Estrogen Receptor (ER)α-regulated Lipocalin 2 Expression in Adipose Tissue Links Obesity with Breast Cancer Progression

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Obesity is associated with increased breast cancer (BrCA) incidence. Considering that inactivation of estrogen receptor (ER)α promotes obesity and metabolic dysfunction in women and female mice, understanding the mechanisms and tissue-specific sites of ERα action to combat metabolic-related disease, including BrCA, is of clinical importance. To study the role of ERα in adipose tissue we generated fat-specific ERα knockout (FERTKO) mice. Herein we show that ERα deletion increased adipocyte size, fat pad weight, and tissue expression and circulating levels of the secreted glycoprotein, lipocalin 2 (Lcn2), an adipokine previously associated with BrCA development. Chromatin immunoprecipitation and luciferase reporter studies showed that ERα binds the Lcn2 promoter to repress its expression. Because adipocytes constitute an important cell type of the breast microenvironment, we examined the impact of adipocyte ERα deletion on cancer cell behavior. Conditioned medium from ERα-null adipocytes and medium containing pure Lcn2 increased proliferation and migration of a subset of BrCA cells in culture. The proliferative and promigratory effects of ERα-deficient adipocyte-conditioned medium on BrCA cells was reversed by Lcn2 deletion. BrCA cell responsiveness to exogenous Lcn2 was heightened in cell types where endogenous Lcn2 expression was minimal, but components of the Lcn2 signaling pathway were enriched, i.e. SLC22A17 and 3-hydroxybutyrate dehydrogenase (BDH2). In breast tumor biopsies from women diagnosed with BrCA we found that BDH2 expression was positively associated with adiposity and circulating Lcn2 levels. Collectively these data suggest that reduction of ERα expression in adipose tissue promotes adiposity and is linked with the progression and severity of BrCA via increased adipocyte-specific Lcn2 production and enhanced tumor cell Lcn2 sensitivity.

The alarming rise in obesity over recent decades is strongly associated with a concomitant increase in chronic disease incidence (1). It is generally appreciated that obesity promotes low-grade systemic inflammation via metabolic dysfunction and immune cell activation (2–3), and these factors, common underpinnings of type 2 diabetes and atherosclerosis, are also associated with increased prevalence of breast cancer (BrCA) in women (4–7).
Breast cancer is a leading cause of mortality in women, and the inability to predict, prevent, and treat metastatic breast cancer currently limits patient care. Obesity is linked with more aggressive forms of cancer with less favorable outcomes as obese women in the highest quintile of body mass index (BMI) have double the death rate from BrCA compared with lean counterparts (4). Although the mechanisms by which obesity and metabolic dysfunction increase BrCA risk remain unclear, recent studies have correlated altered levels of circulating factors including metabolites, hormones, adipokines, and cytokines/chemokines with increased BrCA cell proliferation and migration. In addition to visceral adipose previously linked with higher BrCA incidence, adipocytes are also the most abundant cell type of the mammary tumor stroma, and thus paracrine action on malignant epithelium is thought to impact early stages of carcinogenesis as well as responsiveness of established tumors to adjuvant therapies (8–11).

Although many factors contribute to the development of metabolic dysfunction and obesity in human subjects, rare inactivating mutations in the estrogen receptor α gene, Esr1, and common polymorphisms at this locus, are associated with adiposity, type 2 diabetes, atherosclerosis, and BrCA risk, independent of circulating hormone status (12–15). Moreover, Dahlman-Wright and colleagues (16) showed that Esr1 expression is reduced in adipose tissue from obese women. Consistent with observations in human subjects, mice harboring a homozygous Esr1-null mutation manifest marked obesity, insulin resistance, and heightened tissue inflammation (17, 18). Although a role for ERα in regulating metabolic homeostasis, adiposity, and insulin sensitivity is well established, the molecular targets of ERα action within glucoregulatory cell types remain incompletely understood.

Considering that ERα is markedly reduced in adipose from obese individuals and obesity elevates metastatic breast cancer risk in women, we investigated whether targeted loss of adipose tissue in ERαKO mice could promote increased adiposity, metabolic dysfunction, and a secretory profile promoting BrCA tumorigenesis. Herein we show that adipose tissue deletion of ERα increased adiposity and inflammation in female mice, driven in part by a marked elevation in the adipocyte-derived factor, lipocalin 2 (Lcn2). Conditioned media (CM) containing secreted factors from ERα-deficient adipocytes or pure Lcn2 markedly increased proliferation and motility of a specific set of BrCA cell lines in culture. We found that BrCA responsiveness to exogenous Lcn2 was marked by differential expression of intrinsic Lcn2 signaling components. We identified that expression of 3-hydroxybutyrate dehydrogenase (BDH2), an enzyme responsible for the production of the mammalian sidosphere 2,5-dihydroxybenzoic acid and critical for mitochondrial heme synthesis, as a critical component in determining cellular responsiveness to Lcn2. Importantly, we showed that expression of BDH2 in breast tumor biopsies was positively associated with obesity and circulating Lcn2 levels in women with BrCA. Our findings suggest that adipose tissue ERα expression is an important unifying link between obesity and breast cancer risk in women.

EXPERIMENTAL PROCEDURES

Animals—Male and female flox/flox (f/f) and adipose-specific ERα KO (FERKO) mice on a C57Bl6 background were generated by crossing ERα floxed mice (19) with transgenic lines in which Cre recombinase was driven by the aP2 (FABP4) promoter (20). Lepob mice were from Jackson Laboratories and maintained as previously described (18). The EAAE-ERα DNA-binding domain mutant mice were generated by the Korach laboratory (21, 22) as previously described, and adipose tissue was harvested for subsequent qPCR analyses. Control or 17β-estradiol pellets (0.05 mg; 21 days, Innovative Research) were surgically inserted under the skin of Lepob mice and tissues were harvested after 21 days following a 6-h fast. Female mice from the UCLA hybrid mouse diversity panel (HMDP; supplemental Table S1), including 102 strains of inbred animals (23), were maintained on a high fat (HF)/high sucrose (HS) Western diet (Research Diets, D12266B) with the following composition, 16.8% kcal protein, 51.4% kcal carbohydrate, 31.8% kcal fat. Following fasting, animals were anesthetized with 4% isoflurane and exsanguinated prior to tissue harvest. Blood was collected into tubes containing EDTA, and plasma was separated by centrifugation. All procedures were performed in accordance with the Guide for Care and Use of Laboratory Animals of the National Institutes of Health, and approved by the Animal Research Committee of the University of California, Los Angeles.

Human Subjects—Pre-treatment tumor gene expression data were mined from breast cancer patients participating in the UCLA Translational Oncology Research International (TORI-B02) trial (24).

Circulating Factors—Plasma was analyzed for insulin, leptin, PAI-1 (PAI-1) (Millipore), adiponectin (radioimmunoassay; Millipore), and estradiol (Siemens Diagnostics) as previously described (18). Lipocalin 2 ELISA was performed on plasma from women and female mice as per the manufacturer's instructions (R&D Systems).

Body Composition—Female mice from the HMDP were measured for total body fat mass and lean mass by magnetic resonance imaging (MRI) using Bruker Minispec with software from Eco Medical Systems.

RNA Isolation and Expression Profiling in Adipose from HMDP Mice and BrCA Cell Lines—Total RNA was isolated from tissues using TRIzol (Invitrogen) according to the manufacturer's instructions. Total RNA was isolated from cell cultures using the Qiagen RNeasy columns according to the manufacturer's instructions. For microarrays, adipose tissue and BrCA cell (supplemental Table S2) RNA was hybridized to Affymetrix HT MG-430A arrays and scanned using standard Affymetrix protocols. To reduce the risk of spurious association results, RNA normalization was performed after removing all individual probes with SNPs and all probesets containing 8 or more SNP-containing probes, which resulted in 22,416 remaining probesets.
Adipose Tissue ERα Expression, Obesity, and Breast Cancer Progression

Cell Isolations and Culture—HEK293 cells (ATCC) were maintained and passaged in DMEM containing 10% FBS. ERα stably expressing 3T3-L1 cells were generated by infecting 3T3-L1 pre-adipocytes with fresh retrovirus particles generated in PhoenixE cells transfected with pBABE containing the Esr1-ORF, or empty pBABE for control cells (pB). Stable transformants were selected for 1 week in puromycin (5 μg/ml). For differentiation of 3T3-L1 cells, cells were grown to confluence (day 0) before incubation in the standard DMI (dexamethasone, 3-methyl-1-isobutylxanthine, insulin) differentiation mixture with the addition of rosiglitazone maleate (500 nM, Alexis Biochemicals) where indicated, for 3 days (day 3), then encouraged to lipid load for 4 days in the presence of 10 nM insulin (day 7). Primary white adipocytes were generated from stromal vascular fractions isolated from epididymal adipose tissue beds from both f/f Control and FERKO female mice as previously described (25, 26). After isolation, cells were allowed to proliferate to confluence, then immediately differentiated as stated above for 3T3-L1 cells. Breast cancer cell lines were maintained as previously described (27). MCF7, T47D, ZR75-1, and EFM19 were all grown and maintained in RPMI with 10% FBS. Experiments with CM or Lcn2 were carried out in growth medium for 72 h or Hanks’ balanced salt solution with 1% albumin for 24 h, respectively.

Conditioned Media and Lcn2 Incubation—HEK293 cells were cultured as above until ~70% confluent before transfection with the indicated amounts of HA-Lcn2 or GFP plasmid with Lipofectamine 2000, as per the manufacturer’s instructions (Invitrogen). Cells were allowed to recover in growth medium for 24 h after which the medium was removed and replaced with serum-free medium containing 0.1% fatty acid-free BSA for 6–8 h to generate the CM. Pure Lcn2 (R&D Systems) was reconstituted and bound to iron prior to addition to culture medium as described previously (28).

Breast Cancer Cell Proliferation and Migration—Proliferation and migration experiments were performed in growth medium, unless otherwise specified. For proliferation studies, cells were plated into 24-well culture plates at a density of 20,000 cells per well and allowed to attach overnight before the medium was replaced with the addition of either Lcn2 or GFP CM from HEK293A cells or media from Esr1-KD/D and Esr1-KD/Lcn2-KD cells at a ratio of 1:5 (CM:media). After 48 h cells were washed twice with PBS and stained for 15 min in Hoechst nuclear dye (Invitrogen). Cell number was estimated by plotting Hoechst fluorescence measured in each well, against a predetermined standard curve. Results were confirmed in separate experiments using a Coulter Particle Counter. For migration studies, cells were plated in 6-well plates at subconfluence and allowed to proliferate in the presence of the specified CM until ~95% confluent. Two standardized scratchs were made through each well and images were acquired in at least three wells for each condition as previously described (29, 30). Additional images were acquired 18 and 36 h later.

Quantitative RT-PCR—cDNA synthesis was performed on 1 μg of total RNA using iScript cDNA synthesis kit (Bio-Rad) and qPCR were performed using iQ SYBR Green Supermix (Bio-Rad). Primer sequences for the specific target genes analyzed can be found in supplemental Table S3.

Plasmids and Constructs—Esr1-knockdown (KD) by short hairpin RNAs (shRNAs) cloned into the lentivirus vector pLKO.1-puro were purchased as lentiviral transduction particles from Sigma (MISSION®; Esr1-B). Scrambled (Scr) shRNAs (non-target shRNA vector, catalog number SHC002V; Sigma), used for control experiments, contain a hairpin insert that generates siRNAs but contains 5 base pair mismatches to all known human and mouse genes. Particles were subsequently used to transduce 3T3-L1 preadipocytes (multiplicity of infection 10). Stably expressing cells were selected with puromycin (3 μg/ml) for 1 week. Knockdown efficiency was assessed by qPCR and Western blotting.

ERα Expression Plasmid—A mouse Esr1-ORF cloning vector (OpenBiosystems) was used to subclone esr1 into pcDNA3.1 and delivered to cells using Lipofectamine 2000 as per the manufacturer’s instructions (Invitrogen).

pBABE-Esr1 Retrovirus Plasmid—The Esr1-ORF was cloned into pBABE using Gateway technology (Invitrogen) as described below for Lcn2.

Lcn2 HA-tagged Expression Plasmid—Lcn2 cDNA was PCR amplified with the addition of attB/P Gateway sites from the pmMSC-Lcn2 plasmid (20) (a kind gift from Evan Rosen). The subsequent PCR fragment was gel purified and cloned into a customized N-terminal hemagglutinin (HA) pcDNA backbone vector (kind gift from Thomas Vallim, UCLA) using Gateway technology (Invitrogen), before use in transfection assays as described above. C/EBP plasmids were a kind gift from Stephen Farmer (Boston University). Poly(ADP-ribose) PPARγ 2 (peroxisome proliferator-activated receptor γ 2) plasmid was obtained from Peter Tontonoz (UCLA). All primers used for cloning are presented in supplemental Table S4.

Promoter Luciferase Constructs and Assays—The Lcn2 5‘-upstream promoter (2.7 kb) was cloned by PCR from BAC-clone RP24-290F20 (CHORI) with the primers outlined in supplemental Table S4, designed to add extensions for Gateway BP-cloning and Sacl and Kpn1 sites. PCR fragments were amplified by cloning into pDONR221 using Gateway technology (Invitrogen), transformed, expanded, and isolated by miniprep. Promoter containing plasmids and the luciferase expression plasmid (pGL4-basic, Promega) were separately double digested with Sacl and Kpn1 for 1 h, then precipitated and washed with phenol/chloroform extraction. Digested DNA was resuspended in water and used in ligation reactions with T4 ligase at a ratio of 6:1 (insert: plasmid) according to the manufacturer’s instructions (New England Biolabs) and transformed into competent bacteria. Positive clones were sequenced for confirmation. For luciferase assays, 3T3-L1 cells were transfected with promoter plasmids or empty plasmid (500 ng) and Renilla (1 ng) together with other expression plasmids as indicated (100 ng each) using PLUS-reagent and Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Cells were then placed in phenol red-free DMEM containing 10% charcoal/dextran-stripped FBS for 12 h to recover before treatment with either dimethyl sulfoxide or PPT (100 nM) for 12 h. Luciferase assays were performed according to manufacturer’s instructions (Promega, Dual-Glo Stop & Glo). Data are expressed as a ratio of luciferase to Renilla relative light units and converted to fold-change from basal.
Adipose Tissue ERα Expression, Obesity, and Breast Cancer Progression

Chromatin Immunoprecipitation (ChIP)—Stable ERα-expressing 3T3-L1 cells (described above) were used for ChIP experiments. Cells were grown to confluence and at day 3 of differentiation were harvested and subjected to ChIP analysis as previously described (31) using antibodies against ERα (Santa Cruz Biotechnology). Primers for detection of the presence of the Lcn2 promoter by qPCR are outlined in supplemental Table S4.

Immunoblot Analysis—Animal tissues and cultured cells were homogenized in RIPA lysis buffer containing protease and phosphatase inhibitors. Samples were separated by SDS-PAGE, transferred to PVDF membrane, and probed with the following antibodies for protein detection: pan-actin (Cell Signaling Technologies), ERα (Santa Cruz Biotechnology), and Lcn2 (goat anti-mouse, R&D Systems). Densitometric analysis was performed using Bio-Rad Chemidoc image software, Quantity One (version 4.6).

Adipose Tissue Histology—Periovarian adipose tissue immunohistochemistry was performed on paraformaldehyde (8%) fixed samples by the University of California, San Diego, Moores Cancer Center, Histology and Immunohistochemistry Shared Resource Facility, as previously described (32). Adipocyte size was estimated from H&E-stained sections of adipose tissue by analyzing 10 different sections per genotype, counting 100 adipocytes per section using a Nikon Eclipse 80i Upright microscope and Image-Pro Plus Software (Media Cybernetics).

Statistical Analyses—Associations between biomarkers in human subjects were analyzed using Spearman’s rho correlation. Relationships between expression levels and % body fat for human subjects were analyzed using Spearman’s rho correlation. Associations between biomarkers in male and female mice (Fig. 1, E) were analyzed using Student’s t test. Multiple group by treatment mean comparisons were performed using ANOVA with Tukey’s LSD post-hoc test analyses. Significance was established a priori p < 0.05.

RESULTS

Adipose-specific Deletion of ERα Promotes Obesity—Previous studies have shown that the expression of ESR1 is significantly reduced in adipose tissue from obese women (16). Indeed, we observed reductions in Esr1 expression in adipose tissue from genetically obese LepOb male and female mice (Fig. 1A). We also found that adipose tissue Esr1 expression is inversely correlated with the degree of HFD-induced adiposity in more than 100 genetically diverse strains of inbred female mice, termed the UCLA HMDP (23) (Fig. 1B).

To determine whether impaired ERα action promotes adiposity even during normal chow feeding, we generated fat-specific ERα knock-out mice (FERKO) by mating floxed ERα (if/f) animals (33) with transgenic mice expressing Cre recombinase driven by the aP2 promoter (20). As expected, Esr1 expression levels were significantly reduced in both white adipose tissue (WAT) and brown adipose tissue (BAT) of female (Fig. 1C) and male (Fig. 1D) FERKO mice, compared with if/f Control littermates. Deletion was selective for adipose tissue as Esr1 levels were maintained in skeletal muscle and liver of female FERKO mice (Fig. 1, C and D). Phenotypic analyses revealed that the body mass of female FERKO mice was increased compared with that of if/f Control animals (26.3 ± 0.14 versus 23.0 ± 0.13 g, p < 0.001), a finding also observed in male mice, although the difference did not reach statistical significance (p = 0.116) (Fig. 1E). Consistent with findings for body weight, FERKO female mice had significantly larger gonadal fat pads (0.43 ± 0.01 versus 0.63 ± 0.01 g, p = 0.001; Fig. 1F), BAT deposits (0.046 ± 0.001 versus 0.067 ± 0.001 g, p = 0.029, Fig. 1G), and liver weight (1.32 ± 0.02 versus 1.01 ± 0.01 g, p = 0.002; Fig. 1H) compared with if/f Control. Adipocytes from gonadal fat pads of female FERKO mice were larger in diameter than those from if/f Control mice (Fig. 1I). In contrast to females, WAT (p = 0.03) but not BAT (p = 0.1) and liver (p = 0.4) weights were elevated for male FERKO versus if/f Control.

Analyses of circulating factors were consistent with the observed obesity phenotype in female FERKO mice as plasma levels of leptin (2.4 ± 0.1 versus 1.2 ± 0.1 ng/ml, p = 0.04) and the inflammatory marker PAI-1 (1.0 ± 0.01 versus 0.5 ± 0.04 ng/ml, p = 0.05) were increased over that of if/f Control animals (Table 1). In contrast, no differences in plasma concentrations of insulin, adiponectin, or estradiol were detected between the genotypes (Table 1).

Adipose Tissue ERα Deletion Alters Adipocyte Function and Lipocalin 2 Expression in Female Mice—Considering that the phenotype of female FERKO mice was more prominent than that observed for males, we focused subsequent screening studies on adipose tissue from female animals. Expression of the adipogenic transcription factors C/EBPα and poly(ADP-ribose) polymerase 6 were reduced in FERKO fat (Fig. 2A). With the exception of the inflammatory chemokine MCP1, which was elevated in FERKO ~5-fold above if/f Control (p = 0.001; Fig. 2B), the expression levels of proinflammatory cytokines were similar between the genotypes of female mice. Analysis of adipokine expression from FERKO fat showed no change in adiponectin, leptin, or Rbp4 transcript levels. In contrast, a significant 9-fold elevation in the expression of Lcn2 was observed in adipose tissue from FERKO compared with if/f Control (Fig. 2C). Increased Lcn2 expression was also detected in WAT from LepOb mice of both genders compared with the respective lean controls (Fig. 2D). Of note, a gender dimorphism in adipose tissue Lcn2 expression was also observed as transcript levels were markedly elevated in adipose tissue of lean WT and if/f Control male mice compared with similar genotype female animals (Fig. 2, D and E). Our data suggest that this gender dimorphism was a possible result of higher basal Esr1 expression levels in WAT of female versus male mice (Fig. 1A). Consistent with this observed gender dimorphism, in male LepOb mice an experimental increase in circulating estradiol concentration to levels observed in normally cycling females reduced expression of Lcn2 in epididymal fat below that observed for lean male animals (Fig. 2D).

In gonadal white and subcapsular brown adipose tissue, the loss of ERα promoted increased expression of Lcn2 (5–10-fold) in FERKO compared with if/f Control for female but not male animals (Fig. 2, E and F). Similar to periovarian adipose, reduced Esr1 expression in subcutaneous fat from FERKO female mice (Fig. 2G) was paralleled by a marked increase in Lcn2 expression over if/f Control (Fig. 2H). However, we
Inverse relationship between adipose tissue $Esr1$ expression and adiposity. A, $Esr1$ expression is reduced in adipose tissue from obese $Lep^{ob}$ male and female mice compared with lean animals (2.5 months, $n=6/-group).$ B, inverse relationship between gonadal adipose $Esr1$ expression and % body fat of HF/HS-fed female mice from the UCLA HMDP ($n=102$ strains, 3–6 mice per strain). C and D, $Esr1$ expression determined by qPCR analyses in muscle, liver, WAT, and BAT from female (C) and male (D) FERKO mice compared with littermate $f/f$ Controls (6 months of age, normal chow-fed, $n=6–8/group). E, body weight; F, gonadal WAT weight; $G$, extrascapular BAT weight; and $H$, liver weight in female and male FERKO mice compared with littermate $f/f$ Controls. I, H&E sections and quantification of adipocyte size ($\mu m$) of gonadal WAT from female $f/f$ Control and FERKO mice ($n=4$ mice/genotype). Values are expressed as mean $\pm$ S.E. and differences were detected by Student’s $t$ test and ANOVA where appropriate. *, $p<0.05$ between genotypes; $1$, $p<0.05$ between genders. Correlations were determined by Pearson’s correlation analysis and $p$ values are provided. ND, not detected.

### TABLE 1

| Circulating factor | Female $f/f$ Control | $f/f$ FERKO | $p$ value | Male $f/f$ Control | $f/f$ FERKO | $p$ value |
|--------------------|----------------------|-------------|-----------|----------------------|-------------|-----------|
| Glucose (mg/dl)    | $117 \pm 2.6$        | $138 \pm 3.5$ | 0.07      | $163 \pm 3.7$        | $167 \pm 5.7$ | 0.80      |
| Insulin (ng/ml)    | $0.28 \pm 0.01$      | $0.23 \pm 0.01$ | 0.27      | $0.78 \pm 0.02$      | $1.1 \pm 0.8$ | 0.07      |
| Adiponectin (µg/ml) | $15.3 \pm 0.4$       | $13 \pm 0.3$   | 0.15      | $8.7 \pm 0.3$        | $8.7 \pm 0.2$ | 0.95      |
| Leptin (ng/ml)     | $1.2 \pm 0.1$        | $2.4 \pm 0.1$   | 0.04      | $2.2 \pm 0.2$        | $2.8 \pm 0.1$ | 0.45      |
| PAI-1 (ng/ml)      | $0.50 \pm 0.04$      | $1.0 \pm 0.01$  | 0.05      | $0.50 \pm 0.05$      | $0.53 \pm 0.01$ | 0.86 |
| Estradiol (pg/ml)  | $71 \pm 12$          | $94 \pm 15$    | 0.27      | NA*                  | NA          | NA        |

*NA, not analyzed.*
detected increased Lcn2 expression levels in SC over gonadal fat from all female animals studied. Immunoblot analyses confirmed that the Lcn2 protein was significantly elevated in adipose tissue from obese Lep^ob^ male and female mice compared with lean and estradiol (E2)-treated male animals (n = 6/group). Lcn2 expression in gonadal WAT (E) and extrascapular BAT (F) from female and male f/f Control (open bars) versus FERKO (closed bars) (n = 6–8/group). G and H, similar to gonadal adipose, Esr1 expression is reduced and Lcn2 expression is elevated in subcutaneous (SC) adipose depots from FERKO versus f/f Controls. I, immunoblotting and densitometric analyses on gonadal WAT show increased Lcn2 protein levels in female FERKO mice versus f/f Control, but no change in Lcn2 protein between genotypes of male mice (n = 6/group). J, plasma Lcn2 levels in FERKO versus f/f Control animals (n = 6/group). K–L, adipose tissue Esr1 expression is inversely correlated with Lcn2 expression, and Lcn2 is positively correlated with % body fat in 102 strains of female HMDP animals (n = 3–6/strain). Values are expressed as mean ± S.E. and differences were detected by Student’s t test and ANOVA where appropriate. *, p < 0.05 between genotypes; 1, p < 0.05 between genders; #, p < 0.05 E2-treated versus untreated. Correlations were determined by Pearson’s correlation analysis.
expression in adipose tissue of female mice, we confirmed a strong inverse relationship between \textit{Esr1} and \textit{Lcn2} expression in WAT obtained from 102 unique strains of HF/HS-fed female mice of the UCLA HMDP (Fig. 2K). \textit{Lcn2} expression was highly correlated with body fat percentage (Fig. 2L), similar to the relationship between adiposity and the adipokine leptin. These data confirm that natural genetic variation of \textit{Esr1} expression in adipose tissue among diverse mouse strains is inversely associated with \textit{Lcn2} expression, an adipokine we found tightly correlated with adiposity in female mice.

\textbf{ERα Deletion Impairs Adipocyte Function and Increases \textit{Lcn2} Expression in Culture---Prior studies have identified a functional estrogen response element (ERE) within the \textit{Lcn2} gene promoter that controls its expression in normal breast tissue (38). We investigated the potential for ERα to directly modulate \textit{Lcn2} expression in adipocytes. Our own computational analysis confirmed the existence of a complete palindromic ERE at \textasciitilde2533 bp upstream from the transcriptional start site of the mouse \textit{Lcn2} gene (38). To demonstrate direct action of EROs on \textit{Lcn2} expression independent of secondary in vivo cues including insulin resistance or obesity, we showed that \textit{Lcn2} expression was elevated in cultured primary adipocytes from FERKO WAT compared with primary cells isolated from WAT of f/f Control animals (Fig. 3A). Primary adipocytes lacking ERα (FERKO) released elevated amounts of \textit{Lcn2} into the medium (Day 3 of differentiation) compared with control ERα replete adipocytes (Fig. 3B).

Next we studied the effects of 17βestradiol (E2) on \textit{Lcn2} expression in 3T3-L1 adipocytes. Maximal suppression of \textit{Lcn2} expression was achieved with 1 nM E2 treatment (Fig. 3C). To further support a role for ERα in the regulation of \textit{Lcn2}, we stably overexpressed ERα using retrovirus. \textit{Lcn2} expression was reduced by \textasciitilde80% in cells overexpressing ERα compared with control ERα replete adipocytes (Fig. 3D).

FIGURE 3. Adipocyte \textit{Lcn2} expression is repressed by ERα DNA binding. Primary adipocytes isolated from periorvian WAT of female FERKO mice show reduced \textit{Esr1} and increased \textit{Lcn2} expression as measured by qPCR (A), and release increased amounts of \textit{Lcn2} protein into culture medium (B) compared with adipocytes from f/f Control. C, \textit{Lcn2} expression is reduced in 3T3-L1 adipocytes treated with estradiol (E2, 1 and 10 nM) for 1 h. 3T3-L1 adipocytes treated with retrovirus expressing ERα (pB-ER) show (D) elevated \textit{Esr1} expression levels and a compensatory reduction in \textit{Lcn2} expression compared with control adipocytes expressing empty virus (pB). E, short hairpin-induced knockdown of ERα (observed in three of four stable 3T3-L1 \textit{Esr1}-KD cell lines; open bars for clones C, D, and E), increased \textit{Lcn2} expression (closed bars) (n = 3 observations/clone). F, immunoblotting for ERα in lysates from \textit{Esr1}-KD (D) versus Scr-Control 3T3-L1 adipocytes and for \textit{Lcn2} in adipocyte culture medium (G). Values are expressed as mean \pm S.E. and mean differences were detected by Student’s t test and ANOVA where appropriate. *, p < 0.05 between genotypes; #, p < 0.05 E2 dose-response versus basal (0 nM).
stably expressing $E_{sr1}$ shRNAs (Fig. 3E). Three of the cell lines (C, D, and E) showed significantly reduced $E_{sr1}$ expression, with a concomitant increase in expression of the $Lcn2$ transcript (Fig. 3E). Of note, the KD cell line that showed no reduction in $E_{sr1}$ ($E_{sr1}$-KD-B), also showed no change in $Lcn2$ expression (Fig. 3E). Because of its robust action, $E_{sr1}$-KD D was selected for subsequent analyses.

Similar to our observations for primary adipocytes from FERKO mice, $E_{sr1}$-KD 3T3-L1 adipocytes showed significantly reduced ER$\alpha$ protein expression (Fig. 3F) and increased secretion of $Lcn2$ into the culture media (Fig. 3G). No morphological defects were observed in $E_{sr1}$-KD 3T3-L1 adipocytes, as confluent, non-differentiated pre-adipocytes (day 0, Fig. 4) or as non-lipid loaded differentiating cells (day 3). However, after 4 days of lipid loading in the presence of insulin (day 7, Fig. 4), $E_{sr1}$-KD cells displayed a reduction in lipid accumulation compared with Scr-Control cells. Furthermore, differentiated $E_{sr1}$-KD adipocytes were larger in size and possessed substantially larger intracellular lipid droplets than WT cells (Fig. 4, arrows, bottom panel). These data are consistent with the observation of larger adipocytes and reduced expression of adipocyte transcription factors in FERKO adipose tissue.

ER$\alpha$ Binds the $Lcn2$ Promoter and Competes with C/EBP for Regulation of $Lcn2$ Expression—Considering that previous studies have shown $Lcn2$ expression is induced by the C/EBP family of transcription factors (34), and that ER$\alpha$ forms complexes with members of this family to control their action on specific target genes (39, 40), we hypothesized that interaction of ER$\alpha$ with C/EBP transcription factors may be critical in the regulation of $Lcn2$ expression specifically in adipocytes. The observation that a C/EBP binding site (ccaat) overlaps with the ERE in the $Lcn2$ promoter (−2536 bp), supported our hypothesis and led us to investigate the role of ER$\alpha$ in modulating the function of C/EBP factors in the control of $Lcn2$ expression. To test our hypothesis further, we generated a luciferase reporter construct (pGL4) containing the proximal promoter of
Adipose Tissue ERα Expression, Obesity, and Breast Cancer Progression

FIGURE 5. ERα binds the Lcn2 promoter to repress Lcn2 expression in adipocytes. A, diagram of the 2.7-kb 5′ proximal promoter of Lcn2 cloned into pGL4-luciferase (ERE, estrogen response element) and activation of the promoter indicated by luciferase activity assessment in 3T3-L1 adipocytes transfected with ERα and or C/EBP and treated with the ERα-specific agonist propyl pyrazole triol PPT. C. ChIP analyses show direct binding of ERα to the consensus ERE in the Lcn2 promoter (a, b, and c indicate response elements shown above in A). D, white adipose tissue expression levels of Esr1 (exons 3 and 6–7), C/ebpβ, and Lcn2 assessed by qPCR in WT (open bars) versus ERα DNA binding domain mutant (ERα DBDΔ) mice (n = 6/genotype). Values are expressed as mean ± S.E. and mean differences were detected by Student’s t test and ANOVA where appropriate. *, p < 0.05 between genotypes or from GFP expressing 3T3-L1 adipocytes in panel B; 1, p < 0.05 C/EBP versus C/EBP + ERα ± PPT for panel B.

mouse Lcn2 (2.7 kb) including the ERE (Fig. 5A). Transient transfection of this luciferase reporter construct into 3T3-L1 adipocytes alone or together with ERα and C/EBP factor expression vectors showed that ERα suppressed C/EBP-induced luciferase activity (Fig. 5B). ChIP studies conducted in 3T3-L1 adipocytes confirmed ERα binding to the Lcn2 proximal promoter construct at two sites (Fig. 5C). To confirm the requirement of DNA binding for the repression of Lcn2 by ERα in vivo, we assessed Lcn2 expression in gonadal WAT from the ERα DNA binding incompetent mouse in which ERα protein tethering activity is maintained (21). In gonadal adipose tissue from these DNA binding domain mutant mice (ERα-DBDΔ) we observed a marked increase in C/ebpβ and Lcn2 expression levels over WT control (Fig. 5D). These data confirm the insufficiency of ERα protein tethering, but confirm the requirement of ERα DNA binding in the repression of Lcn2.

Lcn2 Promotes Proliferation and Migration of BrCA Cells—Considering that the phenotype of malignant cells can be influenced by the surrounding stromal microenvironment, and that adipocytes constitute the largest cellular compartment of breast tissue (41), we tested whether Lcn2, an adipocyte-secreted factor, could influence the behavior of BrCA cell lines. We treated SUM159PT BrCA cells with CM from adipocytes where Lcn2 was elevated in response to ERα knockdown, or CM from HEK293A cells transfected with an Lcn2 expression plasmid. Conditioned media from Esr1–KD adipocytes increased the proliferation of the breast carcinoma cell line SUM159PT compared with CM from Scr-Control adipocytes (Fig. 6A). Similarly, CM containing Lcn2 expressed from transfected HEK293A cells promoted increased SUM159PT cell proliferation compared with CM from cells transfected with a GFP expression plasmid (Fig. 6B), suggesting a direct effect of Lcn2 on the proliferation of SUM159PT BrCA cells. Additionally, both Lcn2-CM and Esr1-KD CM increased the migration of SUM159PT cells over scratched monolayers, but had no effect on immortalized, non-transformed breast epithelial, MCF10A cells (Fig. 6C).

To test the role of Lcn2 in mediating the proliferative and promigratory effects of CM from ERα-deficient adipocytes on breast cancer cells, we deleted Lcn2 from ERα-deficient adipocytes (Fig. 7, A and B). Findings for proliferation and migration of SUM159PT BrCA cells treated with CM from adipocytes with a dual KD of Esr1 and Lcn2 mirrored findings for cells treated with CM from Scr-Control ERα replete adipocytes (Fig. 7, C and D). These data indicate that increased Lcn2 released from ERα-deficient adipocytes is a critical mediator of BrCA cell proliferation and migration.

Expression of Lcn2 Signaling Components in BrCA Cells Confer Lcn2 Responsiveness—In an effort to more rigorously interrogate the effect of Lcn2 on BrCA cells in vitro, we studied the Lcn2 responsiveness of additional BrCA cell lines (Fig. 8). We observed a wide variability in the proliferation response to Lcn2 in the different BrCA cell lines (ZR75-1, MCF7, T47D, and EFM19), as well as between lines arising from similar cellular origins. The advantage of studying these specific cell lines is that each is cultured using the same media and environment thus eliminating confounding factors arising from varying culture conditions (27). Lcn2-stimulated proliferation was highest...
in MCF7 and EFM19 and negligible in ZR75-1 cells (data not shown). Interestingly, cellular proliferation correlated well with the expression of BDH2, the intracellular rate-limiting enzyme that catalyzes the production of the mammalian siderophore 2,5-dihydroxybenzoic acid necessary for iron homeostasis and overall Lcn2 responsiveness (42) (Fig. 8A). In contrast to findings showing that LCN2 was expressed in a number of human cancers and associated with ER/progesterone receptor status (43), we screened 55 tumor samples as well as 53 cancer cell lines and found no correlation between ESR1 and LCN2 expression or LCN2 expression and tumor type (supplemental Table 2; cell lines as reviewed in Ref. 27). LCN2 expression levels were ∼100-fold higher in ZR75-1 cells than MCF7 cells, and ∼1000-fold higher than EFM19 cells (Fig. 8B). These findings raise an important question for future study regarding the relative roles of intracellular versus extracellular Lcn2 in driving neoplastic development and metastatic potential.

Differences in components of the Lcn2 signaling pathway were also apparent between lines, with elevated expression of the Lcn2 receptor, SLC22A17 (variant 1), found in the most responsive cell types (MCF7 and EFM19) (Fig. 8C). Exogenous Lcn2 was found to only modulate its receptor expression in MCF7 cells, whereas all other BrCA cell types were unresponsive (Fig. 8D). Expression levels of ferritin light chain (FLT), BNIP3, SNAIL, SLUG, and Vimentin, markers of EMT and apoptosis susceptibility, were elevated in cell lines where ectopic Lcn2 promoted increased proliferation (Fig. 8, E–I). Collectively, these data suggest that intrinsic differences in expression of components of the Lcn2 signaling pathway may underlie the differential responses of BrCA cells to the paracrine actions of Lcn2.

BDH2 Expression in Human BC Tumors Correlates with BMI and Lcn2, but Not Leptin or Adiponectin—To investigate a potential role for components of the Lcn2 signaling pathway in the differential response of primary BrCA tumors to obesity, we analyzed data from microarray studies performed on biopsied tumor samples from women of varying BMI participating in the TORI B02 study (24). Plasma was collected at the time of diagnosis, prior to therapeutic intervention. As expected, plasma adipokines, leptin, and adiponectin were significantly correlated with BMI. Similar to published findings showing
increased plasma Lcn2 levels in obese subjects (34, 36, 37), the mean plasma Lcn2 concentration was elevated 23% in patients with a BMI >30 (Fig. 9A). Additionally, we identified a significant positive association between tumor expression of BDH2 and BMI, as well as tumor BDH2 expression and plasma Lcn2 concentration (Fig. 9, B and C). Unfortunately, probes for the Lcn2 receptor SLC22A17 were not present in the microarray platform so we were unable to assess the relationship between obesity and tumor Lcn2 receptor expression. In contrast to our findings for Lcn2, we found no significant association between tumor BDH2 expression and circulating levels of leptin or adiponectin (Fig. 9, D and E), indicating that tumor BDH2 expression is unlikely influenced by these circulating factors previously associated with BrCA progression (as reviewed in Refs. 44 – 46). Moreover, no significant association between plasma levels of Lcn2 and adiponectin or leptin were detected (Fig. 9, F and G). Thus, when considered in aggregate, these data suggest that adipocyte expression of ESR1, and not simply adiposity per se, may play a greater role in controlling Lcn2 production and breast cancer risk.

**FIGURE 7.** Lcn2 deletion reverses the proliferative and promigratory effects of conditioned media from ERα-KD 3T3-L1 adipocytes on SUM159PT breast cancer cells. A, Lcn2 expression levels in Scr-Control (open bars), Esr1-KD (closed bars), and Esr1-KD/Lcn2-KD (hatched bars) 3T3-L1 adipocytes assessed by qPCR. B, Lcn2 protein levels in conditioned media from Scr-Control, Esr1-KD, and Esr1-KD/Lcn2-KD 3T3L1 adipocytes detected by immunoblotting. C, cellular proliferation in SUM159PT cells after 4 days exposure to CM from Scr-Control, Esr1-KD, and Esr1-KD/Lcn2-KD 3T3L1 adipocytes. D, migration of SUM159PT cells in culture over a standardized scratch site after 18 and 36 h of exposure to CM from Scr-Control, Esr1-KD, and Esr1-KD/Lcn2-KD 3T3-L1 adipocytes. Values are expressed as mean ± S.E. in arbitrary units (AU), and mean differences were detected by Student’s t test and ANOVA where appropriate. For each analysis, three independent experiments were performed in triplicate. *, p < 0.05 between Scr-Control and Esr1-KD CM; #, <0.05 between Esr1-KD and Esr1-KD/Lcn2-KD CM.
Discussion

Obesity is an established risk factor for chronic diseases including atherosclerosis, type 2 diabetes, and cancers of the liver, colon, and breast. Normally cycling females are partially protected against these diseases, but much of this protection is lost following menopause when increased adipose tissue weight gain, tissue inflammation, and metabolic dysfunction manifests (47). Unfortunately, the mechanistic underpinnings linking obesity and metabolic dysfunction to cancer pathobiology remain incompletely understood. Studies by Nilsson et al. (16) implicated a relationship between reduced expression of ERα in adipose tissue and obesity in women. However, whether reduced adipose tissue ESR1 expression is causal for obesity and a disease-promoting secretory profile was unknown.

Herein, we reveal a mechanism whereby genetic ablation of Esr1 in adipose tissue of female mice promotes increased adiposity, tissue inflammation, and increased expression and secretion of the adipokine Lcn2. Considering that the phenotype of malignant cells can be influenced by surrounding stromal cells comprising the tumor microenvironment, and that adipocytes constitute the largest cellular compartment of breast tissue (41), we tested whether adipocyte-secreted factors could influence the behavior of BrCA cells. We showed that adipocytes lacking ERα secrete factors that exert marked effects on BrCA cell proliferation and migration. Because Lcn2 was highly expressed and secreted from ERα-deficient adipocytes, we next determined whether Lcn2 was a critical factor driving the proliferative and migratory response of BrCA cells. Consistent with our hypothesis, deletion of Lcn2 from ERα-deficient adipocytes reversed the proliferative and pro-migratory effects of CM on SUM159PT BrCA cells. These findings suggest that Lcn2 is an important potential mediator promoting tumorigen-
Adipose Tissue ERα Expression, Obesity, and Breast Cancer Progression

Obesity is associated with increased BDH2 expression in primary tumors from breast cancer patients. A, plasma Lcn2 is elevated in obese (BMI > 30; n = 26) compared with lean (BMI < 25; n = 31) women diagnosed with BrCA. BDH2 expression is significantly associated with BMI (B) and plasma (C) Lcn2 concentration in female breast cancer patients from the TORI trial. No significant relationship between BDH2 expression and circulating levels of Lcn2 versus leptin (D and F) or the anti-inflammatory adipokine adiponectin (E and G) were detected. Values are expressed as mean ± S.E., and mean differences were detected by Student’s t test. *, p < 0.05 between groups. Correlation coefficients were determined by Pearson’s r, and p values are provided for each correlation.

Although prior studies have demonstrated a role for Lcn2 in the proliferation and migration of cancer cells in culture (35, 49, 50) and have shown that LCN2 is strongly associated with breast cancer stage in microarray and expression profiling studies, the exact cellular source or sources of Lcn2 have not yet been determined (35, 43, 51). Additionally, although effects of Lcn2 on tumor burden in mouse models of breast cancer have been observed (52–54), these studies in murine models have failed to identify the mechanisms by which Lcn2 promotes tumorigenesis in vivo. Because Lcn2 is now considered a clinical predictor of disease prognosis in human primary breast cancer patients (43, 55), resolution of the mechanisms regulating LCN2 expression, the identification of the cell types involved in its paracrine and endocrine actions, as well as determination of the cell and tissue-specific effects of Lcn2 signal transduction, require greater attention. Our data strongly support a link between ERα-mediated regulation of Lcn2 in adipose tissue and breast cancer progression; however, our studies are limited to the direct impact of Lcn2 on breast cancer cell behavior and do not extend to the potential effects of Lcn2 on the tumor microenvironment, which is likely of equal or greater importance for disease prognosis.

Previous work has shown that Lcn2 expression is up-regulated in differentiating adipocytes in culture, and that circulating levels of Lcn2 are associated with obesity and metabolic dysfunction in wild type male mice (34). Our findings presented herein for female mice now reproduce those initial studies conducted in males. Additionally we have identified ERα as a critical transcriptional regulator of Lcn2 production and adiposity.

In line with the notion that obesity and alteration in adipocyte behavior underlie tumor progression, it is shown that mature breast adipocytes, but not pre-adipocytes, promote breast cancer cell motility (56). Thus it follows that the secretion of soluble factors from differentiated adipocytes may pro-
vide critical cellular cross-talk necessary for disease progression (9). Interestingly, cancer-associated adipocytes located at the tumor invasive front appear delipidated and resemble a de-differentiated phenotype compared with more distal adipocytes. These front-line adipocytes are characterized by a fibroblast-like morphology and down-regulation of key adipogenic markers including poly(ADP-ribose) polymerase γ, C/EBPα, and αp2 (9), a finding reproduced in ERα-deficient adipocytes. These observations suggest that cancer cells may engage in reciprocal talk altering adipocyte phenotype to promote mobilization of substrate, extracellular matrix remodeling, and angiogenesis (41). Scherer and colleagues (57) previously showed that adipocyte-secreted factors have an unparalleled ability to promote increased cell motility, migration, and tumor angiogenesis compared with secreted factors of other stromal cell types. In aggregate, our studies suggest these cancer-promoting effects of adipocytes are accentuated by ERα deficiency.

Although the physiological role of Lcn2 has remained incompletely understood since its initial discovery, it has been primarily associated with iron metabolism and metalloproteinase (MMP-9 specifically) activity (58). More recently, Green and colleagues (28, 48) have identified and characterized both the receptor for Lcn2 (Slc22a17) and enzymes involved in intracellular signaling including Bdh2. We found that Bdh2, an enzyme that catalyzes the formation of the siderophore that binds Lcn2 to control cellular iron metabolism (48), was highly correlated with BrCa cell proliferation. Considering that circulating concentrations of Lcn2 and breast tumor Bdh2 expression levels were elevated in obese subjects and that RNAi-induced Bdh2 deficiency promotes BrCa cell apoptosis susceptibility (48), suggests that targeting this pathway to restrain or reverse breast tumor development in the context of obesity may be of therapeutic benefit.

Additionally, given that adjuvant breast cancer therapies aimed at reducing estrogen production and antagonizing ERα action in mammary tumors also exert whole body effects, the long-term impact of these therapeutic strategies on metabolic function should be monitored, especially in women susceptible for obesity and type 2 diabetes. Moreover, because obesity and type 2 diabetes reduce overall breast cancer survival rates in part by diminishing effectiveness of conventional anti-tumor therapeutics (59, 60), novel approaches to restrain breast cancer progression and reduce mortality in obese populations should be pursued.

In summary, the current investigation provides evidence that loss of ERα signaling in adipose tissue promotes obesity and induces the expression of Lcn2, a glycoprotein implicated in metabolic dysfunction and breast tumorigenesis and metastasis. Our findings suggest that reduced ERα action in adipose tissue, such as that associated with natural genetic variation, menopause, or obesity may be mechanistically linked to the increased prevalence of breast cancer observed under these conditions. Considering that obesity accounts for ~20% of all cancer deaths in women over age 50 (61), and based upon our findings that ERα deletion in adipose is causal of obesity, strategies to maintain ERα action in metabolic tissues including adipose tissue may be of benefit for cancer prevention.

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Note Added in Proof—The version of this article that was published on December 2, 2014 as a Paper in Press was incomplete. Two figures and supplemental Tables S1 through S4 were missing. Supplemental Tables S1 through S4 are now available, and the following figures have been added, revised, or relabeled. Panels C-H were added to Fig. 1. Panels H-K were removed from Fig. 3 and are now presented in Fig. 5. The original Fig. 4 has become Fig. 6 and there is a new Fig. 4. The original Fig. 5 has become Fig. 7. The original Fig. 6 has become Fig. 8. The original Fig. 7 has become Fig. 9 and includes two new panels, F and G. The axis labels in Figs. 2, 5, 8, and 9 have been revised.

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