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# HIF-1α stimulates aromatase expression driven by prostaglandin $E_2$ in breast adipose stroma

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## Abstract

**Introduction:** The majority of postmenopausal breast cancers are estrogen-dependent. Tumor-derived factors such as prostaglandin $E_2$ ($PGE_2$) stimulate CREB1 binding to cAMP response elements (CREs) on aromatase promoter II (PII), leading to the increased expression of aromatase and biosynthesis of estrogens within human breast adipose stromal cells (ASCs). Hypoxia inducible factor-1α (HIF-1α), a key mediator of cellular adaptation to low oxygen levels, is emerging as a novel prognostic marker in breast cancer. We have identified the presence of a consensus HIF-1α binding motif overlapping with the proximal CRE of aromatase PII. However, the regulation of aromatase expression by HIF-1α in breast cancer has not been characterized. This study aimed to characterize the role of HIF-1α in the activation of aromatase PII.

**Methods:** HIF-1α expression and localization were examined in human breast ASCs using QPCR, Western blotting, immunofluorescence and high content screening. QPCR and tritiated water-release assays were performed to assess the effect of HIF-1α on aromatase expression and activity. Reporter assays and ChIP were performed to assess the effect of HIF-1α on PII activity and binding. Treatments included $PGE_2$ or DMOG (HIF-1α stabilizer). Double immunohistochemistry for HIF-1α and aromatase was performed on tissues obtained from breast cancer and cancer-free patients.

**Results:** Results indicate that $PGE_2$ increases HIF-1α transcript and protein expression, nuclear localization and binding to aromatase PII in human breast ASCs. Results also demonstrate that HIF-1α significantly increases PII activity, and aromatase transcript expression and activity, in the presence of DMOG and/or $PGE_2$, and that HIF-1α and CREB1 act co-operatively on PII. There is a significant increase in HIF-1α positive ASCs in breast

cancer patients compared to cancer-free women, and a positive association between HIF-1α and aromatase expression.

**Conclusions:** This study is the first to identify HIF-1α as a modulator of PII-driven aromatase expression in human breast tumor-associated stroma and provides a novel mechanism for estrogen regulation in obesity-related, post-menopausal breast cancer. Together with our on-going studies on the role of AMP-activated protein kinase (AMPK) in the regulation of breast aromatase, this work provides another link between disregulated metabolism and breast cancer.

## Introduction

Epidemiological studies indicate that the proportion of estrogen-dependent breast cancer cases is dramatically increased in postmenopausal women and this, despite low levels of estrogens found in the circulation. Postmenopausally, breast cancer risk also increases with obesity [1]. After menopause, when the ovaries no longer produce measurable amounts of estrogens, an increase in locally produced estrogens within the tumor and surrounding adipose tissue is believed to drive tumor growth via the action of markedly high levels of the aromatase enzyme [reviewed in 2]. The enhanced local expression of aromatase within the breast is mediated via promoter switching from distal promoter I.4 to the alternative proximal promoter II (PII) on the *CYP19A1* gene in response to inflammatory mediators derived from the tumor, such as prostaglandin $E_2$ ($PGE_2$) [3, 4]. A recent study demonstrated that $PGE_2$ is also increased in breast tissues from overweight and obese women and is associated with higher aromatase transcript expression [5]. One of the transcription factors shown to be involved in this process is cAMP response element (CRE) binding protein-1 (CREB1) which binds to the proximal and distal CREs on PII, and stimulates the expression of aromatase [6]. CREB1-coactivators including CRTC2 [7], CBP [8] and p300 [9] are also known to regulate PII-driven aromatase expression.

Many breast cancers are associated with heterogeneously distributed hypoxic tissue areas within the tumor mass [10] and hypoxia inducible factor-1α (HIF-1α) is found to be a key mediator of hypoxia-mediated tumor responses [reviewed in 11]. Previous studies have demonstrated that HIF-1α is a novel prognostic marker in determining the aggressive phenotype of breast cancer [12, 13] and is emerging as a potential target for cancer treatment [14, 15]. HIF-1 consists of 2 subunits, namely HIF1-α and HIF-1β which belong to the basic-helix-loop-helix (bHLH) protein family containing a per-aryl hydrocarbon receptor nuclear

translocator-sim (PAS) domain [16]. HIF-1β is continuously expressed and HIF-1α is continuously synthesized and degraded under normoxic conditions mainly through ubiquitin-proteasome dependent pathways after hydroxylation by prolyl-hydroxylases (PHDs) [17, 18]. Under hypoxic conditions, HIF-1 is stabilized and binds to core hypoxia response elements (HREs) containing the 5′-RCGTG-3′ sequence [19] with other transcription factors such as CBP/p300 via its CH1 domain [20], which results in the transcriptional activation of hypoxia-regulated genes including vascular endothelial growth factor (VEGF), known to promote angiogenesis [reviewed in 21]. In PC-3ML human prostate cancer cells [22] and in HCT116 human colon carcinoma cells [23], it was demonstrated that $PGE_2$ and hypoxia act both independently and synergistically to increase HIF-1α protein levels, and further demonstrated the time-dependent nuclear accumulation of HIF-1α in response to $PGE_2$.

We have identified a putative HRE which overlaps with the proximal CRE of aromatase PII. These findings led us to hypothesize that HIF-1α may have a role in regulating aromatase expression in response to the tumor-derived and obesity-associated factor, $PGE_2$, in breast ASCs.

## Materials and methods

### Plasmids

The *CYP19A1* PII-516 luciferase reporter plasmid, which contains 502bp (-516 to -14) of the proximal promoter PII was generated as previously described [24]. The HA-HIF-1α-pcDNA vector (Addgene plasmid 18949) was generated as previously described [25]. The pCMV.CREB1 plasmid was purchased from Promega (USA).

### Human tissue, cell culture

Primary human breast ASCs were isolated by collagenase digestion of subcutaneous adipose tissue from women undergoing reduction mammoplasty and cultured in Waymouth's medium (Invitrogen, USA), as previously described [26]. The studies have been approved by Southern Health Human Research Ethics Committee B (#B00109). MCF-7 cells (human breast adenocarcinoma cell line) were cultured in dulbecco's modified eagle medium (Invitrogen, USA). Before treatments, cells were serum starved for 24 hours in medium containing 0.1% bovine serum albumin. Treatments included prostaglandin $E_2$ ($PGE_2$, 1μM) and dimethyloxalglycine (DMOG, 100μM; prolyl-4-hydroxylase inhibitor which stabilizes HIF-1α) purchased from Sigma-Aldrich (USA). Sections of formalin-fixed and paraffin-embedded breast tissues from 10 Japanese breast cancer patients (IDC, invasive ductal carcinoma and DCIS: ductal carcinoma *in situ*), with differing hormone receptor status and grade (Additional file 1, Table S1), and 10 cancer-free women were used for double immunohistochemistry studies. Japanese female patients with IDC and DCIS were obtained from St. Luke's International Hospital (Tokyo). The informed consent being obtained from these patients before surgery and the research protocols were approved by the ethics committee at St Luke's International Hospital (2010-509). All the clinical data were retrieved from relevant patient's file and the histological grade was independently evaluated.

**Nuclear extraction and Western blot analysis**

Primary breast ASCs were cultured on 10cm plates and treated as described above. Nuclear extracts were obtained as described in Abcam technical website with minor modifications, including addition of cobalt chloride (1mM) and protease inhibitor cocktail tablet-complete mini from Roche (Germany). BCA protein assay (Thermo Scientific, USA) was performed to quantify protein amount according to manufacturer's instructions. 10 μg of nuclear protein diluted in loading buffer containing β-mercaptoethanol, was run on 10% denaturing

polyacrylamide gel and transferred to nitrocellulose membrane. HIF-1α and histone H3 protein levels were detected using HIF-1α (sc-10790; 1/200 dilution, Santa Cruz Biotechnology, USA), histone H3 (ab1791; 1/10,000 dilution; Abcam, USA) and Alexa Fluor 700 goat anti-rabbit secondary (1/10,000 dilution, Invitrogen, USA) antibodies using the Odyssey infrared imaging system (LI-COR Biosciences, USA). The intensity of the bands detected from Western blotting was quantified using densitometric analysis.

**Reverse transcription and quantitative PCR (QPCR)**

Total RNA was extracted using the RNeasy Mini Kit (QIAGEN, Germany) and 0.3 to 1μg of RNA was reverse transcribed using the AMV RT Kit using oligo-dT primer (Promega, USA) as directed by the manufacturer. DNA was digested using the DNA-free DNase Treatment and Removal Kit (Ambion, USA). QPCR was performed on the LightCycler using LightCycler FastStart DNA Master SYBR Green I kit (Roche, Germany). Quantification of human HIF-1α, human aromatase and β-actin or 18s (housekeeping genes) transcripts was carried out using primers hHIF-1α F: 5'-GTACCCTAACTAGCCGAGGAAGAA-3', hHIF-1α R: 5'-GTGAATGTGGCCTGTGCAGT-3', hArom F: 5'-TTGGAAATGCTGAACCCGAT-3', hArom R: 5'-CAGGAATCTGCCGTGGGGAT-3', β-actin F: 5'-TGCGTGACATTAAGGAGAAG-3', β-actin R: 5'- GCTCGTAGCTCTTCTCCA -3', 18S-F: 5'-CGGCTACCACATCCAAGGAA-3' and 18S-R: 5'GCTGGAATTACCGCGGCT-3'. Cycling conditions were one cycle at 95$^0$C for 10 min followed by 40 cycles of 95$^0$C for 10 sec, 59$^0$C for 6 sec and 72$^0$C for 4 sec for HIF-1α, 40 cycles of 95$^0$C for 10 sec, 60$^0$C for 5 sec and 72$^0$C for 10 sec for aromatase and 30 cycles of 95$^0$C for 10 sec, 59$^0$C for 5 sec and 72$^0$C for 10 sec for β-actin or 18s. All the samples were quantified using standards of known concentrations and corrected for abundance with the housekeeping gene β-actin or 18s.

**Immunofluorescence and confocal imaging**

HIF-1α protein was visualized in primary breast ASCs using immunofluorescence and confocal microscopy. ASCs were plated onto coverslips and cultured until they reached ~70% confluency. Cells were serum-starved overnight and treated for 24hrs. Immunofluorescence was performed as previously described [27], using HIF-1α antibody (sc-10790; 1/2000 dilution, Santa Cruz Biotechnology, USA) and lamin B1+B2 antibody (1/1000 dilution, Abcam, USA) for nuclear stain and visualized using alexa fluor-546 (red) and -488 (green) from Invitrogen (USA), respectively, using confocal microscopy (Olympus Optical Co Ltd, Japan).

**High content screening**

Primary breast ASCs cells were plated in 96-well plates at a density of 6,000 cells per well. One day after plating, the cells were serum-starved for 24 hours and treated with $PGE_2$ for 6 hours. Fixation was carried out in ice-cold methanol for 40 min at -20$^0$C, followed by 2×PBS washes. Cells were blocked in 0.5% BSA/PBS for 30 min and incubated with HIF-1α antibody (sc-10790; 1/2,000 dilution in 0.5% BSA/PBS, Santa Cruz Biotechnology, USA) overnight at 4$^0$C with gentle rocking. After 2×PBS washes, Alexa fluor 488 goat anti-rabbit secondary antibody (1/1,000 dilution, Invitrogen, USA) and Hoechst nuclear counterstain (1/5,000 dilution) in 0.5% BSA/PBS were applied for 90 min. In order to quantitate nuclear fluorescence, images were captured on an ArrayScan VTI instrument (Thermo Fisher Scientific, USA) and analyzed using Cellomics software and the Compartmental Analysis Bioapplication. The analysis algorithm used Hoechst fluorescence to define a mask used to measure nuclear fluorescence. Threshold for nuclear staining was determined by assessing nuclear intensity in negative control samples and set to two standard deviations. Cells with nuclear HIF-1α pixel intensity ≥230 were considered HIF-1α positive.

**Chromatin immunoprecipitation (ChIP)**

Primary breast ASCs treated with $PGE_2$ or DMOG for 45 min were used for ChIP to examine endogenous binding of HIF-1α to aromatase PII using the ChIP-IT express kit (Active Motif, USA) as directed by the manufacturer with minor modifications [7]. Briefly, cells were fixed using 1% formaldehyde to cross-link and preserve endogenous protein-DNA interactions. The DNA was then sheared into small fragments using sonication at 20% amplitude, 7 times for 30 sec pulses. Specific protein-DNA complexes were immunoprecipitated using HIF-1α antibody (Santa Cruz Biotechnology, USA), IgG or water as controls. QPCR was then performed using primers flanking the CREs of *CYP19A1* PII (PII-ChIP-F: 5'-TTTCCACACTACCGTTGGCCG-3' and PII-ChIP-R: 5'-GGCAATCTTCTTCCCTTGAAGC-3'), and normalized to input DNA [7].

**Reporter gene assays**

MCF-7 cells were transfected with wild type or proximal CRE mutated *CYP19A1* PII-516 luciferase constructs, with/without human HIF-1α-pcDNA (Addgene, USA) and/or human CREB1-pcDNA or with HIF-1α siRNA (sc-35561, Santa Cruz Biotechnology, USA) or control siRNA-A (sc-37007, Santa Cruz Biotechnology, USA), using the cell line nucleofector kit V, program E-014 (Lonza Cologne GmbH, Germany), according to the manufacturer's instructions. β-galactosidase was co-transfected and used as a transfection control. After transfection, cells were serum-starved and treated with FSK/PMA and/or DMOG for 24 hours. Luciferase reporter assays were carried out using the Dual-Glo Luciferase Assay System (Promega, USA) according to the manufacturer's protocol and data was normalized to β-galactosidase activity.

**Aromatase activity assay (Tritiated water-release assay)**

Primary breast ASCs and MCF-7 cells were plated in 6-well plates. MCF-7 cells were transfected with 2μg of human HIF-1α-pcDNA (Addgene, USA) or 3μl of HIF-1α siRNA (sc-35561, Santa Cruz Biotechnology, USA) or control siRNA-A (sc-37007, Santa Cruz Biotechnology, USA) using lipofectamine transfection reagent (Invitrogen, USA), as directed by the manufacturer. ASCs and MCF-7 cells were serum-starved for 24 hours and treated with specified reagents. Aromatase activity in these cells was measured using the tritiated water-release assay using androst-4-ene-3, 17-dione (NET926001MC, PerkinElmer, USA) as a substrate, as previously described [28]. Specific activity was normalized to total protein amount.

**Double immunohistochemistry (IHC)**

Formalin-fixed paraffin-embedded tissue sections from breast cancer and cancer-free patients were dewaxed in xylene and were rehydrated through descending concentrations of ethanol solutions to distilled $H_2O$. Tissue sections were incubated in 10% horse serum in CAS-block (Invitrogen, USA) for 30 min. Previously characterized aromatase mouse monoclonal primary antibody 677 (1/250 dilution from 2.6 mg/ml stock in 0.5% BSA/PBS, [29]) was added to the slides and incubated overnight at $4^0$C. After washing in PBS, biotinylated universal secondary antibody (1/200 dilution, Vectastain Universal ABC-AP kit, Vector laboratories, USA) was applied for 30 min and then incubated with Vectastain ABC-AP reagent for 30 min. Alkaline phosphatase substrate (Vector blue alkaline phosphate substrate kit, Vector Laboratories, USA) was added until desired cytoplasmic blue stain intensity developed and the reaction stopped with distilled $H_2O$.

Slides were then subjected to antigen retrieval in an autoclave, at $121^0$C for 5 min in Tris EDTA pH 9 (10 mM Tris base, 1mM EDTA). After cooling for 30 min, sections were washed in PBS and incubated in 10% horse serum in CAS-block (Invitrogen, USA) for 30

min. The HIF-1α primary antibody (sc-10790; 1/250 dilution in 0.5% BSA/PBS, Santa Cruz Biotechnology, USA) was added to the slides and incubated overnight at $4^0$C. After washing in PBS, endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide for 30 minutes. Anti-rabbit IgG secondary antibody (1/1,000 dilution, Vectastain ABC-AP kit-rabbit IgG, Vector laboratories, USA) was applied for 30 min and then incubated with Vectastain ABC-AP reagent for 30 min. Slides were then stained with 3,3′-diaminobenzidine tetrahydrochloride (DAB, Sigma-Aldrich, USA) until desired nuclear brown stain intensity developed and the reaction stopped with distilled $H_2O$. Finally, the sections were mounted with fluorsave reagent (Calbiochem, USA).

Double immunohistochemically stained slides for HIF-1α and aromatase were evaluated independently by two observers (N.U.S. and M.S. or N.U.S. and K.A.B.). The ASCs were examined using systematic random sampling on a stereology microscope (Olympus, Japan) with the aid of CAST-Grid version 1.60 and categorized into four groups including HIF-1α positive and aromatase positive, HIF-1α positive and aromatase negative, HIF-1α negative and aromatase positive, and HIF-1α negative and aromatase negative (Additional file 2, Figure S1).

**Statistical analysis**

All data were expressed as mean ± standard error (SE). Two-tailed Student's t test was performed for experiments comparing two groups. For experiments with multiple comparisons, statistical analysis was done using one-way ANOVA followed by Bonferroni's multiple comparison test. Statistical significance was defined as *, $P<0.05$; **, $P\leq0.005$; ***, $P\leq0.0005$, ****, $P\leq0.0001$. Data analysis was performed using GraphPad Prism version 5.00.

## Results

### $PGE_2$ increases HIF-1α expression and nuclear localization in primary human breast ASCs

The effect of $PGE_2$ on HIF-1α transcript and protein expression was examined in primary breast ASCs. HIF-1α mRNA was significantly increased with $PGE_2$ treatment (Figure 1A). Moreover, Western blotting demonstrated that $PGE_2$ also caused a significant increase in HIF-1α protein abundance in the nucleus (Figure 1B). The subcellular localization of endogenously expressed HIF-1α was examined using immunofluorescence and confocal microscopy after treating with $PGE_2$ and /or DMOG. Results demonstrated that punctate staining for HIF-1α appears in the nucleus after $PGE_2$ treatment (Figure 1C, top right) compared to cytoplasmic localization under basal conditions (Figure 1C, top left). Furthermore, DMOG (Figure 1C, bottom left) and DMOG with $PGE_2$ (Figure 1C, bottom right) resulted in more intense staining for HIF-1α in the nucleus. Results from high content screening also demonstrated that the percentage cells positive for HIF-1α in the nucleus (Figure 1D) was significantly increased in response to $PGE_2$ treatment.

### HIF-1α binds to aromatase promoter PII and increases aromatase expression and activity in primary breast ASCs

The endogenous binding of HIF-1α to aromatase PII was evaluated by ChIP. Treatment with either $PGE_2$ or DMOG resulted in a significant increase in binding of HIF-1α to aromatase PII compared to vehicle control (Figure 2A). Consistent with these findings, treatment of primary human breast ASCs with $PGE_2$ or DMOG also caused a significant increase in aromatase transcript expression (Figure 2B) and aromatase activity (Figure 2C).

Overexpression of HIF-1α in MCF-7 cells treated with $PGE_2$ also resulted in increased aromatase activity (Figure 2D).

**HIF-1α acts cooperatively with CREB1 to increase PII activity breast ASCs**

PROMO 3.0 software analysis was used to identify a putative binding site of HIF-1α on aromatase PII [30, 31]. Results demonstrated that the predicted HRE overlaps with the proximal CRE of aromatase PII (Figure 3A).

To determine the effect of HIF-1α on aromatase PII activity, luciferase reporter assays were performed in MCF-7 cells, transfected with HIF-1α and wild type or proximal CRE mutated *CYP19A1* PII-516 luciferase reporter constructs and treated with DMOG. Cells transfected with HIF-1α treated with DMOG and DMOG alone showed a significant increase in promoter II activity, and this HIF-1α/DMOG-mediated effect on promoter II activity was completely abolished using a proximal CRE-mutated PII reporter construct (Figure 3B). As the putative HRE overlaps with the proximal CRE on aromatase PII, experiments were performed to determine whether HIF-1α acted cooperatively or competitively with CREB1. MCF-7 cells were transfected with HIF-1α and/or CREB1 together with the *CYP19A1* PII-516 luciferase reporter construct, and then treated with DMOG and/or $PGE_2$. PII activity was significantly increased in HIF-1α transfected cells in the presence of $PGE_2$ or DMOG, and treatment with both $PGE_2$ and DMOG led to a further significant increase in PII activity (Figure 3C). Interestingly, cells transfected with both HIF-1α and CREB1 showed a further increase in PII activity ($P \leq 0.005$) with both $PGE_2$ and DMOG compared to cells transfected with either HIF-1α or CREB1 alone (Figure 3C). Hence, HIF-1α and CREB1 act cooperatively to increase PII activity.

**HIF-1α is necessary for the $PGE_2$-mediated induction of aromatase PII**

Reporter assays were also performed to examine requirement of HIF-1α for the $PGE_2$-mediated increase in PII activity. MCF-7 cells transfected with HIF-1α siRNA showed a significant reduction in aromatase PII activity (Figure 3D) and aromatase transcript (Figure 3F), and the $PGE_2$-mediated effect on PII via HIF-1α was suppressed (Figure 3D).

**HIF-1α is increased in ASCs from tumor-bearing breast tissues compared to cancer free breast tissue**

Double immunohistochemistry was performed on formalin-fixed, paraffin-embedded (FFPE) tissues from breast cancer patients and cancer-free women. Results demonstrated that there is a significant increase in the percentage of HIF-1α positive ASCs in breast cancer patients compared to cancer-free women (Figure 4A). Interestingly, ASCs which were HIF-1α and aromatase double-positive, and which were single-positive for HIF-1α or aromatase, were significantly increased in tumor patients compared to cancer-free women (Figure 4B). Furthermore, double negative ASCs were significantly decreased in tumor cases compared to normal (Figure 4B) and the percentage of cells either double-positive or double-negative was significantly higher than single-positive cells (analysis not shown), suggesting a positive association between HIF-1α and aromatase expression.

**Discussion**

In this study, novel evidence is provided for the regulation of HIF-1α and its role in regulating aromatase expression in adipose stromal cells in the context of obesity and breast cancer. Namely, that HIF-1α expression and nuclear localization are increased in response to $PGE_2$ and stimulate the promoter II-driven expression of aromatase in breast ASCs via binding to the proximal CRE (Figure 5).

HIF-1α has been shown to be overexpressed in many different types of tumors including those of the ovary, prostate and breast [32]. However, the expression and regulation of HIF-1α in cancer-associated adipose stromal cells is less well characterized. Here, we demonstrate that HIF-1α transcript, protein expression and protein nuclear localization are increased in breast ASCs in response to the tumor-derived factor $PGE_2$. Consistent with these observations, we also demonstrate that in clinical samples, the number of HIF-1α positive ASCs is increased in breast cancer patient samples compared to cancer-free breast tissue. Despite the limited number of patient samples examined and these being from women with differing tumor grade, the dramatic increase observed was highly conserved amongst all patients. However, the majority of cases were postmenopausal women with hormone receptor positive tumors. Nevertheless, it has been reported that the majority of breast tumors overexpress COX-2 and secrete high levels of $PGE_2$ [33, 34]. Our results are consistent with observations demonstrating that $PGE_2$ causes the stabilization of HIF-1α independent of hypoxia in PC-3ML human prostate cancer cells [22], HCT116 human colon carcinoma cells [23] and AGS gastric carcinoma cells [35]. However, to our knowledge, this is the first report to demonstrate an increase in HIF-1α transcript levels in response to $PGE_2$ in ASCs.

The $PGE_2$-induced upregulation of HIF-1α has been shown to be mediated through EP2 and EP4 receptor activation in PC-3ML cells [22] and the EP1 receptor alone in HCT116 cells [23]. In human embryonic kidney cells expressing the human EP1 receptor, $PGE_2$ has been shown to upregulate HIF-1α protein expression in a time-dependent manner under normoxic conditions [36]. Interestingly, the expression of EP receptors has been demonstrated in breast ASCs and the regulation of aromatase by $PGE_2$ in these cells has been shown to be dependent on activation the EP1 and EP2 receptors [4, 37]. These data suggest that aromatase upregulation via EP receptor activation may involve the induction of HIF-1α. Indeed, HIF-1α increases the activity of aromatase PII, and the increased nuclear localization

and punctuate appearance of HIF-1α in response to $PGE_2$ is also associated with the increased binding of HIF-1α to aromatase PII via the proximal CRE. The regulation of aromatase by HIF-1α has also recently been examined in the context of placental aromatase regulation. In that case, aromatase expression is mediated by distal placental-specific promoter I.1 and is dependent on binding of estrogen-related receptor γ (ERRγ) to the promoter [38]. Hypoxia has been shown to cause the HIF-1α-dependent downregulation of ERRγ [38]. Contrary to our findings, the HIF-1α-mediated effects on PI.1 are inhibitory and appear to be indirect.

Promoter analysis revealed that a putative core HRE sequence is present on the antisense strand of aromatase PII and overlaps with the proximal CRE, suggesting that HIF-1α may interact with CREB1 in ASCs. Previous studies have demonstrated that CREB1 binds to the HRE in the plasminogen activator inhibitor-1 (PAI-1) promoter [39] and HIF-1α can also interact with ATF2/CBP/p300 [40-42]. There is a significant elevation in CREB1 transcript expression in breast tumor tissues compared to non-neoplastic breast tissues and this is positively associated with poor prognosis, metastatic disease and nodal involvement [43, 44]. Interestingly, the present study demonstrates that HIF-1α alone can stimulate aromatase promoter PII and that it also acts cooperatively with CREB1 to increase aromatase PII activity.

The local biosynthesis of estrogens from breast ASCs is considered a key mediator of tumor cell growth in postmenopausal breast cancer, and increased PII activity accounts for the majority of transcripts detected [3]. We have observed that the majority of ASCs in the tumor-bearing tissue are either double-positive or double-negative for HIF-1α and aromatase expression, suggesting an association between the two proteins. These findings support our *in vitro* data demonstrating that HIF-1α directly stimulates aromatase expression.

Collectively, the results obtained in this study show for the first time that HIF-1α activates PII-driven aromatase expression in breast ASCs in response to $PGE_2$, independent of oxygen availability. Hence, this specific association is likely to be an important mechanism for the regulation of estrogen biosynthesis in obesity-related, postmenopausal breast cancer. Third-generation aromatase inhibitors (AIs) are currently the most effective treatment and have been shown to be superior to tamoxifen for hormone receptor positive postmenopausal breast cancer as aromatase catalyses the conversion of circulating androgenic precursors to estrogens [45-47]. However, many women cease use of AIs due to increasingly severe side-effects associated with their use [48-50]. Currently, small molecule inhibitors of HIF-1α are being tested in the clinical setting [reviewed in 51]. We believe that better understanding of the regulation of aromatase PII will allow us to target aromatase expression specifically within the breast, leaving sites such as the bone, brain and heart, where estrogens have beneficial effects, unaffected.

## Conclusions

This study demonstrates that HIF-1α, a master regulator of oxygen homeostasis, stimulates PII-driven aromatase expression in human breast ASCs with other transcription factors including CREB1 in response to tumor-derived and obesity-associated inflammatory mediator $PGE_2$. Our findings of HIF-1α in tumor-associated breast stroma implicate its potential as a therapeutic target in obesity-related, postmenopausal breast cancer.

## Abbreviations

AMPK: AMP-activated protein kinase; ASC: adipose stromal cells; bHLH: basic-helix-loop-helix; CREB1: CRE binding protein 1; CREs: cAMP response elements; HIF-1α: hypoxia inducible factor 1 alpha; HREs: hypoxia response elements; PAS: per-aryl hydrocarbon receptor nuclear translocator-sim; $PGE_2$: Prostaglandin $E_2$; PHDs: prolyl-hydroxylases; PII: promoter II; VEGF: vascular endothelial growth factor.

## Competing interests

The authors declare that they have no competing interests.

## Authors' contributions

NUS and KAB designed all the experiments. NUS conducted all the experiments, with the help from FY, MMD, MS, KMM, HS and SBF. The paper was written by NUS and KAB, ERS, SBF and HS were involved in manuscript revision. KAB and ERS contributed to the conception of project. All authors revised the manuscript and gave their final approval.

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