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

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ABSTRACT

Objective: The endogenous estrogen 17 β -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 ER α increases hepatic expression and production of FGF21 in female mice. *In vivo* activation of ER α 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 ER α increases *Fgf21* transcription and FGF21 production, thus promoting energy expenditure and partially decreasing fat accumulation.

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Keywords Estrogen; ER α ; 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].

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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 17 β -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 ER α promotes hepatic steatosis in female mice [10]. However, the effect of hepatocyte-specific ER α 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 ER α KO [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 ER α lox mice with Albumin-Cre mice (Jackson Laboratories, stock #003574). ER β KO 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 μ g/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 μ g/kg or 160 μ g/kg, dissolved in sesame oil, Steraloids), the ER α agonist propyl-pyrazole-triol (PPT, 8 mg/kg/d) [16], the ER β 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 CCTCAGCAG-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'-GGTCAnnTGACC-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'Éducation 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,

comparisons were made using Bonferroni post-hoc tests. All data are expressed as mean \pm 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 17 β -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 ER α in hepatocytes

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

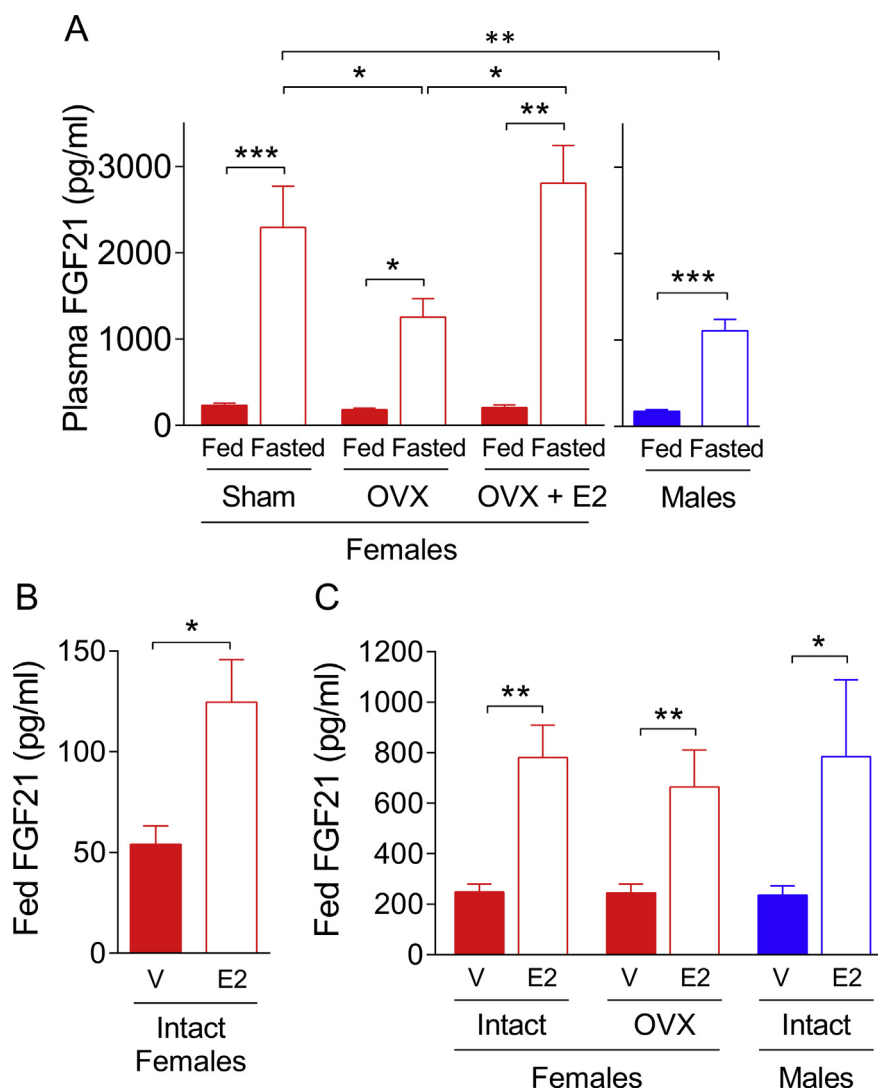


Figure 1: E2 increases serum FGF21 concentrations in female and male mice. (A) Serum FGF21 concentrations in random fed and 40h-fasted sham-operated mice (Sham), ovariectomized mice (OVX), OVX female mice treated with 17 β -estradiol (OVX + E2, 8 μ g/kg/d s.c.) (n = 3–14) and male mice (n = 12–16). (B)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

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 ER α agonist PPT, but not the ER β 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 ER α knockout (ER α KO) mice but was retained in GPER knockout (GPERKO) mice and non-significantly increased in ER β knockout (ER β KO) (Figure 2C). Together, these results indicate that E₂ increases hepatic production of FGF21 via ER α . To explore to what extent E₂ acts directly on ER α in hepatocytes to stimulate FGF21 expression, we generated mice deficient in ER α selectively in hepatocytes (LERKO). Following E₂ treatment, hepatic *Fgf21* mRNA expression and serum FGF21 protein concentrations were increased in ER α lox controls (Figure 2D,E), but this was not observed in LERKO mice, consistent with a role of the hepatocyte ER α 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 extra-hepatic 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 ER α . To assess the contribution of nuclear ER α , we used a knockin mouse with mutant ER α that prevents ER α membrane localization. In this "Nuclear Only ER α ." (NOER)

mouse, ER α is exclusively nuclear, and extranuclear actions of ER α are abolished [13]. In contrast, we also used an ER α deficient mouse re-expressing ER α 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 ER α needs to exhibit a nuclear location to stimulate *Fgf21* mRNA expression and protein secretion. ER α 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 ER α DNA-binding domain mutant mouse (ER α -EAAE), which does not support E₂-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 ER α acting on a NRFE ERE, likely in a distant enhancer, but not directly on the *Fgf21* promoter.

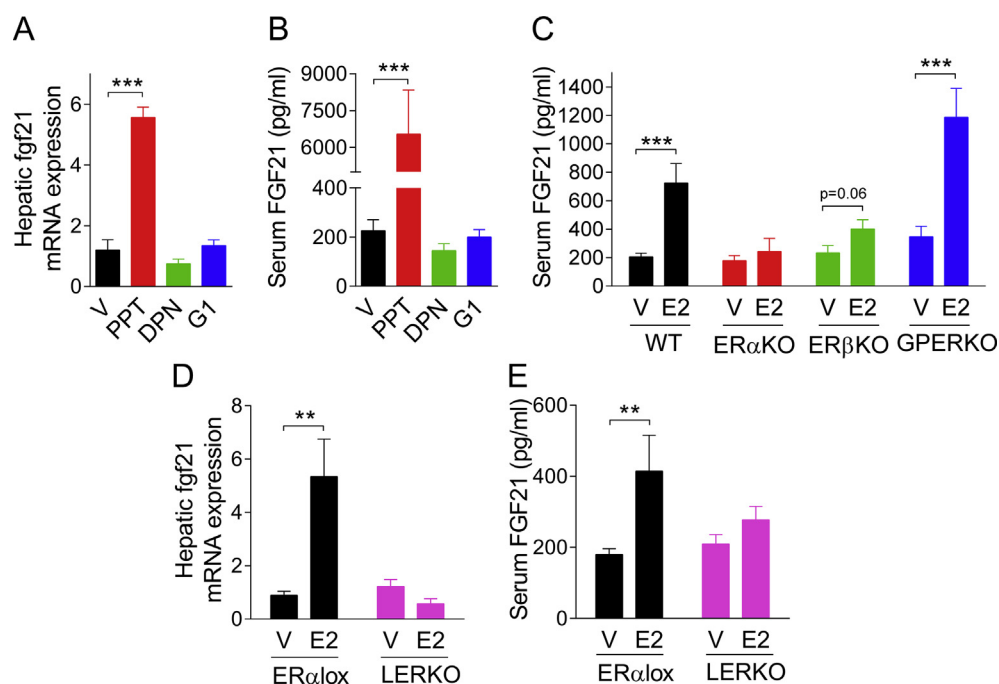


Figure 2: E₂ increases FGF21 production by activating hepatocyte ER α *in vivo*. (A) Liver *Fgf21* mRNA expression and (B) serum FGF21 concentrations in intact female C57Bl6/J mice treated with the indicated compounds (n = 5–6). (C) Serum FGF21 concentrations in the indicated female mice after 3 days 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 μ g/kg/d) (n = 4–9). Data represent the mean \pm SEM. *p < 0.05, **p < 0.01. ***p < 0.001.

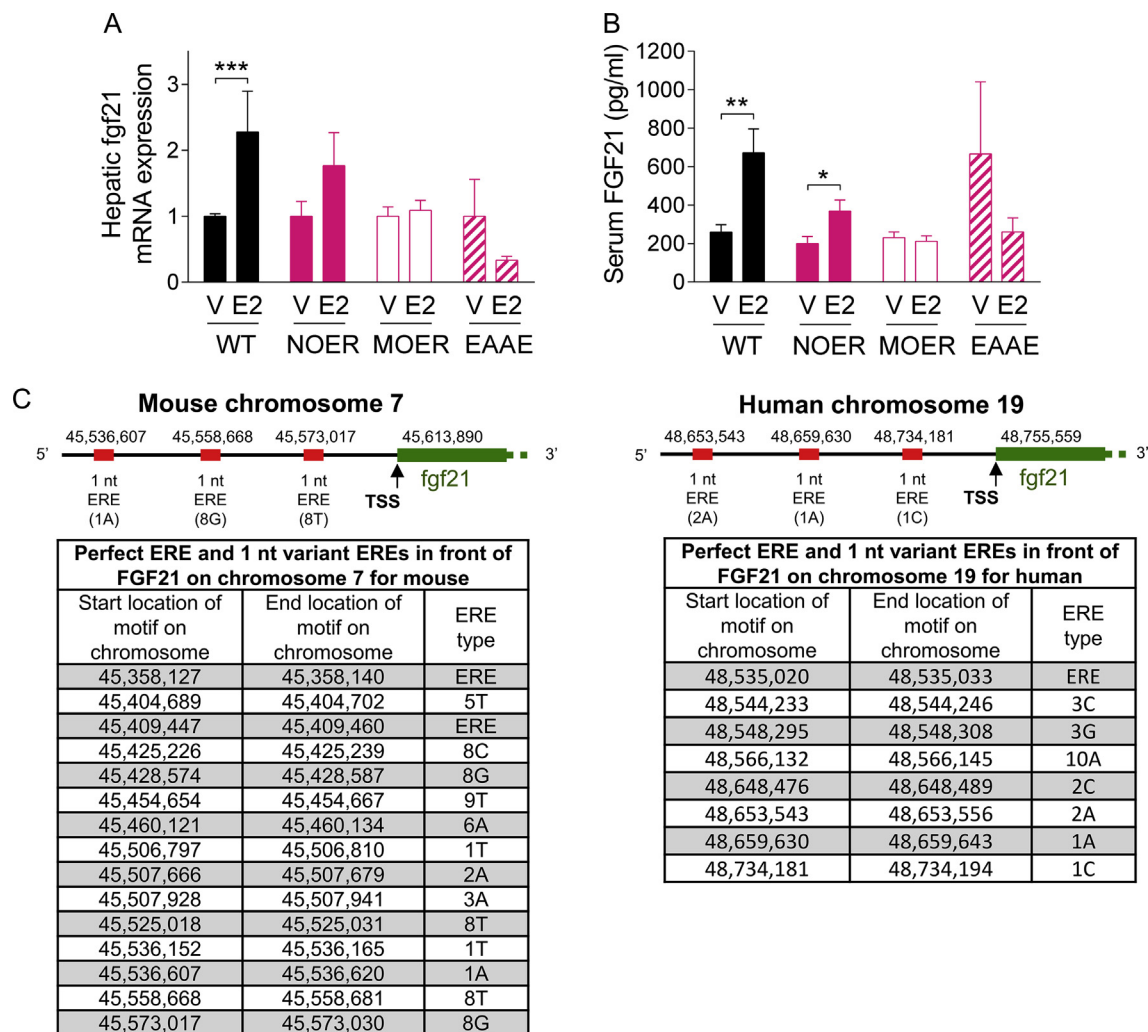


Figure 3: ER α stimulates *Fgf21* gene transcription via an ERE. (A) Hepatic *Fgf21* mRNA expression and (B) serum FGF21 concentrations in intact NOER, MOER and EAAE female mice and control wild-type littermates (WT) after 3 days of treatment with vehicle (V) or 17 β -estradiol (E2, 160 μ g/kg/d) (n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

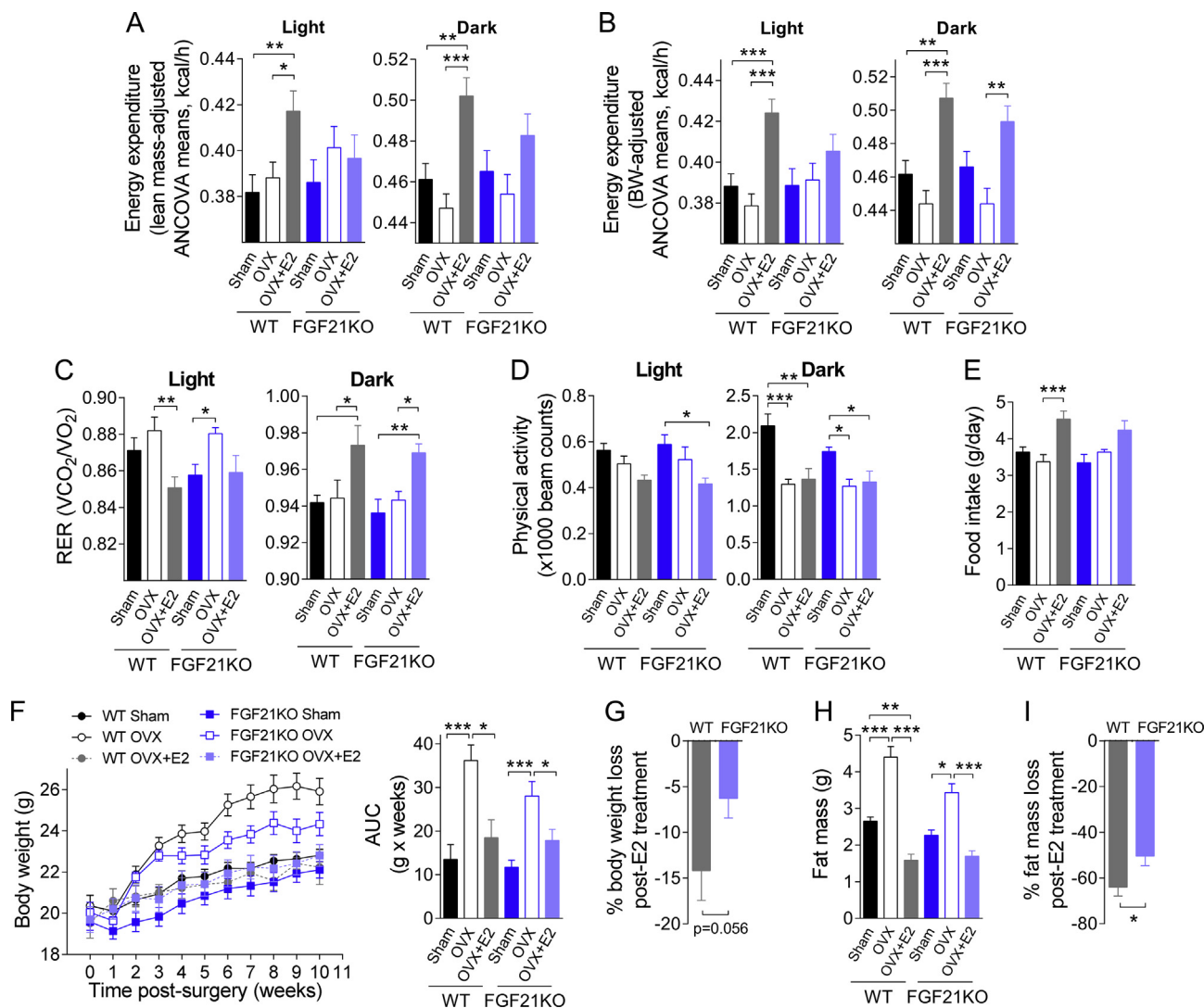


Figure 4: E2 stimulates energy expenditure via FGF21 in OVX-female mice. WT and FGF21KO female mice underwent sham or OVX surgery and after 12 weeks were individually placed in metabolic cages during light (left) and dark (right) phase (n = 5–10). (A) Energy expenditure adjusted to lean mass (ANCOVA 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

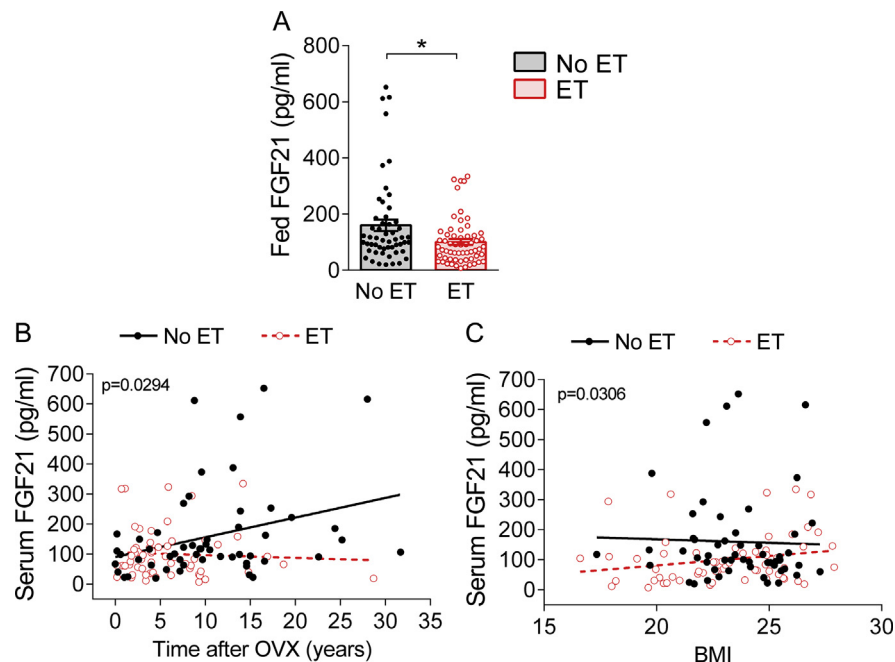


Figure 5: Estrogen treatment in relation to serum FGF21 concentrations in ovariectomized women. (A) Non-adjusted serum FGF21 concentration in women treated or not with estrogen therapy (ET) with E2. (B) Serum FGF21 concentrations in women treated or not with ET with adjustment for body mass index, type 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 \pm 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 ER α in hypothalamic POMC neurons suppresses food intake, while E2 action on ER α 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 PPAR α) via activation of ER α in hepatocytes, not ER β or GPER. Using a combination of genetic mouse models altering the subcellular location of ER α or its binding to perfect consensus EREs on the DNA, we show that ligand-activated ER α increases the transcription of the *Fgf21* gene *in vivo* via a classical nuclear functional ERE. Very likely, nuclear ER α 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 ER α in increasing FGF21 concentrations. Indeed, E2 doesn't stimulate *fgf21* mRNA and FGF21 protein expression in mice expressing ER α selectively in the nucleus (NOER) to the same extent as in control mice.

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 ER α 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.

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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>.

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