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MOLECULAR METABOLISM

Fibroblast growth factor 21 has no direct role in regulating fertility in female mice

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ABSTRACT

Objective: Reproduction is an energetically expensive process. Insufficient calorie reserves, signaled to the brain through peripheral signals such as leptin, suppress fertility. Recently, fibroblast growth factor 21 (FGF21) was implicated as a signal from the liver to the hypothalamus that directly inhibits the hypothalamic–gonadotropin axis during fasting and starvation. However, FGF21 itself increases metabolic rate and can induce weight loss, which suggests that the effects of FGF21 on fertility may not be direct and may reflect changes in energy balance.
Methods: To address this important question, we evaluated fertility in several mouse models with elevated FGF21 levels including ketogenic diet fed mice, fasted mice, mice treated with exogenous FGF21 and transgenic mice over-expressing FGF21.
Results: We find that ketogenic diet fed mice remain fertile despite significant elevation in serum FGF21 levels. Absence of FGF21 does not alter transient infertility induced by fasting. Centrally infused FGF21 does not suppress fertility despite its efficacy in inducing browning of inguinal white adipose tissue. Furthermore, a high fat diet (HFD) can restore fertility of female FGF21-overexpressing mice, a model of growth restriction, even in the presence of supraphysiological serum FGF21 levels.
Conclusions: We conclude that FGF21 is not a direct physiological regulator of fertility in mice. The infertility observed in FGF21 overexpressing mice is likely driven by the increased energy expenditure and consequent excess calorie requirements resulting from high FGF21 levels.

Keywords FGF21; Fertility; Leptin; Hypothalamic action

1. INTRODUCTION

Successful reproduction involves integration of signals originating in the hypothalamus and ultimately targeting the gonads. In the hypothalamus, kisspeptin neurons in the anteroventral periventricular nucleus (AVPV) regulate the secretion of the gonadotropin-releasing hormone (GnRH) in the preoptic area, which, in turn, regulate secretion of the follicle stimulating (FSH) and luteinizing hormones (LH) from the anterior pituitary [1,2]. Kisspeptin neurons also receive input from other neurons, such as vasopressin neurons from the suprachiasmatic nucleus (SCN), to regulate the GnRH release [3]. Furthermore, vasopressin neurons are also known to innervate GnRH neurons directly in the preoptic area, which affects the release of LH [4]. The gonadotropin system is subject to feedback regulation from sex steroids released from the gonads. In addition to neuronal circuitry and subsequent hormonal cascade, successful reproduction requires adequate food availability and caloric stores. Caloric insufficiency can delay puberty and suspend established fertility [5–7]. For example, 48 h starvation inhibits estrus cycling in mice, and women with anorexia nervosa develop amenorrhea [8–10]. In both rodents and humans, low leptin levels play a critical role in signaling insufficiency of calorie supplies to the brain. Indeed, both *ob/ob* mice and leptin-deficient humans fail to enter puberty as, despite significant obesity, the central nervous system perceives starvation [9,11,12]. Furthermore, exogenous leptin corrects this phenotype in calorie-deprived mice and humans [8,9].

Recently, another nutritionally regulated hormone, FGF21, has been proposed as a regulator of fertility in rodents based on the finding that female mice overexpressing FGF21 to supraphysiologic levels (FGF21-Tg) have delayed onset of puberty and remain infertile through adulthood [13]. In this proposed model, FGF21 synthesized and secreted into the circulation by the liver acts on the SCN to suppress the production of vasopressin (*AVP*) which further suppresses kisspeptin-1 (*Kiss-1*), an important regulator of fertility.

We confirmed that FGF21-Tg mice consuming a chow diet are indeed infertile. In contrast to this finding, we observed that female mice consuming a ketogenic diet (KD), a model with dramatic increases in hepatic synthesis of FGF21 and elevated serum levels, demonstrate

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normal estrus cycles and remain fertile. Further infusion of FGF21 into the lateral ventricle had no effect on fertility in wild type (WT) female mice. Mice lacking FGF21 (FGF21-KO) had the same response as WT animals to the fasting induced suppression of cycling [10,14,15]. High fat diet (HFD) feeding to FGF21-Tg mice restored fertility, and this was associated with increased expression of *Kiss-1* and *AVP* in the AVPV and SCN nuclei, respectively.

In aggregate, these results render unlikely the claim that FGF21 has a physiologically relevant influence on fertility in female mice via action on the gonadotropic axis. We propose that the infertility of FGF21-Tg mice results from a relative caloric insufficiency and that the mechanism signaling this deficiency to the brain is neither leptin nor FGF21.

2. MATERIALS AND METHODS

2.1. Animals

Mice overexpressing human FGF21 were generated and generously provided by Lilly Research Laboratories, Indianapolis. Briefly, human apoE promoter including its hepatic control region was used to express the human FGF21 cDNA in transgenic mice [16]. The transgenic vector was linearized and microinjected into C57BL/6NTac eggs by standard methods [17]. Similarly, FGF21-KO mice were also generated and provided by Lilly Research Laboratories as described previously [18].

For other experiments, mice were procured from Jackson Research Laboratories (Bar Harbor, ME) at 8 weeks of age. For diet studies, mice were placed either on obesogenic high-fat/high-sucrose diet (D12451; Research Diets, New Brunswick, NJ) or ketogenic diet (F3666; Bio-Serv, Flemington, NJ). Mice were maintained on a 12:12-h light–dark cycle and an ambient temperature of 22 °C ± 2 °C unless otherwise stated. Food intake and body weights were determined with a tabletop scale measured to the closest 0.1 g. Mice were examined for sexual maturity and euthanized when in diestrus phase with vaporized isoflurane, exsanguinated via cardiac puncture, and serum was collected and frozen immediately. Dissected tissues were weighed and flash-frozen in liquid nitrogen. All animals were allowed *ad libitum* access to food and water. All procedures were in accordance with National Institute of Health Guidelines for the Care and Use of Animals and were approved by the Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committee.

2.2. Recombinant FGF21 protein

In brief, human FGF21 (hFGF21) was expressed in *Escherichia coli,* subsequently refolded in vitro, isolated as previously described [19], and provided by Lilly Research Laboratories (Indianapolis, IN).

2.3. Central FGF21 infusion

Each mouse was anesthetized with ketamine/xylazine and the surgical area shaved and cleaned with an iodine solution and alcohol swab. After being placed into a stereotaxic frame, an incision running dorsally and laterally of 1.5 cm was made to expose the skull, which was cleaned and drilled at the coordinates for the third ventricle: anterioposterior, −0.5 mm and lateral, −1 mm from the bregma. A cannula (Plastics One) was implanted at a cut −2.5 mm below the dura and was secured with cyanoacrylate adhesive Loctite 454 (Durect Corp, Cupertino, CA). Subsequently, an Alzet osmotic minipump (Alzet model 1007D; Durect Corp) filled with saline vehicle or hFGF21 was implanted under the skin between the scapulae and connected to the cannula via vinyl catheter tubing (Durect Corp), and sutures were used to close the wound. The pumps were loaded to deliver 0.4 μg/12 μL/d of hFGF21 or 12 μL/d of saline vehicle alone.

2.4. Vaginal opening and estrus cyclicity

Starting at 3 weeks of age, females were examined for the onset of vaginal opening. Estrus stages were determined by vaginal cytology with techniques that have been previously described [20]. Vaginal smears were collected daily between 9:00 AM and 11:00 AM. Each mouse was held and the vagina was gently flushed with 20 μl PBS or saline using a pipet tip 4–5 times. Cells were then transferred to a slide, allowed to dry and were subsequently stained with 0.1% crystal violet stain for 1 min followed by two washes with tap water, 1 min each. Stained slides were air dried, mounted with permaslip (Alban Scientific, St. Louis, MO), and coverslipped. All the mice were swabbed daily for at least 2–3 weeks, and the estrus stages were plotted as a function of time. Day 1 corresponded to the first day that vaginal smears were collected in each group.

To determine the estrus stage, vaginal smears were categorized based on the 4 different cell types present. Proestrus was identified by the presence of nucleated cells, with or without leukocytes. The estrus stage was characterized by the presence of cornified cells only. Metestrus smears contained mostly lightly stained cornified cells, along with a small number of leukocytes and nucleated cells. Diestrus smears predominantly contained leukocytes, along with some nucleated and cornified cells [20–22].

2.5. Body composition analysis

Body composition was determined using an EchoMRI 3-in-1 quantitative nuclear magnetic resonance (qNMR) system (Echo Medical Systems, Houston, TX). Body fat, lean mass, body fluids, and total body water were measured in live conscious mice with *ad libitum* access to chow or HFD.

2.6. Indirect calorimetry

Mice were maintained on a 12:12-h light–dark cycle and metabolic rate was measured by indirect calorimetry using a Comprehensive Lab Animal Monitoring System (Columbus Instruments, Columbus, OH). Sample air was passed through an O_2 sensor (Columbus Instruments) for determination of O_2 content. O_2 consumption was calculated by examining the difference of O_2 concentration of air entering the chamber compared with air leaving the chamber and heat production on per-animal basis was calculated from the following equation: $(3.82 + 1.23 \times \text{RER}) \times VO_2$, where the respiratory exchange ratio (RER) is the volume of CO_2 produced/volume of O_2 consumed per hour. The sensor was calibrated against a standard gas mix containing defined quantities of O_2, CO_2, and nitrogen. Food and water were available *ad libitum*. The measurement of 24 h of data collection was averaged and binned to create day and night depictions of metabolic rate and normalized to effective mass.

2.7. Ovarian histology

After excision, the ovaries were cleaned of fat, and the right and left ovaries were randomly assigned for histologic evaluation. Ovaries were fixed in 10% formalin overnight and then rinsed with phosphate-buffered saline before being embedded in paraffin. Ovarian follicles were studied by hematoxylin and eosin (H&E) staining and captured by Zeiss Axioplan light microscope and the images were acquired using AxioImager software (Carl Zeiss, Thornwood, NY).

2.8. Leptin studies

Leptin sensitivity was assessed in WT and FGF21-Tg mice by injecting 5 mg/kg leptin ip daily for 5 consecutive days. Food intake and body weight were measured twice daily for 5 days. Leptin infusion was continued for 21 days and vaginal opening was checked daily.

2.9. Hypothalamic tissue dissection

The mice were anaesthetized with isoflurane, and killed by decapitation. Brains were removed and the SCN and AVPV hypothalamic nuclei were micro-dissected (micropunches; 2 mm diameter). The tissues were kept in RNA Later Solution (Ambion, Austin, Texas) at 4 °C for 24 h to preserve the RNA, then removed and kept at −70 °C until RNA isolation. To assess possible changes in hypothalamic gene expression associated with fertility; all the animals were sacrificed in diestrus phase.

2.10. Quantitative RT-PCR

For RNA extraction from hypothalamic sections, Trizol was added before the samples were thawed and 250 μg/mL glycerol was added and samples were homogenized gently with a microcentrifuge pestle. RNA was extracted by routine phenol/chloroform extraction method.
For RNA isolation from other tissues, RNeasy Lipid Tissue Kit (Qiagen, Chatsworth, CA) was used as per the manufacturer's instruction. Complementary DNA was synthesized from 0.5 μg of RNA using a mixture of oligo (dT) and random hexamer primers with Quantiscript Reverse Transcriptase (QuantiTect Reverse Transcription Kit; Qiagen). Quantitative polymerase chain reaction was performed using a 7800HTthermal cycler (Applied Biosystems, Foster City, CA) and SYBR green master mix (Applied Biosystems). Expression of each target gene was quantified by transformation by the $\Delta\Delta Ct$ method and normalized to the expression of a housekeeping gene. TATA box binding protein (TBP) or 36B4 were used as endogenous controls. Primers were designed using Primer3 online software (Open Source) and obtained from Invitrogen (Carlsbad, CA). Primer sequences are available on request.

2.11. Peptides and serum analysis

Blood samples were centrifuged at 2500 rpm for 20 min; and serum was collected and stored at −80 °C. Leptin levels were assayed using a mouse-specific ELISA (Crystal Chem, Downers Grove, IL). For sensitivity studies, Leptin was provided by Dr. Parlow (Harbor-UCLA Medical Center, Torrance, CA) through the National Hormone and Peptide Program. Mouse FGF21 and human FGF21 proteins were measured by ELISA (R & D Systems, MN). Analysis of intact FGF21 was performed by Lilly Research Laboratories by their in-house ELISA and also confirmed by a commercially available ELISA kit (F2131-K01; Eagle Biosciences, Nashua, NH). Plasma levels for insulin like growth factor−1 (IGF-1) were determined using Quantikine ELISA kit for mouse/rat IGF-1 (MG100; R&D Systems, MN).

2.12. Statistical analysis

GraphPad Prism (La Jolla, CA) was utilized to analyze statistical differences using analysis of variance (ANOVA) test where appropriate. For comparisons of two groups, the Student's t-test was used. Where it is shown in figures, *$P < 0.05$; **$P < 0.01$ and ***$P < 0.001$ indicate the levels of significance. Data are displayed as the mean ± Standard error of the mean (SEM).

3. RESULTS

3.1. Mice with high serum FGF21 can maintain normal fertility

We used two physiological models of increased FGF21 expression, ketogenic diet feeding and fasting, to determine the effect of FGF21 on fertility. KD-fed female mice maintained the same body weight as chow fed animals over a three week observation period (Figure 1A). There was a more than 40 fold increase in serum FGF21 levels with KD consumption (Chow 0.27 ± 0.06; KD 11.4 ± 1.5 ng/ml; $p < 0.001$) (Figure 1B). During this period, examination of vaginal cytology revealed that all the mice continued a normal pattern of estrus cycling (Figure 1C). In a separate experiment, KD-fed mice became pregnant even after 8 weeks on the diet (data not shown).
Fasting of female mice for 48 h leads to suppression of estrus cycling and also elevated FGF21 levels [8,10,15]. To assess the role of FGF21 in the suppression of cycling during fasting, we measured the latency of FGF21-KO mice in recovering from fasting-induced inhibition of estrus cycling compared to WT mice. Reduction of body weight was comparable in both groups during the period of 48 h fast (Figure 1D). Serum FGF21 levels increased 10-fold in control mice with fasting (fed 0.25 ± 0.04; fast 2.3 ± 0.45 ng/ml, $p < 0.001$) (Figure 1E). All mice were examined daily for the vaginal opening, and samples were taken for vaginal cytology for at least 21 days. We did not observe any difference in the latency to estrus after a 48 h fast in FGF21-KO mice compared to controls (Figure 1F).

3.2. Central infusion of FGF21 does not inhibit fertility

Peripheral infusions of FGF21 at doses reported to inhibit fertility also lead to a decrease in body weight [23]. To dissociate the secondary effects of FGF21 on fertility from the direct effects of FGF21 on the central nervous system, we infused FGF21 into the lateral ventricle of WT mice for two weeks using osmotic mini pumps. Central infusion of a low dose of FGF21 (0.4 μg/day) did not affect body weight (Figure 2A) or endogenous FGF21 levels (Figure 2B). Since the infused FGF21 is a human protein, we also measured human FGF21 in mouse serum to monitor the circulating levels of centrally-infused FGF21. We found a small increase (Saline 28.1 ± 16.3; FGF21 136.8 ± 22.6 pg/ml, $p = < 0.01$), which is substantially lower than levels achieved with peripheral infusions of FGF21 [23] (Figure 2B right panel). The low level of human FGF21 noted in the saline-treated WT mice results from a 10–30% cross-reactivity of the human ELISA with mouse FGF21, which we tested against human and mouse standards (data not shown).
Centrally infused FGF21 increased the expression of several thermogenic genes such as *UCP-1* and *Cidea* in inguinal white adipose tissue (Figure 2C), confirming that FGF21 is sufficient to activate neuronal circuits leading to browning. We assessed estrus cycling for at least 1 week before the surgery and for 2 weeks during central infusion of FGF21. ICV-FGF21 infusion did not alter the estrus cycle as saline and FGF21-treatment produced similar cycles (Figure 2D). As reported previously, circulating IGF-1 levels were significantly lower in FGF21-Tg mice than in WT mice [24] (Supplementary Figure 2A) and HFD did not increase IGF-1 levels in FGF21-Tg mice compared to chow fed controls (Supplementary Figure 2B). Hepatic expression of *IGF-1* and its binding proteins −1 and −2 also did not alter with HFD feeding (Supplementary Figure 2C).

3.3. Consumption of HFD reverses the infertility of FGF21-Tg mice

We next examined the effect of HFD in the infertile transgenic model of FGF21 overexpression, FGF21-Tg. Chow-fed female FGF21-Tg mice have significantly lower body weight than WT littermates (Figure 3A). Starting at 8 weeks of age, mice were fed with HFD for another 8 weeks. HFD-fed FGF21-Tg mice gained 2 g more than chow-fed FGF21-Tg mice after 8 weeks of duration (FGF21-Tg-Chow 13.8 ± 0.5; FGF21-Tg-HFD 15.9 ± 0.4 gm; $p < 0.05$) (Figure 3A). Most of the body weight increase resulted from increased fat mass (FGF21-Tg-Chow 1.8 ± 0.2; FGF21-Tg-HFD 2.6 ± 0.3 gm; $p < 0.05$) (Figure 3B) without a significant increase in lean mass (Figure 3B). FGF21-Tg mice have significantly higher energy expenditure and hyperphagia compared to WT littermates on either diet (Supplementary Figure 1).

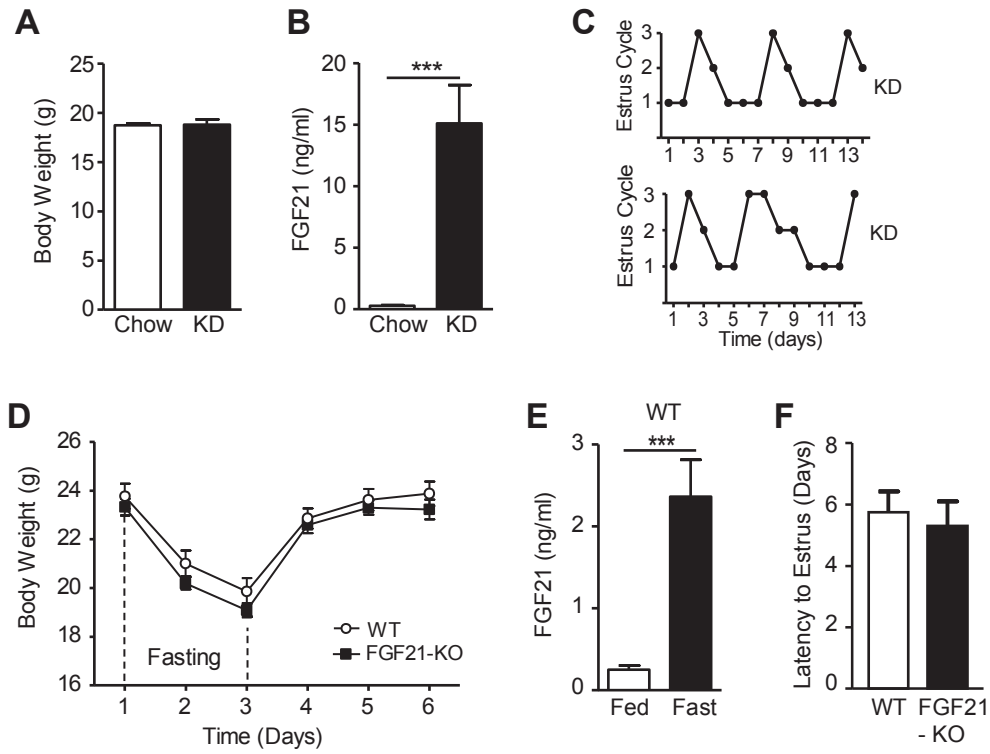

Figure 1: Elevated FGF21 does not alter the estrus cycle in physiological states. A) Body weight and (**B**) Serum FGF21 levels in WT female mice fed chow and KD for three weeks. **C**) Vaginal cytology score representing estrus cycles in WT mice fed KD diet (n = 9). (1: leukocytes [diestrus/metestrus], 2: cornified cells [estrus], 3: nucleated cells [proestrus]). **D**) Body weight of WT and FGF21-KO mice during 48 h fasting and refeeding. **E**) Serum FGF21 in WT mice in fed and fasted state. **F**) Latency to resumption of estrus induced by fasting (n = 10). Statistical significance was evaluated by Student's *t* test; $P^* < 0.05$; **, $P^{**} < 0.01$; $P^{***} < 0.001$.

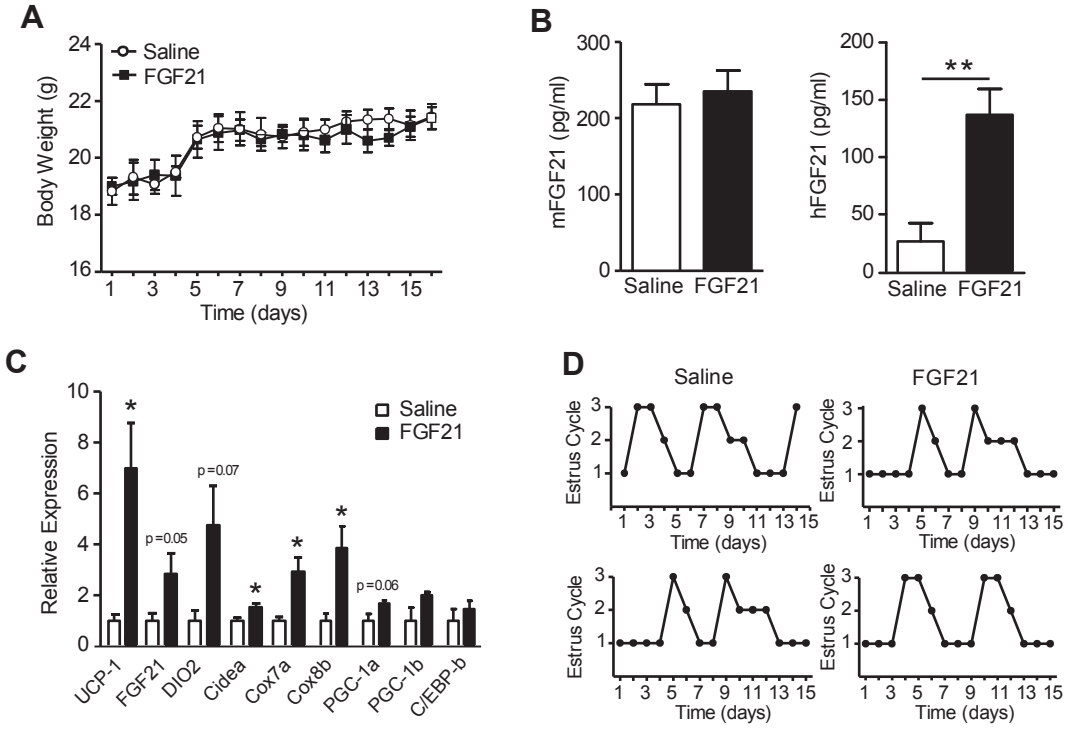

Figure 2: Central infusion of FGF21 does not affect estrus cyclicity. A) Body weight of WT mice after central infusion of either saline or FGF21 (0.4 μg/d) for 2 weeks. Day 1 represents the day of surgical implantation. **B**) Serum levels of mouse FGF21 (left panel) and human FGF21 (right panel). **C**) Gene expression of browning markers in inguinal adipose tissue in response to central FGF21 infusion. **D**) Representation of estrus cycle in mice (n = 4). (1: leukocytes [diestrus/metestrus], 2: cornified cells [estrus], 3: nucleated cells [proestrus]) Statistical significance was evaluated by Student's *t* test; $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

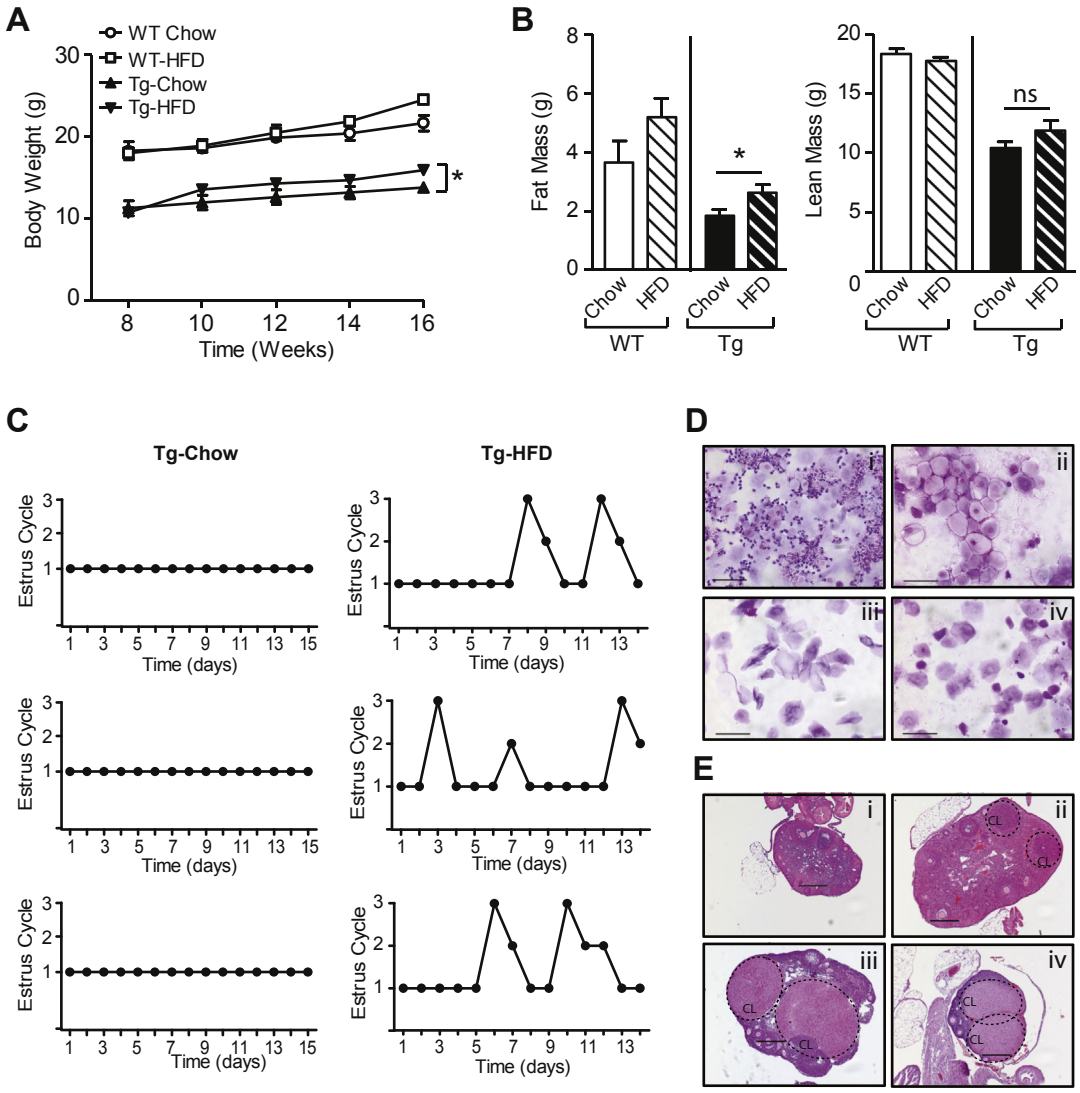

Figure 3: High fat diet reverses the infertility in FGF21-Tg mice. A) Body weight of WT and FGF21-Tg mice fed either with chow or HFD (n = 4–6). **B**) Body composition as represented as absolute fat and lean mass (n = 6–7). **C**) Assessment of estrus cycle in FGF21-Tg mice fed either chow or HFD. (1: leukocytes [diestrus/metestrus], 2: cornified cells [estrus], 3: nucleated cells [proestrus]) **D**) Representative vaginal cytology from FGF21 Tg mice fed with HFD as (i) diestrus, (ii) proestrus, (iii) estrus, (iv) metestrus. Scale: 200 μm (i–iv). (n = 8). **E**) Ovarian histology from FGF21-Tg mice; (i) chow, (ii) HFD, (iii–iv) HFD fed pregnant females. Corpus lutea (CL) are encircled with dotted lines. Scale: 1000 μm. Statistical significance was evaluated by Student's *t* test; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Chow-fed FGF21-Tg mice remain in diestrus phase throughout their adult life and never achieve regular estrus cycle (Figure 3C). Surprisingly, we observed vaginal openings in FGF21-Tg females and onset of cycling by day 11–12 in almost 80% of the FGF21-Tg mice after 1 week of HFD-feeding (Figure 3C). Periodic presence of diestrus phase with predominantly leukocytes (Figure 3D, i) followed by nucleated cells in proestrus (Figure 3D, ii), cornified epithelial cells in estrus phase (Figure 3D, iii) and presence of all three types of cells in metestrus phase (Figure 3D, iv) were observed in at least 8/10 female mice within 15 days observation period. However, 6/8 mice demonstrated at least two estrus cycles while 2/8 mice had only 1 confirmed estrus cycle in that time frame. Further, the average cycle length was about 5.7 days.

Seventy five percent of FGF21-Tg females (6/8) consuming HFD became pregnant. Ovarian histology is shown as an indicator of the presence of corpus luteum (CL) (Figure 3E). The follicles in chow-fed FGF21-Tg mice never mature (Figure 3E, i), while mice on HFD develop mature corpus luteum (CL) (Figure 3E, ii). Presence of an enlarged CL can be seen in pregnant FGF21-Tg mice (Figure 3E, iii–iv). FGF21-Tg females delivered a relatively small litter size of 1–3 pups while a normal litter size (6–7 pups) was observed in WT animals.

3.4. Exogenous leptin does not rescue the infertility of FGF21-Tg mice

Leptin is a known mediator of female fertility in energy deficient states [8–10]. Since FGF21-Tg mice are hypermetabolic and are growth-restricted, we measured circulating leptin levels in WT and FGF21-Tg mice. Circulating leptin levels were similar in both genotypes when fed with chow (Figure 4A). HFD consumption increased the circulating leptin levels in both WT (WT-Chow 4.6 ± 1.2; WT-HFD 11.5 ± 1.8 ng/ml; $p < 0.05$) and FGF21-Tg mice (FGF21-Tg-Chow 4.1 ± 0.3, FGF21-Tg-HFD 6.6 ± 0.9 ng/ml; $p < 0.05$).

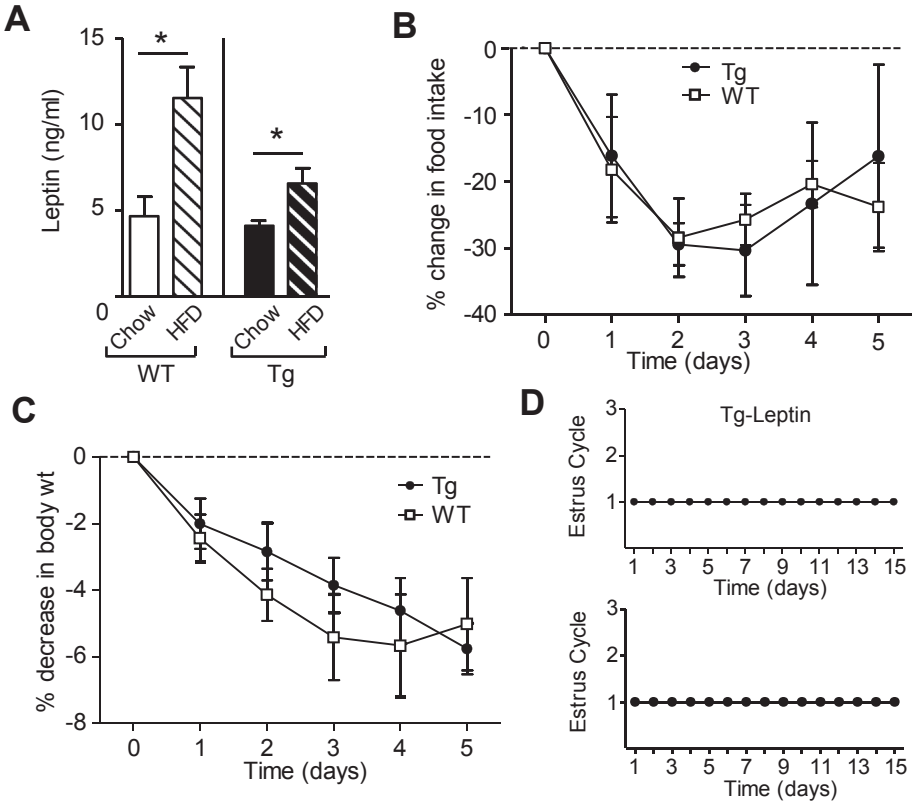

Figure 4: Leptin does not rescue the infertility of FGF21-Tg mice. **A)** Serum leptin levels in WT and FGF21-Tg mice fed with chow or HFD (n = 5–7). **B**) Percentage reduction in food intake and (**C**) body weight after leptin administration (ip 5 mg/kg) (n = 4–6) for 5 days. **D**) Representation of estrus cycle for 2 weeks as determined by vaginal cytology during leptin treatment for 21 days (n = 6). Statistical significance was evaluated by repeated measures ANOVA for food intake and body weight and Student's *t* test for leptin measurement; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

To determine whether the small increase in circulating leptin could be mediating the increased fertility of FGF21-Tg mice and to probe for central leptin resistance in the FGF21-Tg mice, we exogenously administered leptin (5 mg/kg body weight) to mice for 3 weeks. We observed a maximum 30% reduction in food intake (Figure 4B) and 6% reduction in body weight after leptin administration (Figure 4C). No significant difference was observed in FGF21-Tg mice compared to WT mice indicating that FGF21-Tg mice were equally sensitive to exogenous leptin. Hepatic and hypothalamic levels of all three leptin receptor genes remained unchanged (data not shown). We followed the estrus cycling of FGF21-Tg mice with leptin administration for the entire 3 weeks, but we did not observe any signs of sexual maturity by vaginal openings or initiation of estrus cycling as assessed by vaginal cytology (Figure 4D).

3.5. HFD restores hypothalamic vasopressin and kisspeptin expression

HFD feeding did not alter circulating levels of FGF21 in mice as total (Figure 5A) and active (Figure 5B) serum FGF21 levels remained unchanged between the chow and HFD fed FGF21-Tg mice. The proposed central circuitry mediating FGF21 infertility consists of FGF21 suppressing *AVP* expression in the SCN, which leads to decreased expression of *Kiss-1* in the AVPV nucleus [13], which was also observed in our study. However, HFD fed WT mice did not show any alteration in *AVP* or *Kiss-1* expression in SCN and AVPV respectively (Figure 5C,D). Interestingly, HFD increased *AVP* expression in the SCN of FGF21-Tg mice (FGF21-Tg-Chow 0.22 ± 0.10; FGF21-Tg-HFD 0.97 ± 0.49; p = 0.07) (Figure 5C) and *Kiss-1* expression in AVPV of FGF21-Tg mice (FGF21-Tg-Chow 0.37 ± 0.11; FGF21-Tg-HFD 1.94 ± 1.05; p = 0.05) (Figure 5D), although the increase in *AVP* did not reach significance.

4. DISCUSSION

FGF21 is an important metabolic regulator with complex characteristics as it is expressed by multiple organs and regulates diverse metabolic functions including glucose homeostasis, hepatic fatty acid oxidation, brown adipose tissue activity, and browning of white adipose tissue [25,26]. Recently, a potential regulatory role for FGF21 in the reproductive axis has been proposed [13].

FGF21-Tg mice express supraphysiologic levels of FGF21, and females are infertile as reported previously [13] and also as observed by us. However, high FGF21 levels may not necessarily be associated with infertility as female WT mice consuming KD for as long as three months maintain normal cycling and can become pregnant despite the multi-fold rise in serum FGF21. Furthermore, if high FGF21 levels serve as a nutritional signal to suppress fertility in states of relative caloric insufficiency, mice lacking FGF21 should demonstrate either earlier puberty or attenuated suppression of cycling after fasting, a phenomenon which we did not observe. Finally, direct infusion of FGF21 into the brain at doses known to induce peripheral browning failed to have any effects on the estrus cycle.

Intriguingly, HFD feeding rescued the infertility in FGF21-Tg mice. Puberty was initiated as assessed by vaginal opening within 5–6 days

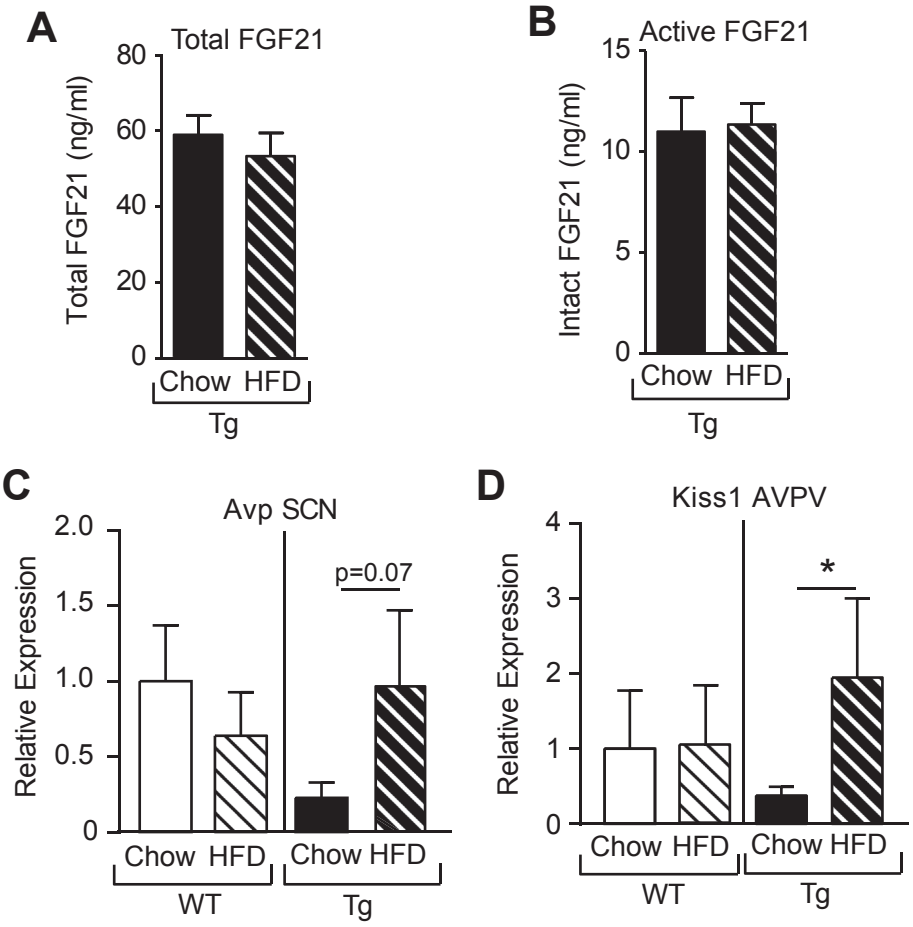

Figure 5: Infertility of FGF21-Tg mice is independent of FGF21. Serum levels of total FGF21 **(A)** and active FGF21 **(B)** in FGF21-Tg mice fed with chow or HFD (n = 9–10). *AVP* and *Kiss-1* gene expression in SCN **(C)** and AVPV **(D)**, respectively of WT and FGF21-Tg mice fed chow or HFD (n = 8–10). All mice were in the diestrus phase. Statistical significance was evaluated by Student's *t* test; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

and was followed by estrus cycling. When FGF21-Tg females consuming HFD were housed with males they became pregnant, and delivered live pups although the litter sizes were small. These changes were observed without any changes in total circulating FGF21 levels. Furthermore, there was no change in circulating intact FGF21, making it unlikely that the rescue of fertility was secondary to changes in FGF21 clearance or processing of the active form. Restoration of fertility in FGF21-Tg mice by deleting β-klotho using brain specific CRE has been suggested as evidence for central regulation of fertility by FGF21 [27]. However, these mice also gain weight, which may be involved in the rescue of their fertility, as seen in FGF21-Tg mice fed HFD [27]. While leptin might be expected to mediate the rescue as animals gained weight and had access to an energy rich diet, in this model, leptin was not involved. Baseline leptin levels and sensitivity to exogenous leptin was the same in FGF21-Tg animals and WT littermates. Furthermore, although leptin both corrects fasting-induced infertility and induces earlier vaginal opening in WT mice, leptin treatment had no effect on infertility of FGF21-Tg mice. We also did not observe any change in expression or circulating levels IGF-1, which rules out the involvement of the somatotropic axis in the restoration of fertility in FGF21-Tg mice.

Specific targets of FGF21 action in the brain are not very well defined and may include both the SCN and the PVN [13,23,27]. In rodents, a circadian signal from the SCN, directly and via kisspeptin neurons, is essential for the proestrus surge of GnRH, which, in turn, induces LH surge and ovulation [28,29]. Indeed, consumption of HFD increases vasopressin levels independent of changes in FGF21.

Further, the results from a wide variety of studies indicate that kisspeptin stimulates gonadotropin secretion via a hypothalamic pathway that activates GnRH neurons [30–32]. Also, significant increase in the number of neurons that express *Kiss-1* and their content of *Kiss-1* expression increases with puberty as seen in many both of the sexes of many species [31–33]. Interestingly, HFD feeding significantly increased kisspeptin expression in FGF21-Tg mice, which may contribute to the restoration of fertility.

The FGF21 pathway is being considered as a potential therapeutic target for humans with obesity and Type II diabetes. Interest in this pathway would certainly be mitigated if FGF21 had direct effects on fertility. Our findings indicate that no such direct effect is likely. Still the mechanism of infertility in FGF21-Tg mice is not clearly understood. We propose that infertility results from relative caloric insufficiency and that the mechanism signaling this insufficiency to the brain is not FGF21.

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CONFLICT OF INTERESTS

The past year EMF has consulted on a one time basis for Novo/Nordisk in the area of diabetes.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molmet.2016.05.010.

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