RESEARCH ARTICLE

CC chemokine ligand 2 (CCL2) stimulates aromatase gene expression in mammary adipose tissue

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Abstract

Obesity is a risk factor for postmenopausal breast cancer. Obesity-related inflammation upregulates aromatase expression, the rate-limiting enzyme for estrogen synthesis, in breast adipose tissue (BAT), increasing estrogen production and cancer risk. The regulation of aromatase gene (*CYP19A1*) in BAT is complex, and the mechanisms linking obesity and aromatase dysregulation are not fully understood. An obesity-associated factor that could regulate aromatase is the CC chemokine ligand (CCL) 2, a pro-inflammatory factor that also activates signaling pathways implicated in *CYP19A1* transcription. By using human primary breast adipose stromal cells (ASCs) and aromatase reporter (hARO-Luc) mouse mammary adipose explants, we demonstrated that CCL2 enhances the glucocorticoid-mediated *CYP19A1* transcription. The potential mechanism involves the activation of PI.4 via ERK1/2 pathway. We also showed that CCL2 contributes to the pro-inflammatory milieu and aromatase expression in obesity, evidenced by increased expression of CCL2 and *CYP19A1* in mammary tissues from obese hARO-Luc mice, and subcutaneous adipose tissue from obese women. In summary, our results indicate that postmenopausal obesity

Abbreviations: ACTB, beta-actin; Akt, protein kinase B; ASCs, adipose stromal cells; BAT, breast adipose tissue; BMI, body mass index; CCL2, CC chemokine ligand 2; CLS, crown-like structures; CCR2, CC-receptor 2; CYP19A1, cytochrome P450 family 19 subfamily A member 1; DEX, dexamethasone; EGR, early growth response protein; ER+, estrogen receptor positive; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GRE, glucocorticoid response element; hARO-Luc, human aromatase-luciferase; HFD, high-fat diet; IL, interleukin; Luc, luciferase; LFD, low-fat diet; MAPK, mitogen-activated protein kinase; MCP-1, monocyte chemotactic protein-1; M2, alternatively activated macrophages; NF-κB, nuclear factor kappa B; OVX, ovariectomized; PI3K, phosphoinositide-3-kinase; PAI-1, plasminogen activator inhibitor 1; PI.4, aromatase promoter I.4; qPCR, quantitative-PCR; TNFα, tumor-necrosis factor alpha; VEGF, vascular endothelial growth factor; WAT, white adipose tissue.

Niina Saarinen and Sari Mäkelä contributed equally to this study.

may promote CCL2 production in BAT, leading to exacerbation of the menopause-related inflammatory state and further stimulation of local aromatase and estrogens. These results provide new insights into the regulation of aromatase and may aid in finding approaches to prevent breast cancer.

1 | INTRODUCTION

Adult obesity is a well-known risk factor for developing the most commonly diagnosed cancer among women, estrogen receptor positive (ER+) postmenopausal breast cancer.[1] After menopause, estrogens are produced from androgens by the enzyme aromatase in peripheral tissues, adipose tissue being the major source. Excessive fat accumulation associates with a chronic low-grade inflammation, and elevated aromatase expression and activity in breast adipose tissue (BAT) of postmenopausal women.[2,3] Combined, these changes are seen as the major driving forces increasing local estrogen production and promoting breast carcinogenesis after menopause.[4,5]

Human aromatase (*CYP19A1*) gene expression is controlled by alternative usage of at least nine tissue-specific promoters, each of which is expressed and regulated in a tissue-selective manner by distinct set of hormones, cytokines, and/or growth factors. In the healthy BAT, basal levels of *CYP19A1* mRNA transcripts are thought to be maintained by the exclusive use of the aromatase promoter I.4 (PI.4) and the balance between the transcriptional inducers and repressors of this promoter within the tissue. Upregulation of PI.4 in adipose tissue is closely linked with the local pro-inflammatory factors, tumor-necrosis factor (TNF)-α, interleukin (IL)-6, IL-1β, and IL-8, produced by hypertrophic adipocytes and resident macrophages.[6-8] Glucocorticoids, always present in BAT, are essential for these factors to induce PI.4 and *CYP19A1* expression in adipose stromal cells.[9,10] On the contrary, anti-inflammatory factors, such as IL-10 produced by, for example, mammary parenchyma and M2 macrophages, mediate its transcriptional repression.[11] In postmenopause, BAT inflammation is a common phenomenon, indicated by increased number of macrophages and crown-like structures (CLS, necrotic adipocytes surrounded by macrophages).[12-14] Although this process is primarily driven by postmenopausal weight gain, it may also occur in women with normal BMI.[15]

CCL2, also known as monocyte chemotactic protein-1 (MCP-1), is a key factor in macrophage-mediated inflammation in the obese white adipose tissue. It is widely recognized by its potent chemoattractant effect when binding to its receptor on monocytes, macrophages, and lymphocytes. High circulating and tissue levels of CCL2 have been found in obese subjects, as well as in women with breast cancer.[16-19] CCL2

and its receptor CCR2, a G-protein coupled receptor, are expressed by a large variety of cells in subcutaneous fat including mature adipocytes, fibroblasts, and immune cells, such as macrophages.[20-23] Interestingly, CCL2-CCR2 interaction involves the activation of the NF-κB and MAPK-ERK1/2 cellular pathways, both of them implicated in the transcription of aromatase PI.4 in BAT.[24,25] Given the importance of CCL2 in supporting the inflammatory environment and its potential in activating molecular pathways that regulate aromatase PI.4, we hypothesize that CCL2 could also play a direct role in modulating the local expression of aromatase in BAT. In this study, we focused on investigating the effects of adiposity and CCL2 on aromatase gene expression in the mammary tissue by using a transgenic human aromatase reporter mouse (hARO-Luc mouse) model, and subcutaneous adipose tissue samples and breast adipose stromal cells from women.

2 | MATERIALS AND METHODS

2.1 | Animals

Animal care and use was conducted in accordance with the Finnish Act on the Use of Animals for Experimental Purposes and EU laws, guidelines, and recommendations. The study protocols and procedures were approved by the national Animal Experiment Board in Finland (ESAVI/7471/04.10.03/2012).

The hARO-Luc mice[26] expressing the full regulatory region of the human aromatase gene (*CYP19A1*) were housed under standard conditions in the Central Animal Laboratory at the University of Turku. The mice were maintained with 12 hours light/dark cycle in constant temperature ($21 \pm 3°C$) and humidity ($55 \pm 15\%$), fed with soy-free RM3 chow (SDS, Whitham, Essex, UK) and tap water ad libitum. Mouse line maintenance and genotyping was done in collaboration with the Turku Center for Disease Modeling (TCDM).

Six-week-old hARO-Luc female mice were allocated into two groups with similar body weights and littermate distribution, and fed with purified low-fat D12450B diet (LFD, 10% of calories from fat) or high-fat D12492 diet (HFD, 60% of calories from fat) obtained from Research Diets Inc (New Brunswick, NJ). Experiments were done in three parts with 4 to 6 animals per group for a final number

of 16 mice on LFD and 17 mice on HFD. After 8 weeks, whole body fat content was measured with EchoMRI (Echo Medical Systems, Houston, TX, USA) and the mice were sacrificed with CO_2 followed by neck dislocation. Four hours fasting tail vein blood was collected for immediate fasting glucose analysis and heart puncture blood for serum. Inguinoabdominal mammary gland (lymph node removed) samples were collected to liquid nitrogen for the aromatase reporter (luciferase) activity measurements. Different adipose tissue depot samples (mammary gland, gonadal fat, and retroperitoneal fat) were collected to culture medium for ex vivo tissue-derived cytokine measurements (described below), and also to 10% of neutral-buffered formalin for histological analyses.

2.2 | Ex vivo mammary adipose tissue cultures

As previously described by McGillicuddy et al,[27] inguinoabdominal mammary gland, gonadal and retroperitoneal fat samples from hARO-Luc mice were divided into explants of approximately 100 mg each, and placed separately on 24-well culture plates containing 1 mL of Modified Eagle Medium (DMEM/F12, Life Technologies Ltd, Grand Island, NY, USA) supplemented with 100 IU/mL of penicillin and 100 μg/mL of streptomycin. In each experiment, 4 to 6 animals were used.

For tissue producing cytokine measurements, tissue culture media were collected after 24 hours incubation at 37°C in a humidified atmosphere with 5% CO_2, and then frozen at −70°C until analyzed. CCL2, leptin, IL-6, and TNFα concentrations were analyzed from tissue exposed media and serum samples with a MILLIPLEX MAP Mouse Adipocyte Magnetic Panel (#MADCYMAG-72K; Merck Millipore, Billerica, MA, USA) according to manufacturer's instructions by using the Luminex 200 analyzer (Luminex Corporation, Austin, TX, USA).

To investigate the potential modulation of luciferase (Luc) aromatase reporter activity by CCL2 in mammary gland explants, the antibiotics containing serum-free DMEM/F12 medium was supplemented with 250 nM dexamethasone (DEX, SERVA Electrophoresis GmbH, Heidelberg, Germany) alone or in combination with 100 ng/mL of CCL2 (PeproTech, Rocky Hill, NJ, USA). After 24 hours incubation, mammary gland tissues were collected and analyzed for Luc activity.

2.3 | Luciferase reporter activity assay

The Luc reporter activity in mammary gland was measured as described before.[26] Shortly, tissue samples were homogenized in buffer containing 25 mM of Tris acetate (pH 7,8), 1.5 of mM EDTA, 10% of glycerol, 1% of Triton X-100, 2 mM of dithiothreitol, and plus 1 x Roche Complete Miniproteinase inhibitor tablets (Roche Diagnostics, Penzberg, Germany). The homogenates were then centrifuged at 800 g for 30 minutes at +4°C. Luc activity was measured from the supernatant with the Luciferase assay kit (BioThema AB, Handen, Sweden) according to manufacturer's instructions by using the Victor2 Multilabel counter (PerkinElmer, Turku, Finland). The results were related to the sample weight or protein content measured by using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL, USA) according to manufacturer's instructions.

2.4 | Subcutaneous adipose tissue

Snap-frozen human abdominal subcutaneous adipose tissue samples were purchased from ZenBIO Inc (Research Triangle Park, NC, USA). Tissue samples were collected from nonobese BMI ($\leq$25) or obese ($\geq$30) women aged 32-47 years.

2.5 | Primary human breast adipose stromal cells (ASCs)

Primary breast ASCs isolated from breast tissues of women undergoing elective breast reduction or mastectomy were purchased from ZenBIO Inc or were obtained from Turku University Hospital (Ethical committee approval ETKM 23/2018). The patients gave their written informed consent for sample usage. ASCs were isolated from dissected human tissue by enzymatic digestion and differential pulse centrifugations as described previously.[28,29] At least three independent experiments using 3 different donors cells each run in triplicates (aged 20-66 y, BMI: 25.1-29.1 kg/m^2) . For expansion, ASCs were plated at a density of $2x10^4$/cm^2 in DMEM/F12 supplemented with 5%-15% heat inactivated fetal bovine serum, 100 IU/mL penicillin and 100 μg/mL streptomycin and were maintained at 37°C humidified atmosphere with 5% CO_2. Cells were passaged once by treating with 0.25% of trypsin-EDTA and seeded (40 000 cells/well) on 12-well plates. At 70% confluency, cells were serum starved for 12 hours in medium containing 0.1% of BSA. Cells were treated with 10 nM DEX alone and in combination with 100 ng/mL CCL2 and with one of the inhibitors (10 μM): U0126 (MEK-ERK1/2 inhibitor, Sigma-Aldrich), BAY 11-7082 (NF-κB inhibitor, Sigma-Aldrich), or RS 504393 (CCR2 inhibitor, Tocris Bioscience, Bristol, UK). The effectiveness and relevance of these inhibitors to inhibit each of their target proteins have been widely demonstrated in different biological systems.[30-35]

2.6 | RNA isolation, cDNA synthesis, and quantitative-PCR

Total RNA was isolated from human ASCs or snap-frozen adipose tissue samples by using TRIsure (Bioline, Luckenwalde, Germany) according to the manufacturer's instructions. One μg of total RNA was treated with deoxyribonuclease I (DNase I amplification grade, Sigma-Aldrich, St Louis, MO, USA) and converted to cDNA by using SensiFAST cDNA synthesis kit (Bioline, London, UK). Quantitative-PCR (qPCR) was performed using the Dynamo Flash SYBR Green qPCR Kit (Thermo Fisher Scientific) on the CFX96 Real-Time C1000 Thermal Cycler (Bio-Rad, Hercules, CA, USA). Primers used were: *CYP19A1* F: 5′-TTGGAAATGCTGACCCGAT-3′, *CYP19A1* R: 5′-CAGGAATCTGCCGTGGGAGA-3′, *aromatase PI.4* F: 5′-GTAGAACGTGACCAACTGG-3, *aromatase PI.4* R: 5′-CACCCGGTTGTAGTAGTTGCAGGCACTGCC-3′, *CCL2* F: 5′-AATGGTCTTGAAGATCACAGCTTC-3′ and *CCL2 R:* 5′-TAGCAGCCACCTTCATTCCCCAAG-3′. Cycling conditions were 95°C for 10 minutes and variable cycles of 95°C for 10 seconds, 59°C for 30 seconds and 72°C for 15 seconds. All samples and standards were run in triplicate. Expression levels of the final products were normalized to human housekeeping genes: *ACTB* F: 5′-TGCGTGACATTAAGGAGAAG-3′, *ACTB* R: 5′- GCTCGTAGCTCTTCTCCA-3′ and *GAPDH* F: 5′-TGGTATCGTGGAAGGACTCATGAC-3′, *GAPDH* R: 5′-ATGCCAGTGAGCTTCCCGTTCAGC-3′.

2.7 | ERK1/2-MAPK activity

ASCs were plated in 24-well plates at the density of 20 000 cells/well. At 80%-90% confluency, cells were treated with one the inhibitors (10 μM): R504393 or U0126. Fifteen min later, 100 ng/mL of CCL2 was added, and after another 15 minutes, endogenous levels of phosphorylated ERK1/2

protein was measured from the cell lysates (containing 10-15 μg of protein per well) by using ERK1/2 (pT202/Y204) SimpleStep ELISA Kit (Abcam). The assay was performed according to the manufacturer's instructions and analyzed at 450 nm using VICTOR Multilabel Plate Reader (Perkin Elmer, Turku, Finland).

2.8 | Statistical analyses

Statistical analyses were performed using GraphPad Prism version 8.0.1 for windows (GraphPad Software Inc, San Diego, CA, USA). To assess the statistical significance between two groups, unpaired parametric t test was used. The differences between multiple treatments groups were tested by one-way analysis of variance (ANOVA) followed by Tukey's or Dunnett's multiple comparisons test. Pearson correlation test was used to evaluate the relationship between two variables. Differences were considered statistically significant at $P \leq .05$. Data are expressed as mean ± standard error of mean.

3 | RESULTS

3.1 | HFD induces weight gain and adiposity and impairs the metabolic status of female hARO-Luc reporter mice

Female hARO-Luc mice fed with HFD gained more weight and accumulated more body fat during the 8-week intervention, compared to the LFD-fed mice (Figure 1A,B). At the end of the experiment, HFD-fed mice were at least 38% heavier and had accumulated 35% more fat than mice fed with LFD and exhibited higher circulating leptin and glucose levels (Figure 1C,D).
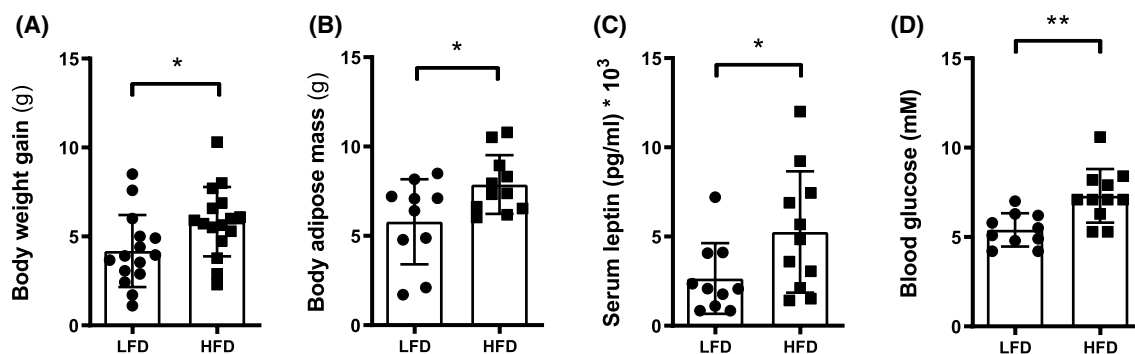
FIGURE 1 HFD induces obesity and obesity-related metabolic disturbances in female hARO-Luc mice. A, Body weight gain. B, Body adipose mass. C, Serum leptin levels. D, Circulating blood glucose levels. LFD N = 16 mice and HFD N = 17 mice. Data are shown as mean ± SEM and was analyzed by unpaired parametric t test. *P < .05, **P < .001

3.2 | Weight gain and adiposity are drivers of both the increased aromatase Luc reporter activity and CCL2 levels in mammary gland of HFD-fed hARO-Luc mice

As has been shown before, HFD induces a variable weight gain in FVB/N mice.[36,37] In our study, the majority of animals on HFD exhibited evident metabolic changes, but three out of 17 mice did not gain weight or, gained less than 20%, after the 8 weeks of dietary intervention. In animals with ≥20% weight gain the expression of aromatase Luc reporter in mammary fat tissue was significantly higher than in the mammary of mice that remained lean (Figure 2A). Furthermore, mammary gland hARO-Luc reporter expression correlated positively with both weight gain and adiposity (Figure 2B,C).

Interestingly, mammary Luc reporter expression correlated positively with tissue pro-inflammatory factors, CCL2 (Figure 2D), IL-6 ($R^2 = 0.72$, $P = .05$), and leptin ($R^2 = 0.60$, $P = .07$). However, only a low number (≤2 per mm^2) of CLS was detected in mammary fat tissue, despite the weight gain and increased adiposity. Additionally, CLS numbers did not correlate with the tissue Luc reporter expression (data not shown). Notably, in the gonadal or retroperitoneal fat tissue no significant correlation was found between CCL2 or IL-6 levels and Luc reporter activity (Supplementary Table

1). The levels of TNFα, IL-1β, IL-1α, adiponectin, VEGF, PAI-1, and resistin did not correlate with aromatase reporter expression in mammary, gonadal, or retroperitoneal fat tissue (Supplementary Table 1).

3.3 | *CCL2* and *CYP19A1* mRNA expressions are upregulated in subcutaneous adipose tissue of obese women

In order to confirm the findings from the mouse model, we investigated whether adipose tissue from obese and lean women show any difference in the expression of *CCL2* or total *CYP19A1* mRNA. Our results show that subcutaneous adipose tissue of obese women exhibited significantly higher aromatase gene expression and almost sixfold increase in *CCL2* gene expression compared to those tissue samples collected from nonobese women (Figure 3A,B).

3.4 | CCL2 upregulates *CYP19A1* in the breast adipose tissue through promoter I.4

To examine whether CCL2 directly modulates glucocorticoid-dependent aromatase gene expression through promoter I.4
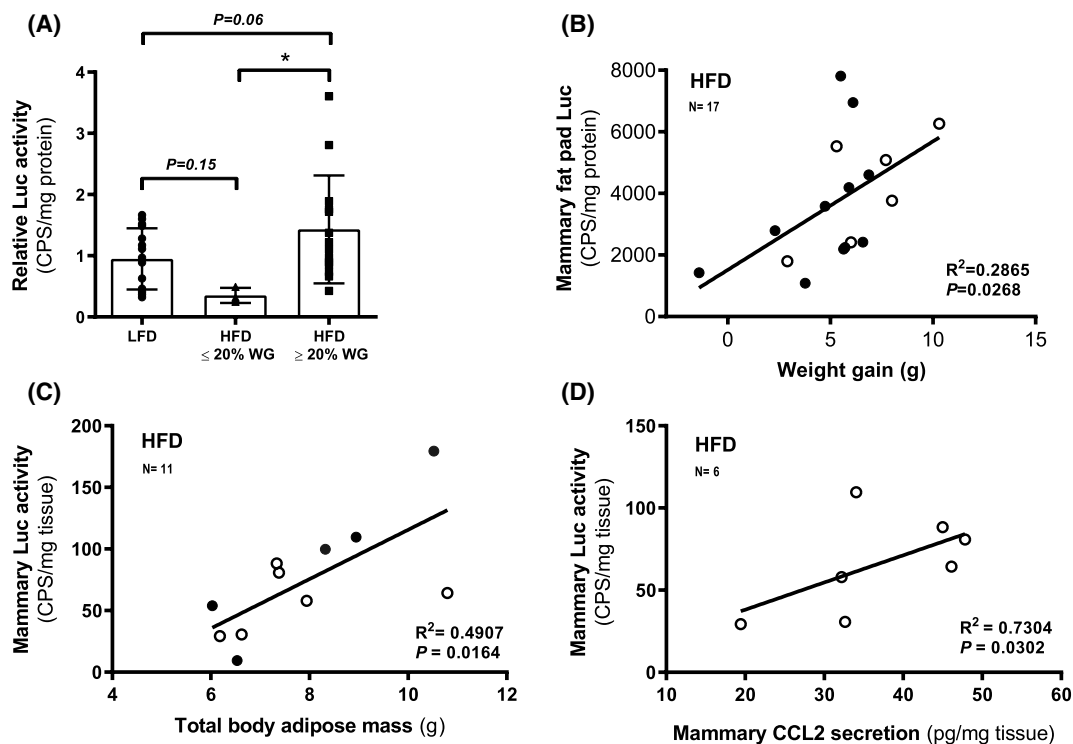
FIGURE 2 In HFD-fed hARO-Luc female mice weight gain and adiposity associate with increased both Luc aromatase reporter activity and CCL2 expression in mammary gland. A, hARO-Luc reporter activity. Data are shown as mean ± SEM and was analyzed by two-way ANOVA with Tukey's multiple comparisons post hoc test. *$P < .05$, no significant (n.s). Correlations between: B, Luc reporter activity in mammary gland and body weight gain, C, Luc reporter activity in mammary gland and body adiposity, and D, Luc reporter activity and CCL2 levels in mammary gland of HFD-fed mice. Open circles in figures B-D denote data from the same six animals. Counts per second (CPS)

(A)

(B)
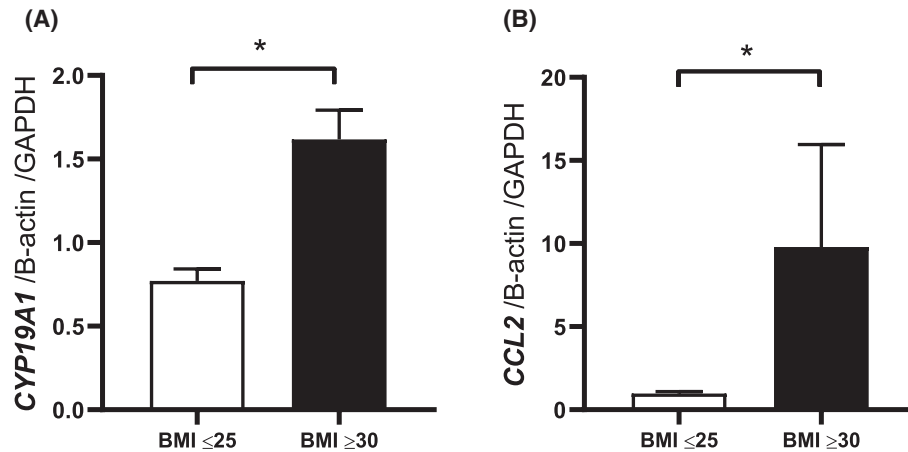
FIGURE 3 Increased expression of *CYP19A1* and *CCL2* in subcutaneous adipose tissue of women with high BMI. Differences in A, *CYP19A1* aromatase and B, *CCL2* gene mRNA expression levels in subcutaneous adipose tissue from nonobese and obese postmenopausal women. Lean women N = 4 and obese women N = 4. Data (mean ± SEM) were analyzed by unpaired parametric t test. *$P < .05$

in mammary adipose tissue, ex vivo cultures of hARO-Luc tissue were performed. Indeed, in the presence of DEX, addition of CCL2 caused a further stimulation of Luc reporter activity in mammary tissue explants from hARO-Luc mice (Figure 4).

Next, we investigated whether CCL2 could also regulate aromatase expression in human breast adipose tissue. In cultured primary human breast ASCs, similar to hARO-Luc mammary gland tissue, CCL2 enhanced total *CYP19A1* gene expression levels (Figure 5A). CCL2-induced aromatase *CYP19A1* gene transcription was prevented by blocking the CCL2 receptor CCR2, with the specific receptor antagonist RS504303 (Figure 5A). Additionally, to examine whether CCL2 mediates aromatase expression via aromatase promoter I.4, transcript levels of this promoter were quantified in ASCs after treatments. Correspondingly, CCL2 enhanced over threefold the effect of glucocorticoids on aromatase promoter I.4 transcripts expression (Figure 5B), thus indicating a specific action through this distal promoter. No significant induction of aromatase promoter II-driven aromatase expression was detected (Supplementary Figure 1).

3.5 | Activation of the MAPK-ERK1/2 signaling pathway is necessary for CCL2 effect on *CYP19A1* gene expression in ASCs

The intracellular signaling pathway displayed upon CCL2-CCR2 activation on the cell surface involves both the NF-κB and MAPK inflammatory cascades. Besides of being important for modulating cell immune responses, the two pathways are also known to regulate aromatase promoter I.4 transcriptional activity in adipose stromal cells.[25,31,38] To determine the potential implication of the two signaling pathways in mediating CCL2 effect on aromatase gene expression,
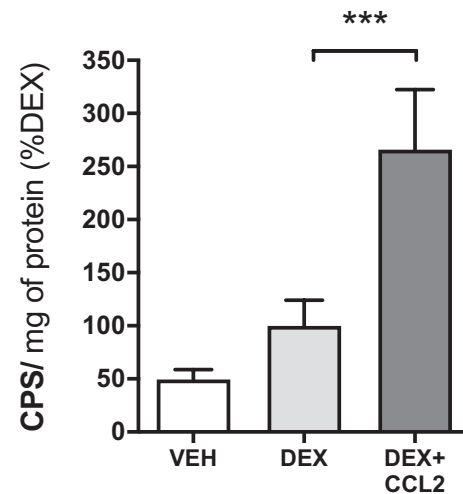
FIGURE 4 In mammary tissue explants of hARO-Luc mice, CCL2 enhances glucocorticoid-dependent aromatase reporter expression. Counts per second (CPS). Data (mean ± SEM) are shown relative to dexamethasone (DEX, set at 100%). Data from three experiments (4 to 6 animals per experiment) analyzed by two-way ANOVA with Tukey's multiple comparisons post hoc test. ***$P < .001$. DEX: 250 nM, CCL2: 100 ng/mL

specific inhibitors of both cascade proteins were tested in ASC cultures in the presence of DEX and CCL2. Our results demonstrate that inhibition of the ERK1/2 pathway with the inhibitor U0126 led to a significant reduction in the CCL2 stimulatory effect on both *PI.4* and *CYP19A1* expression (Figure 5B,C, respectively), whereas inhibition of the NF-κB pathway did not show significant effect on total *CYP19A1* expression (Figure 5C).

The activation of the MAPK-ERK1/2 signaling pathway in primary breast ASCs by CCL2 was further confirmed using a Phospho-ERK1/2 ELISA assay. Phosphorylated ERK1/2 protein levels were measured after treatment with CCL2 alone
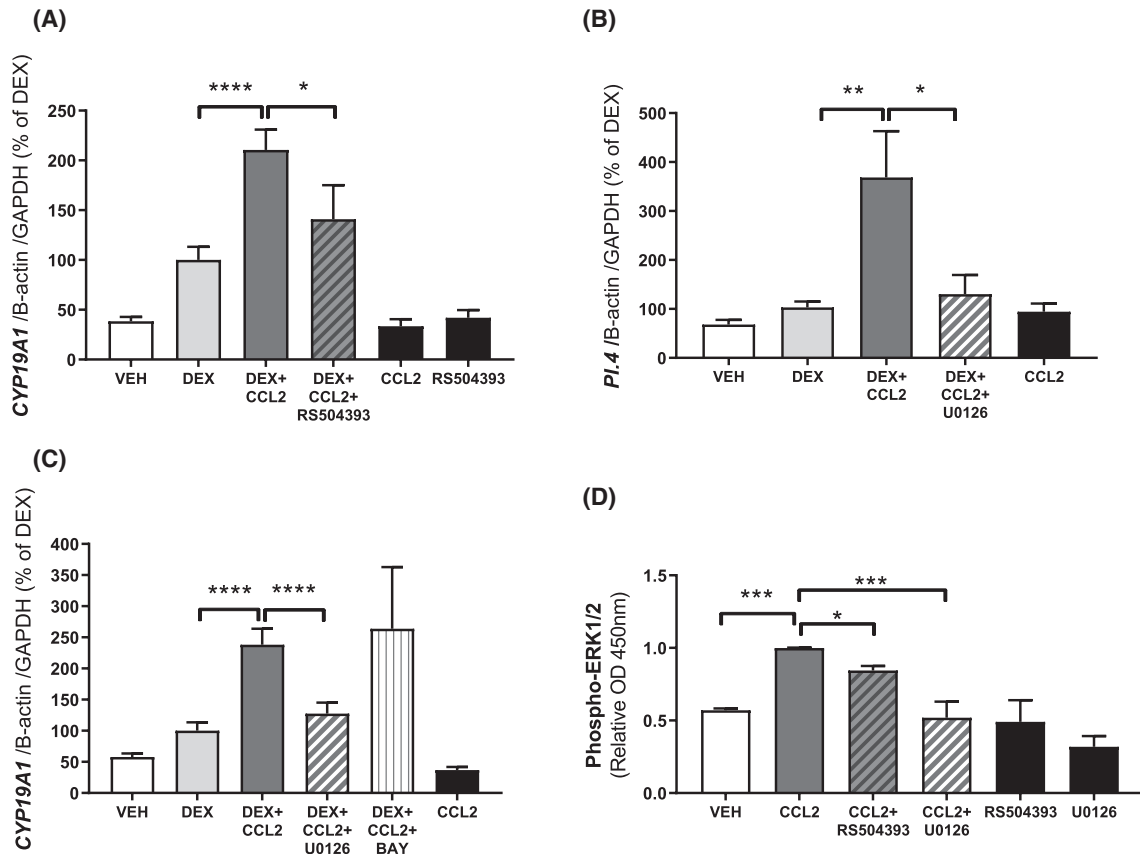
FIGURE 5 CCL2 induces aromatase mRNA expression in primary human breast ASCs through the MAPK-ERK1/2 signaling pathway. A, CCL2 upregulates glucocorticoid-dependent total *CYP19A1* mRNA expression, and the effect of CCL2 is inhibited by the specific CCR2 inhibitor RS504393. B, CCL2 increases glucocorticoid-dependent PI.4-specific transcript expression. C, Inhibition of MAPK-ERK1/2 signaling pathway by the MEK-ERK1/2 inhibitor U0126 blocks CCL2 effect on total *CYP19A1* mRNA expression. D, CCL2 activates ERK1/2 in breast ASCs (measured as phosphorylation of ERK1/2-activated protein by ELISA). Data (mean ± SEM) are shown in A-C relative to dexamethasone (DEX, set at 100%) and in D relative to CCL2 (set at 1). Data are obtained from 3 to 4 experiments (different donors), each treatment with three replicates. Statistical analyses were performed using two-way ANOVA and Dunnett's multiple comparisons post hoc test. $*P < .05$, $**P < .001$, $****P < .0001$. DEX: 10 nM. CCL2: 100 ng/mL. RS504393, U0126, and BAY-11-7082 (BAY, NF-κB inhibitor): 10 μM

or in combination with specific CCR2 inhibitor RS 504393 or with MEK-ERK1/2 inhibitor U0126. As expected, CCL2 stimulated phosphorylation of ERK1/2 intracellular pathway in breast preadipocytes (Figure 5D), while preventive treatment with CCR2 and MEK-ERK1/2 inhibitors reduced CCL2-mediated phosphorylation of ERK1/2 (Figure 5D).

4 | DISCUSSION

Inflammation and increased expression of aromatase are important risk factors for postmenopausal breast cancer. However, the regulation of aromatase (*CYP19A1*) gene expression in breast tissue is complex, and the mechanisms linking adipose tissue inflammation to the increase in aromatase expression are not fully understood. In this study, we demonstrated that CCL2, a chemokine secreted by adipocytes and macrophages, besides being overexpressed in WAT of obese

women, also directly regulates aromatase gene expression in BAT via promoter I.4.

In response to adipose tissue expansion during obesity, hypertrophic adipocytes secrete chemokines, particularly CCL2, to recruit monocytes from the circulation, which facilitates tissue remodeling and adaptation to the altered metabolism. Increased adiposity often associates with abnormally high secretion of CCL2, which in turn, promotes monocyte recruitment and development of CLS, and augments production of pro-inflammatory factors, including TNFα, IL-6, PGE2, and CCL2, and ultimately, increases aromatase expression in BAT.[39,40] Although the most important actions of CCL2 are related to chemotaxis, this cytokine also triggers several intracellular signals implicated in inflammation, angiogenesis, and carcinogenesis.[41-44] Most of these intracellular signals associate with the activation of the PI3K/Akt/NF-κB cascade and the mitogen-activated protein kinase (MAPK) pathway, particularly the Ras/MEK/ERK

cascade.[21,24,25,45] Interestingly, these signaling pathways have also been demonstrated to regulate aromatase promoter I.4 transcript expression in breast ASCs.[31] Given the importance of CCL2 in macrophage-mediated BAT inflammation, as well as its potential to activate NF-κB and MAPK pathways, it is pertinent to propose that CCL2 may also regulate aromatase expression in a transcriptional level. In our study, we showed that, indeed, CCL2 activates aromatase gene transcription in the breast tissue. By utilizing hARO-Luc-derived mammary tissue explants and primary breast ASCs, we have found that CCL2 specifically induces glucocorticoid-dependent *CYP19A1* expression via the activation of the distal promoter I.4. Such activation seems to be particularly mediated through its membrane receptor, CCR2, and by the following stimulation of MAPK/ERK1/2 signaling pathway in primary breast ASCs. However, we cannot exclude the possibility that other pro-inflammatory pathways in addition to MAPK/ERK1/2 and NF-κB, may also be implicated in this stimulatory effect. We then propose that CCL2 may regulate *CYP19A1* transcription in similar manner than TNFα, a well-known inducer of aromatase promoter I.4 in adipose tissue. Prior studies have reported that TNFα acts cooperatively with glucocorticoids to induce *CYP19A1* expression in breast ASCs, acting through NF-κB and MAPK signaling cascades, downstream stimulation of EGR2 and EGR3 transcription factors, and activation of the AP-1 element within PI.4 transcriptional region.[31,46] Nevertheless, further studies are needed to determine whether transcriptional mechanisms of CCL2 involve also the activation of AP-1 responsive element.
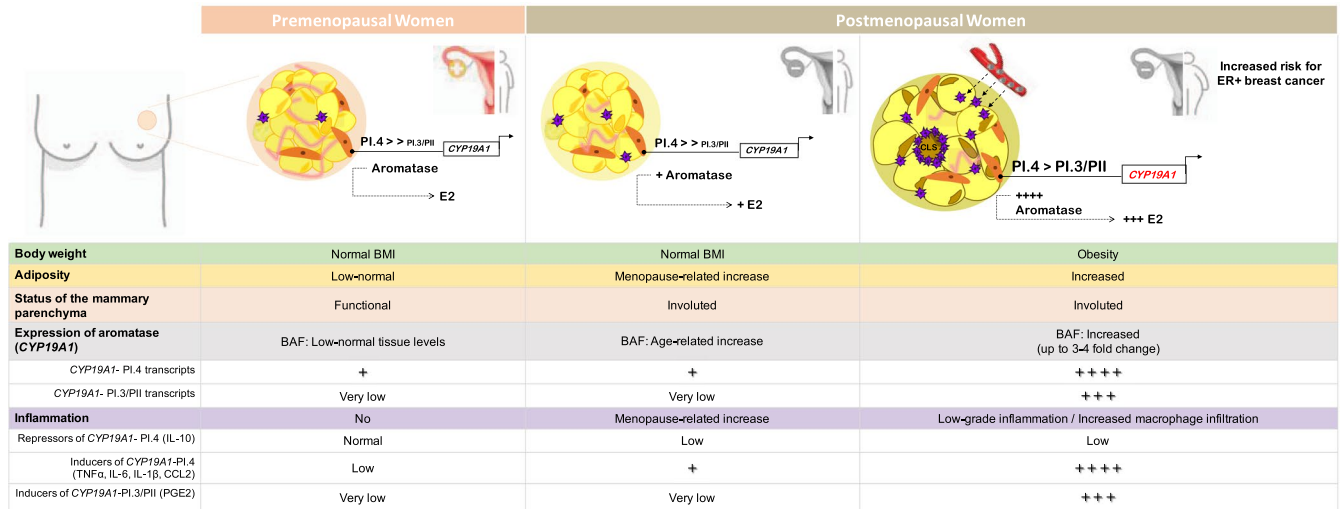
Expression of aromatase in a noncancerous BAT either before or after menopause is primarily driven by the use of the I.4 promoter located approximately 73 kb upstream of the common coding region. In noncancerous conditions, low levels of aromatase transcripts are thought to be maintained via PI.4 thanks to normal tissue levels of pro-inflammatory factors, providing low levels of estrogens in the breast tissue.[7,47-49] As previously established, elevated BMI in postmenopausal women positively associates with higher circulating and adipose tissue levels of estrogens, attributed, in part, to increased inflammation and aromatase expression in WAT.[15,50-52] Adiposity and subsequent adipose tissue inflammation are known to promote a gradual increase in promoter I.4 transcripts. It has also been reported that glucocorticoid levels are higher in the obese subcutaneous adipose tissue.[53,54] The increase in BAT aromatase expression in obese women is likely due to pro-inflammatory factors rather than glucocorticoids as there is no evidence of a direct association between systemic glucocorticoid and aromatase expression levels.[10,55] In the case of morbid obesity and severe inflammation, as well as breast cancer, the increased activity of aberrant promoters I.3 and II in BAT upregulate *CYP19A1* transcription levels up to10-fold higher than those in a healthy BAT.[12,55-58]

The magnitude of aromatase gene upregulation in BAT, however, depends, for example, on the degree of adiposity, the levels of pro-inflammatory factors and the abundance of CLS.[4,13,52,59,60] In line with this, we observed a positive correlation between mammary aromatase reporter activity and weight gain in HFD-fed female hARO-Luc (FVB/N) mice. Interestingly, no weight-related changes in aromatase reporter expression were observed in gonadal, visceral or retroperitoneal fat, indicating that obesity-related aberration in *CYP19A1* regulation is fat depot specific. This is supported by earlier studies with Arom[hum] FVB/N[61] mouse models, showing upregulation of aromatase by HFD feeding in mammary fat pad, but not in intra-abdominal fat depots. In these studies, unlike ours, higher activity of promoters I.4 and II was associated with a significant increase in CLS density, indicating a rather severe inflammatory condition. Interestingly, in our current study, similar to our earlier studies in hARO-Luc males[62] and ovariectomized females,[11] weight gain and adiposity-induced mammary gland aromatase reporter expression via promoter I.4 independent of CLS. This indicates that the pro-inflammatory milieu, prior the onset of CLS, may be sufficient to activate promoter I.4 and upregulate aromatase expression in breast tissue.

The inflammatory milieu and the regulation of aromatase gene transcription in subcutaneous adipose tissue differ from visceral fat depots.[11,61,63,64] Despite the significant increase in fat content in visceral and subcutaneous adipose tissue in our in vivo experiments, aromatase expression and pro-inflammatory factors were significantly altered only in the mammary fat pad. And unlike visceral fat depots, levels of pro-inflammatory cytokines IL-6 and CCL2, and leptin, were significantly higher in mammary fat pad of animals with significant weight gain and correlated positively with mammary aromatase reporter activity. It is important to note that the use of ovary intact mice in this study may have attenuated the inflammation and, in turn, the levels of cytokines and aromatase in the mammary fat pad and other fat tissue depots. In obese women, level of CCL2 is increased in WAT, where it also associates with macrophage infiltration and insulin resistance.[16,39,65,66] Furthermore, circulating CCL2 increases in postmenopause, independent of BMI, as well as in serum and tumors of women with breast cancer.[17-19,66-69] In this study, the expression levels of CCL2 and aromatase transcripts in subcutaneous adipose tissue of obese women were higher, compared to the adipose tissue of lean women, supporting the finding of CCL2 as a factor that induces adipose aromatase expression.

High circulating level of estrogens is accepted as a marker of increased risk for developing postmenopausal breast cancer. Estrogen concentrations in the bloodstream after menopause reflect the metabolism and production rates of estrogens in non-gonadal sites, WAT being the main source

	Premenopausal Women	Postmenopausal Women	
Body weight	Normal BMI	Normal BMI	Obesity
Adiposity	Low-normal	Menopause-related increase	Increased
Status of the mammary parenchyma	Functional	Involuted	Involuted
Expression of aromatase (CYP19A1)	BAF: Low-normal tissue levels	BAF: Age-related increase	BAF: Increased (up to 3-4 fold change)
CYP19A1- PI.4 transcripts	+	+	++++
CYP19A1- PI.3/PII transcripts	Very low	Very low	+++
Inflammation	No	Menopause-related increase	Low-grade inflammation / Increased macrophage infiltration
Repressors of CYP19A1- PI.4 (IL-10)	Normal	Low	Low
Inducers of CYP19A1-PI.4 (TNFα, IL-6, IL-1β, CCL2)	Low	+	++++
Inducers of CYP19A1-PI.3/PII (PGE2)	Very low	Very low	+++

FIGURE 6 Regulation of aromatase expression in the postmenopausal breast adipose stroma. Cessation of ovarian hormone production causes a wide range of changes that lead to increase aromatase expression in BAT. Postmenopausal hormone milieu predisposes women to weight gain, as well as to BAT inflammation, which, eventually, may increase local estrogen biosynthesis and promote breast carcinogenesis. BAF, breast adipose fibroblast

of estrogen biosynthesis. However, it is not known how well blood hormone concentrations reflect the actual local synthesis of estrogens in the breast tissue.[4,5,70-72] Recent studies indicate that postmenopausal women with normal BMI may exhibit signs of WAT inflammation.[15,73] Furthermore, it should be noted that postmenopausal status is associated with increased expression of aromatase in BAT, independent of BMI. Therefore, it is not only the obese women who are at high risk of breast cancer, but also it is well possible that (some) postmenopausal women with normal BMI and blood estrogen levels may present with inflammation and increased aromatase expression in breast tissue, causing an increase in their cancer risk.

Taken together, cessation of ovarian hormone production causes a wide range of changes that lead to increase in BAT aromatase expression (summarized in Figure 6). Postmenopausal hormone milieu stimulates weight gain, as well as development of WAT inflammation. Furthermore, postmenopausal regression of mammary parenchyma, and subsequent loss of local IL-10 production, upregulates aromatase specifically in BAT. Here, we demonstrated that CCL2-CCR2 axis induces aromatase transcription via promoter I.4 in breast tissue, in a process mediated by the activation of its MAPK/ERK1/2-related signaling pathway. This suggests that CCL2 is one of the drivers behind the increased aromatase expression and estrogen synthesis in BAT of obese postmenopausal women.

The key strengths of this study are the use of mouse models, adipose tissues, and primary breast adipose stromal cells from mouse and human females, which facilitate elucidating the mechanisms regulating aromatase in adipose tissue, and translational research aimed at understanding how obesity and adipose tissue inflammation predispose women to breast cancer. Yet, the use of primary cells causes experimental limitations due to poor transfection efficiency and restricted lifespan of cells. To overcome this, we have used the available small molecule inhibitors for the most important mediators along the CCL2 signaling pathway, to provide initial evidence for the involvement of the ERK1/2 signaling cascade in the CCL2-mediated activation of aromatase. However, further studies are needed for more detailed information.

In conclusion, as regards healthy postmenopausal women with normal or moderately elevated BMI, increased transcriptional activity of promoter I.4, regulated by numerous pro- and anti-inflammatory factors, is the underlying force driving aromatase expression, while in women with morbid obesity and /or breast cancer, aromatase expression is mainly driven by the more potent proximal promoters I.3 and PII. Our data suggest an important role for body weight management and control of WAT inflammation in primary breast cancer prevention.

CONFLICT OF INTEREST

The authors declare there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

AUTHOR CONTRIBUTION

GM, EY, NS, and SM designed the experiments and analyzed the data, EP and PH collected breast tissue samples and isolated primary breast ASCs, and GM, EY, LP, NS, and DD performed experiments. All authors participated in the preparation of the manuscript.

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REFERENCES

1. Vrieling A, Buck K, Kaaks R, Chang-Claude J. Adult weight gain in relation to breast cancer risk by estrogen and progesterone receptor status: a meta-analysis. *Breast Cancer Res Treat*. 2010;123:641-649.
2. Thijssen JHH, Longcope C. Post-menopausal estrogen production. In: van Keep PA, Greenblatt RB, Albeaux-Fernet M, eds. *Consensus on Menopause Research: A Summary of International Opinion The Proceedings of the First International Congress on the Menopause held at La Grande Motte, France, in June, 1976, under the auspices of The American Geriatric Society and The Medical Faculty of The University of Montpellier*. Dordrecht: Springer Netherlands; 1976:25-28.
3. Simpson ER, Bulun SE. Competitive reverse transcription-polymerase chain reaction analysis indicates that levels of aromatase cytochrome P450 transcripts in adipose tissue of buttocks, thighs, and abdomen of women increase with advancing age. *J Clin Endocrinol Metab*. 1994;78:428-432.
4. Brown KA. Impact of obesity on mammary gland inflammation and local estrogen production. *J Mammary Gland Biol Neoplasia*. 2014;19:183-189.
5. Simpson ER, Brown KA. Obesity and breast cancer: role of inflammation and aromatase. *J Mol Endocrinol*. 2013;51:T51.
6. Simpson ER, Mahendroo MS, Means GD, et al. Aromatase cytochrome P450, the enzyme responsible for estrogen biosynthesis*. *Endocr Rev*. 1994;15:342-355.
7. Agarwal VR, Bulun SE, Leitch M, Rohrich R, Simpson ER. Use of alternative promoters to express the aromatase cytochrome P450 (CYP19) gene in breast adipose tissues of cancer-free and breast cancer patients. *J Clin Endocrinol Metab*. 1996;81:3843-3849.
8. Zhao Y, Agarwal VR, Mendelson CR, Simpson ER. Transcriptional regulation of CYP19 gene (aromatase) expression in adipose stromal cells in primary culture. *J Steroid Biochem Mol Biol*. 1997;61:203-210.
9. Anwar AJ, Anderson LA, Eggo MC, et al. Glucocorticoid regulation of P450 aromatase activity in human adipose tissue: gender and site differences. *J Clin Endocrinol Metab*. 2002;87:1327-1336.
10. Newton CJ, Samuel DL, James VHT. Aromatase activity and concentrations of cortisol, progesterone and testosterone in breast and abdominal adipose tissue. *J Steroid Biochem*. 1986;24:1033-1039.
11. Martínez-Chacón G, Brown KA, Docanto MM, et al. IL-10 suppresses TNF-α-induced expression of human aromatase gene in mammary adipose tissue. *FASEB J*. 2018;32:3361-3370.
12. Morris PG, Hudis CA, Giri D, et al. Inflammation and increased aromatase expression occur in the breast tissue of obese women with breast cancer. *Cancer Prev Res* (Philadelphia, PA). 2011;4:1021-1029.
13. Subbaramaiah K, Howe LR, Bhardwaj P, et al. Obesity is associated with inflammation and elevated aromatase expression in the mouse mammary gland. *Cancer Prev Res* (Philadelphia, PA). 2011;4:329-346.
14. Iyengar NM, Morris PG, Zhou XK, et al. Menopause is a determinant of breast adipose inflammation. *Cancer Prev Res* (Philadelphia, PA). 2015;8:349-358.
15. Brown KA, Iyengar NM, Zhou XK, et al. Menopause is a determinant of breast aromatase expression and its associations with BMI, inflammation, and systemic markers. *J Clin Endocrinol Metab*. 2017;102:1692-1701.
16. Kim CS, Park HS, Kawada T, et al. Circulating levels of MCP-1 and IL-8 are elevated in human obese subjects and associated with obesity-related parameters. *Int J Obes*. 2006;30:1347-1355.
17. Dutta P, Sarkissyan M, Paico K, Wu Y, Vadgama JV. MCP-1 is overexpressed in triple-negative breast cancers and drives cancer invasiveness and metastasis. *Breast Cancer Res Treat*. 2018;170:477-486.
18. Lubowicka E, Przylipiak A, Zajkowska M, et al. Plasma chemokine CCL2 and its receptor CCR2 concentrations as diagnostic biomarkers for breast cancer patients. *Biomed Res Int*. 2018;2018:9.
19. Svensson S, Abrahamsson A, Rodriguez GV, et al. CCL2 and CCL5 are novel therapeutic targets for estrogen-dependent breast cancer. *Clin Cancer Res*. 2015;21:3794.
20. Deshmane SL, Kremlev S, Amini S, Sawaya BE. Monocyte chemoattractant protein-1 (MCP-1): an overview. *J Interferon Cytokine Res*. 2009;29:313-326.
21. Tsuyada A, Chow A, Wu J, et al. CCL2 mediates cross-talk between cancer cells and stromal fibroblasts that regulates breast cancer stem cells. *Can Res*. 2012;72:2768-2779.
22. Baek SJ, Kang SK, Ra JC. In vitro migration capacity of human adipose tissue-derived mesenchymal stem cells reflects their expression of receptors for chemokines and growth factors. *Exp Mol Med*. 2011;43:596.
23. Ignacio RMC, Gibbs CR, Lee E-S, Son D-S. Differential chemokine signature between human preadipocytes and adipocytes. *Immune Netw*. 2016;16:189-194.
24. Binder NB, Niederreiter B, Hoffmann O, et al. Estrogen-dependent and C-C chemokine receptor-2–dependent pathways determine osteoclast behavior in osteoporosis. *Nat Med*. 2009;15:417.
25. Tang C-H, Tsai C-C. CCL2 increases MMP-9 expression and cell motility in human chondrosarcoma cells via the Ras/Raf/MEK/ERK/NF-κB signaling pathway. *Biochem Pharmacol*. 2012;83:335-344.
26. Strauss L, Rantakari P, Sjögren K, et al. Seminal vesicles and urinary bladder as sites of aromatization of androgens in men, evidenced by a CYP19A1-driven luciferase reporter mouse and human tissue specimens. *FASEB J*. 2012;27:1342-1350.
27. McGillicuddy FC, Harford KA, Reynolds CM, et al. Lack of interleukin-1 receptor I (IL-1RI) protects mice from high-fat

diet-induced adipose tissue inflammation coincident with improved glucose homeostasis. *Diabetes*. 2011;60:1688.

28. Stingl J, Emerman JT, Eaves CJ. Enzymatic dissociation and culture of normal human mammary tissue to detect progenitor activity. In: Helgason CD, Miller CL, eds. *Basic Cell Culture Protocols*. Totowa, NJ: Humana Press; 2005:249-263.

29. Peuhu E, Kaukonen R, Lerche M, et al. SHARPIN regulates collagen architecture and ductal outgrowth in the developing mouse mammary gland. *EMBO J*. 2017;36:165-182.

30. Burkhard K, Shapiro P. Use of inhibitors in the study of MAP kinases. In: Sesger R, ed. *MAP Kinase Signaling Protocols. Methods in Molecular Biology (Methods and Protocols)*. Totowa: Humana Press; 2010:107-122.

31. To SQ, Knower KC, Clyne CD. NFκB and MAPK signalling pathways mediate TNFα-induced early growth response gene transcription leading to aromatase expression. *Biochem Biophys Res Comm*. 2013;433:96-101.

32. Marampon F, Bossi G, Ciccarelli C, et al. MEK/ERK inhibitor U0126 affects in vitro and in vivo growth of embryonal rhabdomyosarcoma. *Mol Cancer Ther*. 2009;8:543.

33. Lee J, Rhee MH, Kim E, Cho JY. BAY 11–7082 is a broad-spectrum inhibitor with anti-inflammatory activity against multiple targets. *Mediators Inflamm*. 2012;2012:416036.

34. Hao Q, Vadgama JV, Wang P. CCL2/CCR2 signaling in cancer pathogenesis. *Cell Commun Signal*. 2020;18:82.

35. Arendt LM, McCready J, Keller PJ, et al. Obesity promotes breast cancer by CCL2-mediated macrophage recruitment and angiogenesis. *Can Res*. 2013;73:6080.

36. Nascimento-Sales M, Fredo-da-Costa I, Borges Mendes ACB, et al. Is the FVB/N mouse strain truly resistant to diet-induced obesity? *Physiol Rep*. 2017;5:e13271.

37. Montgomery MK, Hallahan NL, Brown SH, et al. Mouse strain-dependent variation in obesity and glucose homeostasis in response to high-fat feeding. *Diabetologia*. 2013;56:1129-1139.

38. An J, Xue Y, Long M, Zhang G, Zhang J, Su H. Targeting CCR2 with its antagonist suppresses viability, motility and invasion by downregulating MMP-9 expression in non-small cell lung cancer cells. *Oncotarget*. 2017;8:39230-39240.

39. Sartipy P, Loskutoff DJ. Monocyte chemoattractant protein 1 in obesity and insulin resistance. *Proc Natl Acad Sci USA*. 2003;100:7265-7270.

40. Christiansen T, Richelsen B, Bruun JM. Monocyte chemoattractant protein-1 is produced in isolated adipocytes, associated with adiposity and reduced after weight loss in morbid obese subjects. *Int J Obes*. 2005;29:146-150.

41. Rull A, Camps J, Alonso-Villaverde C, Joven J. Insulin resistance, inflammation, and obesity: role of monocyte chemoattractant protein-1 (or CCL2) in the regulation of metabolism. *Mediators Inflamm*. 2010;2010:326580.

42. Stamatovic SM, Keep RF, Mostarica-Stojkovic M, Andjelkovic AV. CCL2 Regulates angiogenesis via activation of Ets-1 transcription factor. *J Immunol*. 2006;177:2651.

43. Han R, Gu S, Zhang Y, et al. Estrogen promotes progression of hormone-dependent breast cancer through CCL2-CCR2 axis by upregulation of Twist via PI3K/AKT/NF-κB signaling. *Sci Rep*. 2018;8:9575.

44. Chun E, Lavoie S, Michaud M, et al. CCL2 promotes colorectal carcinogenesis by enhancing polymorphonuclear myeloid-derived suppressor cell population and function. *Cell Rep*. 2015;12:244-257.

45. Jiménez-Sainz MC, Fast B, Mayor F, Aragay AM. Signaling pathways for monocyte chemoattractant protein 1-mediated extracellular signal-regulated kinase activation. *Mol Pharmacol*. 2003;64:773.

46. Zhao Y, Nichols J, Valdez R, Mendelson CR, Simpson ER. Tumor necrosis factor-alpha stimulates aromatase gene expression in human adipose stromal cells through use of an activating protein-1 binding site upstream of promoter 1.4. *Mol Endocrinol*. 1996;10:1350-1357.

47. Bulun SE, Lin Z, Imir G, et al. Regulation of aromatase expression in estrogen-responsive breast and uterine disease: from bench to treatment. *Pharmacol Rev*. 2005;57:359.

48. Kulendran M, Salhab M, Mokbel K. Oestrogen-synthesising enzymes and breast cancer. *Anticancer Res*. 2009;29:1095-1109.

49. Bjorntorp P. Adipose tissue distribution and function. *Int J Obes*. 1991;15:67-81.

50. Bulun SE, Chen D, Moy I, Brooks DC, Zhao H. Aromatase, breast cancer and obesity: a complex interaction. *Trends Endocrinol Metab*. 2012;23:83-89.

51. Bezemer ID, Rinaldi S, Dossus L, et al. C-peptide, IGF-I, sex-steroid hormones and adiposity: a cross-sectional study in healthy women within the European Prospective Investigation into Cancer and Nutrition (EPIC). *Cancer Causes Control*. 2005;16:561-572.

52. McTiernan A, Wu L, Chen C, et al. Relation of BMI and physical activity to sex hormones in postmenopausal women. *Obesity*. 2006;14:1662-1677.

53. Rask E, Walker BR, Söderberg S, et al. Tissue-specific changes in peripheral cortisol metabolism in obese women: increased adipose 11β-hydroxysteroid dehydrogenase type 1 activity. *J Clin Endocrinol Metab*. 2002;87:3330-3336.

54. Rask E, Olsson T, Soderberg S, et al. Tissue-specific dysregulation of cortisol metabolism in human obesity. *J Clin Endocrinol Metab*. 2001;86:1418-1421.

55. To SQ, Knower KC, Cheung V, Simpson ER, Clyne CD. Transcriptional control of local estrogen formation by aromatase in the breast. *J Steroid Biochem Mol Biol*. 2015;145:179-186.

56. Grodin JM, Macdonald PC, Siiteri PK. Source of estrogen production in postmenopausal women. *J Clin Endocrinol Metab*. 1973;36:207-214.

57. Simpson ER, Zhao Y, Agarwal VR, et al. Aromatase expression in health and disease. *Recent Prog Horm Res*. 1997;185-213.

58. Bulun SE, Chen D, Lu M, et al. Aromatase excess in cancers of breast, endometrium and ovary. *J Steroid Biochem Mol Biol*. 2007;106:81-96.

59. Subbaramaiah K, Morris PG, Zhou XK, et al. Increased levels of COX-2 and prostaglandin E2; contribute to elevated aromatase expression in inflamed breast tissue of obese women. *Cancer Discov*. 2012;2:356.

60. Howe LR, Subbaramaiah K, Hudis CA, Dannenberg AJ. Molecular pathways: adipose inflammation as a mediator of obesity-associated cancer. *Clin Cancer Res*. 2013;19:6074-6083.

61. Chen D, Zhao H, Coon JS, Ono M, Pearson EK, Bulun SE. Weight gain increases human aromatase expression in mammary gland. *Mol Cell Endocrinol*. 2012;355:114-120.

62. Polari L, Yatkin E, Martínez Chacón MG, et al. Weight gain and inflammation regulate aromatase expression in male adipose tissue, as evidenced by reporter gene activity. *Mol Cell Endocrinol*. 2015;412:123-130.

63. Rice S, Patel B, Bano G, Ugwumadu A, Whitehead SA. Aromatase expression in abdominal omental/visceral and subcutaneous fat

depots: a comparison of pregnant and obese women. *Fertil Steril.* 2012;97:1460-1466.e1461.

64. Hetemäki N, Savolainen-Peltonen H, Tikkanen MJ, et al. Estrogen metabolism in abdominal subcutaneous and visceral adipose tissue in postmenopausal women. *J Clin Endocrinol Metab.* 2017;102:4588-4595.

65. Kanda H, Tateya S, Tamori Y, et al. MCP-1 contributes to macrophage infiltration into adipose tissue, insulin resistance, and hepatic steatosis in obesity. *J Clin Investig.* 2006;116:1494-1505.

66. Westerbacka J, Cornér A, Kolak M, et al. Insulin regulation of MCP-1 in human adipose tissue of obese and lean women. *Am J Physiol Endocrinol Metab.* 2008;294:E841-E845.

67. Basurto L, Gregory MA, Hernández SB, et al. Monocyte chemoattractant protein-1 (MCP-1) and fibroblast growth factor-21 (FGF-21) as biomarkers of subclinical atherosclerosis in women. *Exp Gerontol.* 2019;124:110624.

68. Park K-S, Ahn K-J, Kim B-J, et al. Circulating concentrations of monocyte chemoattractant protein-1 are associated with menopause status in Korean women. *Clin Chim Acta.* 2009;403:92-96.

69. Lim SY, Yuzhalin AE, Gordon-Weeks AN, Muschel RJ. Targeting the CCL2-CCR2 signaling axis in cancer metastasis. *Oncotarget.* 2016;7:28697-28710.

70. Bulun SE, Price TM, Aitken J, Mahendroo MS, Simpson ER. A link between breast cancer and local estrogen biosynthesis suggested by quantification of breast adipose tissue aromatase cytochrome P450 transcripts using competitive polymerase chain reaction after reverse transcription. *J Clin Endocrinol Metab.* 1993;77:1622-1628.

71. van Landeghem AAJ, Poortman J, Nabuurs M, Thijssen JHH. Endogenous concentration and subcellular distribution of estrogens in normal and malignant human breast tissue. *Can Res.* 1985;45:2900.

72. Eliasen AH, Hankinson SE. Endogenous hormone levels and risk of breast, endometrial and ovarian cancers. In: Berstein LM, Santen RJ, eds. *Innovative Endocrinology of Cancer.* New York, New York, NY: Springer; 2008:148-165.

73. Iyengar NM, Brown KA, Zhou XK, et al. Metabolic obesity, adipose inflammation and elevated breast aromatase in women with normal body mass index. *Cancer Prevention Research.* 2017;10:235.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.