Hyperleptinemia in obese state renders luminal breast cancers refractory to tamoxifen by coordinating a crosstalk between Med1, miR205 and ErbB

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Obese women with hormone receptor-positive breast cancer exhibit poor response to therapy and inferior outcomes. However, the underlying molecular mechanisms by which obesity/hyperleptinemia may reduce the efficacy of hormonal therapy remain elusive. Obese mice with hyperleptinemia exhibit increased tumor progression and respond poorly to tamoxifen compared to non-obese mice. Exogenous leptin abrogates tamoxifen-mediated growth inhibition and potentiates breast tumor growth even in the presence of tamoxifen. Mechanistically, leptin induces nuclear translocation of phosphorylated-ER and increases the expression of ER-responsive genes, while reducing tamoxifen-mediated gene repression by abrogating tamoxifen-induced recruitment of corepressors NCoR, SMRT, and Mi2 and potentiating coactivator binding. Furthermore, in silico analysis revealed that coactivator Med1 potentially associates with 48 (out of 74) obesity-signature genes. Interestingly, leptin upregulates Med1 expression by decreasing miR-205, and increases its functional activation via phosphorylation, which is mediated by activation of Her2 and EGFR. It is important to note that Med1 silencing abrogates the negative effects of leptin on tamoxifen efficacy. In addition, honokiol or adiponectin treatment effectively inhibits leptin-induced Med1 expression and improves tamoxifen efficacy in hyperleptinemic state. These studies uncover the mechanistic insights how obese/hyperleptinemic state may contribute to poor response to tamoxifen implicating leptin-miR205-Med1 and leptin-Her2-EGFR-Med1 axes, and present bioactive compound honokiol and adiponectin adipopectine as agents that can block leptin’s negative effect on tamoxifen.

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INTRODUCTION

Obesity is associated with increased risk of multiple types of cancers and notably, accounts for ~55% of all cancers diagnosed in women, including breast cancer1. Multiple epidemiological studies have shown that high body-mass-index (BMI) positively associates with larger tumors, more involved axillary nodes, increased metastatic progression, higher risk of recurrence and worse overall survival though the magnitude of impact may vary based on menopausal status, age, ethnicity as well as breast cancer subtype2. Importantly, women with hormone receptor-positive/ Her2-negative breast cancer show a continuous relation between worst outcomes and increasing BMI even after hormonal therapy. It is imperative to understand the underlying molecular networks that may explain why women in the highest quintile of BMI exhibit double the breast cancer-related mortality rate in comparison to women in the lowest quintile. Obese state may be a surrogate for other oncogenic factors that not only contribute to breast cancer growth and progression but also interfere with responsiveness to endocrine therapy.

Accumulation of excess fat mass owing to the hypertrophy and hyperplasia of adipocytes, which are not only the energy storing cells but also active endocrine organs secreting adipokynes, results in dysregulation of adipokynes in obesity. Hyperleptinemia (high levels of adipokyne leptin) is an important manifestation of obese state and has been shown to mediate myriad biological impacts of obesity including carcinogenesis. Higher serum leptin levels correlate with an increased risk of breast cancer as well as poor clinocopathological tumor characteristics in postmenopausal breast cancer patients. Immuno-histochemical analysis of breast tumors, peritumoral and adjacent normal breast tissues show elevated expression of leptin receptor and leptin in 83 and 92% of breast tumors, respectively, while adjacent normal breast tissue exhibit weak positivity. Also, higher leptin and leptin receptor expression is observed in primary and metastatic invasive ductal breast carcinoma in comparison to normal breast tissue. Previous studies from our group and others have shown the oncogenic effects of leptin on breast cancer. High-fat-diet induced obesity in MMTV-TGF-α mice results in elevated leptin levels as well as shorter latency and higher mammary tumor load. Exogenous leptin administration increases tumor growth in athymic nude mice. Upon leptin exposure, breast cancer cells exhibit increased proliferation, reduced apoptosis, and enhanced angiogenesis to support greater tumor growth. Leptin also supports metastatic progression and tumor recurrence by increasing invasion and migration potential as well as stem-like characteristics in breast cancer cells. However, despite these advances, it is unclear how obesity/hyperleptinemia may reduce the efficacy of hormonal therapy.

In this study, we provide evidence that obese hyperleptinemic mice not only exhibit higher breast tumor burden in comparison...
to lean mice but are also refractory to tamoxifen treatment forming the rationale of our work that the elevated levels of leptin in obese state may modulate the underlying signaling mechanisms to block tamoxifen. Significantly, our studies show that leptin induces ER phosphorylation and transcriptional activation in a ligand-independent manner to abrogate tamoxifen-mediated gene repression. Leptin potentiates the release of corepressors and recruitment of coactivators to ER-responsive gene promoters in tamoxifen-treated breast cancer cells. We present Med1, an important subunit of the human mediator complex, as the key node of leptin action that associates with obesity-signature genes. Subsequent mechanistic studies show that leptin orchestrates Med1 activation via decreasing miR-205 and increasing upstream kinases Her2 and EGFR phosphorylation. Moreover, we show that Med1 silencing inhibits leptin’s negative effects on tamoxifen. We also present bioactive compound honokiol and adipocytokine adiponectin as potential agents to improve tamoxifen efficacy in hyperleptinemia.

**RESULTS**

Hyperleptinemia diminishes the anti-cancer effect of tamoxifen

Inferior disease-free and overall survival observed in obese women with breast cancer is often attributed to poor response to standard therapeutic regimens including endocrine therapy but mechanistic links are unknown. Hence, we queried whether diet-induced obesity (DIO) is capable of impacting the efficacy of tamoxifen in breast cancer. We employed the orthotopic CDX (cell line-derived xenograft) model in obese and non-obese NOD/SCID mice. An obese phenotype was generated using high-fat diet (HFD; 45% kcal fat diet). Obese-mice weighed significantly more than lean-mice (Normal-diet; 10% low-fat control diet) (Fig. 1A). Obese-mice exhibited increased tumor progression and significantly larger tumors compared to lean-mice. Interestingly, obese-mice showed an increase in the tumor progression and tumor load upon tamoxifen treatment and were comparable to vehicle-treated obese-mice whereas lean-mice showed effective tumor regression in response to tamoxifen (Fig. 1B, C). Analysis of tumor sections showed higher expression of PCNA in obese-vehicle as well as obese-tamoxifen group in comparison to tamoxifen-treated lean-mice (Fig. 1D). Hallmarks of obesity include adipocytokine dysregulation where hyperleptinemia (high leptin levels) is closely tied with higher BMI. Indeed, obese-mice exhibited significantly higher circulating leptin levels in comparison to lean-mice (Fig. 1E). Then we analyzed whether the leptin gene expression itself correlated with overall survival in a cohort of ER+ breast cancer patients. Kaplan–Meier analysis indicated that elevated expression of leptin associated with poor overall survival in breast cancer patients (p = 0.003) with estrogen receptor (ER) positive breast cancer (Fig. 1F). In silico analyses of differentially expressed genes (DEGs) in MCF7 cells treated with vehicle vs. leptin20 showed increased levels of several cytokines indicating the importance of leptin as an upstream adipokine (Fig. 1G). We also observed differential regulation of several pathways related to receptor activity (Supplementary Figs 1, 2), estrogen response genes (Supplementary Fig. 3) and EGFR signaling pathway (Supplementary Fig. 4). Collectively, several important genes/pathways are differentially modulated upon leptin treatment. Next, we examined the effect of hyperleptinemia on tamoxifen-mediated growth inhibition of breast cancer cells. Tamoxifen treatment inhibited clonogenic potential (~70% inhibition) and anchorage-independent growth of breast cancer cells as expected but co-exposure of cells with leptin abrogated tamoxifen’s growth-inhibitory effects (Fig. 1H–J). Encouraged by our in vitro findings we investigated whether leptin exposure impacts tamoxifen-mediated tumor inhibition. More rapid tumor progression was observed in leptin-treated group compared to control-group while tamoxifen treatment regressed tumors effectively. Interestingly, mice treated with tamoxifen and leptin exhibited higher tumor growth similar to leptin alone group showing that leptin exposure renders breast tumors unresponsive to tamoxifen (Fig. 1K).

**Leptin exerts ligand-independent activation of estrogen receptor and abrogates tamoxifen-mediated recruitment of corepressors**

Ligand binding or activation of upstream kinase pathways enhances the phosphorylation of estrogen receptor α (ER) at multiple sites leading to its functional activation22. Upon activation, ER undergoes nuclear translocation, and is recruited to the ER-responsive elements (EREs) on the promoters of ER-responsive genes resulting in increased gene expression23. We questioned whether leptin exposure affects phosphorylation and nuclear translocation of ER in breast cancer cells. MCF7 and T47D cells, treated with leptin, showed increased phosphorylation of ER at Ser118 and Ser167 (Fig. 2A). Immunofluorescence analysis with antibodies specific for phosphorylated ER-Ser118 or ER-Ser167 revealed increased nuclear translocation of phosphorylated estrogen receptor upon leptin treatment (Fig. 2B). As expected, tamoxifen treatment decreased the expression of c-Myc, Cathepsin D, EBAG9, TFF1 and XBP1 genes. Leptin exposure, on the other hand, not only increased the expression of ER-responsive genes, but also mitigated tamoxifen-induced gene repression (Fig. 2C, D). Tamoxifen-bound ER undergoes a conformational alteration and preferentially recruits corepressor complexes to the EREs, and inhibits the expression of ER-responsive genes23. Leptin exposure resulted in minimal corepressor binding on the EREs while tamoxifen treatment stimulated increased recruitment of NCoR, SMRT and Mi2 in a chromatin immunoprecipitation assay. Interestingly, leptin exposure significantly reduced the recruitment of NCoR, SMRT and Mi2 on the EREs of EBAG9, c-Myc and Cathepsin D genes in the presence of tamoxifen (Fig. 2E).

In contrast, leptin treatment stimulated the recruitment of coactivators SRC1, TIF2 and A1B1 on the EREs of EBAG9, c-Myc, and Cathepsin D genes whereas tamoxifen treatment inhibited the coactivator binding. Leptin treatment also resulted in coactivator recruitment in the presence of tamoxifen (Fig. 2F). These results present that leptin exerts ligand-independent activation of estrogen receptor and rescues expression of ER-responsive genes from tamoxifen-mediated inhibition via reducing the recruitment of corepressors and increasing the coactivator binding.

**Leptin induces upregulation of coactivator Med1 via inhibition of miR205**

Transcriptional activation by ER involves the recruitment of various coactivators that coordinate chromatin remodeling and facilitate the assembly of RNA polymerase II complex23. Mediator complex has emerged as the key coactivator complex that interacts with ER through Med124. Med1 is a key coactivator responsible for adipogenesis25 and it has also been associated with ER-transactivation24. Protein-protein interaction analysis using
Cytoscape showed that Med1 itself was a major hub that directly interacted with diverse subunits of the mediator complex and beyond (Fig. 3A). Interestingly, highly integrated protein-protein interaction landscape of Med1 involved key leptin signaling pathways including its canonical (Akt, Stat3, and ERK) as well as non-canonical (Wnt, Notch, IGF1R and ErbB kinases) nodes (Fig. 3B). Breast tumors in obese state harbor a distinct obesity-associated gene transcription signature (GSE24185) consisting of 725 downregulated probes and 74 probes that are upregulated with high BMI. Since Med1 is a transcriptional coactivator, we queried whether obesity-associated upregulated-genes in breast cancer are transcriptional targets for Med1. Using Med1 ChIP-seq data from ChIP-Atlas (https://chip-atlas.org/), we noted that among the 74 genes upregulated in obesity-breast cancer signature, 48 genes were putative targets for Med1 recruitment (Fig. 3C, Supplementary Table 1), indicating its importance in obesity-breast cancer connection. Indeed, a strong association between higher Med1 expression and poor overall survival was observed in breast cancer patients with estrogen receptor positive breast cancer (Fig. 3D). Next, we investigated whether obese state and hyperleptinemia influence Med1 expression in breast cancer. Breast cancer cells exposed to leptin showed a temporal increase in Med1 expression (Fig. 3E). IHC analysis showed elevated expression of Med1 in obese state which remained elevated upon tamoxifen treatment (Fig. 3F). Increased expression of Cathepsin D (CATD), cMyc, Cyclin D1, TFF1 and Med1 was noted in MCF7 cells-derived tumors formed in obese-mice in comparison to lean-mice. In addition, their expression remained elevated in breast tumors in obese-mice treated with tamoxifen, whereas tumors in lean-mice...
exhibited lower expression in vehicle as well as tamoxifen-treated groups (Fig. 3G).

Various microRNAs (miRs) act as tumor suppressors or activators, controlling multiple aspects of tumor progression including tumor cell proliferation, apoptosis, invasion, migration and stemness, via modulating the expression of key genes. We queried the involvement of miRs in modulation of Med1 expression in obese and hyperleptinemic state. Using

Fig. 2  Leptin activates estrogen receptor and abrogates tamoxifen-mediated recruitment of corepressors. A MCF7 and T47D cells were grown in dextran charcoal stripped media and phenol red free media prior to stimulation with leptin. Cells were treated with 100 ng/ml leptin for indicated time-intervals and subjected to immunoblot analysis for phosphorylated estrogen receptor proteins as denoted. Actin was used as control. B MCF7 cells were treated with 100 ng/ml leptin followed by immunocytochemical analysis and confocal imaging. Scale bar: 10 µm. C, D Semi-quantitative RT-PCR and real-time quantitative PCR analyses of mRNA expression levels of c-Myc, Cathepsin D (CATD), EBAG9, TFF1, and XBP1 in MCF7 cells treated with 100 ng/ml leptin (L) and/or 1 µM 4-hydroxytamoxifen (T) as indicated. E Chromatin immunoprecipitation assays were performed using antibodies specific for NCoR, SMRT and Mi2 in MCF7 cells treated with 100 ng/ml leptin (L) and/or 1 µM 4-hydroxytamoxifen (T). The purified DNA was analyzed by PCR using specific primers spanning the EREs of EBAG9, cathepsin D (CATD) and c-myc gene promoters. *p < 0.05. F Chromatin immunoprecipitation assays were performed using antibodies specific for SRC1, TIF2, and AIB1 in MCF7 cells treated with 100 ng/ml leptin (L) and/or 1 µM 4-hydroxytamoxifen (T). The purified DNA was analyzed by PCR using specific primers spanning the EREs of EBAG9, cathepsin D (CATD) and c-myc gene promoters. *p < 0.05. Data are means ± SD from three experiments.
Med1, an important node associated with leptin signaling network and obesity-associated signature in breast cancer, is upregulated via miR-205 in hyperleptinemic state. A Protein-protein interaction landscape of Med1 and mediator complex subunits and associated proteins is presented. B An integrated network of proteins involved in leptin signaling network and Med1 is presented. In total, ~70 proteins are represented, 9 of which serve as nodes in the Med1-leptin interaction network. Blue circles represent high confidence leptin-signaling proteins. Pink and purple circles represent additional proteins identified in Med1 protein-protein interaction network. To avoid the overcrowded network, only selected proteins are represented. C Obesity-related gene signature was queried in Med1 ChIP-seq data. 48 out of 75 genes upregulated in breast tumors in obese patients are potential targets of Med1. A summary of Med1 associated proteins that associate with leptin is presented in Supplemental Table S1. D Kaplan–Meier plots of breast cancer survival in relation to mean Med1 (PPARBP) expression (p = 2.6E-03). E MCF7 cells and BT474 cells were treated with 100 ng/ml leptin (L) for various time intervals as indicated and immunoblotted for Med1 expression. F, G MCF7 cells derived tumors from high-fat-diet (HFD) fed obese mice and normal-diet (ND) fed lean mice and treated with 4-hydroxytamoxifen (T) or vehicle (C) were subjected to immunohistochemical (IHC) using Med1 antibody (Scale bars: 50 μm) and immunoblot analysis using Cathepsin D (CATD), cMyc, Cyclin D1, TFF1, and Med1, antibodies. Actin was used as loading control in immunoblot analysis. H The miRNA target pairing sequences were analyzed by bioinformatics tools. I MCF7 cells were transfected with miR-205 mimic and Med1 expression was examined using real-time PCR. J Kaplan–Meier plots of breast cancer survival in relation to mean miR-205 expression (p = 3.6E-03). K Expression of miR-205 was examined in MCF7 cells treated with 100 ng/ml leptin (L) and/or 1 μM 4-hydroxytamoxifen (T) as indicated. L Expression of miR-205 was examined in MCF7 cells-derived tumors from high-fat-diet (HFD) fed obese mice and normal-diet (ND) fed lean mice and treated with 4-hydroxytamoxifen (T) or vehicle (C). M MCF7 cells were transfected with miR-205-mimic and anti-miR-205 followed by treatment with 100 ng/ml leptin (L). Med1, TFF1 and CATD expression was examined using quantitative real-time PCR. ***p < 0.0005, **p < 0.005, *p < 0.05. Data are means ± SD from three experiments.

Bioinformatics algorithms (TargetScan, RNA22 and miRBase), we identified conserved target sites for miR-205 binding site at nucleotides 494–501 of the Med1 3’UTR (Fig. 3H). Ectopic expression of miR-205 (in the form of a mimic molecule) reduced the expression of Med1 (Fig. 3I). Breast cancer patients with estrogen receptor positive breast cancer showed a strong association between higher miR-205 expression and better overall survival (Fig. 3J) in contrast to Med1 whose overexpression associated with poor survival (Fig. 3D). Breast cancer cells exposed to leptin showed decreased levels of miR-205 whereas tamoxifen treatment significantly increased the expression of miR-205. Leptin exposure abrogated tamoxifen-mediated induction of miR-205 (Fig. 3K). Next, we examined whether obese state imparts similar effect on miR-205 expression. Indeed, MCF7 cells-derived tumors formed in vehicle-treated obese-mice showed lower miR-205 expression in comparison to vehicle-treated lean-mice. Tumors from tamoxifen-treated lean-mice exhibited higher levels of miR-205 expression whereas tamoxifen-treated obese-mice showed significant reduction in miR-205 level (Fig. 3L). We further examined the role of miR-205 in leptin-mediated activation of Med1 as well as ER-responsive genes. Breast cancer cells overexpressing miR-205 reduced leptin-mediated Med1, TFF1, and CATD overexpression whereas expression of miR-205-inhibitor potentiated the effect of leptin on Med1 expression (Fig. 3M). Taken together, these results show that Med1 and miR205 exhibit contrasting association with ER-positive breast cancer prognosis and leptin increases the expression of Med1, a key coactivator protein, by inhibiting the expression of miR-205.

Leptin orchestrates phosphorylation, nuclear accumulation and promoter-recruitment of Med1 via activation of EGFR and Her2 kinases

Med1 is a key coactivator protein for ER-mediated transcription; next, we queried whether leptin exposure affects the nuclear localization and coactivation function of Med1. Leptin exposure resulted in higher level of Med1, which was accumulated in nucleus (Fig. 4A, B). Earlier studies have shown that Med1 is essential for the expression of ER-dependent genes and estrogen mediated breast cancer growth. While estrogen-activated ER
Leptin induces phosphorylation, nuclear accumulation and promoter-recruitment of Med1 in breast cancer. 

A Immunoblot analysis of Med1 in cytoplasmic and nuclear fractions of MCF7 cells treated with 100 ng/ml leptin (L). PCNA and actin were used as controls.

B Immunofluorescence analysis of Med1 in MCF7 and BT474 cells treated with 100 ng/ml leptin (L). Scale bar: 10 µm.

C MCF7 cells were treated with 100 ng/ml leptin (L) and/or 1 μM 4-hydroxytamoxifen (T) and chromatin immunoprecipitation assays were performed using antibodies specific for Med1, Ac-H4, and Ac-H3. The purified DNA was analyzed by PCR using specific primers spanning the EREs of EBAG9, PS2, and cathepsin D (CATD) gene promoters.

D Total protein was isolated from MCF7 and BT474 cells followed by immunoblot analysis for phosphorylated-Med1 expression. Actin was used as control.

E Immunoblot analysis of total protein isolated from MCF7 cells-derived tumors from high-fat-diet (HFD) fed obese mice and normal-diet (ND) fed lean mice and treated with 4-hydroxytamoxifen (T) or vehicle (C). Expression of pMed1, pERK and ERK was examined. Actin was used as control.

F Protein lysates from MCF7 and TAM R cells subjected to human phospho-antibody array analyses. Bar diagram shows relative levels of protein phosphorylation as indicated (normalized intensity for each antibody) were calculated for each sample and compared between MCF7 and TAM R cells.

G MCF7 cells were treated with 100 ng/ml leptin as indicated and total lysates were immunoblotted for pEGFR, EGFR, pERK and tERK expression levels.

H Bar diagram shows the ratio of pEGFR/EGFR and pERK /tERK in breast cancer cells treated with leptin as indicated. *p < 0.05. Data are means ± SD from three experiments.
recruits Med1 to facilitate histone acetylation on the promoter region of ER-responsive genes, tamoxifen-bound ER presents a steric hindrance to block coactivator recruitment.23,24 As expected, breast cancer cells treated with tamoxifen showed minimal recruitment of Med1 on the EREs of ER-responsive genes as well as reduced levels of acetylated H3 and H4 binding. Leptin, on the other hand, stimulated significant increase in Med1 recruitment as well as H3/H4 binding on EBAG9, PS2, and CATD genes and also abrogated the inhibitory effects of tamoxifen on coactivator recruitment (Fig. 4C). Phosphorylation of Med1 not only potentiates its interaction with mediator complex, but also required for its transactivation function.29 Interestingly, increased level of phosphorylated Med1 was observed in leptin-treated MCF7 and BT474 cells (Fig. 4D). Furthermore, immunoblot analysis of MCF7 cells-derived tumors formed in obese mice treated with vehicle or tamoxifen exhibited higher expression of phosphorylated-Med1 in comparison to tamoxifen-treated lean mice (Fig. 4E). Since our results indicated that leptin exposure promotes a tamoxifen-refractory breast cancer cell phenotype, we investigated the kinases involved in tamoxifen-resistant breast cancer model to further understand the mechanistic underpinnings. Utilizing a phosphoprotein array comprising of 46 specific Ser/Thr/Tyr phosphorylation sites of 38 selected proteins, we found elevated levels of phosphorylated ERK, EGFR and MSK in tamoxifen-resistant breast cancer cells (Fig. 4F). Shown previously, an increased level of phosphorylated ERK was observed in tumors from obese mice treated with vehicle or tamoxifen (Fig. 4E). Also, elevated levels of phosphorylated EGFR and ERK were observed in leptin-treated breast cancer cells compared to untreated cells (Fig. 4G, H).

Further exploration of the direct involvement of Her2 and EGFR in leptin-mediated Med1 phosphorylation showed increased activation of EGFR, ERK, and Her2 in leptin treated breast cancer cells (Fig. 5A–C). Combined treatment with leptin and EGFR inhibitor, AG1478, inhibited leptin-induced Med1 phosphorylation in comparison to breast cancer cells treated with leptin alone (Fig. 5A, C). In addition, inhibition of Her2 activation with AG825, a selective ATP-competitive inhibitor of the tyrosine kinase activity of HER2/Neu, also abrogated leptin-induced Med1 phosphorylation (Fig. 5B, C). MCF7 cells stably overexpressing Her2 (MCF7Her2) not only exhibited increased activation of Her2, EGFR and ERK, but also showed increased phosphorylation of Med1 compared to MCF7-vector control cells, indicating the direct involvement of Her2 in Med1 phosphorylation (Fig. 5D). Leptin treatment further increased Med1 phosphorylation in MCF7Her2 cells, which was inhibited upon treatment with AG825 in combination with leptin (Fig. 5E, F). Next, we evaluated whether inhibiting upstream kinases impacts leptin-induced nuclear localization of Med1. As expected, immunofluorescence analysis of MCF7 and BT474 cells treated with leptin showed increased nuclear accumulation of Med1 compared to untreated cells. Inhibition of EGFR and Her2 with AG1478 and AG825 treatment alleviated the nuclear localization of Med1 in leptin-exposed cells (Fig. 5G). As Med1 phosphorylation is known to impact its transactivation function, we explored the involvement of EGFR, Her2 and ERK kinases in the recruitment of Med1 to ER promoter. ER itself is an estrogen-responsive gene and its promoter harbors a functional ERE.10 Chromatin immunoprecipitation analysis showed that leptin induced the recruitment of Med1 on ERE region of ER promoter while co-treatment of breast cancer cells with AG825, AG1478, or PD98059 along with leptin resulted in significant reduction in Med1 binding (Fig. 5H). These results explicitly show that leptin induces phosphorylation, nuclear accumulation, promoter recruitment of Med1 in breast cancer cells and inhibition of ERB8 kinases abrogates leptin-stimulated Med1 phosphorylation as well as its transactivation function.

Med1 inhibition using adipocytokine adiponectin and bioactive Honokiol effectively improves the efficacy of tamoxifen in hyperleptinemic state

We further explored whether Med1 is the key node by which leptin interferes with tamoxifen-mediated growth inhibition of breast cancer cells. Towards this, stable pools of MCF7LKO.1 (vector control) and MCF7Med1 (Med1 silenced) cells were selected in puromycin. MCF7Med1 cells showed efficient silencing of Med1 in comparison with MCF7LKO.1 cells (Fig. 6A). Leptin exposure induced whereas tamoxifen treatment decreased the clonogenic potential and anchorage-independent growth of MCF7 cells. Interestingly, leptin exposure alleviated tamoxifen mediated growth inhibition in MCF7LKO.1 cells but no effect of leptin on tamoxifen efficacy was observed in MCF7Med1 cells (Fig. 6B, C). These results indicated that Med1 is integral for leptin-mediated abrogation of tamoxifen effectiveness. Previous studies from our lab have shown that biological effect of hyperleptinemic obese state on breast cancer can be inhibited with adipocytokine adiponectin34 and bioactive compound honokiol34,35, agents known for their anti-breast cancer potential.34,35 We questioned whether adiponectin and/or honokiol can inhibit Med1 in leptin-exposed breast cancer cells. Both honokiol and adiponectin reduced Med1 expression in leptin-exposed cells compared to leptin-alone group (Fig. 6D). In addition, honokiol and adiponectin abrogated the effect of leptin on tamoxifen effectiveness. As expected, breast cancer cells exposed with leptin showed increased clonogenicity and soft-agar colonies even in the presence of tamoxifen treatment. Interestingly, adiponectin and honokiol treatment alleviated the interfering effect of leptin, resulting in effective growth inhibition in the presence of tamoxifen (Fig. 6E, F). Analysis of breast cancer cells-derived tumors from mice treated with leptin, honokiol or a combination of leptin and honokiol showed higher expression of Med1 and phosphorylated Med1 in tumors from leptin-treated mice in comparison to honokiol-treated mice. Honokiol treatment reduced the expression of Med1 in tumors from leptin-treated mice (Fig. 6G). Furthermore, increased Med1 expression was noted in MCF7 cells-derived tumors formed in obese mice in comparison to lean mice. Honokiol treatment could inhibit Med1 expression in tumors formed in lean as well as obese mice. Immunohistochemical analysis of tumors corroborated these findings (Fig. 6H, J). Also, mice treated with adiponectin showed reduced levels of Med1 in MCF7 cells-derived tumors while leptin-treated group exhibited higher Med1 expression which is alleviated with simultaneous treatment with adiponectin. Immunohistochemical analysis of tumor samples from control, leptin, adiponectin or leptin + adiponectin treated mice showed similar findings (Fig. 6I, J). Together, these results show that Med1 is the key node for mediating anti-tamoxifen effects of leptin, and blocking Med1 with adiponectin or honokiol can alleviate the negative effects of leptin and reinstate the effectiveness of tamoxifen in hyperleptinemic state. Next, we interrogated the association of miR-205, Med1 (PPARBP) and leptin with overall survival of breast cancer patients. High expression of miR-205 and low expression of Med1 associated with better overall survival in breast cancer patients. High expression of miR-205 and low expression of Med1 associated with better overall survival in breast cancer patients with estrogen receptor positive breast cancer (Fig. 6K). We also noted that breast cancer patients with estrogen receptor positive breast cancer exhibited better overall survival with higher miR-205, low Med1 and low leptin expression levels (Fig. 6L).

DISCUSSION

Although luminal A (Hormone receptor+/Her2−), a breast cancer subtype accounting for ~70% of all breast cancers, has the highest 5-year survival rate among all subtypes, it is disparately affected by obese state. Obese women with luminal A breast cancer exhibit 1.8-fold (95% CI 1.3–2.5) higher risk of breast cancer related
mortality while no significant associations are observed with Her2-overexpressing or triple-negative tumors. Inferior disease-free and overall survival rates are noted in obese (BMI, ≥ 30 kg/m²) and overweight (BMI, ≥ 25–29.9 kg/m²) women with luminal A breast cancer despite being treated with standard endocrine-based therapy. While most studies support an association between obesity and breast cancer and report a 1.25- to 2.5-fold increase in breast cancer recurrence with increased BMI, obese women with lymph node-negative, ER-positive breast cancer did not show increased recurrence risk and poor response to tamoxifen compared to their lean counterparts. In this clinical trial, inclusion of women with operable tumors and negative axillary lymph nodes only might have led to these results as the study population did not represent a wider spectrum of disease. Nonetheless, the association between luminal A breast cancer and obesity from clinical studies support our observation that obese mice exhibit increased tumor progression which is not alleviated upon tamoxifen treatment. Obese state is a host-centric

Fig. 5  ERBB kinase inhibition abrogates activation of MED1 and recruitment of MED1 to ER promoter in response to leptin. A BT474 cells were treated with Leptin and AG1478 alone or in combination for 24 h. Cell lysates were immunoblotted with indicated antibodies. B BT474 cells were treated with Leptin and AG825 alone or in combination for 24 h. Cell lysates were immunoblotted for pMed1, pHER2, and tHER2. Actin was used as loading control. C Bar graph shows fold change in protein expression in (A) and (B). D Immunoblot analysis of MCF7-vector and MCF7-Her2 O/E cells for indicated proteins. E, F MCF7-Her2 O/E cells were treated with Leptin, AG825 alone or in combination for 24 h. Cell lysates were immunoblotted for pMED1, pHER2, and tHER2. Actin was used as loading control. Bar graph shows the ratio of MED1 and actin. G MCF7 and BT474 cells were treated as in (A) and (B), and subjected to immunofluorescence analysis of pMED1. Scale bar, 10 µm. H MCF7 cells were treated as indicated for 24 h and subjected to ChIP assay using Med1 antibody. The purified DNA was analyzed by using specific primer for ER promoter. **p < 0.005, *p < 0.05. Data are means ± SD from three experiments.
factor that can affect tumor growth and progression via adipocytokine dysregulation. We found that obese/hyperleptinemic tumor bearing mice did not respond to tamoxifen treatment and showed increased tumor progression implying that leptin renders breast tumors refractory to tamoxifen. This observation is supported by a recent study showing that lower levels of circulating leptin achieved via periodic fasting or a fasting mimicking diet potentiates tamoxifen efficacy. It is known that phosphomodiﬁcations of ER can modulate ligand-binding, transactivation-function and response to estrogen-stimulation as well as endocrine therapy. Phosphorylation of ER ensues conformation changes that pose steric hindrance to antiestrogen binding and associated coactivator recruitment. We observed that leptin increases ER phosphorylation at serine 118 and 167 which then responds poorly to tamoxifen and exhibits reduced binding capacity to corepressors proteins, NCoR, SMRT, and Mi2, interfering with tamoxifen-directed ER-corepressors-complex formation. Mechanistic evaluations identify the involvement of mediator protein Med1 whose inhibition leads to blocking leptin’s inhibitory effects on tamoxifen efficacy underscoring its biological importance.

Fig. 6 Bioactive strategies effectively improve the efficacy of tamoxifen in hyperleptinemic state. A Immunoblot analysis of Med1 expression level in stable pools of Med1-depleted (Med1shRNA) and vector control (pLKO.1) MCF7 cells. B Clonogenicity assay performed with MCF7-pLKO.1, MCF7-pMed1shRNA cells in the presence of leptin and 4-hydroxytamoxifen alone or in combination. C Soft agar assay was performed using MCF7-pLKO.1, MCF7-pMed1shRNA cells in the presence of leptin and 4-hydroxytamoxifen alone or in combination. Bar graph shows number of soft agar colonies. D MCF7 cells treated with leptin, adiponectin (10 µg/ml), and Honokiol (HNK) (5 µM) alone or in combination as indicated were subjected to immunoblot analysis for Med1 expression. E, F MCF7 cells were treated with leptin and 4-hydroxytamoxifen alone or combination with adiponectin or HNK as indicated and subjected to (E) clonogenicity assay and (F) soft-agar colony formation assay. G MCF7 cells-derived tumors were developed in nude mice and treated with Leptin, HNK and Combination. At the end of 6 weeks, tumors were collected and tumor samples (n = 3/group) were subjected to immunoblot analysis using Med1 and pMed1 antibodies. H Tumor lysate (n = 3/group) from MCF7-derived lean mice and obese mice treated with HNK were subjected to immunoblot analysis using Med1 and pMed1 antibodies. I Immunoblot analysis of Med1 expression level in stable pools of Med1-depleted (Med1shRNA) and vector control (pLKO.1) MCF7 cells. J Kaplan–Meier plots of breast cancer recurrences in relation to high miR-205 expression level in stable pools of Med1-depleted (Med1shRNA) and vector control (pLKO.1) MCF7 cells. K Kaplan–Meier plots of breast cancer recurrences in relation to high miR-205 expression level in stable pools of Med1-depleted (Med1shRNA) and vector control (pLKO.1) MCF7 cells. L Kaplan–Meier plots of breast cancer recurrences in relation to high miR-205 expression level in stable pools of Med1-depleted (Med1shRNA) and vector control (pLKO.1) MCF7 cells. M Schematic diagram showing the signaling pathways activated in normal leptin and hyperleptinemic state.

It is noteworthy that ER phosphorylation at S118 and S167 can be directed by MAPK, EGFR, and Akt which are integral components of canonical leptin
signaling pathway. Our data shows that leptin stimulates ER phosphorylation leading to reduced binding of corepressors complexes and abrogation of tamoxifen-mediated gene repression.

Med1 (also known as DRIP205, PBP, and TRAPP220) is one of the anchor subunits of Mediator complex, an evolutionary conserved multi-subunit complex consisting of ~25 subunits which is required for the recruitment of the RNA polymerase II-complex to facilitate gene transcription. Med1 is essential for maintaining estrogen-ER function in normal physiological pubertal mammary gland development and luminal progenitor/stem cell differentiation in vivo as well as regulating the expression of ER-responsive genes in breast cancer46,46. Interestingly, Med1 is overexpressed in ~40–50% of breast cancers46,47 and associates with poor clinical response in breast cancer patients treated with endocrine therapy.

Our clinical observations are supported by preclinical studies showing that Med1 plays a significant role in response and resistance to endocrine therapy47. Our study implicates Med1 as an important coactivator mediating the biological effects of obesity, as 48 out of 74 genes coinling the obesity-associated gene signature are putative targets for Med1 recruitment. Indeed, we observed elevated Med1 expression in leptin-treated breast cancer cells and breast tumors developed in obese mice. We found that leptin upregulates Med1 expression via inhibiting miR-205, whose overexpression associates with poor prognosis in ER-positive breast cancer patients. Phosphorylation of Med1 has been shown to enhance its coactivation function by promoting its interaction with mediator complex and nuclear hormone receptors45,47. Our results show that leptin increases Med1 phosphorylation via activation of Her2 and EGFR kinases, whose inhibition not only blocks nuclear accumulation of Med1 but also its interaction with ER upon leptin stimulation. Since our results demonstrate that Med1 activation is key to mediating leptin’s inhibitory effects on tamoxifen efficacy, implication of Her2 and EGFR in leptin-mediated Med1 activation opens up an opportunity to utilize Her2 and EGFR inhibitors to abrogate leptin.

Our results advance the understanding of the molecular mechanisms underlying obese hyperleptinemic state and associated poor response to tamoxifen. In this study, we present miR205 as a regulator of Med1, as well as decipher a ‘phospho-switch’ for Med1 which is regulated by ERβ kinases Her2-EGFR, in leptin stimulated breast cancer. Although discovery of leptin-miR205-Med1 and leptin-Her2-EGFR-Med1 axes presents various approaches to inhibit Med1 in obese hyperleptinemic state using small molecule inhibitors, we examined whether bioactive adipocytokine based modes can be beneficial. Indeed, breast tumors developed in obese hyperleptinemic mice treated with Honokiol, a bioactive compound isolated from Magnolia plants, exhibit reduced Med1 expression. Previous studies from our lab have shown that oral administration of honokiol inhibits breast cancer41. Adiponectin is a guardian angel adipocytokine with anti-breast cancer potential, whose expression is reduced in obese state31,35,40. Adiponectin administration in breast tumor-bearing mice exposed to leptin results in reduced Med1 expression in breast tumors indicating that strategies to elevate adiponectin level in obese state can be beneficial. Various strategies including direct upregulation of adiponectin function using ADIPOR agonists or indirect approaches targeting adiponectin-signaling molecules AMPK and PPAR/PPARG (peroxisome proliferator activated receptor gamma) with AMPK activators and PPAR agonists including ciglitazone, rosiglitazone, pioglitazone and troglitazone48,49 can be potentially utilized to inhibit Leptin-Med1 axis.

Direct targeting of coactivator protein is challenging but a recent study showed successful targeting of Med1 with biosafe pRNA–HER2apt–siMED1 nanoparticle40. Similar strategies could be developed to target Med1 in hyperleptinemic state.

Hyperleptinemic obese state results in a ligand-independent activation of ER which recruits Med1 coactivator to ERs even in the presence of tamoxifen resulting in an elevated expression of ER-responsive genes. Leptin, not only supports an elevated expression of Med1 via inhibiting miR-205, but also induces ErbB kinase-mediated phosphorylation of Med1 which potentiates its nuclear localization and transactivation function (Fig. 6M). Our studies implicate leptin-miR205-Med1 and leptin-Her2-EGFR-Med1 axes by which obese/hyperleptinemic state may contribute to poor response to tamoxifen.

**METHODS**

**Cell culture and reagents**

Human breast cancer cell lines MCF7, BT474 and T47D, purchased from American Type Culture Collection (ATCC), were thawed from early passage liquid nitrogen vapor stocks as required and maintained at 37 °C in 5% CO2 and 95% humidity. TamR (MCF7-OHT) cells were previously developed by our group47. TamR (MCF-OHT) cells show tamoxifen resistance and exhibit no change in ER expression from parent cells47. Cell lines are routinely authenticated with STR profiling. Cells were grown in dextran charcoal stripped media and phenol red free media prior to stimulation with leptin. Antibodies for PCNA (13–1900; 1:1000 dilution), obtained from Cell Signaling Technology (Danvers, MA). Med1 antibody (SC-5334; 1:500 dilution), Cathepsin D (SC-6486; 1:500) and anti-goat secondary antibody (SC-2922; 1:5000) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, Texas). Phosphorylated Med1 antibody (ab64965; 1:1000) was procured from Abcam (Cambridge, UK). Leptin, Adiponectin, 4-Hydroxy-Tamoxifen, estradiol and antibodies targeting β-actin (A5448; 1:5000), anti-mouse secondary (NA931V; 1:5000 dilution) and anti-rabbit secondary (NA934V; 1:5000 dilution) were obtained from Sigma-Aldrich (St. Louis, MO). Inhibitors of epidermal growth factor receptor (AG1478) and ERBB2 (AG825) were purchased from Calbiochem/Millipore Sigma (Burlington, MA). Mimic and inhibitor for miR-205 were purchased from ThermoFisher Scientific (Waltham, MA). Honokiol (HMK) is a natural product extracted from the seed cone of Magnolia grandiflora via HPLC with 98% purity51.

**Obese mice model and breast tumorigenesis assay**

All animal studies were approved by the Johns Hopkins ACUC. NOD/SCID mice (female, 6–8 weeks old) were acquired from SKCCC animal facility and maintained in-house. Mice were fed a 60% kcal fat diet (D12492i, New Brunswick, NJ, USA) to induce diet-induced obesity (DIO) or a 10% low-fat control diet (D12450I, Research Diets Inc., New Brunswick, NJ, USA) to induce diet-induced obesity (DIO) or a 10% low-fat control diet (D12450I, Research Diets Inc., New Brunswick, NJ, USA) for ~12 weeks ad libitum before the experiment and the high-fat-diet (HFD) and control normal diet (ND) groups were maintained on respective diets throughout the experiment. Estrus pellets (60-day slow-release pellet containing 0.72 mg of 17α-estradiol; Innovative Research of America) were subcutaneously implanted 3 days prior to tumor cell inoculation for sustained estrogen supplementation. Exponentially growing MCF7 (5×106 cells in 100 µl matrigel), were implanted in the 4th mammary fat pad on either side. Vehicle and tamoxifen treatment (500 µg of tamoxifen citrate in peanut oil by subcutaneous injection daily Monday to Friday, for 6 weeks) were given to respective treatment groups. For leptin-treated group, mice were treated with recombinant leptin (p.o., dosage of 5 mg/kg), 5 days a week for the duration of the experiment. Mice were regularly monitored for tumor incidence and tumor growth was measured twice a week using calipers for 6 weeks. Tumors were excised and processed for further analysis. For immunohistochemistry, tumors were fixed in 10% formalin, paraffin embedded and sectioned. Tissue sections were deparaffinized and rehydrated followed by antigen retrieval in citrate buffer. IHC for specific proteins was carried out using specific antibodies and Vectastain ABC detection system (Vector Laboratories, Burlingame, CA, USA) according to Vector lab guidelines. Sections were counter stained with hematoxylin, dehydrated and mounted followed by image analyses using Lciea microscope at ×20 magnification. Adiponectin and honokiol treatment: Mice were treated with adiponectin (intratumoral injections of recombinant adenosine10 µg) and/or Honokiol (3 mg/mouse/day in 20% Intralipid [Baxter Healthcare, Deerfield, IL], three times per week) for the duration of the experiment.
Clonogenicity, soft-agar colony-formation assay
For clonogenicity assay, cells were treated, counted and plated in 12-well plates. Colony numbers were assessed using ImageJ after fixing and staining the colonies with crystal violet (0.1% in 20% methanol) (Sigma-Aldrich, C3886). For soft-agar colony-formation assay, 1 × 103 cells were suspended in 1:1 mix of 0.6% agar and complete medium containing treatments and added to 6-well plates containing solidified 0.6% base agar followed by incubation for 3 weeks. Colonies were stained with 0.005% crystal violet and observed using inverted microscope (Eclipse Ti2, Nikon, Melville, NY). Colonies were counted in five randomly selected fields at 10x magnification.

Whole cell lysates, nuclear-cytoplasmic fractionation and immunoblotting
Whole cell lysates of breast cancer cells were prepared using modified RIPA buffer.1 Nuclear-cytoplasmic lysates were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer’s protocol. Equal amounts of whole cell lysates, nuclear extracts and cytoplasmic extracts were subjected to immunoblot analysis.3 All blots are derived from same experiments and were processed in parallel.

Phosphokinase array, immunofluorescence, and confocal imaging
Phosphokinase analysis was performed using the Proteome Profiler Human Phospho-Kinase Array Kit ARV003 (R&D Systems)53. Array images were analyzed using the GeneTools image analysis software (Syngene). For Immunofluorescence, breast cancer cells (5 × 103) were seeded on chambered slides, allowed to grow for 24 h and treated as indicated and subjected to immunofluorescence analysis.53 Immunofluorescently stained cells were imaged using a Zeiss LSM510 Meta (Zeiss, Dublin, California, USA) laser scanning confocal system configured to a Zeiss Axioplan 2 upright microscope (Zeiss, Dublin, CA, USA).

RNA isolation, RT-PCR, transfection and chromatin immunoprecipitation assay
For RT-PCR, cells were lysed in Trizol, RNA was isolated by chloroform-isopropanol method and cDNA was synthesized using iScript cDNA synthesis kit followed by reverse transcription-polymerase chain reaction (RT-PCR).44 For miR assays, cells were transfected with miR-205 mimic or miR-205 inhibitor or control-miR (Applied Biosystems, Ambion, Austin, TX) using Eugene transfection reagent (Promega Corporation, Madison, WI). For qRT-PCR detection of miR-205, miRNA-specific primers, TaqMan miRNA Assay (Applied Biosystems, Ambion, Austin, TX) and Platinum Taq Polymerase Reagents (Invitrogen, Grand Island, NY) were used. Data were calculated by using the standard ΔΔCT method and microRNA expression was represented as fold-difference of each treatment versus vehicle-treated control. Chromatin immunoprecipitation (ChIP) analyses were conducted using our published procedure. Briefly, chromatin lysates were prepared and subjected to immunoprecipitation using specific antibodies.44 Following primers were used. Cathepsin D-RT-PCR: Forward primer: 5’-CCA GCC CCC AAT CCC AAC ACC TCC AG-3; Reverse primer: 5’-CAG TGA AGC TGG GAC GCA AAG GCT ACA AGC-3. c-Myc-RT-PCR: Forward primer: 5’-GCC ACG TCT CAC CCA CAC ATG-3; Reverse primer: 5’-TCC TGG CAG CAG GAT AGT CCT T-3; Reverse primers: 5’-CTT CTT CAT TAG CCG TTG TG-3; XP1-RT-PCR: Forward primer: 5’-CCT TCT ACG CAA GAA GAG-3; Reverse primer: 5’-GGG GCT TGG TAT ATA TGT GG-3. TFF1-RT-PCR: Forward primer: 5’-TTT GGA GCA GAG AGG AGG-3; Reverse primer: 5’-TTG AGT AGT AAA GAG AGC AC-3. β-actin-RT-PCR: Forward primer: 5’-ACT ATG GAT GAT ATC GC-3; Reverse primer: 5’-ACA TGG CCT GGG TGT TAG A-3; Cathepsin D-ChIP: Forward primer: 5’-GCT TTC TCT GGA AGC CCT GTA G-3; Reverse primer: 5’-TCT GCC AGG TCC TTT CTC-3; c-Myc-ChIP: Forward primer: 5’-AGG CGG CCG TAG TTA ATT CAT-3; Reverse primer: 5’-CGG CCT CTG TGT TGA-3; EBAG9-ChIP: Forward primer: 5’-ATT GTC TGC CCT TCG C-3; Reverse primer: 5’-TTTGGAGGCTGCTGCTTT-3.

In silico analyses
Protein-protein interactome (PPI) was constructed using NetworkAnalyst (network-based visual analytics for gene expression profiling, meta-analysis and interpretation).54 Key leptin signaling genes were used as input data to construct the protein-protein interactome of MED1 and its associates using STRING Interactome with a Confidence score cutoff 900.55 Gene Set Enrichment Analysis (GSEA): Utilizing GSEA software available from the Broad Institute.56, GSEA was performed on the data set of GSE58059 and GSE26459 for oncopathic pathways. Gene expression analysis of published data sets: microarray data analyzing gene expression of MCF7 cells treated with Leptin was searched for in NCBI-GEO database. Raw data for data set GSE58059 was retrieved by downloading the Series Matrix File. Sample Sequencing and Sequencing/Array platform information was downloaded and data was arranged as a gene expression matrix in MS Excel. Pre-processed data was then run through web based interface, integrated Differential Expression and Pathway analysis (IDEP).91 Enriched biological processes and molecular pathways were searched for using the “Pathway” application of IDEP and heat maps were constructed. For survival analyses, we performed survival analysis using the METABRIC dataset available in EGA under the study ID EGAS000000000122. The advantage of METABRIC is the simultaneous availability of mRNA and miRNA gene expression data for the same set of patients. The total number of patients with miRNA expression was 1302 and both miRNA expression 1480, all data was available for 1262 patients. Of those, 996 were ER positive when assessed by immunohistochemistry and these patients were used in the analyses. The technology used for determination of miRNA expression was illumina HT 12 and for miRNA expression Agilent ncRNA 60k arrays. In the mRNA dataset, the probes ILLMN_172129, ILLMN_2207505, and ILLMN_2234956 were used to determine PPARGP and LEP, and LEPRI expression, respectively. Survival analysis was performed for overall survival data using Cox proportional hazards regression and by plotting Kaplan–Meier survival plots. Correlation between mRNA expression and miRNA expression was determined using Spearman rank correlation. When combining multiple genes, their mean expression was used as a signature—in this, the expression of PPARGP and LEP were inverted, because these displayed the opposite correlation to survival in the single-gene univariate analysis. The median was used as a cutoff to determine high- and low-expression cohorts. Statistical significance was set at p < 0.05.

Statistical analyses
All experiments were performed thrice in triplicates. Statistical analysis was performed using Microsoft Excel software. Significant differences were analyzed using the Student t test and two-tailed distribution. Results were considered to be statistically significant if p < 0.05. Results were expressed as mean ± standard deviation and all experiments were performed thrice. For animal studies, analysis of variance with repeated measurements was carried out to compare the mean tumor volume between different groups. The overall p value for testing for differences between at least two groups is p < 0.0001.

Reporting summary
Further information on research design is available in the Nature Research Reporting Summary linked to this article.

DATA AVAILABILITY
Analysed data set GSE58059 (GEO accession number) can be accessed on NCBI Gene Expression Omnibus. https://www.ncbi.nlm.nih.gov/geo/query/acc.fcgi?acc=GSE58.

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AUTHOR CONTRIBUTIONS
A.N.: Investigation, Experiments, Methodology, Data Curation, Writing-Original Draft Preparation; S.S.: Experiments, Methodology, Data Curation, Editing; S.P.: Experiments, Methodology, Data Curation, Editing; N.M.: Experiments, Methodology, Data Curation, Editing; D.A.: Experiments, Methodology, Data Curation, Editing; P.K.: Experiments, Methodology, Data Curation; J.E.: Experiments, Methodology, Data Curation; J.L. A.: Experiments, Methodology, Data Curation, Editing; B.G.: Data Curation, Editing; D.S.: Experiments, Methodology, Data Curation, Conceptualization, Data Analysis, Supervision, Writing: Reviewing and Editing.

COMPETING INTERESTS
The authors declare no competing interests.

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