

Involvement of fibroblast growth factor 2 (FGF2) and its receptors in the regulation of mouse sperm physiology

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Abstract

Fibroblast growth factor 2 (FGF2) and its receptors (FGFRs) have been described in several tissues, where they regulate cellular proliferation, differentiation, motility and apoptosis. Although FGF2/FGFRs expression in the male reproductive tract has been reported, there is scarce evidence on their presence in the female reproductive tract and their involvement in the modulation of sperm function. Therefore, the objective of this study was to determine the expression of FGF2 in the female reproductive tract and to assess the role of the FGF2/FGFRs system in the regulation of sperm physiology using the murine model. FGF2 was detected in uterus and oviduct protein extracts, and it was immunolocalized in epithelial cells of the uterus, *isthmus* and *ampulla*, as well as in the *cumulus oophorus-oocyte* complex. The receptors FGFR1, FGFR2, FGFR3 and FGFR4 were immunodetected in the flagellum and acrosomal region of sperm recovered from the *cauda epididymis*. Analysis of testis sections showed the expression of FGFRs in germ cells at different stages of the spermatogenesis, suggesting the testicular origin of the sperm FGFRs. Sperm incubation with recombinant FGF2 (rFGF2) led to increased sperm motility and velocity and to enhanced intracellular Ca²⁺ levels and acrosomal loss compared to the control. In conclusion, this study shows that FGF2 is expressed in tissues of the female reproductive tract. Also, the fact that functional FGFRs are present in mouse sperm and that rFGF2 affects sperm motility and acrosomal exocytosis, suggests the involvement of this system in the *in vivo* regulation of sperm function.

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Introduction

Fibroblast growth factors (FGFs) comprise a family of more than 20 proteins, divided into seven subfamilies. Among them, FGF2 is the best-characterized member (Ornitz & Itoh 2001, Yu *et al.* 2007). The biological activities of FGFs are mediated by their binding to specific receptors (FGFRs), composed of three extracellular immunoglobulin-like domains, a single transmembrane domain, and two cytoplasmic domains containing the catalytic tyrosine kinase core (Givol & Yaron 1992, Johnson & Williams 1993). The most studied FGFRs are known as FGFR1, FGFR2, FGFR3 and FGFR4. Transcripts coding the third extracellular domain of FGFR1, FGFR2 and FGFR3 are subjected to alternative splicing, giving rise to two receptor isoforms (IIIb and IIIc), with different ligand-binding properties and specific tissue expression (Gong 2014). Evidence in somatic cells indicates that interaction of FGFs with heparin or heparan sulfate proteoglycans (HSPGs)

allows their binding to the FGFRs, triggering receptor dimerization and autophosphorylation (Mohammadi *et al.* 2005). FGFR signal transduction results in the activation of several pathways (including STAT, MAPK and PI3K pathways), leading to the phosphorylation of transcription factors and the expression of FGF-target genes (Eswarakumar *et al.* 2005).

Members of the FGFs and FGFRs families have been identified in a variety of tissues (Hughes 1997, Fon Tacer *et al.* 2010), and the FGFs/FGFRs signaling has been implicated in many cellular processes, such as cell proliferation, differentiation, adhesion, survival, apoptosis, motility and chemotaxis. It has been reported that this system is involved in the development and maintenance of normal tissues, but also in tumor progression (Turner & Grose 2010, Belov & Mohammadi 2013, Ornitz & Itoh 2015). Despite the high amount of evidence on the role of FGFs and FGFRs in different tissues, including the reproductive ones, there is not enough

information of their relevance in the regulation of gamete physiology.

Mammalian sperm are produced in the testis by spermatogenesis and undergo a maturation process during their transport through the epididymis. Ejaculated sperm are not able to fertilize an egg; to become fertilization competent, sperm must undergo a series of biochemical modifications in the female reproductive tract, collectively known as capacitation. Capacitation is associated with an increase in sperm intracellular Ca^{2+} levels and in protein tyrosine phosphorylation and with the development of a distinct pattern of motility (hyperactivated motility). Capacitated sperm are also able to respond to chemotactic stimuli from the egg and to undergo acrosomal exocytosis. All these processes are regulated by intrinsic sperm factors, but they also depend on certain stimuli present in the male and female reproductive tracts (Yanagimachi 1994, Wassarman *et al.* 2001, Eisenbach & Giojalas 2006, Vazquez-Levin & Marín-Briggiler 2009).

In previous studies done in the human model, we described the presence of FGF2 and functional FGFRs in the testis and sperm, and our results suggest that this system is involved in the regulation of human spermatogenesis and sperm motility (Saucedo *et al.* 2015, Garbarino Azúa *et al.* 2017). Moreover, human sperm incubation with recombinant FGF2 (rFGF2) led to an increase in the percentage of motile cells (Saucedo *et al.* 2015) and to an improvement in sperm recovery in a selection technique, indicating the potential use of this compound in the human clinical practice (Garbarino Azúa *et al.* 2017).

The present work was aimed to further advance in the knowledge of the FGF2/FGFRs system in the modulation of the reproductive function in mammals, using the murine model. Our objectives were (1) to evaluate the presence of FGF2 in tissues of the female reproductive tract, (2) to describe the expression and localization of FGFRs in the sperm cells and (3) to determine the effect of incubation with rFGF2 upon different sperm parameters.

Materials and methods

Reagents and antibodies

All reagents were of tissue culture grade and molecular biology quality, purchased from Sigma Chemical Co., GE-Amersham Pharmacia (Piscataway, NJ, USA) and Thermo-Life Technologies (Carlsbad, CA, USA), unless indicated.

The following polyclonal antibodies and their corresponding blocking peptides (P) were used: anti-FGF2 (sc-79 and sc-79 P), anti-FGFR1 (sc-121 and sc-121 P), anti-FGFR2 (sc-122 and sc-122 P), anti-FGFR3 (sc-123 and sc-123 P) and anti-FGFR4 (sc-9006) (Santa Cruz Biotechnology Inc.). No blocking peptide was commercially available to anti-FGFR-4. Other antibodies used were rabbit immunoglobulin G (IgG) (Sigma), anti-pTyr monoclonal (clone 4G10; Upstate Biotechnology, Lake Placid, NY, USA), anti-tubulin (Sigma), anti-rabbit

IgG-conjugated with horseradish peroxidase (HRP) (Sigma), with Alexa Fluor 488 (A-11008) (Life Technologies) and with Cy3 (Chemicon-Millipore, Billerica, MA, USA), and anti-mouse conjugated with HRP (Vector Laboratories, Inc., Burlingame, CA, USA).

The rFGF2 was produced in a bacterial expression system (gently provided by Dr. Baldi and Dr. Góngora, IBYME) and was previously shown to activate FGFRs in human sperm (Saucedo *et al.* 2015). BGJ398 (NVP-BGJ398; Selleck Chemicals LLC, Houston, TX, USA), a selective inhibitor of FGFR tyrosine kinase activity (Guagnano *et al.* 2011), was used when indicated.

Animals and experimental procedures

Hybrid F1 (C57Bl/6 x Balb/c) mature (2- to 6-month old) mice were used. Animals were maintained at 23°C with a 12-h light:12-h darkness cycle. Experiments were performed in strict accordance with the Guide for Care and Use of Laboratory Animals approved by the National Institutes of Health. Experimental procedures were reviewed and approved by the Ethics Committee of the Instituto de Biología y Medicina Experimental (Ref: CE 010-2/2013) and the Institutional Animal Care and Use Committee (Approval number 022/2016).

To analyze the expression of FGF2 in tissues of the female reproductive tract, 2- to 4-month-old females were used. The estrous cycle stage was determined every morning by the analysis of vaginal smears as previously described (Caligioni 2009). The uteri and oviducts of females in estrous were removed and stored at -80°C for protein extraction or processed immediately for indirect immunofluorescence. To obtain the *cumulus oophorus*-oocyte complexes (COCs), females were stimulated with hormones. Animals were treated with an i.p. injection of equine chorionic gonadotrophin (eCG; 5 IU; Syntex SA, Buenos Aires, Argentina), followed by an i.p. injection of human chorionic gonadotrophin (hCG; 5 IU, Sigma) 48 h later. After 12–14 h, females were killed, the oviducts were removed and the COCs were collected.

The testes were obtained and stored at -80°C for protein extraction or processed immediately for immunohistochemistry. To recover sperm cells, both *cauda* epididymides from a mouse were collected, dissected and placed in 400 μL of culture medium (Fraser & Drury 1975) for 10 min to allow the release of motile cells (swim-out procedure). When indicated, sperm (at a concentration of $1-10 \times 10^6/\text{mL}$) were resuspended in medium supplemented with 0.3% bovine serum albumin (BSA) and incubated at 37°C in an atmosphere of 5% CO_2 in air to promote capacitation.

Protein extracts, SDS-PAGE and Western immunoblotting

To obtain the protein extracts from uterus, oviduct and testis, small tissue sections were placed in 0.5 mL of homogenization buffer containing 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate in PBS supplemented with protease inhibitors. Tissues were disaggregated with a homogenizer (5 pulses of 3 s at maximum potency) and subjected to centrifugation (15,000 g for 5 min at 4°C).

The supernatants were recovered, supplemented with Laemmli sample buffer, heated at 100°C for 5 min and stored at -70°C until used. To obtain the sperm protein extracts, a suspension of motile cells was centrifuged for 5 min at 600g, the sperm pellets were resuspended with Laemmli sample buffer, heated, centrifuged and the supernatants were recovered. Protein concentration was determined using the Bradford method. Samples were supplemented with 5% 2-mercaptoethanol, boiled for 10 min and subjected to SDS-PAGE in 8 or 15% polyacrylamide gels and Western immunoblotting. Anti-FGF2, anti-FGFR antibodies or rabbit IgG was used at 2 µg/mL and HRP-conjugated secondary antibody was used at 0.5 µg/mL. As a control, each primary antibody was pre-incubated with its blocking peptide at five-fold (by weight) excess following the supplier's recommendations. To analyze protein tyrosine phosphorylation, sperm were incubated under capacitating conditions in the presence or absence of rFGF2 for different periods of time. Proteins were extracted, subjected to SDS-PAGE in 10% polyacrylamide gels and developed with anti-pTyr, anti-tubulin (0.2 µg/mL) and anti-mouse HRP antibody (Marín-Briggiler *et al.* 1999). The reactive bands were detected by enhanced chemiluminescence (ECL kit, GE-Amersham) using standard procedures.

Immunohistochemistry and immunocytochemistry

Uteri and oviducts were dissected, washed and immediately embedded in Optimum Cutting Temperature compound (Biopack, Buenos Aires, Argentina). Sections of 8 µm were cut with a cryostat and kept at -80°C until processed. Slides were fixed in 4% formaldehyde in PBS for 20 min, immersed for 30 min in a 150 mM glycine solution and incubated for 45 min with blocking solution (0.1 M glycine, 1% normal goat serum, 0.5% BSA, 0.01% Triton X-100 in PBS). Slides were then incubated overnight at 4°C with anti-FGF2 (4 µg/mL in PBS with 4% BSA). As a control, the primary antibody was pre-incubated with its blocking peptide at five-fold (by weight) excess following the supplier's recommendations. Following 3 washes with 0.1% Triton X-100 in PBS, tissues were incubated for 1 h with anti-rabbit IgG labeled with Alexa Fluor 488 (10 µg/mL in PBS).

COCs were fixed with 2% paraformaldehyde in PBS for 1 h, washed with PBS and incubated in blocking solution for 2 h. Then, they were exposed to anti-FGF2 (8 µg/mL in 4% BSA in PBS), pre-incubated or not with the blocking peptide. Following 3 washes with PBS containing 0.3% BSA and 0.02% Tween, COCs were incubated for 1 h with anti-rabbit IgG labeled with Alexa Fluor 488 (20 µg/mL). Tissues and COCs were counterstained with propidium iodide (PI, Sigma), slides were mounted with Vectashield (Vector Laboratories, Inc) and were observed with a laser confocal microscope (C1, Nikon). Images were acquired with an objective Plan Apo 40×/0.95 (excitation/emission: 488 nm/515-530 nm and 544 nm/570 LP) and analyzed using standard procedures for fluorescent imaging.

Small portions of mouse testis were fixed and processed for immunohistochemistry, using anti-FGFR antibodies or rabbit IgG (4 µg/mL) and the LSAB+ System HRP kit (K0690, Dako) as previously described (Marín-Briggiler *et al.* 2008).

To determine the specificity of anti-FGFRs, they were pre-incubated with the corresponding blocking peptides. Specimens were counterstained with hematoxylin, dehydrated and mounted. Sections were evaluated at ×600 magnification using an Eclipse E800 microscope (Nikon Instruments Inc.). The expression of FGFRs was assessed in Sertoli and germ cells at different stages of the spermatogenic cycle (Russell *et al.* 1990).

Sperm (non-capacitated or incubated under capacitating conditions for 90 min) were fixed with formaldehyde (2% in PBS) for 4 min and processed for staining with anti-FGFR antibodies, rabbit IgG (20 µg/mL) or the primaries antibodies previously incubated with the blocking peptides and Cy3-conjugated secondary antibody. Sperm were also stained with the lectin *Pisum sativum* agglutinin labeled with FITC (FITC-PSA, Sigma; 50 µg/mL in PBS) to assess the acrosomal status of each cell. A green fluorescent signal on the acrosomal region was indicative of an intact acrosome. At least 200 sperm in duplicate samples were evaluated at ×1000 magnification.

Computer-assisted sperm analysis

To analyze the effect of rFGF2 on sperm motility, samples were incubated for 30, 60 and 90 min with different concentrations of rFGF2. The rFGF2 concentrations used in the present study were similar to those reported with somatic cells (Kubota & Ito 2000, Kamura *et al.* 2010) and with human sperm (Saucedo *et al.* 2015). When indicated, sperm were incubated for 15 min with the FGFR inhibitor (BGJ398, 0.1 µM (Saucedo *et al.* 2015)) prior to the incubation with rFGF2 in capacitating conditions.

Sperm motility parameters were evaluated using the Sperm Class Analyzer system (SCA v.6.2.0.1., Microptic SL, Barcelona, Spain) that acquires 60 frames per s (total frames analyzed: 30). A microscope with a temperature-controlled stage was used to maintain sperm at constant 37°C. For each sample, at least seven microscopic fields were analyzed and more than 300 sperm were evaluated. The following parameters were assessed: curvilinear velocity (VCL, µm/s), straight-line velocity (VSL, µm/s), average path velocity (VAP, µm/s), linearity (LIN, %), amplitude of lateral head displacement (ALH, µm), straightness (STR, %), beat cross frequency (BCF, Hz) and wobble (WOB, %). Sperm motility was measured and classified as follows: rapid progressive (VCL ≥45 µm/s; STR ≥50%), medium progressive (VCL ≥15 µm/s; STR ≥50%), *in situ* (VCL <15 µm/s; VAP ≥5 µm/s) and immotile (VAP <5 µm/s). Percentages of total (rapid progressive+medium progressive+*in situ*) and progressive (rapid+medium progressive) motility were recorded. Sperm were considered hyperactivated when presenting VCL ≥271 µm/s, LIN <50% and ALH ≥3.5 µm.

Sperm accumulation assay

To determine whether rFGF2 stimulates sperm accumulation, the sperm selection assay (SSA) was used (Gatica *et al.* 2013). The device consists of two wells (W1 and W2) connected by a 2 mm-length tube, with a hermetic closing system. An aliquot of 1-h capacitated motile sperm (at a concentration of 3 × 10⁶/mL) was placed in W1, while rFGF2 was loaded in W2,

forming an ascending gradient of the ligand in the connecting tube between the wells. Different concentrations of rFGF2 were tested. Culture medium or 100 pM progesterone (Prg, Sigma) was placed in W2 as negative and positive controls respectively (Guidobaldi *et al.* 2017). After 20 min of incubation at 37°C, 5% CO₂ in air, the content of W2 was recovered and sperm concentration was determined using a Neubauer hemocytometer. The percentage of accumulated sperm was calculated as the number of sperm recovered from W2 after the assay, divided by the number of motile sperm initially placed on W1. The result was multiplied by 100 and each determination was done in duplicate. To discriminate whether sperm accumulation in W2 was due to chemotaxis, chemokinesis (an increase of sperm speed) or trapping (e.g. induced by hyperactivation), the SSA was repeated with homogeneous distribution of rFGF2 in the device (the ligand was present in W1 with the sperm suspension, in the connecting tube and in W2).

Intracellular Ca²⁺ measurements

Intracellular Ca²⁺ levels were measured by flow cytometry as described (Brukman *et al.* 2016). Sperm were incubated in capacitating conditions with different concentrations of rFGF2 for a total of 30 and 60 min, and 2 µM Fluo-4 AM (Invitrogen), diluted in 10% Pluronic F-127 (Invitrogen) was added in the last 30 min. Samples were centrifuged for 3 min at 600g to remove the excess of probe, resuspended in BSA-free medium and exposed to 2.5 µg/mL PI. Fluorescence was detected using a BD FACSCanto™ II analyzer following the manufacturer's indications. Data analysis was performed by the FlowJo 7.6 software (FlowJo LLC, Ashland, OR, USA). Results are shown as mean fluorescence intensity for Fluo-4 AM obtained from at least 10,000 live (PI-negative) sperm. The histograms of a representative experiment were also shown.

Analysis of acrosomal exocytosis

The effect of rFGF2 on acrosomal exocytosis was determined in sperm incubated under capacitating conditions for 90 min in the presence of different concentrations of rFGF2 (0–100 ng/mL). Cells were subjected to Coomassie brilliant blue staining as previously described (Busso *et al.* 2007) and were analyzed under 400× magnification using a Nikon microscope (Nikon Instruments Inc.). At least 200 sperm cells were evaluated. Sperm were scored as acrosome intact when a bright blue staining was found in the dorsal region of the head and as acrosome-reacted when no labeling was observed in this area.

Statistical analysis

Data were expressed as mean ± S.E.M.. To assume normal distribution, percentages were expressed as ratios and subjected to the arcsine square root transformation. Results were compared by one-way ANOVA and the Dunnett's multiple comparison test. Statistical analyses were carried out using the GraphPad InStat program (GraphPad Software). Differences were considered significant at a level of $P < 0.05$.

Results

Presence and localization of FGF2 in the female reproductive tract

The expression of FGF2 protein in the mouse uterus and oviduct was analyzed by Western immunoblotting. Results showed the presence of three FGF2 isoforms (of 18, 21 and 23 kDa) in protein extracts of these tissues (Fig. 1A). Immunofluorescence studies indicated that FGF2 is expressed in the epithelial cells of the uterus, *isthmus* and *ampulla* (Fig. 1B). In addition, a mild immunoreactivity was found in muscle cells of the *isthmus*. FGF2 was also detected in the cytoplasm of

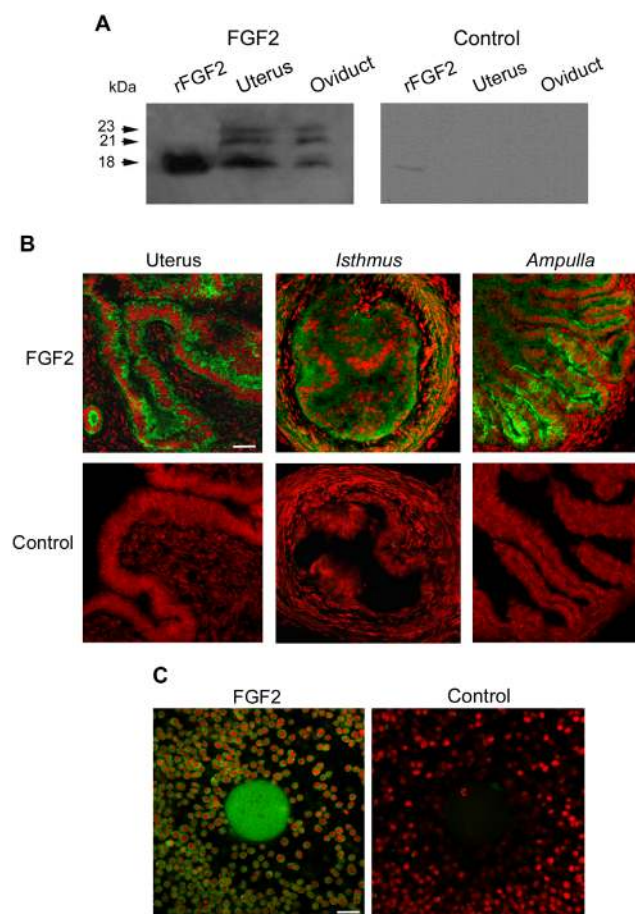


Figure 1 Presence and localization of FGF2 in murine female reproductive tract. (A) Protein extracts from uterus (60 µg) and oviduct (120 µg) were subjected to SDS-PAGE, followed by Western immunoblotting using anti-FGF2. rFGF2 (5 ng) was included. As a control, the primary antibody was pre-incubated with its blocking peptide. A representative result is shown. (B) Sections of uterus, *isthmus* and *ampulla* were subjected to immunofluorescence studies using anti-FGF2 antibody and anti-mouse labeled with Alexa Fluor 488. As a control, the primary antibody was pre-incubated with its blocking peptide. Cell nuclei were counterstained with PI. Bar: 20 µm. Typical results are shown. (C) COCs were recovered from the *ampulla* and subjected to immunofluorescence studies using anti-FGF2 antibody and anti-mouse labeled with Alexa Fluor 488. Cell nuclei were counterstained with PI. Bar: 20 µm.

the oocyte and cumulus oophorus cells (Fig. 1C). The staining was specific, since no signal was observed when the primary antibody was pre-incubated with the blocking peptide as a control (Fig. 1A, B and C).

Presence and localization of FGFR proteins in mouse sperm

Previous studies reported the expression of FGFR1 in mouse sperm (Cotton *et al.* 2006); however, there is no evidence on the expression of other FGFRs in these cells. We analyzed the presence of FGFR1, FGFR2, FGFR3 and FGFR4 in mouse sperm by Western immunoblotting using specific antibodies. A protein form of approximately 100 kDa was detected with anti-FGFR1 and anti-FGFR3. Forms of 127 and 116 kDa were found with anti-FGFR2 and anti-FGFR4, respectively (Fig. 2A). Such bands were not observed when the antibodies against FGFR1, FGFR2 and FGFR3 were pre-incubated with their corresponding blocking peptides or when rabbit IgG was used as control (Fig. 2A).

Indirect immunofluorescence studies revealed the localization of FGFR1, FGFR2, FGFR3 and FGFR4 in the flagellum (middle and principal piece) of all non-

capacitated sperm. FGFR immunoreactivity was also observed in the acrosomal region of non-capacitated, acrosome-intact sperm (for FGFR1: $83 \pm 3\%$, for FGFR2: $93 \pm 1\%$, for FGFR3: $35 \pm 6\%$ and for FGFR4: $83 \pm 1\%$ sperm, $n=5$) (Fig. 2B). After incubation under capacitating conditions, sperm maintained FGFR localization in the flagellum; however, there was a reduction of FGFR signal in the acrosomal region, even when sperm had intact acrosomes (for FGFR1: $14 \pm 1\%$, for FGFR2: $38 \pm 3\%$, for FGFR3, $0 \pm 0\%$ and for FGFR4: $16 \pm 5\%$ sperm) (Supplementary Fig. 1, see section on supplementary data given at the end of this article). FGFR immunodetection was specific, as no staining was observed when the antibodies were pre-incubated with the respective peptides or when primary antibodies were replaced by rabbit IgG (Supplementary Fig. 2).

Expression and immunodetection of FGFR proteins in mouse testis

To analyze the testicular origin of sperm FGFRs, we performed Western immunoblotting of testicular protein extracts, as well as immunohistochemical studies on testicular tissue sections. Protein extracts developed with anti-FGFR1 showed a main protein band of 117 kDa. Bands of approximately 127, 125 and 116 kDa were detected with anti-FGFR2, FGFR3 and FGFR4, respectively. No immunoreactivity was observed when rabbit IgG was used as a control (Fig. 3A).

Immunohistochemical studies allowed the detailed analysis of FGFR expression in the seminiferous epithelium and in different stages of the spermatogenic cycle. Results showed the localization of the four FGFRs in germ cells, in a stage-dependent manner ($n=3$). All receptors were immunolocalized in the flagellum of elongating/elongated spermatids (Fig. 3B). In particular, FGFR1 expression was observed in Sertoli and germ cells, mainly in the adluminal compartment. This protein was localized in pachytene spermatocytes, round and elongated spermatids and the staining was stronger in stages VIII–XII in comparison to stages I–VII. FGFR2 immunoreactivity was moderate in Sertoli cells, but intense in the germ cells, with a higher signal in the cytoplasm of elongating spermatids of stages X–XII. Regarding FGFR3, a weak immunostaining was observed in the seminiferous epithelium, with a mild signal in the cytoplasm of elongating spermatids of stages X–XII. The presence of FGFR3 was also detected in the acrosomal region of round and elongating spermatids of stages VI–XI. A weak staining for FGFR4 was found in germ cells at stages I–V and a moderate signal was detected in the cytoplasm of elongating spermatids (stages X–XII).

Effect of FGF2 in the regulation of sperm physiology

To determine the role of the FGF2/FGFRs system in sperm physiology, cauda epididymal sperm were

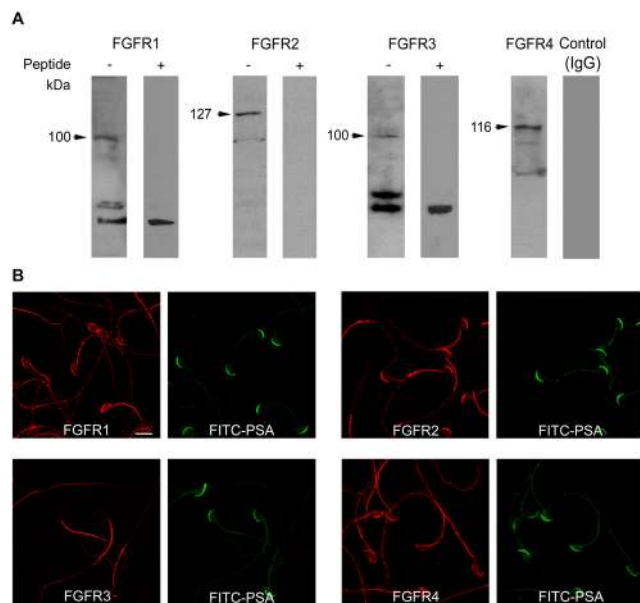


Figure 2 Presence and localization of FGFRs in mouse sperm. (A) Detection of sperm FGFR protein forms using Western immunoblotting. Protein extracts from mouse sperm were subjected to SDS-PAGE and developed using anti-FGFR antibodies. As a control, each primary antibody was pre-incubated with its blocking peptide, or rabbit IgG was used. The estimated molecular weights of the protein bands are indicated on the left. The experiments were performed at least three times obtaining similar results. Typical results are shown. (B) Immunolocalization of FGFRs in mouse sperm. Sperm cells were stained with anti-FGFR1, FGFR2, FGFR3 or FGFR4 and a secondary antibody labeled with Cy3 (left images). Sperm were also stained with FITC-PSA to assess their acrosomal status, and the corresponding fields are shown (right images). Bar: 10 μ m.

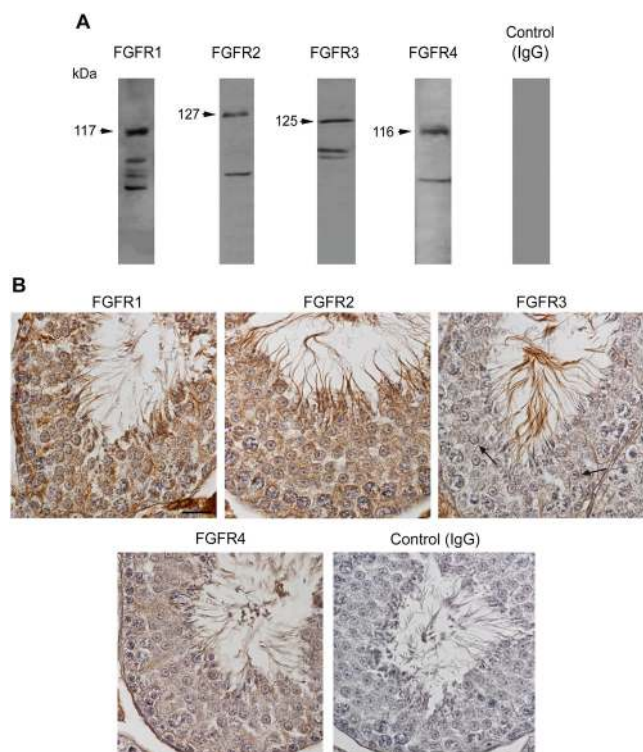


Figure 3 Expression and immunodetection of FGFRs in mouse testis. (A) Detection of sperm FGFR protein forms using Western immunoblotting. Protein extracts from mouse testis were subjected to SDS-PAGE and developed using anti-FGFR antibodies or rabbit IgG as control. The estimated molecular weights of the protein bands are indicated on the left. The experiments were performed at least three times obtaining similar results. Typical results are shown. (B) Immunolocalization of FGFRs in mouse testis. Histological sections were stained with anti-FGFR1, FGFR2, FGFR3 or FGFR4. Rabbit IgG was included as control. The specimens were counterstained with hematoxylin. Images correspond to stages VII or VIII. Arrows indicate immunoreactivity for FGFR3 in spermatid acrosome. Bar: 20 μ m.

incubated with different concentrations of rFGF2 and several parameters were assessed.

Incubation with 0.1 and 1 ng/mL rFGF2 for 30 min led to a significant increase in total and progressive sperm motility. The effect was mediated by FGFR activation, since the response was abolished when the cells were pre-incubated with the inhibitor BGJ398 (Fig. 4A and B). An enhancement ($P < 0.05$) in velocity parameters (VCL and VSL) was also observed after exposure to 0.1 ng/mL rFGF2 for 30 min (Table 1); however, at this time point, no significant changes were observed in other sperm parameters or in the percentage of hyperactivated cells (Table 1). No effect was detected in the percentages of either total, progressive or hyperactivated motility in sperm incubated with rFGF2 for 60 or 90 min (Table 2).

Next, the effect of rFGF2 on sperm accumulation was evaluated using the SSA. When sperm were exposed to an ascending gradient of 0.1 ng/mL rFGF2 in the connecting tube, a significant accumulation of cells was observed in W2 well (Fig. 5A). The percentage of

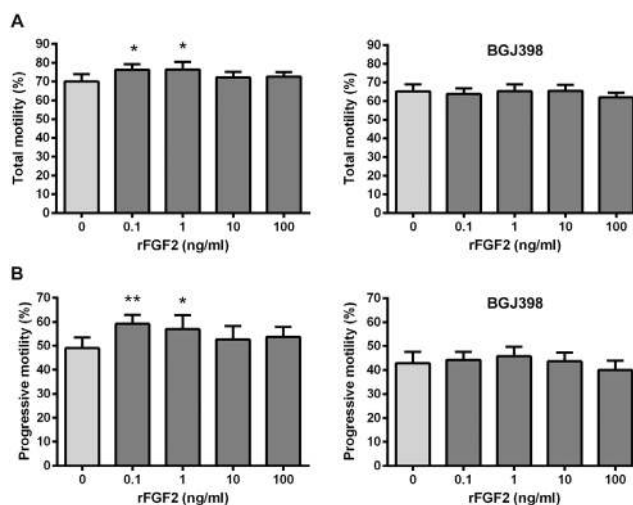


Figure 4 Effect of sperm incubation with rFGF2 on sperm motility. Sperm were incubated with 0, 0.1, 1, 10 and 100 ng/mL rFGF2 in the absence or in the presence of 0.1 μ M BGJ398 (left and right, respectively) and subjected to computer-assisted sperm analysis. (A) Percentages of total motility (rapid progressive + medium progressive + *in situ*). (B) Percentages of progressive motility (rapid + medium progressive). Results are expressed as mean \pm S.E.M., $n = 6$ without BGJ398 and $n = 8$ with BGJ398. * $P < 0.05$, ** $P < 0.01$ compared with 0 ng/mL rFGF2.

accumulated sperm was similar to that obtained with Prg. To distinguish if sperm accumulation was due to an effect of rFGF2 on chemotaxis, chemokinesis or trapping, the assay was repeated in medium with a homogeneous concentration of 0.1 ng/mL rFGF2. Results also showed a significant sperm accumulation under this condition (Fig. 5B), suggesting that the accumulation was not due to a chemotactic response, but to a chemokinetic effect of rFGF2 on the sperm cells. The possibility that rFGF2 has a trapping effect (i.e. hyperactivation) was discarded since under homogeneous rFGF2 distribution, a reduced sperm accumulation would have been expected in W2 well (Giojalas *et al.* 2015).

The effect of sperm incubation with rFGF2 upon capacitation-related events was also determined. Sperm incubated for 30 min with 1, 10 and 100 ng/mL rFGF2 depicted a significant increase in intracellular Ca^{2+} levels. Such effect was not observed after 60-min incubation with rFGF2 (Fig. 6A and Supplementary Fig. 3). In sperm exposed to rFGF2 for 30, 60 or 90 min, no changes in protein tyrosine phosphorylation were detected in comparison to the control (Fig. 6B). However, after 90-min incubation with 0.1, 1 or 10 ng/mL rFGF2 a significant increase in the percentage of acrosome-reacted sperm was observed (Fig. 6C).

Discussion

In the present study, we first aimed to describe the expression of FGF2 in the mouse female reproductive

Table 1 Kinematics and percentage of hyperactivated motility of mouse sperm incubated for 30 min with rFGF2.

	0 ng/mL rFGF2	0.1 ng/mL rFGF2	1 ng/mL rFGF2	10 ng/mL rFGF2	100 ng/mL rFGF2
VCL ($\mu\text{m/s}$)	127 \pm 11	146 \pm 14*	133 \pm 12	120 \pm 16	123 \pm 13
VSL ($\mu\text{m/s}$)	36 \pm 4	45 \pm 6*	34 \pm 4	32 \pm 5	34 \pm 5
VAP ($\mu\text{m/s}$)	63 \pm 4	74 \pm 1	60 \pm 6	60 \pm 6	60 \pm 3
LIN (%)	22 \pm 1	24 \pm 1	22 \pm 1	23 \pm 1	23 \pm 1
ALH (μm)	3 \pm 0	3 \pm 0	3 \pm 0	3 \pm 0	3 \pm 0
STR (%)	47 \pm 1	50 \pm 2	48 \pm 2	48 \pm 2	47 \pm 2
BCF (Hz)	8 \pm 0	9 \pm 0	8 \pm 0	8 \pm 1	8 \pm 0
WOB (%)	42 \pm 1	44 \pm 1	41 \pm 1	43 \pm 1	42 \pm 1
Motility (%)	4 \pm 1	7 \pm 2	5 \pm 2	3 \pm 1	3 \pm 1

Computer-assisted sperm analysis of sperm incubated with 0, 0.1, 1, 10 or 100 ng/mL rFGF2. Parameters measured were curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), linearity (LIN), amplitude of lateral head displacement (ALH), straightness (STR), beat cross frequency (BCF) and wobble (WOB). Results are expressed as mean \pm s.e.m., $n=6$.

* $P < 0.05$ compared with 0 ng/mL rFGF2.

tract. Western immunoblotting analysis allowed the detection of the three FGF2 isoforms, including the 18-kDa secreted form, in the uterus and oviduct. By immunofluorescence, FGF2 was mainly localized in the epithelial cells of these tissues, as well as in the egg and *cumulus oophorus* cells. Previous reports described FGF2 expression in tissues of the bovine and primate female reproductive tracts, and its secretion by oviduct epithelial cells, mainly in the pre-ovulatory stage (Gabler *et al.* 1997, 1998). FGF2 secretion has also been found in human COCs (Malamitsi-Puchner *et al.* 2001, Ben-Haroush *et al.* 2005). Such studies focused in the proliferative, mitogenic and angiogenic effects of FGF2 on cells of these tissues; however, the role of the FGF2 present in the female reproductive tract on the regulation of sperm physiology remains unknown.

To determine the relevance of the FGFs/FGFRs system in the regulation of the mouse male gamete, we initially determine the presence and localization of FGFRs in the sperm cells. By Western immunoblotting, the 4 FGFRs were detected in sperm extracts. Using anti-FGFR1 and anti-FGFR2 antibodies, bands of approximately 100 and 127 kDa, respectively, were found. These bands were similar to those previously reported in mouse testis (Cotton *et al.* 2006, Li *et al.* 2014). Regarding FGFR3, a protein form of approximately 100 kDa was observed, in agreement to that identified in human sperm (Saucedo *et al.* 2015). Anti-FGFR4 revealed a main protein form of 116 kDa, also found in mouse testicular extracts.

The lower molecular weight protein bands obtained for FGFR1 and FGFR3 in sperm in comparison to testicular extracts could be attributed to protein degradation during processing. The same antibodies were used in immunocytochemical studies, and the 4 FGFRs were localized in the flagella of both non-capacitated and capacitated sperm. FGFR1, FGFR2 and FGFR4 were also detected in the acrosomal region of the majority of non-capacitated sperm, but a loss in their immunoreactivity was observed in capacitated cells, as reported for many sperm proteins (Yanagimachi 1994).

In order to determine the testicular origin of the sperm FGFRs, we analyzed the localization of FGFRs in the seminiferous epithelium. The expression of the 4 FGFRs was detected in the germ and Sertoli cells of the mouse testis, in a stage-specific manner, suggesting that FGF signaling is involved in the regulation of murine spermatogenesis. Immunostaining with anti-FGFRs revealed a stronger signal in the adluminal compartment compared to the basal compartment of the seminiferous epithelium, due to lower FGFR expression in the spermatogonia and a higher expression in more differentiated germ cells. In particular, a strong immunoreactivity for FGFRs 1-4 was found in the flagellum of elongating and elongated spermatids. The expression of FGFRs in the testis has been previously described in several mammalian species (Cotton *et al.* 2008), including the human (Steger *et al.* 1998, Saucedo *et al.* 2015). Our results are in accordance to a detailed

Table 2 Mouse sperm motility after incubation for 60 and 90 min with rFGF2.

	0 ng/mL rFGF2	0.1 ng/mL rFGF2	1 ng/mL rFGF2	10 ng/mL rFGF2	100 ng/mL rFGF2
60 min					
Total motility (%)	65 \pm 2	67 \pm 3	68 \pm 4	68 \pm 3	67 \pm 4
Progressive motility (%)	46 \pm 2	47 \pm 4	48 \pm 4	49 \pm 4	47 \pm 6
Hyperactivated motility (%)	7 \pm 3	7 \pm 2	7 \pm 3	8 \pm 2	8 \pm 3
90 min					
Total motility (%)	67 \pm 3	69 \pm 2	68 \pm 2	69 \pm 2	66 \pm 1
Progressive motility (%)	47 \pm 4	49 \pm 3	45 \pm 2	46 \pm 2	43 \pm 3
Hyperactivated motility (%)	8 \pm 2	6 \pm 2	5 \pm 2	5 \pm 1	6 \pm 2

Computer-assisted sperm analysis of sperm incubated for 60 and 90 min with 0, 0.1, 1, 10 or 100 ng/mL rFGF2. Results are expressed as mean \pm s.e.m., $n=7$. At each time point, no significant differences were found for each parameter among the conditions.

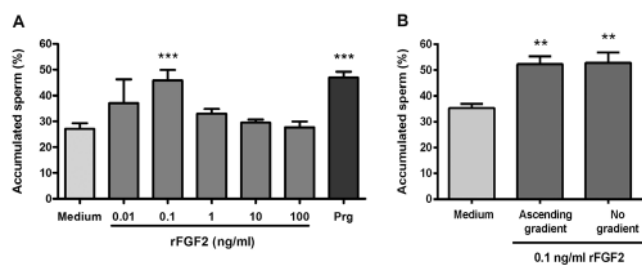


Figure 5 Effect of rFGF2 on sperm accumulation. (A) Sperm were subjected to the sperm selection assay, with different concentrations of rFGF2 in W2 (ascending gradient), or with culture medium and Prg, as negative and positive controls, respectively. (B) Sperm were also exposed to an ascending gradient of 0.1 ng/mL rFGF2 or to homogeneous distribution (no gradient) of the recombinant protein. Results are expressed as mean \pm s.e.m., $n=4$. ** $P<0.01$, *** $P<0.001$ compared with Medium.

study describing the presence and localization of the four FGFRs in the fetal, immature and adult rat testes and that these receptors have preferential localization in the adluminal compartment (Cancilla *et al.* 1998). In addition, the presence of transcripts of several FGFR isoforms (mainly FGFR1c, FGFR2c, FGFR3c and FGFR4) has been reported in the adult mouse testis (Fon Tacer *et al.* 2010). FGFR proteins have been found in the mouse developing gonad (Lai *et al.* 2016), and the signaling mediated by FGFR1 and FGFR8 has been involved in the maintenance of undifferentiated spermatogonia (Hasegawa & Saga 2014). Moreover, the role of testicular FGFRs has been analyzed using some models of transgenic mice. On one hand, it has been described that mice expressing a dominant-negative variant of FGFR1 in the male haploid germ cells are subfertile, show diminished sperm production and alterations in sperm capacitation, suggesting the relevance of this receptor in sperm physiology (Cotton *et al.* 2006). On the other hand, germ-cell-specific conditional knock-out mice for FGFR1 or FGFR2 showed normal fertility (Li *et al.* 2014) and animals lacking FGFR3 or FGFR4 depicted no apparent fertility defects (Deng *et al.* 1996, Weinstein *et al.* 1998, Yu *et al.* 2000). However, in these knock-out animals, a possible functional redundancy with other FGFRs cannot be ruled out.

Considering that mouse sperm showed flagellar localization of FGFRs, it was of interest to analyze the effect of rFGF2 upon sperm motility. Sperm exposure to the recombinant protein led to a significant increase in the percentage of total and progressive motility, in coincidence with the results found in human sperm (Saucedo *et al.* 2015). While in the murine model, the effect was observed with 0.1 and 1 ng/mL rFGF2, an enhancement in human sperm motility was obtained with 10 and 100 ng/mL rFGF2. The differences could be attributed to differences in the physiology of sperm from both species, mainly due to the origin of the cells (epididymal mice sperm vs ejaculated human sperm). When mouse sperm were subjected to the SSA,

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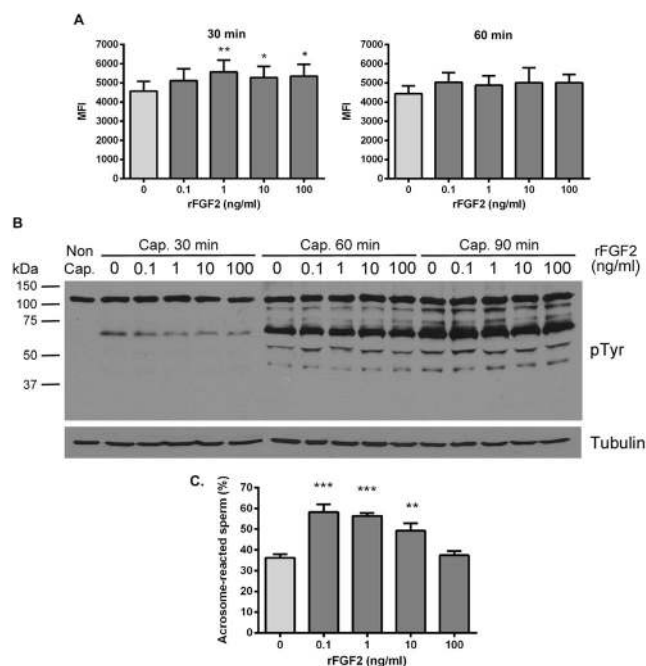


Figure 6 Effect of incubation with rFGF2 on sperm capacitation-related events. (A) Intracellular Ca^{2+} levels evaluated by flow cytometry. Sperm were incubated with rFGF2 for 30 and 60 min and the mean fluorescence intensity (MFI) of Fluo-4 AM was measured in live sperm (PI negative). Results are expressed as mean \pm s.e.m., $n=7$. * $P<0.05$, ** $P<0.01$ compared with 0 ng/mL rFGF2. (B) Protein tyrosine phosphorylation in non-capacitated sperm (Non-Cap.) or in cells incubated with rFGF2 for 30, 60 and 90 min (Cap.). Sperm protein extracts were subjected to SDS-PAGE and Western immunoblotting using anti-phosphotyrosine antibody (pTyr) or anti-tubulin as a control. The molecular weight markers are shown on the left. The experiment was performed three times, obtaining similar results. A typical result is shown. (C) Acrosomal exocytosis in sperm exposed to rFGF2. Sperm were incubated with rFGF2 for 90 min, processed and stained with Coomassie brilliant blue, and the percentage of acrosome-reacted cells was evaluated. Results are expressed as mean \pm s.e.m., $n=5$. ** $P<0.01$, *** $P<0.001$ compared with 0 ng/mL rFGF2.

increased sperm accumulation was observed in the presence of an ascending gradient or homogeneous distribution of rFGF2, suggesting that accumulation was due to a chemokinetic effect of rFGF2. This result agrees with the increment in the velocity determined by computer-assisted sperm analysis. In myoblast cells, it has been observed that FGF2 stimulates migration by chemokinesis, rather than by chemotaxis (Ferreira *et al.* 2015). In line with our findings, a recent work describes that natriuretic peptide type C induces mouse sperm accumulation, due to an increase in progressive motility and velocity, associated with increments in the intracellular levels of cGMP and Ca^{2+} (Kong *et al.* 2017).

Our results also showed that in sperm incubated under capacitating conditions in the presence of rFGF2, there was an increase in intracellular Ca^{2+} levels. The participation of the FGF2/FGFRs system in the regulation of intracellular Ca^{2+} concentrations has been previously

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reported in somatic cells (Munaron *et al.* 1995, Zamburlin *et al.* 2012, Liu & Schneider 2014). In our experimental conditions, sperm incubation with rFGF2 also resulted in an increase in acrosomal exocytosis. Considering that Ca^{2+} ions play a key role in the capacitation process and that an increase in this cation is essential for the occurrence of acrosomal exocytosis (Yanagimachi 1994, Wassarman *et al.* 2001, Vazquez-Levin & Marín-Briggiler 2009), it can be proposed that rFGF2 induces the acrosomal loss by regulating intracellular Ca^{2+} concentrations. However, further studies are needed to understand the mechanisms underlying these effects in sperm cells and their physiological relevance. Regarding the rFGF2 concentrations at which the biological effects were found in the present study, our results are in agreement with the biphasic effect of rFGF2 found in somatic cells (Kanodia *et al.* 2014). It has been proposed that at low/intermediate concentrations of FGF2, there are sufficient free FGFRs available allowing the formation of the FGF2-HSPG-FGFR signaling unit and that at high concentrations of FGF2, FGFR-binding sites become saturated, inhibiting the complex formation.

In summary, our results demonstrate (1) the presence of FGF2 in the mouse uterus, oviduct and COC, (2) the presence of the four FGFRs in sperm cells, mainly localized in the flagellum and acrosomal region and (3) that sperm incubation with rFGF2 led to an increase in sperm motility, in intracellular Ca^{2+} levels and in acrosomal exocytosis. Results from the present study about the presence and function of FGFRs in mouse sperm are similar to those previously found in human sperm (Saucedo *et al.* 2015). Altogether, they suggest the relevance of the FGF2/FGFRs system for the regulation of mammalian sperm physiology and led us to propose that *in vivo*, the FGF2 present in the female reproductive tract would exert a regulatory role on sperm function.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/REP-18-0133>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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