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Altered EGFR Localization and Degradation in Human Breast Cancer Cells with an Amphiregulin/EGFR Autocrine Loop

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Abstract

The epidermal growth factor receptor (EGFR) and its ligand amphiregulin (AR) have been shown to be co-over expressed in breast cancer. We have previously shown that an AR/EGFR autocrine loop is required for SUM149 human breast cancer cell proliferation, motility and invasion. We also demonstrated that AR can induce these altered phenotypes when expressed in the normal mammary epithelial cell line MCF10A, or by exposure of these cells to AR in the medium. In the present studies, we demonstrate that SUM149 cells and immortalized human mammary epithelial MCF10A cells that over express AR (MCF10A AR) or are cultured in the presence of exogenous AR, express higher levels of EGFR protein than MCF10A cells cultured in EGF. Pulse chase analysis showed that EGFR protein remained stable in the presence of AR, yet was degraded in the presence of EGF. Consistent with this observation, tyrosine 1045 on the EGFR, the c-cbl binding site, exhibited less phosphorylation following stimulation with AR than following stimulation with EGF. Ubiquitination of the receptor was also dramatically less following stimulation with AR than following stimulation with EGF. Flow cytometry analysis showed that EGFR remained on the cell surface following stimulation with AR but was rapidly internalized following stimulation with EGF. Immunofluorescence and confocal microscopy confirmed the flow cytometry results. EGFR in MCF10A cells cultured in the presence of EGF exhibited a predominantly intracellular, punctate localization. In stark contrast, SUM149 cells and MCF10A cells growing in the presence of AR expressed EGFR predominantly on the membrane and at cell-cell junctions. We propose that AR alters EGFR internalization and degradation in a way that favors accumulation of EGFR at the cell surface and ultimately leads to changes in EGFR signaling.

Keywords

Amphiregulin; EGFR; degradation; localization; breast cancer

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Introduction

The epidermal growth factor receptor (EGFR) is a transmembrane receptor tyrosine kinase [1]. The seven different ligands that can bind the EGFR are epidermal growth factor (EGF), transforming growth factor alpha (TGF- α), amphiregulin (AR), heparin binding EGF (HB-EGF), betacellulin (BTC), epiregulin (EPR), and epigen [2–8]. Once ligand binds, the receptor dimerizes and becomes activated, leading to propagation of downstream signals [9]. These downstream signals are involved in a number of normal cellular processes such as proliferation, differentiation, migration, and survival. Given the pleiotropic effects of EGFR signaling, the duration and strength of EGFR signals must be tightly controlled as dysregulated EGFR signaling has been demonstrated to contribute to tumorigenesis [10].

Modulation of receptor trafficking and degradation are essential for proper EGFR signaling. It has been shown in several cell types that EGFR is rapidly internalized and degraded following stimulation with EGF [11–13]. The E3 ubiquitin ligase responsible for targeting EGFR for degradation is c-Cbl. c-Cbl contains a phosphotyrosine binding domain at its N-terminus as well as a C3HC4 RING finger [14] and binds EGFR directly via the phosphorylated EGFR Tyr1045 (Y1045) residue or indirectly via Grb2 [15–17]. However, at high but physiological concentrations of EGF, the former interaction is predominant [16,18–20]. c-Cbl recruits ubiquitin conjugating enzymes to transfer ubiquitin on to the receptor via its RING finger domain. In this manner, c-Cbl positively regulates clathrin-dependent endocytosis of EGFR [21]. Ubiquitination of EGFR by c-Cbl is required for directing the EGFR to late endosomes and for subsequent lysosomal degradation [12]. Indeed, over expression of c-Cbl significantly increases EGF-induced EGFR degradation [11]. However, if EGFR is not targeted for ubiquitination and degradation by c-Cbl, it is recycled back to the surface via recycling endosomes where it can be activated again [16]. Therefore, c-Cbl mediated ubiquitination plays a critical role in the regulation of EGFR signaling.

The majority of EGFR trafficking studies have been performed using EGF as the ligand [11, 12,22]. Therefore, much of what we know is based on how EGF interacts with EGFR and regulates its recycling and degradation. However, the diversity of EGF ligands can lead to a unique interaction with the receptor which affects intracellular trafficking of the ligand/EGFR complex [13,23]. In one study, EGF was found to bind tightly to the receptor, resulting in EGFR degradation. In contrast, TGF- α was found to bind less tightly and consequently it completely dissociated at the lower pH of the endosomal compartment which lead to receptor recycling. Single amino acid substitutions in EGF decreased its binding to the receptor and as a result, decreased the degradation rate of EGFR compared with EGFR activated by wild type EGF [13]. Thus, different ligands can have diverse effects on the regulation of EGFR trafficking.

We have previously shown that an AR/EGFR autocrine loop is functioning in the SUM149 human breast cancer cells and is required for their proliferation, motility and invasive capacity [24]. SUM149 cells over express EGFR without gene amplification and the EGFR in these cells is constitutively activated [25]. EGFR and AR have been found to be co-over expressed in aggressive breast cancers [26]. Thus, it is possible that AR is altering EGFR trafficking, which could ultimately affect EGFR expression and signaling in breast cancer cells. Therefore, we have investigated degradation of the EGFR and its localization following stimulation with AR.

Experimental Procedures

Cell Culture

The MCF10A cell line engineered to over express AR (MCF10A AR) was made as previously described [27]. SUM149 cells were maintained in Ham's F-12 with 5% fetal bovine serum (FBS), 5 µg/ml insulin, 2 µg/ml hydrocortisone, 5 µg/ml gentamicin and 2.5 µg/ml fungizone. The serum-free base medium for MCF10A and MCF10A AR cells was SFIH: Ham's F-12, with 1 µg/ml hydrocortisone, 1 mg/ml bovine serum albumin, 10 mM N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid], 5 mM ethanolamine, 5 µg/ml transferrin, 10 nM triiodothyronine, 50 nM sodium selenate, 5 µg/ml gentamicin 2.5 µg/ml fungizone, and 5 µg/ml insulin. The medium for control MCF10A cells also included 10 ng/ml EGF (1.7 nM) from Sigma (SFIHE). MCF10A + AR cells were MCF10A cells grown in SFIH media with 20 ng/ml (1.8 nM) of exogenous AR from R&D Systems (SFIHA), which was found previously to be the biologically equivalent concentration to EGF for these cells [27].

32D myeloid cell lines expressing wild type EGFR under the transcriptional control of the recombinant retroviral expression vector pLXSN (LXSN-WT-EGFR), the EGFR Y1045F mutant (LXSN-Y1045F) or the vector control (LXSN) were established and maintained essentially as described [28]. All cells were maintained in a humidified incubator at 37°C and 10% CO₂.

Required Antibodies

The antibodies used in these studies were: mouse monoclonal anti-EGFR antibody Ab-5 (Oncogene) to immunoprecipitate EGFR for Y1045 and ubiquitin experiments, anti-EGFR antibody MAB108 from a mouse hybridoma (gift from Julie Boerner, Karmanos Cancer Institute) for detecting EGFR in immunofluorescence experiments and for immunoprecipitation of EGFR in the Pulse-Chase experiments, mouse monoclonal anti-EGFR antibody clone 31G7 (Zymed Laboratories, Inc.) to detect EGFR on western blots, rabbit antibody Y1045 (Cell Signaling) to detect EGFR tyrosine phosphorylation at site 1045, and rabbit anti-ubiquitin antibody (Cell Signaling) to detect ubiquitinated EGFR. The anti-phosphotyrosine antibody PY-20 (Calbiochem) was used to detect EGFR tyrosine phosphorylation.

Immunoprecipitation and Immunoblotting

The antibody anti-EGFR Ab-5 (Oncogene Research Products) was used for immunoprecipitation of EGFR for Y1045 and ubiquitin experiments. Immunoprecipitation and western blotting was performed as previously described [29]. Cells were lysed in a buffer containing 20 mM Tris-HCl (pH 8.0), 137 mM NaCl, 1% NP40, 10% glycerol, 1 mM Na₃VO₄, 1 mM phenylmethylsulfonyl fluoride, 1% aprotinin, and 20 µg/ml leupeptin. Protein concentrations were equalized using the Bradford method. For whole cell lysates, Laemmli sample buffer was added and the samples were boiled. For immunoprecipitations, 1 µg of antibody was added to 1 mg of sample and incubated at 4°C for 1 h. Immune complexes were then bound to protein A/G beads for 1 h at 4°C. Immunoprecipitates were washed three times in lysis buffer. Laemmli sample buffer was added and the samples were boiled. EGFR immunoprecipitates were loaded onto 7.5% SDS-polyacrylamide gels. After transferring proteins to PVDF membranes, blots were probed with anti-EGFR antibody 31G7 (Zymed Laboratories), anti-phosphotyrosine antibody Y1045 (Cell Signaling), or anti-ubiquitin antibody (Cell Signaling) and visualized by ECL (Pierce Laboratories).

Pulse-Chase Analysis

MCF10A cells were cultured in SF1H media with either 10 ng/ml EGF or 20 ng/ml AR for 7 days. The media was changed to methionine and cysteine free DMEM supplemented with 1 µg/ml hydrocortisone, 1 mg/ml bovine serum albumin, 10 mM N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid], 5 mM ethanolamine, 5 µg/ml transferrin, 10 nM triiodothyronine, 50 nM sodium selenate, 5 µg/ml gentamicin 2.5 µg/ml fungizone, and 5 µg/ml insulin for 1 hour. 100 µCi/ml ³⁵S-pro-mix (Amersham) was added to the plates for two hours and then washed away and replaced with media containing 200-fold excess unlabeled methionine and cysteine for 4, 6, 8, or 10 hours to chase away labeled EGFR. The cells were lysed and EGFR was immunoprecipitated and run on a 7.5% gel. The gel was dried and exposed to film for 5 days.

Flow Cytometry

MCF10A cells were deprived of EGF for 24 hours. The cells were harvested by trypsinization and then stimulated with equimolar concentrations (16nM) of either EGF or AR for 15, 30, 60, or 90 minutes and fixed with 4% paraformaldehyde in PBS. After fixation, the cells were washed three times in PBS and incubated with EGFR.1 antibody (diluted 1:20 in PBS) for 30 minutes. Cells were washed three times in ice cold PBS and incubated with an Alexa 488-anti-mouse secondary antibody for 30 minutes. Cells were washed three more times with ice cold PBS and then sorted by flow cytometry at the Wayne State University Flow Cytometry core.

Immunofluorescence Assays

Approximately 3.5×10^5 MCF10A, MCF10A+AR, MCF10A AR, and SUM149 cells were seeded on coverslips overnight. The next day, cells were washed 3 times with ice cold PBS, fixed in 4% formalin for 20 minutes at room temperature, washed 3 times in PBS and then permeabilized with ice cold 0.1% Triton-X in PBS for 2 minutes. Cells were washed 3 times, then blocked for 1 hour in 20% Goat Serum. A 1:1000 dilution of 2 µg/µl anti-EGFR antibody MAB108 was incubated with cells for 1 hour. Cells were washed 3 times and incubated for 1 hour with an Alexa488-conjugated anti-mouse secondary antibody (Molecular Probes) at 1:1000 dilution. Cells were then washed 3 times with PBS and once more with distilled water to remove salt. ProLong gold with DAPI was used as a mounting reagent (Molecular Probes) and coverslips were put on slides for microscopy. Pictures were taken with a 65x water lens on a confocal microscope at the Wayne State University Confocal Imaging Facility.

Results

In order to determine if AR was contributing to the high levels of EGFR expression observed in SUM149 human breast cancer cells, we measured EGFR expression by western blot using MCF10A immortalized human mammary epithelial cells cultured in 10 ng/ml EGF (1.7nM), MCF10A cells cultured in 20 ng/ml AR (1.8nM) (MCF10A +AR), MCF10A cells that over express AR (MCF10A AR), and SUM149 cells. The concentration of 20 ng/ml AR was used based on previous published results that demonstrated this concentration was the biological equivalent to EGF for MCF10A cell proliferation [27]. We observed that EGFR protein expression was significantly higher in SUM149 cells, MCF10A AR cells and MCF10A + AR cells than in MCF10A cells cultured in medium containing EGF (Figure 1). Thus, AR stimulation of EGFR results in greater steady-state EGFR expression than does EGF stimulation of EGFR.

Since we observed increased steady-state levels of EGFR protein in cells that are dependent on AR, we hypothesized that EGFR degradation might be impaired when AR is the ligand. Although the effects of EGF on EGFR degradation have been well studied, the effects of AR on EGFR degradation are not well known. In order to determine whether receptor degradation

is impaired when EGFR is stimulated with AR, we measured EGFR half-life using pulse-chase analysis in MCF10A cells cultured in either 10 ng/ml EGF or 20 ng/ml AR. MCF10A cells were deprived of methionine and cysteine for 24 hours, pulsed with media containing ^{35}S labeled methionine/cysteine for 2 hours, and then chased with media containing excess unlabeled methionine and cysteine for 4, 6, 8, or 10 hours. EGFR was immunoprecipitated and analyzed after gel electrophoresis by autoradiography. Figure 2 demonstrates that MCF10A cells cultured in AR displayed little if any EGFR degradation after 10 hours. In contrast, most of the labeled EGFR was degraded at 10 hours in MCF10A cells grown in EGF. Thus, in the presence of AR, EGFR has a significantly longer half-life which can lead to increases in the steady-state levels of EGFR protein when an AR autocrine loop is functioning in breast cancer.

It has been demonstrated that EGFR Y1045 is phosphorylated after EGF activation of EGFR and the E3 ubiquitin ligase c-Cbl binds this phosphorylated residue, thereby mediating EGFR ubiquitination and degradation [16]. Since AR stimulates less EGFR degradation than does EGF, we next examined basal phosphorylation of Y1045 in MCF10A, MCF10A AR, and SUM149 cells. We observed that Y1045 was phosphorylated in all three cell lines (Figure 3A). However, normalization of EGFR phosphorylation at Y1045 to EGFR expression reveals that EGFR in SUM149 cells exhibits almost 50% less phosphorylation at Y1045 than does EGFR in MCF10A cells. Moreover, EGFR in MCF10A AR cells exhibits almost two thirds less phosphorylation at Y1045 than does EGFR in MCF10A cells (Figure 3C). These data suggest that AR stimulates less EGFR Y1045 phosphorylation than does EGF which could contribute to the decreased degradation of the receptor that we observed in the presence of AR.

In order to confirm that the phospho-Y1045 antibody is specific, we used 32D myeloid cells, which do not endogenously express EGFR. Retroviral infection was used to generate 32D myeloid cells that contain the vector control (LXSN), wild type EGFR (LXSN-WT-EGFR) or an EGFR mutant in which the tyrosine at 1045 was replaced with a phenylalanine (LXSN-Y1045F) [28]. Cells were lysed and EGFR was immunoprecipitated and run on a 7.5% gel. Western blots were then probed for either EGFR or pY1045. As shown in figure 3B, phosphorylation of EGFR at Y1045 was detected in cells that express wild-type EGFR. However, no EGFR phosphorylation at Y1045 was detected in the cells that express the EGFR Y1045F mutant, confirming that the phospho-Y1045 antibody is specific for phosphorylation at Y1045.

Based on the significantly reduced phosphorylation of EGFR Y1045 in MCF10A AR cells, we next examined ubiquitination of the receptor following EGF or AR stimulation. First, MCF10A cells were deprived of EGF for 24 hours to reduce EGFR phosphorylation and degradation. We then stimulated the EGF-deprived MCF10A cells with saturating concentrations of either EGF (16.7 nM) or AR (9.09 nM) for 5 or 10 minutes. EGFR immunoprecipitation was performed followed by western blot analysis using an anti-ubiquitin antibody that has been used successfully in other studies [23]. We observed that after 5 minutes of stimulation with EGF, EGFR exhibited high levels of ubiquitination that began to decrease at 10 minutes. In contrast, AR stimulated low levels of EGFR ubiquitination at 5 minutes, which were completely absent by 10 minutes after ligand stimulation (Figure 4). These data are consistent with our pulse-chase data, which showed AR stimulates much less EGFR degradation than does EGF.

When stimulated by EGF, EGFR undergoes rapid endocytosis [16,30]. Therefore, we performed experiments to measure EGF receptor expression on the cell surface following stimulation with AR. MCF10A cells were deprived of EGF for 24 hours and then stimulated with equimolar concentrations of either EGF or AR for 15, 30, 60, or 90 minutes. The cells were then fixed but not permeabilized in order to specifically label EGFR on the cell surface. After fluorescently labeling EGFR on the cells, flow cytometry was performed to quantitate

cell surface EGFR on a per cell basis. This assay was used previously in a study that compared surface expression of wild type EGFR and Y1045F mutant EGFR in porcine aortic endothelial cells. In that report, the amount of wild-type EGFR on the cell surface was greatly diminished upon 5 hours of EGF stimulation. In contrast, the amount of the Y1045F EGFR mutant on the cell surface was unchanged upon 5 hours of EGF stimulation [12]. Figure 5 demonstrates that stimulation of MCF10A cells with EGF resulted in rapid internalization of EGFR from the cell surface as demonstrated by the dramatic leftward shift of the peak. In contrast, AR stimulation induced slight internalization of EGFR with restoration of EGFR cell surface levels by 60 minutes after stimulation. Statistical analysis comparing the mean fluorescence activity of EGF or AR stimulated cells showed that the mean fluorescence of EGF stimulated cells was significantly less than that of AR stimulated cells at all time points ($p < 0.0001$). Thus, AR stimulates much less EGFR internalization than does EGF.

Previous literature has demonstrated that EGFR is primarily localized to endosomes following EGF stimulation [11]. To determine where in the cell EGFR is localized following AR stimulation, we performed EGFR immunofluorescence studies. MCF10A, MCF10A+AR, MCF10A AR and SUM149 cells were seeded on cover slips and then fixed and permeabilized. The fixed cells were fluorescently labeled and examined on a confocal microscope. We observed that EGFR localization in MCF10A cells cultured in EGF was predominantly punctate and intracellular (Figure 6). In contrast, EGFR protein in SUM149 cells, MCF10A +AR, and MCF10A AR cells was primarily localized on the membrane (Figure 6). We also observed EGFR at points of cell-cell contact in these cells (Figure 7). These results suggest that in MCF10A cells, EGF activation of EGFR induces a predominantly intracellular localization. However, AR activation of EGFR in MCF10A cells and SUM149 cells appears to alter localization of the receptor so that it is predominantly expressed on the membrane.

To further verify the importance of the ligand in EGFR internalization and degradation, we took SUM149 breast cancer cells growing in their normal media and compared them to SUM149 cells stimulated with 100ng/ml of EGF for 10 minutes. Examination of EGFR localization under both conditions revealed that EGFR is located on the membrane of SUM149 cells when they are growing in their normal AR containing media. However, when high concentrations of EGF are added to compete out the AR, EGFR then becomes punctate and intracellular indicating that it is being rapidly internalized and degraded in the presence of EGF (Figure 8).

In summary, our data illustrate that AR activation of EGFR results in increased steady-state levels of the receptor that accumulate at the cell surface. This phenomenon is the result of decreased phosphorylation of Y1045 on the EGFR, and the resultant failure to ubiquitinate and internalize the receptor in AR-stimulated cells. We propose that accumulation of EGFR at the cell surface alters EGFR signaling in ways that induce motile and invasive properties of breast cancer cells with an AR/EGFR autocrine loop.

Discussion

Tight regulation of epidermal growth factor receptor (EGFR) signaling is critical for normal mammary gland development and growth [31]. While an absence of EGFR signaling can severely impair growth, excessive and uncontrolled EGFR signaling is associated with breast cancer [31–34]. Research aimed at identifying the mechanisms used by cells to regulate EGFR suggests that the modulation of receptor trafficking and degradation are essential for appropriate EGFR signaling [35–40]. EGFR degradation is regulated in part via the E3 ubiquitin ligase c-Cbl. EGF activation of EGFR leads to phosphorylation of Y1045. The ubiquitin ligase c-Cbl binds this site and targets EGFR for ubiquitination [12,16]. EGFR is then rapidly endocytosed via a clathrin-dependent mechanism and the receptor is degraded in

the lysosomes [14,16,41]. Alterations in this mechanism, such as those observed in Y1045F EGFR mutant cells and c-Cbl deficient cells, leads to decreased degradation of the receptor and increased recycling of EGFR to the membrane [16,30].

Although degradation is widely accepted as the ultimate fate of EGF-activated EGFR, the rate of receptor degradation has been found to be significantly slower than the rate of internalization [42]. Thus, significant intracellular pools of activated receptor can accumulate and it has been demonstrated that the majority of EGF stimulated EGFR is localized in the endosomes of many cell types [11,12]. Our data are consistent with this, as we observed punctate EGFR staining within the MCF10A cells stimulated with EGF. Although the degree to which the receptor remains active after internalization is not clear, it has been shown that EGFR in the endosomes proceeds to signal intracellularly. For example, it has been demonstrated that following administration of a saturating dose of EGF, intracellular EGFR was associated with signaling molecules such as Shc, Grb2 and SOS for up to 60 minutes [43]. Furthermore, another study showed that ERK activation was strongly reduced in cells where EGFR endocytosis was inhibited by dominant-negative dynamin [44]. Thus, it is conceivable that EGF-activated EGFR in MCF10A cells is signaling predominantly from the endosomes rather than from the membrane.

We have previously published that SUM149 breast cancer cells have an AR autocrine loop that is required for their proliferation [24]. The majority of studies on EGFR trafficking and degradation have involved EGF as the activating ligand. We show that AR activated EGFR in MCF10A AR and SUM149 breast cancer cells exhibits decreased Y1045 phosphorylation as compared with EGF stimulated EGFR in MCF10A cells. In addition, EGFR is ubiquitinated at only very low levels compared with EGF. The low levels of ubiquitinated EGFR we observed in AR-stimulated cells could be explained by the ability of c-Cbl to target EGFR indirectly for degradation by binding to Grb2. However, this does not appear to be the predominant interaction between c-Cbl and EGFR [12]. The low levels of EGFR ubiquitination exhibited by AR stimulation of EGFR resemble findings in another study where TGF- α induced only low levels of EGFR ubiquitination relative to EGF [23]. Furthermore, EGFR protein remained stable for 10 hours rather than degraded as it was in the presence of EGF. Thus, from our results it appears that although both ligands bind and activate the same receptor, AR stimulates significantly less EGFR ubiquitination and degradation than EGF.

Our results demonstrate that EGFR is not effectively internalized from the cell surface when MCF10A cells are stimulated with AR. In addition, large amounts of AR-stimulated EGFR are localized predominantly at the cell membrane and cell-cell junctions likely due to a lack of internalization and degradation. This altered EGFR protein expression and degradation could have significant effects on downstream signaling. We have previously demonstrated that AR and EGF induce different patterns of gene expression in MCF10A cells which leads to an increase in cell motility and invasion in the presence of AR [24]. If more receptors are present at the cell surface and are available for activation, this will likely result in sustained EGFR signaling from the membrane that could increase proliferation, migration, and survival. Also, it has been demonstrated that signaling through PLC- γ and PI3K pathways is restricted to the cell surface and therefore these pathways are not activated by EGFR in the endosomes [36, 37]. Thus, based on EGFR localization, EGF and AR could potentially signal via different pathways and this could explain the different phenotypes we observed when MCF10A cells are stimulated with AR versus EGF [24].

In summary, we have demonstrated in MCF10A cells and SUM149 breast cancer cells that AR activation of EGFR results in increased steady-state levels of EGFR protein, less EGFR phosphorylation at Y1045 and less ubiquitination of the receptor compared with EGFR that is activated by EGF. Consistent with these data, EGFR degradation is reduced when AR is the

ligand. In addition, localization of EGFR is punctate and intracellular when stimulated by EGF but is predominantly on the membrane and cell-cell junctions when stimulated by AR. Altered receptor expression and localization induced by AR binding may ultimately make EGFR a more potent signal transducer. Also, decreased degradation of activated EGFR could contribute to the over expression of EGFR protein observed in aggressive, triple-negative breast cancer [45]. Thus, our data suggest a mechanism by which an AR autocrine loop can contribute to breast cancer progression.

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Abbreviations

AR	amphiregulin
BTC	betacellulin
EGF	epidermal growth factor
EGFR	epidermal growth factor
EPR	epiregulin
FBS	fetal bovine serum
HB-EGF	heparin binding epidermal growth factor
IP	immunoprecipitation
PI3K	phosphoinositide 3-kinase
PLC-γ	phospholipase C-gamma
PVDF	polyvinylidene difluoride
TGF-α	transforming growth factor alpha

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Figure 1.

High levels of EGFR protein in SUM149 cells and MCF10A cells with AR activated EGFR. MCF10A, SUM149, MCF10A AR, and MCF10A+AR cells were lysed and 50, 100, or 200 µg of whole cell lysate protein was separated by SDS-PAGE and then transferred onto a PVDF membrane. Membranes were probed for EGFR using Zymed monoclonal anti-EGFR antibody. A non-specific protein band is shown to demonstrate relative loading.

Figure 2.

EGFR degradation is decreased in the presence of AR versus EGF during steady-state growth conditions. MCF10A cells were cultured in either 10ng/ml EGF (1.7nM) or 20ng/ml AR (1.8nM). MCF10A cells were then incubated in methionine/cysteine free media for 1 hour. Cells were pulsed with ^{35}S labeled methionine and cysteine for 2 hours and then chased with media containing 200x more unlabeled methionine and cysteine for 4, 6, 8, or 10 hours. NC are cells that were not chased after ^{35}S incorporation.

Figure 3.

Decreased phosphorylation of EGFR Y1045 when AR is the ligand. A) MCF10A cells, SUM149 cells and MCF10A AR cells were lysed and EGFR was immunoprecipitated using the MAB108 antibody. Membranes were immunoblotted for pY1045 (Cell Signaling), EGFR (Zymed), or p-tyr (Calbiochem). Band densities were quantitated and are displayed relative to MCF10A. Actin was used as a loading control. C) Band density values for pY1045 were divided by the total amount of EGFR relative to MCF10A. This graph displays the average of three different experiments. B) Western blot showing Y1045 phosphorylation of EGFR in 32D myeloid cells transfected with either vector (LXSN), wild type EGFR (LXSN-WT-EGFR) or Y1045 mutant EGFR (LXSN-Y1045F).

Figure 4.

Decreased ubiquitination of EGFR when MCF10A cells are spiked with AR compared with MCF10A cells spiked with EGF. MCF10A cells were deprived of EGF for 24 hours and then stimulated with saturating concentrations of EGF and AR (100ng/ml) for 5 or 10 minutes. Cells were then lysed and EGFR was immunoprecipitated. Membranes were immunoblotted with either an anti-ubiquitin (anti-Ub) antibody (Cell Signaling) or an anti-EGFR antibody (Zymed).

Figure 5.

Impairment of EGFR internalization in the presence of AR. MCF10A cells were deprived of EGF for 24 hours and then spiked with equimolar concentrations of either EGF or AR (16nM) for 15, 30, 60 or 90 minutes. EGFR on the membrane was fluorescently labeled and analyzed using flow cytometry. A) Flow cytometry graphs where the empty peak represents cell surface EGFR before the addition of ligand and the green peaks represent EGFR after ligand is added. B) A graphical representation of the experiments in 3A indicating a time course of mean fluorescence intensity. Vertical bars indicate the standard deviation at each time point. C) Statistical analysis of flow cytometry data. A two sample student t-test was used to compare fluorescence intensity of AR or EGF treated cells. Mean fluorescence of EGF treated cells is significantly less than that of AR treated cells at each time point. ($p < 0.0001$ for each).

Figure 6.

EGFR localization in MCF10A, MCF10A AR, MCF10A + AR, and SUM149 cells. MCF10A (A,B), MCF10A+AR (C,D), MCF10A AR (E,F) and SUM149 (G,H) cells were seeded on coverslips. Cells were then washed, fixed, permeabilized and incubated with anti-EGFR antibody Mab108 for 1 hour followed by incubation with an anti-mouse Alexa 488 conjugated secondary antibody for 1 hour. Coverslips were then mounted on slides and viewed using a 65x water objective on a confocal microscope.

Figure 7. EGFR is localized at the membrane and cell-cell junctions of cells stimulated with AR. Higher magnification of MCF10A, MCF10A+AR, MCF10A AR and SUM149 cells from figure 6 to show more detailed EGFR localization on the membrane and at cell-cell junctions.

Figure 8.

EGFR localization in SUM149 cells stimulated with EGF. SUM149 cells were seeded on coverslips and then treated with either 100ng/ml of EGF or vehicle for 10 minutes. Cells were then washed, fixed, permeabilized and incubated with anti-EGFR antibody Mab108 for 1 hour followed by incubation with an anti-mouse Alexa 488 conjugated secondary antibody for 1 hour. Coverslips were then mounted on slides and viewed using a 65x water objective on a confocal microscope.