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ORIGINAL PAPER

17Beta-Estradiol Promotes Aggressive Laryngeal Cancer Through Membrane-Associated Estrogen Receptor-Alpha 36

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Abstract 17β-estradiol (E_2) plays a key role in tumorigenesis by enhancing cell survivability and metastasis through its cytoplasmic receptors. Recently, a variant of estrogen receptor alpha, ERα36 has been implicated as a substantial mediator of E_2's proliferative and antiapoptotic effects through rapid membrane-associated signaling, and cancers previously regarded as hormone-independent due to the absence of traditional receptors, may in fact be susceptible to E_2. Despite rising from a secondary sex organ and having a clear gender disposition, laryngeal cancer is not uniformly accepted as hormone dependent, even in the face of compelling evidence of E_2 responsiveness. The aim of this study was to further elucidate the role of E_2 in the tumorigenesis of laryngeal cancer, both in vitro and in vivo. ERα36 presence was evaluated in membranes of the laryngeal carcinoma cell line, Hep2, as well as in laryngeal tumor samples. In vitro ERα36 was found to mediate rapid activation of protein kinase C and phospholipase D by E_2, leading to increased proliferation and protection against chemotherapy-induced apoptosis. Furthermore, in response to E_2 activation of ERα36, an upregulation of angiogenic and metastatic factors was observed. Clinical analysis of laryngeal tumors revealed a similar association between the amount of ERα36 and VEGF and indicated a role in lymph node metastasis. These findings present compelling evidence of ERα36-dependent E_2 signaling in laryngeal cancer. Thus, targeting ERα36 may reduce the deleterious effects of E_2 in laryngeal cancer, ultimately suggesting the importance of antiestrogen therapy or the production of novel drugs that specifically target ERα36.

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Introduction

The mechanisms responsible for tumor growth and metastasis are complex and are only partially understood. One of the factors contributing to this process that has major clinical implications for both prognosis and treatment is the presence of hormone and growth factor receptors in cancer cells. 17β-estradiol (E_2) traditionally acts as a sex hormone, involved in sexual development and the reproductive systems of males and females, but it has also many other effects both in nonreproductive tissues such as bone and in cancerous tissue. The importance of E_2 in the pathogenesis of breast and prostate cancer is well established [1, 2]. E_2 is known to enhance cell proliferation, survivability, and metastasis [3–5]. This deleterious effect of E_2 is in fact the target of one of the main treatments in breast cancer, the antiestrogen tamoxifen.

Classically, the mechanism of E_2 action is mediated through interaction with its receptors, ERα and ERβ, which are found mainly in the nucleus and act as transcription factors [6]. There is increasing evidence that E_2 also acts via plasma membrane receptor(s), which rapidly activate signal transduction pathways, thus enhancing proliferation and attenuating apoptosis [7, 8]. This membrane-mediated mechanism does not necessitate new gene expression or protein synthesis and is thought to be responsible for the E_2 activation of protein kinase C (PKC) [9].

One of the key candidates for the membrane-mediated action of E_2 is a novel ERα variant, with a molecular mass of ~36 kDa (ERα36). It differs from the classical ERα66 by lacking both transcriptional activation domains (AF1/AF2), but retains the DNA-binding domain as well as partial dimerization and an intact ligand-binding domain [10, 11]. It also contains a novel C-terminal exon that encodes 27 amino acids of unknown function. We have previously shown that this alternative ER resides in the plasma membranes of breast cancer cells and mediates E_2's proliferative and antiapoptotic effects [12].

Epidemiological studies have implied that sex hormones may be involved in the tumorigenesis of laryngeal carcinomas, due to the vast differences in gender susceptibility, with a male/female ratio of 11:1 [13]. The larynx is a secondary sex organ, which undergoes trophic changes in response to hormonal changes during puberty, and morphostructural changes during adulthood. Nonetheless, while other tumors of secondary sex organs have been accepted as hormone dependent cancers, laryngeal carcinoma is still a subject of controversy, and as such, its treatment is not oriented to counteract the hormonal effect.

The presence of classical cytosolic ERs is controversial in laryngeal cancer [14, 15]. Despite this, laryngeal cancer cells display E_2 responsiveness, as E_2 was found to increase proliferation of laryngeal cancer cells [16], and a non-genomic mechanism was implicated [17]. Furthermore, the effects of E_2 were inhibited by tamoxifen [18]. These findings advocate a role for E_2 in the tumorigenesis of laryngeal cancer and warrants further exploration of its effects.

In order to further improve the understanding of the role of E_2 in the tumorigenesis and metastasis of laryngeal cancer, we examined the underlying mechanism of E_2's effect in vitro using laryngeal cancer cell lines as the model. We hypothesized that the effect exerted by E_2 on laryngeal cancer is similar to that found in breast cancer and that ERα36-associated E_2 membrane signaling mediated by PKC is involved in promoting proliferation, protecting against apoptosis, and stimulating gene expression resulting in enhanced aggressiveness and metastasis of laryngeal cancer. In addition, we used immunohistochemistry to assess expression of ERα36 and vascular endothelial growth factor (VEGF) in histological samples from human laryngeal tumors in order to help clarify the role of E_2 in vivo.

Materials and Methods

Reagents The Hep2 laryngeal carcinoma, TT thyroid carcinoma, HeLa cervical carcinoma, and HCC38 breast cancer cell lines were validated and obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Minimum essential medium (MEM) and Roswell Park Memorial Institute (RPMI) 1640, both lacking phenol red, were purchased from Invitrogen (Grand Island, NY, USA), and F12K nutrient mixture was purchased from Cellgro (Manassas, VA, USA). Charcoal/dextran-filtered fetal bovine serum (FBS) was purchased from Gemini Bio-Products (Sacramento, CA, USA). The estradiol enantiomer ent-17β-estradiol (Ent-E_2) was kindly provided as a gift from Douglas Covey (Washington University, St. Louis, MO, USA) [19]. E_2, E_2 conjugated to bovine serum albumin (E_2-BSA), and taxol were purchased from Sigma (St. Louis, MO, USA). The phospholipase D (PLD) inhibitor wortmannin was purchased from EMD chemicals (Billerica, MA, USA). Protein content of samples was measured using the Macro BCA reagent kit from Pierce/Thermo Scientific (Rockford, IL, USA). ERα36 antibodies were purchased from Cell Applications Inc. (San Diego, CA, USA), GAPDH antibodies were purchased from EMD-Millipore (Billerica, MA, USA), and caveolin-1 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). VEGF antibodies were purchased from Abcam (Cambridge, MA, USA). Details on the use of the antibodies in this study can be seen in Supplemental Table 1. The PKC assay kit was purchased from GE Lifesciences (Pittsburgh, PA, USA). The Amplex Red phospholipase D assay kit was purchased from Life Technologies (Grand Island, NY, USA). The Titertacs TUNEL assay kit was purchased from R&D Systems (Minneapolis, MN, USA). The CaspAce assay kit was purchased from Promega (Madison, WI, USA). All primers were purchased from Eurofins (Huntsville, AL, USA). The IRB-approved larynx tissue microarray was purchased from Imgenex (San Diego, CA, USA).

Cell Culture Hep2 and HeLa cells were cultured in MEM-based media, as specified by the ATCC, containing 10 % charcoal/dextran-filtered FBS and lacking phenol red, which can mimic the effects of E_2 at low levels. HCC38 cells were maintained in RPMI 1640-based media, while TT cells were cultured in F12K media.

Presence of ER Isoforms In order to determine whether laryngeal cancer cells express ERα36, sequence-specific primers, designed in our lab previously [12], were used to selectively identify the traditional ERα66 and the alternatively spliced variant ERα36, as well as the G-protein coupled estrogen receptor, GPR30. RNA was extracted from Hep2, HeLa, HCC38, and TT thyroid medullary carcinoma cell lines cells

using the TRIzol method. Reverse transcription was performed to produce cDNA for ERs from the laryngeal cell line. PCR was then performed to determine whether mRNAs for the receptors were expressed. Primer sequences for all mRNAs examined in this study can be seen in Supplemental Table 2. In addition, cell lysates were fractionated according to the method previously described by Smart et al. [20] to obtain whole cell lysates, crude nuclear fractions, and plasma membrane fractions. Western blots were then used to identify the subcellular localization of ERα36 in the cell lines.

PKC and PLD Activity Subconfluent cultures of Hep2 cells were treated with E_2, Ent-E_2, and E_2-BSA at the indicated time points and at a concentration of 10^{-8} M unless otherwise specified. Ent-E_2 was used in order to determine if E_2 response relies on a stereospecific receptor response. The use of E_2-BSA, which cannot pass through the plasma membrane and reach the nuclear receptor [21–23], enables confirmation that the effects elicited by E_2 are membrane mediated. The concentration of E_2 in E_2-BSA was calculated as follows: the E_2-BSA provided has a molar ratio of 30 mol of E_2 to 1 mol of BSA. Based on this, using a molecular weight for E_2 equal to 272.4 g/mol and a molecular weight of BSA equal to 66,463 g/mol, we calculated a mass percentage of E_2 in a given mass of E_2-BSA conjugate equal to 10.95 %. Using this percentage, we created a stock concentration of E_2-BSA containing 10^{-3} mol of E_2 per liter of vehicle, in this case ethanol. We then passed the solution through a centrifugal filtration unit with a molecular weight cutoff of 50kDa, allowing all free E_2 to pass through the filter and allowing us to recover a solution from the filter theoretically containing only pure conjugated E_2-BSA. From this stock concentration, we created the dilutions used in the study.

The PLD inhibitor, wortmannin, was used at a concentration of 10^{-5} M. ERα36 antibodies were used to block the effect of E_2, in order to determine the role of ERα36 in the E_2 activation of PKC and PLD. Wortmannin and ERα36 antibodies were used 15 min prior to E_2 treatment. Cells were washed twice with phosphate buffered saline and lysed in RIPA buffer at time of harvest. PKC activity was measured using the Biotrak PKC assay kit from GE Lifesciences. PLD activity was measured using the Amplex Red PLD assay from Life Technologies and normalized using the PicoGreen DNA quantification assay. ERα36 antibodies were used to block the effect of E_2, in order to determine the role of ERα36 in the E_2 activation of the PLD signal cascade.

Cell Proliferation, Viability, and Apoptosis In order to establish whether signaling via ERα36 can protect against apoptosis, the Hep2 cell line was treated with taxol, a known chemotherapeutic agent that induces cell death, as previously demonstrated in breast cell lines [12]. Subconfluent cultures were pretreated with E_2 for 90 min followed by taxol treatment for 4 h, after which cells were assayed for caspase-3 activity, using the CaspAce assay kit from Promega (Madison, WI) and DNA fragmentation via TUNEL. Furthermore, cell proliferation was assessed as a function of DNA synthesis, using the Click-iT EdU assay from Invitrogen (Grand Island, NY). ERα36 antibodies were used to block the effect of E_2, thus verifying the role of ERα36 in the E_2 signal cascade.

Expression of Angiogenic and Metastatic Factors Quantitative RT-PCR was used to determine the effect of E_2 on expression of VEGF and fibroblast growth factor 2 (FGF2), which have been implicated in pathological angiogenesis associated with tumors [24–26]. Snail1 and e-cadherin (CDH1), which are metastatic factors leading to epithelial to mesenchymal transitions [27] and the metastatic factor CXC chemokine receptor type 4 (CXCR4) [28], were also measured in the Hep2 cell line. RT-PCR was performed with Applied Biosystems Fast SYBR Mastermix in the Applied Biosystems StepOne real-time system.

Immunohistochemistry A laryngeal cancer tissue microarray containing 39 histological slices of laryngeal cancer tissue was obtained from Imgenex Corp. Clinical information for the 39 patients with laryngeal carcinoma is presented in Table 1. Prior to immunohistochemistry, slides were placed in a 60 °C oven for 30 min, deparaffinized, and rehydrated, and antigen presentation

Table 1 Laryngeal cancer patient characteristics ($n=39$)

Age					
Mean (SD)	60 (8.68)				
>50	36				
≤50	3				
Sex					
Male	38				
Female	1				
Tissue type					
Supraglottic	16				
Glottic	7				
Transglottic	6				
Pyriform sinus	5				
Subglottic	5				
TNM classification	0	1	2	3	4
Tumor size	0	1	1	16	21
Lymph node metastasis	16	4	17	1	N/A
Metastasis	38	1	N/A	N/A	N/A
Stage	I	II	III	IV (A, B, C)	
	1	1	12	25 (23, 1, 1)	

A total of 39 laryngeal cancer subject tissues were examined by immunohistochemistry and blindly scored by three independent observers. The characteristics in the table above describe the age, sex, tissue type, TNM classification, and staging characteristics for these 39 subjects

was achieved by heating in a pressure cooker for 3 min in 0.1 M sodium citrate buffer. ERα36 and VEGF presence was examined by immunohistochemistry using the LSAB immunohistological staining kit from Santa Cruz Biotechnology. Slides were counterstained with hematoxylin. A negative control for antibody detection can be seen in Supplemental Fig. 1.

Imaging and Scoring Slides were imaged using a Leica DMLB microscope system with a 63× objective oil immersion lens. Images were taken with ImagePro for all samples and blindly scored by three independent observers according to the scoring method listed in Table 2. ERα36 presence in all tissues was scored according to two classifications as follows: ERα36 number describes the number of cells in the tissue that are positive for ERα36; ERα36 intensity describes the intensity of the staining, which describes cells that express either low amounts of ERα36 or high amounts of ERα36. VEGF was scored in a similar manner to ERα36 for VEGF number and VEGF intensity.

Statistical Analyses For all in vitro experiments, data are represented as the mean and standard error among six individual samples. Statistical analyses were performed by analysis of variance with Bonferroni's correction for Student's *t* test at a significance level of $\alpha \leq 0.05$. Histological samples were scored blindly by three independent observers, and correlations of ERα36 number and intensity to clinicopathological variables were performed using Fisher's exact test.

Table 2 Immunohistological scoring method

	Score	Representation
ERα36 number	0	<1 % cells positive
	1	1<10 % cells positive
	2	10<50 % cells positive
	3	≥50 % cells positive
ERα36 location	0	Nuclear
	1	Non-nuclear
ERα36 intensity	0	Low
	1	High
VEGF number	0	<1 % cells positive
	1	1<10 % cells positive
	2	10<50 % cells positive
	3	≥50 % cells positive
VEGF intensity	0	Low
	1	High

All samples were scored blindly by three independent observers. Scores were then compared and for samples that were not scored the same by all three observers, the two most common scores were used. If no scores matched, a fourth observer blindly determined the score

ER estrogen receptor and *VEGF* vascular endothelial growth factor

Results

RT-PCR results showed that Hep2 laryngeal carcinoma epithelial cells expressed the alternative splicing variant to the traditional ERα, ERα36 (Fig. 1a). The cells also expressed mRNA for the classical ERα66, as well as the nonclassical G-

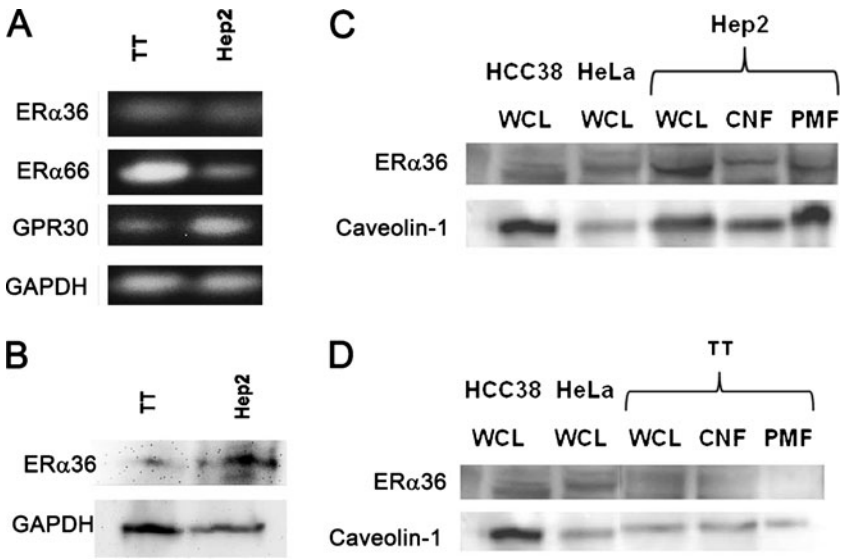

Fig. 1 Presence of ERs in laryngeal and thyroid cell lines. RT-PCR shows that both the TT thyroid medullary and Hep2 laryngeal carcinoma cell lines express both the traditional ER, ERα66, and the novel variant, ERα36, as well as the nontraditional G-protein coupled ER, GPR30 (**a**). Western blots of whole cell lysates from both the TT and Hep2 cell lines show that they both contain protein for the novel receptor, ERα36, but it appears at least qualitatively that the Hep2 cell line contains more (**b**). Western blots on plasma membrane fractions show the presence of ERα36 in the plasma membranes of Hep2 cells, but not in TT cells(**c**, **d**). *WCL* whole cell lysate, *CNF* crude nuclear fraction, and *PMF* plasma membrane fraction

protein coupled estrogen receptor GPR30 (Fig. 1a). The presence of ERα36 protein was confirmed by Western blot analysis, which demonstrated that Hep2 cells contained protein for ERα36. While ERα36 was also identified in the TT thyroid cancer cell line, it was more pronounced in the Hep2 laryngeal cells (Fig. 1b). Plasma membrane isolation of Hep2 cells and Western blot analysis showed that ERα36 was present in Hep2 plasma membranes along with caveolin-1, a protein found in the caveolae, which are specifically invaginated compartments of the plasma membrane that are known to house many integral membrane receptors (Fig. 1c); however, TT cells did not appear to have detectable levels of plasma membrane-associated ERα36, although caveolin-1 was found in this fraction (Fig. 1d). HCC38 breast cancer cells and HeLa cervical cancer cells were used as positive controls for ERα36 present in the plasma membrane (Fig. 1c, d). This is in agreement with our previous results, which placed this receptor in the plasma membrane of different cell lines [19].

E_2 caused a significant increase in PKC activity after 30 min of treatment (Fig. 2a). The effect of E_2 was biphasic, with a significant increase in PKC at 10^{-9} and 10^{-8} M but with no effect at 10^{-7} M E_2 (Fig. 2b). E_2's effect was abolished with the specific PLD inhibitor wortmannin (Fig. 2c) or by antibodies specifically against ERα36 (Fig. 2d). Furthermore, E_2 stimulated PLD activity. This effect appeared after 60 min (Fig. 3a), between the concentrations 5×10^{-9}–10^{-8} M E_2 (Fig. 3b). The enantiomer of E_2 (Ent-E_2) had no effect on PLD activity (Fig. 3c). In addition, E_2-BSA had a stimulatory effect at a concentration of 10^{-9} M on PLD activity in the Hep2 cells (Fig. 3d). Using antibodies specifically against ERα36 to block the receptor prior to E_2 treatment abolished the effect of E_2 on PLD activity (Fig. 3e).

In order to determine the physiological effects of E_2 signaling mediated by ERα36, we further examined cell survivability and downstream gene expression related to angiogenesis and metastasis. Initially, taxol was used to induce apoptosis, in order to assess the antiapoptotic effects of E_2 in cancer cells. The TUNEL assay, which measures DNA nicks associated with apoptosis, showed that taxol enhanced TUNEL, while pretreatment with 10^{-8} and 10^{-7} M E_2 prevented this effect (Fig. 4a). While taxol additionally enhanced caspase-3 activity, an enzyme that plays a major role in apoptosis, 10^{-8} M E_2 also blocked this effect (Fig. 4b). E_2 also exhibited a proliferative effect in Hep2 cells, as evident from the enhanced DNA synthesis (Fig. 4c). Pretreatment of Hep2 cells with ERα36 antibodies (1:500 dilution) blocked these antiapoptotic and proliferative effects of E_2 (Fig. 4b, c).

Quantitative RT-PCR analysis showed that 10^{-8} M E_2 increased mRNA levels of the angiogenic factors VEGF (Fig. 5a) and FGF2 (Fig. 5b), while ERα36 antibodies (1:500 dilution) blocked this effect. E_2 enhanced the metastatic factor Snail1 (Fig. 5c), while downregulating the

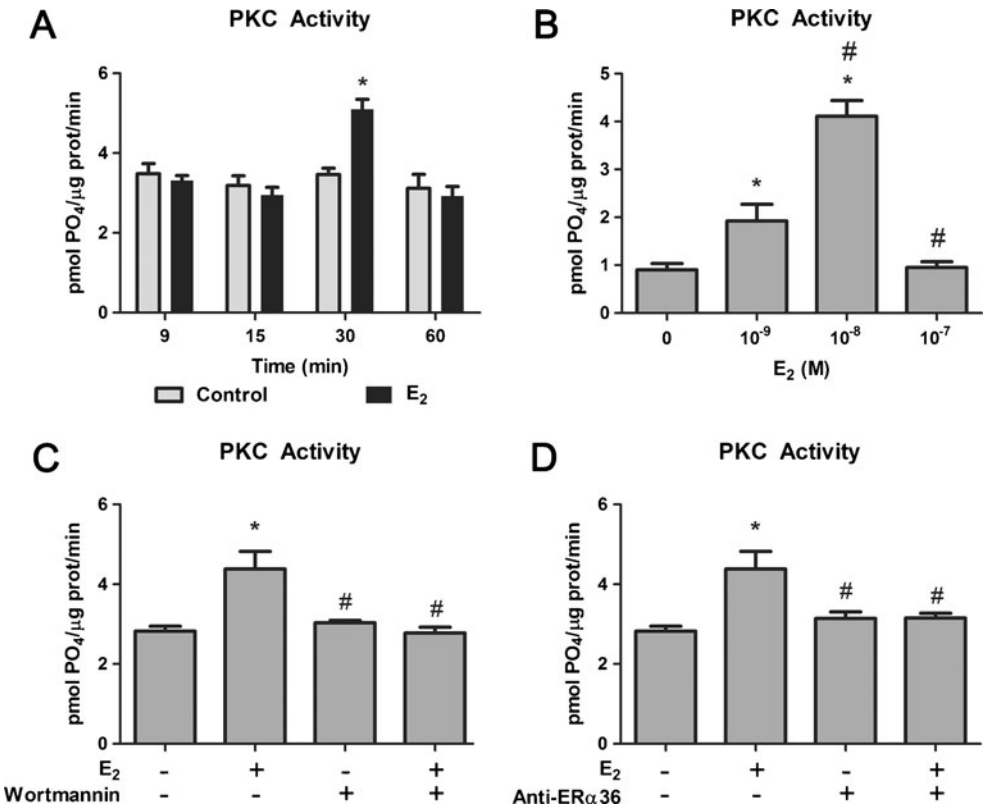

Fig. 2 Estradiol effects on PKC activity in Hep2 laryngeal carcinoma cells. A time-course evaluation of PKC activity in response to 10^{-8} M E_2 treatment of Hep2 cells shows that PKC activity is enhanced 30 min after treatment (**a**), while in response to different doses of E_2, a biphasic effect is observed (**b**). Pretreatment for 15 min with 10^{-5} M wortmannin and 1:500 ERα36 antibody prevent the effect of E_2 on PKC (Fig. 2c, d, respectively). *$p<0.05$ versus corresponding control; #$p<0.05$ compared to 10^{-9} M E_2

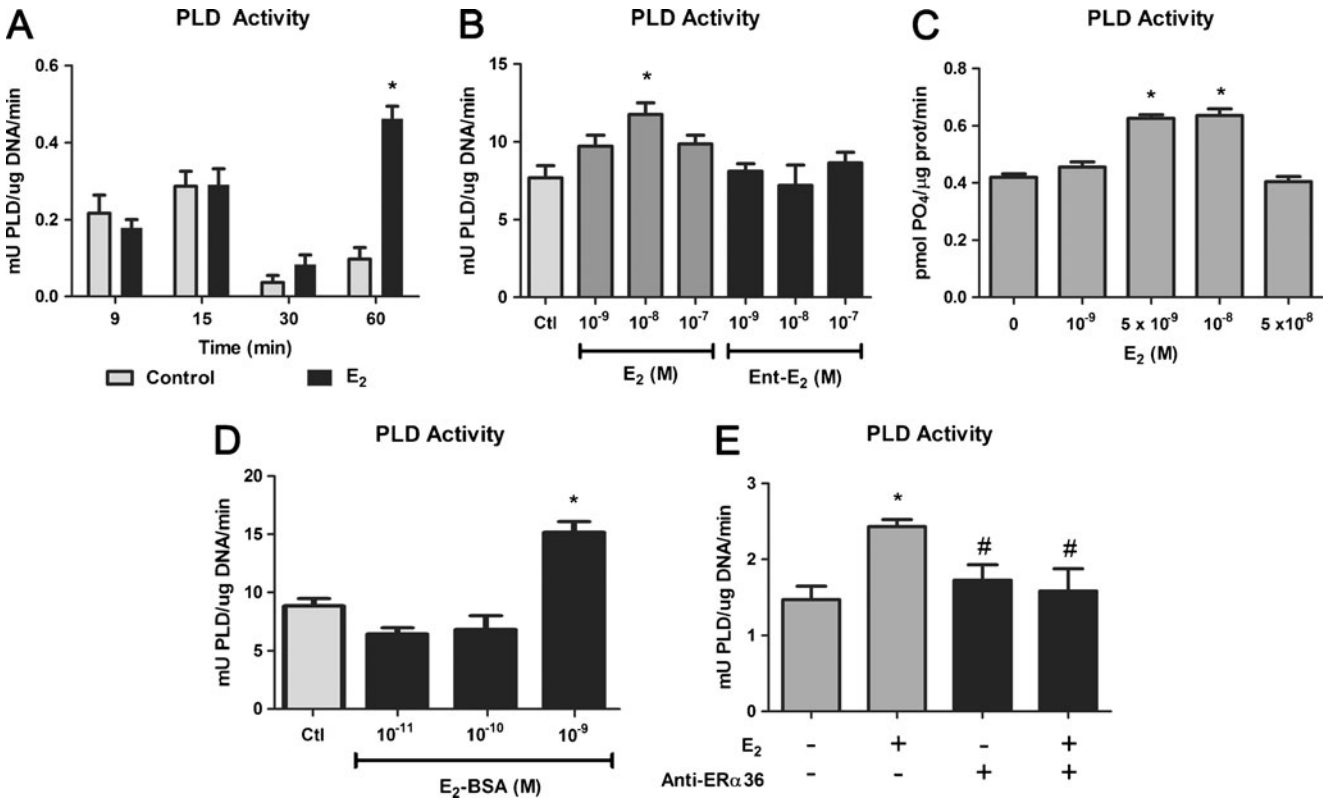

Fig. 3 Estradiol effects on PLD activity in Hep2 laryngeal carcinoma cells. A time-course evaluation of PLD activity in response to 10^{-8} M E_2 treatment of Hep2 cells shows that PLD activity is enhanced 60 min after treatment (**a**), while a biphasic effect is observed in response to different doses (**b**). When the enantiomer for E_2, Ent-E_2, is used to treat Hep2 cells, no increase in PLD activity is observed (**c**). When E_2-BSA is used to treat Hep2 cells, an increase in PLD activity is only observed with 10^{-9} M E_2-BSA (**d**). When Hep2 cells were pretreated with antibody against ERα36, the effect of E_2 on PLD activity was abolished (**e**). *$p<0.05$ versus corresponding control; #$p<0.05$ compared to E_2 only

expression of e-cadherin (CDH1) (Fig. 5d), and both of these effects by E_2 were blocked by ERα36 antibodies. Furthermore, the migratory factor CXCR4 (Fig. 5e) showed a similar enhancement with E_2 treatment, and this was again blocked with ERα36 antibodies. mRNA levels were all normalized to GAPDH (Fig. 5).

The blinded analysis of laryngeal cancer immunohistochemical staining for ERα36 demonstrated that all laryngeal cancer patient tissues were positive for the presence of the ERα36. There was a variance in the distribution of the receptor and in the degree of positive staining for the receptor. Tissues containing less ERα36 also appeared to contain less VEGF, and vice versa (Fig. 6). Samples with relatively low ERα36 appeared to also contain less VEGF (Fig. 6a). Conversely, samples that exhibited strong punctate staining of ERα36 also had punctate staining of VEGF, which appeared to occur around blood vessels (Fig. 6b). While some samples with relatively high, ERα36 exhibited moderate amounts of VEGF (Fig. 6c), others appeared to stain strongly for VEGF (Fig. 6d). Amongst the study sample, all samples exhibited positive staining for ERα36 and VEGF, and a correlation was found between the number of ERα36 receptors and the number of VEGF ($p=0.0178$) (Table 3). ERα36 number and intensity was further found to correlate with metastasis to regional lymph nodes ($p=0.0263$ and $p=0.0119$, respectively). However, we did not observe a correlation between ERα36 number and intensity with other variables such as age, tumor size, or VEGF intensity (Table 3).

Discussion

E_2 has been studied in multiple ER expressing cells as a potential factor that influences tumorigenesis. In breast cancer, the prototype of ER expressing cancer cells, ER-negative tumors have been found to respond to E_2 with increases in PKC activity, which correlates with enhanced tumorigenicity [12]. The discovery of a novel ER variant, ERα36, opened the possibility that cancers previously labeled as non-hormone dependent and ER negative might in fact be susceptible to the effects of E_2 via this membrane receptor, as was demonstrated in ER negative breast cancers [9, 12]. In breast cancer, ERα36 was found to be a key cellular and transcriptional regulator of proliferation and enhanced aggressiveness [11, 12], thus emphasizing the importance of characterizing further

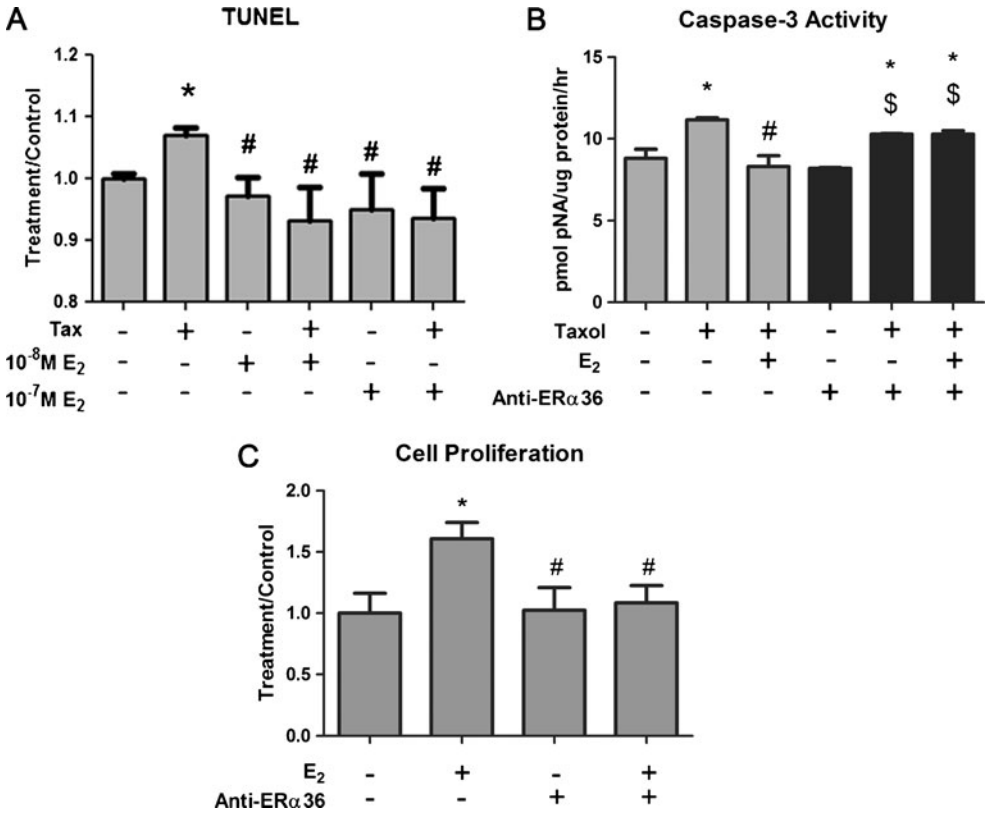

Fig. 4 Role of E_2 in Hep2 laryngeal carcinoma cell survival. Taxol increased apoptosis and this was blocked by 10^{-8}–10^{-7} M E_2 (**a**). 10^{-8} M E_2 also inhibited the ability of taxol to increase caspase-3 activity, while antibodies against ERα36 reduced this effect of E_2 (**b**). 10^{-8}–10^{-7} M E_2 increased cell proliferation, but in the presence of ERα36 antibodies, this effect was not evident (**c**). *p <0.05 versus corresponding control; #p <0.05 compared to taxol only; \$$p$ <0.05 compared to anti-ERα36 only

its presence and role in other cancers that are subject to the influences of sex hormones.

Previous work has suggested that laryngeal squamous cell carcinomas exhibit sex hormone-dependent behavior [15]. Here, we show that laryngeal carcinoma cell lines possess functional ERα36, and it is present in the plasma membranes. The existence of ERα36 in the plasma membrane was not uniform in all cancer cells, however, as demonstrated by the lack of ERα36 in TT thyroid cancer cells.

Previous studies in breast cancer demonstrated that ERα36 is localized specifically in the caveolae and is responsible for the E_2 activation of the PKC signaling cascade [12]. This was also the case for Hep2 cells; ERα36 was localized with caveolin-1, indicating its present in caveolae. Moreover, E_2 stimulated PKC activity in Hep2 cells via a mechanism comparable to that seen in the breast cancer cells. E_2 caused an increase in PKC activity that was dependent on PLD, based on its inhibition by wortmannin. E_2 activated PLD via a receptor-mediated mechanism; the enantiomer of E_2 had no effect, and antibodies to ERα36 blocked E_2-dependent increases in PLD activity. It was also membrane-dependent as E_2-BSA was able to elicit the response. GPR30, which is a well-studied alternative receptor for estrogen, was also expressed in Hep2 cells. Previously, we demonstrated that antibodies against GPR30 did not block the effect of E_2 nor E_2-BSA [12], indicating that GPR30 does not mediate the membrane-associated responses to E_2 examined in the present study. Furthermore, blocking ERα36 signaling with antibodies abolished the protective and proliferative effects of E_2, indicating that ERα36 has a role in tumorigenesis of laryngeal cancer and in the antiapoptotic effect of E_2 against chemotherapeutics.

PKC has a key role in promoting tumorigenesis [29]. Higher PKC activity correlates in vivo to enhanced tumor aggressiveness and progression [12, 29, 30], while in vitro, activation of the PKC signaling pathway enhances cell proliferation and survival via an antiapoptotic mechanism [9, 12]. Activation of PKC initiates a signaling cascade that results in activation of the ERK1/2 family of mitogen-activated protein kinases, providing an alternative method for steroid hormones to modulate gene expression rather than by traditional nuclear receptor-mediated pathways [12].

PKC expression in laryngeal cancer was reported to be significantly higher than that found in normal laryngeal epithelium and adjacent noncancerous laryngeal epithelium and was correlated with clinical stage and cervical lymph node metastasis [31]. In our study, in response to E_2, Hep2 cells demonstrated an increase in PKC activity followed by an upregulation of angiogenic and metastatic factors. Clinical analysis of patients with laryngeal cancer revealed an association between the number and concentration of ERα36 and metastasis to regional lymph nodes. A similar relationship was observed in other cancer cells, such as breast [12] and gastric carcinoma [32]. Previous studies found that E_2 activation of PKC mediates the expression of several metastatic factors

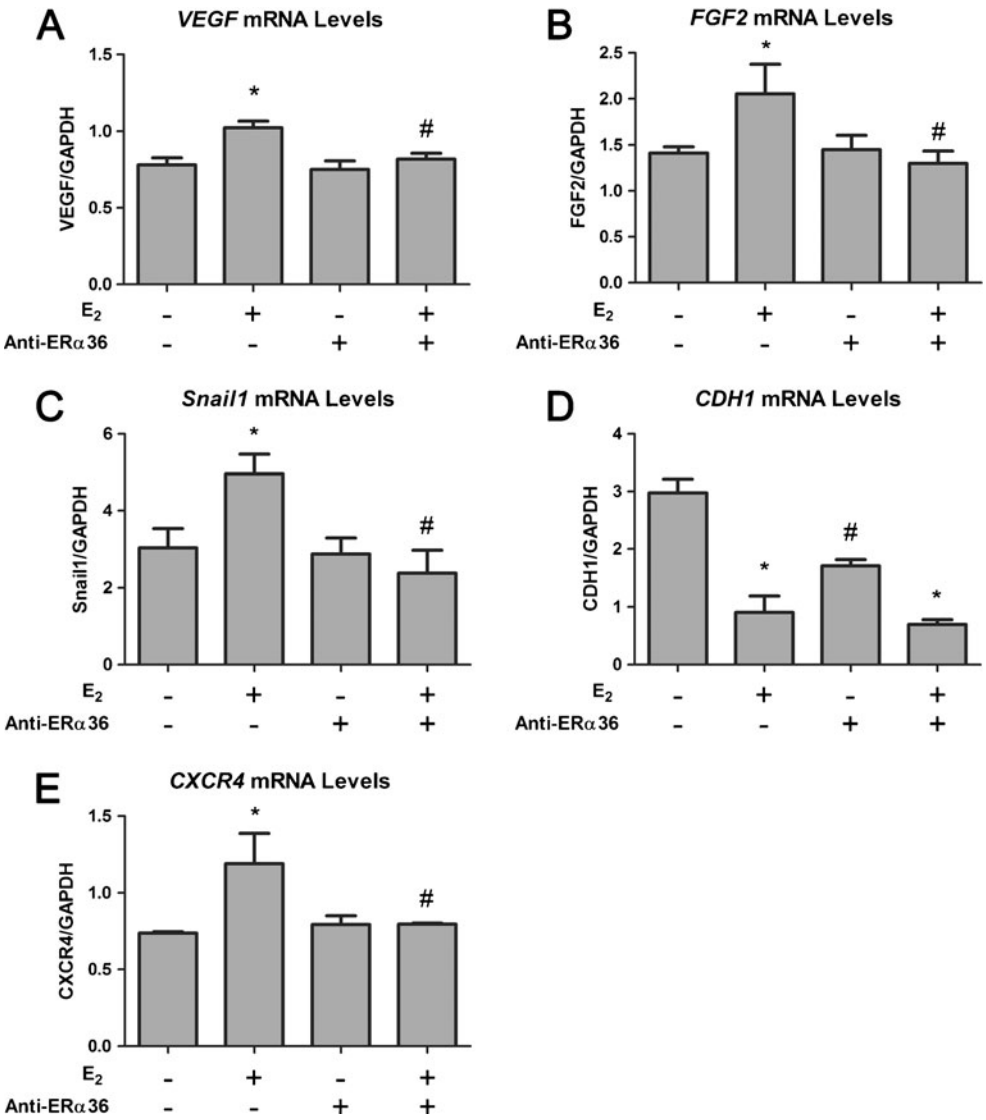

Fig. 5 Role of ERα36 in E_2's effect on angiogenic and metastatic factor expression. 10^{-8} M E_2 increased mRNA levels of proangiogenic factors *VEGF* (**a**) and *FGF2* (**b**), and the metastatic factors *CXCR4* (**c**) and Snail1 (**d**), while antibodies to ERα36 blocked this effect. The effects on Snail1 were accompanied by a corresponding decrease in E-Cadherin (*CDH1*) mRNA levels that was also inhibited by ERα36 antibodies (**e**). All values were normalized to *GAPDH* mRNA levels. *$p < 0.05$ versus corresponding control; #$p < 0.05$ versus E_2 only

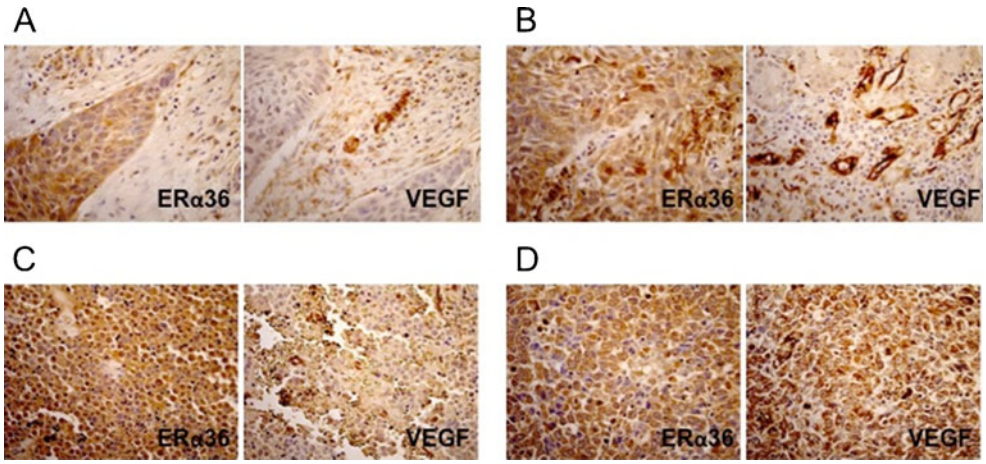

Fig. 6 Larynx TMA ERα36 and VEGF immunohistochemistry. Sample with low ERα36 and low VEGF levels (**a**). Samples with strong punctate staining of ERα36 accompanied by strong punctate staining of VEGF in blood vessels (**b**). Sample with high levels of ERα36 and moderate amounts of VEGF (**c**). Sample with strong ERα36 and VEGF staining (**d**)

Table 3 Larynx TMA statistics

Association of:	With:	Significant?	*P* value
ERα36 Number	Age (≤50 vs. >50)	No	0.6057
	Tumor size by TNM staging	No	0.6057
	Metastasis to regional lymph nodes	Yes	0.0263
	VEGF intensity	No	0.2156
	VEGF number	Yes	0.0178
ERα36 Intensity	Age (<50 vs. >50)	No	0.4520
	Tumor size by TNM staging	No	0.3698
	Metastasis to regional lymph nodes	Yes	0.0119
	VEGF number	No	0.7250
	VEGF intensity	No	0.2973

P values were obtained by Fisher's exact test

TMA tissue microarray, *ER* estrogen receptor, and *VEGF* vascular endothelial growth factor

such as Snail1 and Snail2, which downregulate the cell–cell interaction proteins such as cadherins, leading to what is known as an epithelial to mesenchymal transition [33], thus promoting tumor metastasis [12]. Tamoxifen was found to exert its effect, among others, by inhibiting the PKC signal pathway [9, 34], thus opening the possibility that tamoxifen may be considered in the treatment of laryngeal cancer.

There are a number of alternative mechanisms by which PKC is activated and regulated via a membrane receptor, of which phospholipase A2, phospholipase C, and phospholipase D have a prominent role. The mechanism by which this family of phospholipases cleaves different membrane phospholipids, such as phosphatidylcholine or phosphatidylinositol, to form diacylglycerol, thus activating PKC is well studied [35]. However, there is increasing evidence that this mechanism is not one directional, as in the case of PLD, in which PKC was found to be an upstream regulator of PLD [31]. Moreover, PLD in itself has antiapoptotic effects, through the production of lysophosphatidic acid, which in turn leads to decreased caspase-3 activity [35]. Our findings support that in laryngeal cancer, E_2 exerts its effects through the PLD–PKC pathway. Further research is needed in order to elucidate the mechanism by which this pathway renders laryngeal cancer cells sensitive to the effects of E_2.

These effects can explain the ability of E_2 to enhance tumor aggressiveness directly by allowing cells to proliferate and evade the effects of common chemotherapeutics. Not only can these cells proliferate and evade apoptosis in the presence of E_2, but E_2 may also lead to a more aggressive cancer by leading the primary tumor and metastasizing to other secondary sites in the patient. As tumors grow, cells within the tumor secrete VEGF, leading to vascularization of the tumor and providing a network through which these tumor cells can enter the vasculature and travel throughout the body. Our previous results showed that E_2 enhanced VEGF and FGF expression in vitro, and the in vivo correlation between ERα36 and VEGF suggest that patients with high levels of ERα36 may have a more aggressive tumor phenotype as evidenced by the correlation to lymph node metastasis. These patients may benefit from treatments such as tamoxifen, which can block signaling of E_2 through ERα36, regardless of whether they contain traditional ERs.

As opposed to breast cancer in which the role of E_2 signaling has been studied mainly in female patients, in this study, we observed that E_2 plays also a major role in development of cancer in male patients. Our findings suggest that male patients with laryngeal cancer, and possibly other cancers that are hormone dependent, may benefit from ER-targeting treatments. Originally, we sought to determine if the role of ERα36 in laryngeal cancer is sex dependent, but were unable to do so due to the low frequency of laryngeal cancer in women, and thus our sample of tissues contained samples from mostly male patients.

The findings of this study present compelling evidence that laryngeal cancer is hormone responsive, specifically to the effects of E_2, via ERα36. This membrane receptor activates several pathways, which were found both in vitro and in vivo, to correlate with laryngeal cancer progression and aggressiveness. E_2 also elicits antiapoptotic effects that can oppose the effects of chemotherapeutic agents, such as taxol, and might, thus, confer resistance to treatment. Further investigation is warranted in order to elucidate the role of E_2 and this membrane-mediated mechanism and to shed light on its impact on the treatment and management of laryngeal cancer clinically.

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References

1. Yager JD, Liehr JG (1996) Molecular mechanisms of estrogen carcinogenesis. Annu Rev Pharmacol Toxicol 36:203–232. doi:10.1146/annurev.pa.36.040196.001223
2. Kampa M, Pelekanou V, Castanas E (2008) Membrane-initiated steroid action in breast and prostate cancer. Steroids 73(9–10):953–960. doi:10.1016/j.steroids.2007.12.009
3. Katzenellenbogen BS, Kendra KL, Norman MJ, Berthois Y (1987) Proliferation, hormonal responsiveness, and estrogen receptor content of MCF-7 human breast cancer cells grown in the short-term and long-term absence of estrogens. Cancer Res 47(16):4355–4360
4. van den Brule FA, Engel J, Stetler-Stevenson WG, Liu FT, Sobel ME, Castronovo V (1992) Genes involved in tumor invasion and metastasis are differentially modulated by estradiol and progestin in human breast-cancer cells. Int J Cancer 52(4):653–657
5. Carruba G (2007) Estrogen and prostate cancer: an eclipsed truth in an androgen-dominated scenario. J Cell Biochem 102(4):899–911. doi:10.1002/jcb.21529
6. Enmark E, Gustafsson JA (1999) Estrogen receptors—an overview. J Intern Med 246(2):133–138
7. Levin ER (2001) Cell localization, physiology, and nongenomic actions of estrogen receptors. J Appl Physiol 91(4):1860–1867
8. Revankar CM, Cimino DF, Sklar LA, Arterburn JB, Prossnitz ER (2005) A transmembrane intracellular estrogen receptor mediates rapid cell signaling. Science 307(5715):1625–1630. doi:10.1126/science.1106943
9. Boyan BD, Sylvia VL, Frambach T, Lohmann CH, Dietl J, Dean DD, Schwartz Z (2003) Estrogen-dependent rapid activation of protein kinase C in estrogen receptor-positive MCF-7 breast cancer cells and estrogen receptor-negative HCC38 cells is membrane-mediated and inhibited by tamoxifen. Endocrinology 144(5):1812–1824
10. Wang Z, Zhang X, Shen P, Loggie BW, Chang Y, Deuel TF (2005) Identification, cloning, and expression of human estrogen receptor-alpha36, a novel variant of human estrogen receptor-alpha66. Biochem Biophys Res Commun 336(4):1023–1027. doi:10.1016/j.bbrc.2005.08.226
11. Wang Z, Zhang X, Shen P, Loggie BW, Chang Y, Deuel TF (2006) A variant of estrogen receptor-{alpha}, hER-{alpha}36: transduction of estrogen- and antiestrogen-dependent membrane-initiated mitogenic signaling. Proc Natl Acad Sci U S A 103(24):9063–9068. doi:10.1073/pnas.0603339103
12. Chaudhri RA, Olivares-Navarrete R, Cuenca N, Hadadi A, Boyan BD, Schwartz Z (2012) Membrane estrogen signaling enhances tumorigenesis and metastatic potential of breast cancer cells via estrogen receptor-alpha36 (ERalpha36). J Biol Chem 287(10):7169–7181. doi:10.1074/jbc.M111.292946
13. Cook MB, Dawsey SM, Freedman ND, Inskip PD, Wichner SM, Quraishi SM, Devesa SS, McGlynn KA (2009) Sex disparities in cancer incidence by period and age. Cancer Epidemiol Biomarkers Prev 18(4):1174–1182. doi:10.1158/1055-9965.EPI-08-1118
14. Reiner Z, Cvrtila D, Petric V (1988) Cytoplasmic steroid receptors in cancer of the larynx. Arch Otorhinolaryngol 245(1):47–49
15. Hagedorn HG, Nerlich AG (2002) Analysis of sex-hormone-receptor expression in laryngeal carcinoma. Eur Arch Otorhinolaryngol 259(4):205–210
16. Dorsey M, Benghuzzi H, Tucci M, Cason Z (2005) Growth and cell viability of estradiol and IP-6 treated Hep-2 laryngeal carcinoma cells. Biomed Sci Instrum 41:205–210
17. Robbins KT, Vu TP, Diaz A, Varki NM (1994) Growth effects of tamoxifen and estradiol on laryngeal carcinoma cell lines. Arch Otolaryngol Head Neck Surg 120(11):1261–1266
18. Ferrandina G, Almadori G, Maggiano N, Lanza P, Ferlini C, Cattani P, Piantelli M, Scambia G, Ranelletti FO (1998) Growth-inhibitory effect of tamoxifen and quercetin and presence of type II estrogen binding sites in human laryngeal cancer cell lines and primary laryngeal tumors. Int J Cancer 77(5):747–754. doi:10.1002/(SICI)1097-0215(19980831)77:5<747::AID-IJC14>3.0.CO;2-Z
19. Green PS, Yang SH, Nilsson KR, Kumar AS, Covey DF, Simpkins JW (2001) The nonfeminizing enantiomer of 17beta-estradiol exerts protective effects in neuronal cultures and a rat model of cerebral ischemia. Endocrinology 142(1):400–406
20. Smart EJ, Ying YS, Mineo C, Anderson RG (1995) A detergent-free method for purifying caveolae membrane from tissue culture cells. Proc Natl Acad Sci U S A 92(22):10104–10108
21. Sylvia VL, Walton J, Lopez D, Dean DD, Boyan BD, Schwartz Z (2001) 17 beta-estradiol-BSA conjugates and 17 beta-estradiol regulate growth plate chondrocytes by common membrane-associated mechanisms involving PKC dependent and independent signal transduction. J Cell Biochem 81(3):413–429. doi:10.1002/1097-4644(20010601)81:3<413::AID-JCB1055>3.0.CO;2-M
22. Filardo E, Quinn J, Pang Y, Graeber C, Shaw S, Dong J, Thomas P (2007) Activation of the novel estrogen receptor G protein-coupled receptor 30 (GPR30) at the plasma membrane. Endocrinology 148(7):3236–3245. doi:10.1210/en.2006-1605
23. Stevis PE, Deecher DC, Suhadolnik L, Mallis LM, Frail DE (1999) Differential effects of estradiol and estradiol-BSA conjugates. Endocrinology 140(11):5455–5458
24. Pinto MP, Badtke MM, Dudevoir ML, Harrell JC, Jacobsen BM, Horwitz KB (2010) Vascular endothelial growth factor secreted by activated stroma enhances angiogenesis and hormone-independent growth of estrogen receptor-positive breast cancer. Cancer Res 70(7):2655–2664. doi:10.1158/0008-5472.CAN-09-4373
25. Yamasaki T, Tsuda H, Imazeki N, Matsubara O (2005) Vascular endothelial growth factor mRNA levels quantified by reverse transcription-polymerase chain reaction in microdissected breast carcinoma tissues are correlated with histological type and grade of both invasive and intraductal components. Pathol Int 55(5):255–263. doi:10.1111/j.1440-1827.2005.01822.x
26. Van der Auwera I, Van Laere SJ, Van den Eynden GG, Benoy I, van Dam P, Colpaert CG, Fox SB et al (2004) Increased angiogenesis and lymphangiogenesis in inflammatory versus non-inflammatory breast cancer by real-time reverse transcriptase-PCR gene expression quantification. Clin Cancer Res 10(23):7965–7971. doi:10.1158/1078-0432.CCR-04-0063
27. Herranz N, Pasini D, Diaz VM, Franci C, Gutierrez A, Dave N, Escriva M et al (2008) Polycomb complex 2 is required for E-cadherin repression by the Snail1 transcription factor. Mol Cell Biol 28(15):4772–4781. doi:10.1128/MCB.00323-08
28. Mukherjee D, Zhao J (2013) The role of chemokine receptor CXCR4 in breast cancer metastasis. Am J Cancer Res 3(1):46–57
29. Blobe GC, Obeid LM, Hannun YA (1994) Regulation of protein kinase C and role in cancer biology. Cancer Metastasis Rev 13(3–4):411–431
30. Ways DK, Kukoly CA, deVente J, Hooker JL, Bryant WO, Posekany KJ, Fletcher DJ, Cook PP, Parker PJ (1995) MCF-7 breast cancer cells transfected with protein kinase C-alpha exhibit altered expression of other protein kinase C isoforms and display a more aggressive neoplastic phenotype. J Clin Invest 95(4):1906–1915. doi:10.1172/JCI117872
31. Liu J, Pan S, Zhou Y, Yu Z, Liu L (2010) Expression and significance of IGF-IR and PKC in laryngeal squamous cell carcinoma. Lin Chung Er Bi Yan Hou Tou Jing Wai Ke Za Zhi 24(4):152–155
32. Deng H, Huang X, Fan J, Wang L, Xia Q, Yang X, Wang Z, Liu L (2010) A variant of estrogen receptor-alpha, ER-alpha36 is expressed in human gastric cancer and is highly correlated with lymph node metastasis. Oncol Rep 24(1):171–176
33. Tomaskovic-Crook E, Thompson EW, Thiery JP (2009) Epithelial to mesenchymal transition and breast cancer. Breast Cancer Res 11(6):213. doi:10.1186/bcr2416

34. Ahn SJ, Yoon MS, Hyuk S, Han W, Yoon YD, Han JS, Noh DY (2003) Phospholipase C-protein kinase C-mediated phospholipase D activation pathway is involved in tamoxifen-induced apoptosis. J Cell Biochem 89(3):520–528. doi:10.1002/jcb.10532

35. Hurst-Kennedy J, Zhong M, Gupta V, Boyan BD, Schwartz Z (2010) 24R,25-Dihydroxyvitamin D3, lysophosphatidic acid, and p53: a signaling axis in the inhibition of phosphate-induced chondrocyte apoptosis. J Steroid Biochem Mol Biol. doi:10.1016/j.jsbmb.2010.05.010