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Insulin-Like 3 Signaling Is Important for Testicular Descent but Dispensable for Spermatogenesis and Germ Cell Survival in Adult Mice[1]

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ABSTRACT

Relaxin family peptide receptor 2 (RXFP2) is the cognate receptor of a peptide hormone insulin-like 3 (INSL3). INSL3 is expressed at high levels in both fetal and adult Leydig cells. Deletion of *Insl3* or *Rxfp2* genes in mice caused cryptorchidism resulting from a failure of gubernaculum development. Using a novel mouse transgenic line with a knock-in LacZ reporter in the *Rxfp2* locus, we detected a robust *Rxfp2* expression in embryonic and early postnatal gubernaculum in males and in postmeiotic spermatogenic cells in adult testis. To study the role of INSL3/RXFP2 signaling in male reproduction, we produced a floxed *Rxfp2* allele and used the Cre/loxP approach to delete *Rxfp2* in different tissues. Using *Cre* transgene driven by retinoic acid receptor beta promoter, conditional gene targeting in gubernacular mesenchymal cells at early embryonic stages caused high intraabdominal cryptorchidism as in males with a global deletion of *Rxfp2*. However, when the *Rxfp2* was deleted in gubernacular smooth or striated muscle cells, no abnormalities of testicular descent or testis development were found. Specific ablation of *Rxfp2* in male germ cells using *Stra8-icre* transgene did not affect testis descent, spermatogenesis, or fertility in adult males. No significant change in germ cell apoptosis was detected in mutant males. In summary, our data indicate that the INSL3/RXFP2 signaling is important for testicular descent but dispensable for spermatogenesis and fertility in adult males.

INSL3, RXFP2, spermatogenesis, testicular descent

INTRODUCTION

During embryonic development in mammals, both male and female gonads are located at a high pararenal position at the time of sex differentiation. The gonads are connected by two ligaments to the body wall: the cranial mesonephric ligament and the caudal genitoinguinal ligament or the gubernaculum [1, 2]. After sex determination, male gonads start the process of testicular descent resulting in testes positioned in the scrotum while female gonads remain in the high abdominal position. Testicular descent can be divided into two phases: the transabdominal phase (in mice from embryonic day 14.5 [E14.5] to E17.5) and the inguinoscrotal phase (in mice from E18 to postnatal day 21 [P21]) [3]. During testicular descent, the cranial ligament regresses while the gubernaculum undergoes a series of differentiation steps. At the transabdominal phase, the gubernaculum differentiates into two structures: the gubernacular cord and the bulb. This is followed by the differentiation of the muscle layers of the bulb, composed primarily of striated and some smooth muscle. Further development of the gubernaculum involves the enlargement of the bulb through cell proliferation and extracellular matrix deposition. In the beginning of the inguinoscrotal phase, the processus vaginalis forms via the outgrowth and invagination of the gubernacular bulb. The components of the fetal gubernaculum or abdominal body wall further differentiate into cremaster muscle and the wall of a cremasteric sac. By P12, the distal end of the processus vaginalis reaches the end of the scrotum, and the epididymis and testis are located inside the scrotum [4].

Insulin-like 3 (INSL3) hormone signaling via its cognate G protein-coupled receptor, relaxin family peptide receptor 2 (RXFP2), and androgen hormone signaling via androgen receptor play crucial roles in the transabdominal and inguinoscrotal phases, respectively [5, 6]. INSL3 is produced in fetal and adult Leydig cells and is first detected at E13.5 in mouse just before the start of testicular descent [7]. Coincidently, the mRNA of *Rxfp2* expression was clearly detected at E14.5 specifically in gubernacular tissue [8]. The *Insl3* or *Rxfp2* global knockout in mice resulted in the same phenotype of high intraabdominal cryptorchidism and male infertility caused by spermatogenesis arrest [9–12]. In contrast, transgenic overexpression of *Insl3* induced gubernaculum development and ovary descent in female mice [13]. Quantitative RT-PCR (QRT-PCR) showed an increase in mouse *Rxfp2* expression in the postnatal adult cremasteric sac [14]. We also detected strong positive staining in adult cremaster muscles using several commercially available RXFP2 antibodies [14]. There are however some discrepancies in the RXFP2 expression data in the testis. In situ hybridization indicated that *Rxfp2* is expressed in rat postmeiotic germ cells of the seminiferous tubules but not in adult Leydig cells [15], whereas the RT-PCR and immunohistochemistry showed the *Rxfp2* expression in human, rat, and mouse postnatal germ and interstitial somatic cells [14, 16, 17].

The global deletion of *Rxfp2* gene in existing conventional mutants and the somewhat conflicting data on tissue and cellular gene expression make it difficult to identify direct targets of INSL3/RXFP2 signaling in the gubernaculum and testis. In adult male mice, INSL3 is strongly expressed in Leydig cells, but its role in spermatogenesis remains unclear. While the absence of INSL3/RXFP2 signaling in mutant mice could contribute to male infertility, the suppression of spermatogenesis in cryptorchid mutants may be in fact the direct result of abnormal testis position and environment.

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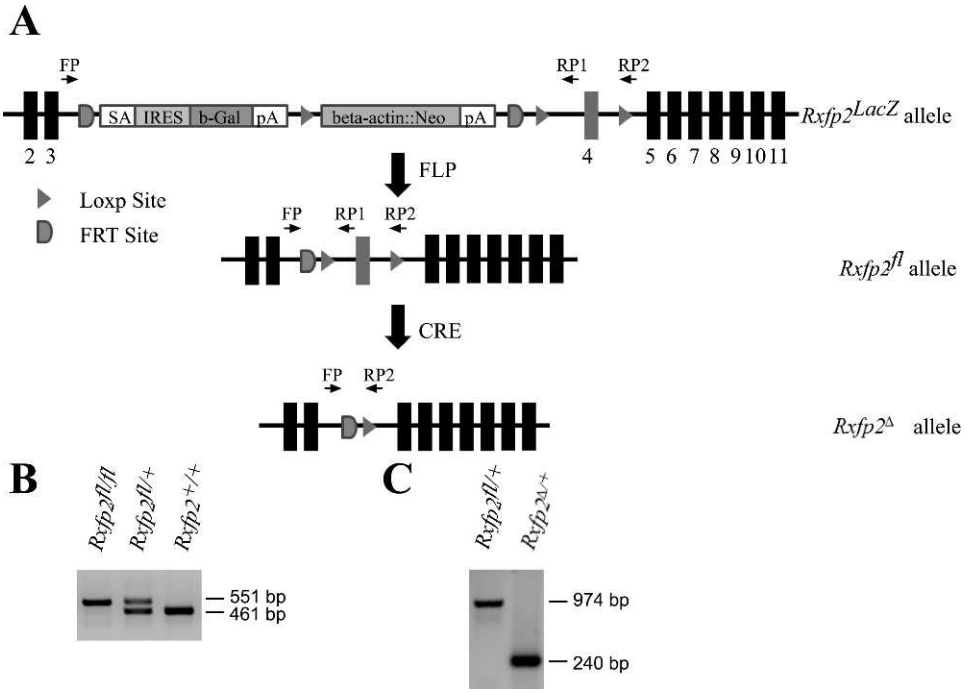

FIG. 1. LacZ knock-in and floxed alleles of *Rxfp2*. **A**) Schematic representation of *Rfxp2*^{LacZ}, *Rxfp2*^{fl}, and *Rxfp2*$^{\Delta}$ alleles. Exons are shown as solid black bars and marked by a number underneath. SA is a splicing acceptor; IRES is an internal ribosome entry site; b-Gal is a beta-galactosidase gene; pA is a poly (A) signal, beta-actin::Neo is a neomycine resistant gene driven by β-actin promoter; FLP is *flp* recombinase; and CRE is *cre* recombinase. *Rxfp2*^{fl} allele is produced by *flp*-induced recombination, and the deleted allele is produced by *cre*-induced recombination as shown. The position of the primers used for genotyping is shown with arrows. **B**) Detection of *Rxfp2*^{fl} floxed allele with primers FP/RP1. The floxed allele produced a 551-bp band, and the wild-type allele produced a 461-bp band. **C**) Detection of *Rxfp2*$^{\Delta}$ deleted allele with FP/RP2 primers. The floxed allele produced a 974-bp band, and the deleted allele produced a 240-bp band. The wild-type allele is not detectable with this primer pair.

Indeed, orchiopexy, or surgical correction of the cryptorchid testis position, restored spermatogenesis in mutant mice [10, 11, 18]. On the other hand, it was reported that INSL3 suppressed testis germ cell apoptosis induced by gonadotropin-releasing hormone (GnRH) antagonist injections in rats, suggesting a role for this hormone in germ cell survival [15].

In this study we generated two new alleles of *Rxfp2* in mice. To map the expression pattern of *Rxfp2*, we produced a LacZ knock-in allele. The expression of the LacZ reporter was used to identify potential target cells of INSL3/RXFP2 signaling at different stages of prenatal and postnatal development and in adult male reproductive organs. We also produced a floxed allele of *Rxfp2* and analyzed the consequences of *Rxfp2* deletion in different cells of the gubernacular ligament and in postnatal male germ cells. The results of this study indicate that INSL3/RXFP2 signaling is important for early stages of testicular descent but is dispensable for spermatogenesis and germ cell survival in adult males.

MATERIALS AND METHODS

Production of New Alleles of Rxfp2 *and Mouse Breeding*

All the animal studies were approved by the Institutional Animal Care and Use Committees at Florida International University. The embryonic stem (ES) cells with a targeted *Rxfp2* allele were obtained from the EuCOMM/EuMMCR collection. The recombinant allele contains an *frt*-flanked LacZ/neo cassette inserted into the third intron and a floxed fourth exon. This allele is therefore a knock-in, null allele of the gene (*Rxfp2*^{LacZ}) (Fig. 1). Chimeric mice were produced at the University of Miami Transgenic Core Facility, Miami, FL. Confirmation of germ line transmission of the mutant allele was performed by PCR using the genomic DNA isolated from ear pieces with primers specific for LacZ: LacZF, CAGACGATGGTGCAGGATAT, and LacZR, ATACAGCGCGTCGTGAT. Mice with the floxed *Rxfp2* allele (*Rxfp2*^{fl}) were produced by breeding *Rxfp2*^{LacZ} females with *ROSA-FLP* (*Gt [ROSA] 26Sor*$^{tm1[FLP1]Dym}$*/RainJ*) transgenic mice [19] obtained from The Jackson Laboratory. The PCR primers used for genotyping of *Rxfp2*^{fl} allele were: FP, CCAAATCAAATATCCATAGGATCAGC, and RP1, TTAGTGGATCCAC TGAGCCCTTCC. Excision of the floxed fourth exon resulted in a deleted allele, *Rxfp2*$^{\Delta}$, which was detected by PCR using primers FP and RP2, TGAACTGATGGCGAGCTCAGACC. The latter combination of primers did not detect wild-type allele.

Conditional inactivation of *Rxfp2* was achieved by crossing *Rxfp2*$^{fl/fl}$ mice with Cre-transgenic mice and backcrossing to *Rxfp2*$^{-/-}$ [9] or *crsp*/*crsp* (deletion alleles of *Rxfp2*) [10] female mice. Four different Cre-transgenic strains were used: *Rarb-cre* (*Tg [Rarb-cre]1Bhr*) [20], a generous gift from Dr. Richard Behringer (MD Anderson Cancer Center), *ACTA1-cre* (*Tg [ACTA1-cre] 79Jme/J*) [21], *Tagln-cre* (*Tg [Tagln-cre] 1Her/J*) [22], and *Stra8-icre* (*Tg [Stra8-icre] 1Reb/J*) [23], all from The Jackson Laboratory. Animal genotyping was performed with *cre* primers creF, ATCAACGTTTTCTTTTCGG, and creR, ATTTGCCTGCATTACCGGTC, and the primers for different *Rxfp2* alleles and *icre* described in the original publications.

For fertility testing, individual mutant male mice and control male mice were each mated continuously with two wild-type CD-1 (Charles River) females for 2 mo. The number of litters and the litter sizes were recorded and compared. At least three males of each genotype were used in the analysis. To assess sperm count and motility, sperm was released from the cauda epididymis into M2 medium (Millipore), and the motility and numbers were scored using microscopic observations.

Histology, Immunohistochemistry, and Apoptosis Detection

Adult mouse organs were collected and fixed in 4% paraformaldehyde or Bouin solution, washed with phosphate-buffered saline (PBS), stored in 70% ethanol at 4°C overnight, embedded in paraffin, and sectioned at 6 or 7 μm using standard protocols. Hematoxylin and eosin (H&E) staining was performed for routine analysis of the sections. A TUNEL assay was performed using an ApopTag Plus peroxidase in situ apoptosis detection kit (Millipore). Stained slides were examined with a Carl Zeiss Axio A1 microscope, and images were captured by an AxioCam MRc5 CCD camera. Three animals were used for each genotype, and for each animal testis section, over 200 tubules

were counted and the number of apoptotic cells per tubule was used for comparison.

RNA Isolation and cDNA Synthesis and Real-Time QRT-PCR

Total RNA was isolated from mouse testis with Trizol (Invitrogen) according to the manufacturer's protocol. Complementary DNA was synthesized using Verso cDNA kit (Thermo Scientific) according to the manufacturer's protocol. The expression of *Rxfp2* gene was evaluated by conventional RT-PCR and real-time QRT-PCR. GoTaq qPCR master mix (Promega) kit was used for real-time QRT-PCR. Primers were designed to span a long intron to avoid amplification from contaminating genomic DNA. The primer sequences are: 1) for β-actin (*Actb*) gene used in RT-PCR and QRT-PCR: ActbF, CTAAGGCCAACCGTGAAAAG, and ActbR, ACCAGAGG CATACAGGGACA; 2) for *Rxfp2* gene used in RT-PCR: mRxfp2ex2F, GTGGGAATCTCACCAAATGC, and mRxfp2ex4R, GTGCTGTGGA TACTGGCTGA; and 3) for *Rxfp2* gene used in QRT-PCR: mRxfp2ex3F, CCATGGGAATGTCAATAAAGTG, and mRxfp2ex4R, TCTGCAGTAA CAGTGCTGTGG. The SybrGreen real-time protocol was run on an Eppendorf Mastercycler ep realplex instrument (Eppendorf). The relative fold change in mRNA level was calculated by the comparative C_t ($2^{-\Delta\Delta}C_t$) method, where the β-actin expression was used for normalization of the SybrGreen data. Student *t*-test for two group comparisons was used to assess the significance of differences. Differences were expressed as a mean ± SEM; $P < 0.05$ was considered significant. All the analyses were performed using the GraphPad Software package (GraphPad Software).

X-Gal Staining

Briefly, after fixing for 60 min in 4% formaldehyde in PBS at 4°C, tissues were rinsed for 45 min in 0.02% Nonidet P-40, 2 mM $MgCl_2$, and 0.01% sodium deoxycholate with shaking, and stained with X-gal staining solution (1 mg/ml) at 37°C in a humid chamber overnight [24]. The tissue was then washed with PBS, postfixed overnight in 4% formaldehyde in PBS, and then processed through increasing concentrations of ethanol, dehydrated, and paraffin wax embedded. Seven-micrometer sections were cut and counterstained with eosin.

Propidium Iodide Staining and Flow Cytometry of Mouse Testis Cells

The tunica albuginea was removed from the mouse testis, and decapsulated testes were incubated in Gey balance salt solution (Sigma-Aldrich) containing 0.33 mg/ml collagenase and 3.3 μg/ml DNase (Sigma-Aldrich) for 15 min at 32°C with shaking. The digested tubules were washed twice with PBS and incubated with 1.0 μg/ml trypsin and DNAse in PBS for 15 min at 32°C. Fetal bovine serum was added to stop the digestion and the suspension was mixed by gentle pipetting for 4 min. The cell suspension was filtered through a 100 μm nylon mesh (Fisher Scientific). The filtered single cell suspension was washed twice with PBS, and the cells were counted. After counting, the cells were fixed in 70% ice-cold ethanol and stored at 4°C until flow cytometry analysis. The fixed cells were washed twice in PBS and stained with staining solution containing 25 μg/ml propidium iodide, 40 μg/ml RNase, and 0.3% Tween-20 in PBS at room temperature for 20 min. Later the stained cells were analyzed in an AccuriC6 flow cytometer (Becton-Dickinson Immunocytometry). The fluorescent signals of propidium iodide-stained cells were recorded, and a histogram of DNA intensity versus cell count was used to compare cell populations from different samples. A total of 500 000 events were recorded for each histogram. The relative numbers of cells (1N = haploid, 2N = diploid, 4N = tetraploid, and cells in the S-phase) were calculated using the software of the flow cytometer. Three animals were analyzed for control and mutant groups. Student *t*-test for two group comparisons was used to assess the significance of the differences. Differences were expressed as a mean ± SEM; $P < 0.05$ was considered significant. All the analyses were performed using the GraphPad Software package.

RESULTS

Production of Rxfp2^{LacZ} and Rxfp2^{fl} Alleles

To produce the *Rxfp2*^{LacZ} mouse line, we obtained an ES cell clone with a targeted allele of *Rxfp2* from EuCOMM/EuMMCR. The targeting allele contains two FRT sites for flippase recombination. The two FRT sites flank a LacZ gene with an internal ribosome entry site (IRES) and a neomycin resistance (*neo*) cassette. The target allele also contains three LoxP sites for Cre recombinase on the 5′ and 3′ ends of *neo* cassette and within intron 4 (Fig. 1A). As the LacZ reporter is transcribed with *Rxfp2* mRNA, it faithfully mirrors the expression of the *Rxfp2* gene, whereas the expression of endogenous full-length *Rxfp2* mRNA is disrupted. Germline transmission of the targeted allele from chimeric males allowed the establishment of the *Rxfp2*^{LacZ} mutant line. After interbreeding heterozygotes, we obtained homozygous *Rxfp2*^{LacZ}/*Rxfp2*^{LacZ} males. We also produced males with the *Rxfp2*^{LacZ} allele and previously generated *Rxfp2*$^{-}$ knockout [9] or *crsp* (complete deletion of *Rxfp2* locus) [10] alleles. Males with all three genotypes showed the same high intraabdominal cryptorchidism as *Rxfp2*$^{-/-}$, demonstrating the absence of *Rxfp2*$^{-}$ complementation by *Rxfp2*^{LacZ}. These results confirmed the loss of function of *Rxfp2*^{LacZ} allele and the correct targeting of *Rxfp2* locus. To produce the *Rxfp2*^{fl} mice, we bred the *Rxfp2*^{LacZ} mice with a transgenic mouse line containing a ubiquitously expressed FLP recombinase. In the double mutant mice, the LacZ/*neo* cassette was removed by flippase-induced recombination in all the tissues including the germ line (Fig. 1A). All the tested progeny of such males had a recombinant *Rxfp2*^{fl} allele regardless of flippase transgene presence. Males that were homozygous for *Rxfp2*$^{fl/fl}$ had a normal testis position, indicating that the floxed *Rxfp2*^{fl} allele was fully functional. Cre-induced recombination of the *Rxfp2*^{fl} allele led to the deletion of exon 4, a reading frameshift and premature stop codon in *Rxfp2*$^{\Delta}$ allele (Fig. 1).

Rxfp2 Expression in Male Reproductive Tissues

To monitor the *Rxfp2* gene expression pattern in male reproductive tissues, we used X-gal staining of tissues obtained from phenotypically wild-type males heterozygous for *Rxfp2*^{LacZ} allele (Fig. 2). As noted above, the LacZ reporter was driven by the endogenous *Rxfp2* promoter as part of *Rxfp2* mRNA and was translated through an IRES site. In this study, we focused mainly on the testis and gubernaculum of male mice, the two major sites of *Rxfp2* expression. In E17.5 embryos and in newborn males at P1, *Rxfp2* was expressed in both the gubernacular cord and the gubernacular bulb (Fig. 2, A and B). In the gubernacular bulb, blue staining was clearly detected in the fibroblast core of the bulb as well as in the muscle cell layer at the rims of this structure (Fig. 2, A2 and B2). No detectable *Rxfp2* expression was found at this age in testis. The blue staining was also observed in the stromal and surface epithelial cells of the mutant but not control epididymis (Fig. 2, A2 and B2). In P7 and P14 gubernacula, the overall *Rxfp2* expression pattern remained the same (Fig. 2, C1 and C2). At that stage, the gubernacular cord is represented by a small ligament with strong blue staining. It connects developing cremaster muscles with the cauda epididymis. No *Rxfp2* expression was detected by LacZ staining in testis (data not shown). In P21, P30, and adult males, no expression of *Rxfp2* was found in differentiated cremaster muscle. However, blue staining was still observed in the mesenchymal fibroblast-like cells located between muscle cells of cremasteric sac (Fig. 2, C3–C5) and in epithelial cells surrounding the sac. In testis of P21, P30, and adult males, we detected *Rxfp2* expression in postmeiotic spermatogenic cells, but not in the basal layer of spermatogonial or spermatocyte cells, somatic Sertoli, or peritubular myoid cells of testicular seminiferous tubules (Fig. 2, D2, E2, and F2). Strong expression of the endogenous β-galactosidase gene in adult Leydig cells (Fig. 2F1) and the epithelial cells of epididymis (data not shown) from P14 prevented detailed analysis of *Rxfp2* expression in these cells at later stages of development.

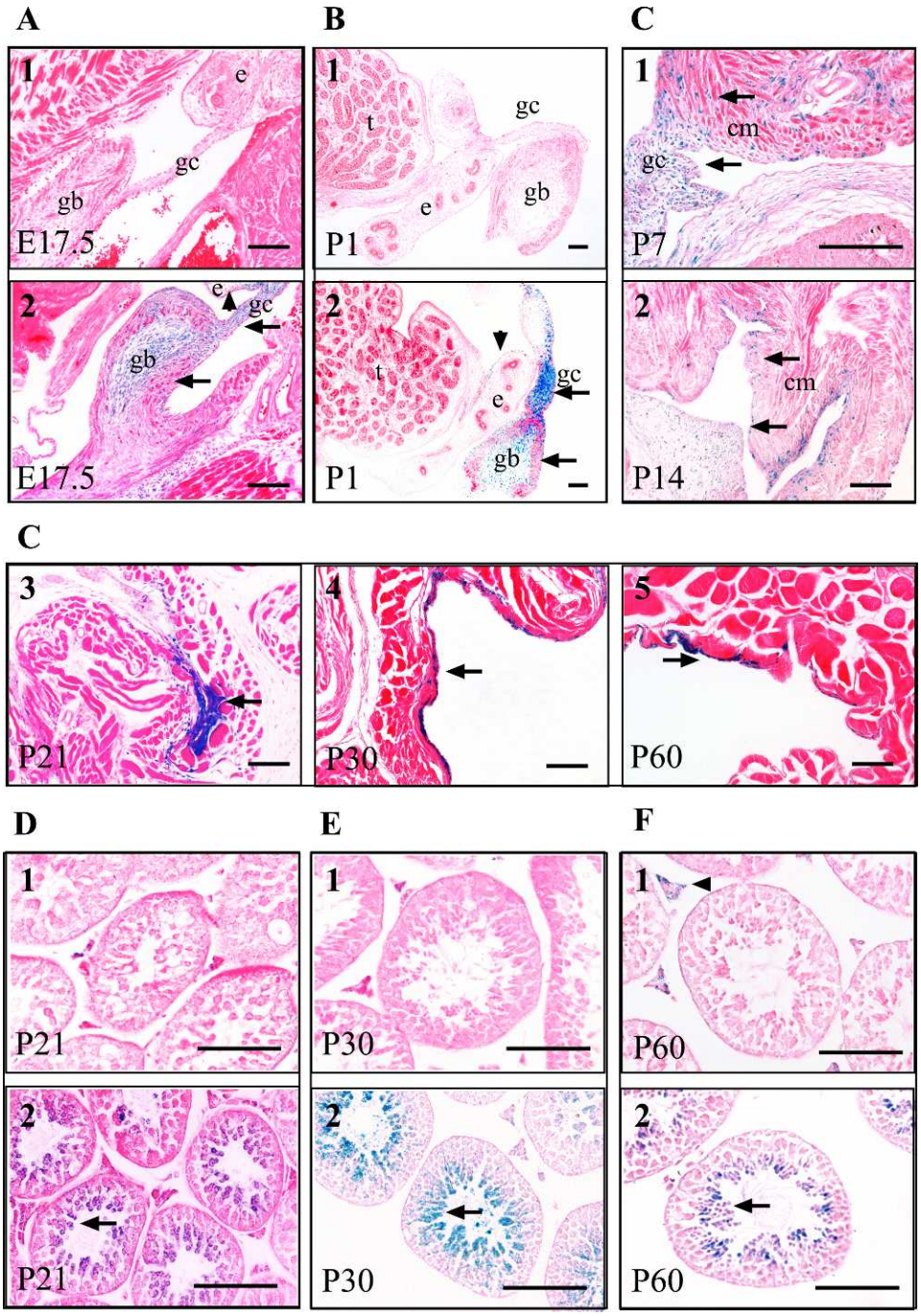

FIG. 2. *Rxfp2* expression in the gubernaculum and testis. The representative images of X-gal staining of $Rfxp2^{LacZ/+}$ male reproductive organs. Blue staining indicates gene expression. **A**–**C**) *Rxfp2* expression in gubernaculum of pre- and postnatal mice. **A**) E17.5 control (image no. **1**) and mutant (**2**). **B**) P1 control (**1**) and mutant (**2**). **C**) Cremasteric sacs of mutants at P7 (**1**), P14 (**2**), P21 (**3**), P30 (**4**), and P60 (**5**). **D**–**F**) *Rxfp2* expression in postnatal testes. **D**) P21 control (**1**) and mutant (**2**). **E**) P30 control (**1**) and mutant (**2**). **F**) P60 control (**1**) and mutant (**2**). Bars = 100 μm. The positive staining is labeled by arrows or arrowheads (in epididymis in **A2** and **B2**). Endogenous expression of β-Gal in adult Leydig cells is indicated by arrowhead in **F1**. e, epididymis; t, testis; gc, gubernacular cord; gb, gubernacular bulb; cm, cremaster muscle.

Conditional Knockout of Rxfp2 *in Gubernacular Embryonic Mesenchymal but Not in Striated or Smooth Muscle Cells Causes Cryptorchidism*

To analyze the target cell population of INSL3/RXFP2 signaling in the gubernaculum and the significance of *Rxfp2* expression in different cellular components of this organ for testicular descent, we produced three different groups of males with a conditional ablation of *Rxfp2*. All such males had one floxed $Rxfp2^{fl}$ allele, one $Rxfp2^{-}$ allele, and the tissue-specific Cre transgene. The $Rxfp2^{fl}/Rxfp2^{-}$ males without the Cre transgene from the same crosses were used as controls. The first Cre transgene, *Rarb-cre*, was previously shown to be expressed in metanephric mesenchyme and its derivatives, including gubernaculum [20, 25, 26]. Conditional ablation of *Rxfp2* before or at early stages of gubernacular formation using *Rarb-cre* led to cryptorchidism with adult testes located in a high intraabdominal position below the kidney (Fig. 3A). Next,

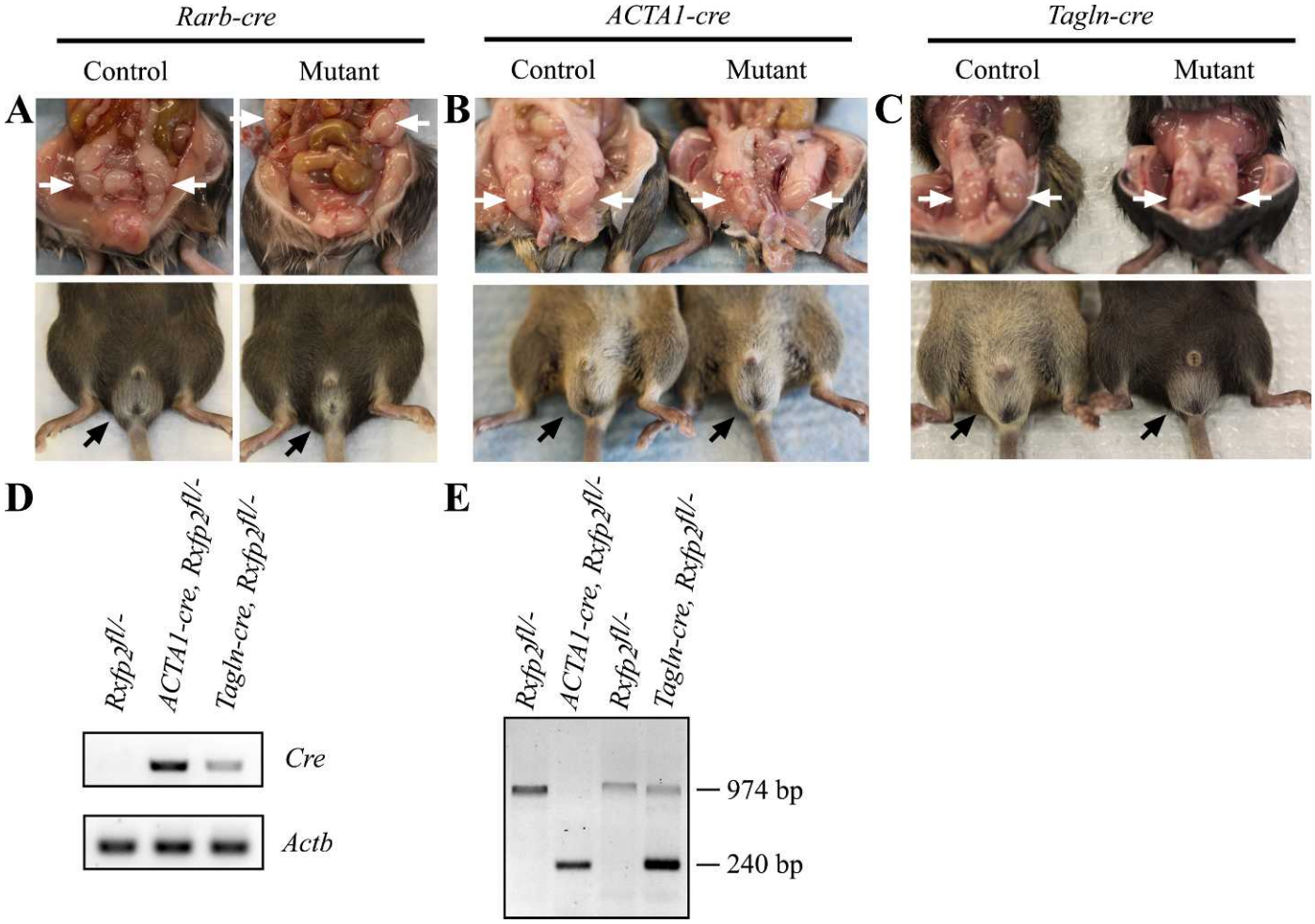

FIG. 3. Conditional knockout of *Rxfp2* in embryonic gubernaculum and in gubernacular striated or smooth muscle cells. **A**–**C**) Testicular position in adult mice with conditional knockout of *Rxfp2* in gubernaculum using the *Rarb-cre* transgene, in striated muscle cells (*ACTA1-cre*), or smooth muscle cells (*Tagln-cre*). The white arrow points to the testis position in the upper row, and the black arrow points to the scrotum in the lower row. Note the high intraabdominal testis position in $Rxfp2^{fl}/Rxfp2^{-}$, *Rarb-cre* mutant, but normal scrotal position in the other mutants. **D**) RT-PCR analysis of *cre* expression in cremaster muscles in mice with *ACTA1-cre* and *Tagln-cre*. The *Actb* gene expression was used as a positive loading control. **E**) PCR analysis of genomic DNA isolated from adult cremasteric sac of control $Rxfp2^{fl}/Rxfp2^{-}$; *ACTA1-cre,* $Rxfp2^{fl}/Rxfp2^{-}$; and *Tagln-cre,* $Rxfp2^{fl}/Rxfp2^{-}$ mice. The lower band corresponds to the deleted allele, and the higher band is the floxed allele as indicated in Figure 1.

we produced mice with conditional ablation of *Rxfp2* in striated or smooth muscle cells of the gubernaculum (Fig. 3, B and C). Both *ACTA1-cre* (striated muscle-specific Cre expression) and *Tagln-cre* (smooth muscle-specific Cre expression) transgenes were expressed in adult cremaster muscle as detected by RT-PCR (Fig. 3D). As shown in Figure 3E, the analysis of genomic DNA isolated from adult cremasteric sac of both $Rxfp2^{fl}/Rxfp2^{-}$, *ACTA1-cre* and $Rxfp2^{fl}/Rxfp2^{-}$, *Tagln-cre* revealed the presence of recombinant $Rxfp2^{\Delta}$ allele. In the striated muscle-specific knockout, the upper amplicon—corresponding to a floxed nonrecombined allele—was not detected, suggesting that the majority of cells in cremaster muscle are striated muscle cells. Because the smooth muscle cells represent only a fraction of the adult cremasteric sac, both small ($Rxfp2^{\Delta}$) and big ($Rxfp2^{fl}$) fragments were detected in DNA isolated from $Rxfp2^{fl}/Rxfp2^{-}$, *Tagln-cre* males. Despite successful targeting of the *Rxfp2* gene in striated and smooth muscle cells, both mutants had a normal testis position (Fig. 3, B and C) and well-developed cremaster muscle (data not shown). The testes were located inside the scrotum of these mice, just as in wild-type control males of the same age (Fig. 3, B and C). The QRT-PCR of the total testis RNA isolated from three to five mutant and control 30-day-old males showed no difference in *Rxfp2* gene expression between the two groups (data not shown). The latter result was expected because of the *Rxfp2* gene silencing in adult muscle cells.

Conditional Knockout of Rxfp2 *Gene in Germ Cells*

The strong *Rxfp2* expression in postmeotic germ cells described above suggested a possible role of INSL3/RXFP2 signaling in spermatogenesis and male fertility in mice. To study the effect of the *Rxfp2* gene ablation in a normally positioned scrotal testis, we produced germ cell-specific *Rxfp2* conditional knockout mice using a *Stra8-icre* transgene. The Cre expression in this transgenic mice is specific for postnatal germ cells and first detected in premeiotic cells [23]. The mutant $Rxfp2^{fl}/Rxfp2^{fl}$, *Stra8-icre* males showed no visible reproductive organ or behavioral abnormalities. Testicular histology was the same in mutants and controls suggesting normal spermatogenesis (Fig. 4A). There were no differences in epididymis, seminal vesicle, and testis weights (Fig. 4, B–D), sperm count (Fig. 4E), and sperm motility (data not shown) between mutant and control males. The fertility testing found no difference in fecundity, with identical litter size (Fig. 4F)

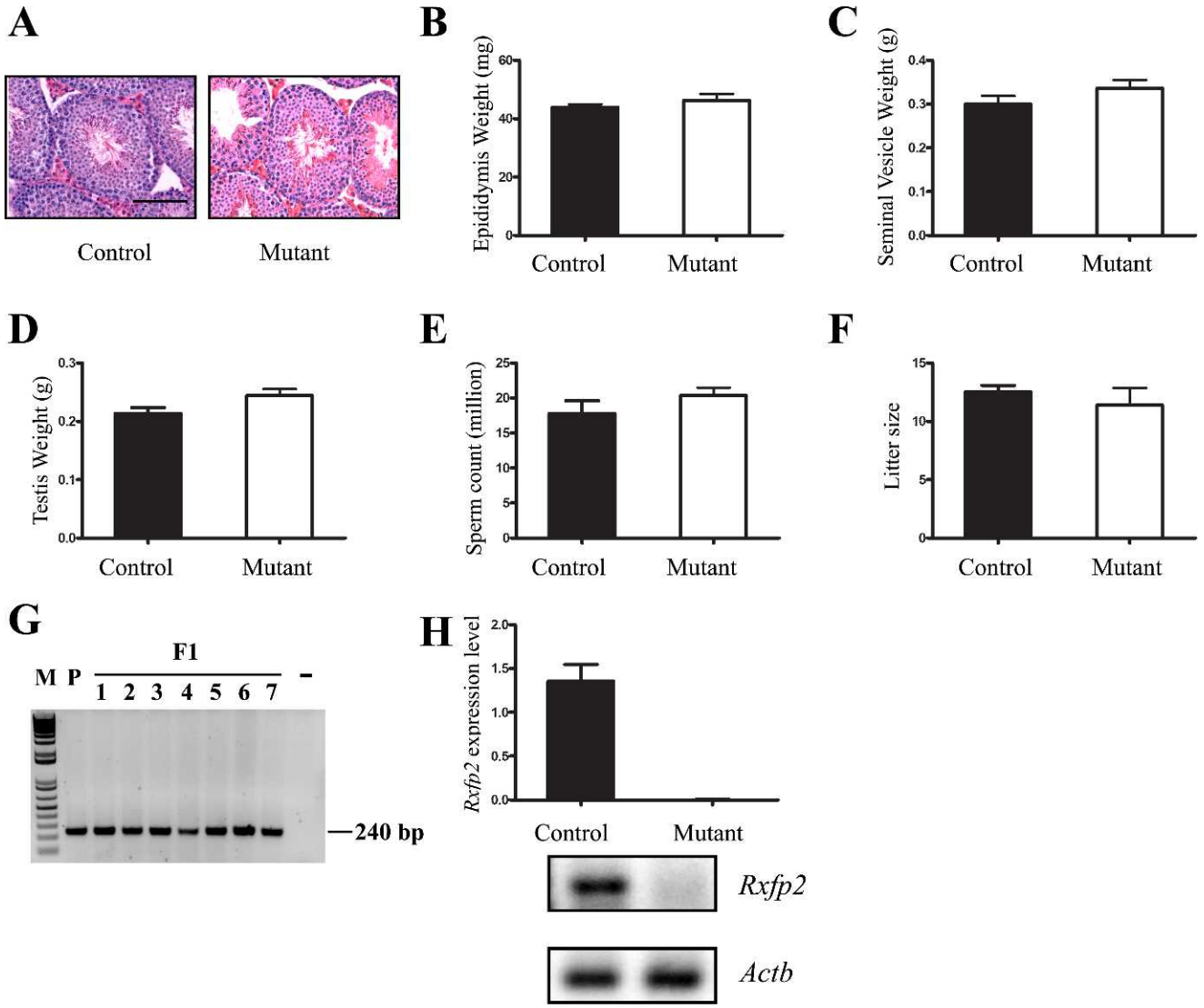

FIG. 4. Normal phenotype of mice with conditional deletion of *Rxfp2* in male germ cells. **A**–**F**) Comparisons of spermatogenesis (H&E staining), epididymis weight, seminal vesicle weight, testis weight, sperm count, and the litter size in 4-mo-old control and *Rxfp2*^{fl}/*Rxfp2*^{fl}, *Stra8-icre* mutant males. Bar in **A** = 100 μm. No differences were detected between the two groups. **G**) Analysis of genomic DNA isolated from the testis of *Rxfp2*^{fl}/*Rxfp2*^{fl}, *Stra8-icre* male (P) and from the ear genomic DNA of his F1 progeny. A band corresponding to a deleted allele was detected in all the samples, indicating efficient recombination in mutant germ cells. M, 1-kb marker; - indicates a negative control. **H**) QRT-PCR and conventional RT-PCR (image below) analysis of *Rxfp2* expression in adult control and mutant testes. No *Rxfp2* expression was detected in mutant testis. Data are shown as mean ± SEM. The analysis of *Actb* expression was used as a positive control.

and number of litters in the progeny of mutant and control males. PCR analysis of testis genomic DNA PCR confirmed the presence of the *Rxfp2*$^{\Delta}$ allele (Fig. 4G). Genetic testing of the mutant males progeny indicated the presence of *Rxfp2*$^{\Delta}$ allele in all the pups regardless of the presence of *Rarb-cre* transgene in their genotype (Fig. 4G). QRT-PCR of testis RNA indicated a dramatic decrease in the level of *Rxfp2* gene expression in mutant testis versus control (Fig. 4H). Conventional RT-PCR of the total RNA isolated from the mutant testis failed to detect the *Rxfp2* expression. The results confirmed that the Cre-induced recombination in germ cells was very efficient and that in postnatal adult testis the *Rxfp2* gene is expressed only in germ cells. DNA staining and fluorescence-activated cell sorting (FACS) was performed to analyze quantitatively the possible changes in different germ cells during spermatogenesis in mutant testis. The FACS histogram suggested that the germ cell populations of haploid, diploid, S-phase, and tetraploid cells were not different both in ratio and numbers between control and mutant mice (Fig. 5). Finally, we conducted a TUNEL assay for analysis of cell apoptosis in seminiferous tubules to study the possible role of INSL3/RXFP2 signaling in germ cell survival. The results indicated no significant difference in germ cell apoptosis between control and mutant mice under normal conditions (Fig. 6).

DISCUSSION

The role of INSL3/RXFP2 signaling in testicular descent was first recognized based on the cryptorchid phenotype of mutant mice with *Insl3* or *Rxfp2* ablation [9–12]. It was shown that the gubernaculum, the inguinoscrotal ligament connecting the epididymis with the caudal abdominal wall, was a primary target of INSL3/RXFP2 signaling; in the mutant males this structure failed to differentiate. It was suggested that INSL3/RXFP2 signaling might induce myogenic differentiation of gubernacular mesenchymal cells leading to the formation of

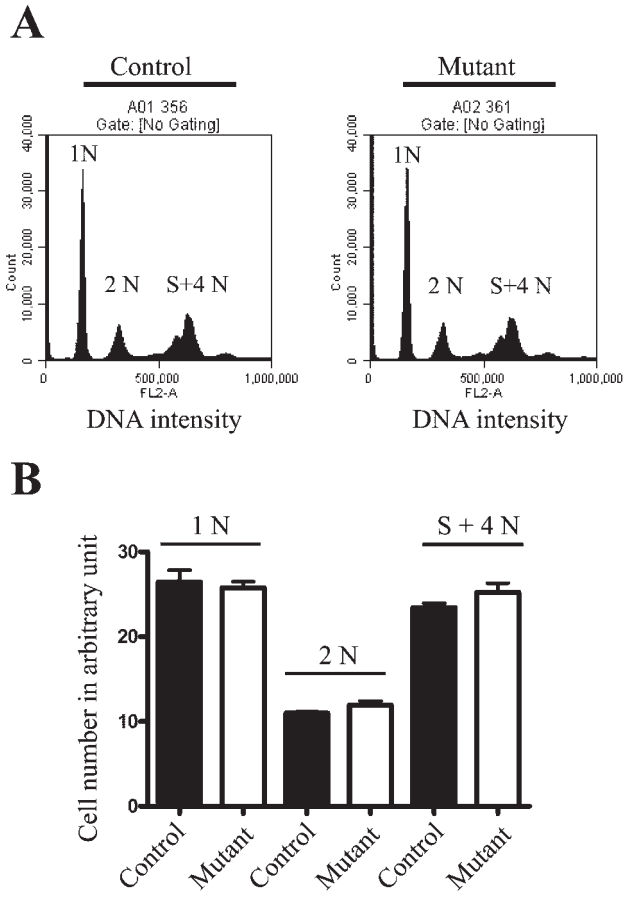

FIG. 5. Flow cytometry analysis of testis from males with germ cell-specific inactivation of *Rxfp2*. **A**) Flow cytometry histograms of the control and mutant testis. The y axis is the cell count, and the x axis represents the DNA intensity. Testis cells have three cell populations distinguished by DNA intensity: 1N (haploid), 2N (diploid), and S (S-phase) plus 4N (quadriploid). A representative image of one of three males analyzed in each group is presented. **B**) Quantitative analysis of different germ cell populations in the control and mutant testis. No statistically significant differences were detected. Data are shown as mean ± SEM.

cremaster muscle [26]. Interestingly, while the INSL3 peptide is highly expressed in both fetal and adult Leydig cells, its role in postnatal reproductive organs is not quite clear. In this report, we describe the generation of two new alleles of *Rxfp2*. The first, with the knock-in LacZ reporter, was used to examine which cells express *Rxfp2* at different stages of reproductive organs development. Using the floxed allele of *Rxfp2,* we have shown that while conditional deletion of the gene in metanephric mesenchyme, the gubernaculum anlage, caused cryptorchidism, the *Rxfp2* ablation in differentiated muscle cells did not affect testicular descent. Surprisingly, despite strong expression of *Rxfp2* in postmeiotic germ cells, conditional inactivation of the gene in germ cells had no effect on spermatogenesis, germ cell survival, and male fertility.

Previously, *Rxfp2* expression in male reproductive organs was studied using an array of methods. The different approaches led, however, to somewhat different results and conclusions. In situ hybridization showed a specific expression in the mouse gubernacular ligament and perhaps in at least parts of the Wolffian duct derivatives at E14.5 [8]. The RT-PCR data and the use of transgenic *Rxfp2-cre* mice, where Cre expression was driven by the partial *Rxfp2* promoter, suggested a wider gene expression at prenatal stages including testis [14]. However, the available RXFP2 antibodies failed to detect protein expression during embryonic development [14]. On the other hand, a strong reactivity to the RXFP2 antibodies was detected in the cremaster muscle and in the testicular interstitial compartment in addition to the germ cells [14, 16, 17].

The use of a reporter LacZ knock-in allele enables the direct visualization of endogenous gene expression. Our data confirmed an early and ubiquitous expression of *Rxfp2* in mesenchymal cells of the embryonic gubernaculum, but we did not detect any reporter activity in adult cremaster muscle cells. In adults, the *Rxfp2* gene was expressed in nondifferentiated interstitial cells located between muscle layers and in epithelial cells lining the cremasteric sac. One possibility is that the interstitial cells represent a stem cell population supplying new myoblasts. Indeed, we have previously shown a strong LacZ expression in adult cremaster muscle cells in mice with *Rxfp2-cre* and *ROSA26* reporter [14]. Because *Rxfp2* is not expressed in adult muscle cells, this suggests that the progenitor cells, marked by the *Rxfp2-cre*-activated ROSA26 allele, differentiated into the muscle cells. We have previously shown that in mice with a partial suppression of *Rxfp2* gene expression the cremaster muscle was poorly differentiated [26]. This suggests that the INSL3/RXFP2 signaling might provide some stimuli for myogenic differentiation of mesenchymal cells. Indeed, as we have demonstrated here, the early conditional deletion of *Rxfp2* caused by *Rarb-cre* transgene completely disrupted gubernaculum differentiation. While the *Rarb-cre* expression is not limited to gubernaculum, it is strongly expressed there [20, 25, 26]. On the other hand, the inactivation of *Rxfp2* in smooth

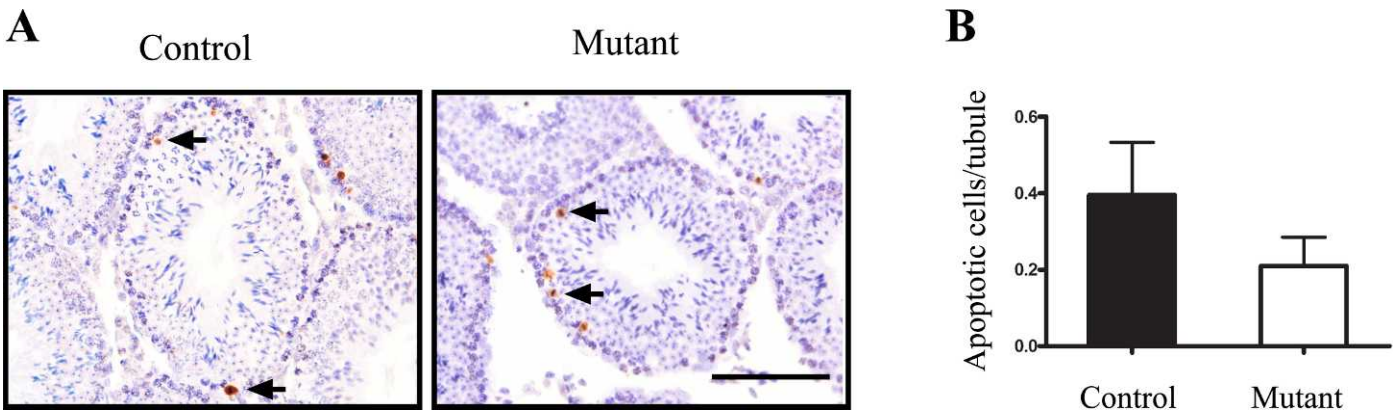

FIG. 6. Analysis of germ cell apoptosis in males with germ cell-specific inactivation of *Rxfp2*. **A**) Representative images of control and mutant testis TUNEL analysis. Apoptotic cells are indicated by black arrows. Bar = 100 μm. **B**) Apoptotic cell number per seminiferous tubule was used for comparison between control and mutant groups. The difference is not statistically significant. Data are shown as mean ± SEM.

or striated muscle cells had no effect on testicular descent. No visible changes in cremaster muscle development were detected. In our experiments, we used Cre transgenes driven by promoters of early markers of striated or smooth muscle cells, ACTA1 and TANGL. Although the exact cell type where the ablation of the gene occurred was not defined in our experiments, both transgenes were expressed in cremaster muscle leading to the recombination of floxed allele. Thus, while *Rxfp2* expression might be important for initiation of myogenic differentiation pathway, the muscle-specific ablation of INSL3/RXFP2 signaling appears to be not required for normal testicular descent.

In the adult testis, we detected strong *Rxfp2* expression in postmeiotic germ cells, confirming previously published data [14–17]. Indeed, the expression of the knock-in LacZ reporter was first detected in P21 testis, when the first wave of spermatogenesis reached the postmeiotic stage. It was suggested that INSL3/RXFP2 signaling had a prosurvival effect on germ cells, as the cotreatment of male rats with a GnRH antagonist and INSL3 reduced germ cell apoptosis compared to the treatment with GnRH antagonist alone [15]. The INSL3/RXFP2 signaling, however, appears to be not essential for spermatogenesis because the surgical correction of cryptorchid testes in *Rxfp2*- or *Insl3*-deficient mice rescued spermatogenesis [10, 11, 18]. To directly investigate the role of *Rxfp2* deficiency in testis, we used a *Stra8-icre* transgene to target germ cells in fully descended testes. Conditional inactivation of the *Rxfp2* gene was highly efficient as was demonstrated by the presence of only deleted, but not floxed, alleles in the progeny of mutant males. Absence of the *Rxfp2* gene expression in mutant testes pinpoints germ cells as the source of *Rxfp2* in male gonads. Germ cell *Rxfp2* deficiency had no effect on fertility, reproductive organ weights, spermatogenesis, sperm count, motility, or germ cell survival in the mutant testis. Thus, our data indicate that under normal conditions, INSL3/RXFP2 signaling has no effect on germ cell differentiation and development in adult testis. It is possible, however, that INSL3/RXFP2 signaling still might be required for germ cell survival under GnRH antagonist treatment or some other assaults, such as the use of antiandrogens or testicular heat, or even during the establishment of spermatogenesis in puberty. It is also possible that INSL3 may be used as germ cell antiapoptotic treatment at high pharmacological doses. The availability of previously designed conventional and now conditional mutants of *Rxfp2* will allow for the further investigation of these questions.

In conclusion, we produced the first LacZ knock-in reporter and floxed *Rxfp2* alleles in mice. Analysis of gene expression combined with the conditional targeting of *Rxfp2* in gubernacular cell populations and in testicular germ cells demonstrated the importance of INSL3/RXFP2 signaling in testicular descent. Germ-cell deletion of *Rxfp2* did not affect spermatogenesis, germ cell survival, or male fertility in adult mice.

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