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 spermatogenesis 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 *Fgfr*s in male germ cells for spermatogenesis and fertility.

Keywords: Conditional gene knockout, Fertility, FGF, *Fgfr*, Spermatogenesis, Testis.
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Introduction

Fibroblast 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 receptor isoforms that are expressed in a tissue-specific 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 region 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 cell-specific 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 Tex101-iCre;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*$^{\text{flox/flox}}$ or *Fgfr2*$^{\text{flox/flox}}$ males to obtain bigenic heterozygous females (*i.e.*,*Tex101-iCre*;*Fgfr1*$^{\text{flox/+}}$ and *Tex101-iCre*;*Fgfr2*$^{\text{flox/+}}$). Then, these heterozygous females were bred with *Fgfr1*$^{\text{flox/flox}}$ or *Fgfr2* $^{\text{flox/flox}}$ to generate male germ cell-specific *Fgfr1* or *Fgfr2* mutant mice (male *Tex101-iCre*;*Fgfr1*$^{\text{flox/flox}}$ or *Tex101-iCre*;*Fgfr2*$^{\text{flox/flox}}$). Floxed *Fgfr1* and *Fgfr2* mice were kindly provided by Dr. Juha Partanen (floxed *Fgfr1* mice, University of Helsinki, Finland) and Dr. David M. Ornitz (floxed *Fgfr2* mice, 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 *Fgfr1*$^{\Delta}$ and *Fgfr2*$^{\Delta}$ were used to determine the deletion of floxed *Fgfr1* 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 *μg/ml* DNase I (Sigma) at 32^oC 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^oC 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-

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

Genes	Primer sequences (5`-3`)	PCR cycles
RT-PCR primers		
Fgfr1	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 primers		
Fgfr1	F: TTGACCGGATCTACACACACC R: AACCACCCCCACACCAAA	32
Fgfr2	F: GTCAATTCTAAGCCACTGTCTGCC R: CTCCACTGATTACATCTAAAGAGC	32
Fgfr1$^{\Delta}$	F: GGACCTCTGGAAGAGCAGTG R: AGGTTCCCTCCTCTTGGATGA	32
Fgfr2$^{\Delta}$	F: ATAGGAGCAACAGGCGG R: CATAGCACAGGCCAGGTTG	32
iCre	F: TCTGATGAAGTCAGGAAGAACC R: GAGATGTCCTTCACTCTGATTC	33

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 *μg/μl*. 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 β-actin or β-tubulin 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% H_2O_2 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 anti-rabbit 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 3'3-diaminobenzidine. 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*$^{\text{flox/flox}}$ and four *Tex101-iCre*;*Fgfr2*$^{\text{flox/flox}}$ 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

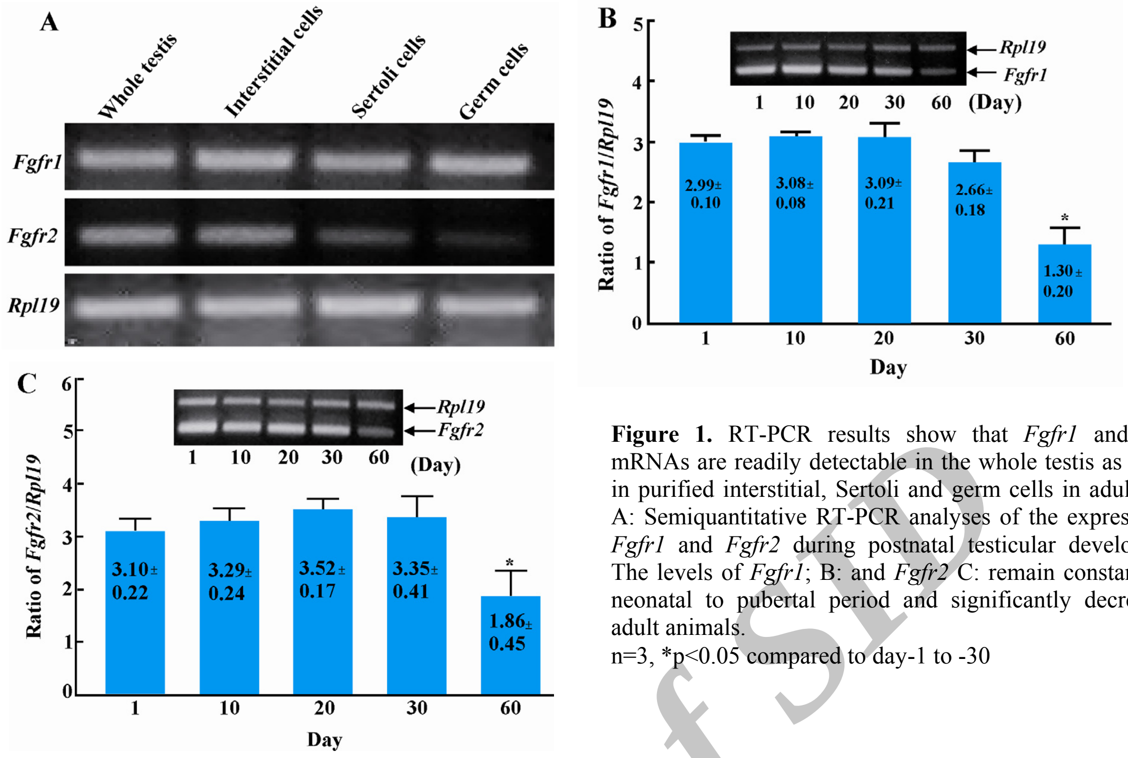

Figure 1. RT-PCR results show that *Fgfr1* and *Fgfr2* mRNAs are readily detectable in the whole testis as well as in purified interstitial, Sertoli and germ cells in adult mice. A: Semiquantitative RT-PCR analyses of the expression of *Fgfr1* and *Fgfr2* during postnatal testicular development. The 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

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, immunoblotting 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

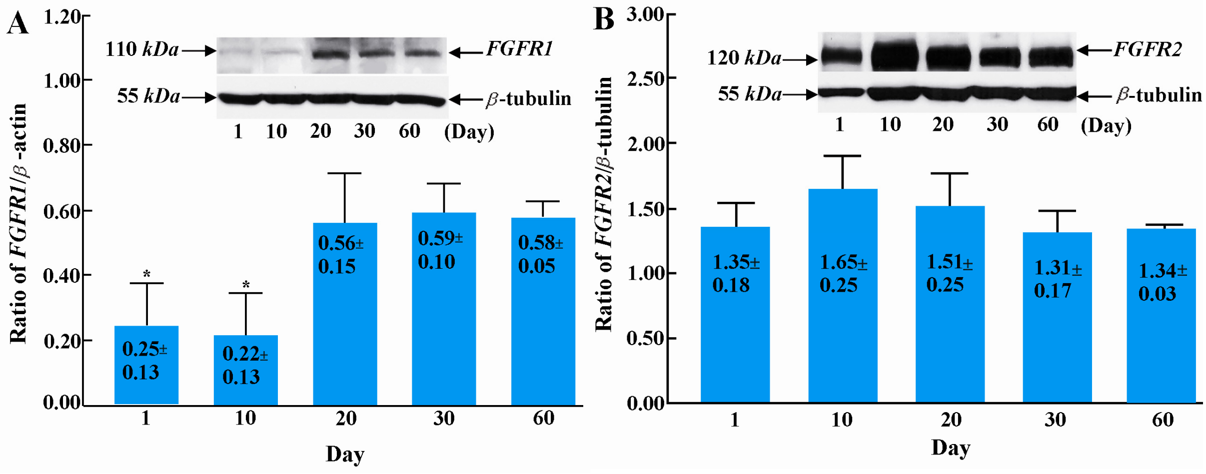

Figure 2. Western blot analyses of FGFR1 and FGFR2 during postnatal testicular development. The protein levels of FGFR1; A: are low at the neonatal and premature period, then increase from the peripubertal stage to adulthood. FGFR2; B: remains unchanged throughout entire postnatal period.
n=3, * p<0.05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

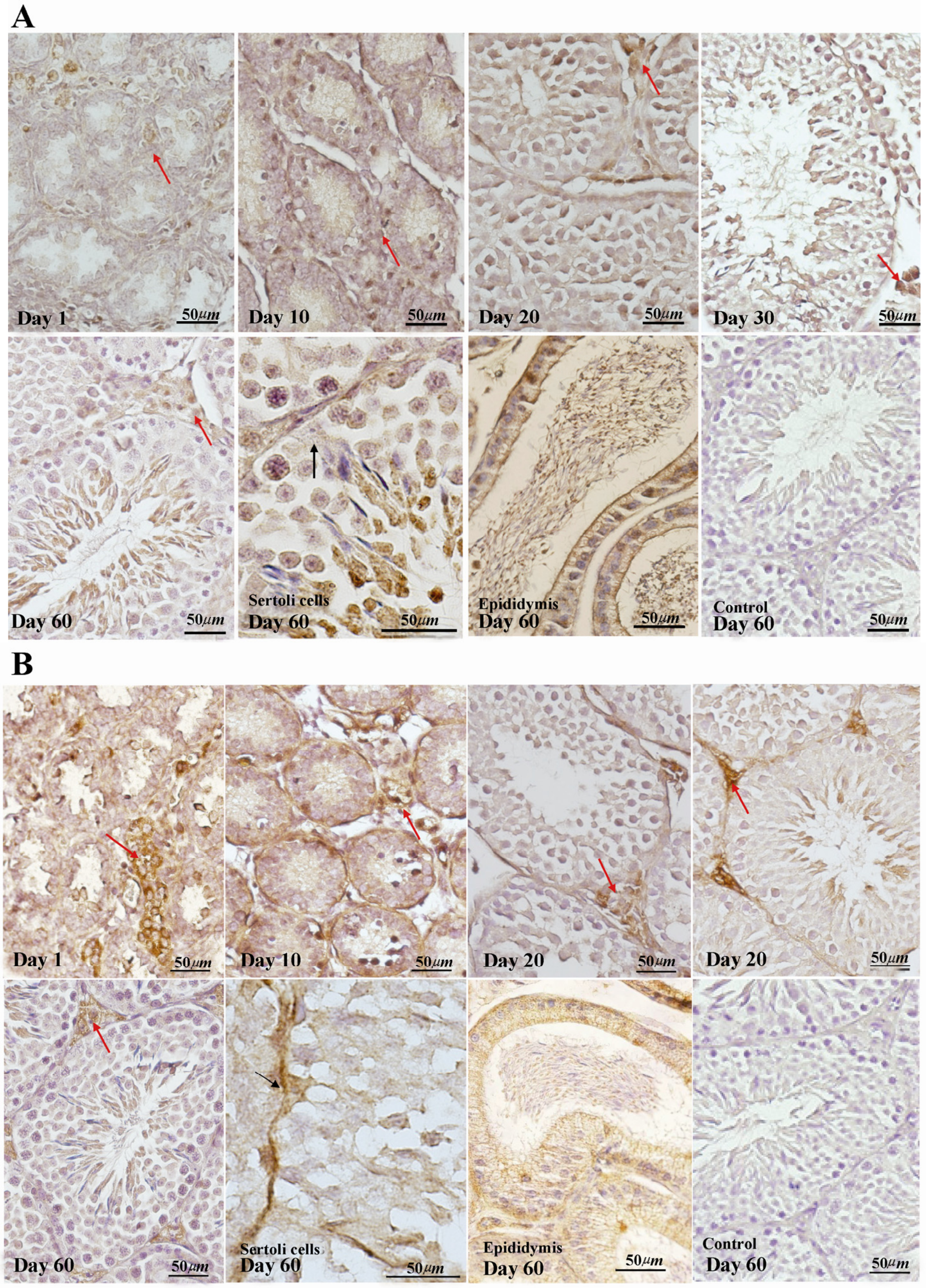

Figure 3. Immunohistochemical staining of FGFR1; A: and FGFR2 B: during postnatal testicular development. Prominent immunostaining of FGFR1 and FGFR2 are observed in interstitial (red arrows) and peritubular cells with no significant changes among all age groups. In the seminiferous tubular compartment, 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

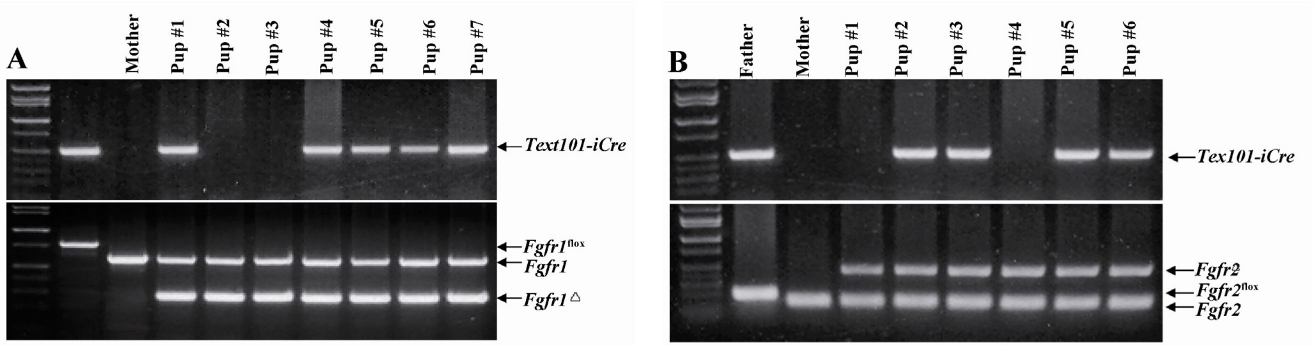

Figure 4. Deletion of the floxed *Fgfr1*; A) and *Fgfr2*; B) alleles by *Tex101-iCre* in male germline. Representative PCR genotyping results of a litter of pups from breeding of a *Tex101-iCre;Fgfr1* $^{flox/flox}$ (A) or *Tex101-iCre;Fgfr2*$^{flox/flox}$ (B) male with a wild-type female, respectively. Note the lack of the *Fgfr1*^{fl} (A) and *Fgfr2*^{fl} (B) alleles and the presence of the *Fgfr1*$^{\Delta}$ and *Fgfr2*$^{\Delta}$ 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 *Tex101-iCre*;*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*$^{\Delta}$ and *Fgfr2*$^{\Delta}$ 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*$^{\Delta}$ and *Fgfr2*$^{\Delta}$ 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 *Tex 101-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*; *Fgfr*$^{flox/flox}$ (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*;*Fgfr2*$^{flox/flox}$ (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

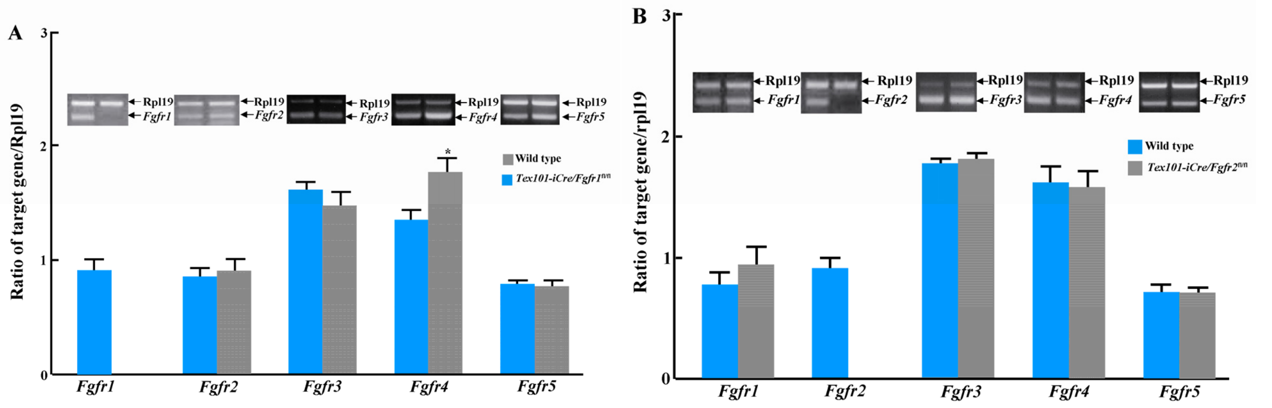

Figure 5. Complete lack of *Fgfr1*; A: or *Fgfr2*; B: expression in isolated testicular germ cells of *Tex101-iCre*;*Fgfr1*$^{flox/flox}$; A) and *Tex101-iCre;Fgfr2*$^{flox/flox}$; B) adult mice. Semiquantitative RT-PCR shows that deletion of *Fgfr1* or *Fgfr2* gene did not significantly influence the 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

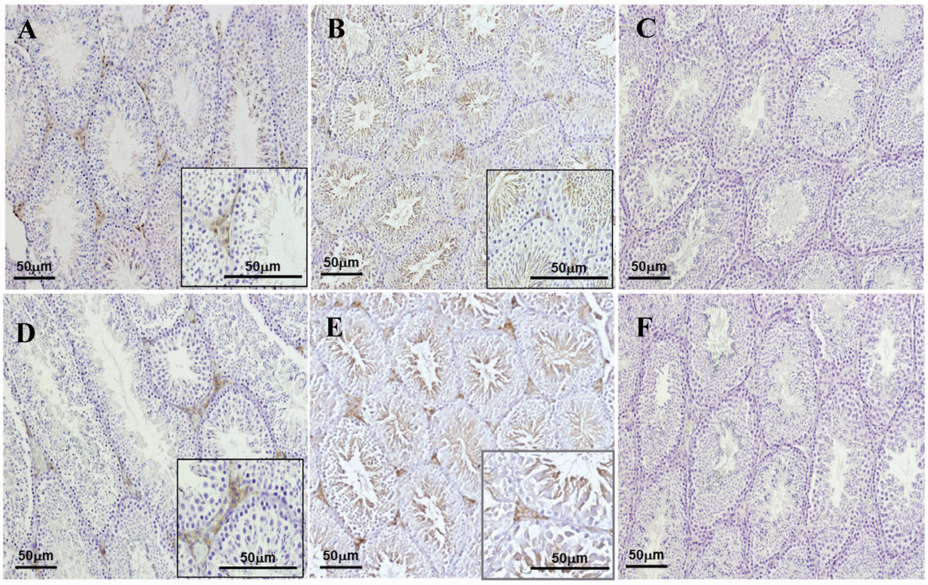

Figure 6. Immunohistochemistry reveals the absence of FGFR1 (A) and FGFR2 (D) in spermatogonia, spermatocytes and spermatids of *Tex101-iCre;Fgfr1*$^{flox/flox}$ (A) and *Tex101-iCre;Fgfr2*$^{flox/flox}$ (D) adult mice, while immunostaining of FGFR1 (A) and FGFR2 (D) in interstitial and peritubular cells 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 cell-selective 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 wild-type (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
Tex101-iCre/Fgfr1$^{fl/fl}$**×wild type**	4	8.6±1.7
Tex101-iCre fgfr2$^{fl/fl}$**×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-

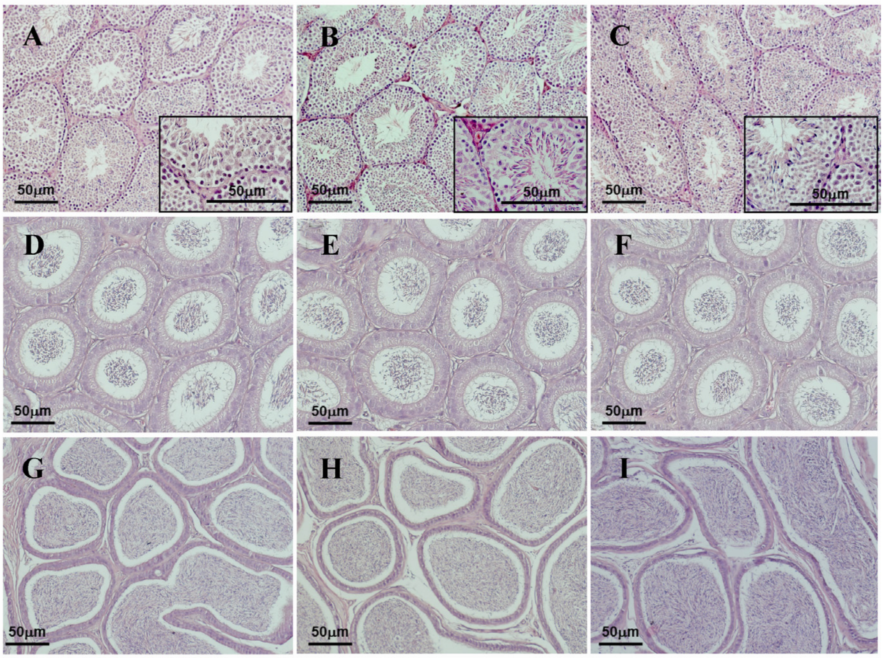

Figure 7. Morphological analyses of H & E stained sections of the testes; A-C: and caput D-F: and cauda G-I: epididymis do not show any abnormalities in either *Tex101-iCre;Fgfr1*$^{flox/flox}$ (A, D & G) or *Tex101-iCre;Fgfr2*$^{flox/flox}$ (B, E & H) adult mice as compared to wild-type siblings (C, 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 *Fgfr*4 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 *Fgfr*s in these cells. These results show that the transcripts of the other four *Fgfr*s 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*$^{\text{flox/flox}}$ 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.

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