FGRF-1 signaling is involved in spermiogenesis and sperm capacitation

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Accepted 21 September 2005
Journal of Cell Science 119, 75-84 Published by The Company of Biologists 2006
doi:10.1242/jcs.02704

Summary
Cloning of the fibroblast growth factor receptor (FGFR) adaptor Snt-2 cDNA and the identification of FGFR-1 protein in association with sperm tails, suggested that FGFR-1 signaling was involved in either sperm tail development or function. This hypothesis was tested by the creation of transgenic mice that specifically expressed a dominant-negative variant of FGFR-1 in male haploid germ cells. Mating of transgenic mice showed a significant reduction in pups per litter compared with wild-type littermates. Further analysis demonstrated that this subfertility was driven by a combination of reduced daily sperm output and a severely compromised ability of those sperm that were produced to undergo capacitation prior to fertilization. An analysis of key signal transduction proteins indicated that FGFR-1 is functional on wild-type sperm and probably signals via the phosphatidylinositol 3-kinase pathway. FGFR-1 activation also resulted in the downstream suppression of mitogen activated protein kinase signaling. These data demonstrate the FGFR-1 is required for quantitatively and qualitatively normal spermatogenesis and has a key role in the regulation of the global tyrosine phosphorylation events associated with sperm capacitation.

Supplementary material available online at http://jcs.biologists.org/cgi/content/full/119/1/75/DC1

Key words: FGFR-1, Sperm, Capacitation, Fertility, Spermatogenesis

Introduction
Fibroblast growth factors (FGFs) are a large family of multifunctional peptide growth factors of which there are at least 22 known members (Ornitz and Itoh, 2001), that have pivotal roles in many cellular processes including mitogenesis, differentiation, migration and cell survival [reviewed in (Goldfarb, 1996)]. FGF action is mediated through high-affinity binding to receptor tyrosine kinases (RTKs), which are designated FGF receptors 1-5 (FGFR-1 to FGFR-5), however, most FGF actions are mediated through FGFR1-4 (Powers et al., 2000; Schlessinger, 2000; Sleeman et al., 2001). Functional FGFRs are transmembrane proteins characterized by an extracellular ligand-binding domain that contains up to three extracellular immunoglobulin (Ig)-like domains and an intracellular domain, which consists of a juxtamembrane region and a split tyrosine kinase domain (Johnson and Williams, 1993). Alternative mRNA splicing of the Ig loops in FGFR-1 through FGFR-3, leads to distinct functional variants of the receptor (Cancilla et al., 2001; Ornitz et al., 1996). FGF binding induces FGFR dimerization, which is followed by intramolecular transphosphorylation of the receptor subunits and the initiation of intracellular signal transduction cascades (Ornitz et al., 1996; Powers et al., 2000; Robinson et al., 1995).

Genetic and biochemical studies have shown that the docking proteins suc-1 associated neurotrophic factor target 1 and 2 (Snt-1 and Snt-2) function as key mediators of cellular signaling from FGFRs (Kouhara et al., 1997). It has been shown that Snts localize in the cell membrane through a myristyl anchor and interact with the FGFRs through a phosphotyrosine-binding (PTB) domain (Guy et al., 2002; Kouhara et al., 1997; Xu et al., 1998). Upon FGF stimulation, Snts are phosphorylated on multiple tyrosine residues to provide binding sites for the adaptor protein Grb2 and for the protein tyrosine phosphatase (PTP) Shp2 (Hadari et al., 1998; Kouhara et al., 1997), which in turn leads to the activation of the ERK arm of the mitogen activated protein kinase (MAPK) cascade or the phosphatidylinositol 3-kinase (PI3K) cascade and downstream changes in cell function or phenotype (Gotoh et al., 2004; Lamothe et al., 2004; Lax et al., 2002).

The conservation of FGF/FGFR proteins throughout evolution, and the extreme phenotypes of constitutive knockout models (Counou and Deng, 2003; Ornitz and Itoh, 2001; Powers et al., 2000), is indicative of them having important roles during development. While a previous study has localized FGFR-1 to the haploid germ cell using immunohistochemistry (Cancilla and Risbridger, 1998), there are few data relating to the role of FGFR signaling in spermatogenesis and male fertility. As such, the aim of the current study was to assess the role of FGFR-1 in spermiogenesis and male fertility using dominant-negative transgenic mouse models. These models and in vitro sperm function assays conclusively show that FGFR-1 is a functional requirement, which contributes to sperm production and function.
Results
FGFR-1 signaling components in sperm development
During the process of screening a rat testis expression library for sperm tail components, a 1.2 kb, 5' fragment of Snt-2, a major component of the FGFR-1 signaling complex (Kouhara et al., 1997), was cloned. The remaining 796 bp fragment was subsequently cloned using 3'RACE and the entire sequence deposited in the NCBI database as accession number AY972083 (data not shown). In the absence of Snt-2 antisera, northern blotting of testes samples of defined ages was used as an indicator of cellular expression. Minimal amounts of Snt-2 mRNA was detected in 10 day old testes. Expression dramatically increased at 25 days and was maintained in 90 days (adult) (supplementary material, Fig. S2A). As a result of the strictly controlled developmental processes of spermatogenesis and the sequential appearance of more mature germ cell subtypes, this pattern was highly suggestive of expression within round through to elongated spermatids.

The expression pattern of Snt-2 in spermatis, co-incident with the expression of FGFR-1 protein (Cancilla and Risbridger, 1998) strongly suggested a functional role for this receptor complex in spermiogenesis and/or sperm function. To determine if FGFR-1 was expressed in mature sperm, sperm and subsequently sperm tails, were isolated from both rat and mouse cauda epididymides and probed for FGFR-1 protein. Consistent with the testicular expression, ~150 kDa and ~110 kDa FGFR-1 protein was expressed in rat sperm tails (supplementary material, Fig. S2B) i.e. the long and short forms and the ~150 kDa long form was seen in mouse sperm tails (Dionne et al., 1990; Johnson et al., 1990).

The expression of FGFR-1 was further validated on mouse sperm tails using an immunofluorescence technique where weak, but distinct staining was seen on the mid-piece and principal piece of sperm tails (supplementary material, Fig. S2D). The absence of staining on sperm incubated in parallel with non-immune Ig indicates staining specificity.

Transgene expression
FGFR-1 dominant-negative mouse lines were produced to ascertain the role(s) of FGFR-1 signaling in spermiogenesis and male fertility. The relative expression of wild-type and transgenic mice nFGFR-1 and tFGFR-1 mRNA is shown in Fig. 1. Hemizygous mice showed a significant induction of tFGFR-1 mRNA over nFGFR-1 mRNA in wild-type mice (up to 80-fold). However, within tFGFR-1 mice there was also a significant upregulation of nFGFR-1 mRNA levels suggesting a feedback loop controlling FGFR-1 signaling. The driver(s) of this loop are not currently known and have not been previously reported. Cumulatively line 4 and line 9 mice showed a sixfold and fivefold overexpression of tFGFR-1:nFGFR-1 respectively (Fig. 1B). A third band at approximately 6.0 kb was noted in transgenic mice (Fig. 1A).

While the longest FGFR-1 transcript reported within the literature or publicly available databases is 3.8 kb (Harari et al., 1997), this may represent a longer alternatively spliced version of FGFR-1 mRNA and as such the ratios calculated above should be treated with caution. If this is indeed a FGFR-1 variant, it represents a novel isoform.

To increase transgene expression in the hope of saturating the upregulation of nFGFR-1 mRNA, line 4 and 9 mice were intercrossed. As can be seen in Fig. 1B, DH4/9 mice showed a 120-fold over-expression of tFGFR-1 mRNA expression compared with nFGFR-1 expression in wild-type mice. nFGFR-1 mRNA expression was not proportionally upregulated in DH4/9 testes. Within transgenic DH4/9 a 19-fold over-expression of tFGFR-1:nFGFR-1 mRNA was calculated.

To confirm transgene expression to the appropriate cell types, Bouin’s fixed transgenic and wild-type mouse testes were immunohistochemically labeled using a flag-tag antibody. Consistent with previously published data on the Pml promoter (Braun, 2000), transgenic protein was expressed in elongating and elongated spermatids (supplementary material, Fig. S3). No staining was observed in wild-type testes confirming the specificity of labeling. Consistent with mRNA expression levels, qualitatively stronger staining was observed in DH4/9 testes compared with line 4 and line 9 testes (supplementary material, Fig. S2), i.e. DH4/9>4>9.

Fertility analysis
Breeding experiments using age matched transgenic and wild-type males showed that suppressed haploid germ cell FGFR-1 signaling resulted in a significant reduction in pups per litter in

Fig. 1. FGFR-1 mRNA expression in wild-type and transgenic testes. (A) A representative northern blot from transgenic mouse line 4. Testis RNA from five transgenic and wild-type mice was probed with a cDNA probe designed to detect both the native (n) FGFR-1 mRNA and the transgenic (t) FGFR-1 mRNA at 3.8 kb and 1.4 kb, respectively. A band was also observed at 6.0 kb, which may be representative of another FGFR-1 splice variant. (B) The relative FGFR-1 mRNA expression levels in wild-type and transgenic mice. Ratios indicate the fold over-expression of tFGFR-1 mRNA compared with nFGFR-1.
FGFR-1 in sperm development and function

Both the line 4 and DH4/9 mice (5.7 versus 9, and 2.5 versus 9, respectively) (Fig. 2A). Pups per litter in line 9 did not differ significantly from wild-type numbers.

Consistent with the absence of any significant differences in testis weights between transgenic and wild-type mice (Fig. 2B), testis histology appeared qualitatively normal in all tFGFR-1 mice assessed (supplementary material, Fig. S3). To determine the origin of tFGFR-1 subfertility the DSP for each line was calculated. Both the line 4 and DH4/9 mice showed a significant reduction in daily sperm output compared with wild-type age matched males (30% and 41% respectively) (Fig. 2C). The DSP of line 9 male mice did not differ significantly from wild-type values. Reduced elongated sperm numbers were not caused by an increase in classic apoptosis as indicated by the absence of an increase in TUNEL positive cells (data not shown) in transgenic compared with wild-type testis sections. The exacerbation of the phenotype in the DH4/9 line compared with line 4 strongly argues against a transgene insertion effect on male fertility. Collectively these date indicate that germ cells were disappearing at a relatively late stage in spermiogenesis in a non-classic apoptosis process.

Serum hormone levels of activin A, inhibin and FSH were unchanged compared with wild-type animals in all tFGFR-1 lines (supplementary material, Fig. S4). Normal seminal vesicle and prostate weights strongly suggested that testosterone levels were unperturbed (data not shown).

While significant and no doubt a contributing factor in the observed subfertility, a 41% and 30% reduction in sperm output was unlikely to be solely responsible for the 70% and 37% reduction in average litter size in the DH4/9 and line 4 mice. Based on previous observations that during sperm capacitation, there is a marked increase in phosphorylation within the sperm tail (Aitken et al., 1995; Asquith et al., 2004; de Lamirande and Gagnon, 2002; Visconti et al., 1995), we propose that FGFR-1 signaling had a role in the initiation or regulation of capacitation. As such, wild-type and line 4 transgenic sperm were subjected to capacitating conditions and changes in global tyrosine phosphorylation measured as a hallmark of capacitation. As expected, wild-type sperm incubated in BWW underwent a time-dependent increase in global tyrosine phosphorylation (Fig. 3A). Tyrosine phosphorylation levels were however not changed in line 4 tFGFR-1 sperm even following 90 minutes incubations under capacitating conditions (Fig. 3A). These data strongly suggest that suppression of FGFR-1 signaling during spermiogenesis renders sperm functionally compromised in terms of pre-fertilization maturation events.

Changes in MAPK and PI3K signaling

To determine the pathway(s) involved in FGFR-1 signaling within sperm, wild-type and transgenic sperm incubated under capacitating conditions were assessed for activation of the two FGFR-1 induced signaling cascades, the MAPK and PI3K pathways. As shown in Fig. 3B, and consistent with previously published data on human sperm (de Lamirande and Gagnon, 2002; Luconi et al., 1998), the MAPK pathway, as indicated by an increase in ERK1/2 phosphorylation, became activated during BWW induced capacitation of wild-type sperm ie. time 0 minutes versus time 90 minutes. Conversely, tFGFR-1 sperm when compared with wild-type, contained elevated levels of phospho-ERK1/2 in the non-capacitated (time=0) state and showed a suppression of MAPK activation when incubated under capacitating conditions ie. time 0 minutes versus time 90 minutes (Fig. 3B).

Similarly, the incubation of wild-type sperm under capacitating conditions resulted in an induction of PI3K signaling as evidenced by an increase in p85 phosphorylation (Fig. 3C). Transgenic sperm, however, displayed a reduction in PI3K signaling over the period of capacitation.

Notice that BWW does not contain bFGF or any other growth factor. As such, MAPK and PI3K activation in wild-type, or suppression in transgenic sperm, was not induced directly by FGFR-1 signaling. This data indicates, however, that a disruption of FGFR-1 signaling during spermiogenesis renders sperm functionally compromised in terms of MAPK and PI3K activation during capacitation (Fig. 3B).

Is bFGF a ‘decapacitation factor’?

The above data strongly suggested that FGFR-1 signaling during spermiogenesis is involved in the establishment of the machinery required for the global tyrosine phosphorylation associated with sperm capacitation. This raised the possibility...
that FGFR-1 signaling is also involved in the regulation of capacitation in wild-type mice. To test this hypothesis, wild-type sperm were incubated under capacitating conditions in the presence or absence of the main FGFR-1 ligand, bFGF (Ornitz and Itoh, 2001; Powers et al., 2000). In contrast to sperm incubated in BWW alone, those incubated in the presence of 10 ng/ml bFGF showed a complete absence of global tyrosine phosphorylation even following 90 minutes incubation (Fig. 4A). These data conclusively showed that the FGFR-1 receptor is present and functional on wild-type sperm and strongly suggest that FGFR-1 signaling is a negative regulator of capacitation as judged by its capacity to prevent the increase in tyrosine phosphorylation. The addition and incubation of bFGF did not affect membrane integrity of caudal sperm in BWW, thus ruling out the possibility of bFGF toxicity leading to suppressed global tyrosine phosphorylation (data not shown).

Fig. 3. Capacitation induced changes in tyrosine phosphorylation in wild-type and tFGFR-1 mice. (A) A comparison of tyrosine phosphorylation patterns of wild-type and transgenic, line 4, mouse sperm under capacitating conditions for the times indicated. The relative absorbance of tyrosine phosphorylation for each assay was determined and all data was normalized to β-tubulin. The relative change in tyrosine phosphorylation was determined and plotted, n=3. (B) A comparison of ERK 1/2 activation in wild-type and transgenic mouse sperm under capacitating conditions for the times indicated. The relative absorbance of ERK phosphorylation for each assay was determined and normalized to the expression of β-tubulin. The relative changes are plotted, n=3. (C) A comparison of p85 activation in wild-type and transgenic mouse sperm under capacitating conditions for the times indicated. The relative absorbance of p85 phosphorylation for each assay was determined and normalized to β-tubulin expression. The relative changes in p85 activation are plotted, n=3. *P<0.05; **P<0.01; ***P<0.001.
To determine the pathway(s) involved in FGFR-1 signaling within wild-type sperm, sperm were incubated in the presence or absence of bFGF. As described above, both the MAPK and PI3K pathways became activated during capacitation in BWW (Fig. 4B,C). Similarly, sperm incubated in the presence of bFGF showed a time-dependent activation of the PI3K pathway (Fig. 4C). By contrast, no activation of the MAPK was observed (Fig. 4B). These data suggest that within wild-type sperm FGFR-1 signals via the PI3K pathway and results in the downstream suppression of the MAPK pathway. The suppression of the MAPK pathway occurs within 10 minutes, but at present it is unknown if this is a direct consequence of PI3K activation or mediated via an intermediary pathway.

**Discussion**

Through the creation of dominant-negative transgenic mouse lines, we have clearly demonstrated that FGFR-1 signaling is required for full male fertility. Compromised FGFR-1 signaling during spermiogenesis not only resulted in a significant decrease in daily sperm output, but rendered those sperm that were produced functionally compromised in terms of their ability to undergo capacitation.

**Fig. 4.** The effect of FGFR-1 activation on wild-type and tFGFR-1 sperm capacitation induced tyrosine phosphorylation. (A) A comparison of tyrosine phosphorylation patterns of wild-type sperm +/– 10 ng/ml bFGF under capacitating conditions for the times indicated. The relative absorbance of tyrosine phosphorylation was determined and all data was normalized to β-tubulin, n=3. (B) A comparison of ERK 1/2 activation in wild-type mouse sperm +/– 10 ng/ml bFGF under capacitating conditions. The relative absorbance of ERK phosphorylation for each sample was determined and all data was normalized to the expression of β-tubulin, n=3. (C) A comparison of PI3K activation in wild-type mouse sperm +/– 10 ng/ml bFGF under capacitating conditions. The relative absorbance of p85 phosphorylation for each sample was determined and all data normalized to the expression β-tubulin. *P<0.05, **P<0.01, ***P<0.001.
Further, our data strongly suggest that FGFR-1 signaling can act to suppress capacitation in vivo. Capacitation is an essential series of processes, which sperm undergo following entry into the female reproductive tract. Hallmarks of capacitation include the acquisition of the ability to bind to the zona pellucida, the ability to undergo the acrosome reaction following zona binding, and a time-dependent increase in tyrosine phosphorylation in a range of sperm proteins associated largely, but not exclusively, with the tail (Aitken et al., 1995; Asquith et al., 2004; Visconti et al., 1995). The absolute functional significance of increased tyrosine phosphorylation has not yet to be conclusively demonstrated; however, the results of many studies strongly suggest that it is required for fertilizing ability (Baker et al., 2003; de Lamirande and Gagnon, 2002; Urner et al., 2001; Urner and Sakkas, 2003; Visconti et al., 1995). In vivo fertilization in the absence of global tyrosine phosphorylation has not been reported. Hyperactivation, which is required for sperm to penetrate the outer vestments of the oocyte in vivo, is generally considered a functional extension of capacitation however, data particularly from the bull and Catsper knockout mice, suggest it is that possible to uncouple capacitation and hyperactivation (Carlson et al., 2003; Ho et al., 2002; Marquez and Suarez, 2004; Okunade et al., 2004).

The regulation of global sperm tyrosine phosphorylation associated with capacitation is a complex process that probably involves input from several signaling pathways including the MAPK pathway, PKA, A1 adenosine receptor, and the platelet activating factor (PAF) receptor signaling (de Lamirande and Gagnon, 2002; Luconi et al., 1998; Minelli et al., 2004; Muguruma and Johnston, 1997; Rouldebusch et al., 2005; Wu et al., 2001). It is absolutely dependent upon cholesterol efflux (and an associated increase in membrane fluidity), bicarbonate and Ca\textsuperscript{2+} influxes (Carlson et al., 2003; Davis et al., 1979; Davis and Gergely, 1979; Gadella and Harrison, 2000; Ho and Suarez, 2001). The transgenic mice presented herein are the only mice reported to show an absence of global tyrosine phosphorylation. These data and the associated suppression of wild-type sperm tyrosine phosphorylation in the presence of bFGF strongly suggest that FGFR-1 signaling, probably via the PI3K pathway, has a key regulatory role in capacitation associated with fertility; specifically in the suppression of premature capacitation.

Fig. 5. A proposed mechanism for the regulation of events leading to sperm capacitation. Ejaculated sperm entering the female reproductive tract are exposed to a FGFR-1 ligand. The ligand induces a FGFR-1 signaling cascade. Activation of the PI3K pathway results in the phosphorylation of a ‘master controller of capacitation’ (MCC), which suppresses downstream tyrosine phosphorylation of signal transduction pathways including protein kinase A (Konopka et al., 1984) activation. Following the disengagement of FGFR-1 from its ligand, or the suppression of the PI3K pathway, PTPs dephosphorylate the MCC, allowing the tyrosine phosphorylation and activation of target proteins leading to the correlates of sperm capacitation. Furthermore, activation of the PI3K pathway results in the suppression of the MAPK pathway. The level at which this suppression occurs is not known, however, it is upstream of ERK 1/2.

A role for FGFR-1 signaling in suppressing premature capacitation is plausible as bFGF has been identified in the uterus of many species including humans, monkeys, pigs, mice and rats (Ferriani et al., 1993; Gupta et al., 1997; Reynolds et al., 1998; Rider et al., 1997; Samathanam et al., 1998; Sangha et al., 1997; Wordinger et al., 1994). We hypothesize that binding of bFGF (or an equivalent ligand) to FGFR-1 leads to activation of the PI3K pathway and the subsequent phosphorylation of an as yet to be identified ‘master controller of capacitation’ (MCC) (Fig. 5). The MCC may be a single kinase or a complex of proteins containing a kinase, which when phosphorylated following FGFR-1 signaling, is rendered inactive. Conversely, when the MCC is dephosphorylated by protein tyrosine phosphatases (PTPs), the MCC can activate downstream pathways including the PKA pathway, leading to global tyrosine phosphorylation and the functional changes associated with capacitation. The uncoupling of FGFR-1 signaling by either the disengagement of the FGF ligand from the receptor, or the inhibition of an upstream PI3K component, may allow for the dephosphorylation of the MCC and the subsequent activation of capacitation associated tyrosine phosphorylation events. In support of this hypothesis, Luconi and colleagues have recently demonstrated that the inhibition of PI3K in human sperm increases cAMP levels and the subsequent phosphorylation of AKAP3 and recruitment of PKA in sperm tails (Luconi et al., 2004). Functionally, this leads to an increase in the number of sperm displaying hyperactivated motility and enhanced zona pellucida binding (Du Plessis et al., 2004; Luconi et al., 2001). Further, PTPs have been demonstrated in sperm and have been shown to be involved in the tyrosine phosphorylation, dephosphorylation, and re-phosphorylation of a hamster sperm protein implicated in the regulation of hyperactivation (Si, 1999; Si and Okuno, 1999).

Our data demonstrate that FGFR-1 signaling leads to the suppression of the MAPK pathway in association with
suppressed capacitation and is consistent with previously published data indicating that the MAPK activation is involved in capacitation (Ashizawa et al., 1997; de Lamirande and Gagnon, 2002; Luconi et al., 1998; O’Flaherty et al., 2005). It suggests that FGFR-1/Pi3K activation acts either directly or indirectly on MAPK activation. These data suggest that Pi3K activation serves to prevent premature MAPK activation and capacitation. The level at which cross-talk between the Pi3K and MAPK pathways remains, however, to be defined.

Our interpretation that FGFR-1 signals via the Pi3K pathway must be interpreted with caution because of the similar activation profile observed in the presence or absence of FGFR-1 ligand in wild-type sperm. Clearly signal transduction within sperm is complex and highly interconnected and the activation of signal transduction pathways classically associated with growth factor signaling can occur within sperm even in the absence of growth factor binding. The upstream source of this activation is currently unknown. Regardless, the data strongly suggest that the observed activation of FGFR-1 signaling is mediated via the Pi3K pathway.

Similarly, as with all genetically modified animal models, caution must be exercised in directly translating the abnormal function of transgenic sperm to wild-type sperm function. On the face of it, the inhibitory action of FGFR-1 signaling on wild-type sperm capacitation and the suppressed capacitation in FGFR-1 dominant-negative sperm appear contradictory. Clearly, however, FGFR-1 signaling is required for spermiogenesis as indicated by a significant reduction in the DSPs of FGFR-1 dominant-negative mice. The most simplistic explanation of the capacitation data is that FGFR-1 is involved in the establishment or packaging of signal transduction machinery into sperm for use while in the female reproductive tract – a time which sperm are transcriptionally and translationally silent. This packaging was abnormal in FGFR-1 transgenic haploid germ cells, resulting in an inability to activate both the MAPK and Pi3K pathways under capacitating conditions. In wild-type sperm, however, in which the signal transduction machinery is intact, FGFR-1 activation resulted in the activation of the Pi3K pathway and the downstream suppression of the MAPK pathway.

The dominant-negative tFGFR-1 lines described should be treated as knockdown models of FGFR-1 function, rather than knockout models. Consistent with the possibility that functional nFGFR-1-nFGFR-1 dimers form and signal normally, DH4/9 mice retained some degree of fertility, albeit at a severely compromised level. Herein lays the utility of dominant-negative models. A complete ablation of FGFR-1 signaling within haploid germ cells, i.e. a tissue specific knockout, would in all likelihood have resulted in the production of sterile mice and an inability to discern the more subtle role of FGFR-1 signaling in capacitation. The point at which germ cells were lost from the transgenic testes can only be from the spermatid population. Should this have taken place at the spermatogonial or spermatocyte stages, there would be a significant decrease in testis weight and the appearance of increased levels of apoptosis in these cell types. Consistent with this, FGFR-1 transgenic protein expression was restricted to the spermatid population. The stage at which germ cells were being lost during spermiad maturation (spermiogenesis) is less clear, but it probably is within the elongating and elongated spermatid population, consistent with transgenic protein expression. Spermatid loss at the early stages of spermiogenesis, such as that occurring following in androgen withdrawal (Beardsley and O’Donnell, 2003), results in the sloughing of round spermatids into the lumen and their passage into the epididymis. Loss at later stages is more difficult to distinguish phenotypically due to the intricate relationship of later spermatids and the Sertoli cells. Furthermore, there was no evidence of failed spermiation or germ cell sloughing within transgenic testes (data not shown).

The data presented herein, definitively illustrate roles for FGFR-1 signaling in the establishment of quantitatively and qualitatively normal spermiogenesis, the presence of functional FGFR-1 complexes on sperm and their signaling – probably via the Pi3K pathway. The data also strongly suggest that FGFR-1 serves to suppress the MAPK pathway within sperm and prevents premature capacitation. As premature capacitation is a significant problem in semen programs (e.g. bovine), particularly following cryopreservation, these discoveries will probably lead to increased productivity in addition to a greater understanding of signaling within sperm.

Materials and Methods

Reagents
Antisera were obtained from the following sources: G410 mouse monoclonal antibody and a Pi3K polyclonal antibody (which recognizes the p85 subunit of Pi3K) from Upstate (Lake Placid, NY); a flag-tag mouse monoclonal antibody from Sigma-Aldrich (Sydney, Australia); FGFR-1 sera from Santa Cruz Biotechnology (Santa Cruz, CA); and an activated ERK1/2 (p42/44) polyclonal antibody from Cell Signaling Technology (Beverly, MA).

The identification of FGFR signaling components in sperm development

During the process of cloning components of the mouse sperm tail, as described previously (Hickox et al., 2002; O’Bryan et al., 1998), a partial fragment of suc1-associated neurotrophic factor target 2 (snt-2) was identified. The missing 3‘ end was cloned using 3‘ rapid amplification of cDNA ends (RACE), utilizing the FirstChoice™ RLM-RACE kit (Ambion, Texas) as previously described (O’Bryan et al., 2000b; Troutt et al., 1992), using the primers F1701A 5‘-AGC-CCAAATGCACCATTAG3‘, F1701B 5‘-CTTCCCTCTTGTTGAGAGA3‘ and the supplied adaptor primer, AP3.2. The sites of cellular expression of snt-2 within the developing testis was determined by Northern blotting using a 1.2 kb (5‘ fragment) snt-2 specific probe (O’Bryan et al., 1998).

A previous report showed that FGFR-1 was localized to elongated spermatids within the testis, including the tail, during spermiogenesis (Cancilla and Risbridger, 1998). To confirm the validity of this observation, testis homogenates and isolated cauda epididymal sperm and sperm tails (O’Bryan et al., 2001) were solubilized in RIPA buffer and 2× SDS-PAGE buffer, respectively, and run into 8% SDS-PAGE gels using standard methods. Following transfer to PVDF membrane, the presence or absence of FGFR-1 was determined using a FGFR-1 specific antisera at a concentration of 4 μg/ml. FGFR-1 protein was also localized to cauda epididymal sperm using immunofluorescence. Following the extraction of sperm from the cauda epididymis and dilution to a concentration of approximately 1×10⁹ per ml, sperm were air dried onto Superfrost Plus slides (Biolab Scientific, Melbourne, Australia). Slides were subsequently incubated in 0.2% Triton X-100 for 30 minutes, washed in Tris-buffered saline (TBS; 0.05 M Tris, 0.15 M NaCl) twice for 5 minutes each then blocked in 10% non-immune goat serum, 1% BSA, 0.01 M Na₂SO₄ in TBS for 30 minutes. FGFR-1 protein was bound using 10 μg/ml of anti-FGFR-1 serum overnight at room temperature, washed, then detected using Texas Red-conjugated goat anti-rabbit Ig (Amersham) at a dilution of 1 in 100. Nuclear DNA was visualized using DAPI in mounting solution (Zymed).

Transgenic mouse production

FVB/N mice were obtained from the Animal Resources Centre (Perth, WA, Australia) and were maintained under standardized conditions of lighting (12L:12D) and nutrition (food and water ad libitum). Studies were performed in accordance with the National Health and Medical Research Council’s (NHMRC) Guidelines on Ethics in Animal Experimentation and were approved by the Monash Medical Centre Animal Experimentation Ethics Committee.

A haploid male germ cell specific FGFR-1 dominant-negative construct was prepared by conjugating the extracellular domain and transmembrane region of
FGFR-1 (tFGFR-1) cDNA to the protamine 1 (Prm1) promoter and the SV40 polyadenylation signal (supplementary material, Fig. S1A). The absence of the FGFR-1 intracellular domain results in an absence of signal transduction following dimerization with either native or tFGFR-1 protein. The tFGFR-1 cDNA was amplified from the vector described in (Harari et al., 1997), using primers 5'-CCTCGATGGCCAATGTGGAGGTACGTACGGAG-3' and 5'-AGTACATCGTCTGCTGCTA-3'. The second PCR amplified a 300 bp region of the Prm1 promoter and part of the tFGFR-1 promoter, using primers 634: 5'-CCAAGATCCCTCGCTCAGC-3' and 652: 5'-GGGCTCTTGGCCATTACGC-3'. To assure DNA quality, a third 500 bp band from an irrelevant gene (Pebp1, NCBI database accession # NM_98858) was amplified using primers GG33/H9262 and GG16/H9252. Following confirmation of mRNA and transgene tag expression, two lines were selected for further analysis (line 4 and 9). To increase transgene transcription/translation and further suppress FGFR-1 signaling, line 4 and 9 mice were intercrossed to produce the double heterozygous (DH) 4/9 mice. DH4/9 mice were selected using the above PCR screening followed by Southern blotting using the tFGFR-1 sequence was gel purified, ethidium bromide/high salt extracted, and microdialysed (Stemmer, 1991). 1-2 ng of the construct was microinjected into fertilized oocytes and implanted into pseudopregnant FVB recipients of a Monash University Post-graduate student award. This research was supported in part by funding from the NHMRC (Grant 334011) and the Australian Research Council (ARC, Grant C3E0348239) to M.K.O.B. and D.M.D.K. M.K.O.B. is the recipient of a senior research fellowship from Monash University. L.C. is the recipient of a Monash University Post-graduate student award.

References


Journal of Cell Science

Transgene mRNA expression

Transgene mRNA expression was assessed by northern blotting as described above. Both native (nFGFR-1) and transgene (tFGFR-1) derived FGFR-1 mRNA expression were detected using a 400 bp cDNA probe directed to the 5' end of FGFR-1 mRNA. Within individual samples, both native and transgene derived mRNA expression were measured at 3.8 kb and 1.4 kb, respectively. Sample loading was normalized to 26S expression. The relative expression levels of transgenic lines was determined using Image Gauge software and compared with the expression of the nFGFR-1 in wild-type mice, which was set to an arbitrary value of 1.

Transgene-derived protein within testis sections was detected by immunohistochemistry using a flag-tag monoclonal antibody at a concentration of 10 µg/ml using the DAKO EnVision+ System (DakoCytomation, Glostrup, Denmark) as outlined by the manufacturer, following antigen retrieval as described earlier (O’Bryan et al., 2001). Specificity was assured by the absence of staining in wild-type testes processed in parallel.

Fertility analysis

Wild-type, single heterozygous and double heterozygous (DH) males (11-32 weeks), were housed with two to three wild-type female mice (aged 8 weeks) each. Following confirmation using females were removed and the number of pups resulting recorded. A minimum of eight males from each genotype and line were used. Testes from 9-12 week-old animals were dissected free of fat and epididymal tissue, weighed and used for histological analyses, mRNA and protein extraction, and daily sperm count (DSP) analysis. To assess testicular morphology, testes were immediately immersion fixed in Bouin’s fluid and processed over paraffin using standard methods. 5 µm sections were cut and stained using either Periodic Acid Schiff/fuchsin or for apoptotic cells using the Apoptag kit (TUNEL assay (Intergen Company, Purchase, NY) as outlined previously (O’Bryan et al., 2000a). DSPs were determined from a minimum of four animals per genotype

using the method of Robb et al. (Robb et al., 1978). Briefly, samples of known mass were homogenized in SMT buffer (0.15 M NaCl, 0.1 mM NaN3, 0.05% Triton X-100), which served to lyse all cells except the condensed spermatid nuclei. The number of elongated spermatids per testis was determined using a hemocytometer and the DSP estimated by dividing the total elongated spermatid number per testis by 4.84 (the number of days an elongated spermatid is resident within the testis) (Robb et al., 1978).

Hormone assays

The key reproductive hormones follicle stimulating hormone (FSH), activin A, and inhibin were measured from five animals per genotype, aged 9-12 weeks. FSH concentrations in mouse serum were determined using radioimmunoassay reagents kindly donated by A. Parlow (NIDDK, Bethesda, MD). Activin A was measured using a specific enzyme linked immunosorbent assay (ELISA) (Knight et al., 1996) according to the manufacturer’s instructions (Oxford Bio-Innovations, Oxfordshire, UK). Immunoreactive inhibin was measured by heterologous radioimmunoassay as described previously (Robertson et al., 1988).

Capacitation assays

Sperm were collected from wild-type and transgene line 4 males, aged 9-12 weeks, and subjected to capacitating conditions in Buggers, Whitten and Whittington medium (BWW) as outlined previously (Aitken et al., 1995) in the presence or absence of 10 ng/ml BFGF for a period of 90 minutes. Component proteins were separated on 7% reducing SDS-PAGE gels in the case of global tyrosine phosphorylation and MAPK activation, and on Ready Gel Tris-HCl 4-15% gradient gels (Bio-Rad Laboratories Inc., Regents Park, NSW, Australia) in the case of phospho-p85. Following transfer to a nitrocellulose membrane, changes in global tyrosine phosphorylation as a hallmark of capacitation were detected using 0.3 µg/ml of the G410 antibody (Aitken et al., 1995). Similarly, the activation of the MAPK pathway was monitored using a phospho-ERK specific antiseraum at a concentration of 0.2 µg/ml. Activation of the PI3K pathway was monitored through the presence of phospho-(Tyr)-p85 PI3K using a phospho antisera at a concentration of 0.2 µg/ml. Western blots were carried out using standard methods. Sample loading was normalized using β-tubulin expression. It is of note that sperm are both transcriptionally and translationally silent. Therefore, any changes in phosphoprotein expression were solely due to post-translational modification, rather than changes in total protein expression. Capacitation assays were carried out a minimum of three times per treatment group.

The live-to-dead ratio of sperm at time=0 and time=90 minutes under capacitating conditions was determined using the Live/Dead® sperm viability kit as recommended by the manufacturer (Molecular Probes, Inc., Eugene, OR). Briefly, 1 ml sperm samples containing 3-4×10^9 sperm/ml were treated with 1 µl of a working solution of SYBR 14 (100 nM) incubated at 37°C in the dark for 10 minutes. Samples were then treated with 5 µl of propidium iodide (12 µM) and incubated further for 10 minutes in the dark. Sperm were washed twice in PBS and resuspended in formaldehyde (1%) and stored at 4°C for up to 1 hour before cytometric analysis. A minimum of 7000 cells per sample were counted by flow-cytometric analyses to determine the proportion of sperm with intact membranes.

Statistics

All data was analyzed using one-way ANOVA, with results presented as mean ± s.e.m., with significance indicated by P<0.05. Analysis was undertaken using the GraphPad Prism® version 4 software.

We thank Ora Bernard (WEHI) and Robert Braun (University of Washington) for the provision of the tFGFR-1 and Prm1 promoter constructs, respectively. We are grateful to Amy Herlihy, Anne O’Connor, Sue Haywood and Ann Davies for technical assistance. This research was supported in part by funding from the NHMRC (Grant 334011) and the Australian Research Council (ARC, Grant C3E0348239) to M.K.O.B. and D.M.D.K. M.K.O.B. is the recipient of a senior research fellowship from Monash University. L.C. is the recipient of a Monash University Post-graduate student award.
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