ERα 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.

ERα | HIF-1α | 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 (5). 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 chemotherapy (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 ERα-dependent transcription (16). Further studies revealed that in ERα-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/3JMJD2B, 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 role independent of ERα is unknown. The purpose of this study is to determine the direct interaction between ERα and HIF-1α pathways and to elucidate the mechanism by which they function in breast cancer development.

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.
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-1α signaling and how HIF-1α 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 enrichment analysis (ChEA) of transcription factors binding to the dual responsive genes. (ChIP) enrichment analysis of transcription factors (ChEA) analyzed these genes through chromatin-immunoprecipitation (ChIP) enrichment analysis of transcription factors (ChEA) of transcription factor binding to the dual responsive genes.

Fig. 1. ERα signaling regulates hypoxia/HIF pathway. (A) MCF7 cells were treated with 1 μM of ICI182780 in normoxia and hypoxia (1% O2) 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.
more commonly mutated in triple negative (TN) 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 transcription. Most studies focus on the regulation of protein stability of ERα signaling pathway is involved in hypoxia/ERα 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 dinitrophenol (DNP) (Fig. 2A), we found that HIF-1α mRNA level was significantly reduced after ICI182780 treatment, indicating an
ER-mediated transcriptional mechanism regulates HIF-1α transcription.

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 ERα 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. When ERα was depleted in these cells, HIF-1α function through different mechanisms.

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 corepressors 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 expression 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 IC182780. Although the parental and HIF-1α expressing cells responded to IC182780 similarly after one week treatment (Fig. 5F), long-term treatment (18 days or 4 weeks) with 10 nM or 100 nM led to development of more resistant colonies (Fig. 5C and D). Though 1000 nM of IC182780 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 IC182780 (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-1α 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=0.0011) (Fig. 5A). A significant difference in outcome for those treated with tamoxifen remained (P=0.03) (Fig. 5A). Then we analyzed whether the HIF-1α 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-1α gene expression had a poorer relapse-free survival to endocrine therapy or tamoxifen treatment alone (P=0.0009, Fig. 5G and Fig. SSB) although overall survival was not significantly different (Fig. SSC). When chemotherapy was included for those patients who received tamoxifen, HIF-1α is also associated with poor overall survival (P=0.017, Fig. 5H). These data further indicate that HIF-1α may be directly involved in modulating tamoxifen response in ER positive patients. Interestingly, high HIF-2α is associated with better survival in ER positive breast cancer patients who received endocrine therapy (Fig. SSD). Although not statistically significant, HIF-2α tends to be associated with better survival in tamoxifen treated patients (Fig. SSE). These results were consistent with our findings that HIF-2α overexpression was harmful for ERα positive cancer cells and the clinical data that HIF-2α 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-1α is not well studied except in one report showing that the NF-kB pathway regulates HIF-1α gene expression (35). Here we show that HIF-1α 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 IC182780 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 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 IC182780. Although the parental and HIF-1α expressing cells responded to IC182780 similarly after one week treatment (Fig. 5F), long-term treatment (18 days or 4 weeks) with 10 nM or 100 nM led to development of more resistant colonies (Fig. 5C and D). Though 1000 nM of IC182780 efficiently suppressed both cell lines, HIF-1α expressing cells gave rise to more large resistant colonies (Fig. 5D).

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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 normoxic 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 stress (ER) stress (36). Interest in T3-ER+ breast cells 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% glucose, 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 ≤ 0.05 considered statistically significant.

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