Role of Postnatal Expression of *Fgfr1* and *Fgfr2* in Testicular Germ Cells on Spermatogenesis and Fertility in Mice

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Abstract

Background: Fibroblast growth factor (FGF) signaling is thought to play diverse roles in the male reproductive system. However, its role in testicular cells for sper-matogenesis and fertility remains unclear.

Methods: In this study, the expression and localization of *Fgfr 1* (FGF Receptor) and *Fgfr 2* in the postnatal mouse testes were examined by RT-PCR, Western blotting and immunohistochemistry. The *in vivo* function of each receptor in testicular germ cells was determined using germ cell-specific *Fgfr* mutant animals, *Tex101-iCre;Fgfr*^{flox/flox} and *Tex101-iCre;Fgfr*^{flox/flox} mice. The results were analyzed by Kruskal-Wallis test and Dunn's Post-test.

Results: Both Fgfr1 and Fgfr2 were expressed in the testis throughout the entire postnatal development. Prominent immunostaining of these FGFRs was observed in interstitial and peritubular cells with little or no changes in all phases during postnatal development. Positive staining of these receptors was also detected in germ cells including elongated spermatids and spermatozoa. Germ cell-specific Fgfr1 or Fgfr2 mutant mice were viable with no developmental abnormalities in the testes and accessory sex organs. Fertility studies showed that the fecundity of both mutant mouse lines did not significantly differ from wild-type siblings (n=4, p>0.05). Further analysis indicated the presence of other Fgfrs in testicular germ cells including Fgfr 3, 4 and 5.

Conclusion: The results demonstrated that Fgfr1 and 2 are expressed in all testicular cell types and that neither Fgfr1 nor Fgfr2 in testicular germ cells is essential for spermatogenesis and fertility. Future studies are needed to investigate the potential functional redundancy among five Fgfrs in male germ cells for spermatogenesis and fertility.

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Introduction

F ibroblast growth factors (FGFs) are a large family of structurally-related, widely expressed, multifunctional heparin-binding polypeptides, which contain 23 members in vertebrates. The biological processes of FGFs are mediated by binding to and activating a group of high-affinity FGF receptors (*FGFRs*), which are encoded by five distinct genes. Differential splicing of *Fgfr* mRNA also gives rise to several re-

ceptor isoforms that are expressed in a tissuespecific fashion (1). Overwhelming data demonstrate that *FGF/FGFR* signaling cascades play an important role in many cellular processes including mitogenesis, differentiation, migration, survival and polarity (1-4).

FGFRs are evolutionarily conserved transmembrane proteins that are composed of an extracellular ligand-binding domain, a transmembrane re-

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Received: Dec. 23, 2013 **Accepted:** Apr. 19, 2014 gion and a cytoplasmic portion containing the catalytic protein tyrosine kinase domain (1, 2, 5). Studies have demonstrated that *FGFRs* possess broad ligand binding affinity and specificity that can interact with multiple FGFs for signal transduction. *FGFR1* to *FGFR4* are known to propagate the highest level of FGF signals in a wide range of tissues. The binding of FGFs to these receptors activates multiple signaling cascades, which include STAT, MAPK and PI3K pathways (6). The role of *FGFR5* (also known as *FGFR* like-1), which is a short form of *FGFR* lacking the catalytic protein tyrosine kinase domain, is currently less understood (7, 8).

A number of FGFs and FGFRs are detected in the male reproductive system of different mammalian species including mouse, rat, bovine and human (1, 9-16). The most characterized gonads are the rat testes. Previous studies have demonstrated that the transcript variants of Fgfr1 IIIb and IIIc, Fgfr2 IIIc, Fgfr3 IIIc and Fgfr4 are expressed in fetal, immature and adult rat testes (10). However, only *FGFR1* and *FGFR3* but not *FGFR2* and FGFR4 proteins are detected in the fetal rat testes (10). In immature testes, all four *FGFRs* are present in the germ and Leydig cells but not in Sertoli cells. FGFR1 to FGFR4 are found in the seminiferous epithelium and interstitium of adult rat testes (11, 17). It is reported that all four FGFRs are immunolocalized in germ cells including elongated spermatids, while only FGFR4 is present in Sertoli cells (10). Furthermore, the expression pattern of each Fgfr in the germ cells during spermatogenesis exhibits a stage-specific change (10-12).

The presence of multiple FGFs and FGFRs in multiple cell types of pre- and post-natal testes implies that these factors are important in regulation of the fetal testicular development, maturation of sperm, inducing the capability of male to produce functional gametes and affecting male fertility (1). To investigate the function of FGFs/ FGFRs signaling in vivo, several mutant mice have been created. Conventional gene knockout of either *Fgfr1* or *Fgfr2* results in an early death in utero, suggesting the vital role of these receptors during embryonic development (18, 19). Fgfr3 and Fgfr4 null mutant mice, on the other hand, are viable with no reproductive phenotype reported (20-22). Conditional gene knockout of Fgfrs in specific organs or cells in mice (16, 19, 21, 23-29) have been generated to circumvent the embryonic lethality. The crucial role of FGFR2 during testicular development has been elegantly demonstrated by Kim et al. Using two different transgenic Cre mouse lines that induce either a temporal or a cellspecific ablation of this receptor reveal that *FGFR2* mediated FGF9 signaling is essential for proliferation and Sertoli differentiation during testis determination (16).

Despite extensive studies in the last decades, the temporal and spatial expression of Fgfr1 and Fgfr2 in mouse testes during the postnatal development is not well defined and their exact roles in spermatogenesis and male fertility are not unequivocally demonstrated. The aim of this study was to determine the localization of FGFR1 and FGFR2 in the mouse testes during postnatal development, and to elucidate the effect of each Fgfr1 and Fgfr2 on spermatogenesis and fertility using mouse models with postnatal germ cell-specific deletion.

Methods

Animals: All animals were housed under 12 hr light-dark cycles with food and water provided ad libitum. All mice were maintained as required under the National Institutes of Health guidelines for the Care and Use of Laboratory Animals. All studies have been approved by the Animal Care and Use Committee of the University of Louisville. All the mice were sacrificed under ketamine anesthesia and all efforts were made to minimize their suffering.

Generation of Tex101-iCre; Fgfr1^{flox/flox} and Tex101iCre;Fgfr2^{flox/flox} mice: To specifically investigate the role of each Fgfr1 and Fgfr2 in germ cells, a transgenic Cre mouse line expressing an improved Cre (*iCre*) recombinase driven by the mouse Tex101 promoter (Tex101-iCre) was used. This transgenic line was previously generated in our laboratory (30). The expression of *iCre* was specifically detected in the prespermtogonia within seminiferous tubules of postnatal eight-day-old testes. In adult mice, there were robust *iCre* activities in spermatocytes and spermatids and a weak activity in spermatogonia. For germ cell selective deletion of Fgfr1 or Fgfr2, Tex101-iCre female mice were first bred with Fgfr1^{flox/flox} or Fgfr2^{flox/flox} males to obtain bigenic heterozygous females (i.e., Tex101-iCre; Fgfr1^{flox/+} and Tex101 $iCre;Fgfr2^{flox/+}$). Then, these heterozygous females were bred with $FgfrI^{flox/flox}$ or $Fgfr2^{flox/flox}$ to generate male germ cell-specific Fgfr1 or Fgfr2 mutant mice (male Tex101- $iCre;Fgfr1^{flox/flox}$ or Tex101- $iCre;Fgfr2^{flox/flox}$). Floxed Fgfr1 and Fgfr2 JRI Germ Cell Fgfr1 and Fgfr2 in Male Fertility

mice were kindly provided by Dr. Juha Partanen (floxed Fgfr1 mice, University of Helsinki, Finland) and Dr. David M. Ornitz (floxed Fgfr2mice, Washington University in St Louis) and details were described elsewhere (23-25). To determine the efficiency of Tex101-*iCre* in excision of floxed Fgfr1 and Fgfr2 alleles, Tex101*iCre;Fgfr1*^{flox/flox} and Tex101-*iCre;Fgfr*^{flox/flox} male mice were mated with wild-type females.

Genotyping: Genomic DNA was isolated from mouse tails using proteinase K and phenol chloroform extraction method as described previously (30). The presence of *iCre* or *LacZ* was determined by PCR using the primer pairs listed in table 1. The primer sets $Fgfrl^{\Delta}$ and $Fgfr2^{\Delta}$ were used to determine the deletion of floxed Fgfrl and Fgfr2 alleles.

Isolation of testicular cells: Testicular cells were isolated from three 2-month-old mice using the procedure described previously (31) with a little modification. Briefly, the testes were decapsulated and incubated with a collagenase type II solution (0.5 mg/ml, Sigma, St. Louis, MO) to separate interstitial cells and seminiferous tubules. The interstitial cells were pelleted by centrifugation. To obtain the germ cells and Sertoli cells, the dispersed seminiferous tubules were cut into small pieces and digested with a solution containing 1 mg/ml trypsin (Sigma) and 10 $\mu g/ml$ DNase I (Sigma) at $32^{\circ}C$ for 30 min. The reaction was stopped by adding trypsin inhibitor (Sigma) and Hank's Balanced Salt Solution (HBSS, Invitrogen, Carlsbad, CA). The supernatant that contained germ cells was collected after precipitation by unit gravity. The pellet was incubated with a collagenase type II solution at $32^{\circ}C$ for 15 min and settled down by unit gravity. The cell pellet that contained Sertoli cells was rinsed with HBSS three times and cultured with Dulbecco's Modified Eagle's Nutrient Mixture/F12 Ham Medium supplemented with 10% fetal bovine serum (Invitrogen) overnight. Sertoli cells were harvested the next day after residual germ cells were hypotonically removed. The purity of isolated interstitial, Sertoli and germ cells was evaluated by performing RT-PCR using several putative marker genes, which included cholesterol side-chain cleavage enzyme and 17α -hydroxylase (interstitial cells), follicle stimulating hormone receptor and Pem homeobox gene (Sertoli cells), alkaline phosphatase and fibronectin (myoid cells) and protamine 2 and stimulated by retinoic acid gene 8 homolog (germ cells) (32, 33). The results showed that the contamination of each cell type by the others was minimal (data not shown).

Semiquantitative RT-PCR: Total RNA was ex-

Genes	Primer sequences (5'-3')	PCR cycles	
RT-PCR prime	rs		
Fgfrl	F: AAGAGAGACCAGCTGTGATG R: ATATTCGGAGACTCCAGCCA	31	
Fgfr2	F: AGAAGGAGATCACGGCTTCC R: TACTCGGAGACCCCTGCTAG	31	
Fgfr3	F: CTGTGCCAGCCGCAAACACT R: AGAATGGCTGTCTGGTTGGC	31	
Fgfr4	F: TCCCAGCCAACACCACAGCT R: TCTTCCTCTGGCAGCACCGT	31	
Fgfrl1	F: GGACGCCACAACTCCACCAT R: GAAGACAGCACCAGCTGGGA	31	
Rpl19	F: GAGTATGCTCAGGCTTCAGA R: TTCCTTGGTCTTAGACCTGC	Co-amplified with target genes	
Genotyping pri	mers		
Fgfr1	F: TTGACCGGATCTACACACACC R: AACCACCCCCACACAAA	32	
Fgfr2	F: GTCAATTCTAAGCCACTGTCTGCC R: CTCCACTGATTACATCTAAAGAGC	32	
$Fgfrl^{\Delta}$	F: GGACCTCTGGAAGAGCAGTG R: AGGTTCCCTCCTCTTGGATGA	32	
$Fgfr2^{\Delta}$	F: ATAGGAGCAACAGGCGG R: CATAGCACAGGCCAGGTTG	32	
iCre	F: TCTGATGAAGTCAGGAAGAACC R: GAGATGTCCTTCACTCTGATTC	33	

Table 1. Oligonucleotide primers for genotyping and semiquantitative RT-PCR (F, forward; R, reverse)

tracted from the testes and the isolated testicular cells using Trizol Reagent (Invitrogen) according to manufacturer's instructions. Total RNA was adjusted to a concentration of approximately 1.0 $\mu g/\mu l$. Two microgram of total RNA was reverse transcribed into cDNA with random primers (Invitrogen) and avian myeloblastosis virus (AMV) reverse transcriptase (Promega Corporation, Madison, WI). The cDNA was amplified by PCR with the primer sets of the target gene and a housekeeping gene, ribosomal protein large subunit 19 (Rpl19). PCR primers, as listed in table 1, were designed according to the sequences obtained from GenBank using the Vector NTI 12.0 program (Invitrogen) and synthesized by Operon Technologies (Alameda, CA). All primers were designed to amplify all variants of *Fgfr1* and *Fgfr2* and the products covered one or more exons. The amplified products were separated by electrophoresis and the intensity of specific bands was scanned and semi-quantified using the image analysis software, TotalLab (Nonlinear USA Inc, Durham, NC). The results were presented as the ratio of target gene over Rpl19.

Western blot analysis: The testes were homogenized by sonication in an ice-cold lysis buffer. The protein concentrations were measured by the Bradford method (Bio-Rad laboratories, Hercules, CA). Protein aliquots were separated on SDS-PAGE gels, transferred to PVDF membranes, blocked with 3% non-fat milk, and then incubated overnight with rabbit polyclonal antibodies against FGFR1 (sc-121, 1:400) and FGFR2 (sc-122, 1:600) (Santa Cruz Biotechnology, Santa Cruz, CA), respectively. Peroxidase-conjugated anti-rabbit IgG (1:2000, Vector Laboratories, Burlingame, CA) was used as the secondary antibody. Immunoblotting signals were detected by Amersham ECL plus Western blotting detection system (GE healthcare Biosciences, Pittsburgh, PA). All membranes were re-blotted with B-actin or Btubulin antibodies (Sigma) as the loading control. The intensity of specific bands was scanned using image analysis software, TotalLab (Nonlinear USA Inc). The results were presented as the ratio of target protein over β -actin or β -tubulin.

Immunohistochemistry: Tissues were fixed in 10% formalin and embedded in paraffin. The procedure was performed by an avidin-biotin immunoperoxidase method as described previously (34). Briefly, sections were de-waxed, rehydrated and then incubated with 1% H₂O₂ for 30 *min*. After rinsing with phosphate buffered saline (PBS),

sections were treated with 0.025% trypsin (Sigma) for 30 min at room temperature and incubated with rabbit polyclonal antibodies against FGFR1 (1:50) and FGFR2 (1:200) (Santa Cruz Biotechnology) overnight at $4 \,\%$, respectively. Sections were then incubated with biotinylated goat antirabbit IgG (Vector Laboratories, Burlingame, CA) for 1 hr. After rinsing with PBS, sections were incubated with avidin-biotin-horseradish peroxidase complex using a Vectastain ABC kit (PK-4000, Vector Laboratories) for 1 hr and rinsed with PBS. Immunostaining was detected by incubation of the sections with the substrate 3'3diaminobenzidine. All sections were counterstained with hematoxylin. Replacement of the primary antibody with PBS was performed at the same time as a procedure control. We also used irrelevant rabbit IgG instead of the primary antibody later to check the specificity of the immunostaining. Immunostained sections were evaluated by two researchers independently. The stage of the seminiferous epithelium cycle was determined by the morphology and localization of spermatocytes and spermatids within the seminiferous tubule as described by Hess and Renato de Franca (35).

Fertility test: Four Tex101- $iCre;Fgfr1^{flox/flox}$ and four Tex101- $iCre;Fgfr2^{flox/flox}$ male mice at age of 3 months were housed individually with proven female breeders. The females were separated from males once they were pregnant. The breeding test for males continued for a period of 4 months. The resultant pregnancies were noted and the number of pups for each litter was recorded.

Statistical analysis: The data presented are the means±SEM. All results were analyzed by Kruskal-Wallis test and Dunn's post-test using a version 3.06 Instat program (Graphpad Software, San Diego, CA). A p-value <0.05 was considered statistically significant.

Results

Expression of Fgfr1 and Fgfr2 in mouse testes during postnatal development: To identify which cell types expressed *Fgfr1* and *Fgfr2* in adult mouse testes and whether the expression of these two receptors changed during postnatal development, RT-PCR was performed. The results indicated that the transcripts of *Fgfr1* and *Fgfr1* were present in germ cells, Sertoli cells and interstitial cells in adult mouse testes (Figure 1A). The mRNAs of these two receptors were readily detected in the testes during the entire postnatal



development period examined ranging from neonatal (day-1), immature (day-10), peripubertal (day-20), pubertal (day-30) to adulthood (day-60) (Figures 1B and C). The testicular mRNA levels of both *Fgfr1* (Figure 1B) and *Fgfr2* (Figure 1C) remained constant from neonatal to pubertal period and then significantly decreased in adult testes (n=3, p<0.05 compared to day-1 to -30).

To determine whether the levels of FGFR1 and *FGFR2* proteins were also postnatally regulated in the testes, Western blot analyses were carried out. In contrast to their mRNA profiles, immuno-



Figure 1. RT-PCR results show that Fgfr1 and Fgfr2 mRNAs are readily detectable in the whole testis as well as in purified interstitial, Sertoli and germ cells in adult mice. A: Semiquantitative RT-PCR analyses of the expression of Fgfr1 and Fgfr2 during postnatal testicular development. The levels of Fgfr1; B: and Fgfr2 C: remain constant from neonatal to pubertal period and significantly decrease in adult animals.

n=3, *p<0.05 compared to day-1 to -30

blotting demonstrated that the protein levels of *FGFR1* were low during the neonatal and immature stage, increased in the peripubertal period and then maintained a steady level to adulthood (n=3, p<0.05 compared to day-20 to-60, Figure 2A). The protein levels of *FGFR2*, on the other hand, showed no apparent changes from the neonatal period to adulthood (n=3, p>0.05, Figure 2B). The mechanism by which the mRNA and protein levels of *Fgfr1* and *Fgfr2* in sexually mature animals is differentially regulated is currently unknown.



Localization of FGFR1 and FGFR2 in mouse testes

Figure 2. Western blot analyses of FGFR1 and FGFR2 during postnatal testicular development. The protein levels of FGFR1; A: are low at the neonatal and premature period, then increase from the peripubertal stage to adulthood. FGFR2; B: remains unchanged throughout entire postnatal period. n=3, * p<0.05 compared with day-20 to -60

during postnatal development: Immunohistochemical staining of testicular sections revealed that *FGFR1* (Figure 3A) and *FGFR2* (Figure 3B) were detected in both the interstitial and seminiferous tubular compartments from neonatal to adulthood. The most prominent immunostaining of *FGFR1* and *FGFR2* was observed in interstitial and peritubular cells with little or no changes in all phases of postnatal development. In the seminiferous tubular compartment, weak immunostaining of both receptors was present in spermatogonia, spermatocytes and Sertoli cells throughout all phases of postnatal development. The immunostaining for *FGFR1* and *FGFR2* was also evident in elongated spermatid, spermatozoa, the seminiferous tubules and sperm in the epididymis but weak



Figure 3. Immunohistochemical staining of FGFR1; A: and FGFR2 B: during postnatal testicular development. Prominent immunostaining of FGFR1 and FGFR2 are observed in interstitial (red arrows) and peritubular cells with no significant changes among all age groups. In the seminiferous tubular compartment, spermatogonia, spermatocytes, round spermatids and Sertoli cells (black arrows) exhibit weak immunostaining for these FGFRs in all age groups. The immunostaining for these FGFRs was also evident in elo/ngated spermatid, spermatozoa and sperm in the epididymis. In adult testes, the weakest immunostaining for FGFR1 and FGFR2 were found in stages IX and X of the seminiferous epithelial cycle, and other stages displayed no significant changes. The primary antibodies replaced by irrelevant rabbit IgG served as a procedural control. A 60-day old control picture is presented. The control pictures for other age groups are not shown

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Figure 4. Deletion of the floxed *Fgfr1*; A) and *Fgfr2*; B) alleles by *Tex101-iCre* in male germline. Representative PCR genotyping results of a litter of pups from breeding of a *Tex101-iCre;Fgfr1* flox/flox (A) or *Tex101-iCre;Fgfr2* flox/flox (B) male with a wild-type female, respectively. Note the lack of the *Fgfr1* (A) and *Fgfr2* (B) alleles and the presence of the *Fgfr1* and *Fgfr2* alleles in all pups, indicating complete deletion of the floxed *Fgfr1* and *Fgfr2* alleles in the male germline, regardless of the presence of *iCre* transgene in the progeny

immunostaining was seen in round spermatid in adult testes (Figures 3A and B). Differential immunostaining intensity of *FGFR1* and *FGFR2* was observed in seminiferous epithelial cycle. It appeared to be lower in stages IX and X and higher in stages I-VIII.

Deletion of Fgfr1 and Fgfr2 in testicular germ cells: First, the efficiency of *Tex101-iCre* in deletion of floxed *Fgfr1* and *Fgfr2* was evaluated by breeding *Tex101-iCre;Fgfr1*^{flox/flox} and *Tex101iCre;Fgfr2*^{flox/flox} males with wild-type females, respectively, and genotyping analysis of the progenies was performed. If *iCre* recombinase is active in the spermatogenic cells, the floxed *Fgfr1* and *Fgfr2* alleles will be converted to the recombined *Fgfr1*^{Δ} and *Fgfr2*^{Δ} allele regardless of the presence or absence of *Tex101-iCre* transgene in the progenies. The results showed that lack of *Fgfr1*^{flox} (Figure 4A) and *Fgfr2*^{flox} (Figure 4B) alleles and presence of *Fgfr1*^{Δ} and *Fgfr2*^{Δ} alleles in all pups indicated complete deletion of the floxed Fgfr1 or Fgfr2 alleles in the male germline. Complete deletion of Fgfr1 and Fgfr2 in the germ cells of Tex101- $iCre;Fgfr1^{flox/flox}$ and Tex101- $iCre;Fgfr2^{flox/flox}$ males was further confirmed by performing RT-PCR and immunohistochemistry. RT-PCR showed that the transcripts of testicular germ cells of Fgfr1 in Tex101-iCre; $Fgfr2^{flox/flox}$ (Figure 5A) and Fgfr2 in Tex101-iCre; $Fgfr2^{flox/flox}$ (Figure 5B) animals were not detectable. Immunohistochemistry demonstrated the absence of immunostaining of Tex101-iCre; $Fgfr1^{flox/flox}$ (Figure 6A) and FGFR2 in Tex101-iCre; $Fgfr1^{flox/flox}$ (Figure 6D) mice, while the immunostaining of these two proteins in testicular somatic cells for either genotype animal was comparable to wild-type siblings (Figures 6B and E).

Male fertility and testicular phenotype in the absence of either Fgfr1 or Fgfr2 in germ cells: Both Tex101-iCre; $Fgfr1^{flox/flox}$ and Tex101-iCre; $Fgfr2^{flox/flox}$ mice were viable with no apparent developmental defects. To test the fertility of



Figure 5. Complete lack of Fgfr1; A: or Fgfr2; B: expression in isolated testicular germ cells of Tex101- $iCre;Fgfr1^{flox/flox}$; A) and Tex101- $iCre;Fgfr2^{flox/flox}$; B) adult mice. Semiquantitative RT-PCR shows that deletion of Fgfr1 or Fgfr2 gene did not significantly influence the expression of other Fgfrs in the germ cells of mutant mice (A & B) except that Fgfr4 expression is significantly elevated in Tex101- $iCre;Fgfr1^{flox/flox}$ mice (A), n=3.

* P<0.05 as compared to wild-type



Figure 6. Immunohistochemistry reveals the absence of FGFR1 (A) and FGFR2 (D) in spermatogonia, spermatocytes and spermatids of *Tex101-iCre;Fgfr1*^{flox/flox} (A) and *Tex101-iCre;Fgfr2*^{flox/flox} (D) adult mice, while immunostaining of FGFR1 (A) and FGFR2 (D) in interstitial and peritubular cells was comparable to wild-type animals (B & E). Omission of the FGFR1 (C) and FGFR2 (F) primary antibodies served as a procedural control.

Fgfr1 and *Fgfr2* mutant male mice, sexually mature *Tex101-iCre;Fgfr1*^{flox/flox} and *Tex101-iCre; Fgfr2*^{flox/flox} male mice were mated with wild-type female mice, respectively. All of the wild-type mice tested delivered pups with normal litter sizes. Average litters sired by *Tex101-iCre; Fgfr1*^{flox/flox} and *Tex101-iCre;Fgfr2* ^{flox/flox} male mice during four months of fertility tests did not significantly differ from wild-type siblings (n=4, p>0.05, Table 2), indicating that germ cellselective ablation of individual *Fgfr1* or *Fgfr2* in mice does not affect male fertility.

There were no gross abnormalities and size difference in the testes and accessary sex organs in either *Fgfr1* or *Fgfr2* mutant mice. Light microscopy revealed that in wild-type and mutant mice, all stages of spermatogenesis were present. The size of the seminiferous tubules, the histological structures of the testes and epididymides of wildtype (Figures 7C, F, I), *Tex101-iCre;Fgfr1*^{flox/flox} (Figures. 7A, D, G) and *Tex101-iCre;Fgfr2*^{flox/flox} (Figures 7B, E, H) were essentially indistinguishable.

Table 2. Breeding performance of mature male mice

Male×Female	n	Litter size
Wild type×wild type	4	9.0±2.2
<i>Tex101-iCre/Fgfr1^{///¶×}</i> wild type	4	8.6±1.7
<i>Tex101-iCre fgfr2^{fl/fl}×</i> wild type	4	8.8±1.5

To explore possible effects of germ cell-selective deletion of Fgfr1 and Fgfr2 on the expression of other Fgfrs, RT-PCR was carried out to determine their mRNA levels in adult testes. The results showed that Fgfr1 to Fgfr5 were expressed in the germ cells of adult testes. Deletion of Fgfr1 in germ cells led to a moderate elevation of Fgfr4 mRNA levels, while the expression of Fgfr3 and Fgfr5 was not affected (Figure 5A). Semiquantitative RT-PCR also showed that deletion of Fgfr2 in germ cells did not significantly influence the expression of Fgfr1, Fgfr3, Fgfr4 and Fgfr5 in the adult testes (Figure 5B).

Discussion

More than seven FGFs including FGF1 to FGF5, FGF8 and FGF9 are known to be expressed in the fetal and adult testes (1, 29, 36-38). It is well established that the signals evoked by FGF family members are converted by four major *FGFRs*, namely *FGFR1* to *FGFR4*, to exert myriad biological effects on embryonic development and homeostasis in the adult for a wide range of tissues (1-4). In this study, it was reported that in the neonatal (day-1), immature (day-10), peripubertal (day-20), pubertal (day-30) and sexually mature (day-60) mouse testes, both *FGFR1* and *FGFR2* were present within the seminiferous tubules and the interstitial compartment, and the expression pat-

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Figure 7. Morphological analyses of H & E stained sections of the testes; A-C: and caput D-F: and cauda G-I: epididymis do not show any abnormalities in either *Tex101-iCre;Fgfr1*^{flox/flox} (A, D & G) or *Tex101-iCre;Fgfr2*^{flox/flox} (B, E & H) adult mice as compared to wild-type siblings (C, F & I)

terns changed based on the stages of spermatogenesis in sexually mature animals. The present study demonstrated that prominent immunostaining of FGFR1 and FGFR2 was observed in interstitial and peritubular cells in all phases during postnatal development. In the seminiferous tubular compartment, weak immunostaining for FGFR1 and FGFR2 was found in spermatogonia, spermatocytes and Sertoli cells throughout all phases during postnatal development. The immunostaining of these two receptors was also observed in sperm in the epididymis. The findings are essentially consistent with previous studies (1, 9-11, 16). However, the current report did not specify what FGFR1 and FGFR2 variant proteins were present in germ cells, Sertoli cells and interstitial cells due to lack of proper antibodies to detect these variants. The broad expression of *Fgfr1* and *Fgfr2* in the testes throughout the entire postnatal development implies that these two major receptors may transduce diverse signals of FGFs in modulation of postnatal testis development and spermatogenesis. For example, FGFR1 in mouse sperm has been reported to mediate FGF signal for modulating sperm capacitation by differentially influencing the downstream PI3K and MAPK activity (39).

Although Fgfr1 to Fgfr4 are expressed in the fetal as well as adult testes, Fgfr3 or Fgfr4 is neither essential for prenatal testis development nor crucial for spermatogenesis in the adult (20-22). Both *Fgfr1* and *Fgfr2* are critical for embryonic development. Fgfr1 null mutant embryos die during gastrulation and segmentation, while homozygous embryos of Fgfr2 knockout die before gonad formation (18, 19). The postnatal roles of *Fgfr1* and Fgfr2 in regulation of male reproductive functions remain obscure due to lack of viable Fgfr1 or Fgfr2 null mutant animal models. To bypass the early lethal phenotype of *Fgfr1* null mutation, embryonic stem (ES) cells with Fgfr1 null mutant have been used to generate chimeric mice that develop to adulthood. Despite the fact that these chimeric mice exhibit various defects in neural tube and limb development, no morphological abnormalities of the testes and functional defects of male fertility are detected in these animals with varying contributions of Fgfr1 null mutant ES cells (Embryonic Stem cells) (27). However, another study reported that transgenic mice overexpressing a truncated Fgfr1 that lacks a signal transduction domain in elongated spermatids displayed a reduction of daily sperm production and capacitation (39). As such, the function of Fgfr1 in adult testes is still contentious. More recent studies, in which the loxp-Cre system is adapted to conditional knockout of Fgfr2 in somatic progenitor cells of embryonic gonads, reveal that Fgfr2 is crucial for male sex determination (16, 29). However, whether Fgfr2 plays a role in postnatal testes remains to be established.

To elucidate the contribution of each *Fgfr1* and Fgfr2 in testicular germ cells to spermatogenesis, floxed Fgfr1 and floxed Fgfr2 and Tex101-iCre transgenic mice were used in this study (24, 25, 30) to overcome embryonic lethality and to achieve selective deletion of Fgfr1 and Fgfr2 in postnatal testicular germ cells. The data clearly demonstrated that Tex101-iCre mediated ablation of floxed Fgfr1 and floxed Fgfr2 in testicular germ cells was specific and complete, which excised regions including the transmembrane and most of the intracellular portions of Fgfr1 and the ligand binding and transmembrane domains of Fgfr2 and produced functional inactive Fgfr1 and Fgfr2 alleles, respectively (24, 25). However, spermatogenesis and fertility of mature males were well preserved. No morphological changes in the testes and epididymis were observed. These findings indicate that each germ cell Fgfr1 or Fgfr2 is postnatally dispensable. The current study and published data convincingly demonstrate the presence of all five Fgfrs in the postnatal testes. Moreover, almost all testicular cell types express multiple Fgfrs. The results of this study do not rule out the possibility that Fgfrs expressing in the testicular cell types other than germ cells convert the FGF signals which indirectly influence spermatogenesis. Using the loxp-Cre system to selectively delete either Fgfr1 or Fgfr2 in other testicular cell types will help to verify this speculation.

Given the facts that numerous FGF ligands are present in the germ cells of adult testes and that each FGF can interact with multiple FGFRs for signal activation (9-16), it is plausible that the lack of individual Fgfr1 or Fgfr2 in testicular germ cells is compensated by the presence of other Fgfrs in these cells. These results show that the transcripts of the other four *Fgfrs* in germ cells of both genotype testes were not significantly altered except that the mRNA levels of Fgfr4 were moderately elevated in these cells of Tex101*iCre:Fgfr1*^{flox/flox} testes. Indeed, a compensatory function between Fgfr3 and Fgfr4 in modulating postnatal lung development has been demonstrated. Although Fgfr3/Fgfr4 double null mutant mice were viable, only a few animals sired and the growth of these animals was severely retarded (21), which were not observed in individual Fgfr3 or Fgfr4 null mutant animals (20, 21). In future studies, creating compound mutations of Fgfr1 and Fgfr2 in the germ cells will allow us to address this issue.

Conclusion

In summary, this study demonstrated that (1) Fgfr1 and Fgfr2 in mouse testes were present in germ, Sertoli and interstitial cells throughout entire postnatal development; (2) male germ cell-selective individual ablation of Fgfr1 or Fgfr2 did not affect mouse reproductive capability and suggested possible presence of redundant FGF/FGFR signal pathways in adult male germ cells.

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Conflict of Interest

The authors declare that there are no conflicts of interest that could be perceived as prejudicing the impartially of the research reported.

References

- 1. Cotton LM, O'Bryan MK, Hinton BT. Cellular signaling by fibroblast growth factors (FGFs) and their receptors (FGFRs) in male reproduction. Endocr Rev. 2008;29(2):193-216.
- Johnson DE1, Williams LT. Structural and functional diversity in the FGF receptor multigene family. Adv Cancer Res. 1993;60:1-41.
- Ornitz DM1, Xu J, Colvin JS, McEwen DG, MacArthur CA, Coulier F, et al. Receptor specificity of the fibroblast growth factor family. J Biol Chem. 1996; 271(25):15292-7.
- 4. Brooks AN, Kilgour E, Smith PD. Molecular pathways: fibroblast growth factor signaling: a new therapeutic opportunity in cancer. Clin Cancer Res. 2012;18(7):1855-62.
- 5. Schlessinger J. Cell signaling by receptor tyrosine kinases. Cell. 2000;103(2):211-25.
- 6. Eswarakumar VP, Lax I, Schlessinger J. Cellular signaling by fibroblast growth factor receptors. Cytokine Growth Factor Rev. 2005;16(2):139-49.
- Powers CJ, McLeskey SW, Wellstein A. Fibroblast growth factors, their receptors and signaling. Endocr Relat Cancer. 2000;7(3):165-97.

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- Sleeman M, Fraser J, McDonald M, Yuan S, White D, Grandison P, et al. Identification of a new fibroblast growth factor receptor, FGFR5. Gene. 2001; 271(2):171-82.
- 9. Steger K, Tetens F, Seitz J, Grothe C, Bergmann M. Localization of fibroblast growth factor 2 (FGF-2) protein and the receptors FGFR 1-4 in normal human seminiferous epithelium. Histochem Cell Biol. 1998;110(1):57-62.
- Cancilla B, Risbridger GP. Differential localization of fibroblast growth factor receptor-1, -2, -3, and -4 in fetal, immature, and adult rat testes. Biol Reprod. 1998;58(5):1138-45.
- Cancilla B, Davies A, Ford-Perriss M, Risbridger GP. Discrete cell- and stage-specific localisation of fibroblast growth factors and receptor expression during testis development. J Endocrinol. 2000;164 (2):149-59.
- 12. Kirby JL, Yang L, Labus JC, Hinton BT. Characterization of fibroblast growth factor receptors expressed in principal cells in the initial segment of the rat epididymis. Biol Reprod. 2003;68(6):2314-21.
- Hirai K, Sasaki H, Yamamoto H, Sakamoto H, Kubota Y, Kakizoe T, et al. HST-1/FGF-4 protects male germ cells from apoptosis under heat-stress condition. Exp Cell Res. 2004;294(1):77-85.
- El Ramy R, Verot A, Mazaud S, Odet F, Magre S, Le Magueresse-Battistoni B. Fibroblast growth factor (FGF) 2 and FGF9 mediate mesenchymal epithelial interactions of peritubular and Sertoli cells in the rat testis. J Endocrinol. 2005;187(1):135-47.
- Abd-Elmaksoud A, Sinowatz F. Expression and localization of growth factors and their receptors in the mammalian testis. Part I: Fibroblast growth factors and insulin-like growth factors. Anat Histol Embryol. 2005;34(5):319-34.
- Kim Y, Bingham N, Sekido R, Parker KL, Lovell-Badge R, Capel B. Fibroblast growth factor receptor 2 regulates proliferation and Sertoli differentiation during male sex determination. Proc Natl Acad Sci USA. 2007;104(42):16558-63.
- Han IS, Sylvester SR, Kim KH, Schelling ME, Venkateswaran S, Blanckaert VD, et al. Basic fibroblast growth factor is a testicular germ cell product which may regulate Sertoli cell function. Mol Endocrinol. 1993;7(7):889-97.
- Deng CX, Wynshaw-Boris A, Shen MM, Daugherty C, Ornitz DM, Leder P. Murine FGFR-1 is required for early postimplantation growth and axial organization. Genes Dev. 1994;8(24):3045-57.
- 19. Arman E, Haffner-Krausz R, Chen Y, Heath JK, Lonai P. Targeted disruption of fibroblast growth

factor (FGF) receptor 2 suggests a role for FGF signaling in pregastrulation mammalian development. Proc Natl Acad Sci USA. 1998;95(9):5082-7.

- Deng C, Wynshaw-Boris A, Zhou F, Kuo A, Leder P. Fibroblast growth factor receptor 3 is a negative regulator of bone growth. Cell. 1996;84(6):911-21.
- Weinstein M, Xu X, Ohyama K, Deng CX. FGFR-3 and FGFR-4 function cooperatively to direct alveogenesis in the murine lung. Development. 1998;125(18):3615-23.
- 22. Yu C, Wang F, Kan M, Jin C, Jones RB, Weinstein M, et al. Elevated cholesterol metabolism and bile acid synthesis in mice lacking membrane tyrosine kinase receptor FGFR4. J Biol Chem. 2000;275 (20):15482-9.
- 23. Trokovic N, Trokovic R, Mai P, Partanen J. Fgfr1 regulates patterning of the pharyngeal region. Genes Dev. 2003;17(1):141-53.
- 24. Trokovic R, Trokovic N, Hernesniemi S, Pirvola U, Vogt Weisenhorn DM, Rossant J, et al. FGFR1 is independently required in both developing midand hindbrain for sustained response to isthmic signals. EMBO J. 2003;22(8):1811-23.
- 25. Yu K, Xu J, Liu Z, Sosic D, Shao J, Olson EN, et al. Conditional inactivation of FGF receptor 2 reveals an essential role for FGF signaling in the regulation of osteoblast function and bone growth. Development. 2003;130(13):3063-74.
- 26. Blak AA, Naserke T, Saarimäki-Vire J, Peltopuro P, Giraldo-Velasquez M, Vogt Weisenhorn DM, et al. Fgfr2 and Fgfr3 are not required for patterning and maintenance of the midbrain and anterior hindbrain. Dev Biol. 2007;303(1):231-43.
- Deng C, Bedford M, Li C, Xu X, Yang X, Dunmore J, et al. Fibroblast growth factor receptor-1 (FGFR-1) is essential for normal neural tube and limb development. Dev Biol. 1997;185(1):42-54.
- Xu X, Qiao W, Li C, Deng CX. Generation of Fgfr1 conditional knockout mice. Genesis. 2002;32 (2):85-6.
- Schmahl J, Kim Y, Colvin JS, Ornitz DM, Capel B. Fgf9 induces proliferation and nuclear localization of FGFR2 in Sertoli precursors during male sex determination. Development. 2004;131(15): 3627-36.
- Lei Z, Lin J, Li X, Li S, Zhou H, Araki Y, et al. Postnatal male germ-cell expression of cre recombinase in Tex101-iCre transgenic mice. Genesis. 2010;48(12):717-22.
- Boucheron C, Baxendale V. Isolation and purification of murine male germ cells. Methods Mol Biol. 2012;825:59-66.

- 32. Mruk DD, Lau AS. RAB13 participates in ectoplasmic specialization dynamics in the rat testis. Biol Reprod. 2009;80(3):590-601.
- 33. Iwanami Y, Kobayashi T, Kato M, Hirabayashi M, Hochi S. Characteristics of rat round spermatids differentiated from spermatogonial cells during coculture with Sertoli cells, assessed by flow cytometry, microinsemination and RT-PCR. Theriogenology. 2006;65(2):288-98.
- 34. Lei ZM, Mishra S, Zou W, Xu B, Foltz M, Li X, et al. Targeted disruption of luteinizing hormone/ human chorionic gonadotropin receptor gene. Mol Endocrinol. 2001;15(1):184-200.
- 35. Hess RA, Renato de Franca L. Spermatogenesis and cycle of the seminiferous epithelium. Adv Exp Med Biol. 2008;636:1-15.
- 36. Yamamoto H, Ochiya T, Takahama Y, Ishii Y, Osumi N, Sakamoto H, et al. Detection of spatial

localization of Hst-1/Fgf-4 gene expression in brain and testis from adult mice. Oncogene. 2000; 19(33):3805-10.

- 37. Elo T, Sipila P, Valve E, Kujala P, Toppari J, Poutanen M, et al. Fibroblast growth factor 8b causes progressive stromal and epithelial changes in the epididymis and degeneration of the seminiferous epithelium in the testis of transgenic mice. Biol Reprod. 2012;86(5):157, 1-12.
- Valve E, Penttila TL, Paranko J, Harkonen P. FGF-8 is expressed during specific phases of rodent oocyte and spermatogonium development. Biochem Biophys Res Commun. 1997;232(1):173-7.
- Cotton L, Gibbs GM, Sanchez-Partida LG, Morrison JR, de Kretser DM, O'Bryan MK. FGFR-1 [corrected] signaling is involved in spermiogenesis and sperm capacitation. J Cell Sci. 2006;119(Pt 1): 75-84.