6F). The staining of the immature germ cells (DazL), proliferating cell nuclear antigen (PCNA) (Fig. 6C). In sections from both WT and Rex1−/− mice we observed positive staining for PCNA within well organized spermatogonia (Fig. 6D). Consistent with our observation that Rex1−/− mice display depletion of their germ cell compartment, PCNA staining was often absent from spermatogonia within the tubules of the sections from the Rex1−/− mice (Fig. 6D).

The structural design of the testis is well defined, with germ cell differentiation and maturation occurring in well synchronized steps. Sox9 expression appears to be tightly associated with Sertoli cell development (Kent et al., 1996; Morais da Silva et al., 1996; Vidal et al., 2001). Sox9 expression remains in Sertoli cells exclusively after testis cord formation and is used as a Sertoli cell marker (Hemendinger et al., 2002; Morais da Silva et al., 1996). To investigate the status of Sertoli cells in Rex1−/− mice, we examined Sox9 protein in WT and Rex1−/− mice, nuclear staining was predominantly restricted to spermatogonia and was evenly distributed within this population along the outer edges of the seminiferous tubules (Figs. 6A, B). This indicates that Rex1 expression is not necessary for maintenance of the most immature type of germ cells, the spermatogonia.

To evaluate the proliferation of germ cells, sections were stained with proliferating cell nuclear antigen (PCNA) (Fig. 6C). In sections from both WT and Rex1−/− mice we observed positive staining for PCNA within well organized spermatogonia (Fig. 6D). Consistent with our observation that Rex1−/− mice display depletion of their germ cell compartment, PCNA staining was often absent from spermatogonia within the tubules of the sections from the Rex1−/− mice (Fig. 6D).

The mature testis is composed of many different cell types, including germ cells and Sertoli cells. Germ cells are closely associated with Sertoli cells throughout their development and differentiation, and in wild type mice Rex1 is predominantly expressed in spermatocytes undergoing meiosis (Rogers et al., 1991). To examine how the lack of Rex1 affects testis architecture, histological sections of testicular tissues from WT and Rex1−/− mice were immunostained with markers of specific cell types. First, sections were stained with DazL, a protein known to be expressed in immature germ cells (Saunders et al., 2003). In both WT and Rex1−/− mice, nuclear staining was predominantly restricted to spermatogonia and was evenly distributed within this population along the outer edges of the seminiferous tubules (Figs. 6A, B). This indicates that Rex1 expression is not necessary for maintenance of the most immature type of germ cells, the spermatogonia.

Discussion

Rex1 (Zfp42) is a zinc finger transcription factor (Hosler et al., 1989) and is expressed in human and murine stem cells (Boyer et al., 2006; Brivanlou et al., 2003; Loh et al., 2006; Masui et al., 2007; Mongan et al., 2006; Rogers et al., 1991). Although Rex1 is not necessary for the maintenance of ES cell pluripotency, Rex1−/−
deficient ES cells do show a greater tendency to differentiate towards somatic lineages (Scotland et al., 2009; Toyooka et al., 2008). To understand the function of Rex1 in stem cells, we made a conditional knockout mouse that lacks this gene. We show here that mutant mice deficient in Rex1 are viable and fertile. This is the first conditional Rex1 knockout mouse line to be reported.

Since the testis expresses a high level of Rex1 RNA (Rogers et al., 1991), we examined Rex1−/− mutant mice testes and found mild to moderate germ cell depletion (Figs. 2B, C, D, E), with early/late spermatids being the population mostly affected. Additionally, Rex1−/− mutant mice displayed abnormal sperm morphology. These data could indicate defects in DNA replication or chromosome segregation in meiosis, a phase during which differentiation/maturation of germ cells occurs and when Rex1 is highly expressed (Kristensen et al., 2008; Rogers et al., 1991). Alternatively, this phenotype could indicate that Rex1−/− mice are undergoing accelerated aging (Zhang et al., 2006). Yet another possibility is that the phenotypes can be attributed to defects in X chromosome inactivation. X chromosome inactivation is an example of an epigenetic regulatory mechanism used to equalize the X linked gene dosage. During mammalian germ cell development the X

---

**Fig. 4.** Cell cycle analysis. Testicular cells were prepared from the testes of 4–6 month old mice (WT, n = 6, Rex1−/−, n = 7) following the protocol described in Materials & methods. ~2 × 10⁶ cells were stained with propidium iodide (PI) to analyze the cell cycle. *p < 0.05, t-test.
chromosome is transiently silenced at meiosis (Namekawa et al., 2006; Nguyen and Disteche, 2006), presumably to equalize the X linked gene dosage during those critical steps of development. In cultured mES cells Rex1 was recently identified as a required factor for efficient elongation of Tsix, a key non-coding transcript involved in X-inactivation (Navarro et al., 2010), and we and others have identified key regulators of X-inactivation as differentially expressed genes in global gene expression analysis (Masui et al., 2008; Scotland et al., 2009). Kim et al. recently characterized a mouse line in which the β-geo gene trap vector was inserted into the third intron of the Rex1 gene; since the entire open reading frame for Rex1 protein is present in the fourth exon (Hosler et al., 1989, Hosler et al., 1993), we would predict that the entire Rex1 protein would still be produced in this β-geo gene trap mouse line (Kim et al., 2011).

Spermatogenesis is a highly organized process that takes place in three distinct phases in the seminiferous epithelium: spermatogonial proliferation (spermatogoniogenesis), meiosis of spermatocytes, and differentiation of haploid round spermatids into elongated spermatids (spermiogenesis) (Leblond and Clermont, 1952). Each phase is accompanied by morphological and biochemical changes (Johnston et al., 2008) and changes in regulation of the cell cycle (Kierszenbaum, 2006). The entire process can be subdivided into 12 stages. Although we have not analyzed each step in depth, we studied cellular pathways relevant to the observed phenotype. We did not observe statistically significant differences between populations with 1N, 2N and 4N DNA content within the testes of 4–6 month old mice, indicating that Rex1 ablation does not interfere with cell cycle distribution in male germ cells (Fig. 3). Similarly, the evaluation of apoptosis in the testis does not show a statistically significant difference between WT and Rex1−/− mice, although there was high variability within individual testes. The above phenotypes of Rex1−/− mice are variable, suggesting incomplete penetrance. One possible explanation for this is that the ubiquitous polycomb transcription factor Yin Yang 1 (YY1) may be compensating for Rex1 to some extent (Kim et al., 2007; Mongan et al., 2006). A high degree of homology exists between Rex1 and YY1, and although YY1 gene knockout results in embryonic lethality during peri-implantation (Donohoe et al., 1999), a conditional knockout of YY1 showed defects in spermiogenesis (Wu et al., 2009). When YY1 expression was lost in...
the testes, testis size was reduced to a third the size of YY1 heterozygous mice (YY1f/+\text{Cre}), and the testes had abnormal and vacuolated seminiferous tubules and less pachytene spermatocytes (Wu et al., 2009). Stat3 is an essential mammalian protein with well-defined roles in the maintenance of mES cell pluripotency (Raz et al., 1999). Phosphorylation of the STAT3 protein leads to the formation of active STAT3 dimers, which translocate to the nucleus and work as active transcription factors (Zhang et al., 1995). Rex1\textsuperscript{−/−} mice show strong cytoplasmic staining of total Stat3 in Sertoli cells, spermatogonia, and spermatocytes, particularly in the seminiferous tubules with obvious atrophy/germ cell depletion (Fig. 5G). Characterization of the more immature germ cells (DazL), proliferating germ cells (PCNA), and Sertoli cells (Sox9) (Figs. 6A-F) indicates that those populations are unaffected by the lack of Rex1 expression. These results, along with the abnormal sperm morphologies (Fig. 2G) and spermatid depletion in Rex1\textsuperscript{−/−} mice, indicate that Rex1 expression affects the later stages of spermatogenesis.

Lastly, from the microarray analysis, we identified a growth hormone regulated gene, GRTPI, that was transcriptionally downregulated in Rex1\textsuperscript{−/−} germ cells. Although hormones such as follicle stimulating hormone (FSH), luteinizing hormone (LH), and testosterone have all been shown to play important roles in germ cell biology, it is generally accepted that hormone triggered physiology effects occur via signaling from the neighboring Sertoli cells, as germ cells do not contain receptors for hormones (Ruwanpura et al., 2010). It is unclear if the lack of Rex1 leads to a cell intrinsic defect in germ cell hormonal response. In mouse ES cells the key regulators of mammalian X-inactivation, Tsix and Xist, were respectively identified as a 3.6 fold downregulated gene in Rex1\textsuperscript{−/−} (Masui et al., 2008), and a ~2.9 fold upregulated gene in the presence and absence of LIF (Scotland et al., 2009). We have previously studied in detail the mechanisms underlying the transcriptional activation of the suppressor of cytokine signaling 3 (SOCS3) gene by a combination treatment of retinoic acid, theophylline and dibutyryl cyclic AMP in F9 cells. By promoter deletion, mutation and transient transfection analysis we identified this transcriptional increase to be mediated by STAT3 DNA binding elements in the SOCS-3 promoter. We also observed activation of phospho-STAT3 in the context of differentiation when a retinoic acid, theophylline and dibutyryl cyclic AMP combination treatment was used; we concluded that Rex1 plays an indirect role in the Janus Kinase (JAK)/signal transducer and activator pathway.

Our results provide in vivo genetic evidence demonstrating that Rex1 plays an important role within the testis. We are currently investigating if loss of Rex1 leads to defects in other adult stem cell compartments and/or misregulation of adult stem cell differentiation.

Supplementary materials related to this article can be found online at doi:10.1016/j.ydbio.2011.05.664.
Fig. 7. Evaluation of Germ cell, proliferating, and Sertoli cell populations immunohistological evaluation. Six week old mice were assessed by immunohistochemistry using antibodies to Dazl, PCNA and Sox9. (A, B) Dazl staining of both wild type and Rex1−/− mice (33 wt, 28 Rex1−/−). Black arrows highlight examples of the most immature, Dazl positive germ cells within the WT and Rex1−/− testes. (C, D) PCNA staining of WT and Rex1−/− testes (33 wt, 28 Rex1−/−). Black arrows highlight examples of proliferating germ cells. (E, F) Sox9 staining of WT and Rex1−/− testes (34 wt, 26 Rex1−/−). Black arrows highlight examples of Sertoli cells of both WT and Rex1−/− mice.

Acknowledgments

We thank all of the members of the Gudas laboratory for scientific discussions, Tamara Weissman for editorial assistance, the WCMC Genomics Core Facility and the Histology Core of Weill Cornell Medical College for section preparation, and the Transgenic Core Facility for ES cell injections into blastocysts. We would like to thank Sergei Rudchenko and Stanka Semova for their assistance with the FACS analysis in the WCMC FACS Core Facility, and Jacqueline Bromberg from Memorial Sloan Kettering Cancer Center for guidance with STAT3 evaluation in the WCMC FACS Core Facility and the Histology Core of Weill Cornell Medical College for section preparation, and the Transgenic Core Facility for ES cell injections into blastocysts. We would like to thank Sergei Rudchenko and Stanka Semova for their assistance with the FACS analysis in the WCMC FACS Core Facility, and Jacqueline Bromberg from Memorial Sloan Kettering Cancer Center for guidance with STAT3 analysis in the WCMC FACS Core Facility and the Histology Core of Weill Cornell Medical College for section preparation, and the Transgenic Core Facility for ES cell injections into blastocysts. We would like to thank Sergei Rudchenko and Stanka Semova for their assistance with the FACS analysis in the WCMC FACS Core Facility, and Jacqueline Bromberg from Memorial Sloan Kettering Cancer Center for guidance with STAT3 analysis in the WCMC FACS Core Facility and the Histology Core of Weill Cornell Medical College for section preparation, and the Transgenic Core Facility for ES cell injections into blastocysts. We would like to thank Sergei Rudchenko and Stanka Semova for their assistance with the FACS analysis in the WCMC FACS Core Facility, and Jacqueline Bromberg from Memorial Sloan Kettering Cancer Center for guidance with STAT3 analysis in the WCMC FACS Core Facility and the Histology Core of Weill Cornell Medical College for section preparation, and the Transgenic Core Facility for ES cell injections into blastocysts. We would like to thank Sergei Rudchenko and Stanka Semova for their assistance with the FACS analysis in the WCMC FACS Core Facility, and Jacqueline Bromberg from Memorial Sloan Kettering Cancer Center for guidance with STAT3 analysis in the WCMC FACS Core Facility and the Histology Core of Weill Cornell Medical College for section preparation, and the Transgenic Core Facility for ES cell injections into blastocysts. We would like to thank Sergei Rudchenko and Stanka Semova for their assistance with the FACS analysis in the WCMC FACS Core Facility, and Jacqueline Bromberg from Memorial Sloan Kettering Cancer Center for guidance with STAT3 analysis in the WCMC FACS Core Facility and the Histology Core of Weill Cornell Medical College for section preparation, and the Transgenic Core Facility for ES cell injections into blastocysts. We would like to thank Sergei Rudchenko and Stanka Semova for their assistance with the FACS analysis in the WCMC FACS Core Facility, and Jacqueline Bromberg from Memorial Sloan Kettering Cancer Center for guidance with STAT3 analysis in the WCMC FACS Core Facility and the Histology Core of Weill Cornell Medical College for section preparation, and the Transgenic Core Facility for ES cell injections into blastocysts. We would like to thank Sergei Rudchenko and Stanka Semova for their assistance with the FACS analysis in the WCMC FACS Core Facility, and Jacqueline Bromberg from Memorial Sloan Kettering Cancer Center for guidance with STAT3 analysis in the WCMC FACS Core Facility and the Histology Core of Weill Cornell Medical College for section preparation, and the Transgenic Core Facility for ES cell injections into blastocysts. We would like to thank Sergei Rudchenko and Stanka Semova for their assistance with the FACS analysis in the WCMC FACS Core Facility, and Jacqueline Bromberg from Memorial Sloan Kettering Cancer Center for guidance with STAT3 analysis in the WCMC FACS Core Facility and the Histology Core of Weill Cornell Medical College for section preparation, and the Transgenic Core Facility for ES cell injections into blastocysts. We would like to thank Sergei Rudchenko and Stanka Semova for their assistance with the FACS analysis in the WCMC FACS Core Facility, and Jacqueline Bromberg from Memorial Sloan Kettering Cancer Center for guidance with STAT3 analysis in the WCMC FACS Core Facility and the Histology Core of Weill Cornell Medical College for section preparation, and the Transgenic Core Facility for ES cell injections into blastocysts. We would like to thank Sergei Rudchenko and Stanka Semova for their assistance with the FACS analysis in the WCMC FACS Core Facility, and Jacqueline Bromberg from Memorial Sloan Kettering Cancer Center for guidance with STAT3 analysis in the WCMC FACS Core Facility and the Histology Core of Weill Cornell Medical College for section preparation, and the Transgenic Core Facility for ES cell injections into blastocysts. We would like to thank Sergei Rudchenko and Stanka Semova for their assistance with the FACS analysis in the WCMC FACS Core Facility, and Jacqueline Bromberg from Memorial Sloan Kettering Cancer Center for guidance with STAT3 analysis in the WCMC FACS Core Facility and the Histology Core of Weill Cornell Medical College for section preparation, and the Transgenic Core Facility for ES cell injections into blastocysts.

References


