Hormone signaling and fatty liver in females: analysis of estrogen receptor α mutant mice

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Abstract

BACKGROUND—Treatment with estrogen in early menopausal women protects against development of hepatic steatosis and NAFLD but estrogen has undesirable side effects which negate its beneficial effects in pre- and post-menopausal women. Targeted therapies require better understanding of the target sites and mechanisms by which estrogen signaling exerts its protective effects in women. ERα is thought to be the primary mediator for estrogen signaling to protect against hepatic steatosis. ERα has several mechanisms for signal transduction: 1) inducing gene transcription by direct binding to specific DNA sequences 2) inducing tethered transcription with other DNA binding factors 3) stimulating nongenomic action through membrane associated ERα. However, it is still unclear which mechanisms mediate ERα dependent protection against hepatic steatosis.

METHODS—To understand the mechanisms of estrogen signaling for protection against hepatic steatosis in females, we analyzed the global ERα knockout mouse (αERKO), ERα DNA binding domain mutant mouse (KIKO), and liver-specific ERα knockout mouse (LERKO) fed high fat diets (HFD). The KIKO mouse disrupts the direct DNA binding transcription activity but retains tethered transcription regulation and nongenomic action. Hepatic steatosis was evaluated by scoring the macrovesicular and microvesicular steatosis as well as serum ALT levels. We analyzed serum testosterone to assess its correlation with hepatic steatosis.

RESULTS—Liver fat accumulation was far greater in HFD-fed αERKO and KIKO females than in HFD-fed WT controls. Conversely, HFD-fed LERKO females did not accumulate excess liver

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fat. HFD-fed αERKO and KIKO females showed higher microvesicular steatosis and ALT levels than WT controls which correlated with increased serum testosterone levels.

CONCLUSIONS—ERα mediated direct transcription in non-hepatic tissues is essential for estrogen mediated protection against hepatic steatosis in HFD-fed females. The balance between non-hepatic estrogen signaling and hepatic or non-hepatic testosterone action may control hepatic steatosis.

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD), defined as the accumulation of excess liver triglyceride (>5% of liver weight), is the most common cause of non-alcohol-related liver disease in adults as well as children [1]. NAFLD is often asymptomatic at presentation but may progress with time to steatohepatitis and cirrhosis requiring liver transplant. NAFLD is associated with the metabolic syndrome (central adiposity, hypertension, insulin resistance, and dyslipidemia), which predisposes to type 2 diabetes mellitus and coronary artery disease. Metabolic syndrome is known to be related to lifestyle factors as well as genetic, hormonal, and environmental inputs [2,3].

Several lines of evidence suggest that estrogen signaling modulates the risk of NAFLD. Women with Turner's syndrome, who lack endogenous estrogen production, are at higher risk for development of NAFLD [4]. Likewise, treatment with tamoxifen, an estrogen receptor antagonist, is associated with development of NAFLD [5]. Women have lower risk than men until menopause, at which point rates of metabolic syndrome and fatty liver disease rise to meet those of age-matched males [6] Treatment with estrogen in early menopause protects against development of metabolic syndrome and NAFLD [7] but it has undesirable side effects which may negate its benefits in some post-menopausal women [8]. Targeted therapies will require better understanding of the mechanisms by which estrogen signaling exerts its protective effects.

Several animal models have been developed to explore mechanisms by which estrogen protects against development of NAFLD. Ovariectomized (OVX) mice lacking estrogen production develop increased liver fat content; these effects are reversible by administration of estradiol (E2) [9]. Similar results have been seen in *Cyp19* (aromatase) knockout (KO) mice unable to synthesize E2 from androgen precursors. Namely, treatment with exogenous E2 protects *Cyp19*KO male mice against the development of metabolic syndrome and NAFLD [10,11]. Interestingly, *Cyp19*KO females were obese but did not develop hepatic steatosis [10], suggesting sexual dimorphism of hepatic steatosis regulation. In humans, mutations in the aromatase gene are associated with central adiposity, elevated lipids, and insulin resistance; furthermore the treatment with E2 resolved these defects in an aromatase mutant male [12]. Similar features were seen in an adult male with a mutation in the ERα gene [13].

Animals lacking functional estrogen receptor alpha (ERα) have been shown to have a similar metabolic phenotype to OVX animals, with increased body weight, visceral adiposity, and insulin resistance [14,15]. In contrast, estrogen receptor beta (ERβ) KO mice show no discernable metabolic or hepatic phenotype [15]. Together, this body of evidence

suggests that estrogen action through ERα is the primary protection mechanism of reproductive-aged females against metabolic syndrome and NAFLD.

ERα-dependent transcription is mediated through direct binding to specific DNA sequences termed the estrogen responsive element (ERE) [16]. In addition, ERα may activate ligand dependent transcription through indirect DNA binding with other DNA binding factors such as AP-1, which is referred to as tethered transcription regulation [17]. Furthermore, a small amount of ERα protein associates with the cellular membrane and transmits E2 mediated signal transduction independent of transcription, called nongenomic action [18]. It is still unclear which mechanisms mediate estrogen dependent protection against development of central adiposity, weight gain, insulin resistance, and NAFLD. An understanding of the pathways by which estrogenic signaling exerts its protective effects is crucial for the future development of targeted therapies. To that end, we analyzed the ERα DNA binding domain (DBD) mutant mouse model (KIKO), in which a mutation disrupts the ERE direct binding activity but retains ERE independent functions, including tethered transcription regulation and nongenomic action [19].

It is also important to understand the tissue specific functionality of ERα for the development of targeted therapies. We have observed striking differences in the degree of liver fat infiltration seen in ERα KO females compared to wild type (WT) females. Currently, it is unclear if these changes in liver fat accumulation are mediated through hepatic ERα signaling or through ERα actions in non-hepatic tissues. Although several groups have described metabolic dysfunction in ERα KO mice, the role of ERα in liver remains controversial [20,21]. To further our understanding of hepatic ERα action, we generated and analyzed a liver-specific ERα KO mouse (LERKO).

In this report, we propose that protection against liver fat infiltration in high fat diet (HFD) fed females is regulated by ERα mediated direct transcription in non-hepatic tissues. Furthermore, we postulate that hepatic steatosis is controlled by the balance between non-hepatic estrogen signaling and hepatic or non-hepatic androgen actions.

MATERIALS AND METHODS

Animals

All experiments involving animals were carried out according to US Public Health Service guidelines. Studies were approved by the National Institute of Environmental Health Sciences Institutional Animal Care and Use Committee. The generation of mice lacking exon 3 of *Esr1* (Ex3αERKO) has been reported [22]. The generation of KIKO mice has been described previously [19]. To generate the liver specific *Esr1* KO mouse (LERKO), *Esr1*f/f mice were crossed with Tg(Alb-cre) mice. The F1 offspring were genotyped and *Esr1*f/f-Tg(Alb-cre) mice were used. Animals were maintained on a regular diet (NIH-31, Harlan Laboratory). On the high fat diet (HFD) feeding protocol, animals were maintained with a diet containing 60% kcal% fat (#12492, Research Diets) beginning at age 9–11 weeks for 10 weeks. Animals were allowed ad libitum access to food during that period. The body weight was determined every week. Glucose tolerance tests and insulin tolerance tests were assessed near the end of the HFD feeding period. For the 5α-dihydrotestosterone (DHT)

treatment experiment, 6-week-old C57BL/6 females were ovariectomized (OVX) 2 weeks prior to feeding HFD. OVX mice and intact (non-OVX) females were maintained on the HFD for 4 weeks. One week after beginning the HFD feeding, the mice were treated with DHT (0.5mg/21days release, Innovative Research of America) or placebo pellet for 3 weeks. Animals were randomly selected for each experimental group. The number of animals for experimental studies was estimated by statistical calculations based on a power requirement with 80% confidence interval width for histomorphology. HFD feeding experiment was performed in singlicate in eight mice for each genotype.

Sample collection

The animals were fasted for 4hr before euthanasia with CO_2. Liver was weighed and a piece of the left lateral lobe was fixed in 10% neutral buffered formalin for histological assessment. For RNA extraction and liver triglyceride measurement, the remaining left lateral lobe was frozen in liquid N_2 and stored in $-70°C$. Blood was collected from the descending aorta for measuring serum lipid, hormone and ALT levels.

Assessment of liver fat content

Formalin-fixed, paraffin-embedded liver sections were stained with hematoxylin and eosin. Samples were rated with the assistance of a pathologist blinded to genotype for degree of macrovesicular steatosis, in which the cytoplasm is filled by a large fat droplet that displaces the nucleus to the edge of the cell and microvesicular steatosis, in which small fat vacuoles fill in the cytoplasm without displacing the nucleus [23]. The rating is scaled from 0, no appreciable lipid vesicles to 4, severe steatosis. Liver triglycerides for DHT experiment were measured as follows: crushed frozen liver (100-120 mg) was homogenized in 1ml of 5% NP-40 solution. The homogenates were incubated for 5 min at 80-100˚C then cooled to room temperature. The soluble fraction after centrifugation was used for the assay. Triglycerides were measured by the Triglyceride Quantification Assay kit (Abcam). The values were normalized to sample weight.

Glucose tolerance tests and insulin tolerance tests

Mice were fasted overnight (16hr) and injected intraperitoneally (IP) with 2g/kg glucose for glucose tolerance tests. Fasted mice received IP injections of 0.75 units/kg Humulin R (Eli Lilly) for insulin tolerance tests. Tail vein blood was taken to measure the blood glucose levels prior to injection and 20, 40, 60, 120, and 180 minutes after injection using a Nova Max glucometer (Nova Biomedical).

Serum assays

UniCel DxC600 Synchron Clinical Systems (Beckman Coulter) was used for measurement of ALT, cholesterol and triglyceride levels. The level of non-esterified fatty acids was measured by the colorimetric determination reagent (Wako USA). The level of adiponectin, insulin and leptin were measured by SECTOR Imager 2400 (MSD) using the Meso Scale Discovery kits (Mouse Adiponectin Kit, Mouse Metabolic Kit for insulin and leptin, MSD). Total free testosterone levels were analyzed by enzyme immunoassay assay using a kit (CLIA Kit for Testosterone; UCSN).

Tissue protein extractions and Western blot analyses

Total protein homogenates from liver were prepared and 20 μg of protein was used for Western blot analysis. Proteins were resolved by SDS-PAGE and subsequently transferred to nitrocellulose membranes. Blots were incubated overnight in 4 °C with primary antibody for ERα (1:650; MC-20; Santa Cruz Biotechnology) or β-tubulin (1:1000; H235; Santa Cruz Biotechnology). The blots were washed then incubated with HRP-conjugated anti-rabbit IgG (1:5000). Signal was developed using ECL Plus reagent (GE Healthcare).

Microarray

Total RNA was extracted from liver tissue using TRIzol (Invitrogen). Microarray analysis was performed by NIEHS molecular genomics core laboratory using the Affymetrix Mouse Genome 430 2.0 GeneChip arrays (Affymetrix). Arrays were scanned in an Affymetrix Scanner 3000 and data was obtained using the GeneChip Expression Console Software (Ver. 1.2) using the MAS5 algorithm to generate CHP files. The resulting data were processed using OmicSoft Array Studio (Ver. 6.0) software. In order to identify differentially expressed probes, ANOVA was used to determine if there was a statistical difference between the means of groups. The analysis was performed with setting parameters of at least 2-fold difference and significance at $p < 0.01$. Pathway analysis was performed using the Ingenuity software (Qiagen).

Statistical analysis

All data are represented as mean ± s.e.m. Statistical analyses were performed by GraphPad Prism 6 (GraphPad Software, Inc.). Analytical methods used for each experiment were specified in figure legends. Significance level set at $p < 0.05$ for every analysis.

RESULTS

ERα null females develop hepatic steatosis after HFD feeding while WT female mice are resistant

High fat diet (HFD)-fed ERα null ($Esr1^{-/-}$; αERKO) females had significantly higher body weights than wild type ($Esr1^{+/+}$; WT) females (Figure 1a, circle symbols). In contrast, the body weights of males did not differ between genotypes (Figure 1a, triangle symbols). After 4 weeks of HFD feeding, the body weights of αERKO females were comparable to those of males. While WT females did not develop liver steatosis after prolonged HFD feeding, αERKO females showed significant liver fat infiltration, comparable in magnitude to WT and αERKO males (Figure 1b). Blinded histologic ratings of macrovesicular steatosis (Figure 1c) and microvesicular steatosis (Figure 1d) were performed to evaluate the severity of liver steatosis. The αERKO females have higher steatosis ratings than WT female controls, while no difference is seen between genotypes in males. Total liver weights of αERKO females were significantly higher than those of WT controls (Figure 1e) and the proportion of liver weight to body weight (relative liver weight) was also higher (Figure 1f). Serum alanine aminotransferase (ALT) level, a marker of hepatic dysfunction, trended higher in αERKO females than WT controls. ALT level in WT males was higher than in WT

females (Figure 1g). ALT was elevated in αERKO females fed a HFD but not a regular diet (RD) (Figure 1h).

ERα DNA binding domain mutant females develop hepatic steatosis after HFD feeding

To determine if protection against hepatic steatosis in WT females requires ERα-mediated transcription through direct ERE binding, we analyzed ERα DNA binding domain mutant (*Esr1*[AA/−]; KIKO) females and heterozygous littermate females (*Esr1*^{+/−}; control). No statistical differences in body weight were noted between KIKO females and controls until 4 weeks after beginning HFD feeding (Figure 2a). The rate of increase in body weight of KIKO females was not as dramatic as the rate of increase in αERKO females, which was noted within 2 weeks of the dietary change (Figure 1a). The accumulation of liver fat in KIKO females was significant compared to controls (Figure 2b). In blinded histologic ratings, KIKO females had significantly higher ratings of macrovesicular steatosis (Figure 2c) and microvesicular steatosis (Figure 2d) compared to controls. Total liver weights (Figure 2e) and relative liver weights (Figure 2f) were significantly higher in KIKO females than controls. Serum ALT levels in KIKO females, like αERKO females, were increased compared to controls (Figure 2g). These results suggest that transcriptional activity through direct ERE binding of ERα mediates the estrogen dependent protection against hepatic steatosis.

The levels of serum lipids were not changed in ERα mutant females

Consistent with the hepatic steatosis phenotype, liver triglyceride (TG) content was significantly higher in HFD-fed αERKO and KIKO females compared to WT/control females (supplemental material; Figure S1a and Figure S1b). In contrast, serum TG (Figure S1c) and cholesterol (Figure S1d) levels were not different among these three genotypes. Serum non-esterified fatty acids were lower in ERα mutant females compared to WT controls (Figure S1e).

αERKO females demonstrate impaired glucose tolerance

Since clinically hepatic steatosis is often accompanied by hepatic insulin resistance [24], we performed glucose tolerance tests and insulin tolerance testing in HFD-fed αERKO and KIKO females to evaluate the effect of ERα signaling on whole body glucose metabolism. Fasting serum glucose levels of αERKO and KIKO females were significantly higher than control females (Figure 3e). When subjected to an IP glucose challenge, αERKO females had higher glucose values than WT control by 120 min post-injection (Figure 3a). KIKO females showed higher glucose levels than control females at 20 min but similar levels by 60 min (Figure 3b). Following an IP injection of insulin the magnitude of glucose level decline in αERKO females was significantly less than in control females, suggesting insulin resistance (Figure 3c). The trend was similar but less striking in KIKO females than in αERKO females (Figure 3d). Fasting insulin level, which generally reflects hepatic insulin sensitivity, was significantly higher in αERKO females but not KIKO females (Figure 3f). These results suggest that ERE independent ERα action is involved in insulin mediated glucose homeostasis.

Evaluation of hepatic ERα involvement in protection against hepatic steatosis using microarray

Since the observations of KIKO females suggested that the ERE-dependent transcription regulation of ERα is essential for protection against hepatic steatosis during HFD feeding, we performed gene profiling by microarray to analyze ERα-regulated hepatic genes in HFD-fed females. We focused on the gene set that was differentially expressed in RD- and HFD-fed WT liver but not differentially expressed in RD- and HFD-fed αERKO liver (supplemental material; Figure S2a). Theoretically, this gene set includes ERα associated HFD regulated genes in the liver. We performed INGENUITY pathway analysis (IPA) for this gene cluster (849 genes) to discover the potential upstream regulators. IPA predicted that β-catenin (CTNNB1) was the top rated upstream regulator (p-value; 3.94E-07). Interestingly, 5α-dihydrotestrosterone (DHT), the active endogenous ligand for the androgen receptor, appeared as the second rated regulator (p-value; 2.24E-06). Surprisingly, IPA prediction for a role of ERα (ESR1) and estrogen were rated very low (p-values; 1.04E-03 and 4.82E-04 respectively) (Figure S2b).

Liver-specific ERα knockout females do not develop hepatic steatosis after HFD feeding

To determine whether hepatic ERα transactivation is essential for estrogen-dependent protection against steatosis, we generated liver-specific ERα KO mice ($Esr1^{f/f}$-Tg(Alb-cre); LERKO). ERα protein was detected in the uterus of control ($Esr1^{f/f}$) and LERKO females but no appreciable signal was visible in LERKO liver tissue (Figure 4a). During the HFD feeding, LERKO females gained weight at rates comparable to controls (Figure 4b). After HFD feeding, very little steatosis was present in either LERKO or control females (Figure 4c). In blinded histologic ratings, there were no differences between the two genotypes in macrovesicular steatosis (Figure 4d) and microvesicular steatosis (Figure 4e). This is in marked contrast to the steatosis results detected in αERKO (Figure 1) and KIKO (Figure 2) females. The total liver weight and relative liver weight of LERKO females were similar to controls (Figure 4f and Figure 4g). Moreover, ALT levels did not differ between the two genotypes (Figure 4h). In addition, the levels of triglycerides, total cholesterol and non-esterified fatty acids did not differ between genotypes (Figure 4i). HFD-fed LERKO and control females showed indistinguishable profiles of glucose tolerance (Figure 4j) and insulin sensitivity assessed by IP insulin tolerance testing (Figure 4k). In addition, fasting plasma insulin levels did not differ between these genotypes (Figure 4l). These results suggest that hepatic ERα signaling plays a limited role in hepatic fat accumulation or systemic glucose metabolism.

Adipokine levels in ERα mutant females

Since leptin and adiponectin, the adipocyte secreted metabolic effectors, have been implicated in NAFLD [25], we analyzed serum leptin and adiponectin levels in HFD-fed WT and ERα mutant females and RD-fed WT and αERKO females. Although serum leptin levels were elevated in HFD-fed females compared to RD-fed females regardless of genotype, the leptin levels of HFD-fed αERKO and KIKO females were higher than HFD-fed LERKO and WT controls (Figure 5a). The leptin level correlated positively with adiposity. The serum adiponectin level was suppressed in αERKO and KIKO females

compared to WT controls. The lower adiponectin level in αERKO females was observed regardless of diet (Figure 5b). The adiponectin level correlated inversely with adiposity. It has been reported that testosterone (T) is a possible regulator of adiponectin levels [26], so we analyzed serum T levels in the mice. The profile of serum T level correlated inversely with serum adiponectin level (Figure 5c).

The balance between E2 and T correlates with HFD induced hepatic steatosis

Because we found a correlation between serum T and adiposity in the ERα mutants, we analyzed the effect of T on the early phase of hepatic steatosis in females. HFD-fed OVX and intact WT females were implanted with a pellet containing DHT or placebo for 3 weeks. The body weight of HFD-fed OVX mice was significantly increased compared to intact females. Treatment with DHT enhanced the body weight gain of OVX females but not intact females (Figure 6a). Notably, macrovesicular steatosis was not observed after 4 weeks HFD feeding; however, microvesicular steatosis was observed in all DHT treated OVX females which was different from placebo treated OVX mice or intact females (Figure 6b and 6c). Consistent with the score of microvesicular steatosis, the liver TG content was higher in the HFD-fed OVX mice compared to intact females. Furthermore, the hepatic TG level in OVX mice was significantly increased by DHT treatment but not in the intact females (Figure 6d). The serum ALT level was significantly increased in OVX mice compared to intact females. The ALT level in the DHT treated OVX mice was slightly higher than placebo treated OVX mice but not statistically significant (Figure 6e).

DISCUSSION

Previous studies by Park et al. reported that KIKO females fed RD and HFD did not become obese compared to αERKO females [27], suggesting that ERE independent E2-ERα signaling can prevent obesity. The report suggested that differential locomotor activities mediated by hypothalamic ERα function between αERKO and KIKO may explain the differential regulation of obesity [27]. In contrast, our studies of KIKO mice suggest that ERE independent E2-ERα signaling does not prevent HFD mediated obesity and hepatic steatosis, though the initial body weight gain of KIKO mice is slower than that of αERKO mice (Figure 1a and Figure 2a). It should be noted that the composition of diet differed between our study and Park et al. [27]. We used a 60% kcal% fat diet (D12492; Research Diets) while the other group used a 45% kcal% fat diet (D12451; Research Diets). These two high fat diets (D12492 and D12451) contain different amount of fat (34.9% and 24%) and carbohydrate (26.3%, and 41%). Notably, D12492 diet, which we used, contains 1.5 times more cholesterol than D12451. Our results suggest that an excess of dietary lipid may diminish the preventive effect of ERE independent E2-ERα signaling for obesity.

In contrast to αERKO mice, previous reports found normal insulin sensitivity in KIKO females [27,28]. We observed that the insulin response in HFD-fed KIKO female was not statistically different from the control mice and fasted KIKO females had lower insulin than αERKO, even though the fasting blood glucose level in KIKO females was higher than control mice (Figure 3e). Overall, our findings and the previous reported results suggested that KIKO females are more sensitive to insulin than αERKO. This result suggests that ERE

independent E2-ERα signaling is involved in the maintenance of insulin mediated glucose homeostasis and that it may be independent of its protection against obesity.

We previously reported that the mutated DBD of KIKO can bind to progesterone receptor (PR), glucocorticoid receptor and androgen receptor (AR) binding DNA sequences, and the KIKO mutant ERα binds to PR binding elements in the uterus and compromises progesterone signaling [29]. At the present time, we have no evidence that the KIKO mutant ERα bound to AR binding sequences in the liver or other tissues that would compromise androgen signaling. However, it would be interesting to reevaluate the ERE independent ERα action in future studies using another ERα DBD mutant mouse model, such as *Esr1*EAAE/EAAE mouse (B6;129P2-*Esr1*tm2.1Gsc) [29,30].

Several groups reported the functionality of hepatic ERα using the LERKO mouse [20,21]. Zhu et al. reported that hepatic ERα signaling is required for HFD mediated insulin resistance and prevention of liver fat accumulation following OVX [20]. On the other hand, Matic et al. used intact mice to conclude that hepatic ERα action is not the responsible factor for the insulin resistance observed in αERKO females [21]. Consistent with the observations of Matic et al., the phenotypes of our LERKO mouse were indistinguishable from control females (Figure 4). The discrepancy between our results and Zhu et al., who used OVX mice, would suggest that hepatic ERα may have some functionality, but that non-hepatic ERα mediated signaling plays the primary role in preventing hepatic steatosis under the control of gonadal hormones.

We demonstrated that HFD-fed αERKO females showed severe hepatic steatosis compared to WT females. In contrast, severe liver steatosis was observed in both genotypes of HFD-fed males compared with females (Figure 1b). It has been postulated that an insufficiency of estrogen rather than an excess of testosterone is likely to induce hepatic steatosis because E2 treatment normalized body fat content in the *Cyp19*KO male mice [10]. Additionally, fat accumulation in OVX rodents is reversible with reintroduction of E2 [9]. We have shown that αERKO females have high serum T similar to males. However, it was unclear whether the higher T level in αERKO females affects hepatic steatosis. As we demonstrated here, DHT treatment enhanced liver fat accumulation in OVX mice but not in intact females (Figure 6). These observations suggest that T can stimulate hepatic steatosis in females but E2-mediated protective effect overcomes that event. Interestingly, IPA analysis suggested that DHT is a high rated upstream regulator in differentially regulated genes in HFD- and RD-fed WT female liver, while ESR1 and estrogen were lower rated factors. Furthermore, DHT induced liver fat infiltration but not serum ALT in the early phase of hepatic steatosis. Together with our findings in the LERKO mice, these results suggest that T-mediated hepatic signaling enhanced HFD derived fat accumulation in the liver, leading to hepatocellular inflammation whereas E2 protected hepatic steatosis through the non-hepatic tissue derived factor(s).

Beta-catenin was ranked as the highest upstream factor in our IPA analysis. Recently, Behari et al. reported that liver specific β-catenin overexpressing transgenic [Tg(Alb-β-cateninS45D)] male mice showed hepatic steatosis and increased hepatic triglyceride levels when fed HFD but not RD. In contrast, the liver specific β-catenin KO male mice showed

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lower hepatic triglyceride levels even in the HFD-fed state [31]. These results support our IPA analysis result. It would informative to evaluate how E2-ERα signaling might regulate Wnt/β-catenin signal in the HFD-fed mouse liver for protecting hepatic steatosis in future studies.

We demonstrated the importance of ERE dependent ERα transcription regulation in protecting against hepatic fat accumulation in HFD-fed female mice. However, hepatic ERα signaling does not play a critical role in this protection mechanism. Rather, our results suggest that hepatic steatosis is controlled by a balance between non-hepatic estrogen signaling and hepatic or non-hepatic androgen actions. The balance between E2 and T levels in perimenopausal and menopausal women may be an important parameter to control the risk of metabolic syndrome and NAFLD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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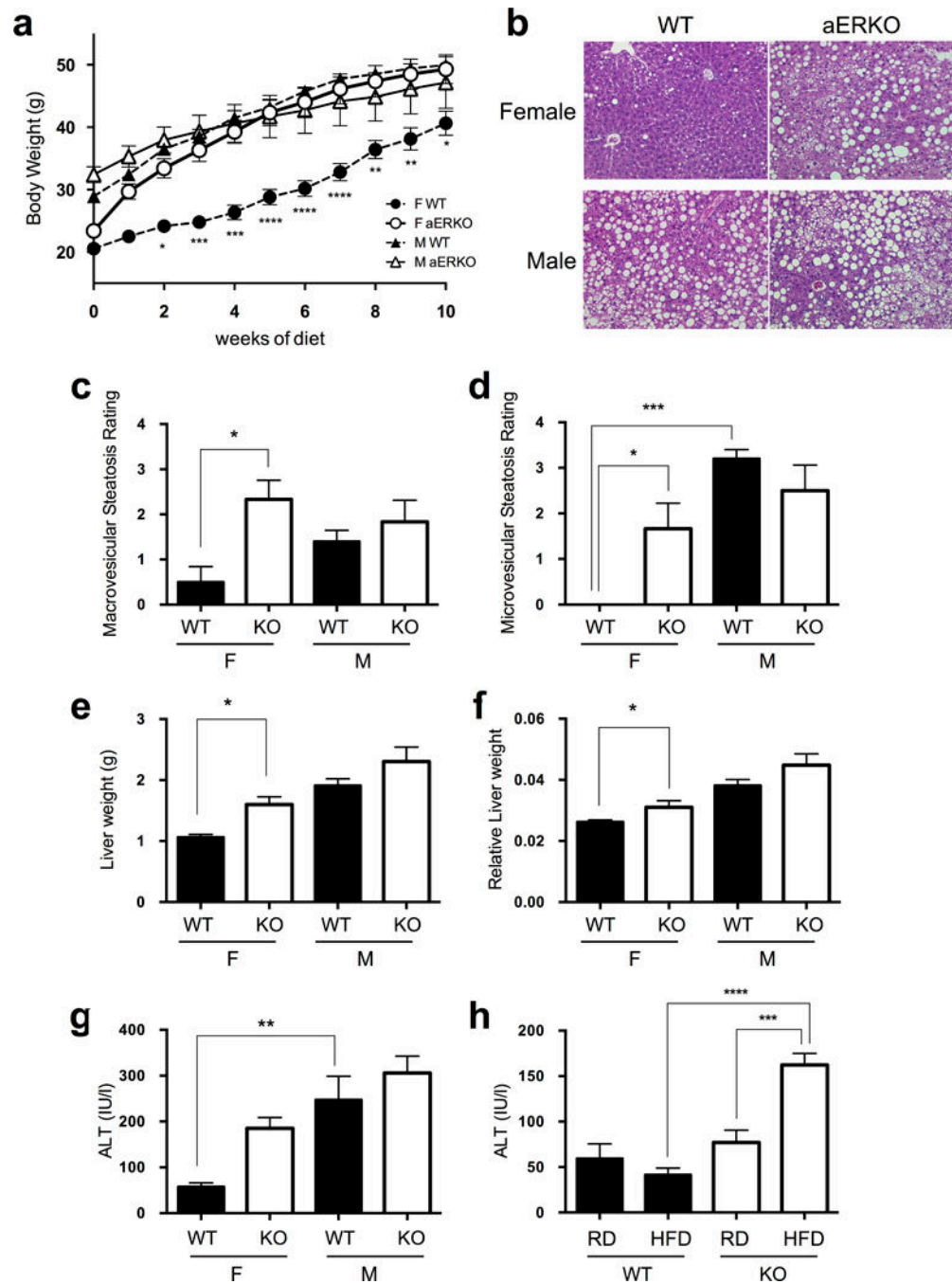
Figure 1.
(**a**) body weight in wild-type (WT) and ERα knockout (αERKO) female (F) (WT n=8, αERKO n=8) and male (M) mice (WT n=8, αERKO n=8) fed high fat diet (HFD) for 10 weeks. Two-way ANOVA with Sidak's multiple comparisons test were performed to compare the difference between WT and αERKO in each sex. (**b**) representative liver sections from HFD-fed WT and αERKO (KO) female and male mice. The blinded histologic ratings of (**c**) macrovesicular steatosis and (**d**) microvesicular steatosis in HFD-fed WT and αERKO (KO) female and male liver. Rating shows 0, no appreciable lipid vesicles

to 4, severe steatosis. (**e**) total liver weight and (**f**) relative liver weight of HFD-fed WT and αERKO (KO) female and male. (**g**) serum alanine aminotransferase (ALT) level in HFD-fed WT and αERKO (KO) female and male. (**h**) serum ALT level in regular diet (RD) or 2 weeks HFD-fed WT and αERKO (KO) female. One-way ANOVA with Tukey's multiple comparisons test were performed to compare the difference between groups (*c*, *d*, *g* and *h*). Two-tailed t-test (unpaired) was performed for comparing the difference between WT and KO (*e* and *f*). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. All data are represented as mean ± s.e.m..
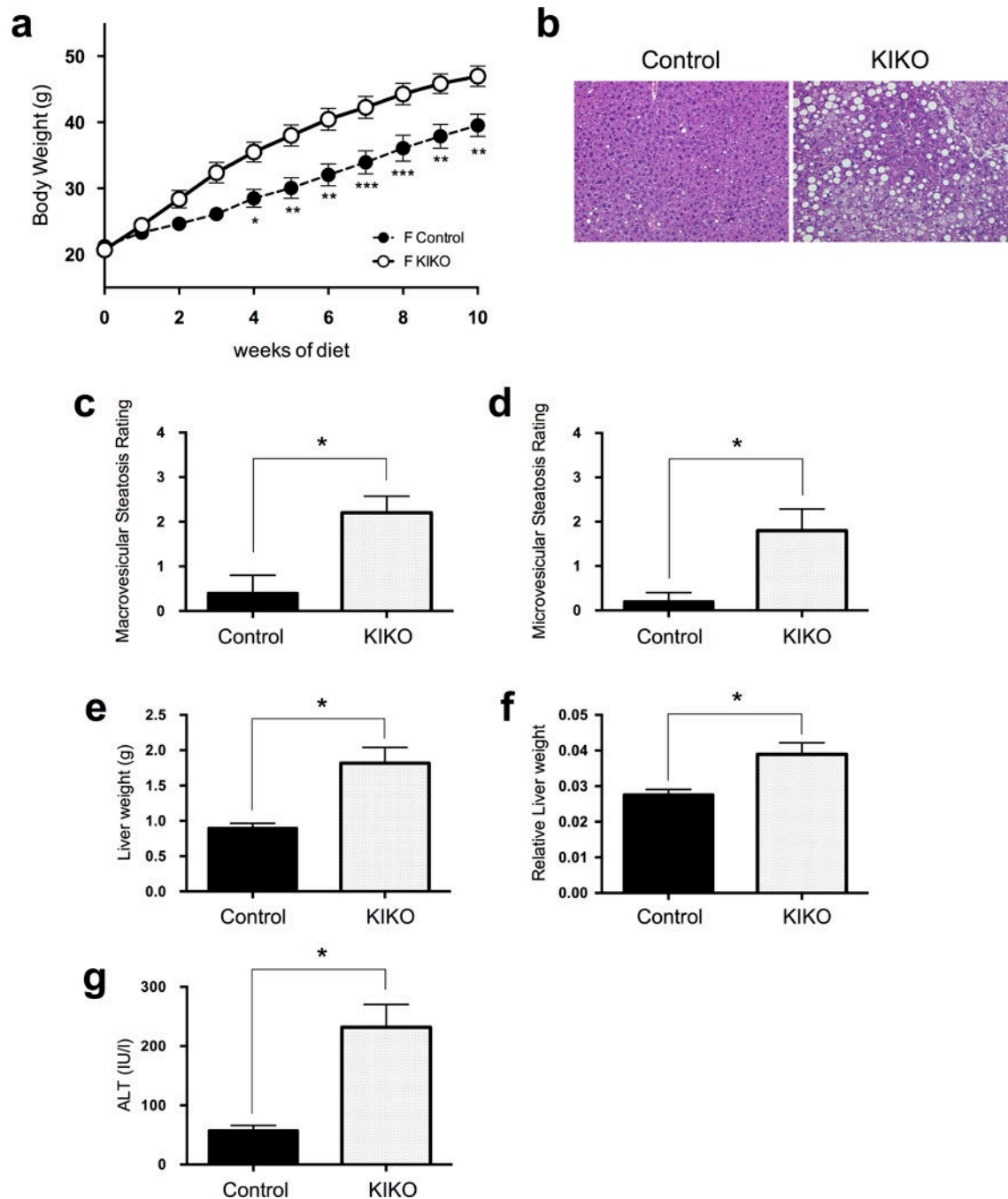
Figure 2.
(**a**) body weight in control (n=8) and ERα DNA binding domain mutant (KIKO) female mice (n=8) fed HFD for 10 weeks. Two-way ANOVA with Sidak's multiple comparisons test were performed to compare the difference between Control and KIKO. (**b**) representative liver sections from HFD-fed control and KIKO females. The blinded histologic ratings of (**c**) macrovesicular steatosis and (**d**) microvesicular steatosis in HFD-fed WT and KIKO female liver. Rating shows 0 – 4 scale. (**e**) total liver weight and (**f**) relative liver weight of HFD-fed WT and KIKO females. (**g**) serum ALT level in HFD-fed WT and
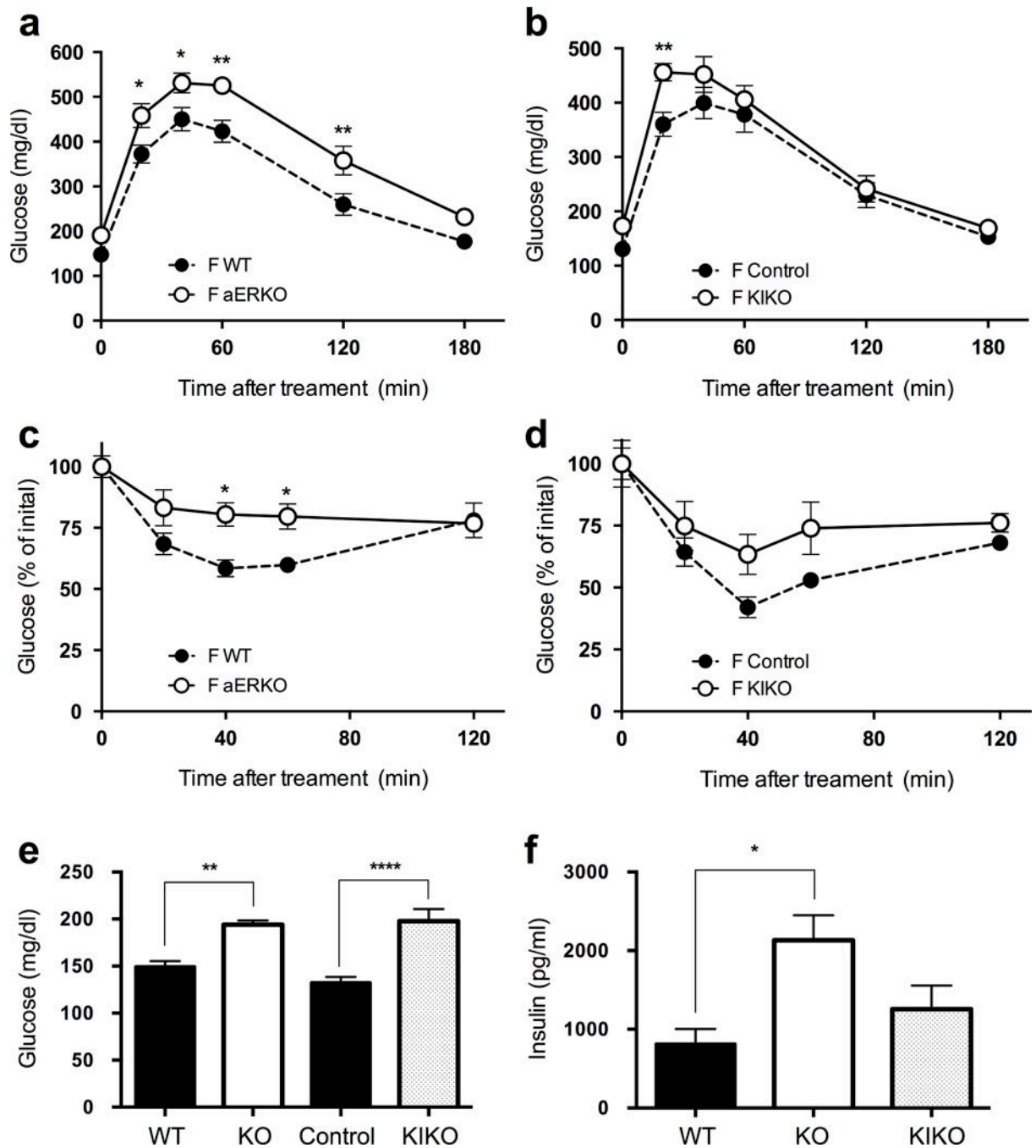
KIKO females. Two-tailed t-test (unpaired) was performed for comparing the difference between Control and KIKO (*c*, *d*, *e*, *f* and *g*). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. All data are represented as mean ± s.e.m..

Figure 3.
(**a**) glucose tolerance test in HFD-fed WT and αERKO (KO) females. (**b**) glucose tolerance test in HFD-fed control and KIKO females. (**c**) insulin tolerance test in HFD-fed WT and αERKO (KO) females. (**d**) insulin tolerance test in HFD-fed control and KIKO females. Two-way ANOVA with Sidak's multiple comparisons test were performed for comparing the difference between genotypes (*a*, *b*, *c* and *d*). (**e**) serum glucose level of WT, αERKO (KO), control and KIKO females in overnight fasting (16 hr) after HFD feeding. Two-tailed t-test (unpaired) was performed for comparing the difference between WT and KO or
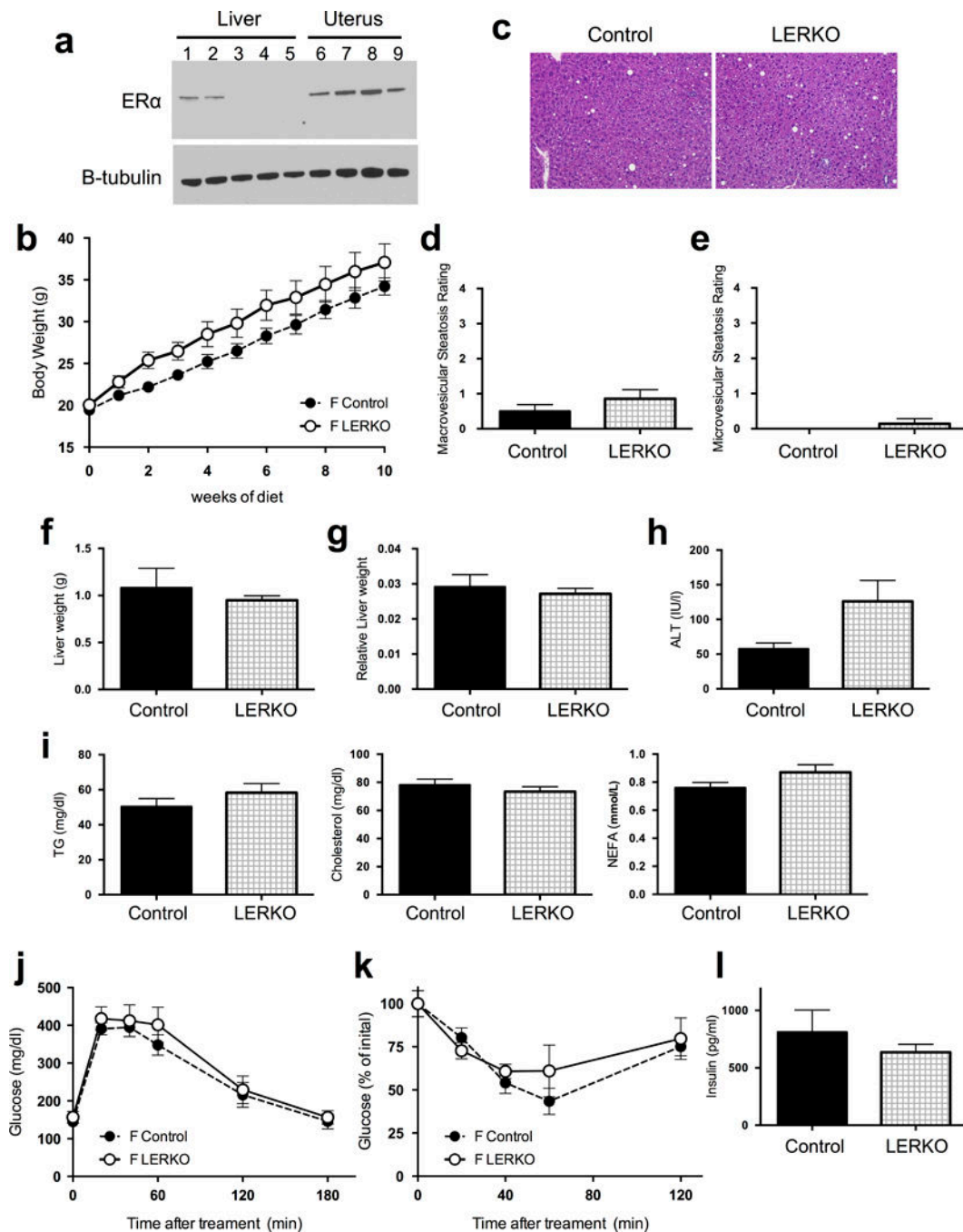
between Control and KIKO. (**f**) serum insulin level of WT, αERKO (KO) and KIKO females in overnight fasting (16 hr) after HFD feeding. One-way ANOVA with Tukey's multiple comparisons test were performed for comparing the difference between groups. $*p < 0.05$, $**p < 0.01$, $****p < 0.0001$. All data are represented as mean ± s.e.m..

Figure 4.
(**a**) whole tissue lysates extracted from control or liver specific ERα knockout (LERKO) liver and uterus were analyzed by immunoblotting with anti-ERα antibody. β-tubulin was used as a loading control. 1, 2, 8 and 9 are control; 3, 4, 6, and 7 are LERKO; 5 is αERKO. A representative Western blot analysis is shown. (**b**) body weight in control (n=8) and LERKO female mice (n=8) fed HFD for 10 weeks. (**c**) representative liver sections from HFD-fed control and LERKO females. The blinded histologic ratings of (**d**) macrovesicular steatosis and (**e**) microvesicular steatosis in HFD-fed control and LERKO female liver.

Rating shows 0 – 4 scale. (**f**) total liver weight and (**g**) relative liver weight of HFD-fed control and LERKO females. (**h**) serum ALT level in HFD-fed control and LERKO females. (**i**) serum triglyceride, cholesterol and non-esterified fatty acids levels in HFD-fed control and LERKO females. (**j**) glucose tolerance test in HFD-fed control and LERKO females. (**k**) insulin tolerance test in HFD-fed control and LERKO females. (**l**) serum insulin level of control and LERKO females in overnight fasting (16 hr) after HFD feeding. Two-way ANOVA with Sidak's multiple comparisons test were performed to compare the difference between Control and LERKO (*b*, *j* and *k*). Two-tailed t-test (unpaired) was performed for comparing the difference between Control and LERKO (*d*, *e*, *f*, *g*, *h*, *i* and *l*). All data are represented as mean ± s.e.m..

Figure 5.
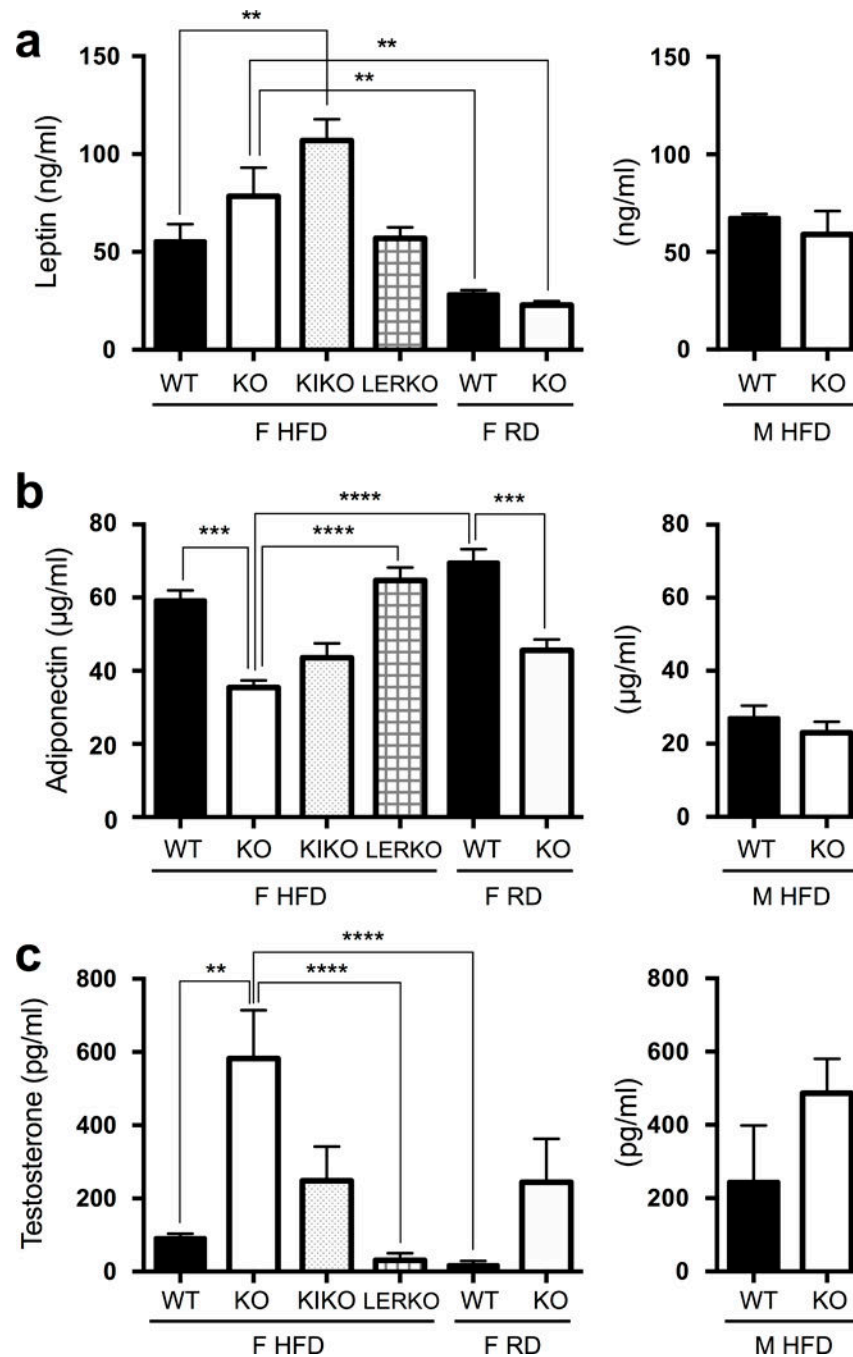(**a**) serum leptin levels of WT, αERKO (KO), KIKO and LERKO females (F) after 10 weeks HFD feeding and RD-fed WT and KO females. (**b**) serum adiponectin levels of WT, αERKO (KO), KIKO and LERKO females after 10 weeks HFD feeding and RD-fed WT and KO females. (**c**) serum testosterone levels of WT, αERKO (KO), KIKO and LERKO females after 10 weeks HFD feeding and RD-fed WT and KO females. One-way ANOVA with Tukey's multiple comparisons test were performed for comparing the difference between groups. The right panel in each graph shows the value of WT and KO males (M)
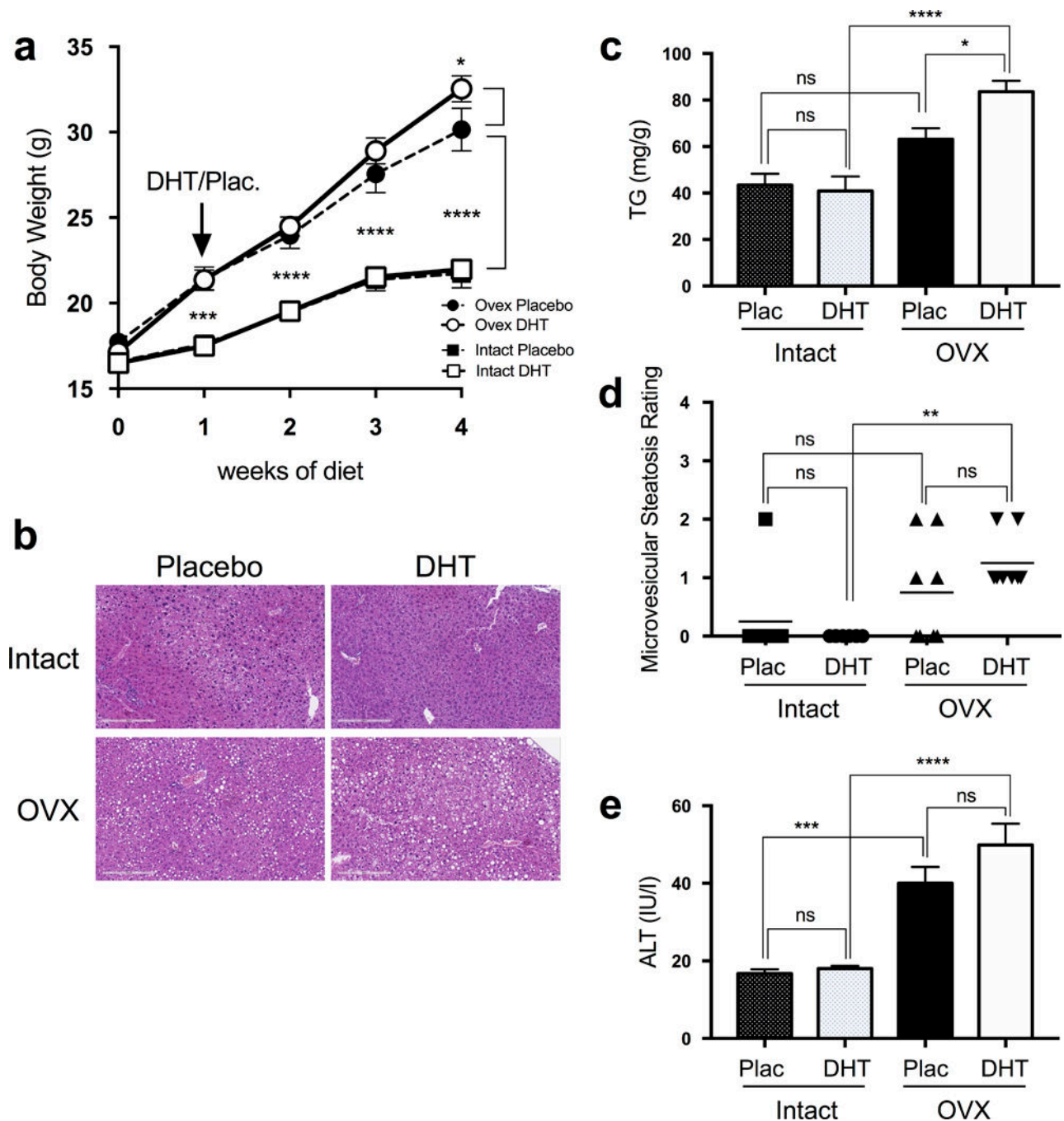
after 10 weeks HFD feeding. Two-tailed t-test (unpaired) was performed for comparing the difference between WT and KO males. **p < 0.01, ***p < 0.001, ****p < 0.0001. All data are represented as mean ± s.e.m..

Figure 6.
(**a**) body weight in intact female with placebo (closed square, n=8), intact female with DHT (open square, n=8), OVX female with placebo (closed circle, n=8) and OVX female with DHT (open circle, n=8) fed HFD for 4 weeks. Arrow indicates the date of placebo (Plac) or DHT pellet implantation. Two-way ANOVA with Tukey's multiple comparisons test were performed to compare the difference between the groups. (**b**) the liver sections depict the most severe steatosis in each group. (**c**) liver triglyceride content. (**d**) the blinded histologic ratings of microvesicular steatosis. Rating shows 0 – 4 scale. The bar indicates average of

the score. (**e**) serum ALT level. One-way ANOVA with Tukey's multiple comparisons test were performed for comparing the difference between groups (*c* and *e*). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. ns denotes no significant difference. All data are represented as mean ± s.e.m.