ERa directly regulates the hypoxia-inducible factor 1 pathway associated with anti-estrogen response in breast cancer

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A majority of breast cancers are driven by estrogen via ERα. Our previous studies indicate that HIF-1α cooperates with ERα in breast cancer cells. However, whether ERα is implicated in the direct regulation of HIF-1α and the role of HIF-1α in endocrine therapy response are unknown. In this study we found that a subpopulation of HIF-1α targets, many of them bearing both hypoxia response elements and estrogen response elements, are regulated by ERα in normoxia and hypoxia. Interestingly, the HIF-1α gene itself also bears an estrogen response element, and its expression is directly regulated by ERα. Clinical data revealed that expression of the HIF-1α gene or a hypoxia metagene signature is associated with a poor outcome to endocrine treatment in ERα-positive breast cancer. HIF-1α was able to confer endocrine therapy resistance to ERα-positive breast cancer cells. Our findings for the first time define a direct regulatory pathway between ERα and HIF-1α, which might modulate hormone response in treatment.

ERa | HIF-1a | tamoxifen

Introduction

Estrogen receptor alpha (ERα) is an estrogen-dependent nuclear transcription factor that is critical for mammary epithelial cell division and breast cancer progression (1, 2). ERα is expressed in approximately 70% of breast tumors (3), the majority of which depend on estrogen signaling, thereby providing the rationale for using anti-estrogens as adjuvant therapy to treat breast cancer (4). Tamoxifen is a first generation selective ER modulator (SERM) and has been widely used in breast cancer prevention and treatment (4). Although now replaced by aromatase inhibitors (AI) as first line treatment in post-menopausal women, it still remains important in premenopausal breast cancer and after failure of AIs. Tamoxifen acts as an antagonist in breast cancer cells by competing with estrogen for the ER. Tamoxifen-bound ER recruits the nuclear receptor co-repressor (NCOR) and histone deacetylase (HDAC), as opposed to co-activators, leading to transcriptional repression (5). Although hundreds of thousands of patients have benefited from tamoxifen treatment, its efficacy is limited to an average time of 15 months in patients with metastatic disease (6), as resistance often develops (7). Many mechanisms have been proposed to account for tamoxifen resistance (8), including loss of ERα expression or expression of truncated ER isoforms, posttranslational modification of ERα, deregulation of ERα co-activators, and increased receptor tyrosine kinase signaling. Recent studies further indicate that somatic ERα mutation (9, 10), as well as genomic amplification of distant ER response elements (11) could contribute to hormone therapy resistance.

Our clinical studies suggest that the in vivo tumor environment may play a role in tamoxifen resistance, as HIF-1α protein expression was associated with tamoxifen resistance in neoadjuvant, primary therapy of ERα-positive breast cancers (12) as well as resistance to chemoendocrine therapy (13).

HIF-1α is a master regulator of oxygen homeostasis, which is rapidly degraded in normoxia by the tumor suppressor, von Hippel–Lindau protein (VHL), but is stabilized in hypoxia (14). This process is mainly determined by the hydroxylation of HIF-1α catalyzed by prolyl hydroxylases (PHD). HIF-1α has been associated with an aggressive phenotype of breast cancer, i.e. large tumor size, high grade, high proliferation and lymph node metastasis (15). Increased HIF-1α is also associated with ERα positivity (15) whilst HIF-1β, the partner of HIF-1α, has been shown to function as a potent co-activator of ER-dependent transcription (16). Further studies revealed that in ER-positive T47D breast cancer cells, combined hypoxia and E2 treatment had additive effects on expression of some genes (17), although the mechanism is not clear. We have previously shown that HIF-1α and ERα can coordinate expression of genes such as KDM4B/JMJD2B, an H3K9me3/me2 histone demethylase, which is targeted by both ERα and HIF-1α and epigenetically regulates cell cycle progression (18). The genomic locus of KDM4B bears both HIF-1α and ERα binding elements (18, 19). These data collectively suggest that HIF-1α and ERα are functionally associated. However, how these two important oncogenic pathways interact has not yet been defined. In addition, whether HIF-1α plays an autonomous

Significance

1.7 million new cases of breast cancer occur every year, 70% of which are ERα positive. Anti-estrogen therapy to block ERα function is the most important approach in treatment of ERα positive patients. However, resistance eventually will develop for various reasons. Here we demonstrate that HIF-1α is a direct transcriptional target of ERα, which may compensate for ERα function loss as many other ERα targets are also HIF-1α targets. We further show that HIF-1α is able to confer cancer cell resistance to ERα antagonists; and the expression of HIF-1α is associated with poor survival to endocrine therapy in ERα positive patients. Our findings thus have revealed a new mechanism for anti-estrogen resistance.

Reserved for Publication Footnotes

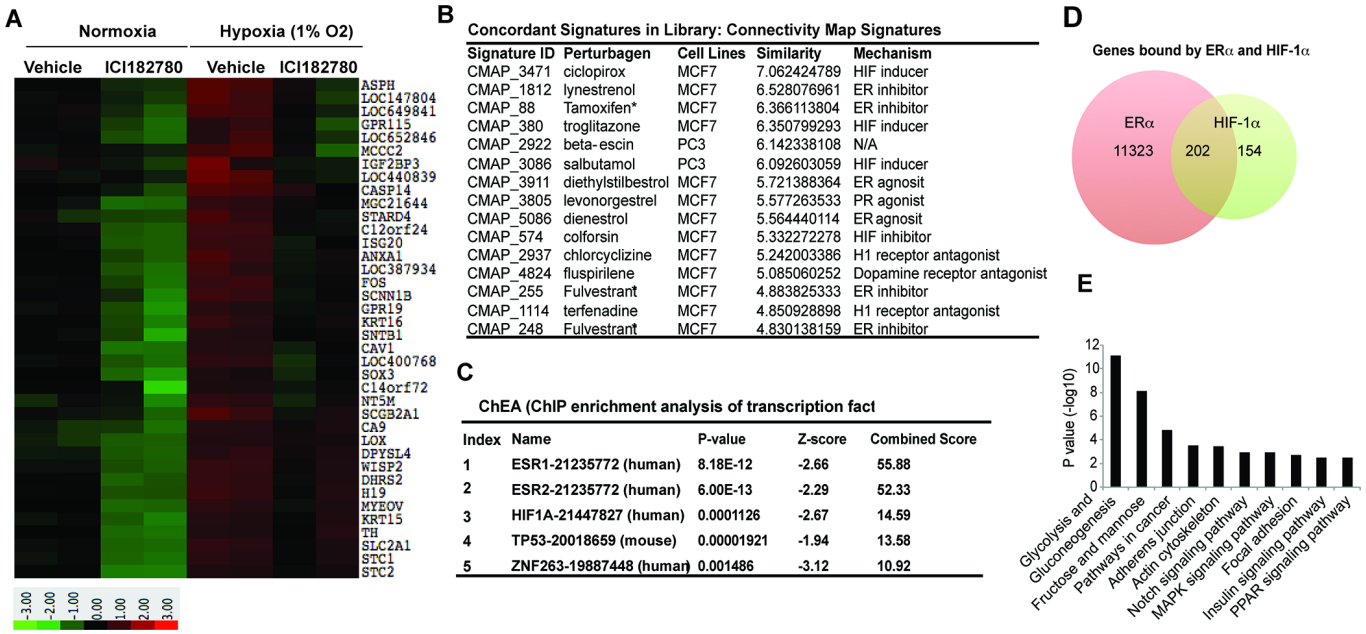

Fig. 1. ERα signaling regulates hypoxia/HIF pathway. **(A) MCF7 cells were treated with 1uM of ICI182780 in normoxia and hypoxia (1% O_2) for 24 hours. Extracted RNA from duplicated biological samples was subject to microarray analysis. Heatmap shows a subgroup of genes that are dual responsive to hormone and oxygen. (B) The dually responsive genes were queried with LINCS program to search Connectivity MAP (CMAP) for compounds that induced a similar pattern to ICI182780. (C) Chromatin-immunoprecipitation enrichment analysis (ChEA) of transcription factor binding to the dual responsive genes. (D) Venn diagram shows the common gene bound by both ERα** and HIF-1α. **(E) KEGG pathway annotation of the common genes from Figure 1D.**

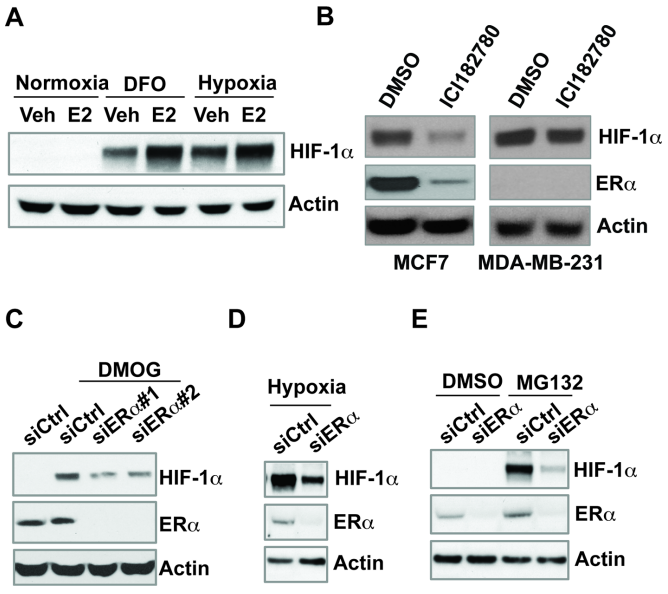

Fig. 2. ERα signaling regulates HIF-1αexpression (A) After 4 days hormonal starvation, MCF7 cells were treated with 500 μM of DFO or 1% O_2 for 6 hours in the presence or absence of 100 nM of E2. The whole cell lysates were subject to western blotting with indicated antibodies. V=Vehicle. (B) MCF7 and MDA-MB-231 cells were treated with 1uM of ICI182780 in hypoxia (1% O_2) for 24 hours. The whole cell lysates were subject to western blotting with indicated antibodies.(C-D) ERα in MCF7 cells was knockdown with two different siRNAs (C) or SMARTpool (D), which were treated with 200 μM of DMOG (C) or hypoxia (D). The whole cell lysates were subject to western blotting with indicated antibodies.(E) MCF7 cells were treated with siRNA SMARTpool against ERα, treated with 10 μMG132 for 16 hours. The whole cell lysates were subject to western blotting with indicated antibodies.

role in modulating endocrine therapy efficacy such as tamoxifen resistance is unknown. In this study, we investigated the role of ERα in the regulation of HIF-1 signaling and how HIF-1 signaling is involved in endocrine drug response.

Results

ERα signaling regulates hypoxia/ HIF-1α pathway

We have previously shown that knockdown of ERα significantly downregulated histone demethylase KDM4B expression (18), a HIF-1α transcriptional target, suggesting that HIF-1α function is compromised by loss of ERα even in hypoxia. To study whether ERα signaling is involved in the regulation of the hypoxia/HIF pathway, we used a chemical genetics approach in which the ERα positive breast cancer cell line MCF7 was treated with ICI182780 (Fulvestrant) in normoxia and hypoxia to perform a global gene expression profile analysis (Fig. 1A). ICI182780 is an estrogen receptor antagonist with no agonist effects, which works by down-regulating the ERα expression. Clinically, ICI182780 has been used in hormone receptor positive metastatic breast cancer in postmenopausal women with disease progression following anti-estrogen therapy. The gene expression profiling results showed that a cluster of genes such as *STC2*, *STC1, SLC2A1* (also known as *Glut-1*) and *LOX* that were induced in hypoxia were down-regulated by ICI182780 in both normoxia and hypoxia (Fig. 1A). We then queried iLINCS (integrative LINCS) genomics data portal to search compounds that regulate similar gene pattern as ICI182780 induced in MCF7 cells. Among the top 15 hits most are HIF-1α inducers or ERα modulators including Tamoxifen and ICI182780 (Fig. 1B). We analyzed these genes through chromatin-immunoprecipitation (ChIP) enrichment analysis of transcription factors (ChEA) through LINCS canvas browser II to examine what transcriptional factors directly regulate their expression. The top hits were ERα and HIF-1α (Fig. 1C). Thus, these data indicate that a subgroup of genes that are targeted by hypoxia/HIF-1α is also regulated by ERα signaling, which are dual responsive to hormone and oxygen. Interestingly, some of these genes can be bound by p53 in murine embryonic stem cells although the biological function is unclear (20). Cancer genomic sequencing reveals that p53 is

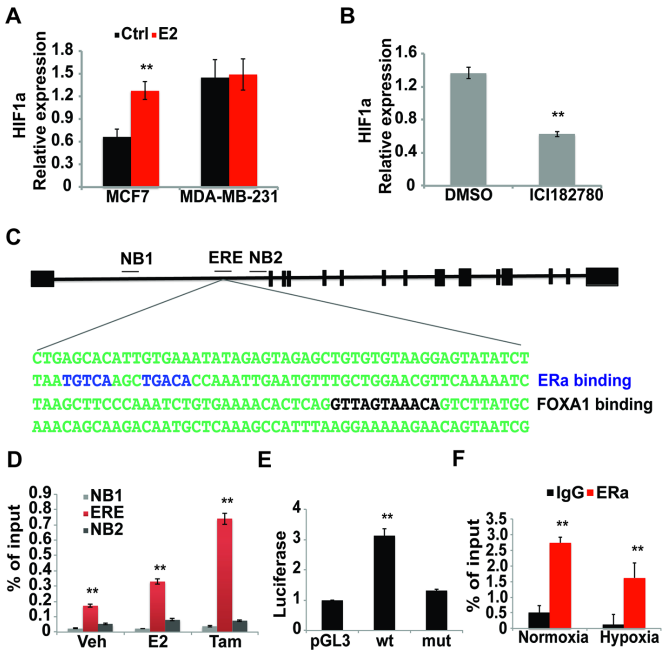

Fig. 3. ERα directly binds estrogen response elements on HIF-1α gene to enhance HIF-1αtranscription(A) After 3 days hormonal starvation, MCF7 cells were treated with 100 nM of E2 for 24 hours. Real-time PCR was performed to assess *HIF-1*α gene expression.(B) MCF7 cells were treated with 1 μM of ICI182780 for 24 hours. Real-time PCR was performed to assess *HIF-1*α gene expression.(C) The gene structure of *HIF-1*α and the sequence that bears ERα binding sites (ERE) and FOXA1 binding sites as indicated. The black bar represents each exon while the black line represents each intron of *HIF-1*α. NB1 and NB2 represent the primer location for ChIP PCR to assess ERα binding, as negative controls as shown in Figure 3D.(D) After 3 days hormonal starvation, MCF7 cells were treated with 100 nM of E2 or tamoxifen for 24 hours. ChIP PCR was performed to assess ERα binding among the regions as indicated in Figure 3C.(E) The ERE region and the mutant were cloned into pGL3 luciferase reporter. The plasmids and the pRL Renilla Luciferase control vector (to normalize the ERE reporter) were transfected into MCF7 cells. Dual luciferase assay was performed.(F) MCF7 cells were incubated in hypoxia for 8 hours. ChIP PCR was performed to assess ERα binding at ERE of *HIF-1*α.

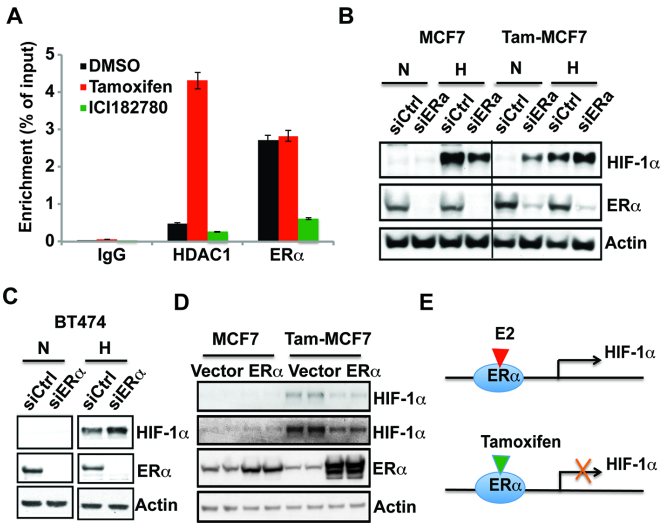

Fig. 4. Tamoxifen-bound ERα inhibits HIF-1α expression(A) MCF7 cells were cultured in standard DMEM and treated with 1000 nM of tamoxifen or ICI182780 for 48 hours.ChIP PCR was performed to assess HDAC1 and ERα binding the ERE of *HIF-1*α. (B) MCF7 and TamR-MCF7 cells were transfected with siRNA SMARTpool against ERα. After 36 hours transfection, cells were incubated in normoxia and hypoxia for 16 hours. Western blotting was used to assess the indicated proteins. (C)BT474 cells were treated as in (B).(D) MCF7 and TamR-MCF7 cells were transfected with ERα expression plasmids. MCF7 cells were cultured in normoxia in standard DMEM medium while Tam-MCF7 cells were cultured in phenol-free DMEM with 100 nM of tamoxifen. After 48 hours transfection cells were harvested for western blotting. The two HIF-1α blots were for short and long exposure, respectively.(E) The cartoon shows the E2-bound and tamoxifen-bound ERαexert opposite effect on *HIF-1*α transcription.

more commonly mutated in triple negative (TRN) breast cancers than ERα− positive patients (21) while MCF7 is p53 wild-type. ZNF263 is a transcription factor that regulates FoxA1 expression (22). FoxA1 is a pioneer factor that facilitates ERα for genomic binding (23), which further suggests that estrogen-ER signaling pathway is involved in hypoxia/HIF response.

To further confirm that ERα and HIF-1α directly bind their response elements in a subgroup of genes, we re-analyzed published ChIP sequencing data (24, 25). We found that among the 356 genes bound by HIF-1α, 202 (57%) of them were identified as the common genes bound by ERα as well (Fig. 1D, Table S1). *KDM4B* was one of the targets of both ERα and HIF-1α (Table S1), consistent with our previous studies (18, 26). Pathway analysis reveals that these common genes are involved in metabolism, cancer and important signaling pathways including Notch, MAPK and insulin pathways (Fig. 1E).

ERα signaling regulates HIF-1α expression

Some genes such as *STC2, VEGFA* and *KDM4B* bear both ERα and HIF-1α binding elements (18, 19, 27-30), and thus we initially hypothesized that blockade of the ER pathway might disrupt HIF-1α binding to its target genes. Surprisingly, we found that ERα signaling actually directly regulates HIF-1α expression. When MCF7 cells were grown without estrogen for 4 days and then placed in hypoxia or treated with the hypoxia mimetic deferoximine, 17-β-estradiol (E2) greatly enhanced HIF-1α expression (Fig. 2A). However, ICI182780 significantly reduced HIF-1α and ERα expression in hypoxia, (Fig. 2B), consistent with its biological function in suppressing HIF-1α targets (Figure 1). In addition, ICI1820780 treatment did not affect HIF-1α expression in MDA-MD-231 cell (Fig. 2B), an ERα negative cell line. Knockdown of ERα with two separate siRNA oligos or a smartpool of siRNA greatly reduced HIF-1α induction under hypoxia or hypoxic mimetic DMOG treatment (Fig. 2, C and D). The proteasome inhibitor MG132, which stabilizes HIF-1α protein expression by preventing protein degradation, failed to rescue ERα knockdown-mediated down-regulation of HIF-1α (Fig. 2E). These data clearly show that the ERα signaling pathway directly regulates HIF-1α expression.

HIF-1α gene bears estrogen response element and ERα enhances HIF-1α transcription

HIF-1α is labile and subject to rapid proteasomal degradation. Most studies focus on the regulation of protein stability of HIF-1α while how HIF-1α is transcriptionally regulated is not well understood. The down-regulation of HIF-1α by knockdown of ERα was not rescued by the proteasome inhibitor MG132, indicating that ERα regulates HIF-1α expression in a protein stability independent manner (Fig. 2E). Supporting this hypothesis, the ERα antagonist tamoxifen treatment resulted in downregulation of HIF-1α expression in MCF7 but not MDA-MB-231 cells, although tamoxifen caused ERα protein upregulation (Fig. S1A), suggesting impaired ERα transcriptional function is involved. To determine whether ERα signaling directly regulates *HIF-1*α gene expression, we treated hormone starved MCF7 and MDA-MB-231 cells with estrogen. RT PCR data revealed that *HIF-1*α, □*KDM4B* and *STC2* mRNA was significantly upregulated in MCF7 but not MDA-MB-231 cells (Fig. 3A; S1, B and C). RT PCR results further showed that *HIF-1*α mRNA level was significantly reduced after ICI182780 treatment, indicating an

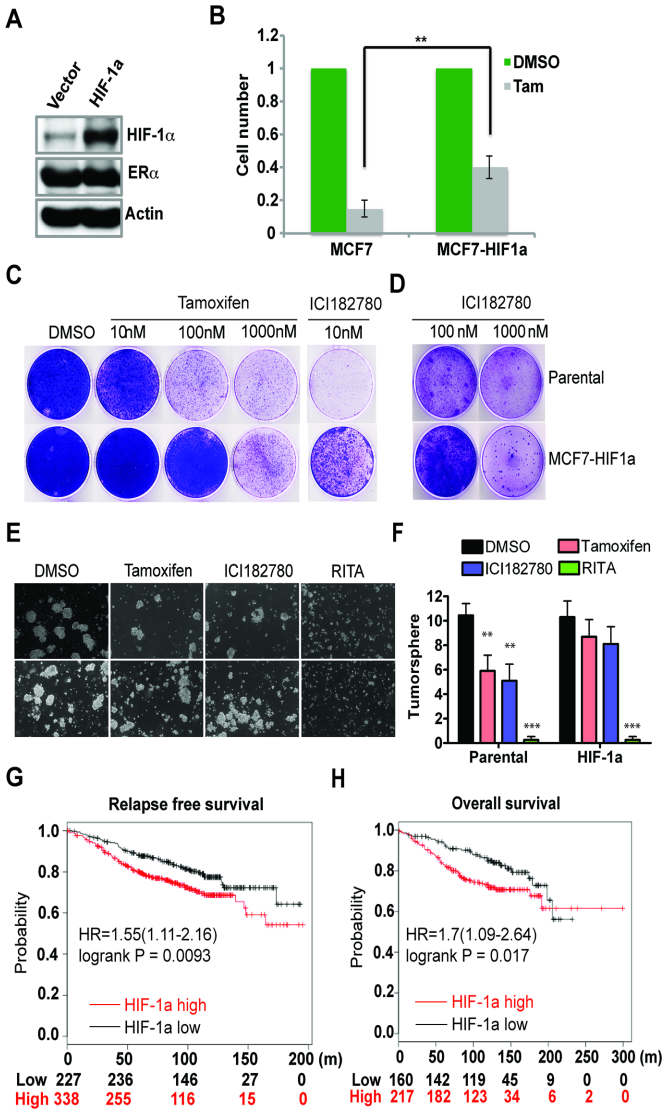

Fig. 5. HIF-1α confers tamoxifen resistance to ER-positive breast cancer cells (A) Western blotting to assess MCF7 cells expressing HIF-1α.(B) MCF7 control and MCF7-HIF-1α cells were treated with 100 nM of tamoxifen for 96 hours. Cell were stained with crystal violet and counted under microscope, cell number was normalized with control treatment. (C) The MCF7 control and MCF7-HIF-1α cells were treated with tamoxifen or ICI182780 with indicated concentration for 18 days. Cell colonies were stained with crystal violet.(D) The MCF7 control and MCF7-HIF-1α cells were treated with ICI182780 for 4 weeks. Cell colonies were stained with crystal violet.(E) Tumorspheres generated from the parental (top panel) and HIF-1α expressing (bottom panel) MCF7 cells were treated for 5 days with 1μM of tamoxifen, 1μM of ICI182780 and 0.5μM of RITA. Photos were taken under microscope (10X).(F) After counting 10 different 10X fields, the tumorsphere counts were averaged. (G) Kaplan-Meier analyses for relapse free survival of the cohort of patients with ER positivity, receiving tamoxifen treatment only without chemotherapy.Affymetrix ID for *HIF-1α* used was 200989_at. The cutoff value used in analysis was 3043 and the expression range of the probe was 439–17198. Patient number for low *HIF-1α* (black) and high *HIF-1α*(red) is presented under the following months.(H) Kaplan-Meier analyses for overall survival of the cohort of patients with ER positivity, receiving tamoxifen treatment with chemotherapy.The cutoff value was 3035 and the expression range was 456–11726.Patient number for low *HIF-1α* (black) and high *HIF-1α*(red) is presented under the following months.

ER-mediated transcriptional mechanism regulates *HIF-1α* transcription.

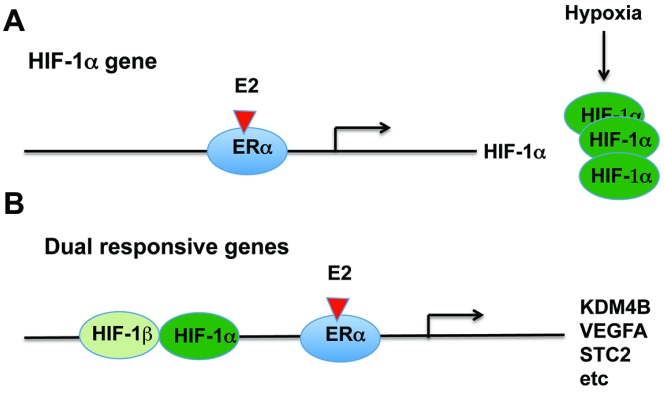

Fig. 6. The working model between ERα and HIF-1 pathway. **(A)** ***HIF-1α*** gene bears ERα binding site whose transcription is directly regulated by ERa signaling pathway. Under hypoxia, produced HIF-1α protein is stabilized. **(B) Dual responsive genes bear both ERα** and HIF-1α binding sites, which can be regulated by two signaling pathways.

By analyzing the *HIF-1α* genomic sequence that bears 15 exons and 14 introns (Fig. 3C), we found that there is a canonical estrogen response element (ERE) located in the first intron. Interestingly, there is also a FOXA1 binding site that is 64 nucleotides downstream of ERE (Fig. 3C), further supporting it as a bona fide ERα binding element because FOXA1 is a pioneer factor that facilitates ERα recruitment (23). In fact, all ER-chromatin interactions and gene expression changes depend on the presence of FOXA1(23). To test whether ERα is able to bind the ERE of *HIF-1α* gene, we treated MCF7 cells with E2 or tamoxifen and then performed a chromatin immunoprecipitation (ChIP) and real time PCR assays. The results clearly showed that ERα bound to this ERE compared to the surrounding negative regions, and E2 and tamoxifen treatment further enhanced ERα binding (Fig. 3D). Next, we cloned the ERE sequences (Fig. S2, A and B) into a luciferase reporter and performed a luciferase assay to assess the regulatory function of ERα. Indeed, the luciferase activity was significantly high while the ERE mutant abrogated the activity in MCF7 cells (Fig. 3E). We also tested the binding of ERα in hypoxia condition. The results showed that ERα still bound at ERE of *HIF-1α* under hypoxia (Figure 3F). Thus, these data demonstrate that we have identified *HIF-1α* as a direct target of ERα, potentially explaining why HIF-1α is associated with ER positivity in breast cancer samples.

Interestingly, we did not see positive correlation between HIF-1α and ERα at transcript levels from tumor cohorts; instead we found a negative correlation between them (Fig. S3, A and B). This may be explained by the hypothesis that ER- breast cancer has been epigenetically remodeled to express high HIF-1α transcript. This led us to further speculate that ERα loss need to be compensated by high HIF-1α activity.

Tamoxifen-bound ERα inhibits HIF-1α expression

Tamoxifen is an antagonist of ERα, competing with estrogen for the ER binding. Tamoxifen-bound ERα recruits the co-repressors NCOR and HDAC to silence gene transcription of ERα targets(5). The discovery of enhancing HIF-1α by E2-bound ERα prompted us to examine the effect of tamoxifen-bound ERα. Tamoxifen treatment of MCF7 cells greatly increased HDAC1 binding on the ERE of *HIF-1α*. Interestingly, ICI182780 did not affect HDAC1 binding although remarkably reduced the ERα binding (Figure 4A). These data indicate that the two compounds inhibit ERα function through different mechanisms.

We then used an in vitro established tamoxifen-resistant MCF7 (TamR-MCF7) cell line and BT474 cells which are intrinsically resistant to tamoxifen (Fig. 4, B and C). These cells were cultured in media containing tamoxifen for propagating. When ERα was depleted in these cells, HIF-1α expression was

upregulated (Fig. 4, B and C). Overexpression of ERα in TamR-MCF7 cells significantly reduced HIF-1α expression but not for the parental MCF7 cells in normoxia (Fig. 4D). Longer exposure of the film showed that overexpression of ERα enhanced HIF-1α in parental cells (Fig. 4D). Interestingly, we noticed that the basal levels of HIF-1α expression in Tam-MCF7 cells is higher than in parental cells, indicating epigenetic effects are involved after cells acquire tamoxifen resistance. These data indicate that E2-bound ERα induces but tamoxifen-bound ERα suppresses HIF-1α expression (Fig. 4E).

HIF-1α confers tamoxifen resistance on ERα-positive breast cancer cells.

Since HIF-1α was a downstream target of ERα and enhanced ERα target expression, we hypothesized that HIF-1α may modulate endocrine efficacy in ER-positive breast cancers. We therefore assessed the role of HIF in regulation of breast cancer cell survival or proliferation in response to inhibitors of ERα. We first used retroviral vector-mediated transduction to stably introduce HIF-1α and HIF-2α into T47D and MCF7 cells. HIF-2α expression significantly suppressed proliferation of both cell lines, while expression of HIF-1α did not. Thus, we were unable to generate stable cell lines with HIF-2α. Although both parental MCF7 and T47D and their derivative HIF-1α expressing cells responded to tamoxifen treatment, the HIF-1α expressing cells were at least 2-fold more resistant in normoxia (Fig. 5B and Fig. S4) and long term treatment showed more remarkable effect (Fig. 5C), demonstrating that HIF-1α is able to confer tamoxifen resistance. We also treated the cells with ICI182780. Although the parental and HIF-1α expressing cells responded to ICI182780 similarly after one week treatment (Fig. S4), long-term treatment (18 days or 4 weeks) with 10 nM or 100 nM led to development of more resistant colonies (Fig. 5 C and D). Though 1000 nM of ICI182780 efficiently suppressed both cell lines, HIF-1α expressing cells gave rise to more large resistant colonies (Fig. 5D).

We further tested the capacity for tumorsphere formation of the HIF-1α expressing and parental cells using a mammosphere 3D culture system. HIF-1α did not affect tumorsphere formation (Fig. 5, E and F); however, HIF-1α conferred significant resistance to tamoxifen and ICI182780 (Fig. 5, E and F). We also tested another drug, RITA, which inhibits HIF-1α expression and induces apoptosis of MCF7 cells (31). The results showed that RITA equally reduced the tumorsphere formation in both HIF-1α expressing and parental cells (Fig. 5, E and F).

Hypoxia meta-gene signature and high *HIF-1*α gene expression show a poor response to tamoxifen treatment in ERα-positive breast cancer

To determine whether hypoxia/HIF-1α is associated with tamoxifen effectiveness in patients with breast cancer, we first examined the hypoxia status of 2 groups of breast cancer patients by a hypoxia/HIF-1α meta-gene signature (32). We then compared relapse-free survival of ERα-positive breast cancers classified as hypoxic or normoxic by their gene expression profiles with this hypoxia/HIF-1α meta-gene signature. In those without adjuvant tamoxifen therapy, hypoxic cases had a significantly worse outcome than the normoxic breast cancers (P=0.001) (Fig. S5A). A significant difference in outcome for those treated with tamoxifen remained (P=0.03) (Fig. S5A). Then we analyzed whether the *HIF-1*α gene expression itself correlated with tamoxifen response in a large cohort of ER positive patients from public data (33). Kaplan-Meier analysis results showed that patients with high level of *HIF-1*α gene expression had a poorer relapse-free survival to endocrine therapy or tamoxifen treatment alone (P=0.0093, Fig. 5G and Fig. S5B) although overall survival was not significantly different (Fig. S5C). When chemotherapy was included for those patients who received tamoxifen, *HIF-1*α is also associated with poor overall survival (P=0.017, Fig. 5H). These data further indicate that *HIF-1*α may be directly involved in modulating tamoxifen response in ER positive patients. Interestingly, high *HIF-2*α is associated with better survival in ER positive breast cancer patients who received endocrine therapy (Fig. S5D). Although statistically not significant, *HIF-2*α tends to be associated with better survival in tamoxifen treated patients (Fig. S5E). These results were consistent with our findings that HIF-2α overexpression was harmful for ERα positive cancer cells and the clinical data that *HIF-2*α is significantly lower in breast cancer tissues than the normal breast tissue (Fig. S6).

Discussion

To develop novel therapeutics to treat breast cancer, a deeper understanding of the molecular mechanism of ER-driven cancer is important, as the most common type of metastatic breast cancer is endocrine receptor positive. The association of ERα positivity and HIF-1α from clinical studies (12, 15, 34) supports our findings that these two pathways may act in cooperation to promote breast cancer progression. However, the basis of these previous clinical observations was unclear since ER-negative tumors have a greater proliferation rate and are more hypoxic than ER-positive tumors. Hence it was unclear why HIF-1α should be more highly expressed in ER-positive tumors (15, 34). Our data, which demonstrate that ERα regulates HIF-1α expression, provide a mechanism for these clinical observations. HIF-1α is a labile protein that is rapidly degraded by VHL-mediated proteasomal degradation in normoxia but stabilized in hypoxia. However, the transcriptional regulation of *HIF-1*α is not well studied except in one report showing that the NF-kB pathway regulates *HIF-1*α gene expression (35). Here we show that *HIF-1*α gene bears a canonical ER binding element that responds to estrogen signaling, demonstrating a direct regulatory link between the ERα and HIF-1α pathways in breast cancer (Fig. 6A).

Interestingly, we found a subgroup of genes that are dually responsive to hormone and oxygen (Fig. 1). These genes were upregulated by hypoxia but the ERα antagonist ICI182780 significantly reduced their expression. Previous studies also show that some genes such as KDM4B, STC2 and VEGFA bear both a hypoxia response element and estrogen response element (18, 19, 26-30). This may indicate that the estrogen receptor signaling pathway can be enhanced by HIF-1α induction and vice versa (Fig. 6B). The physiological significance of the crosstalk between these two pathways in breast or ovary development warrants further studies. Nevertheless, we envisage that both pathways form a positive feedback loop to enhance the common downstream target gene expression. But is HIF-1 required for ERα activity? We previously showed that depletion of HIF-1α only partially affected KDM4B expression in hypoxia while depletion of ERα nearly abrogated KDM4B expression (18), indicating that HIF-1 might not be required for ERα activity but synergizes with ERα. Here we further tested this by knocking down HIF-1α in tamoxifen resistant MCF7 and BT474 cells and obtained a similar result that KDM4B expression was only partially reduced (Fig. S7), further suggesting that ERα function may not rely on HIF-1α. However, we cannot exclude out the possibility that ERα may require HIF-1α function for activation of certain specific genes or under specific conditions.

Another important conclusion of our findings is that over-active HIF-1α function may partially compensate for estrogen signaling when ERα function is compromised, such as under the cirmumstances of hormone therapy. When ERα positive breast cancer cells were transduced with HIF-1α, the cancer cells became much more resistant to tamoxifen and ICI182780 treatment (Fig. 5). This is consistent with the fact that HIF-1α and hypoxia gene signature were correlated with poorer survival in response to hormone therapy. Although the molecular mechanism by which HIF-1α confers tamoxifen resistance needs to

be further defined, our recent findings indicate that the histone demethylase KDM4B is important in coordinating HIF-1α and ERα. KDM4B is a direct target of both HIF-1α and ERα□ and regulates expression of many genes in normoxia and hypoxia and cell cycle progression (18). Thus, HIF-1α may drive gene expression of KDM4B and other genes to compensate for tamoxifen inhibition of ERα signaling. This may be more important in vivo as the common factors are secreted extracellular signaling molecules. These results complement recent data showing the importance of HIF-1α in TRN breast cancer that is driven by XBP1 in response to unfolded protein (UPR) or endoplasmic reticulum (ER) stress (36). Interestingly, XBP1 has been shown to confer both estrogen independence and anti-estrogen resistance in breast cancer cell lines (37). Thus, it is also possible that cellular stress induced-XBP1 might interact with HIF-1α to confer anti-estrogen resistance. Therefore, our data suggest that targeting the HIF-1α signaling pathway might increase efficacy of endocrine therapy in breast cancers. In the future, we will use genetic models and xenografts to modulate HIF-1α activity in hormone therapy.

Materials and methods

Cells were maintained in DMEM supplemented with 10% FCS, 1% glutamine, and 1% penicillin-streptomycin. Western blot analysis, qRT-PCR, tumorsphere formation, chromatin immunoprecipitation (ChIP), retroviruses and plasmids, siRNA transfection, cell viability and colony formation assays, luciferase reporter assay, gene expression profiling, data mining, and patient details and gene expression profiling for patients sample are described in SI Materials and Methods. Statistical analyses were two-tailed *t* tests, with $P \leq 0.05$ considered statistically significant.

Authors' contributions

JY, AMD and ALH conceived and designed the study, and drafted the manuscript. JY performed RT-PCR, western blotting, ChIP, Luciferase reporter assay, cell viability assay and data analysis with help of AA, NV, RI and DJ. FMB performed microarray data analysis. CQ performed analysis of ChIP-seq data. JLL made retrovirus. RIN provided TamR-MCF7 cell. JB and JR performed microarray.

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