Expression of EGFR isoform D is regulated by HER receptor activators in breast cancer cells

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ARTICLE INFO	ABSTRACT
	Human epidermal growth factor receptor isoform D (EGFR; isoform D) is a soluble protein from a 3 kb alternate mRNA transcript that arises from the human EGFR gene. Several studies have identified this circulating isoform of EGFR as a potential diagnostic biomarker for the detection of early stage of cancers. While the expression of the full-length EGFR (isoform A) is regulated by its cognate ligand, EGF, as well as by phorbol myristate acetate (PMA), no studies have examined the factors regulating the expression of EGFR isoform D. In this study, using breast cancer cell lines, we show that the HER receptor ligands, EGF and neuregulin (NRG-1β), as well as the phorbol ester, PMA, can increase the expression of EGFR isoform D, as well as isoform A. Our results, based on measurement of mRNA levels, suggest that EGF induced expression of both isoform A and isoform D occur through a mitogen activated protein kinase (MAPK)-dependent mechanism, and also suggest that protein kinase C is involved in PMA-induced regulation of both isoforms. We also demonstrate that NRG-1β increases isoform A and isoform D expression via the MAPK-dependent pathway, but this regulation occurs independently of phosphatidylinositol 3-kinase/Akt activation. These results suggest that regulation of EGFR isoform A and isoform D expression occur using similar mechanisms. Despite commonalities in the transcriptional regulation of these two EGFR isoforms, the half-lives of these two transcripts is quite different. Moreover, EGFR isoform D, unlike isoform A, is not post-transcriptionally modulated by EGFR activators in the breast cancer cell line MDA-MB-468.

1. Introduction

Epidermal growth factor receptor (EGFR) is the prototypic member of the ErbB/HER family of receptor tyrosine kinases which consists of four members: EGFR (ErbB1/HER1), ErbB2 (Neu/HER2), ErbB3 (HER3) and ErbB4 (HER4). All four members of this receptor family are structurally similar, and contain an extracellular ligand-binding domain, a single membrane-spanning domain and a cytoplasmic tyrosine kinase domain [1,2]. Two families of ligands bind to these receptors: the epidermal growth factor (EGF) family and the neuregulin (NRG; also called heregulin) family. The EGF family includes EGF, transforming growth factor-α (TGF-α), amphiregulin, β-cellulin, heparin-binding EGF and epiregulin. The two NRG family members, NRG-1 and NRG-2 bind to HER3 and HER4, whereas NRG-3 and NRG-4 bind only to HER4. All EGF ligand family members bind to EGFR, but no soluble ligand has been identified for HER2 [3]. Receptor heterodimerization occurs following ligand binding, and the identity of the ligand dictates the nature of the partners in the dimerization complex, as well as the signal transduction pathway(s) activated [4]. Ligand-dependent activation of various signaling cascades by HER family members results in diverse downstream cellular responses including cell proliferation, differentiation, apoptosis, adhesion and migration [2].

In addition to the full-length ErbB/HER receptors, normal and

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Abbreviations	
EGFR	epidermal growth factor receptor
PMA	phorbol myristate acetate
NRG-1β	neuregulin
PKC	protein kinase C
MAPK	mitogen-activated protein kinase
PI3K	phosphoinositide 3-kinase
ER	estrogen receptor

malignant cells express soluble ErbB/HER receptor isoforms [5–10]. Aberrant expression or activation of EGFR and its isoforms, has been detected in diverse human malignancies including breast cancer [11–13]. These isoforms are comprised of the receptor's extracellular domain, and are generated either by limited proteolytic cleavage (HER2 [5], HER4 [14] and EGFR [6]), or via translation of diverse alternative transcripts (EGFR [7,8], HER2 [9] and HER3 [10]). Reiter et al. initially identified a 3 kb alternate transcript of EGFR in human placenta and in two carcinoma-derived cell lines that overexpress EGFR, i.e., MDA-MB-468 and A431 [7,15,16]. This 3 kb transcript encodes an 110-kDa protein, designated isoform D (Genbank 'EGFR isoform D'; NM_201284). This isoform D protein contains subdomains I, II, III and subdomain IV up to amino acid residue 603 of EGFR isoform A plus 78 unique C-terminal amino acids (Fig. 1). EGFR isoform D is associated with the plasma membrane [17] and is commonly expressed in human tissues and cell lines that also express the full-length EGFR isoform, isoform A [18]. Wilken et al. have shown that EGFR isoform D is a cell surface protein that is proteolytically released from the cell surface via a TACE-dependent mechanism [17]. Isoform D contains a series of hydrophobic amino acid as part of its unique c-terminus and it is possible that this short terminal sequence is embedded in the membrane. It is also possible that this hydrophobic sequence is the site of an, as yet, unidentified, post-translational modification [19]. Multiple physiological roles have been described for the soluble EGFR/HER isoforms. For example, Herstatin, an alternate product of HER2, reduces tyrosine phosphorylation and uncouples intracellular signaling mediated by EGF and TGF-α *in vitro* [20]. Similarly, two soluble isoforms of HER3, p45-and p85-sHER3, bind to neuregulin with high affinity, and inhibit neuregulin-induced activation of HER2, HER3 and HER4 in breast and ovarian cancer-derived cell lines [21]. EGFR isoform D also binds to EGF [17], and antibodies against the unique carboxy-terminus of isoform D
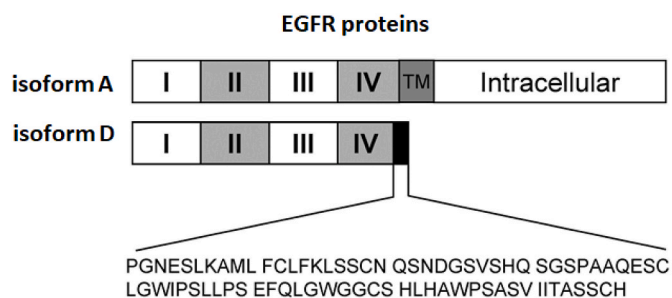

Fig. 1. Comparative diagram of EGFR isoform A and D protein structures. The structure of EGFR contains three domains: an extracellular ligand-binding domain (aa 1–621), a transmembrane domain (aa 622–644), and an intracellular tyrosine kinase domain (aa 645–1186). The extracellular domain of the EGFR isoform A consists of four subdomains (I–IV). EGFR isoform D shares complete sequence identity with isoform An until amino acid residue 603. At this point isoform D diverges into a unique 78 amino acid sequence encoded by the alternate exon 15 B[11]. EGFR isoform D lacks 18 amino acids of the extracellular domain of the full-length receptor, its transmembrane, and the entire intracellular domain.

have been shown to induce cell cohesion, resulting in compaction of cultured cells [22].

The regulation of EGFR isoform A expression has been well characterized. Cellular regulators, such as EGF family members, phorbol esters, and steroid hormone have been shown to modulate the expression of EGFR isoform A at both the mRNA and protein levels in several cell lines including breast cancer cells [23–34]. EGF binding to isoform A activates the MAPK pathway [35] resulting in an increase in both mRNA and protein expression of EGFR isoform A in various cell lines including breast cancer-derived cells [23,25–28,30,31]. Similarly, phorbol 12-myristate 13-acetate (PMA) increases mRNA and protein expression of EGFR isoform A in multiple cell types [25–27,30]. PMA activates protein kinase C (PKC), a family of serine/threonine kinases, resulting in activation of the mitogen-activated protein kinase (MAPK) pathway [36], leading to an increase in EGFR transcription (isoform A) [37]. In contrast, the transcriptional regulation of EGFR isoform D has not been investigated previously, even though its expression in cancer cells as well as in normal human tissues and blood suggest it may play critical roles in both tissue homeostasis and pathogenesis. In this study using breast cancer-derived cell lines, we have investigated the effects of various ErbB/HER ligands (EGF and NRG-1β), as well as the phorbol ester PMA, on the expression of the 3 kb transcript encoding EGFR isoform D, and have compared the mechanisms underlying regulation of expression of these two EGFR receptor isoforms.

2. Materials and methods

2.1. Reagents

EGF, Phorbol 12-myristate 13-acetate and NRG-1β were purchased from Sigma (St. Louis, MO). The inhibitors LY294002 and U0126, were obtained from Calbiochem. The monoclonal antibody directed against the ligand-binding domain of EGFR (15E11) have been described by us previously [38]. The following antibodies were purchased from Cell Signaling: Akt, phospho-Akt (Ser473), phospho-p44/42MAPK (Tyr202/204) (E10), phospho-EGFR (Tyr1148), phospho-HER2 (Tyr1221/1222), phospho-HER3 (Tyr1289) (21D3), phospho-EGFR (Tyr1148) and α/β-tubulin. Anti-MAPK antibody was obtained from Calbiochem, MAb EGFR.1 were purchased from Lab Vision. All breast carcinoma cell lines (MCF7, MDA-MB-468, ZR-75-1, BT-20, MDA-MB-231) used in this study were obtained from American Type Culture Collection.

2.2. Cell culture

MCF-7 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM)-F12 and all other cells were grown in DMEM (Bio Whittaker). The culture media were supplemented with 10% Fetal Bovine Serum (Sigma); 2 nM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin and 0.3 μg/ml fungizone (Gibco). Cell cultures were maintained at 37 °C in an atmosphere of 5% CO_2. Cells were grown until approximately 90% confluence and incubated in serum-free media overnight prior to the experiments. For the kinetic studies, cells were treated with 60 ng/ml EGF, 500 nM PMA, 50 ng/ml NRG-1β or vehicle alone (8 μM acetic acid, 0.1% ethanol or PBS respectively) for 0, 2, 4, 6, 8 and 24 h. For the inhibitors studies, cells were pre-incubated for 30 min with 1 μM Gö6983 prior to 4hr's PMA treatment (500 nM), for 45min with 50 μM U0126 before 4hr's treatment of 60 ng/ml EGF (or 50 ng/ml NRG-1β), or for 30 min with 15 μM LY294002 before the 4hr's treatment with 50 ng/ml NRG-1β.

2.3. Immunoblot analysis

Cells were lysed with NP-40 lysis buffer containing 20 mM Tris-HCl pH 7.4, 150 mM NaCl, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 1 mM phenylmethylsulphonyl fluoride, 1 mM orthovanadate and 20 mM NaF.

Cell lysates were sonicated and centrifuged for 10 min at 13,000 rpm at 4 °C. Protein concentration was determined using Bio-Rad DC Protein Assay (Bio-Rad Laboratories). Equal amounts of protein were mixed with 4X Laemmli sample buffer (250 mM Tris-HCl pH 6.8, 8% SDS, 40% glycerol, 20% β-mercaptoethanol, 4 mM EDTA, 0.08% bromophenol blue), boiled for 5 min and resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE; 7.5% polyacrylamide). Proteins were electrophoretically transferred to polyvinylidene difluoride membranes (PVDF) (Millipore). Membranes were blocked with 1X Western Blot Reagent (Roche Diagnostics) for 1 h at room temperature, washed three times with T-TBS (10 mM tris-HCl pH 7.4, 150 mM NaCl, 0.1% Tween), probed with primary antibody, washed three times with T-TBS, probed with goat anti-rabbit- or goat anti-mouse-horseradish peroxidase conjugated (Millipore) and washed three times with T-TBS. Proteins were visualized by chemiluminescence with Visualizer™ WB Detection kit (Millipore) using the Bio-Rad Quantity One ChemiDoc (Bio-Rad). Membranes for phospho-MAPK detection were incubated overnight at 4C with Phoshpo-p44/42 MAPK antibody 1:2000 dilution. All other antibodies were diluted 1:1000 dilution.

2.4. Isolation of RNA and RT-qPCR analysis

Total RNA was isolated using RNeasy Mini Kit (Qiagen), followed by DNase I treatment (DNAfree, Ambion) according to the manufacturer's instructions. RNA was quantified by UV spectrophotometry (UV Du® 530 DNA/Protein Analyzer, Beckman) at 260 nm or in a Nanodrop 8000. RNA purity was confirmed at a 260/280 nm, ratio above 1.8. RNA quality and integrity was assessed by 1% agarose gel electrophoresis. Reverse transcription and amplification were performed using the TaqMan One-step Real Time PCR (Applied Biosystems) and the iCycler iQ™ Real-Time Detection System Thermal Cycler (Bio-Rad). Reaction mix contained 1X TaqMan Buffer, MgCl$_2$, dNTPs, AmpliTaq Gold DNA Polymerase, 0.25 U/μl Multiscribe Reverse Transcriptase, 0.4 U/μl RNAse Inhibitor, 250 nM forward and reverse primers, 200 nM probe and 100 ng of total RNA. RT-qPCR reactions were performed in duplicate 25 μl mixtures as follows: a single cycle of 30 min at 48 °C (reverse transcription) and 10 min at 95 °C, 40 cycles of 15 s at 95 °C (denaturation), 30 s at 48 °C (annealing) and 60 s at 60 °C (extension). Real-Time quantification was performed using the Bio-Rad iCycler iQ System Software Version 3.0a (Bio-Rad). EGFR isoform A forward primer: 5′-ACCAGTCCGTTCCCAAAAGG-3′; reverse primer: 5′-AGGCTGATTGT-GATAGACAGGATTC-3′; and probe: FAM5′-CGCTGGCTCTGTGC-3′ (Eclipse Dark Quencher). Predicted EGFR isoform A RT-qPCR product size is 62bp. isoform D's forward primer: 5′-CAGGAGTCATGCTTAG-GATGGAT-3′; reverse primer: 5′-GCCGTGATGATCACAGATGC-3′; and probe: FAM5′-CTTCTCTTCTGCCGTCAGAGTTTCAGC-3′ (Eclipse Dark Quencher). Predicted isoform D RT-qPCR product size is 113 bp. TATA-binding protein (TBP) forward primer: 5′-CACGAACCACGGCACTGATT-3′, reverse primer: 5′-TTTTCTTGCTGCCAGTC TGGAC-3′ and probe: FAM5′-TGTGCACAGGAGCCAAGAGTGAAGA-3′ Eclipse Dark Quencher. TBP RT-qPCR product size is 89 bp [39]. The size of the RT-qPCR products was confirmed by 4% agarose gel electrophoresis. EGFR isoform A primers and probes are directed to the intracellular domain; therefore these primers do not recognize EGFR isoform D. EGFR isoform D primers amplified a sequence within the region that encodes the unique 78 amino acids. Sequences of EGFR isoform A and isoform D were obtained from GenBank accession numbers AF288738 and NM-201284, respectively. Primers and probes were designed using Primer Express® Software Version 2.0 (Applied Biosystems) and Net-Primer (Premier Biosoft International) programs and prepared by Eurogentec North America. TBP expression is constitutive and was used as a control to normalize levels of expression of EGFR isoform A and D. The levels of expression of EGFR isoform A and D were determined and compared between treated vs. untreated cells. mRNA expression levels were calculated using the ΔΔCt method where Ct is the value at which amplification is first observed [40]. The calculations used were as follows: 1) $\Delta Ct = Ct_{sample} - Ct_{TBP}$, 2) $\Delta\Delta Ct = \Delta Ct_{treated} - \Delta Ct_{untreated}$, 3) $2-\Delta\Delta Ct$ = fold change in gene expression.

2.5. Determination of the half-life of isoform D

MDA-MB-468 cells were incubated with 1 μg/ml actinomycin D (or 0.1% DMSO) for 0, 0.5, 1, 2, 4, 6 and 8 h. Alternatively, MDA-MB-468 cells were incubated with 60 ng/ml EGF (or vehicle alone, 8 μM acetic acid) or 500 nM PMA (or vehicle alone, 0.1% EtOH) for 4 h followed by incubation with 1 μg/ml actinomycin D (or vehicle alone, 0.1% DMSO) for 0, 0.5, 1, 2, 4, and 8 h. Total RNA was isolated and analyzed by RT-qPCR as previously described. To determine total copy number, a calibration curve was prepared using a quantified amount of EGFR isoform D pcDNA plasmid in a 10-fold serial dilution ranging from 10^2 -10^8 final copy number. After RT-qPCR analysis, EGFR isoform D transcript levels were compared between actinomycin D treated cells vs. cells treated with vehicle alone. Data were fitted to a one phase exponential decay equation to calculate the half-lives.

2.6. Statistical analysis

Data were analyzed with GraphPad Prism version 8 (GraphPad Software Inc). Values were expressed as mean (±SE), and experiments were replicated at least three times. For the inhibitor studies, data were evaluated using paired *t*-test. For the kinetic studies, data were evaluated using one-way ANOVA and Tukey's multiple comparison test was also used to test the fold-inductions of NRG-1 β-treated cells. $p < 0.05$ was considered statistically significant.

3. Results

3.1. EGF and PMA increase expression of the transcripts encoding EGFR isoform A and D in MDA-MB-468 and MCF-7 cells

EGF and phorbol esters, such as PMA, have been shown to increase EGFR mRNA and protein levels in various cell lines including MDA-MB-468 [23,25–28,30,31]. To determine if EGF or PMA also affects the expression of EGFR isoform D in breast cancer cells, MDA-MB-468 and MCF-7 were incubated with EGF or PMA for various time periods before their RNA were harvested. RT-qPCR analysis showed that EGF treatment resulted in a 4.4-fold increase in EGFR isoform D levels after 4 h of incubation in MDA-MB-468, and a 3.9-fold increase in MCF-7 cells after 2 h of incubation (Fig. 2a and b). PMA treatment resulted in a 6.6-fold induction in EGFR isoform D transcript levels after 6 h of incubation in MDA-MB-468 cells, and a 27-fold induction in MCF-7 cells after 4 h of incubation (Fig. 2c and d). As previously reported, we also confirmed that EGF and PMA effectively increased the expression of EGFR isoform A in both cell lines (Fig. 2a through d).

3.2. NRG-1β increases expression of EGFR isoform A and D transcripts in MCF-7 but not MDA-MB-468 cells

Previous reports have shown that two EGF family ligands, EGF and TGF-α, increase EGFR levels in various cell lines including breast cancer-derived cells [23,26,27]. To investigate the effects of neuregulin (NRG-1β) on the expression of EGFR isoform D in breast cancer cell lines, MDA-MB-468 and MCF7 were activated by NRG-1β and their isoform D expression were examined by RT-qPCR. 50 ng/ml of NRG-1β increased both EGFR isoform A and D transcript levels by 9.7 and 7.7-fold respectively in MCF-7 cells (Fig. 2f), while it had any significant effects on neither isoform A nor isoform D transcript expression in MDA-MB-468 cells (Fig. 2e). These findings prompted us to analyze the effect of NRG-1β on EGFR isoform A and D expression in additional breast cancer cell lines (Fig. 3). NRG-1β increased EGFR isoform A transcript levels by approximately 8.6-fold and EGFR isoform D transcript levels by 2.7-fold in ZR-75-1 cells. On the other hand, the HER3
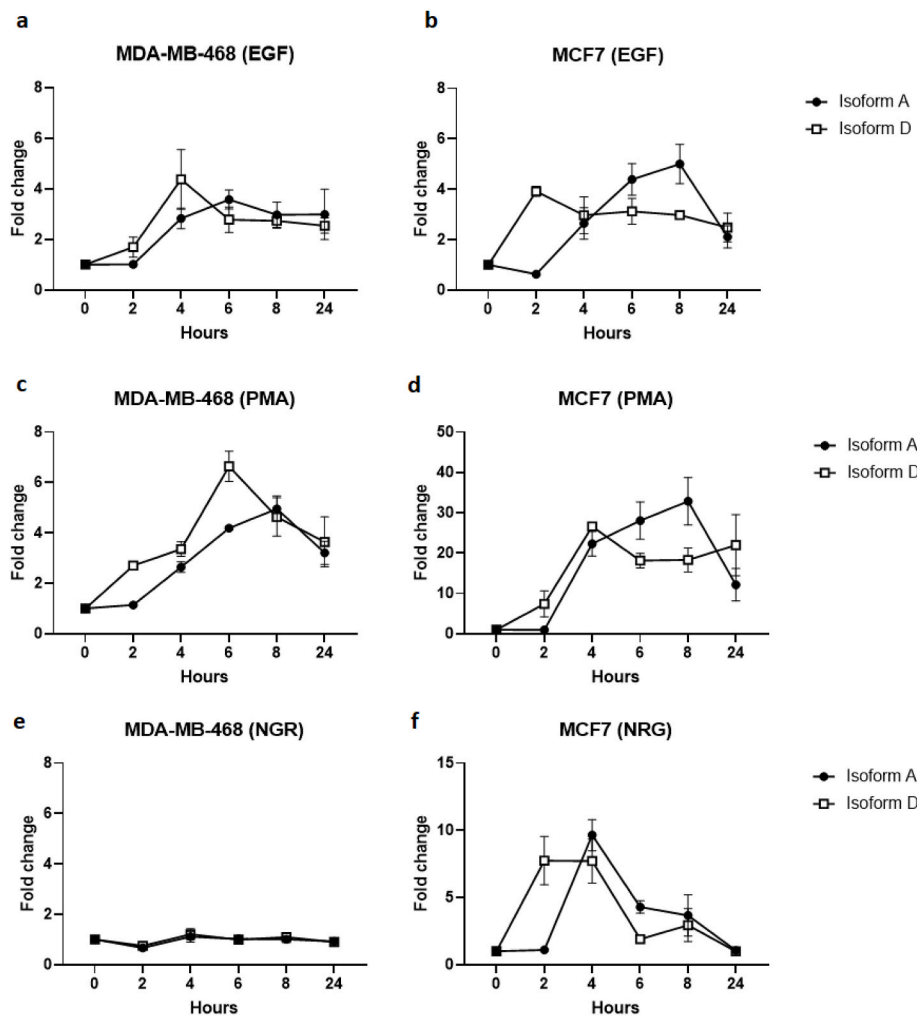
Fig. 2. The effect of ErbB receptor activators on expression of EGFR isoform A and D mRNA.

EGF and PMA increase the expression of the isoform D transcript in both MDA-MB-468 and MCF-7 cells while MDA-MB-468 cells do not respond to Neuregulin-1β. Serum-starved cells were incubated with 60 ng/ml EGF, 500 nM PMA, 50 ng/ml NRG-1β or vehicle alone for the indicated times before RT-qPCR was performed. Fold-induction of EGFR isoform A and D transcripts was expressed as ratios to the vehicle group. Data are represented as Mean ± SE.
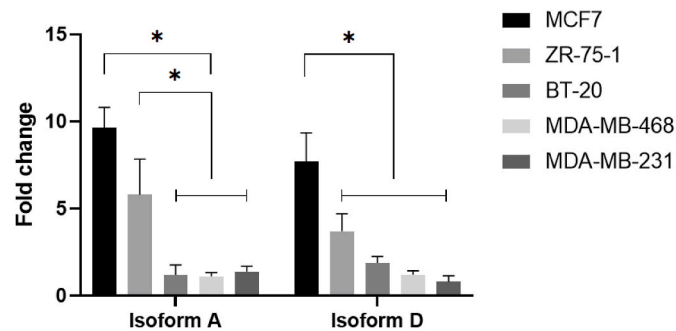

Fig. 3. Effect of Neuregulin 1β on the expression of EGFR isoform A and D in breast cancer-derived cell lines

NRG-1β significantly induced EGFR isoform A and D in ER-positive MCF7. RNA were collected 4 h after the cells were treated with 50 ng/ml NRG-1β. RT-qPCR was performed as described in the Method section. Data are represented as mean ± SE, ANOVA followed by a Tuckey test was used for statistical analysis, *: p < 0.05.

ligand modestly increased EGFR isoform A transcript levels (2.2-fold), and isoform D transcript levels (2.0-fold) in BT-20 cells. Similar to our findings in MDA-MB-468 cells, NRG-1β did show no effect on either EGFR isoform A or D transcript expression in MDA-MB-231 cells.

3.3. MAPK pathway is involved in the expression of EGFR isoform D

EGF binding to EGFR activates several signaling cascades including the MAPK pathway [1]. To test if MAPK signaling is required for EGFR isoform D expression, MDA-MB-468 and MCF-7 cells were pre-incubated with the MAPK inhibitor U0126 prior to EGF treatment (Fig. 4). U0126 decreased EGF-induced expression of EGFR isoform A by 61% in MDA-MB-468 cells (Fig. 4a) and by 78% in MCF-7 cells (Fig. 4b). Similarly, EGF-induced expression of EGFR isoform D was decreased by 66% in both cell lines by U0126. The EGFR isoform A and D expression were also correlated with increase in phosphorylation of MAPK in both cell lines (Fig. 4c and d). Together, these findings suggest that MAPK signaling is involved in EGF-induced expression of EGFR isoform D.

3.4. Inhibition of PKC decreases the expression of EGFR isoform D

Along with MAPK pathways, PKC has been reported to mediate PMA-induced the synthesis of EGFR [25,26]. The involvement of PKC in the expression of EGFR isoform D was also examined in MDA-MB-468 and MCF-7 cells treated with a PKC inhibitor Gö6983 and PMA, a PKC activator. RT-qPCR analyses demonstrate that Gö6983 decreased PMA-induced expression of EGFR isoform A by 76% in MDA-MB-468 cells, and by 64% in MCF-7 cells while EGFR isoform D was decreased by the MAPK inhibitor by 61% in MDA-MB-468 cells and by 68% in MCF-7 cells (Fig. 5a and b). The PKC activities in the treated cells were confirmed with phosphorylation of MAPK as shown in Fig. 5c and d. These results suggest that the expression EGFR isoform D is also
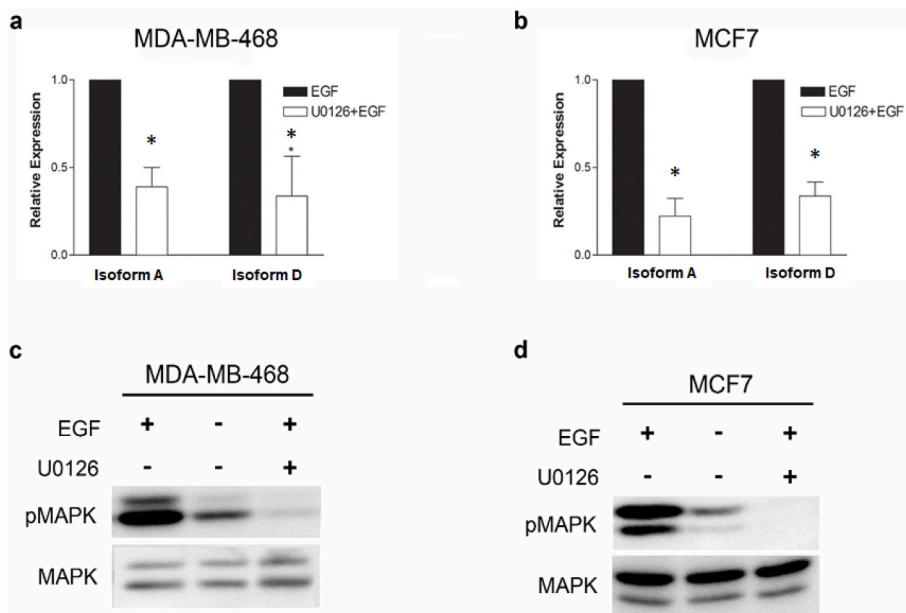
Fig. 4. The MAPK inhibitor U0126 inhibits EGF-induced expression of EGFR isoform A and D in MDA-MB-468 and MCF-7 cells.

Cells were serum-starved overnight, followed by incubation (45 min) with 20 or 50 μM inhibitor U0126, or with vehicle alone at 37 °C. Cells were incubated for 4 h with 60 ng/ml EGF or vehicle alone. Total RNA was isolated and analyzed by RT-qPCR. In a and b, relative EGFR isoform A and D transcript expression was measured in cells treated with EGF and inhibitor U0126 relative to cells treated with EGF alone. In c and d, total protein in cell lysates from MDA-MB-468 or MCF-7 cells was resolved using SDS-PAGE followed by immunoblot analysis using antibodies specific for either phospho-MAPK or total MAPK. Data are represented as mean ± SD. *: $p < 0.05$.
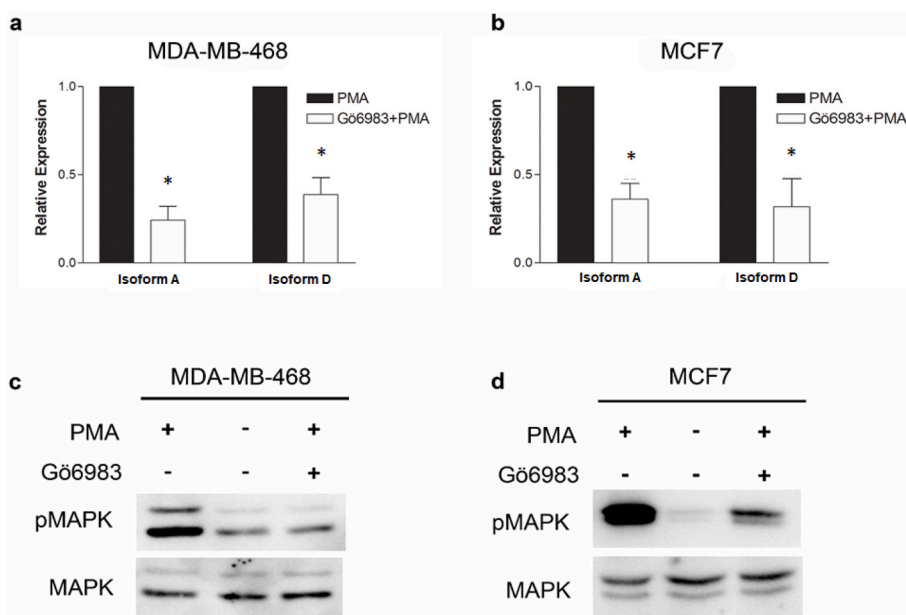

Fig. 5. The PKC inhibitor Gö6983 inhibits PMA-induced expression of EGFR isoform A and D in MDA-MB-468 and MCF-7 cells.

MDA-MB-468 and MCF-7 cells were serum-starved overnight. Then cells were incubated for 30 min with 1 μM inhibitor Gö6983 or with vehicle alone followed by incubation for 4 h with 500 nM PMA or vehicle at 37 °C. Total RNA was isolated and analyzed by RT-qPCR. In a and b, relative expression of EGFR isoform A and D was determined in cells treated with PMA and inhibitor Gö6983 vs cells treated with PMA alone. In c and d, total protein in cell lysates from MDA-MB-468 or MCF-7 cells was resolved by SDS-PAGE followed by immunoblot analysis using antibodies specific for either phospho-MAPK or total MAPK. Data are represented as mean (±SD). *: $p < 0.05$.

mediated by PKC-MAPK pathway.

3.5. NRG-1β increases EGFR isoform D via a MAPK-dependent but phosphoinositide 3-kinase/Akt-independent mechanism

Since it has been shown that NRG-1β activates both the phosphoinositide 3-kinase (PI3K) and MAPK pathways in MCF-7 cells [41], we chose to examine the potential role of MAPK in NRG-1β regulation of EGFR isoform A and D mRNA expression (Fig. 6a and c). As predicted, NRG-1β indeed activates the MAPK-pathway in MCF7. Pre-incubation with the MAPK inhibitor U0126 decreased the NRG-1β-induced expression of EGFR isoform A and D by 74% and 64%, respectively (Fig. 5a). U0126 abolishes NRG-1β-induced phosphorylation of MAPK (Fig. 5c). However, interestingly, the enhanced expression of EGFR isoform A and D by NRG-1β were not altered, but rather increased by a PI3K inhibitor, LY294002 (Fig. 6b). As shown in Fig. 6d, LY294002

effectively inhibited the Akt phosphorylation by NRG-1β while significantly increased phosho-MAPK possibly through a reciprocal crosstalk between extracellular signal-regulated kinases (ERK) and PI3K [42–44]. Thus, the decrease in phosph-Akt was completely compensated by the hike of phosho-MAPK in the MCF7, leading to such increase in EGFR isoform D expression as well as isoform A as shown in Fig. 6b. Together, these results suggest that NRG-1β-induced expression of both EGFR isoform A and D transcripts in MCF-7 cells is mediated via a MAPK-dependent pathway but not PI3K-Akt pathway.

3.6. EGF and PMA *does* not post-transcriptionally regulate EGFR isoform D

To examine the effect of EGF and PMA on stability of the EGFR isoform D transcript, we determined the half-life of the isoform D in MDA-MB-468 cells. The levels of EGFR isoform D transcripts were
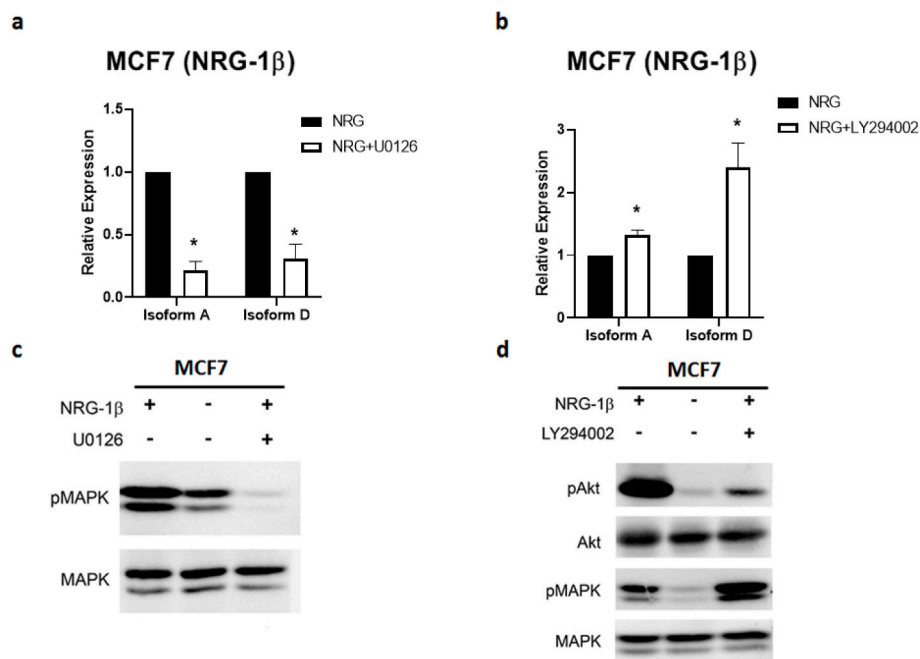
Fig. 6. LY294002 enhances NRG-1β-induced expression of EGFR isoform A and D transcripts, while the MAPK inhibitor inhibits both isoforms in MCF-7 cells. In a and b, relative expression of EGFR isoform A and D in cells treated with NRG-1β and MAPK inhibitor U0126 or PI3K inhibitor LY294002. Cells serum-starved overnight were incubated for 45 min with each inhibitor or vehicle alone followed by treatment with 50 ng/ml NRG-1β or vehicle alone for 4 h at 37 °C. In c and d, total protein in cell lysates was resolved using SDS-PAGE followed by immunoblot analysis using antibodies specific for phospho-Akt, total-Akt, phospho-MAPK or total-MAPK. Data are represented as mean ± SD. *: p < 0.05.

determined in actinomycin D treated cells relative to cells treated with vehicle alone. Our results show that the basal half-life of the isoform D transcript was 0.83 h in MDA-MB-468 cells (Fig. 7) and the half-lives were not significantly changed when the cells were treated with EGF (0.89 h) or PMA (0.78 h) (Fig. 7). These results demonstrate that neither EGF nor PMA does not affect the stability of EGFR isoform D transcripts.

4. Discussion

In this study, we demonstrate the regulation of expression of an alternately spliced EGFR transcript [7] that encodes an emerging serum biomarker, designated EGFR isoform D [45]. A series of studies with epithelial ovarian cancer patients has shown that women with low isoform D serum concentrations have a higher risk of disease when compared to women with normal serum levels of isoform D [46,47].
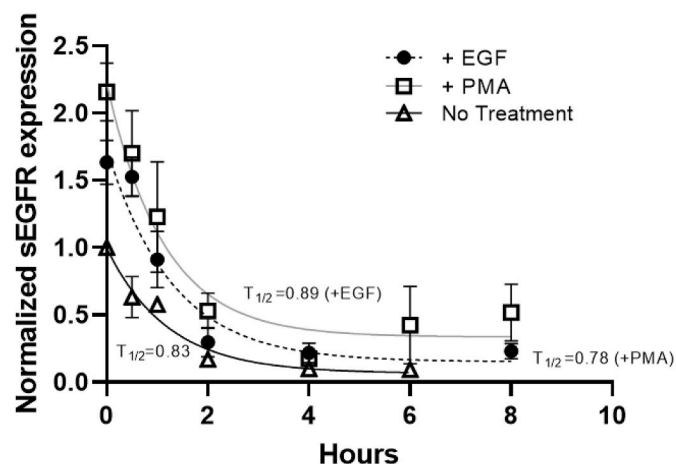

Fig. 7. Half-life of EGFR isoform D in MDA-MB-468
Cells were treated with 1 μg/ml Actinomycin D or 0.1% DMSO. Total RNA was isolated at different time points, and 100 ng samples were analyzed by RT-qPCR. Copy number was determined using a calibration curve ranging from 10^2 to 10^8 copies of isoform D. isoform D copy numbers from cells treated with actinomycin D relative to cells treated with vehicle alone were fitted using one-phase exponential decay equation.

Also, in hormonally responsive endometrial cancer, serum EGFR isoform D concentrations have been correlated with overall survival in patients treated with the EGFR-directed kinase inhibitor, gefitinib [48]. On the other hand, a study from the Women's Health Initiative have shown that serum EGFR was the only biomarker that can prospectively predict breast cancer up to 17 months prior to diagnosis of the cancer [13], while serum EGFR isoform D is decreased in 76% of postmenopausal breast cancer patients treated with letrozole, an aromatase inhibitor, when compared to pretreatment samples [49]. In addition, more recently, there have been growing numbers of studies reporting the relationship of EGFR isoform D with head and neck squamous cell carcinomas [50–52]. Given both the diagnostic and theragnostic potential of EGFR isoform D in cancer patients [45], we investigated the mechanisms governing the synthesis of EGFR isoform D vs. isoform A (full-length receptor isoform). Since EGFR isoform A and D share identical promoters and transcription start sites, it has been hypothesized that the mechanisms governing the expression of these two isoforms may be similar. Therefore, we have tested this hypothesis using several breast cancer cell lines and the findings are summarized as follows.

First, the expression of both EGFR isoform A and D in breast cancer cells is stimulated by EGF, PMA and NRG-1β. Similar to previous studies showing that EGF and PMA increase both EGFR isoform A mRNA and protein levels in various cell lines [25–28,31], we also have observed that both EGF and PMA induces EGFR isoform A transcription (Fig. 2). And in support of our hypothesis, elevation of EGFR isoform D mRNA was also observed following EGF or PMA treatment of both MDA-MB-468 and MCF-7 cells even though basal expression of EGFR in MDA-MB-468 is higher than EGFR expression in MCF-7 [6,53,54]. In contrast, treatment with NRG-1β demonstrates a somewhat more complex result; NRG-1β significantly increases both EGFR isoform A and D expression in MCF-7 while there was no substantial change observed in MDA-MB-468. Given that both cell lines express similar levels of HER2 and HER3 [53], we speculate that the differential activities of NRG-1β in these two cell lines may reflect their 'estrogen receptor (ER)' levels. It is well documented that ER expression is inversely associated with both NRG-1β and EGFR expression in breast cancer cell lines [2,53] and in breast tumors [11,55]. Also, ER-positive breast cancer cells, in general, express lower levels of EGFR while ER-negative cells express high levels of EGFR. Thus, it is possible that the lack (or low level) of NRG-1β expression in the ER positive cell lines may make them more sensitive to

exogenous NRG-1β. Furthermore, there is cross talk between ER and HER2. Shou et al. have reported that overexpression of HER2 in MCF7 increases the phosphorylation of ER and activation of downstream signaling such as MAPK leading to resistance to tamoxifen [56]. In addition, it has been reported that hyperactive HER2 may reduce ER expression but result in phosphorylation and potentiation of the transcriptional activity of ER [57]. Consistent with these reports, our results also suggest that ER-positive MCF7 cells respond to NRG-β1 resulting in increased expressed of both EGFR isoforms A and D, while MDA-MB-468, an ER-negative cell line, poorly expresses either receptor isoform (Fig. 2e and f). We have further confirmed these observations with additional breast cancer cell lines which express low levels of ER, such as MDA-MB-231, BT-20 and ZR-75-1. In these follow-up studies, NRG-β1 does not substantially induce either EGFR isoform A or D expression, except in ZR-75-1 in which only EGFR isoform A is induced by NRG-β1 treatment (Fig. 3). Speculation that estrogen may be involved in isoform D expression is consonant with at least one previous report demonstrating the effect of letrozole, an aromatase inhibitor, on circulating EGFR isoform D concentrations in metastatic breast cancer patients [49].

Second, we suggest that both EGFR isoform A and D expression are regulated by common pathways, MAPK and PKC. As is known, MAPK, PKC and PI3K/Akt signaling pathways are the primary mechanisms utilized for signal transduction by EGFR/HER receptors [1,58,59], and the EGFR activators, EGF and PMA, and NRG-1β, HER3/4 agonists, also signal through all three pathways [59,60]. In this study, we show that both EGFR isoform A and D expression are activated by NRG-1β through MAPK or PKC signaling, while the PI3K-Akt pathway has no effect. Pharmacological inhibition of either MAPK or PKC successfully suppressed both EGFR isoform A and D induction by these HER receptor activators (Figs. 4–6). Paradoxically, however, treatment with a PI3K inhibitor further increased the EGFR isoform A and D expression induced by NRG-1β (Fig. 6). This unexpected outcome may come from unusual but reciprocal cross talk between the MAPK and PI3K pathways. In support of this concept, one study has shown that LY294002 can augment NRG-1β-induced MMP1 expression in breast cancer cell lines through ERK1/2 activation [43]. In addition, knockdown of PI3K has been associated with activation of MAPK in rhabdomyosarcomas [44]. Through reciprocal interactions between these two pathways, the inhibition of PI3K/Akt pathway may further enhance MAPK phosphorylation to compensate for the loss of NRG-1β signaling, leading to increases in the expression of EGFR isoforms A and D. Based on these observations and speculation, we conclude that MAPK and PKC are the primary mechanisms governing expression of both the EGFR isoform A and D transcripts. We summarized our findings and hypothesis in Fig. 8.

It is notable, however, that post-transcriptionally, EGFR isoform D transcripts respond to EGF in a different way than do isoform A transcripts. Multiple studies have shown that EGF enhances EGFR mRNA levels by increasing EGFR mRNA stability as well as by up-regulating EGFR transcription by more than two-fold in various cell types including breast cancer-derived cell lines such as MDA-MB-468 and BT-20 [24,27,29]. However, our preliminary data using MDA-MB-468 cells suggest that the basal half-life of the EGFR isoform D transcript is much shorter (0.83 h) than that previously observed for isoform A in this cell line (~6.5 h) [23], and that neither EGF nor PMA affects the basal half-life of the isoform D transcript (Fig. 7). Given the observation that EGF increases the stability of EGFR isoform A mRNA in MDA-MB-468 cells [23], these results suggest that isoform D transcripts may be under different mechanisms of post-transcriptional regulation from those observed for EGFR isoform A. One possible explanation for these half-life differences is the difference between the 3′ untranslated (3′-UTR) sequences of these two isoforms. While EGFR isoform D shares identity in sequence through its first 15 exons with the EGFR transcript, the unique 3′-UTR of isoform D is encoded by an alternate exon (15B), and hence shares no sequence homology with the 3′ UTR of EGFR isoform A [7]. Since the 3′UTR region of EGFR isoform A has been shown to
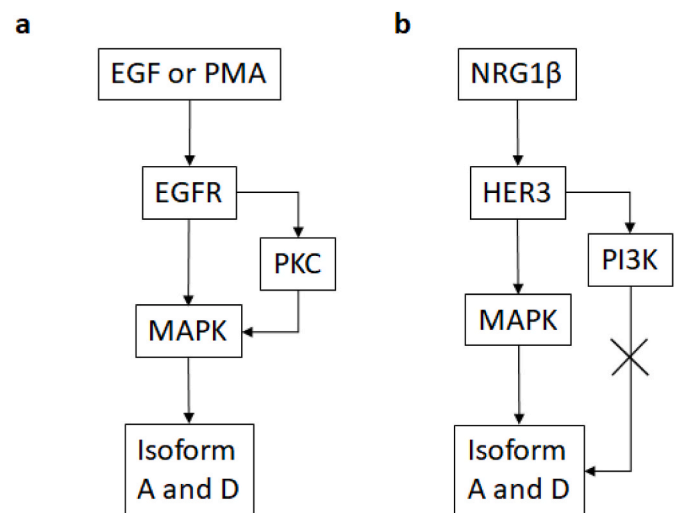
Fig. 8. Diagram summarizing regulation of expression of EGFR Isoforms A and D

a. MAPK and PKC medicates the expression of isoform A and D by EGF or PMA.
b. NRG1β induce isoform A and D expression through MAPK pathway.

play a critical role in the regulation of EGFR mRNA stability [23,32], perhaps the 3′ UTR of isoform D similarly plays a role in regulating the half-life of this alternate transcript. These observations may also provide a potential mechanistic basis for the differential expression of isoform D vs isoform A observed in certain rodent tissues, such as the exclusive expression of isoform D in the luminal epithelium of the mouse pre-implantation uterus [61], as well as in the supra-ameloblast cells of the enamel pre-secretion stage of the developing tooth [62].

5. Conclusion

We show that regulation of EGFR isoform D expression, when compared to the regulation of isoform A expression, shares similar mechanisms of transcriptional regulation, but exhibits apparently distinct, and as yet undefined, mechanisms of post-transcriptional regulation. Validation of these results in human tissues as well as their relevance to EGFR/HER targeted cancer therapeutics and diagnostics will become possible, once isoform-specific immuno-reagents suitable for human tissue analysis become available.

Ethical standards

The authors declare that the experiments described in this manuscript comply with the current laws of Puerto Rico.

CRediT authorship contribution statement

Lisandra Negrón-Vega: Conceptualization, Methodology, Investigation, Funding acquisition, Writing – original draft. **Elsa M. Cora:** Conceptualization, Methodology, Investigation, Funding acquisition, Writing – original draft. **Marianela Pérez-Torres:** Conceptualization, Methodology, Investigation, Funding acquisition, Writing – original draft. **Shou-Ching Tang:** Writing – review & editing. **Nita J. Maihle:** Conceptualization, Investigation, Funding acquisition, Writing – review & editing. **Jung Su Ryu:** Funding acquisition, Investigation, Writing – review & editing.

Declaration of competing interest

The authors have no conflict of interest to declare.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2022.101326.

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