Pubertal Onset and Maintenance in FGFR3 Global Knockout Mice

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Abstract:

Vertebrate reproduction is driven by hormones from the hypothalamic-pituitary-gonadal axis. Previous studies showed that neurons from the hypothalamus secreting gonadotropin-releasing hormone (GnRH) require fibroblast growth factors (FGF) to develop and mature, but the specific role of fibroblast growth factor receptor 3 (FGFR3) in overall reproduction is unknown. This study aims to examine the role of FGFR3 in gonadal maturation and function by characterizing mice with a global inactivating mutation in the *FGFR3* gene. Pubertal onset was assessed by sex steroid-mediated events such as vaginal opening (VO) in females and balanopreputial separation (BPS) in males. Adult mice were also examined for estrous cyclicity in females and motile sperm concentrations in males. Female FGFR3 heterozygous global knockout mice (*FGFR3*$^{+/-}$) exhibited delayed VO and abnormal estrous cyclicity with reduced time spent in diestrus. On the contrary, male *FGFR3*$^{+/-}$ mice exhibited normal timing of BPS and motile sperm concentration compared to WT mice. These results indicated that females were more adversely affected by FGFR3 deficiency than males. It is currently unclear if these female-specific adverse effects are exerted at the level of the hypothalamus, pituitary, or gonad. These results provide initial evidence that FGFR3 deficiency can disrupt female reproduction. Further, inactivating *FGFR3* mutations may contribute to human reproductive disorders such as hypogonadotropic hypogonadism.

Introduction:

Vertebrate reproduction requires proper development and function of the endocrine organs within the hypothalamic-pituitary-gonadal (HPG) axis. The cascade of hormones produced by this axis begins with the pulsatile release of gonadotropin-releasing

hormone (GnRH) from the neuroendocrine hypothalamus. GnRH travels through the portal system where it acts on gonadotropes of the anterior pituitary and induces the release of the gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH). Finally, the gonadotropins travel through the general circulation and stimulate the gonads (ovaries and testes) to increase gametogenesis and the production of sex steroids. Effective communication within the HPG axis is essential to ensure adequate pubertal onset and fecundity.

Because GnRH is the most upstream activator of the HPG axis, defects in the GnRH system and HPG axis present a severe obstacle to fertility and reproduction. Therefore, it is of utmost importance to identify the factors needed for the development and maintenance of the GnRH system. There is growing evidence suggesting that the formation of the GnRH system is largely dependent on a class of signaling molecules, fibroblast growth factors (FGFs), and their receptors, fibroblast growth factor receptors (FGFRs), which collectively mediate the genesis, differentiation and migration of GnRH neurons (Chung et al. 2008). The FGF signaling family consists of 22 FGF ligands and 4 tyrosine kinase FGFRs. When an FGF binds the extracellular domain of the FGFR, the receptors dimerize, resulting in the cross phosphorylation of the intracellular domain. The cross phosphorylation induces a phosphorylation cascade, resulting in a variety of cellular effects.

FGF signaling molecules and FGFRs are present in the birthplace of GnRH neurons, the olfactory placode, at the time of neuron fate specification (Tsai et al. 2011). GnRH neurons emerge in the olfactory placode and migrate along the olfactory axons through the cribriform plate, where they reach their final destination, the preoptic area (POA) (Wierman et al. 2011). GnRH neuronal cell bodies remain in the POA while their axons extend to the median eminence.

GnRH axon terminals release GnRH peptide into the hypophyseal portal system in a pulsatile fashion to induce the onset and maintenance of reproduction.

Although a critical relationship exists between the ligand FGF8 and receptor FGFR1 to allow proper GnRH neuron development, FGFR3, another FGF receptor present on GnRH neurons, has not been studied extensively (Chung et al. 2008). In humans, loss-of-function mutations in *FGFR1* are causally linked to hypogonadotropic hypogonadism (HH), a condition characterized by insufficient gonadotropins and therefore incomplete/absent puberty and infertility (Fraietta et al. 2013). However, a correlation between *FGFR3* mutations and HH has not been established in humans. There has been only one clinical report on a loss-of-function mutation in *FGFR3*, and no reproductive deficits were noted in the two male subjects studied (Makrythanasis et al. 2014). The correlation between FGFR3 and reproduction in rodents was similarly incomplete. One study suggested that FGFR3 did not affect the development of GnRH neurons but may be needed to maintain postnatal GnRH neurons, but it did not study the reproductive consequence of *FGFR3* deficiency (Chung et al. 2010).

The objectives of this study were to examine the timing of pubertal onset and adult reproductive function in *FGFR3* global knockout male and female mice. To assess the timing of pubertal onset, external indicators of puberty were examined: vaginal opening and first estrus in females and balanopreputial separation in males. Following pubertal onset, general reproductive functions including estrous cyclicity in females and motile sperm concentration in males were assessed by vaginal cytology and epididymal squash, respectively. These results should establish, for the first time, a direct correlation between *FGFR3* deficiency and reproduction that may be extrapolated to humans harboring inactivating *FGFR3* mutations.

Materials and Methods:

Transgenic animals

C57BL/6 mice with a global heterozygous deletion in the *FGFR3* gene were obtained from the Jackson Laboratories in Bar Harbor, ME and further propagated by breeding. Animals were produced by placing a neomycin cassette in embryonic stem cells, creating a 3 kb deletion of the genomic exon encoding the Ig-domain II and extending through the exon encoding the transmembrane domain (Colvin et al. 1996). Deletion of these components resulted in a truncated nonfunctional FGFR3. The mice were housed in the Gold Conventional animal facility under a 12-h light:12-h dark cycle and given rodent chow and water *ad libitum*. Day of birth is designated as postnatal day (PN) 0. At PN20, pups were weaned and had a tail biopsy obtained for genomic DNA isolation. All animals were genotyped using polymerase chain reactions (PCR) described by Colvin et al. (1996). Although my breeding scheme generated wild type (WT), FGFR3 heterozygous global knockout mice (*FGFR3*$^{+/-}$) and FGFR3 homozygous global knockout mice (*FGFR3*$^{-/-}$), only the first two genotypes were used in this study. *FGFR3*$^{-/-}$ mice produced in our colony exhibited severe musculoskeletal deformities and rarely lived past PN40.

Phenotypic pubertal assessment

Male mice were checked for balanopreputial separation (BPS) by gently retracting the prepuce from the glans penis from PN20 until sufficient separation was apparent. Mice were examined every 24 hours for BPS after weaning.

Female mice were examined for the age of vaginal opening (VO) by visual examination of the vulva from PN20 until a clear opening is present. Mice were examined every 24 hours after weaning.

Vaginal cytology

Once VO in female peripubertal mice (around PN30) had occurred, females were examined daily for vaginal cytology to determine their estrous cycle stages. For this, mice were gently grasped by the nape of their neck and flipped onto their backs. The tail of the mouse was secured between ring and index fingers, allowing the exposure of the vulva. The tip of a pipette was gently inserted into the vaginal opening and flushed with 9% saline solution to obtain vaginal epithelial cells. The solution was placed on a glass slide, sealed with a cover slip, and examined for different cell types under a light microscope at x10 magnification. Vaginal cytology assessment began the day after VO and lasted for 15 days.

The estrous cycle is characterized by four stages, each represented by different ratios of four types of vaginal epithelial cells (Long et al. 1922). Diestrus is part of the reproductive cycle in which the remaining corpora lutea are degraded and new follicles begin to develop. This stage is characterized by a majority of leukocytes and a few nucleated epithelial cells. Following diestrus is proestrus, a period of rapid follicular growth characterized by a high ratio of nucleated epithelial cells. A surge of gonadotropins (mainly LH) required for ovulation occurs in the afternoon of proestrus. Following proestrus, estrus ensues and is characterized predominantly by cornified epithelial cells. Ovulation and sexual receptivity occur for up to 13 hours during estrus. Lastly, metestrus follows estrus and is characterized by the formation of corpora lutea and contains all three cell types. The estrous cycle typically lasts 4-5 days (Caligioni et al. 2001) and repeats itself if the mouse does not become pregnant.

Motile sperm concentration measurement by epididymal squash

At PN60, male mice were anaesthetized using isoflurane and rapidly decapitated. A V-shaped abdominal incision was made, and epididymal fat pads were gently pulled to expose the testes and epididymis. The epididymis was isolated from each testis and placed in a petri dish with 2 mL of prewarmed (37 °C) phosphate-buffered saline (PBS). Both epididymides were used for this procedure. The epididymal cauda was isolated from the rest of the epididymis and vas deferens, minced with two scalpel blades and incubated in 2 ml of PBS at 35 °C for 15 minutes with gentle agitation. Following, 500 µl of sperm-containing PBS was mixed with 500 µl of 3% NaCl to immobilize sperm for consistent counting. The solution containing immobilized sperm was then vortexed and a 10-µl aliquot was used for counting by a hemacytometer. Of the 25 grids, four corner grids and one center grid were counted for number of sperm. Sperm that were not fully enclosed in the grid were only counted if they passed over the top or left line of the grid. Average number of sperm contained in the 5 grids was calculated. The average number of sperm was multiplied by the dilution factor, the number of total grids, and the volume of the hemocytometer to result in a final concentration of sperm per mL.

Statistical analysis

Homoscedasticity was observed for all data, thus differences between genotypes were determined by Students *t*-test. Differences were considered significant when $p<0.05$.

Results:

Phenotypic pubertal assessment

To determine the onset of puberty in both male and female mice, VO and BPS were examined. The age of VO in *FGFR3*$^{+/-}$ females was significantly increased compared to WT female ($p<0.05$) (Figure 1A). *FGFR3*$^{+/-}$ females took approximately 4.71 days longer to achieve VO compared to WT females (Figure 1A). In contrast, there was no significant difference in the age of BPS between *FGFR3*$^{+/-}$ and WT males (Figure 1B).

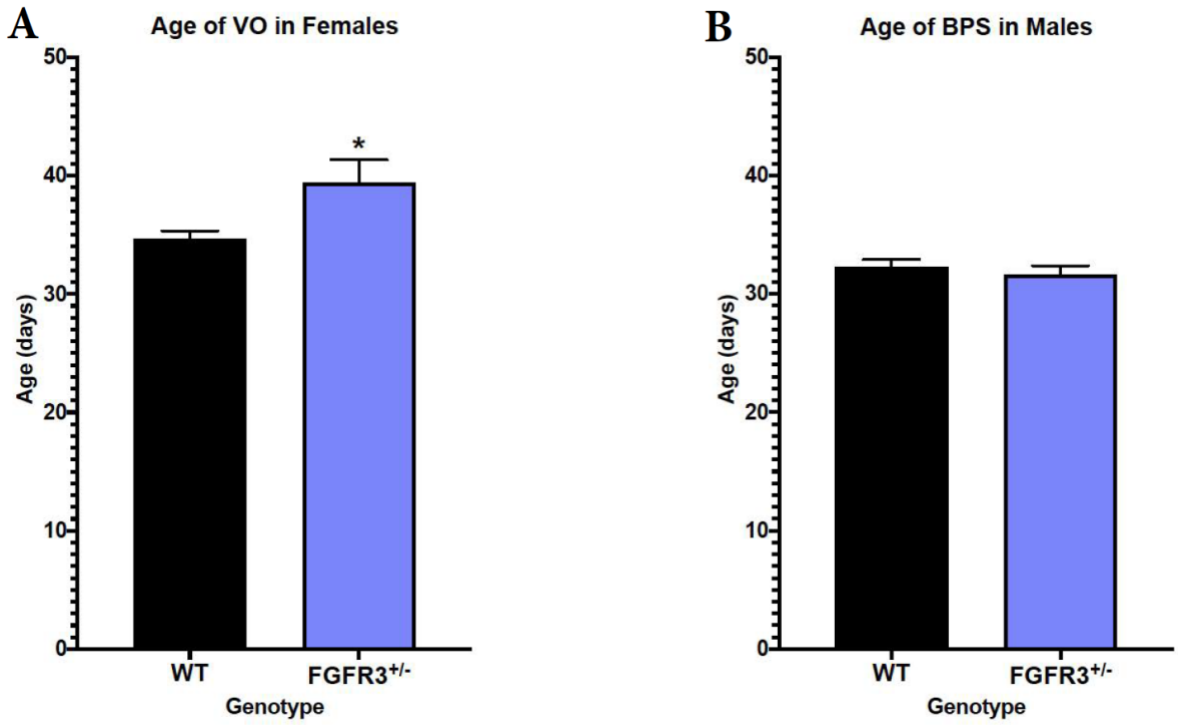

Figure 1. (A) Age of VO in *FGFR3*$^{+/-}$ females is significantly delayed compared to WT females (N=8-9). (B) Age of BPS is not significantly different between *FGFR3*$^{+/-}$ and WT males (N=4-5). All data represent mean ± SE.

First estrus

To evaluate pubertal transition to adulthood, both WT and *FGFR3*$^{+/-}$ females were examined for the age of first estrus. Although the age of VO is often considered to be an early sign of estrogen increase, the age of first estrus is a more consistent predictor of gonadal function

initiation. There was no significant difference between the age of first estrus between the genotypes (Figure 2). Despite a delay in age of VO among *FGFR3*$^{+/-}$ mice, they resumed normal reproductive development and underwent their first estrus in a similar time frame as the control mice.

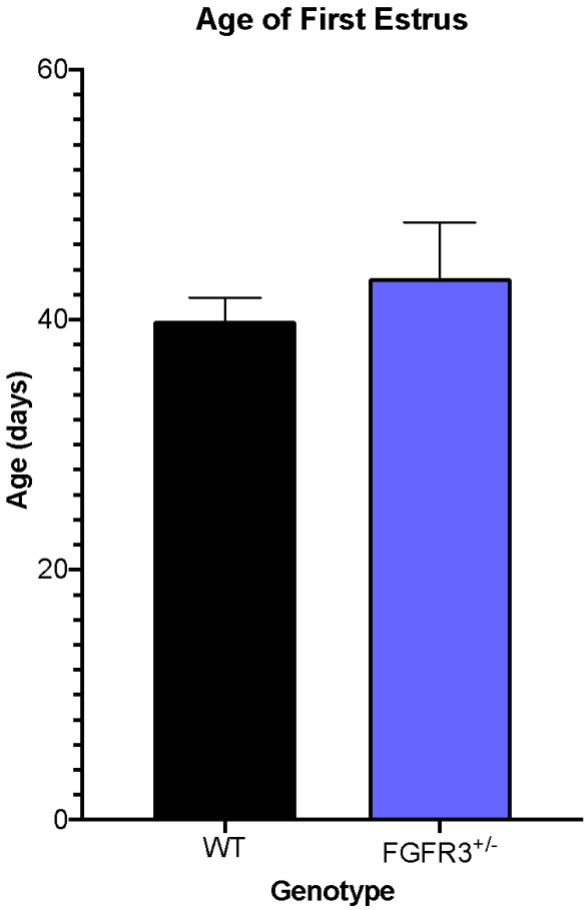

Figure 2. Age of first estrus is not significantly different between WT and *FGFR3*$^{+/-}$ females (N=6-9). All data represent mean ± SE.

Days in estrus

Evaluating estrous cyclicity provides important information on the fertility and receptivity of mammals. In particular, days in estrus indicates the number of days that a mouse is sexually receptive, or able to copulate and become pregnant (Byers et al. 2012). Percent of time

spent in estrus is not significantly different between WT and *FGFR3*$^{+/-}$ mice (Figure 3B). Additionally, there was no difference in percentage of time spent in proestrus and metestrus between WT and *FGFR3*$^{+/-}$ mice (Figure 3A, 3C). Despite average percentage for three phases, the average number of days in diestrus in *FGFR3*$^{+/-}$ female mice were significantly different WT female mice ($p<0.05$) (Figure 3D). Wild type mice spent an average of 24.44% of their cycle in diestrus, whereas *FGFR3*$^{+/-}$ mice spent an average of 15.56% of their cycle in estrus.

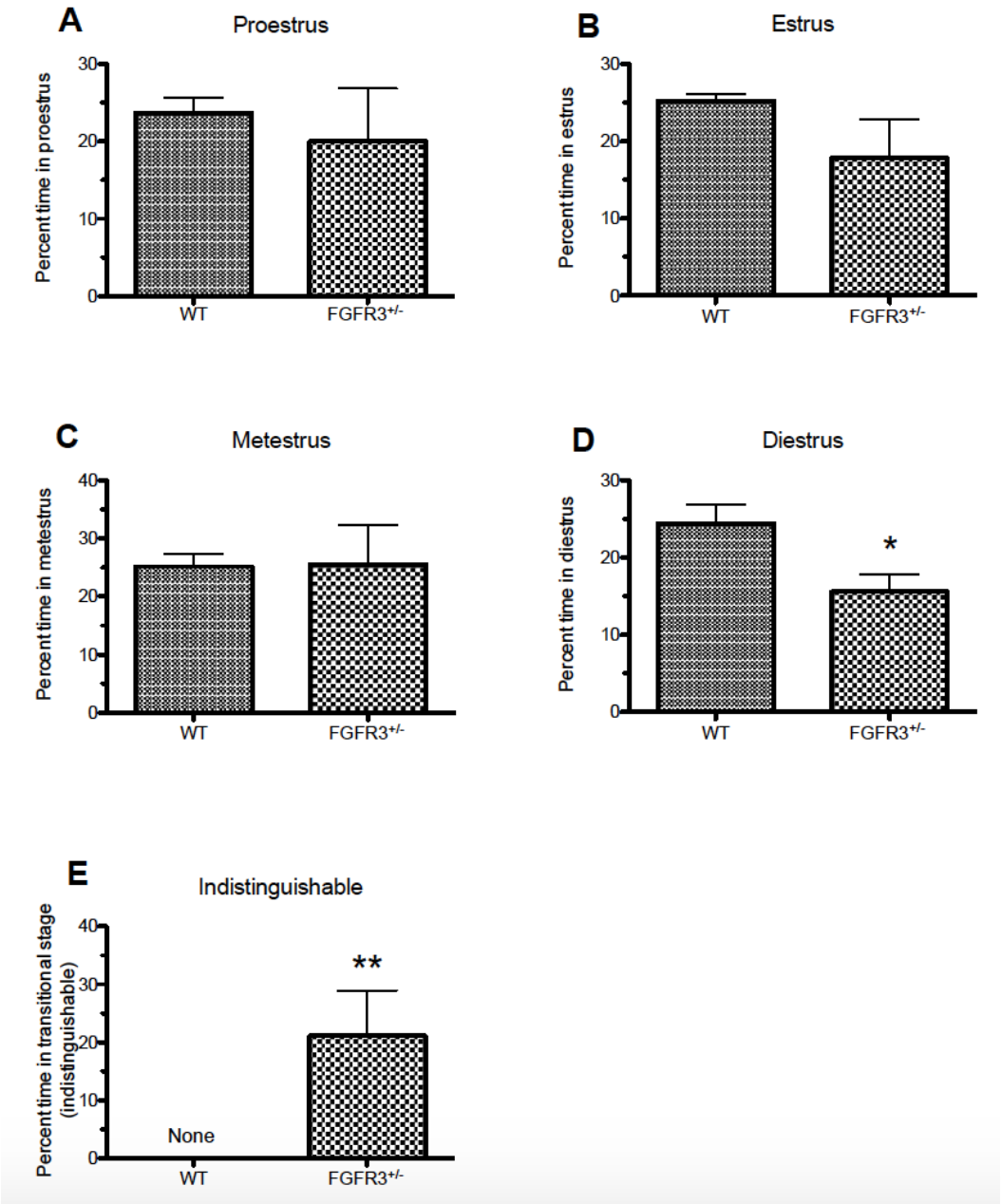

Figure 3. (A) Percent of time in proestrus over 15 days is not significantly different between WT and *FGFR3*$^{+/-}$ mice (N=9, N=5). (B) Percent of time in estrus over 15 days is not significantly different between WT and *FGFR3*$^{+/-}$ mice (N=9, N=5). (C) Percent of time in metestrus over 15 days is not significantly different between WT and *FGFR3*$^{+/-}$ mice (N=9, N=5). (D) Percent of time in diestrus over 15 days is significantly different between WT and *FGFR3*$^{+/-}$ mice (N=9, N=5) ($p<0.05$). (E) Percent of time in an indistinguishable stage over 15 days is significantly different between WT and *FGFR3*$^{+/-}$ mice (N=9, N=5) ($p<0.05$). All data represent the mean ± SE.

Motile Sperm Concentration

During early adulthood (PN60), male mice were evaluated for their relative fertility by examining the concentration of motile sperm from the cauda epididymis. Incubation of cauda epididymis, instead of testes, was employed in order to exclude non-motile sperm from the testes and focus only on fully motile sperm capable of fertilizing oocytes. At PN60, there was no significant difference in motile sperm concentration between *FGFR3*$^{+/-}$ compared to WT males (Figure 4).

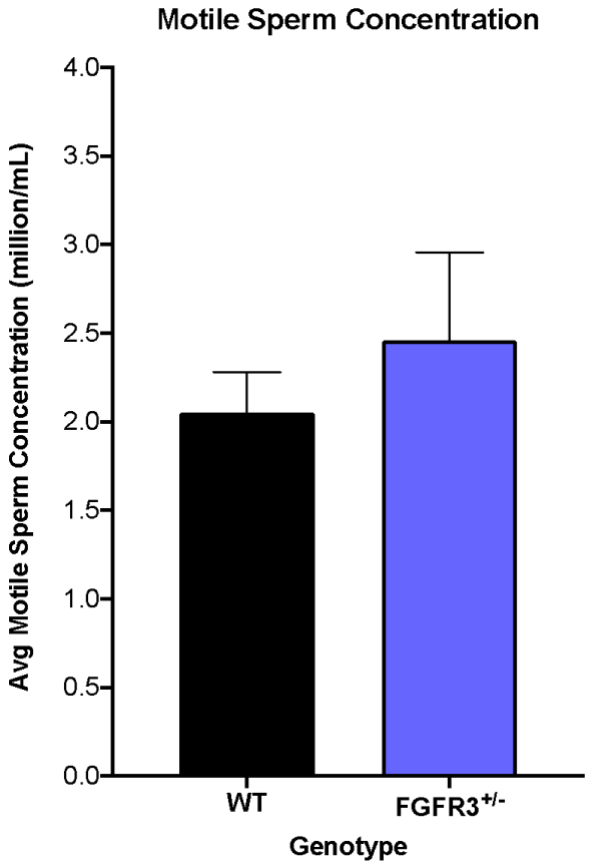

Figure 4. Motile sperm concentration obtained by epididymal squash of the cauda epididymis was not significantly different between PN60 WT and *FGFR3*$^{+/-}$ males (N=3-5 respectively). All data represent mean ± SE.

Discussion:

Characterization of the reproductive function is fundamental to understanding the effects of *FGFR3* deficiency on the HPG axis. Previously studies showed that *FGFR3*$^{+/-}$ mice were born with a normal set of GnRH neurons but later exhibited a postnatal loss (PN60) of these neurons (Chung et al. 2010). However, the reproductive consequence of this neuronal loss was not examined. In addition, *FGFR3* is also expressed in the pituitary and gonad (Sharma et al. 2019, Kaminskas et al. 2019), suggesting it may directly mediate the functions of these two

downstream organs. This study aimed to examine the onset of puberty in young FGFR3-deficient mice as well as the maintenance of reproduction in older adults.

External pubertal markers for both female and male mice were assessed. BPS is an androgen-dependent event that coincides with the maturation of the testes and is an external indicator of puberty (Korenbrot et al. 1977). Our results showed that WT and *FGFR3*$^{+/-}$ males attained BPS at the same time (Figure 1B), suggesting their circulating androgen levels were likely to be similar. These results suggest that the postnatal loss of GnRH neurons in *FGFR3*$^{+/-}$ males may not have yet occurred at the time of BPS (around PN30).

Another indication of puberty, VO, was determined after weaning (PN20) of female mice. Vaginal opening is initiated by a surge of estrogen occurring around the time of puberty in female mice (Ojeda et al. 1994). Although their male counterparts achieved puberty at a normal age, *FGFR3*$^{+/-}$ females exhibited delayed VO compared to WT (Figure 1A). These results suggest a potential lack of adequate estrogen production to induce apoptosis of lower vaginal mucosa (Rodriguez et al. 1997), leading to delayed VO. Decreased estrogen levels could result from decreased GnRH neurons found in *FGFR3*$^{+/-}$ mice at PN60. Interestingly, *FGFR3*$^{-/-}$ mice (N=3) never achieved VO even 72 days after birth (data not shown). These mice died at various ages with the oldest being PN72, yet never underwent VO at the time of death. Absence of VO in these mice could be due to a complete absence of functional *FGFR3* allele, resulting in absent or insufficient GnRH neurons and therefore no pubertal onset. That being said, *FGFR3*$^{-/-}$ mice suffer significant musculoskeletal deformities and may experience issues unrelated to primary GnRH deficiency, such as reduced feeding or enhanced stress, that could suppress the HPG axis (Rivier et al. 1991).

Evaluating the age of first estrus is particularly relevant to fertility in females because it indicates not just an estrogen surge sufficient to induce VO, but the beginning of ovarian cyclicity. Despite a delayed VO, *FGFR3*$^{+/-}$ and WT females attained the first estrus at the same time. These results suggest that like humans, puberty in mice consists of many stages, and FGFR3 deficiency may influence the earlier (VO) but not the later (first estrus) stages.

Over a 15-day period, females were examined for estrous cyclicity, including the number of days spent in each stage. WT and *FGFR3*$^{+/-}$ mice spent the same percentage of time in proestrus, estrus, and metestrus (Figure 3A, 3B, 3C). Interestingly, WT spent 8.88% more time in diestrus compared to *FGFR3*$^{+/-}$ mice (Figure 3D). Additionally, *FGFR3*$^{+/-}$ mice spent 21.11% of their cycle in an indistinguishable phase consisting of little to no cells and severely crenated cells (Figure 3E). Abnormal GnRH numbers could disrupt HPG axis signaling and interfere with normal estrous cyclicity. Lack of gonadotropins and sex steroids due to a GnRH deficiency could produce the indistinguishable estrous phase seen in these mice. Ovarian analyses should be performed to examine the morphology of a follicle at this indistinguishable phase. Additionally, levels of gonadotropins and sex steroids should be examined for a potential discrepancy. Abnormal estrous cyclicity can be seen in *FGFR3*$^{+/-}$ mice and may cause a decrease in fertility as compared to WT mice.

Male mice were evaluated for fertility by examining motile sperm concentration in adulthood (PN60). Despite the previously shown reduction in GnRH neurons in PN60 *FGFR3*$^{+/-}$ males (Chung et al. 2010), motile sperm concentration at PN60 was not significantly different between WT and *FGFR3*$^{+/-}$ males (Figure 4). Overall, male mice appear to be less affected by *FGFR3* deficiency than female mice. The cause of this sex difference is unknown. Multiple studies have reported that females are, in general, more vulnerable to HPG axis disruption due to

a need for robust hormonal changes to drive the female cycle. The precise locus along the HPG axis leading to this sex difference remains to be investigated.

Our results showed that FGFR3 deficiency did not impact male puberty and reproductive function, but significantly disrupted female pubertal onset and cyclicity. These results are novel and suggest FGFR3 inactivating mutations in humans may similarly impact certain aspects of women's reproduction. Future studies should examine tissue-specific *FGFR3*$^{+/-}$ knockout mice to ensure reproductive defects are due to a suboptimal HPG axis and not secondarily to defects in musculoskeletal functions (Kubota et al. 2020). Additionally, the precise extra-hypothalamic loci of the HPG axis impacted by *FGFR3* deficiency, such as the pituitary and the gonad, should be investigated.

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