Original Article

MOLECULAR METABOLISM

Activation of hepatic estrogen receptor-α increases energy expenditure by stimulating the production of fibroblast growth factor 21 in female mice

Camille Allard [1], Fabrice Bonnet [5], Beibei Xu [1], Laurel Coons [3], Diana Albarado [4], Cristal Hill [4], Guy Fagherazzi [5], Kenneth S. Korach [3], Ellis R. Levin [6,7], John Lefante [8], Christopher Morrison [4], Franck Mauvais-Jarvis [1,2,*]

ABSTRACT

Objective: The endogenous estrogen 17β-estradiol (E2) promotes metabolic homeostasis in premenopausal women. In a mouse model of postmenopausal metabolic syndrome, we reported that estrogens increased energy expenditure, thus preventing estrogen deficiency-induced adiposity. Estrogens' prevention of fat accumulation was associated with increased serum concentrations of fibroblast growth factor 21 (FGF21), suggesting that FGF21 participates in estrogens' promotion of energy expenditure.

Methods: We studied the effect of E2 on FGF21 production and the role of FGF21 in E2 stimulation of energy expenditure and prevention of adiposity, using female estrogen receptor (ER)- and FGF21-deficient mice fed a normal chow and a cohort of ovariectomized women from the French E3N prospective cohort study.

Results: E2 acting on the hepatocyte ERα increases hepatic expression and production of FGF21 in female mice. *In vivo* activation of ERα increases the transcription of *Fgf21* via an estrogen response element outside the promoter of *Fgf21*. Treatment with E2 increases oxygen consumption and energy expenditure and prevents whole body fat accumulation in ovariectomized female WT mice. The effect of E2 on energy expenditure is not observed in FGF21-deficient mice. While E2 treatment still prevents fat accumulation in FGF21-deficient mice, this effect is decreased compared to WT mice. In an observational cohort of ovariectomized women, E2 treatment was associated with lower serum FGF21 concentrations, which may reflect a healthier metabolic profile.

Conclusions: In female mice, E2 action on the hepatocyte ERα increases *Fgf21* transcription and FGF21 production, thus promoting energy expenditure and partially decreasing fat accumulation.

© 2019 Published by Elsevier GmbH. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Keywords Estrogen; ERα; FGF21; Menopause; Obesity; Metabolic syndrome

1. INTRODUCTION

Estrogens help maintain energy homeostasis in both male and female rodents and humans [1]. After menopause, estrogen-deficient women are predisposed to metabolic dysfunction, including metabolic syndrome, obesity, and type 2 diabetes. Large randomized controlled trials and observational studies have suggested that menopausal therapy with estrogens reduces the incidence of metabolic syndrome, obesity, and type 2 diabetes in women [2]. However, because of its complex balance of risks and benefits, estrogen therapy is neither appropriate nor approved for the prevention of metabolic dysfunction in menopausal women. We must explore new therapeutic approaches to improve metabolic outcomes in menopausal women while reducing oncogenic risk associated with estrogen therapy.

Fibroblast growth factor 21 (FGF21), a hormone mainly produced by the liver during fasting [3], has emerged as a therapeutic avenue for treatment of type 2 diabetes and obesity [4]. In diabetic rodents, primates, and humans, FGF21 increases energy expenditure, reduces body weight, and improves insulin sensitivity [5]. Several FGF21 analogs are currently in clinical trials for the treatment of metabolic disorders [6], and are a major focus of therapies for non-alcoholic fatty liver disease (NAFLD) [7].

[1]Diabetes Discovery Research and Sex-Based Medicine Laboratory, Department of Medicine, Section of Endocrinology and Metabolism, Tulane University Health Sciences Center, School of Medicine, USA [2]Southeast Louisiana Veterans Healthcare System Medical Center, New Orleans, LA 70112, USA [3]Receptor Biology Section, Reproductive and Developmental Biology Laboratory, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, Durham, NC 27709, USA [4]Pennington Biomedical Research Center, Louisiana State University System, Baton Rouge, LA 70803, USA [5]LACESP, INSERM U1018, Université Paris-Sud, UVSQ, Université Paris-Saclay, Gustave Roussy, Villejuif Cedex, F-94805, France [6]Division of Endocrinology, Veterans Affairs Medical Center, Long Beach, CA 90822, USA [7]Department of Medicine and Biochemistry, University of California, Irvine, CA 92717, USA [8]Department of Global Biostatistics and Data Science, Tulane University School of Public Health and Tropical Medicine, New Orleans, LA 70112, USA

*Corresponding author. Section of Endocrinology, Tulane University Health Sciences Center, 1430 Tulane Avenue, New Orleans, LA 70112, USA. Fax: +1 504 988 6271. E-mail: fmauvais@tulane.edu (F. Mauvais-Jarvis).

Received December 12, 2018 • Revision received February 7, 2019 • Accepted February 10, 2019 • Available online 14 February 2019

https://doi.org/10.1016/j.molmet.2019.02.002

 © 2019 Published by Elsevier GmbH. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Female rats fed a high-fat high-fructose diet developed NAFLD which was aggravated by ovariectomy and associated with decreased serum FGF21 concentrations [8]. Estrogen supplementation restored serum FGF21 concentrations and reduced NAFLD in these female rats. Using a mouse model of menopause exposed to a high-fat, high-fructose diet, we reported that treatment with either subcutaneous 17β-estradiol (E2) or oral conjugated estrogens similarly improved metabolic homeostasis by increasing energy expenditure, thus preventing diet- and estrogen deficiency-induced obesity and NAFLD [9]. These effects were associated with increased hepatic *fgf21* mRNA expression and elevated serum FGF21 concentrations. Together, these studies suggest that the beneficial effects of estrogen therapy on energy expenditure and NAFLD are mediated, at least in part, via FGF21 production. Consistent with this possibility, hepatocyte-specific knockout of ERα promotes hepatic steatosis in female mice [10], However, the effect of hepatocyte-specific ERα deficiency on estrogens stimulation of FGF21 production is unknown.

The goal of this study was to investigate the mechanism by which estrogens increase FGF21 production, and the role of FGF21 in estrogen-mediated increase in energy expenditure and decrease in adiposity.

2. MATERIAL AND METHODS

2.1. Animals

Animal experiments were performed with male and female C57Bl/6J mice, 8–12 weeks old, fed a chow diet ad libitum. The generation of ERαKO [11], Membrane-Only and Nuclear-Only Estrogen Receptor α (MOER and NOER respectively) [12,13], DNA-binding domain mutant (EAAE) mice [14], and FGF21KO mice [15] have been previously described. Liver specific Estrogen Receptor α Knock-Out mice (LERKO) were generated by breeding ERαlox mice with Albumin-Cre mice (Jackson Laboratories, stock #003574). ERβKO were bought from Jackson Laboratories (stock #004745). Wild-type littermates were used as controls. If not available, C57Bl/6J mice were bought from Jackson Laboratories. All experiments performed were approved by the Institutional Animal Care and Use Committees of Tulane University and Pennington Biomedical Research Center, and were in accordance with NIH guidelines.

2.2. Metabolic tests

Blood glucose was measured using a True Result glucose meter (Nitro Diagnostics). Random-fed serum collection was performed in the morning, 3h after the beginning of the light period. For prolonged fasting experiments, food was removed for a total of 40h. Blood sampling was performed either by collecting blood from the tail vein (awake mice) or from the inferior vena cava (after anesthesia with 200 mg/kg ketamine, 16 mg/kg xylazine). Aprotinin was added to the blood as a protease inhibitor. Blood was centrifuged at 4,000 g for 15 min at 4 °C. Serum was then collected and frozen at −80 °C. Tissues were dissected, snap-frozen in liquid nitrogen, and stored at −80 °C until further analysis. Metabolic tests were performed after 6h fasting. Glucose tolerance test (GTT) and insulin tolerance test (ITT) consisted of measuring blood glucose after an i.p. injection of 2 g/kg glucose and 0.5 U/kg insulin (Humulin, Lilly), respectively. For the FGF21KO mice, energy expenditure, food intake, and physical activity were measured using metabolic chambers (PhenoMaster/LabMaster; TSE Systems). Body composition was measured via TD-NMR (Bruker Minispec). Hormones were measured by ELISA: Insulin (Millipore) and FGF21 (R&D systems). Hepatic triglycerides were measured with a colorimetric assay (Cayman) following manufacturer's guidelines.

2.3. Ovariectomy

Ovariectomy (OVX) consisted of removing both ovaries in 8–12 week-old female mice using a bilateral approach, under isoflurane anesthesia and sterile conditions. Briefly, after opening the peritoneal cavity, ovaries were ligatured and dissected. The muscle layer was sutured and the skin closed using clips. Sham-operated animals were incised as for the OVX, but the ovaries were left in place. Short term E2 treatment (8 or 160 μg/kg/d, 3 days) was initiated 3 weeks post-surgery. Long term E2 treatment (0.5 mg s. c. pellet, 12 weeks) was started immediately post-surgery.

2.4. *In vivo* treatment with estrogens

Mice were treated for 3 days with vehicle (10% ethanol in sesame oil), E2 (8 μg/kg or 160 μg/kg, dissolved in sesame oil, Steraloids), the ERα agonist propyl-pyrazole-triol (PPT, 8 mg/kg/d) [16], the ERβ agonist diarylpropionitrile (DPN, 1 mg/kg/d) [17], or the GPER agonist G1 (1 mg/kg/d) [18] via subcutaneous injection. The last day of treatment, mice were fasted for 6h after injection before sacrifice. PPT and DPN were obtained from Dr John Katzenellenbogen (University of Illinois) and G1 was obtained from Dr Eric Prossnitz (University of New Mexico). Wild type and FGF21KO mice were implanted with an E2 pellet inserted s.c. in the back of the neck (0.5 mg/pellet; 90-day release, Innovative Research of America).

2.5. RT-qPCR

RNA was extracted using TRIzol RNA Isolation method (Invitrogen) following the provider's instructions. cDNA was synthesized using iScript reverse transcription kit (Biorad). For real time qPCR performed with SYBRgreen (Biorad), primers for the following genes were used: fgf21, 5-GCTCTCTATGGATCGCCTCA-3 (forward) and 5-TTGTAACCGT CCTCCAGCAG-3 (reverse); fas, 5- CACAGATGATGACAGGAGATGG-3 (forward) and 5- TCGGAGTGAGGCTGGGTTGAT-3 (reverse); β-actin, 5-GATGTATGAAGGCTTTGGTC-3 (forward) and 5-TGTGCAC TTTTATTGGTCTC-3 (reverse). For real time qPCR performed with TaqMan (Thermo Scientific), the following probes were used: fgf21, Mm00840165_g1; β-actin, Mm00607939_s1. Real-time qPCR was performed using 25–50 ng of cDNA on a Roche Lightcycler. Target gene expression level was calculated using the $\Delta\Delta C_T$ method, with β-actin as a reference gene, and normalized according to the control condition.

2.6. Identification of estrogen response elements (EREs) in mouse and human genomes

The location coordinates of the perfect and 1 nt variant EREs were identified in the mouse and human genomes using OligoMatch (UCSC) as previously described [19]. Here, the ERE is the motif 5'-GGTCAnnnTGACC-3'.

2.7. E3N cohort

We obtained serum samples from the prospective French cohort of the Etude Epidémiologique de Femmes de la Mutuelle Générale de l'Education Nationale (E3N) [20]. We selected a sample of ovariectomized post-menopausal women treated with estrogen (estrogen therapy, ET group) or not (no ET group).

2.8. Statistical analysis

Mouse. Data were analyzed using GraphPad Prism software. Normal distribution was tested using Kolmogorov–Smirnov test. Depending on the experiments, either Student t-test (Mann–Whitney test if non-parametric distribution) or one-way ANOVA (Kruskal Wallis if non-parametric distribution) were used. When results were significant,

 © 2019 Published by Elsevier GmbH. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

comparisons were made using Bonferroni post-hoc tests. All data are expressed as mean +/− SEM, with a probability value of 0.05 considered statically significant.

E3N cohort. Mean serum FGF21 levels were compared between treated and untreated women using the two-sample t-test. Multiple linear regression was used and analysis of covariance was performed to estimate the difference in group FGF21 levels, adjusting for differences in covariates. Results are presented on the original FGF21 scale. Analyses were also performed on the log-transformed values of FGF21 to account for sensitivity to possible non-normality of the FGF21 values. Data were analyzed using SAS 9.4.

3. RESULTS

3.1. E2 increases circulating FGF21 concentrations in female mice

We measured serum FGF21 concentrations in mice of both sexes fed a normal chow. We used prolonged fasting as a powerful inducer of FGF21 in mice [21,22]. Fasting increased serum FGF21 concentrations in male and female mice (Figure 1A) and fasted female mice exhibited higher serum concentrations of FGF21 than fasted males (Figure 1A). Ovariectomy (OVX) reduced the fasting-induced rise in serum FGF21 concentrations in female mice, and treatment with 17β-estradiol (E2) at a replacement dose increased FGF21 serum concentrations to levels of ovary-intact, sham operated female mice (Figure 1A). At this low dose, E2 did not increase FGF21 serum concentrations in fed OVX female (Figure 1A), but E2 increased FGF21 serum concentrations in fed intact female mice (Figure 1B). At higher doses leading to physiological concentrations corresponding to pregnancy levels [9], E2 increased serum FGF21 concentrations in both intact and OVX female mice as well as males (Figure 1C). Thus, E2 increases fasting and fed FGF21 serum concentrations in intact and OVX female mice.

3.2. E2 increases circulating FGF21 concentrations of via nuclear ERα in hepatocytes

FGF21 is a liver-derived circulating hormone (hepatokine) [3] and hepatocytes express three estrogen receptors (ERs): ERα, ERβ and the

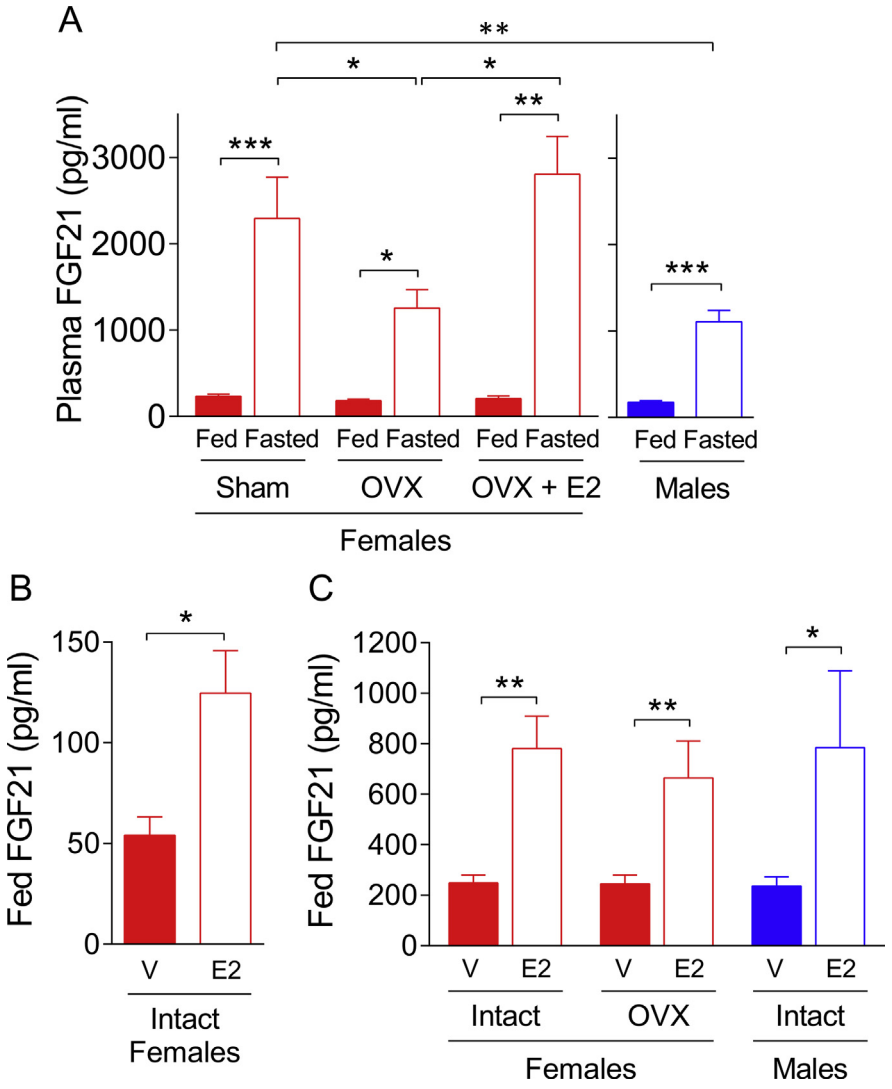


Figure 1: E2 increases serum FGF21 concentrations in female and male mice. (**A**) Serum FGF21 concentrations in random fed and 40h-fasted sham-operated mice (Sham), ovariectomized mice (OVX), OVX female mice treated with 17β-estradiol (OVX + E2, 8 μg/kg/d s.c.) (n = 3–14) and male mice (n = 12–16). (**B**) Fed serum FGF21 concentrations in intact female mice after s.c. treatment with vehicle or E2 (8 μg/kg/d) (n = 4–6). (**C**) Fed serum FGF21 concentrations in the indicated female and male mice after s.c. treatment with V or E2 (160 μg/kg/d) (n = 6–25). All mice are C57Bl/6J. Data represent the mean ± SEM. *p < 0.05, **p < 0.01.

 © 2019 Published by Elsevier GmbH. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

G protein-coupled ER (GPER). To determine which ER(s) is (are) involved in the E2-induced increase in serum FGF21 concentrations, we treated wild-type mice with ER specific agonists. The ERα agonist PPT, but not the ERβ agonist DPN or the GPER agonist G1, significantly increased hepatic *Fgf21* mRNA expression and serum FGF21 concentrations (Figure 2A,B). Consistent with results obtained with pharmacological agonists, the effect of E2 to increase serum FGF21 concentrations in WT female mice was abolished in female ERα knockout (ERαKO) mice but was retained GPER knockout (GPERKO) mice and non-significant increased in ERβ knockout (ERβKO) (Figure 2C). Together, these results indicate that E2 increases hepatic production of FGF21 via ERα. To explore to what extent E2 acts directly on ERα in hepatocytes to stimulate FGF21 expression, we generated mice deficient in ERα selectively in hepatocytes (LERKO). Following E2 treatment, hepatic *Fgf21* mRNA expression and serum FGF21 protein concentrations were increased in ERαlox controls (Figure 2D,E), but this was not observed in LERKO mice, consistent with a role of the hepatocyte ERα in increasing *Fgf21* mRNA expression and FGF21 protein secretion (Figure 2D,E). In mouse models, manipulations inducing metabolic dysregulation can promote *Fgf21* mRNA expression in inguinal white adipose tissue (iWAT) [23], brown adipose tissue (BAT) [24,25] or skeletal muscle [22,26], which can increase circulating FGF21 serum concentrations. Thus, we sought to determine whether E2 could increase *Fgf21* mRNA production by these extrahepatic tissues in LERKO mice. However, *Fgf21* mRNA was not detectable in iWAT, BAT, and gastrocnemius muscle from E2-treated LERKO mice (data not shown).

Estrogens' actions in hepatocytes are mediated by pools of nuclear or membrane ERα. To assess the contribution of nuclear ERα, we used a knockin mouse with mutant ERα that prevents ERα membrane localization. In this "Nuclear Only ERα" (NOER) mouse, ERα is exclusively nuclear, and extranuclear actions of ERα are abolished [13]. In contrast, we also used an ERα deficient mouse re-expressing ERα selectively at the plasma membrane, the Membrane Only Estrogen Receptor-α (MOER) mouse [12].

E2 treatment increased hepatic fgf21 mRNA expression and serum FGF21 concentrations (Figure 3A,B) in WT and NOER mice (note that fgf21 mRNA was non-significantly increased in NOER). In contrast, E2 had no effect on hepatic *Fgf21* mRNA expression and serum FGF21 concentrations in MOER mice (Figure 3A,B), suggesting that ERα needs to exhibit a nuclear location to stimulate *Fgf21* mRNA expression and protein secretion. ERα is a ligand-activated transcription factor that regulates gene expression by binding estrogen response elements (ERE) present on the DNA. In order to test whether E2 requires an ERE on the *Fgf21* promoter to stimulate *Fgf21* mRNA expression, we used the ERα DNA-binding domain mutant mouse (ERα-EAAE), which does not support E_2-mediated transcription and lacks the ability to bind perfect consensus EREs, 1 nt variant EREs, and a small subset of 2 nt variant EREs [14]. We observed that E2-induced *Fgf21* hepatic mRNA expression and FGF21 serum concentrations were blunted in the EAAE female mouse (Figure 3A,B), demonstrating its dependence on an ERE. We next looked at locations of EREs on the chromosome that contains the *Fgf21* gene in the mouse and human genome. No ERE was present in the promoter of *Fgf21* in either the mouse or human genome (Figure 3C). The closest perfect ERE is located ~200kb upstream of the transcription start site (TSS) of *Fgf21* in the mouse genome and ~220kb in the human genome. The closest 1nt variant EREs are located 41 and 21kb upstream of the TSS of *Fgf21* (Figure 3C) respectively. Thus, E2 increases FGF21 transcription via ERα acting on a NRFE ERE, likely in a distant enhancer, but not directly on the *Fgf21* promoter.

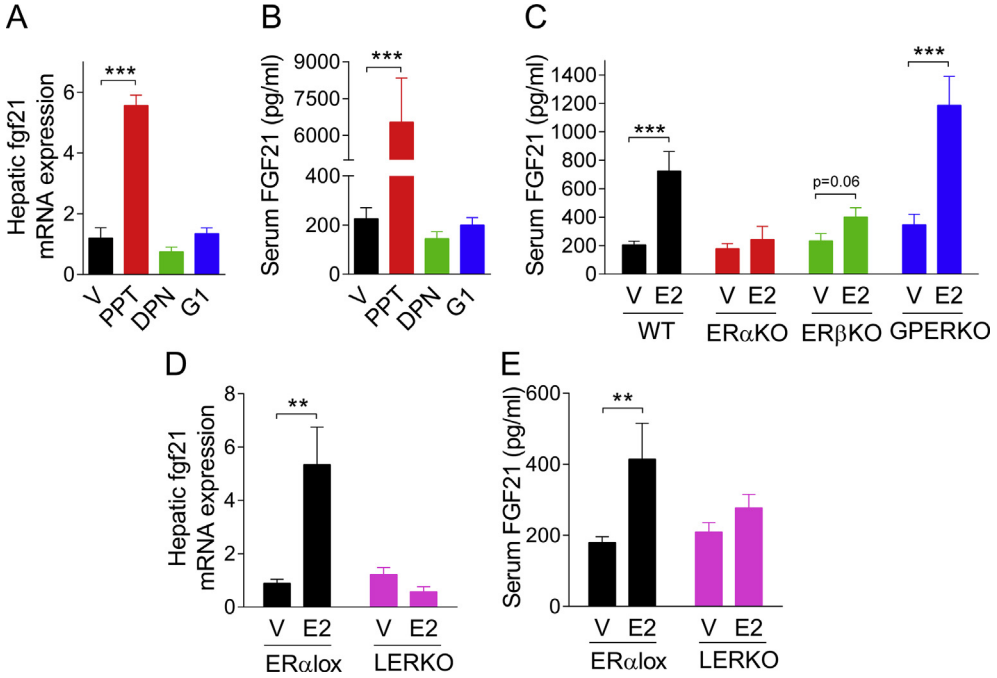
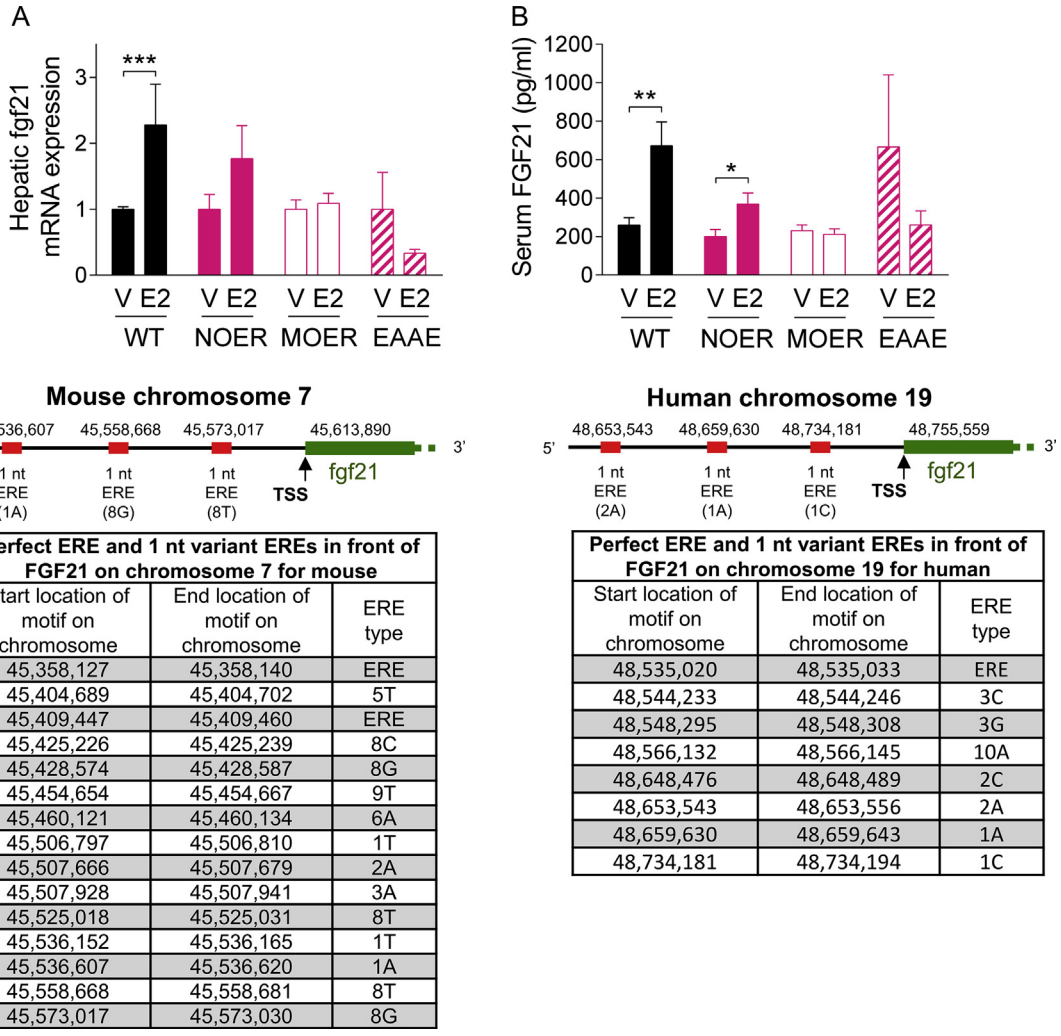


Figure 2: E2 increases FGF21 production by activating hepatocyte ERα *in vivo*. (**A**) Liver *Fgf21* mRNA expression and (**B**) serum FGF21 concentrations in intact female C57Bl6/J mice treated with the indicated compounds (n = 5–6). (**C**) Serum FGF21 concentrations in the indicated female mice after 3 days of treatment with the indicated compounds (n = 5–22). Wild-type (WT) littermates from each group were pooled. (**D**) Liver *Fgf21* mRNA expression and (**E**) serum FGF21 concentrations in the indicated female mice treated for 3 days with vehicle (V) or E2 (160 μg/kg/d) (n = 4–9). Data represent the mean ± SEM. *p < 0.05, **p < 0.01. ***p < 0.001.

 © 2019 Published by Elsevier GmbH. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).



Perfect ERE and 1 nt variant EREs in front of FGF21 on chromosome 7 for mouse

Start location of motif on chromosome	End location of motif on chromosome	ERE type
45,358,127	45,358,140	ERE
45,404,689	45,404,702	5T
45,409,447	45,409,460	ERE
45,425,226	45,425,239	8C
45,428,574	45,428,587	8G
45,454,654	45,454,667	9T
45,460,121	45,460,134	6A
45,506,797	45,506,810	1T
45,507,666	45,507,679	2A
45,507,928	45,507,941	3A
45,525,018	45,525,031	8T
45,536,152	45,536,165	1T
45,536,607	45,536,620	1A
45,558,668	45,558,681	8T
45,573,017	45,573,030	8G

Perfect ERE and 1 nt variant EREs in front of FGF21 on chromosome 19 for human

Start location of motif on chromosome	End location of motif on chromosome	ERE type
48,535,020	48,535,033	ERE
48,544,233	48,544,246	3C
48,548,295	48,548,308	3G
48,566,132	48,566,145	10A
48,648,476	48,648,489	2C
48,653,543	48,653,556	2A
48,659,630	48,659,643	1A
48,734,181	48,734,194	1C

Figure 3: ERα stimulates *Fgf21* gene transcription via an ERE. (**A**) Hepatic *Fgf21* mRNA expression and (**B**) serum FGF21 concentrations in intact NOER, MOER and EAAE female mice and control wild-type littermates (WT) after 3 days of treatment with vehicle (V) or 17β-estradiol (E2, 160 μg/kg/d) (n = 3–15). (**C**) Position of perfect EREs and 1nt variant EREs in front of *Fgf21* gene in the mouse (left) and human (right) genomes. Data correspond to the mean values ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001.

3.3. E2 increases energy expenditure via FGF21

We previously reported that E2 treatment of OVX mice augmented serum FGF21 concentrations, which was associated with increased energy expenditure and decreased fat mass [9], suggesting that the increased energy expenditure in E2-treated OVX mice was due to FGF21 action. To explore this possibility, we measured energy expenditure in E2-treated WT and FGF21-deficient mice. OVX non-significantly decreased, and E2 increased, circulating FGF21 concentrations in the WT group (Suppl figure 1A). In WT mice, OVX decreased energy expenditure in the dark phase (Figure 4A,B, non-significant), and, accordingly, E2 treatment increased energy expenditure in both phases. In contrast, in the FGF21KO mice, OVX and E2 treatment failed to increase energy expenditure corrected for lean mass in both phases (Figure 4A,B), and corrected for body weight in the light phase only (Figure 4C). E2 treatment still increased energy expenditure in FGF21KO mice in the dark phase. E2 decreased the respiratory exchange ratio (RER, Figure 4C) during the light phase and increased it during the dark phase to a similar extent in WT and FGF21KO mice, consistent with a stimulatory effect for E2 on lipid and carbohydrate oxidation in both groups, independent from the increase in FGF21. Physical activity was reduced during the dark phase in WT and FGF21KO mice and was not affected by E2 treatment (Figure 4D). Food intake was increased in E2-treated WT OVX mice (Figure 4E), which we interpret as a failed attempt to compensate for the large increase in energy expenditure induced by high doses of E2 (as confirmed by E2-induced large increase in uterus weight Suppl Figure 1B).

Surprisingly, OVX increased body weight and fat pad weights in WT and FGF21KO mice, and E2 treatment reduced body weight and fat pad weight in WT and FGF21KO mice (Figure 4F–H). However, the percentage of weight (non-significant) and fat mass loss was more pronounced in WT compared to FGF21KO mice (Figure 4G and I).

3.4. E2 therapy is associated with decreased serum FGF21 concentrations in post-menopausal women

To assess whether E2 treatment also increases FGF21 production in women, we measured serum FGF21 concentrations in a cohort of post-menopausal women from the French E3N observational study. We selected a sample of women who were ovariectomized, some of whom were treated with E2 and some whom were not. The characteristics of the women are shown in Table 1. In contrast to what we saw in rodents, we observed that serum FGF21 concentrations were lower in women who received E2 therapy (ET) compared to those who

 © 2019 Published by Elsevier GmbH. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

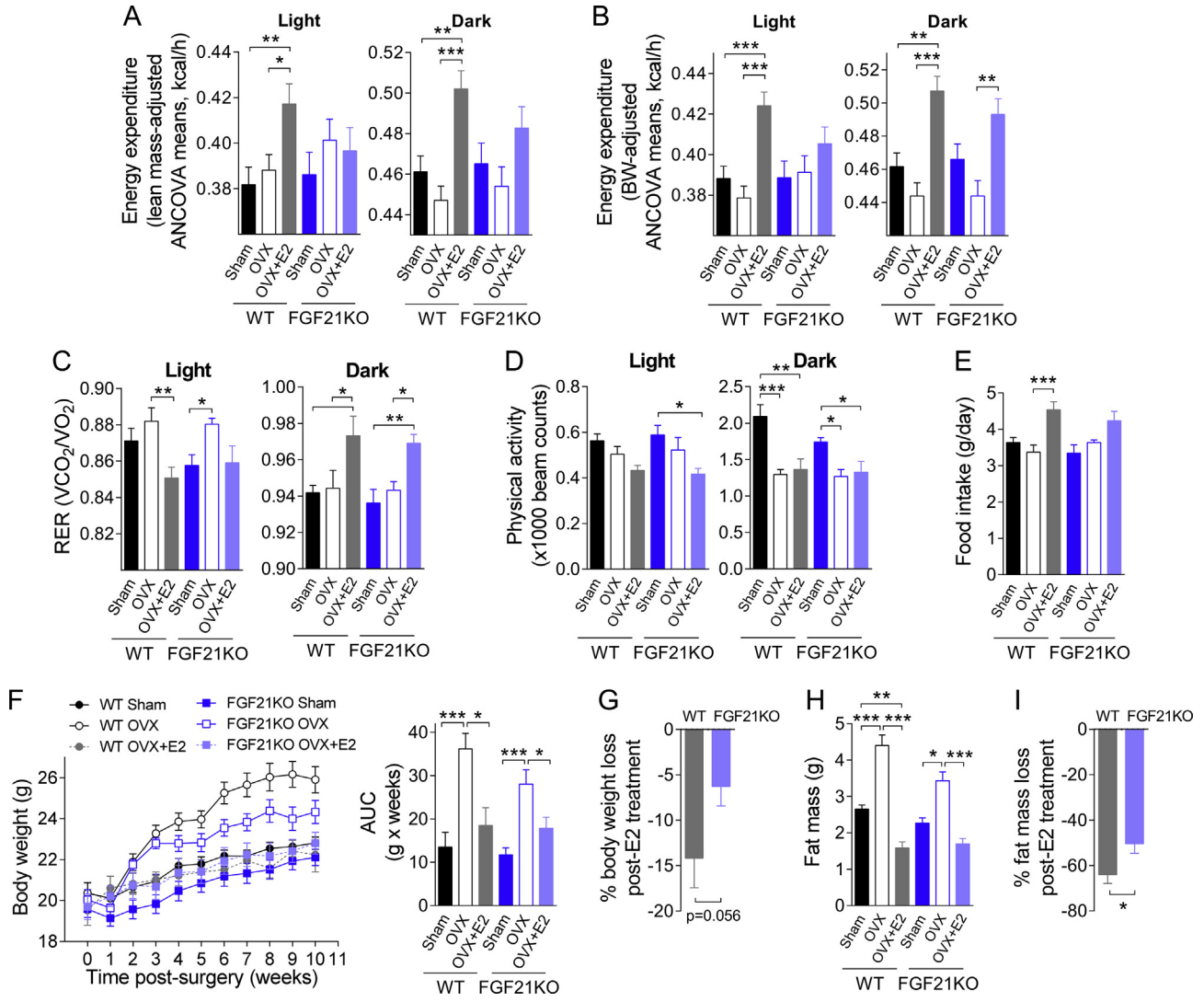


Figure 4: E2 stimulates energy expenditure via FGF21 in OVX-female mice. WT and FGF21KO female mice underwent sham or OVX surgery and after 12 weeks were individually placed in metabolic cages during light (left) and dark (right) phase (n = 5–10). (**A**) Energy expenditure adjusted to lean mass (ANCOVA means). (**B**) Energy expenditure adjusted to body weight (ANCOVA means). (**C**) Respiratory Exchange Ratio (RER). (**D**) Physical activity. (**E**) Food intake. (**F**) Post-surgery body weight (left) and its calculated AUC (right). (**G**) Comparison of relative body weights between ovariectomized E2 treated and untreated animals from Figure 4F expressed in % loss (two cohorts of animals). (**H**) Fat mass measured by NMR at week 10 from F. (**I**) Difference in fat mass between OVX and OVX + E2 group from Figure 4H expressed in % loss. Data correspond to the mean values ± SEM (2 independent experiments). For (F) significance was assessed by repeated measures ANOVA * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Table 1 – Metabolic characteristics of E3N women (Average ± sem). These women never took hormonal contraception in their life. The ovariectomy (OVX) occurred before or after the natural menopause (natural estrogen levels drop). Mets: Metabolic Equivalent of Task, index of averaged physical activity of participants.

	No ET	ET	p value
Number	54	60	
Body Mass Index	23,6 ± 0,3	23,1 ± 0,4	0,2532
Age at menopause	45,7 ± 0,9	48,7 ± 0,7	0,0025
Age at OVX	46,4 ± 0,9	49,4 ± 0,7	0,0055
% of natural menopause	14,8	23,3	
Age when blood sampling	57,0 ± 0,4	55,3 ± 0,4	0,0039
Treatment duration (years)	11,3 ± 1,0	6,7 ± 0,6	<0,0001
Mets	45,7 ± 6,8	41,1 ± 5,1	0,82

never received E2 therapy (no ET) (Figure 5A). Following adjustment for body mass index, type of menopause (surgical or natural), age at the time of menopause, duration of treatment, and physical activity, the difference was not statistically significant (No ET: 149.81 ± 18.93, ET: 109.63 ± 16.93 pg/ml, p = 0.098). Among the covariates, the duration of OVX, and thus the duration of treatment for the ET group, had a significant effect: In women who did not receive ET, serum FGF21 concentrations were positively correlated with the number of years post-OVX. In contrast, in women who received ET, serum FGF21 concentrations remained constant over time (Figure 5B). We also observed a relationship between serum FGF21 concentrations and BMI in the two groups (Figure 5C): In women who did not receive ET, BMI was inversely correlated with serum FGF21 concentrations. Conversely, in women who received ET, BMI was positively correlated

 © 2019 Published by Elsevier GmbH. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

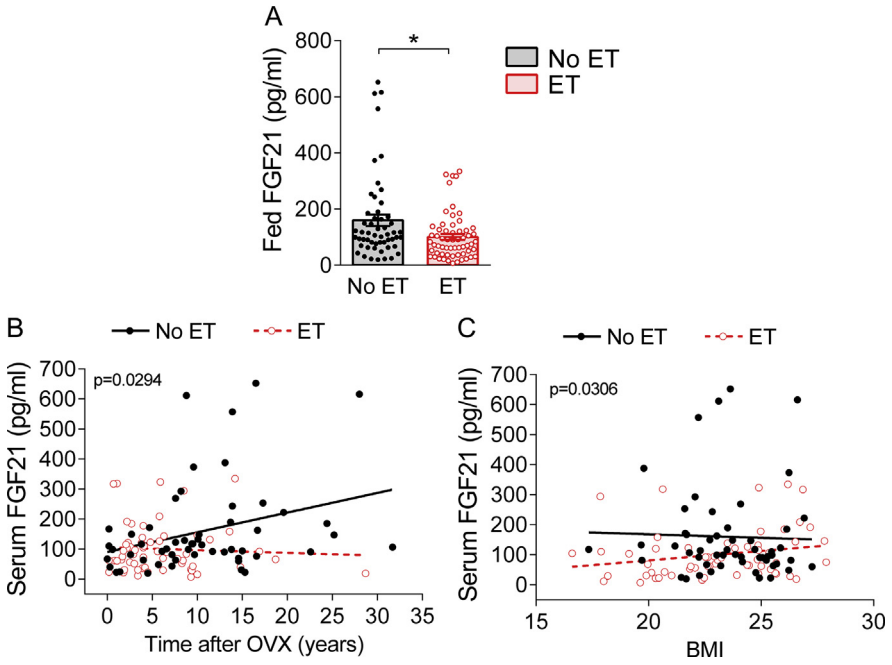


Figure 5: Estrogen treatment in relation to serum FGF21 concentrations in ovariectomized women. (**A**) Non-adjusted serum FGF21 concentration in women treated or not with estrogen therapy (ET) with E2. (**B**) Serum FGF21 concentrations in women treated or not with ET with adjustment for body mass index, type of menopause (surgical or natural), age at the time of menopause, duration of treatment, and physical activity. (**C**) Analysis of covariance between serum FGF21 and treatment duration post-ovariectomy. (**D**) Analysis of covariance between the serum FGF21 and body mass index (BMI). Data correspond to the mean values ± SEM (n = 54 for no ET, n = 60 for ET). **p < 0.01.

with serum FGF21 concentrations. Analyses performed on the log of FGF21 produced similar results, with significantly lower log FGF21 levels for treated women and similar trends in serum FGF21 levels across BMI and treatment duration by treatment groups.

4. DISCUSSION

In mice, hormonal cues stimulating hepatic *Fgf21* expression include growth hormone [27], thyroid hormones [28], and glucocorticoids [29]. We show that treatment with E2 (the main female hormone) at doses leading to high physiological concentrations increases hepatic FGF21 production in the fasting and fed states in intact or ovariectomized female mice on a normal chow. Treatment with E2 increases energy expenditure in ovariectomized female mice at least partially via FGF21-dependent mechanisms. The stimulatory effect of exogenous E2 on energy expenditure is not observed in ovariectomized FGF21-deficient female mice. However, the ability of exogenous E2 to reduce body weight and fat mass in ovariectomized wild-type mice is only minimally impaired in FGF21-deficient mice. Thus, FGF21 is not essential to the effect of exogenous E2 in controlling fat mass. Indeed, it is well documented that E2 controls body weight via non-FGF21 means in female mice. E2 action on ERα in hypothalamic POMC neurons suppresses food intake, while E2 action on ERα in hypothalamic SF-1 neurons increases energy expenditure [30]. It is likely that E2-induced FGF21 increases energy expenditure via central mechanisms, as central FGF21 action is needed for sympathetic nerve activity and energy expenditure in mice [31,32]. One should keep in mind that we used exogenous E2 at high therapeutic doses, and that these results cannot be extrapolated to the physiological effect of endogenous, cyclical E2. Unlike E2 effects on energy expenditure, the ability of exogenous E2 to stimulate lipid and carbohydrate oxidation is independent from FGF21, since we observed no difference in RER between wild-type and FGF21-deficient female mice.

FGF21 is a circulating hormone produced by hepatocytes (hepatokine). In mice, FGF21 serum concentrations are dramatically increased by conditions such as fasting or ketogenic diet that increase circulating free fatty acids (FFA) and thus activate hepatic peroxisome proliferator-activated receptor (PPAR)α-dependent *Fgf21* gene transcription [21,33]. In addition, in mice and humans, hepatic FGF21 expression is under complex nutritional regulation including protein restriction via the amino acid sensor GCN2 [34], fructose via the carbohydrate response element binding protein [35], bile acids via farnesoid X receptor [36], all-trans-retinoic acid via retinoic acid receptor β [37], α-lipoic acid via cAMP response element binding protein H [38], and resveratrol via SIRT1 [39]. During pregnancy, E2 concentrations are high [40], and late in pregnancy, hepatic FGF21 production is increased in mice, followed by a drop in the final days that matches E2 levels [41]. We show that in female mice, exogenous E2 increases FGF21 production in the fasting and fed states (thus independently from PPARα) via activation of ERα in hepatocytes, not ERβ or GPER. Using a combination of genetic mouse models altering the subcellular location of ERα or its binding to perfect consensus EREs on the DNA, we show that ligand-activated ERα increases the transcription of the *Fgf21* gene *in vivo* via a classical nuclear functional ERE. Very likely, nuclear ERα binds an ERE in an upstream enhancer of *Fgf21*, since no ERE is present in the promoter of *Fgf21* in either the mouse or human genome, but perfect or 1nt variant EREs are located upstream to the transcription start site of *Fgf21* in the mouse and human genomes. Note that we cannot exclude a partial role of membrane pools of ERα in increasing FGF21 concentrations. Indeed, E2 doesn't stimulate fgf21 mRNA and FGF21 protein expression in mice expressing ERα selectively in the nucleus (NOER) to the same extent as in control mice.

 © 2019 Published by Elsevier GmbH. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

In order to determine whether these findings can be translated to menopausal women treated with estrogens, we studied the French E3N prospective cohort study initiated in 1990 to investigate the risk factors associated with cancer and other major non-communicable diseases in women. E3N is one of the largest observational studies of postmenopausal women [20]. We selected ovariectomized postmenopausal women who either had or had not received E2 therapy and measured serum FGF21 concentrations. In contrast to results obtained in female mice, E2 therapy was associated with lower serum FGF21 concentrations in ovariectomized postmenopausal women compared to no treatment. Several limitations can account for this difference between women and mice: *First*, FGF21 regulation is different in mice and humans. For example, FGF21 is induced by FFAs in mice, but conversely, elevation of plasma FFAs in humans decreases circulating FGF21 concentrations [42,43]. Similarly, a ketogenic diet induces FGF21 in liver and increases its circulating levels in mice [21]. In contrast, a ketogenic diet does not increase FGF21 serum concentrations in healthy, obese, or diabetic humans [44,45] and even decreases FGF21 concentrations in obese patients [46]. *Second*, in humans, FGF21 concentrations are positively correlated with BMI and total fat mass (Reviewed in [47]). In fact, FGF21 concentrations are even higher in metabolically unhealthy obese individuals compared to body-fat-matched healthy obese subjects [48]. Further, FGF21 serum concentrations are elevated in subjects with nonalcoholic fatty liver disease independently of BMI [47]. Therefore, the observed decreased FGF21 concentrations in ovariectomized postmenopausal women who received E2 therapy may reflect a state of metabolic improvement (decreased FGF21 resistance). Consistent with this possibility, we observe that FGF21 concentrations decrease with time in E2-treated ovariectomized postmenopausal women compared to non-treated women. *Finally*, the E3N cohort study is an observational study, not a randomized controlled trial and other differences may exist between the E2-treated and non-treated groups. Indeed, E2-treated women were older at menopause/ovariectomy and younger when blood was drawn to measure FGF21 than non-treated controls. Further analysis of the effect of estrogens on serum FGF21 concentrations in menopausal women in a large randomized, placebo-controlled trial cohort, like the Women's Health Initiative (WHI) Hormone Trial, is warranted.

5. CONCLUSIONS

In female mice, treatment with exogenous E2 acting on the hepatocyte ERα increases *Fgf21* transcription and liver FGF21 production, thus raising serum FGF21 concentrations. In these mice, exogenous E2 stimulates energy expenditure at least partially via FGF21. These results are not reproduced in an observational cohort of menopausal women who received E2 therapy.

FUNDING

This work was supported by grants from the National Institutes of Health (NIH, R01 DK074970, and DK107444), a Department of Veterans Affairs Merit Review Award (#BX003725), and the Price-Goldsmith Endowed Chair at Tulane University School of Medicine to FMJ. C.A. was supported by American Diabetes Association Post-Doctoral Fellowship (1-16-PDF-004). CDM was supported by NIH R01DK105032 and CMH was supported by F32 DK11513. JL was supported in part by 1 U54 GM104940 from the National Institute of General Medical Sciences of the NIH, which funds the Louisiana Clinical and Translational Science Center. This project/work used facilities within the Pennington Biomedical Animal Metabolism & Behavior Core that are supported in part by NIH COBRE (P30GM118430) and NORC (P30DK072476) center grants.

ACKNOWLEDGEMENTS

The authors thank Madeleine Dehner for assisting in mouse metabolic phenotyping experiments.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.molmet.2019.02.002.

REFERENCES

[1] Mauvais-Jarvis, F., Clegg, D.J., Hevener, A.L., 2013. The role of estrogens in control of energy balance and glucose homeostasis. Endocrine Reviews (C), 1–30.
[2] Mauvais-Jarvis, F., Manson, J.E., Stevenson, J.C., Fonseca, V.A., 2017. Menopausal hormone therapy and type 2 diabetes prevention: evidence, mechanisms, and clinical implications. Endocrine Reviews 38(3):173–188.
[3] Markan, K., Naber, M., Ameka, M., 2014. Circulating FGF21 is liver derived and enhances glucose uptake during refeeding and overfeeding. Diabetes 63(April):1–21.
[4] Kharitonenkov, A., Adams, A.C., 2014. Inventing new medicines: the FGF21 story. Molecular Metabolism 3(3):221–229.
[5] Gaich, G., Chien, J.Y., Fu, H., Glass, L.C., Deeg, M.A., Holland, W.L., et al., 2013. The effects of LY2405319, an FGF21 analog, in obese human subjects with type 2 diabetes. Cell Metabolism 18(3):333–340.
[6] Dong, J.Q., Rossulek, M., Somayaji, V.R., Baltrukonis, D., Liang, Y., Hudson, K., et al., 2015. Pharmacokinetics and pharmacodynamics of PF-05231023, a novel long-acting FGF21 mimetic, in a first-in-human study. British Journal of Clinical Pharmacology 80(5):1051–1063.
[7] Sanyal, A., Charles, E.D., Neuschwander-Tetri, B.A., Loomba, R., Harrison, S.A., Abdelmalek, M.F., et al., 2019. Pegbelfermin (BMS-986036), a PEGylated fibroblast growth factor 21 analogue, in patients with non-alcoholic steatohepatitis: a randomised, double-blind, placebo-controlled, phase 2a trial. Lancet (London, England) 392(10165):2705–2717.
[8] Chukijrungroat, N., Khamphaya, T., Weerachayaphorn, J., Songserm, T., Saengsirisuwan, V., 2017. Hepatic FGF21 mediates sex differences in high-fat high-fructose diet-induced fatty liver. American Journal of Physiology Endocrinology and Metabolism 313(2):E203–E212.
[9] Kim, J.H., Meyers, M.S., Khuder, S.S., Abdallah, S.L., Muturi, H.T., Russo, L., et al., 2014. Tissue-selective estrogen complexes with bazedoxifene prevent metabolic dysfunction in female mice. Molecular Metabolism 3(2):177–190.
[10] Zhu, L., Brown, W.C., Cai, Q., Krust, A., Chambon, P., McGuinness, O.P., et al., 2013. Estrogen treatment after ovariectomy protects against fatty liver and may improve pathway-selective insulin resistance. Diabetes 62(2):424–434.
[11] Hewitt, S.C., Kissling, G.E., Fieselman, K.E., Jayes, F.L., Gerrish, K.E., Korach, K.S., 2010. Biological and biochemical consequences of global deletion of exon 3 from the ER gene. The FASEB Journal 24(12):4660–4667.
[12] Pedram, A., Razandi, M., Kim, J.K., O'Mahony, F., Lee, E.Y., Luderer, U., et al., 2009. Developmental phenotype of a membrane only estrogen receptor alpha (MOER) mouse. The Journal of Biological Chemistry 284(6):3488–3495.
[13] Pedram, A., Razandi, M., Lewis, M., Hammes, S., Levin, E.R., 2014. Membrane-localized estrogen receptor α is required for normal organ development and function. Developmental Cell 29(4):482–490.

 © 2019 Published by Elsevier GmbH. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

[14] Hewitt, S.C., Li, L., Grimm, S.A., Winuthayanon, W., Hamilton, K.J., Pockette, B., et al., 2014. Novel DNA motif binding activity observed in vivo with an estrogen receptor α mutant mouse. Molecular Endocrinology (Baltimore, Md.) 28(6):899–911.

[15] Potthoff, M.J., Inagaki, T., Satapati, S., Ding, X., He, T., Goetz, R., et al., 2009. FGF21 induces PGC-1alpha and regulates carbohydrate and fatty acid metabolism during the adaptive starvation response. Proceedings of the National Academy of Sciences of the United States of America 106(26):10853–10858.

[16] Stauffer, S.R., Coletta, C.J., Tedesco, R., Nishiguchi, G., Carlson, K., Sun, J., et al., 2000. Pyrazole ligands: structure–Affinity/activity relationships and estrogen receptor-α-selective agonists. Journal of Medicinal Chemistry 43(26):4934–4947.

[17] Meyers, M.J., Sun, J., Carlson, K.E., Marriner, G.A., Katzenellenbogen, B.S., Katzenellenbogen, J.A., 2001. Estrogen receptor-β potency-selective ligands: structure–Activity relationship studies of diarylpropionitriles and their acetylene and polar analogues. Journal of Medicinal Chemistry 44(24):4230–4251.

[18] Bologa, C.G., Revankar, C.M., Young, S.M., Edwards, B.S., Arterburn, J.B., Kiselyov, A.S., et al., 2006. Virtual and biomolecular screening converge on a selective agonist for GPR30. Nature Chemical Biology 2(4):207–212.

[19] Coons, L.A., Hewitt, S.C., Burkholder, A.B., McDonnell, D.P., Korach, K.S., 2017. DNA sequence constraints define functionally active steroid nuclear receptor binding sites in chromatin. Endocrinology 158(10):3212–3234.

[20] de Lauzon-Guillain, B., Fournier, A., Fabre, A., Simon, N., Mesrine, S., Boutron-Ruault, M.-C., et al., 2009. Menopausal hormone therapy and new-onset diabetes in the French Etude Epidemiologique de Femmes de la Mutuelle Générale de l'Education Nationale (E3N) cohort. Diabetologia 52(10):2092–2100.

[21] Badman, M.K., Pissios, P., Kennedy, A.R., Koukos, G., Flier, J.S., Maratos-Flier, E., 2007. Hepatic fibroblast growth factor 21 is regulated by PPARα and is a key mediator of hepatic lipid metabolism in ketotic states. Cell Metabolism 5:426–437.

[22] Izumiya, Y., Bina, H.A., Ouchi, N., Akasaki, Y., Kharitonenkov, A., Walsh, K., 2008. FGF21 is an Akt-regulated myokine. FEBS Letters 582(27):3805–3810.

[23] Fisher, F.M., Kleiner, S., Douris, N., Fox, E.C., Mepani, R.J., Verdeguer, F., et al., 2012. FGF21 regulates PGC-1α and browning of white adipose tissues in adaptive thermogenesis. Genes & Development 26(3):271–281.

[24] Hondares, E., Iglesias, R., Giralt, A., Gonzalez, F.J., Giralt, M., Mampel, T., et al., 2011. Thermogenic activation induces FGF21 expression and release in brown adipose tissue. Journal of Biological Chemistry 286(15):12983–12990.

[25] Keipert, S., Kutschke, M., Lamp, D., Brachthäuser, L., Neff, F., Meyer, C.W., et al., 2015. Genetic disruption of uncoupling protein 1 in mice renders brown adipose tissue a significant source of FGF21 secretion. Molecular Metabolism 4(7):537–542.

[26] Kim, K.H., Jeong, Y.T., Oh, H., Kim, S.H., Cho, J.M., Kim, Y.-N., et al., 2013. Autophagy deficiency leads to protection from obesity and insulin resistance by inducing Fgf21 as a mitokine. Nature Medicine 19(1):83–92.

[27] Chen, W., Hoo, R.L., Konishi, M., Itoh, N., Lee, P., Ye, H., et al., 2011. Growth hormone induces hepatic production of fibroblast growth factor 21 through a mechanism dependent on lipolysis in adipocytes. Journal of Biological Chemistry 286(40):34559–34566.

[28] Adams, A.C., Astapova, I., Fisher, F.M., Badman, M.K., Kurgansky, K.E., Flier, J.S., et al., 2010. Thyroid hormone regulates hepatic expression of fibroblast growth factor 21 in a PPARalpha-dependent manner. The Journal of Biological Chemistry 285(19):14078–14082.

[29] Patel, R., Bookout, A.L., Magomedova, L., Owen, B.M., Consiglio, G.P., Shimizu, M., et al., 2015. Glucocorticoids regulate the metabolic hormone FGF21 in a feed-forward loop. Molecular Endocrinology (Baltimore, Md.) 29(2):213–223.

[30] Xu, Y., Nedungadi, T.P., Zhu, L., Sobhani, N., Irani, B.G., Davis, K.E., et al., 2011. Distinct hypothalamic neurons mediate estrogenic effects on energy homeostasis and reproduction. Cell Metabolism 14(4):453–465.

[31] Owen, B.M., Ding, X., Morgan, D.A., Coate, K.C., Bookout, A.L., Rahmouni, K., et al., 2014. FGF21 acts centrally to induce sympathetic nerve activity, energy expenditure, and weight loss. Cell Metabolism 20(4):670–677.

[32] Douris, N., Stevanovic, D., Fisher, F.M., Cisu, T.I., Chee, M.J., Ly Nguyen, N., et al., 2015. Central fibroblast growth factor 21 browns white fat via sympathetic action in male mice. Endocrinology(May) en.2014-2001.

[33] Inagaki, T., Dutchak, P., Zhao, G., Ding, X., Gautron, L., Parameswara, V., et al., 2007. Endocrine regulation of the fasting response by PPARα-mediated induction of fibroblast growth factor 21. Cell Metabolism 5(June):415–425.

[34] Laeger, T., Albarado, D.C., Burke, S.J., Trosclair, L., Hedgepeth, J.W., Berthoud, H.-R., et al., 2016. Metabolic responses to dietary protein restriction require an increase in FGF21 that is delayed by the absence of GCN2. Cell Reports 16(3):707–716.

[35] Fisher, F.M., Kim, M., Doridot, L., Cunniff, J.C., Parker, T.S., Levine, D.M., et al., 2017. A critical role for ChREBP-mediated FGF21 secretion in hepatic fructose metabolism. Molecular Metabolism 6(1):14–21.

[36] Cyphert, H.A., Ge, X., Kohan, A.B., Salati, L.M., Zhang, Y., Hillgartner, F.B., 2012. Activation of the farnesoid X receptor induces hepatic expression and secretion of fibroblast growth factor 21. The Journal of Biological Chemistry 287(30):25123–25138.

[37] Li, Y., Wong, K., Walsh, K., Gao, B., Zang, M., 2013. Retinoic acid receptor β stimulates hepatic induction of fibroblast growth factor 21 to promote fatty acid oxidation and control whole-body energy homeostasis in mice. The Journal of Biological Chemistry 288(15):10490–10504.

[38] Bae, K.-H., Min, A.-K., Kim, J.-G., Lee, I.-K., Park, K.-G., 2014. Alpha lipoic acid induces hepatic fibroblast growth factor 21 expression via up-regulation of CREBH. Biochemical and Biophysical Research Communications 455(3–4):212–217.

[39] Li, Y., Wong, K., Giles, A., Jiang, J., Lee, J.W., Adams, A.C., et al., 2014. Hepatic SIRT1 attenuates hepatic steatosis and controls energy balance in mice by inducing fibroblast growth factor 21. Gastroenterology 146(2):539–549 e7.

[40] Barkley, M.S., Geschwind, I.I., Bradford, G.E., 1979. The gestational pattern of estradiol, testosterone and progesterone secretion in selected strains of mice. Biology of Reproduction 20(4):733–738.

[41] Cui, Y., Giesy, S.L., Hassan, M., Davis, K., Zhao, S., Boisclair, Y.R., 2014. Hepatic FGF21 production is increased in late pregnancy in the mouse. American Journal of Physiology Regulatory Integrative and Comparative Physiology 307(3):R290–R298.

[42] Schmid, A., Leszczak, S., Ober, I., Karrasch, T., Schäffler, A., 2015. Short-term and divergent regulation of FGF-19 and FGF-21 during oral lipid tolerance test but not oral glucose tolerance test. Experimental and Clinical Endocrinology & Diabetes Official Journal German Society of Endocrinology and German Diabetes Association 123(2):88–94.

[43] Matikainen, N., Taskinen, M.-R., Stennabb, S., Lundbom, N., Hakkarainen, A., Vaaralahti, K., et al., 2012. Decrease in circulating fibroblast growth factor 21 after an oral fat load is related to postprandial triglyceride-rich lipoproteins and liver fat. European Journal of Endocrinology 166(3):487–492.

[44] Christodoulides, C., Dyson, P., Sprecher, D., Tsintzas, K., Karpe, F., 2009. Circulating fibroblast growth factor 21 is induced by peroxisome proliferator-activated receptor agonists but not ketosis in man. The Journal of Clinical Endocrinology and Metabolism 94(9):3594–3601.

[45] Dushay, J., Chui, P.C., Gopalakrishnan, G.S., Varela-Rey, M., Crawley, M., Fisher, F.M., et al., 2010. Increased fibroblast growth factor 21 in obesity and nonalcoholic fatty liver disease. Gastroenterology 139(2):456–463.

[46] Crujeiras, A.B., Gomez-Arbelaez, D., Zulet, M.A., Carreira, M.C., Sajoux, I., de Luis, D., et al., 2017. Plasma FGF21 levels in obese patients undergoing energy-restricted diets or bariatric surgery: a marker of metabolic stress?, 2005 International Journal of Obesity 41(10):1570–1578.

[47] Staiger, H., Keuper, M., Berti, L., Hrabe de Angelis, M., Häring, H.-U., 2017. Fibroblast growth factor 21-metabolic role in mice and men. Endocrine Reviews 38(5):468–488.

[48] Berti, L., Irmler, M., Zdichavsky, M., Meile, T., Böhm, A., Stefan, N., et al., 2015. Fibroblast growth factor 21 is elevated in metabolically unhealthy obesity and affects lipid deposition, adipogenesis, and adipokine secretion of human abdominal subcutaneous adipocytes. Molecular Metabolism 4(7):519–527.

 © 2019 Published by Elsevier GmbH. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).