FGF21 contributes to neuroendocrine control of female reproduction

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Preventing reproduction during nutritional deprivation is an adaptive process that is conserved and essential for the survival of species. In mammals, the mechanisms that inhibit fertility during starvation are complex and incompletely understood1–7. Here we show that exposure of female mice to fibroblast growth factor 21 (FGF21), a fasting-induced hepatokine, mimics infertility secondary to starvation. Mechanistically, FGF21 acts on the suprachiasmatic nucleus (SCN) in the hypothalamus to suppress the vasopressin-kisspeptin signaling cascade, thereby inhibiting the proestrus surge in luteinizing hormone. Mice lacking the FGF21 co-receptor, β-Klotho, in the SCN are refractory to the inhibitory effect of FGF21 on female fertility. Thus, FGF21 defines an important liver-neuroendocrine axis that modulates female reproduction in response to nutritional challenge.

FGF21 is an atypical member of the FGF superfamily that can enter the circulation and function as a hormone8. Physiologically, plasma FGF21 levels are increased during starvation as a result of hepatic PPARα-mediated gene transcription9,10. FGF21 coordinates a systemic response to fasting by increasing ketogenesis, suppressing growth and promoting torpor9,11–13. Pharmacologically, FGF21 has beneficial metabolic effects in obese and diabetic animal models as an insulin sensitizer12, and therefore considerable effort is being devoted to understanding its mechanism of action14.

We previously reported that female, but not male, mice engineered to transgenically overexpress FGF21 (Tg(Fgf21)) are infertile9. Although the female Tg(Fgf21) mice are smaller and have higher insulin sensitivity, their body fat percentage and plasma adiponectin and leptin concentrations do not differ from their wild-type counterparts15. Initial characterization of the cause of infertility revealed a delay in the onset of puberty (Fig. 1a) and a failure to mate with proven stud males (Fig. 1b). Vaginal cytology and ovarian histology showed abnormalities consistent with anovulatory hypogonadism. Tg(Fgf21) mice rarely entered the ovulatory estrus phase of the cycle and displayed a prolonged diestrus (Fig. 1c). Ovarian histology revealed the presence of mature follicles in Tg(Fgf21) mice, but there were few, if any, post-ovulation corpora lutea (Fig. 1d). The abnormal estrous cycles in female Tg(Fgf21) mice were concordant with altered plasma gonadotropin concentrations: whereas plasma follicle stimulating hormone (FSH) concentrations were normal, concentrations of ovulation-inducing luteinizing hormone were significantly lower than those of wild-type mice (Fig. 1e). These analyses demonstrate that female Tg(Fgf21) mice exhibit hypogonadotropic hypogonadism.

To assess the function of the hypothalamic-pituitary-gonadal axis in female Tg(Fgf21) mice, we performed a series of hormone challenge tests. In response to exogenous gonadotropin (PMSG, gonadotropin from pregnant mare serum), plasma estradiol levels increased normally in both wild-type and Tg(Fgf21) mice (Fig. 2a). However, ovariectomized Tg(Fgf21) mice had a markedly reduced luteinizing...
hormone surge in response to exogenously administered estradiol, compared to wild-type mice (Fig. 2b), suggesting a defect at the level of the hypothalamus or pituitary. When stimulated with a synthetic gonadotropin–releasing hormone (GnRH) receptor agonist (leuprolide), the pituitary of Tg(Fgf21) mice produced a luteinizing hormone surge of similar magnitude to that of wild-type mice (Fig. 2c).

Therefore, the hypothalami of Tg(Fgf21) mice does not elicit an appropriate GnRH signal to the pituitary in response to a surge of estradiol. Consistent with this interpretation, transplantation of ovaries from Tg(Fgf21) mice into wild-type recipients was sufficient to rescue ovarian function (Supplementary Fig. 1).

The luteinizing hormone surge is controlled by a hypothalamic-neuroendocrine axis. Kisspeptin, the product of the Kiss1 gene, is expressed in the arcuate and anteroventral periventricular (AVPV) nuclei of the hypothalamus, where it communicates changes in plasma estradiol to GnRH neurons that, in turn, regulate pituitary gonadotropin secretion. Upstream, the SCN has an essential role in ovulation by communicating, through vasopressinergic neurons, to the kisspeptin axis in the AVPV nucleus and gating the luteinizing hormone surge. In the arcuate, a nucleus that mediates negative feedback effects of sex steroids on gonadotropin release, Kiss1 gene expression is similar in female wild-type and Tg(Fgf21) mice (Fig. 2d). However, in the AVPV nucleus, where kisspeptin exerts positive feedback effects of estradiol, and stimulates the preovulatory luteinizing hormone surge, Kiss1 gene expression was significantly lower in female Tg(Fgf21) mice compared to wild-type mice (Fig. 2d).

Likewise, vasopressin (AVP) mRNA levels were lower in the SCN of Tg(Fgf21) mice compared to wild-type mice (Fig. 2d). Notably, intracerebroventricular (i.c.v.) administration of either kisspeptin or vasopressin to Tg(Fgf21) mice was sufficient to restore the ability of the hypothalamus to induce a luteinizing hormone surge (Fig. 2e,f). Together, these data demonstrate that FGF21 perturbs the hypothalamic-neuroendocrine axis that is essential for ovulation. Our finding that FGF21 affects AVP nucleus kisspeptin neurons without affecting the arcuate nucleus population provides a possible explanation for the lack of effect of FGF21 on male fertility, given that male mice are virtually devoid of kisspeptin neurons in the AVPV nucleus.

β-Klotho is an essential co-receptor for FGF21 (ref. 22), and, in the forebrain, Klb expression is restricted to the SCN. To examine the involvement of the forebrain in the reproductive effects of FGF21, we crossed the Tg(Fgf21) mice with homozygous Klb-floxed (loxP-flanked) mice (Klb<sup>floxed</sup>) expressing Cre recombinase from the calcium/calmodulin-dependent kinase IIa (Camk2a) promoter. This strategy created a mouse strain (Klb<sup>floxed</sup>Camk2a<sup>Cre</sup>) that selectively eliminated Klb expression in the SCN of Tg(Fgf21) mice. Notably, the deletion of Klb in the forebrain of female Tg(Fgf21) mice resulted in higher expression of Kiss1 in the AVPV nucleus and Avp in the SCN compared to Klb<sup>floxed</sup> control mice (Fig. 3a). Estrous cycles were restored in 13 out of 14 Klb<sup>floxed</sup>Camk2a<sup>Cre</sup>, Tg(Fgf21) mice (Fig. 3b). Furthermore, all of the Klb<sup>floxed</sup>Camk2a<sup>Cre</sup>, Tg(Fgf21) mice became pregnant after mating with stud males (Fig. 3c). As shown in our companion paper<sup>23</sup>, Klb is also expressed in the dorsal vagal complex of the hindbrain. Eliminating Klb in the hindbrain of Tg(Fgf21) mice using a Phox2b-Cre line<sup>23</sup> did not restore estrus in any of the female mice (Supplementary Fig. 2). These results show that β-Klotho–mediated FGF21 signaling in the forebrain leads to an abnormal gonadotropic response to estradiol and anovulation.

Multiple redundant mechanisms have evolved to prevent reproduction during nutritional deprivation<sup>1–7</sup>. To determine whether FGF21 contributes to this important adaptive process, we first tested whether Klb<sup>floxed</sup>Camk2a<sup>Cre</sup> mice are resistant to fasting-induced infertility. Whereas fasting (48 h) perturbed the estrous cycle in all mice, the delay in ovulation upon refeeding was significantly shorter in Klb<sup>floxed</sup>Camk2a<sup>Cre</sup> compared to Klb<sup>floxed</sup> mice (Fig. 4a). In addition, the expression of Kiss1 in the AVPV nucleus and Avp in the SCN was significantly higher in Klb<sup>floxed</sup>Camk2a<sup>Cre</sup> mice than in Klb<sup>floxed</sup> mice following a 48-h fast (Fig. 4b). The finding that β-Klotho in the SCN is required for the normal reproductive response to starvation in female mice strongly supports the notion of a physiologic FGF21-neuroendocrine axis.

Finally, using subcutaneous osmotic minipumps, we assessed the effect of long-term exposure to fasting levels of FGF21 on female reproduction. Continuous infusion of recombinant human FGF21 at
Figure 4  Evidence that FGF21 modulates female reproduction as part of the adaptive starvation response. (a) Delayed ovulation caused by a 48-h fast in Klbtm1 mice in the presence (+Cre) or absence (−Cre) of Camk2a-Cre (n = 6). (b) Hypothalamic gene expression in in Klbtm1 mice in the presence (+Cre) or absence (−Cre) of Camk2a-Cre following a 48-h fast (n = 6). Cycle-time (CT) values are shown for −Cre controls. (c) Plasma human FGF21 concentrations achieved by osmotic minipump infusion of vehicle (Veh) or human FGF21 (n = 6). (d) Effects of minipump administration of FGF21 on estrus cycles and (e) hypothalamic gene expression in Klbtm1 mice in the presence (+Cre) or absence (−Cre) of Camk2a-Cre (n = 6). C1 values are shown for vehicle controls. (f) Schematic diagram illustrating FGF21 action on the hypothalamic-pituitary-ovarian axis. Data are expressed as mean ± s.e.m. *P < 0.05 compared to −Cre controls (a,b) or compared to vehicle (e).

a rate of 1.1 µg h⁻¹ resulted in stable plasma concentrations between 5 and 10 ng ml⁻¹ (Fig. 4c), which are similar to physiologic levels achieved during fasting (as observed in our accompanying paper23 and previously24). At these concentrations, FGF21 had no effect on body weight, body composition or plasma leptin concentrations (Supplementary Fig. 3). However, this treatment was sufficient to terminate estrous cycles in five out of six female Klbtm1 mice (Fig. 4d).

By contrast, estrous cycles were maintained in FGF21-treated Klbtm1(Camk2a) mice (Fig. 4d). Similarly, Kiss1 expression in the AVPV nucleus and Avp expression in the SCN were lower in FGF21-treated mice than in vehicle-treated mice, an effect that was also dependent on Klb expression in the SCN (Fig. 4e). Together, these findings demonstrate that exposure to fasting levels of FGF21 is sufficient to cause female infertility in adult mice and that β-Klotho–dependent FGF21 signaling in the SCN may contribute to suppressing female fertility as part of the adaptive starvation response.

In summary, these studies define a liver-neuroendocrine signaling pathway in which FGF21, acting through the SCN in the hypothalamus, contributes to the suppression of ovulation during starvation (Fig. 4f). In an accompanying paper23, we show that FGF21 action on the hypothalamus also increases corticosterone concentrations, suppresses growth and alters behavior. Although the effects of FGF21 on metabolic parameters, including plasma insulin and glucocorticoid concentrations, may also contribute to female infertility, our finding that direct i.c.v. injection of either vasopressin or kispeptin into female Tg(Fgf21) mice rapidly induced an luteinizing hormone surge indicates that FGF21 causes infertility in part by dampening the output of the SCN, which in turn governs the kispeptin-GnRH–luteinizing hormone neuroendocrine circuit (Fig. 4f). Notably, SCN-derived vasopressin is part of the neuroendocrine pathway that also suppresses corticosterone secretion from the adrenal glands25. Thus, the suppression of vasopressin expression in the SCN may be the mechanism unifying the effects of FGF21 on circulating glucocorticoid levels, female reproduction and possibly other physiologic processes. Finally, the observation that FGF21 can also be elevated systemically by obesity26 suggests that FGF21 might contribute to the reproductive anomalies that have been associated with the metabolic syndrome27.

METHODS
Methods and any associated references are available in the online version of the paper.
Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS
B.M.O. designed and performed all experiments, analyzed data and wrote the paper. A.L.B. generated Klbtm1(Camk2a); Tg(Fgf21) and Klbtm1(Phox2b); Tg(Fgf21) mice and designed and performed experiments. X.D. generated Klbtm1 mice. V.Y.L., S.D.A. and L.G. performed experiments and analyzed data. D.J.M. and S.A.K. supervised the project and wrote the paper.

COMPETING FINANCIAL INTERESTS
The authors declare competing financial interests: details are available in the online version of the paper.

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Luteinizing hormone surge experiments. A standard protocol was followed to induce an estrogen-stimulated luteinizing hormone surge. Briefly, mice were ovarioctomized and implanted with a Silastic pellet containing estradiol (1 cm pellet containing 0.1 mg ml⁻¹ 17β-estradiol in sesame oil). Mice were allowed to recover for 1 week and then received an intraperitoneal injection of estradiol benzoate (0.05 mg per kg body weight) or vehicle (sesame oil) at ZT2. Blood was collected for luteinizing hormone measurement at ZT13.5 the following day.

The ability of leuprolide to stimulate pituitary luteinizing hormone secretion was tested in wild-type and Tg(Fgf21) mice. Mice were ovarioctomized and implanted with a Silastic pellet containing estradiol (as above). The mice were allowed to recover for 1 week and then received an intraperitoneal injection of vehicle (saline) or leuprolide (1 µg per kg body weight) at ZT9. Blood was collected for luteinizing hormone measurement 1 h after injection.

The ability of kisspeptin to induce a luteinizing hormone surge was tested in intact Tg(Fgf21) mice. Mice underwent surgery for the implantation of a guide cannula (coordinates from the bregma: anterior-posterior −0.34 mm, L/R +1 mm, D −2.3 mm). Following recovery for 1 week, mice received a single i.c.v. injection of vehicle (artificial cerebral spinal fluid) or kisspeptin (1 nM in 1 µl) at ZT9. Blood was collected for luteinizing hormone measurement 1 h after injection.

The ability of vasopressin to rescue an estrogen-stimulated luteinizing hormone surge was tested in Tg(Fgf21) mice. Mice underwent guide cannula surgery; ovarioctomy; Silastic pellet implantation and estradiol benzoate injection as described above. A single i.c.v. injection of vehicle (artificial cerebral spinal fluid) or vasopressin (3 ng in 1.5 µl) was administered at ZT6 on the day after estradiol injection and blood was collected at ZT13 for luteinizing hormone measurement.

Brain dissection. The brain was rapidly removed from the skull and placed on ice. Punches from the anteroventral periventricular (AVPV), suprachiasmatic nucleus (SCN) and arcuate nucleus of the hypothalamus were cut away from the surrounding tissue of 0.5-mm-thick coronal slices under RNase-free conditions. Care was taken to avoid adjacent nuclei. The location of nuclei was determined in relation to white matter landmarks (anterior commissure and optic chiasm) and the third ventricle, with the aid of the Paxinos Mouse Brain Atlas.

Gene expression analysis. Standard protocols were followed for quantifying gene expression of mouse tissue. Primers for detecting Fgf21, Kiss1 and Avp mRNA are available upon request.

Statistical analyses. Data are expressed as mean ± s.e.m. Statistical significance was calculated using a two-tailed Student’s t-test with P < 0.05 considered significant.

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