Involvement of estrogen-related receptors in transcriptional response to hypoxia and growth of solid tumors

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The development of intratumoral hypoxia is a universal hallmark of rapidly growing solid tumors. Adaptation to the hypoxic environment, which is critical for tumor cell survival and growth, is mediated primarily through a hypoxia-inducible factor (HIF)-dependent transcriptional program. HIF activates genes that facilitate crucial adaptive mechanisms including increased glucose uptake and glycolysis and tumor angiogenesis, making it an important therapeutic target. However, the HIF-dependent transcriptional mechanism remains incompletely understood, and targeting HIF is a difficult endeavor. Here, we show that the orphan nuclear receptor estrogen-related receptors (ERRs) physically interact with HIF and stimulate HIF-induced transcription. Importantly, ERRs appear to be essential for HIF's function. Transcriptional activation of hypoxic genes in cells cultured under hypoxia is largely blocked by suppression of ERRs through expression of a dominant negative form of ERR or treatment with a pharmacological ERR inhibitor, diethylstilbestrol. Systematic administration of diethylstilbestrol severely diminished growth and angiogenesis of tumor xenografts *in vivo*. Because nuclear receptors are outstanding targets for drug discovery, the findings not only may offer mechanistic insights into HIF-mediated transcription but also may open new avenues for targeting the HIF pathway for cancer therapy.

angiogenesis | energy metabolism | nuclear hormone receptor

Rapidly growing solid tumors tend to outstrip the supply of oxygen and nutrients provided by blood vessels, resulting in regions of low oxygen levels (hypoxia). Cancer cells undergo adaptation to persist in the hostile hypoxic environment. The adaptive response to hypoxia is controlled primarily by the hypoxia-inducible factor (HIF), a master regulator of hypoxic gene expression and oxygen homeostasis (1–3). HIF is a heterodimeric transcription factor comprising an oxygen-regulated α-subunit (HIF1α or -2α) and a constitutively expressed and stable β-subunit (HIFβ). Under normal oxygen tension, the HIFα subunit is subjected to prolyl hydroxylation catalyzed by a set of oxygen- and ferrous ion-dependent prolyl hydroxylases. Hydroxylated HIFα is recognized by the tumor-suppressor protein von Hippel–Lindau (VHL), a component of an E3 ubiquitin ligase complex. Subsequently, HIFα becomes polyubiquitinated and is targeted for rapid degradation by the proteasomal system. Oxygen deprivation, or administration of iron chelators or cobaltous ion (a classic hypoxia mimetic, which competes with ferrous ion), suppresses hydroxylase activity, allowing HIFα to escape the VHL-mediated destruction and to accumulate and dimerize with the constitutively present HIFβ. The binding of the HIFα-HIFβ heterodimer along with the transcriptional coactivator p300/CBP to the cognate hypoxia-responsive element (HRE) augments the expression of a plethora of hypoxic genes that carry such elements within their promoters or enhancers (4). The HIF-orchestrated transcriptional program is directly responsible for adaptive mechanisms, such as glycolytic metabolism, angiogenesis, tumor cell survival, invasion, and metastasis.

Cancer cells commonly rely on glycolysis for their energy needs (5, 6). Glycolysis breaks down glucose to generate pyruvate. Pyruvate is then reduced to lactic acid by lactate dehydrogenase (LDH) or, in the presence of oxygen, is oxidized to acetyl-CoA by the pyruvate dehydrogenase (PDH) enzymatic complex. This step represents an important regulatory point in cellular energy metabolism, because PDH can be switched off by the pyruvate dehydrogenase kinases (PDKs). Acetyl-CoA enters the tricarboxylic acid (TCA) cycle, leading to efficient production of ATP through oxidative phosphorylation (OXPHOS). Under hypoxia, lack of oxygen, the substrate for OXPHOS, restrains cells from the mitochondrial respiratory pathway for ATP generation. To meet tumor cells' high energy demand, HIF strongly stimulates the expression of genes that encode glucose transporters and glycolytic enzymes (including LDH) to accelerate glucose uptake and the anaerobic glycolytic ATP production. In addition, HIF actively attenuates both the TCA cycle and OXPHOS via direct induction of PDK1 (one of the four PDKs) to block conversion of pyruvate to acetyl CoA (7, 8). This HIF-mediated metabolic switch allows tumor cells to survive and grow under hypoxic environment. Furthermore, HIF activates several angiogenesis-promoting factors (9), including VEGF, which is a dominant growth factor in the tumor angiogenesis cascade. Ultimately, neovascularization may correct the hypoxia and nutrient depletion and consequently aid tumor growth and metastasis.

The HIF-coordinated systemic adaptation contributes substantially to the malignant phenotype. There is mounting clinical evidence correlating tumor hypoxia and HIF overexpression to aggressive tumor behavior, diminished therapeutic response, and increased patient mortality in a broad range of sporadic cancer types (2). Experimental attenuation of HIF activity by genetic or pharmacological approaches in tumor xenografts and transgenic models leads to markedly reduced tumor size, demonstrating a causal role of HIF in tumor growth and progression (2, 10). Disruption of individual HIF downstream events such as angiogenesis and glycolysis has shown promising efficacy (6, 11). Interventions to decrease global HIF activity may even more profoundly prevent tumor cells from adapting to hypoxia, impede angiogenesis, and cause tumor cell death through metabolic derangement.

Given the presence of hypoxia in nearly all solid tumors and the centrality of the HIF pathway in malignancy, pharmacological inhibition of HIF for therapeutics has attracted a great deal of attention. However, transcription factors (except nuclear

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receptors) are generally not suitable targets for drug discovery, because, unlike enzymes, it is not feasible to identify active site inhibitors. Several cancer drugs that inhibit oncogenic signaling pathways are able to turn down the hypoxic response by indirectly reducing HIF protein synthesis/stability (1, 2). Recently, the proof of principle for targeting HIF coactivators has been established. Chetomin, a natural compound capable of binding to p300 and causing structural changes, perturbs association of p300 with HIF and induction of hypoxic genes (12). However, the exact mechanism for HIF-induced transcriptional activation is still uncertain. Genetic studies demonstrate that p300/CBP are responsible for a subset of HIF-stimulated genes and suggest the existence of additional coactivating mechanisms (13). Moreover, although HIF stabilization and activation under hypoxia are evident in almost all cell types, expression of many HIF target genes exhibits a cell-type-specific pattern. HIF alone cannot account for this cell type specificity. Recent *in vivo* characterization of a hypoxia-inducible promoter suggested that HRE is required but not sufficient for the hypoxic response. Additional ancillary *cis* elements are essential in the process, even though the corresponding *trans* factor(s) has not been identified (14). Together, these observations further point out the important contributions of potential HIF cofactors to HIF-dependent transcriptional regulation.

Hormone therapy has been used for decades to treat breast cancer. Antiestrogen administration [e.g., the estrogen receptor (ER) antagonist tamoxifen] is applied on the rationale that estrogens stimulate proliferation of breast cancer cells. Paradoxically, the synthetic estrogen diethylstilbestrol (DES) was used clinically to achieve cancer remission (15). High-dose DES was generally considered the endocrine therapy of choice in women with advanced breast cancer before the introduction of tamoxifen in the 1970s. Tamoxifen became preferable to DES not because of a superior efficacy but rather because of its lower incidence of side effects. The efficacy of the ER antagonist tamoxifen and the ER agonist DES was surprisingly comparable (15, 16). Survival was even modestly but significantly better for women treated with DES (16). However, the basis for the beneficial effect of DES is largely elusive.

In the present study, we sought to identify HIF-interacting factors. The estrogen-related receptors (ERRs) (17), a subgroup of nuclear receptors (NRs), were found to serve as essential cofactors of HIF in mediating the hypoxic response. NR proteins share similar modular structures, including the central DNA-binding domain (DBD) and C-terminal ligand-binding domain (LBD). The DBDs of ERRs are closely related to those of ERs, but the LBDs are more divergent. ERRs are constitutively active orphan receptors. DES can bind and inhibit ERRs (18). Here, we show that DES blocks the HIF-dependent hypoxic response and tumor xenograft growth.

Results

ERRs Interact with HIF. Response to hypoxia is a fundamental property of living cells. The HIF pathway is highly conserved from fly to human. From a large-scale yeast two-hybrid-based interaction analysis of *Drosophila* proteins (19), the fly HIFα homolog Sima showed association with an uncharacterized protein CG7404. Based on sequence similarity, CG7404 is the homolog of mammalian ERRs.

ERRs are crucial regulators of energy metabolism (17). Given the importance of the metabolic switch in adaptation to hypoxia, we decided to further investigate this interaction. We first examined whether mammalian HIF and ERR proteins interacted with each other *in vitro* by the GST pulldown assay. There are three *ERR* genes (*ERR*α, -β, and -γ) in human. We fused full-length cDNAs of each ERR to GST and generated fusion proteins in bacteria. We assayed them for binding to *in vitro* translated full-length HIF1α, -2α, and -1β. All GST ERRs, but not GST itself, exhibited interactions with premixed HIF1α-HIF1β and HIF2α-HIF1β (Fig. 1*A*). However, GST ERRs did not bind to single HIF1β (Fig. 1*A*) or HIF1α (data not shown) when the two HIF subunits were separated, suggesting that ERRs recognized only the functional HIF heterodimers. Given the homology between ERRs and ERs, we examined whether ERα might interact with HIF as well. As shown in supporting information (SI) Fig. S1*A*, the ERα–HIF interaction was barely detectable and was much weaker than the ERR–HIF interaction, suggesting that the HIF–ERR interaction is rather specific.

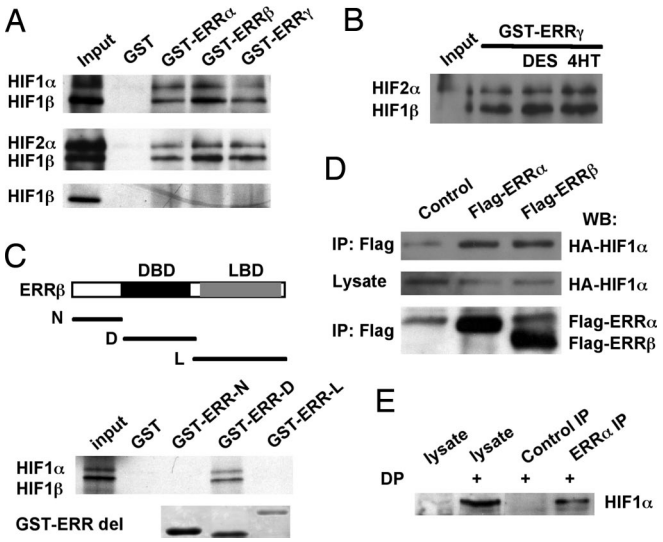

Fig. 1. HIF-ERR interaction *in vitro* and *in vivo*. (*A*) HIF heterodimers bind to all three ERRs. *In vitro* translated HIF proteins were mixed with GST or GST-ERR fusions and assayed for binding. (*B*) HIF-ERR interaction is resistant to DES and 4HT treatment. The binding assay was performed in the presence of DES (50 μM) or 4HT (10 μM). (*C*) The DBD of ERR is responsible for HIF association. (*Top*) Diagram of deletion mutants of ERRβ. N, N terminus; D, DBD; L, LBD. (*Middle*) Heterodimeric HIF associates with ERR DBD. (*Bottom*) Gel showing loading of GST-ERR fusion proteins. (*D*) Exogenous HIF and ERR interact *in vivo*. Flag-ERRs and HA-HIF1α were coexpressed in COS cells. The cellular lysates were subjected to anti-Flag immunoprecipitation. HA-HIF1α was enriched in the ERR precipitates. (*E*) Endogenous ERR and HIF are present in the same complex. Cellular lysates from MDA-MB-435 cells treated with DP were subjected to immunoprecipitation with anti-ERRα or a control antibody (anti-HA). The pellets were analyzed for the presence of HIF1α by blotting with the anti-HIF1α antibody.

The conformation and activity of NRs can often be modulated by small chemical molecules. The synthetic estrogen DES has been found to act as an antagonist of all three ERRs by disrupting their interactions with coactivators such as p160/SRC (18), whereas the ER antagonist 4-hydroxytamoxifen (4HT) selectively inhibits ERRγ (17). We tested whether these compounds might interfere with the ERR–HIF interaction. It was apparent that the ERRγ–HIF association was insensitive to the treatments with DES and 4HT (Fig. 1*B*). Similarly, the interactions of HIF with ERRα and ERRβ were not affected by DES either (Fig. 1S*B*). These results indicate that HIF may bind to a domain in ERRs, which is different from the one responsible for ERRs' coactivator binding. To map the detailed structural requirements for ERR binding to HIF, we made a series of ERRβ truncation mutants covering the N terminus, DBD, and LBD. We found that the DBD was involved in HIF binding (Fig. 1*C*). This observation is consistent with the chemical treatment result as DES and 4HT bind to the LBD.

We asked whether the ERR–HIF interaction occurred in intact cells. HIF1α and ERRs, tagged with the epitopes HA and Flag, respectively, were coexpressed in mammalian cells by

transient transfection. When the ERR proteins were immunoprecipitated with the anti-Flag antibodies, HIF1α was enriched in the pellets as revealed by Western blot analysis with the anti-HA antibodies (Fig. 1*D*). This observation supports that the HIF and ERR proteins form complexes *in vivo*. We further investigated whether endogenous HIF and ERR proteins might exist in the same complex. The human breast cancer MDA-MB-435 cells were treated with the iron chelator dipyridyl (DP) to stabilize the endogenous HIFα, and the cellular lysates were subjected to immunoprecipitation with anti-ERRα to enrich ERR-associated proteins. HIF1α was readily detected in the immunoprecipitates by anti-HIF1α Western blot analysis (Fig. 1*E*). Together, these results suggest that HIF and ERR proteins physically associate with each other both *in vitro* and *in vivo*.

HIF exerts its function by binding to the HREs present in the promoter/enhancer of many hypoxic genes (4). Given the physical association between HIF and ERR, we questioned whether the ERR proteins would be present at the HIF-bound genes. We chose a few established HIF target genes with well defined HREs, such as VEGF, PDK1, and phosphoglycerate kinase 1 (PGK1), and analyzed potential *in vivo* promoter binding of HIF and ERR by the ChIP assay. In normoxic cells, chromatin precipitated with the anti-HIF1α and -ERRα antibodies showed no enrichment of the HIF-dependent genes (Fig. 2*A* and Fig. S2). However, in DP-treated cells in which HIFα was stabilized, occupancy of both HIF1α and ERRα at the HIF target gene promoters was detected (Fig. 2 *A* and Fig. S2), suggesting that in response to hypoxia, the ERR proteins are recruited to HIF-regulated genes.

ERRs Augment Hypoxic Gene Expression. The presence of ERR at HIF target genes raised the possibility that ERR might directly modulate HIF-mediated gene transcription. We tested this idea by reporter-based assays. A HIF-dependent luciferase reporter containing multiple HREs was readily activated by transfection of the HIF1α or -2α expression vectors (Fig. 2*B*). Although ERRα or -β alone did not notably affect the reporter, they strongly enhanced the transcriptional activity of HIFα (Fig. 2*B*). The reporter also responded robustly to increased endogenous HIF activity in cells treated with hypoxic mimetic DP or cobalt (Fig. 2*C*). Expression of ERRα or -β further potentiated reporter activation in these HIF-stabilized cells (Fig. 2*C*). Synergistic effects on the hypoxic response by ERR were also observed by using reporters carrying a natural hypoxia-responsive enhancer [e.g., erythropoietin (EPO)] or promoter (e.g., PGK1) (Fig. 2 *D* and *E*). Therefore, the ERR proteins apparently serve as a positive cofactor of HIF. Consistent with the lack of a strong interaction between ER and HIF, cotransfection of ERα (in the presence or absence of its ligand E2) had no effect on HIF's transcriptional activity (data not shown).

Although ERRs are capable of strengthening HIF activity, a critical question is whether they are required for the hypoxic regulation. Genetic studies in mice have shown that mouse mutants deficient for HIFα, HIFβ, or ERRβ exhibit strikingly similar phenotypes (20–22), suggesting that HIF and ERR may function in the same pathway, and ERR may be an essential component. ERRs are constitutively active orphan receptors. Even in the absence of ligands, ERRs can recruit SRC-1, -2, and -3; PGC1α and -β (17); and likely other typical NR coactivators as well (e.g., p300/CBP, mediator, and SWI/SNF chromatin remodeling complexes). It is conceivable that ERRs bring these coactivator complexes into close proximity of HIF and boost HIF activity. Because association of ERRs with their coactivators can be regulated by small chemical inhibitors (ref. 17 and Fig. S3), we asked whether inhibition of ERRs could serve as a point of intervention in the HIF-mediated hypoxia-responsive pathway. Indeed, activation of the HIF-dependent reporter by DP-induced HIF stabilization was severely impaired by the ERR inhibitor DES (Fig. 2*F*), indicating that ERRs may be required for HIF's full activity.

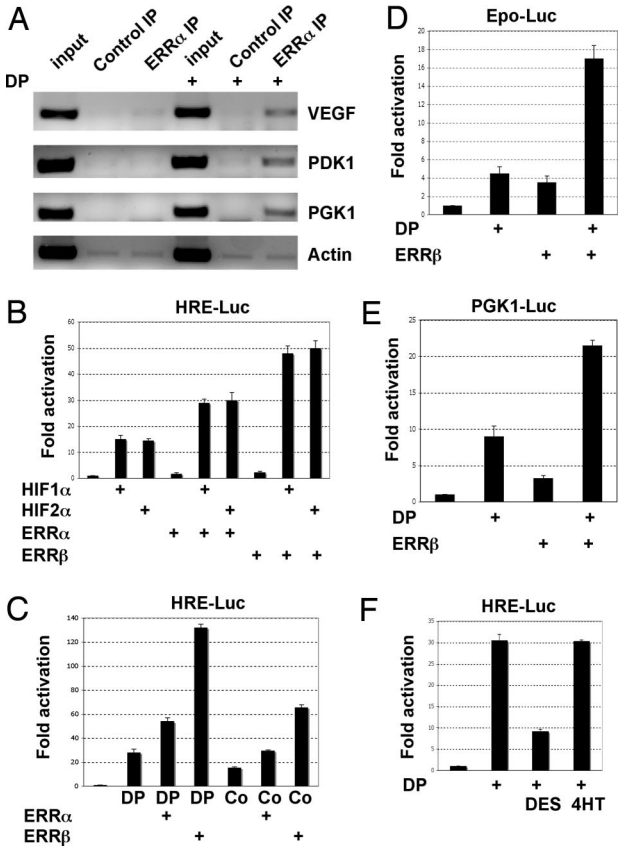

Fig. 2. ERRs enhance HIF-mediated gene transcription. (*A*) ERRα is recruited to HIF-bound genes under hypoxia. ChIP assay was carried out for VEGF, PDK1, PGK1 (HIF target genes), and β-actin (negative control). Sheared chromatin from normoxic or DP-treated MDA-MB-435 cells was precipitated with polyclonal anti-ERRα or control antibody (anti-HA). Enrichment of the indicated gene promoters was analyzed by PCR amplification with primers encompassing the defined HREs. (*B*) ERRs potentiate the transcriptional activity of exogenous HIFα. HEK293 cells were transfected with a HIF-dependent firefly luciferase reporter (HRE-Luc), HIFα, and ERRs. In each transfection, we included a *Renilla* luciferase (Rlu) reporter driven by SV40 promoter (SV40-Rlu) to serve as a normalization control. Error bars represent standard deviations. (*C*) ERRs enhance endogenous HIFα activity. HEK293 cells were transfected with HRE-Luc, SV40-Rlu (for normalization) and ERRs, and followed by administration of hypoxic mimetics. (*D* and *E*) ERRβ and endogenous HIF synergistically activate EPO and PGK1. Luciferase reporters driven by the EPO enhancer or PGK1 promoter were introduced into HEK293 cells together with ERRβ and SV40-Rlu, cells were subsequently treated with DP. (*F*) DES attenuates HIF activity. Breast cancer SKBR3 cells were transfected with HRE-Luc and SV40-Rlu and subsequently treated with DP, DES, or 4HT. Lack of effects by 4HT may be due to the redundant function of ERRα and ERRβ, or its failure to dissociate a specific coactivator(s) of ERR.

The HIF-mediated hypoxic response is defined by transcriptional activation of many hypoxic genes. We asked whether inhibition of ERR was capable of blocking hypoxia-induced transcription of endogenous genes. As expected, when breast cancer cells (MDA-MB-435 and SKBR3) were cultured under severe hypoxia or treated with DP, known direct target genes of HIF, such as the key angiogenic factor VEGF and glycolytic enzymes, were robustly up-regulated (Fig. 3 *A* and *B*). Interestingly, in the presence of DES, this transcriptional induction was largely abolished (Fig. 3 *A* and *B*). A similar phenomenon was observed in melanoma cells (Fig. 3*C*). We examined whether the effect caused by DES was due to its possible influence on HIFα

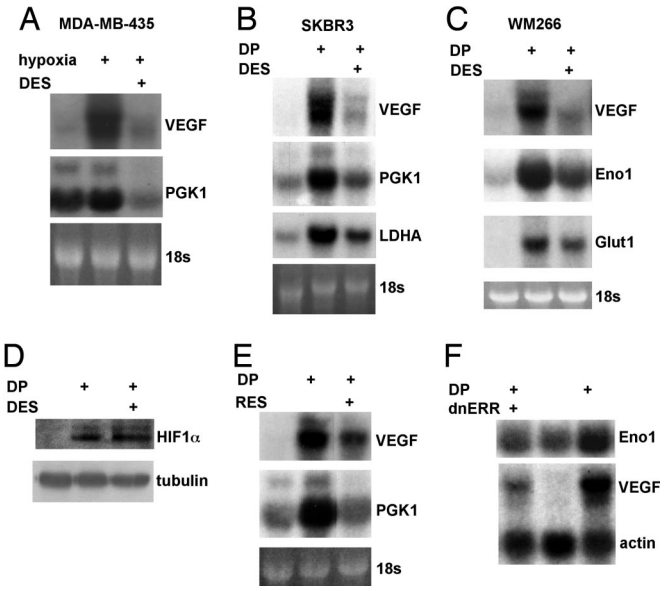

Fig. 3. Inhibition of ERR attenuates the hypoxic response. For Northern blot analysis, nylon membranes carrying total RNA samples were hybridized with probes made from cDNA fragments encoding for VEGF, PGK1, lactate dehydrogenase A (LDHA), Enolase 1 (Eno1), and Glucose transporter 1 (Glut1). The 18s rRNA and β-actin served as loading controls. (*A*) Northern blot analysis of MDA-MB-435 breast cancer cells cultured under severe hypoxic condition in the absence or presence of DES. (*B*) Northern blot analysis of SKBR3 breast cancer cells exposed to DP and DES. (*C*) Northern blot analysis of melanoma WM266 cells treated with DP and DES. (*D*) Western blot analysis for HIF1α in SKBR3 cells treated with DP and DES. HIF1α was detected with anti-HIF1α antibody. The same blot was reprobed with an antitubulin antibody. Tubulin served as a loading control. (*E*) Resveratrol (RES)-reduced hypoxic gene expression in A549 lung cancer cells treated with DP and RES. (*F*) Compromised hypoxic gene induction in MDA-MB-435 cells expressing dnERR.

protein stability. It seemed that under DES treatment, HIF1α was stabilized normally by DP (Fig. 3*D*) but remained transcriptionally incompetent. This unique effect of DES distinguished it from several HIF pathway inhibitors, which mainly decrease HIF protein levels (1, 2). We conclude that inhibition of ERRs by DES attenuates the transcriptional activity of HIF.

We explored whether other ERR inhibitors might interfere with the hypoxic response as well. An interesting candidate is resveratrol, which is known to exert a wide range of activities from extending life span to suppressing tumor growth and neovascularization (23). Resveratrol is capable of dissociating coactivator complexes from all three ERRs, albeit at higher concentrations than DES (18). Indeed, we found that resveratrol manifested inhibitory activities on the hypoxia-induced gene transcription (Fig. 3*E*). This finding also raises the possibility that some of the resveratrol's effects might be attributable to its ability of inhibiting ERRs and the hypoxic response.

To further attest that inhibition of ERRs was accountable for the observed effect of DES on the hypoxic response, we adopted a molecular approach to inactivate ERR. We designed a dominant negative form of ERR (dnERR) to selectively antagonize the activities of all three ERRs. We fused the DBD of ERRβ, which is sufficient for HIF binding (Fig. 1*C*), to a transcriptional repression domain of the *Drosophila* Engrailed protein (24) (Fig. S4). The Engrailed domain possesses potent repressive activity when fused to heterologous proteins and has frequently been used to create artificial transcriptional repressors. The ERR-engrailed chimeric protein was intended to suppress HIF-mediated transcription. We expressed dnERR in the MDA-MB-435 cells by stable transfection. Although the activity of endogenous ERRs was down-regulated (Fig. S4), the dnERR-expressing cells did not display obvious defects in gross morphology and growth. Under the hypoxic stimulus, however, induction of hypoxic genes was greatly compromised in the dnERR-expressing cells when compared with the control parental cells (Fig. 3*F*). Together, this finding supports that blockade of the hypoxic response pathway by pharmacological ERR inhibitors is likely a result of specific inhibition of ERRs.

Systematic Application of DES Inhibits Tumor Xenograft Growth and Angiogenesis. The HIF-mediated hypoxic response has been shown to be critical for tumor survival and growth *in vivo*. To extend our studies in cultured cells, we evaluated the effect of ERR inhibition on human tumor cells in a mouse xenograft model. Human breast cancer MDA-MB-435 cells were implanted s.c. into immunodeficient mice and allowed to grow for 2 weeks to establish xenografts before initiation of DES treatment. DES at a dose of 500 μg per day per mouse has been shown to cause placental defects in pregnant female mice (18), which mimic the ERRβ knockout mutants (20), strongly suggesting that this dosage is sufficient for inactivation of ERRs *in vivo*. In our study, each mouse was fed with either vehicle (sesame oil) or 750 μg of DES daily, a slightly higher dose than previously reported (18). There was a dramatic reduction in tumor volumes and growth rate in the DES-administrated mice when compared with the vehicle-treated control animals (Fig. 4*A*). At the end of treatment, tumors were excised and analyzed. Compared with tumors in vehicle-treated mice, DES-treated xenografts were much smaller and weighed approximately three times less ($P < 0.0001$) (Fig. 4*B*). Because angiogenesis is a key part of the hypoxic response and is required to support tumor growth beyond a certain threshold size, we assessed tumor vascularization by performing immunohistochemical staining by using anti-CD34 antibody. Mouse CD34 is present on endothelial cells and hematopoietic progenitor cells. The antibody recognizes endothelium *in vivo*, particularly on small vessels and newly formed capillaries and developing vascular structures. CD34 staining revealed abundant vascular capillary formation within the control tumors (Fig. 4*C*) and a clear reduction of micro vascular density in tumors from the DES-treated animals ($P < 0.001$) (Fig. 4*D*). Therefore, inhibition of ERR results in retarded tumor growth and diminished tumor angiogenesis *in vivo*.

Discussion

In the current study, the orphan nuclear receptor ERRs were found to be a positive and essential component of the HIF transcriptional complexes controlling hypoxia-inducible gene expression. The functional collaboration between ERR and HIF is further supported by genetic studies in mice. Physiological hypoxia plays an important role in placental development, and HIF regulates placental morphogenesis, angiogenesis, and cell fate determination. Placentas from mouse embryos lacking the HIFα (*HIF1*$\alpha-/-$*HIF2*$\alpha-/-$ double mutants) or HIFβ subunits exhibit aberrant placental morphology and decreased vascularization (21, 22). Hypoxia and HIF promote the *in vitro* differentiation of trophoblast stem cells into spongiotrophoblasts as opposed to polyploid giant cells (21). Accordingly, HIF-null placentas display greatly reduced labyrinthine and spongiotrophoblast layers and increased numbers of giant cells (21, 22). Intriguingly, these phenotypes closely resemble those seen in the ERRβ-deficient placentas (20). Moreover, treatment of trophoblast stem cells *in vitro* with DES results in their differentiation toward the giant cell lineage (18). Hence the genetic and cellular evidence indicates that HIF and ERR may act in a common pathway, supporting our biochemical model that the two factors may form a regulatory complex. The genetic studies also suggest that both HIF and ERR are essential for the pathway. This is in agreement of the blockade of the HIF-dependent response by

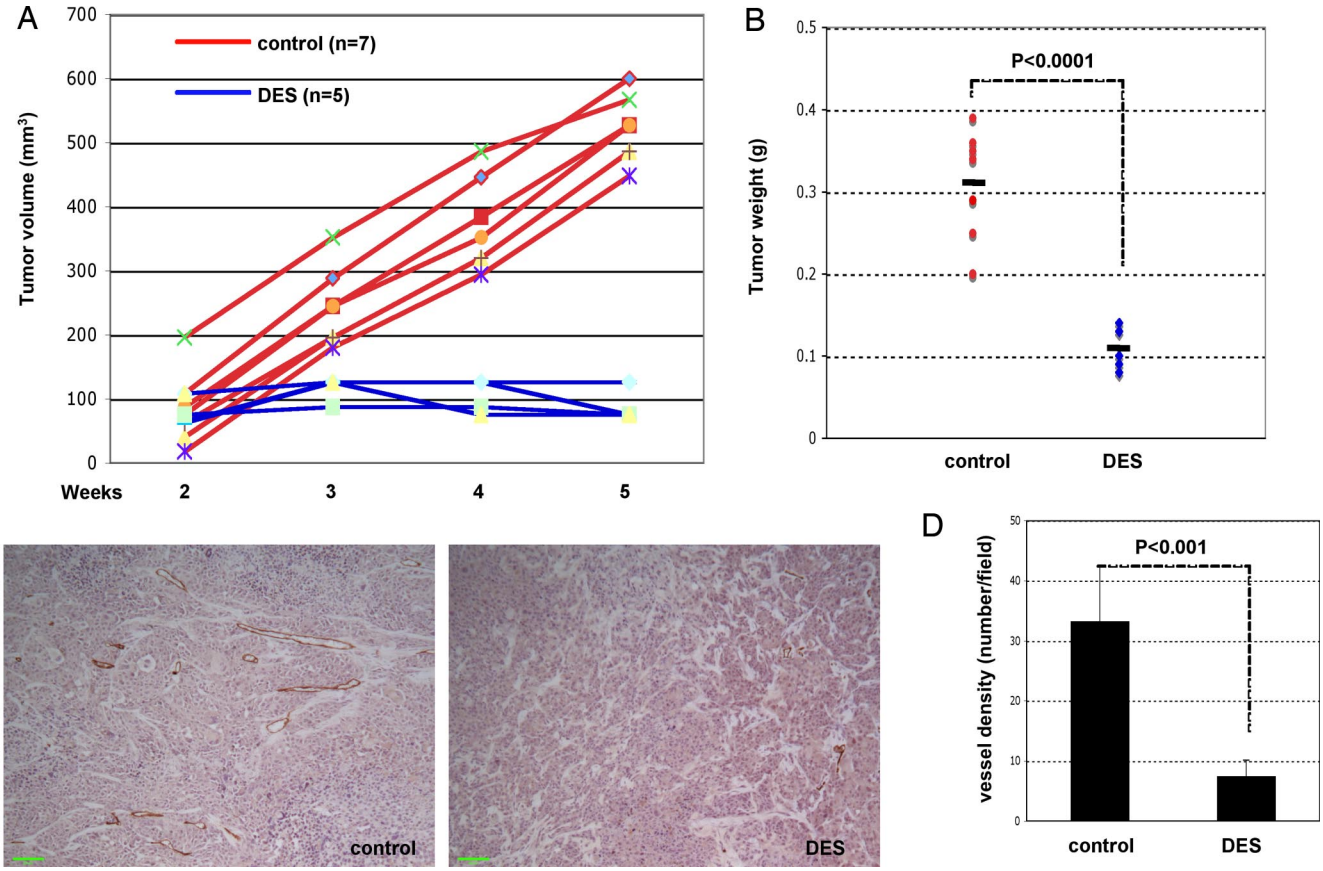

Fig. 4. Growth and angiogenesis of human breast cancer xenograft are sensitive to DES treatment *in vivo*. (*A*) DES treatment affects kinetics of tumor growth. Two groups of mice bearing established tumor xenografts of human MDA-MB-435 breast cancer cells were treated daily with DES (dissolved in sesame oil) or control (sesame oil). Tumor volume was measured weekly. (*B*) DES treatment leads to reduced tumor mass. Tumor xenografts were harvested at time of necropsy (after 3 weeks of treatment) and weighed. DES-treated tumors evidently weighed much less than the control samples. (Scale bars represent average tumor weight.) (*C*) DES treatment diminishes tumor angiogenesis. Tumor transplants were processed and subjected to CD34 immunostaining for blood vessels (brown staining). (*Left*) Tumor section from the control group exhibits high microvessel density. (*Right*) DES-treated tumor contains few blood vessels. (Scale bars, 65 μm.) (*D*) Blood vessel density in tumors is reduced by DES treatment. Microvessel density was determined by counting the number of CD34 positive blood vessels from four randomly chosen visual fields from each group of tumors. The average number in the fields was taken as the mean of vessel density of the group.

pharmacological ERR inhibitors or a dominant negative form of ERR.

The unique ERR-HIF connection is consistent with their known functions as established key metabolic regulators. ERRs stimulate genes involved in mitochondrial biogenesis and OXPHOS (17). However, this enhanced mitochondrial oxidative capacity is mainly directed at fatty acid metabolism, because ERRs activate medium chain acetyl CoA dehydrogenase (17), a pivotal enzyme involved in fat oxidation, and up-regulate PDK4 to prevent glucose oxidative metabolism (25–27). The latter is reminiscent of HIF's activation of PDK1 (7, 8), which is crucial for the switch from glucose oxidation via the TCA cycle to glycolysis under hypoxia. This shared function on inhibition of glucose respiratory metabolism may represent an intrinsic link between ERR and HIF.

The HIF-dependent hypoxic response has a causal role in tumor growth and progression (1, 2). Disrupting the ability of tumors to adapt to hypoxia via inactivating key components in the hypoxic response pathway is an attractive antitumor strategy. Inhibition of HIF activity may be effective irrespective of the mode of pathway activation by upstream events. However, HIF itself is difficult to block directly by small-molecule drugs. The interdependence of HIF and ERR suggests that inhibition of ERRs may be a tractable approach to abrogating hypoxia-induced gene expression and malignant progression. The ERR inhibitor DES has exhibited clinically proven antitumor efficacy for advanced breast cancer, although the mechanisms underlying cancer regression are poorly understood (15). Breast cancer often progresses from an early ER-positive stage to an advanced ER-negative stage. Hypoxia can promote cancer progression by cellular dedifferentiation and down-regulation of ER expression (28, 29). Highly hypoxic tumors are most likely to be ER-negative (30). However, increased ERRα levels appear to be associated with ER-negative tumor status. In fact, ERRα is also a potent prognosis indicator in human breast carcinoma and is significantly associated with an increased risk of recurrence and adverse clinical outcome (31, 32). However, it remains unclear whether ERR plays a pathogenic role. It is possible that ERR enhances the HIF-mediated hypoxic response and results in a more malignant phenotype. DES treatment may hence block the HIF pathway in advanced cancer cells.

NRs, including ERRs, are generally outstanding therapeutic targets because of their inherent structure to interact with small chemical compounds. The activity of NRs is modulated by ligand binding to their LBDs that results in a conformational change leading to either up- or down-regulation of gene expression. The ligands include many natural and synthetic small lipophilic substances, which can diffuse across the cell membrane. The anticancer efficacy of the existing ERR inhibitor (i.e., DES) holds great promise for developing new ligands with increased

potency and selectivity. The crystal structures of ERR LBDs have revealed how inhibitors can promote dissociation of coactivators and potential recruitment of corepressors (17, 33). This detailed knowledge of the structural mechanism sets the stage for the rational design and rapid identification of new compounds with desired properties for cancer therapy.

Materials and Methods

Plasmids and Antibodies. The HIF-dependent reporter HRE-Luc was generated by inserting two copies of synthetic HRE into the pGL3 luciferase vector (Promega). Epo-Luc and PGK1-Luc were created by cloning the PCR-amplified HIF-dependent enhancer and promoter regions of Epo and PGK1, respectively, into pGL3. Full-length EST clones encoding ERR-α, -β, -γ, HIF-1α, -2α, and -1β were used for *in vitro* translation (Promega TNT kit), production of GST fusions by cloning into the pGEX vector, and mammalian expression by cloning into modified pcDNA3 vectors with Flag or HA epitope tags. ERR deletion mutants were generated by PCR. dnERR was constructed by PCR amplification of ERRβ DBD and Engrailed repression domain, followed by a three-way ligation into pcDNA3. Anti-Flag, -HA, -ERRα, and -HIF1α antibodies were obtained from Sigma, Covance, Upstate, BD Biosciences, and Novus, respectively.

Cell Lines and Cell Cultures. All human cancer cells were maintained in the following basal media (Invitrogen) supplemented with 10% FBS: breast cancer cell line MDA-MB-435 in DMEM/F12, melanoma WM266 cells in McCoy's 5A, breast cancer SKBR3 in DMEM. Cells were treated with the indicated chemicals (purchased from Sigma): $CoCl_2$ (300 μM), DES (50 μM), DP (100 μM), and 4HT (10 μM). The antibiotic G418 was added in the medium (800 μg/ml) for selection of stable dnERR transfectants. A severe hypoxic culture environment was achieved by using a GasPak Pouch System (BD Biosciences), which was capable of reducing the oxygen concentration to less than or equal to 1% (mean percentage: 0.7%).

Northern Blot, GST Binding, Western Blot, Immunoprecipitation (IP), and ChIP. All procedures followed standard molecular biology techniques. For Northern blot assays, total RNA was prepared by using TRIzol (Invitrogen). For GST binding and IP assays, we used the following binding buffer [20 mM Tris, pH 7.5; 150 mM NaCl; 0.5% Nonidet P-40; and protease inhibitor mixture (Roche)]. HIFα was found to be not stable under this condition. In later experiments, the proteasome inhibitor MG132 (10 μM) was included in the buffer. ChIP assays were performed by using a kit from Upstate, according to the manufacturer's instructions.

Tumor Xenograft Experiment. MDA-MB-435 cells (5×10^6) were resuspended in a mixture of 100 μl of PBS and Matrigel (BD Biosciences; 2:1 ratio) and injected s.c. into the fourth mammary gland fat pad of 6- to 8-week-old female severe combined immunodeficient mice. Two weeks after injection, tumor xenografts became palpable. The mice bearing tumor xenografts were randomly divided into two groups. One group was orally fed daily with 30 μl of DES (25 μg/μl in sesame oil) per mouse, and the control group received 30 μl of sesame oil. Tumors were serially measured weekly with a ruler, and the tumor volumes were calculated with the following formula: volume (mm^3) = width^2 $\times$ length/2. After 3 weeks of treatment, the mice were killed. All animal studies were approved by the Institutional Animal Care and Use Committee at the University of Florida.

Immunohistochemistry. Tumors were dissected at time of necropsy, weighed, and fixed in 4% paraformaldehyde in PBS overnight. Samples were dehydrated in 70% ethanol, paraffin-embedded, and sectioned (5 mm). Deparaffinized sections were stained for CD34 to assess blood vessel density. Briefly, samples were microwaved in sodium citrate (pH 6) for 20 min for antigen retrieval and treated with 3% H_2O_2 at room temperature for 20 min to quench endogenous peroxidase activity. Sections were then blocked in 1.5% rabbit serum and incubated with rat anti-mouse CD34 antibody (1:100 dilution, clone MEC14.7, BioLegend), followed by biotinylated rabbit anti-rat IgG (1:200 dilution; Vector Laboratories). Detection was done with avidin-biotin-HRP complex (Vector Laboratories) and di-aminobenzidin as chromogen. Nuclei were counterstained with hematoxylin. Student's *t* test was performed by a Web tool from the University of Miami.

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