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Activated EGFR Stimulates MUC1 Expression in Human Uterine and Pancreatic Cancer Cell Lines

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ABSTRACT

MUC1 is a large cell surface mucin glycoprotein that plays diverse roles in both normal and tumor cell biology. These roles include mucosal hydration and protection, inhibition of embryo implantation, protection of tumor cells from the immune system and reduction of cytotoxic drug uptake. Similarly, the EGFR family of cell surface receptors drives many normal developmental processes as well as various aspects of tumor growth and gene expression. EGFR family members have been demonstrated to form complexes with MUC1 in various cellular contexts. Nonetheless, the role that EGFR activation plays in modulating MUC1 levels has not been considered. In this study, we demonstrate that activated EGFR drives high level MUC1 expression in multiple cell lines of uterine adenocarcinoma and pancreatic cancer origins. In some cells, addition of exogenous EGFR ligands (EGF or HB-EGF) elevates MUC1 levels while addition of the EGFR tyrosine kinase inhibitor, AG1478, reduces MUC1 levels. The thiazolidinedione, rosiglitazone, previously shown to reduce progesterone-stimulated MUC1 expression, also blocks EGFR ligand-driven MUC1 expression. This activity was observed at relatively high rosiglitazone concentrations (above 10 μM) and appeared to be largely PPARγ independent indicating a novel utility of this drug to reduce mucin-expression in various tumor settings. Collectively, these data demonstrate that: (1) activation of EGFR stimulates MUC1 expression in multiple cellular contexts and (2) it may be possible to develop useful interventions to reduce MUC1 expression as a complementary strategy for tumor therapy. J. Cell. Biochem. 114: 2314–2322, 2013. © 2013 Wiley Periodicals, Inc.

KEY WORDS: MUC1; EGF; EGFR; ROSIGLITAZONE

High molecular weight mucin glycoproteins (>200 kDa) are characterized by a large number of tandem repeat domains enriched in serine and threonine that serve as oligosaccharide attachment sites and proline residues responsible for the highly rigid and extended protein structure. The type I transmembrane mucin, MUC1, is abundantly expressed in normal simple epithelial cells of the uterus, pancreas and many other epithelial tissues [Gendler and Spicer, 1995; Gendler, 2001; Brayman et al., 2004; Hollingsworth and Swanson, 2004; Yonezawa et al., 2008]. MUC1 and other membrane-tethered and gel-forming mucins contribute to the protective barrier function of epithelial cells by lubricating and hydrating cell surfaces against microbial and proteolytic attack [Gendler, 2001; McAuley et al., 2007]. In the context of embryo implantation, the highly extended ectodomain of MUC1 serves as a barricade to embryo attachment and implantation [Carson et al., 2000]. In most epithelial carcinomas, MUC1 is highly expressed, underglycosylated and loses its restricted apical localization [Hollingsworth and Swanson, 2004; Kufe, 2008]. As a consequence, MUC1-expressing tumor cells become poorly adherent and metastatic. The barrier function of MUC1 also protects tumor cells from killing by the host immune system and a variety of cytotoxic drugs normally used in cancer chemotherapies [Ren et al., 2004]. Cancer chemotherapies often rely upon triggering apoptosis in tumor cells. In this regard, MUC1 also protects tumor cells from apoptosis via interactions of its cytoplasmic tail with key proteins involved in apoptotic cascades [Carson, 2008]. As a result, MUC1 overexpression is believed to be particularly insidious by promoting tumor cell metastasis and protecting these cells from killing via multiple mechanisms. Consequently, high level MUC1 expression by tumors is frequently associated with a poor prognosis [Luttges et al., 2002]. Expression of mucins also serve as prognostic markers in pancreatic cancer [Pantano et al., 2009; Torres et al., 2012].

Mucin expression increases in response to proinflammatory cytokines and progesterone, factors also prevalent in the perimplan-

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tation-stage uterine milieu as well as tumor microenvironments [Behrens et al., 2010]. The *cis* regulatory elements that control MUC1 expression by these factors fall within the 1.4 kb proximal MUC1 promoter [Kovarik et al., 1993; Lagow and Carson, 2002; Shalom-Barak et al., 2004; Carson et al., 2008; Dharmaraj et al., 2010]. These include tumor necrosis factor α (TNFα), interferon γ (IFNγ) and progesterone receptor [Carson et al., 2008]. There is evidence suggesting the important involvement of EGF, HB-EGF, and EGFR in embryo implantation and development [Birdsall et al., 1996; Lessey et al., 2002]. In addition, EGFR-family members and their ligands are important drivers of various cancers [Yarden, 2001]. Nonetheless, studies on the regulation of MUC1 expression by EGF family members have not been reported.

In normal and cancer cells, EGFR regulates cell survival and growth. EGFR belongs to the ErbB family of transmembrane tyrosine kinase growth factor receptors. Ligand-induced phosphorylation of EGFR results in activation of signaling pathways, thereby triggering multiple cellular processes. Activation of EGFR can be blocked by tyrosine kinase inhibitors of EGFR, such as AG1478, leading to cancer cell death [Levitzki and Gazit, 1995]. An association of MUC1 with EGFR has been demonstrated in several carcinomas including those of the pancreas [Hollingsworth and Swanson, 2004] and previous reports have verified that expression of MUC1 inhibits EGFR degradation [Li et al., 2005; Pochampalli et al., 2007; Bitler et al., 2010]. MUC1 also promotes the nuclear accumulation of EGFR independent of the addition of exogenous ligand [Bitler et al., 2010]. We sought to determine if EGFR family ligands increase MUC1 expression in uterine and pancreatic cancer cell lines. We also examined if rosiglitazone, a thiazolidinedione, previously reported to reduce progesterone-stimulated MUC1 expression in a Peroxisome Proliferator Activated Receptor-γ (PPARγ) dependent fashion [Wang et al., 2010] reduces EGFR family ligand-stimulated MUC1 expression.

MATERIALS AND METHODS

ANTIBODIES AND REAGENTS

CT-1, an antibody that recognizes all cell-associated forms of MUC1 was used at a final dilution of 1:2,000 as previously described [Dharmaraj et al., 2010]. β-Actin antibody was purchased from Abcam (Cambridge, MA; catalog no. ab8226-100). Total EGFR (Clone H9B4) antibody was purchased from Invitrogen (Carlsbad, CA; catalog no.44798). Antibody specific for the EGFR phosphorylated at Tyr1068 was purchased from Invitrogen (catalog no.44788). Hsp70 and Hsp90 antibodies were purchased from Abcam (Catalog no. ab69412; 13495). Rosiglitazone was purchased from Cayman Chemical (Ann Arbor, MI). GW9662, AG1478, bafilomycin, EGF, and HB-EGF were purchased from Sigma (St. Louis, MO).

CELL CULTURE

The pancreatic cancer cell lines HPAF-II and CAPAN-2 were kindly provided by Dr. Kenneth Van Golen (University of Delaware, Newark, DE). Cells were maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; Atlanta Biologicals, Lawrenceville, GA), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen). HES cells stably transfected with human progesterone receptor B (HES-PRB) were created and maintained as described previously [Dharmaraj et al., 2010]. KLE cells were kindly provided by Dr. Russell Broaddus (MD Anderson Cancer Center, Houston, TX) and were maintained in DMEM-F12 supplemented with 10% (v/v) heat-inactivated FBS and 100 U/ml penicillin and 100 μg/ml streptomycin. All cells were routinely maintained at 37°C in a humidified atmosphere of air:CO_2 (95:5 v/v). Prior to experiments, cells were seeded in RPMI, DMEM-F12, or DMEM media supplemented with 10% (v/v) charcoal stripped FBS (Hyclone, Logan, UT) for 48 h.

WESTERN BLOT ANALYSES

KLE, HES–PRB, CAPAN-2, or HPAF-II cells were plated in 24-well plates and maintained as described above until reaching 80% confluence. Cells then were serum-starved for 24 h prior to treatment. Cells then were incubated in fresh serum free medium with phosphate-buffered saline (PBS) vehicle (0.01% w/v BSA in PBS), EGF (50 ng/ml), HB-EGF (100 ng/ml), or rosiglitazone (50 or 100 μM), or GW9662 (50 μm) as indicated for 48 h. Cell lysates were collected with sample extraction buffer (SEB; 0.05 M Tris pH 7, 8 M urea, 1% w/v urea, 1% v/v β-mercaptoethanol and protease inhibitor cocktail mix at 1:100 dilution [Sigma]). Ten percent of the total protein extract was separated by SDS–PAGE using a 5% (w/v) Laemmli stacking gel and a 10% (v/v) Porzio and Pearson resolving gel [Laemmli, 1970; Porzio and Pearson, 1977]. Proteins then were transferred to nitrocellulose membranes at 4°C. Blots were blocked at 4°C in PBS plus 0.1% (v/v) Tween-20 and 5% (w/v) non-fat dry milk (PBS-T20) or 3% (w/v) BSA for 6 h and probed with the MUC1 primary antibody CT-1 (1:2,000), β-actin antibody (1:10,000), total EGFR antibody (1:1,000), antibody specific for the EGFR phosphorylated at Tyr1068 (1:1,000), HSP70 antibody (1:500), or HSP90 antibody (1:500), in incubation buffer (3% w/v BSA or 5% w/v non-fat dry milk in PBST) overnight at 4°C. Blots were rinsed three times for 5 min each at room temperature and incubated for 2 h at 4°C with horseradish peroxidase-conjugated sheep-anti-mouse IgG (Jackson Immunoresearch) or donkey anti-rabbit (Sigma) at final dilutions of 1:200,000 in blocking solution. Finally, the blots were rinsed three times for 5 min each and signal intensities were detected using the ECL system (Pierce) as described by the manufacturer. Blots were exposed to x-ray film, and signal intensities were quantitated using Image J software (NIH, Bethesda, MD).

STATISTICAL ANALYSIS

Data are shown as the means ± SD of triplicate samples and are representative of at least two independent experiments. All data were analyzed by one-way ANOVA followed by the Tukey–Kramer multiple comparisons test or Student's *t*-test using the GraphPad InStat software, version 3.05 (GraphPad Software, San Diego, CA).

RESULTS

EGFR LIGANDS STIMULATE MUC1 PROTEIN EXPRESSION VIA ACTIVATED EGFR

Previous reports investigated the role of MUC1 in EGFR stability, cellular and nuclear localization [Pochampalli et al., 2007; Bitler et al., 2010; Merlin et al., 2011]. To evaluate regulation of MUC1

protein expression by EGFR family ligands, we examined the effects of EGF in the uterine adenocarcinoma cell line, KLE, as well as the pancreatic cancer cell lines, CAPAN-2 and HPAF-II. The relative levels of cell-associated MUC1 were determined by western blot analysis using a cytoplasmic-domain specific antibody, CT-1. Recognition of the MUC1 cytoplasmic domain with CT-1 antibody often appears as a smear due to variation in *N*-glycosylation [Parry et al., 2006; Julian et al., 2009] (Fig. 1 panel A). In KLE, CAPAN-2 and HPAF-II, addition of EGFR ligands stimulated MUC1 levels. Similar results were obtained with HES-PRB cells (data not shown). Endogenous EGFR phosphorylation at Tyr-1068 was evident in all cases (data not shown). In HPAF-II cells, treatment with the specific EGFR kinase inhibitor AG1478 confirmed that increase in MUC1-expression was driven by activated EGFR (Fig. 1 panel B). Another EGFR kinase inhibitor, erlotinib [Grunwald and Hidalgo, 2003], also inhibited EGF-stimulated MUC1 expression (Supplementary Fig. 2). Thus, while addition of exogenous ligand stimulated MUC1 expression, inhibition of EGFR activity greatly reduced MUC1 expression. This suggests that chronic activation of EGFR in these cells drives MUC1 expression. This interpretation was born out in later experiments showing constitutive EGFR phosphorylation at Tyr-1068 (Fig. 3 below). Collectively, we concluded that activation of EGFR stimulated MUC1 expression.

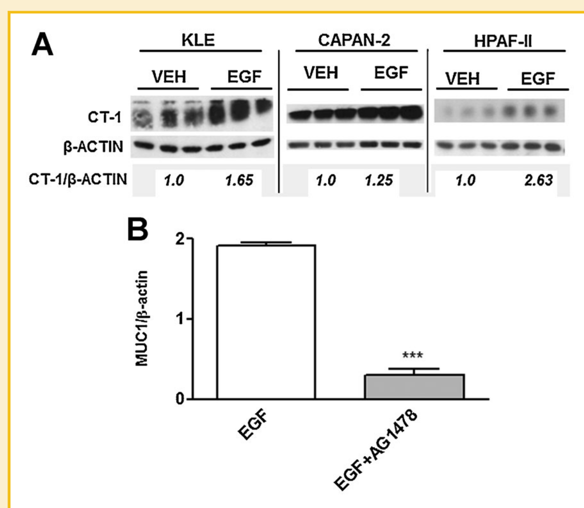


Fig. 1. EGFR activation stimulates MUC1 expression. Panel A: EGF (50 ng/ml) treatments for 48 h stimulate MUC1 expression in uterine adenocarcinoma cells, KLE, and pancreatic cancer cells, CAPAN-2 and HPAF-II. Triplicate independently derived cell lysates in each case were subjected to SDS–PAGE and western blot analyses for MUC1 (CT-1) and β-actin antibodies as described in Materials and Methods Section. Numerical values below the blots indicate the mean ratio of CT-1/β-actin with the value for the vehicle control arbitrarily set to 1. Panel B: HPAF-II cells were treated with EGF (50 ng/ml) with or without AG1478 (10 μM) for 48 h. Bar graphs represent densitometric analysis of MUC1 expression probed with CT-1 antibody from triplicate independently derived cell lysates. The bars indicate mean ± SD values representative of at least two experiments performed in triplicates in each case and are expressed relative to β-actin. $^{***}P < 0.001$ versus EGF only.

ROSIGLITAZONE ANTAGONIZES EGFR-STIMULATED MUC1 PROTEIN EXPRESSION

We previously reported that rosiglitazone antagonizes progesterone-stimulated MUC1 expression in a PPARγ-dependent fashion [Wang et al., 2010]. Based on these studies, we tested the possibility that rosiglitazone also might antagonize EGF-stimulated MUC1 expression. Rosiglitazone treatments (1–100 μM) performed in the presence of EGF (50 ng/ml) in HPAF-II cells indicated that 50 μM rosiglitazone concentration was necessary to antagonize EGF-stimulated MUC1 expression. Lower concentrations did not reduce MUC1 expression (Supplementary Fig. 1). This concentration was similar to that needed to efficiently reduce progesterone-stimulated MUC1 expression [Wang et al., 2010]. To assess the effect of rosiglitazone on MUC1 protein expression, KLE, HES-PRB, CAPAN-2, and HPAF-II were treated with rosiglitazone (50 or 100 μM) in the presence or absence of HB-EGF (100 ng/ml) or EGF (50 ng/ml) for 48 h. The relative levels of cell-associated MUC1 were determined by western blot analysis. Rosiglitazone treatments antagonized EGF-stimulated MUC1 expression in KLE, HES-PRB cells, CAPAN-2 and HPAF-II (Fig. 2). In most cases, rosiglitazone alone reduced MUC1 below levels observed in untreated cells.

ROSIGLITAZONE REDUCES EGFR LEVELS AND PHOSPHORYLATION

Enhanced degradation of cyclin D1, estrogen receptor α and Aβ precursor protein [Qin et al., 2003; d'Abramo et al., 2005] in response to PPARγ activation has been previously reported. Previously, we found that one aspect of rosiglitazone antagonism of progesterone-stimulated MUC1 expression was enhanced PRB degradation [Wang et al., 2010]. Therefore, we considered that rosiglitazone also might reduce EGFR levels. Experiments in three cell lines (CAPAN-2, HPAF-II, and HES-PRB) indicated that rosiglitazone alone reduced basal levels of EGFR by 40–50% (data not shown). When rosiglitazone was added in the presence of EGFR ligand total EGFR levels were severely reduced (>90%) in all cell lines (data not shown). Thus, events associated with ligand-activated EGFR degradation [Alwan et al., 2003; Stoorvogel et al., 2004] appear to be activated by rosiglitazone. Previous work also indicated that rosiglitazone inhibited ligand-activated progesterone receptor phosphorylation [Wang et al., 2010]. We noted that EGFR phosphorylation at Y-1068 was severely inhibited (>80%) by rosiglitazone both in the absence or presence of EGF in HPAF-II (Fig. 3). Therefore, like its effect on progesterone receptor, rosiglitazone action on EGFR was twofold by: (1) by reducing EGFR levels and (2) inhibiting EGFR phosphorylation.

To determine the kinetics of EGFR loss, HPAF-II cells were incubated with EGF and rosiglitazone, samples collected at various time points up to 48 h and EGFR levels assessed by western blotting (Fig. 4). After an initial lag period of approximately 13 h, EGFR levels declined steadily reaching 50% of control levels at approximately 32 h of treatment. If the lag period is subtracted the time required for a 50% EGFR reduction is approximately 15 h.

ROSIGLITAZONE ANTAGONISM OF EGF STIMULATED MUC1 EXPRESSION IS NOT A DRUG INDUCED STRESS RESPONSE AND IS PPARγ INDEPENDENT

We considered that rosiglitazone may have caused a drug induced stress response as reported in other studies [Weber et al., 2004];

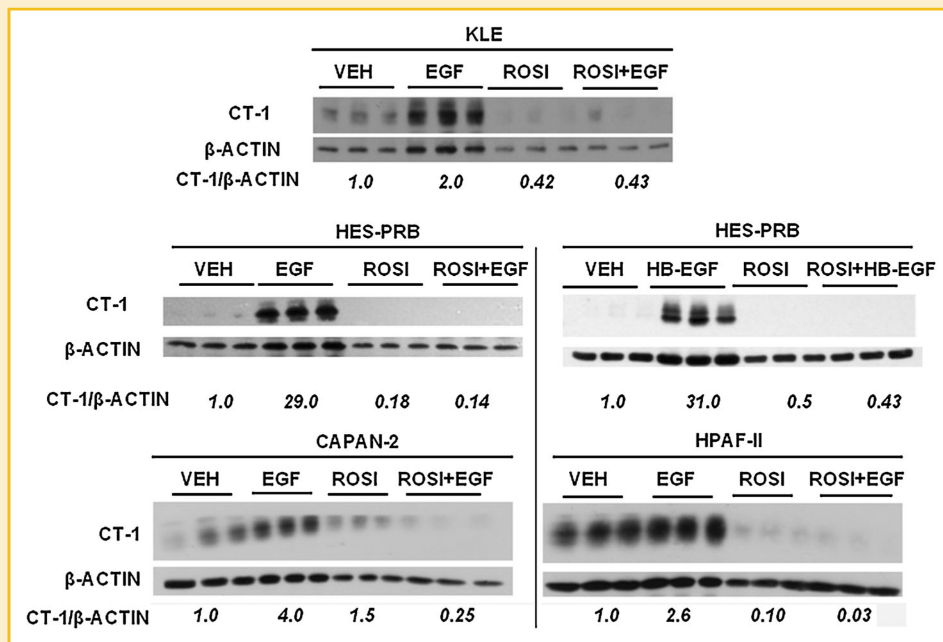


Fig. 2. Rosiglitazone antagonizes EGF or HB-EGF-stimulated MUC1 expression. KLE cells were treated with vehicle (VEH) or EGF (50 ng/ml) in the presence or absence of rosiglitazone (ROSI; 50 μM), for 48 h. Cell lysates from triplicate independent samples were probed with the MUC1 antibody CT-1 or β-actin by western blotting as described in Materials and Methods Section. HES cells stably expressing PRB were treated with vehicle (VEH), EGF (50 ng/ml) or HB-EGF (100 ng/ml) in the presence or absence of rosiglitazone (ROSI; 100 μM) as indicated for 48 h. Cell lysates from triplicate independent samples were subjected to SDS–PAGE and western blotting with the MUC1 antibody, CT-1 or β-actin as described in Materials and Methods Section. CAPAN-2 or HPAF-II cells were treated with vehicle (VEH) or EGF (50 ng/ml) in the presence or absence of rosiglitazone (ROSI; 100 μM) for 48 h. Cell lysates were subjected to SDS–PAGE and analyzed by western blotting with CT-1 and β-actin as described in Materials and Methods Section. The numerical values below the blots represent the mean values of the CT-1/β-actin signal with the value obtained for the vehicle control arbitrarily set to 1 in each case.

however, no significant increase in the levels of heat shock proteins 70 and 90 were observed by western blot analysis indicating a stress response was not involved (Fig. 5A,B). We then assessed the mechanism underlying rosiglitazone actions with the PPARγ antagonist, GW9662, in HPAF-II cells in the presence of EGF and/or rosiglitazone. We found that GW9662 did not antagonize rosiglitazone's suppression of EGF-stimulated MUC1 expression (Fig. 6). PPARγ agonists including rosiglitazone can function via both PPARγ-dependent and -independent mechanisms [Galli et al., 2004; Sun et al., 2009; Akinyeke and Stewart, 2011]. Consequently, while rosiglitazone proved to effectively reduce both MUC1 and EGFR levels these responses appear to be neither PPARγ dependent nor a drug stress response.

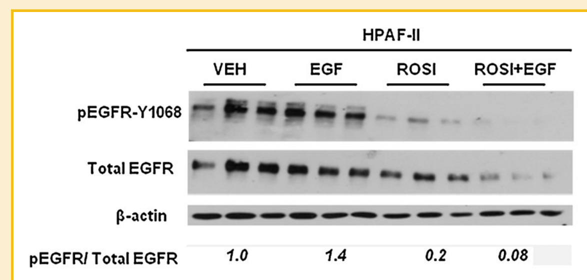


Fig. 3. Rosiglitazone lowers EGFR levels and EGFR phosphorylation. HPAF-II cells were incubated with vehicle (VEH) or EGF (50 ng/ml) in the presence or absence of rosiglitazone (ROSI; 100 μM) as indicated for 48 h. Cell lysates were subjected to SDS–PAGE and western blotting with antibodies to phospho-EGFR (Y1068), total EGFR, and β-actin as described in Materials and Methods Section. Combined treatments with EGF and rosiglitazone markedly reduced EGFR levels. EGFR phosphorylation was even more severely reduced. The numerical values below the blots represent the mean values of the phospho-EGFR/total EGFR signal with the value obtained for the vehicle control set to 1.

DISCUSSION

The EGFR family and its ligands, EGF and HB-EGF, are important mediators of embryo-uterine interactions during implantation [Das et al., 1994; Schneider and Wolf, 2008; Singh et al., 2011]. Expression of these receptors and ligands are spatio-temporally regulated in endometrial epithelial cells suggesting a critical role in implantation [Hofmann et al., 1991; Yoo et al., 1997]. Human MUC1 expression is of great importance in many normal and pathological processes, including mucosal lubrication and protection from pathogenic attack, embryo implantation and cancer [Gendler, 2001; Brayman et al., 2004; McAuley et al., 2007]. Many adenocarcinomas, such as pancreatic and endometrial carcinomas aberrantly express MUC1, a probable consequence of a tumor environment enriched with growth factors, cytokines and hormones [Hollingsworth and Swanson, 2004; Di Cristofano and Ellenson, 2007]. The EGFR family and their ligands play a vital role in normal cellular and

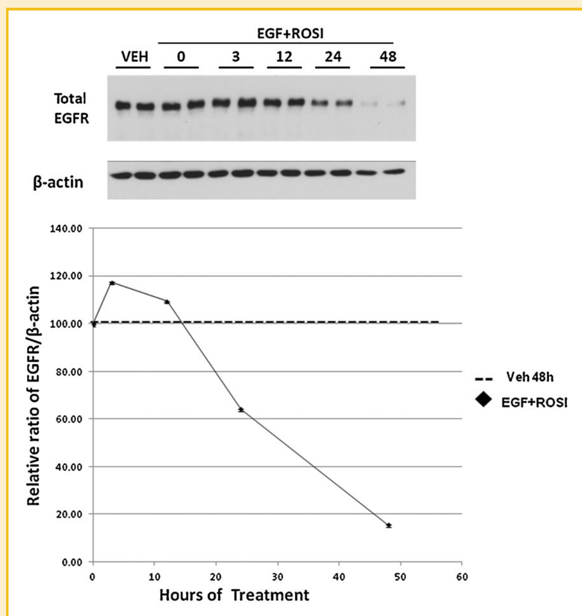


Fig. 4. Kinetics of EGFR loss in response to rosiglitazone. HPAF-II cells were treated with EGF (50 ng/ml) in the presence or absence of rosiglitazone (50 μM) and cell lysates were collected at the indicated time points. One set of samples received vehicle (VEH) for 48 h. Duplicate cell lysates were subjected to SDS–PAGE and western blotting with antibodies to total EGFR and β-actin as described in Materials and Methods Section (inset figure). The zero time value for the EGFR/β-actin ratio was set to 100 and other values compared to that. A half time for EGFR loss was calculated to be approximately 32 h.

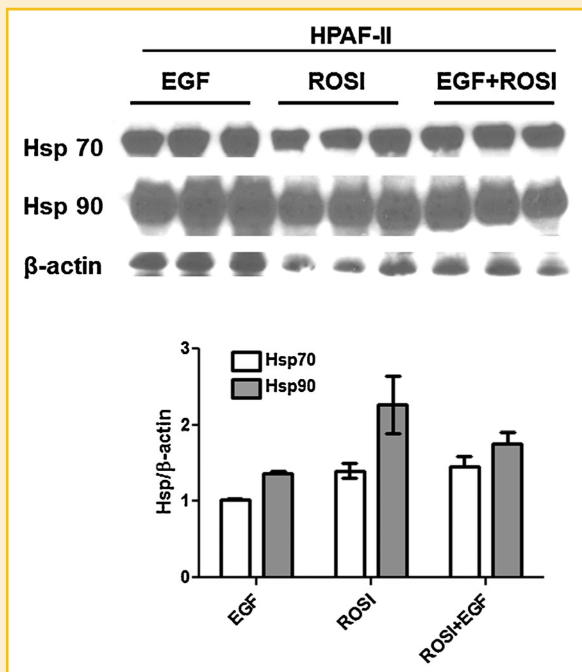
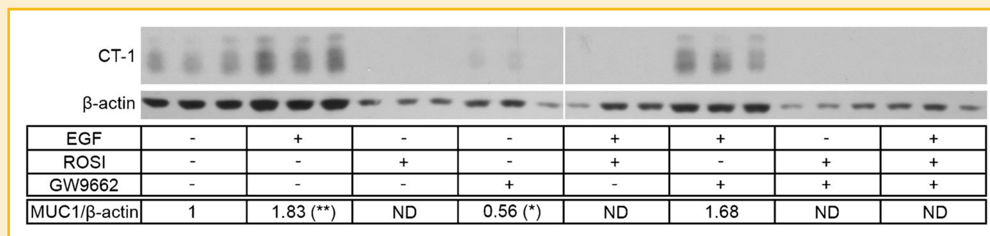


Fig. 5. Rosiglitazone antagonism of EGF-stimulated MUC1 expression is not a drug-induced stress response. HPAF-II cells were treated with EGF (50 ng/ml) in the presence or absence of rosiglitazone (50 μM) for 48 h as indicated. Triplicate cell lysates were subjected to SDS–PAGE and western blotting probing for hsp70, hsp90, and β-actin. No significant change in hsp70 or hsp90 levels was observed.

developmental processes and their abnormal activity in cancer supports tumor growth and metastasis [Yarden, 2001; Schneider and Wolf, 2009; Mitsudomi and Yatabe, 2010]. MUC1 is known to interact with EGFR [Schroeder et al., 2001] and regulate EGFR stability, cellular and nuclear localization [Pochampalli et al., 2007; Bitler et al., 2010; Merlin et al., 2011].

In this current study, we report the regulation of MUC1 protein expression by EGFR and its ligands, EGF and HB-EGF. In the endometrial adenocarcinoma cell line, KLE, pancreatic cancer cell lines, HPAF-II and CAPAN-2, and also in HES cells stably-transfected with progesterone receptor B, liganded EGFR stimulated MUC1 expression (Fig. 7). By contrast, we observed that other endometrial carcinoma cell lines including, Hec50, An3CA, HEC1-A as well as an immortalized human endometrial epithelial cell line (hTERT-ECC) did not respond to EGFR ligands by increasing MUC1 levels (data not shown). The EGFR tyrosine kinase activity inhibitors, AG1478 [Levitzki and Gazit, 1995], and erlotinib [Grunwald and Hidalgo, 2003] blocked EGF-mediated induction of MUC1 expression indicating that EGFR activation regulates MUC1 expression. A recent study reported that PI3K/AKT/mTORC1 activated by EGF or heregulin (HRG) in a breast cancer line, MCF-10A, induced MUC1-C translation [Jin et al., 2012]. No apparent stimulation of MUC1 mRNA levels by EGF was observed in CAPAN-2 and KLE cells (data not shown) indicating a post-transcriptional regulation. Similar observations were reported by Jin et al. [2012] in the MCF-10A cells. It has been previously reported that MUC1-EGFR interactions lead to increased MAPK activation [Schroeder et al., 2001] and phosphorylation of MUC1 cytoplasmic domain by EGFR leads to PI3K-Akt activation [Li et al., 2001; Ramasamy et al., 2007]. Most studies have only focused on MUC1 cytoplasmic domain and EGFR interactions. EGF induces Src-1 dependent MUC1 cleavage leading to the expression of genes involved in metastasis [Lau et al., 2012]. The current studies expand the relationship between MUC1 and EGFR by demonstrating the ability of activated EGFR to stimulate MUC1 expression.

Thiazolidinediones (TZDs), including rosiglitazone and pioglitazone, are PPARγ agonists used clinically to increase insulin sensitization and treat type 2 diabetes [Rangwala and Lazar, 2004]. Rosiglitazone stimulates murine MUC1 expression [Shalom-Barak et al., 2004] whereas progesterone-stimulated human MUC1 expression is antagonized by rosiglitazone [Wang et al., 2010]. These responses appear to be due to structural differences between the human and mouse MUC1 promoters [Wang et al., 2010]. In the current study, rosiglitazone antagonized liganded-EGFR stimulation of MUC1 protein expression in uterine adenocarcinoma and pancreatic cancer cell lines. Rosiglitazone antagonism of progesterone-stimulated human MUC1 expression reflects a combination of enhanced liganded PRB degradation and decreased PRB phosphorylation [Wang et al., 2010]. Other studies have demonstrated enhanced degradation of cyclin D1 and estrogen receptor α in response to PPARγ agonists [Qin et al., 2003]. Normal EGFR function involves its



EGF	-	+	-	-	+	+	-	+
ROSI	-	-	+	-	+	-	+	+
GW9662	-	-	-	+	-	+	+	+
MUC1/β-actin	1	1.83 (**)	ND	0.56 (*)	ND	1.68	ND	ND

Fig. 6. Rosiglitazone antagonism of EGF-stimulated MUC1 expression is not PPARγ dependent. HPAF-II cells were treated with the PPARγ antagonist, GW9662 (50 μM) in the presence of EGF (50 ng/ml) and/or rosiglitazone (50 μM) as indicated. Triplicate cell lysates were subjected to SDS–PAGE and western blotting probing for CT-1 and β-actin as described in Materials and Methods Section. Numerical values below the blots indicate the mean ratio of CT-1/β-actin with the value for the vehicle control arbitrarily set to 1. Note that treatments with GW9662 does not antagonize rosiglitazone actions on MUC1 expression. ROSI, rosiglitazone; ND, not detected.

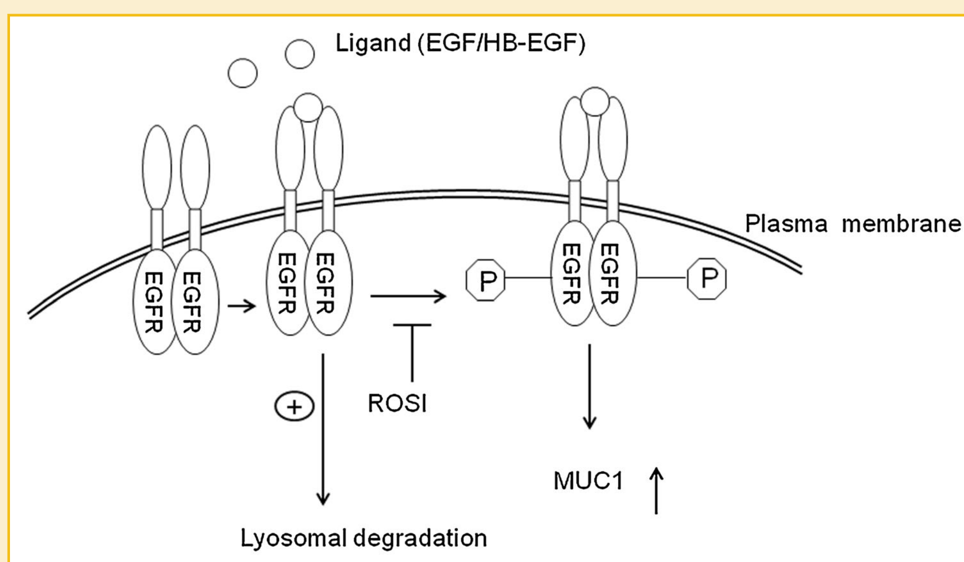


Fig. 7. Model of MUC1 regulation by EGFR and rosiglitazone. Liganded EGFR induces EGFR phosphorylation and activates downstream pathways leading to an increase in MUC1 expression. Rosiglitazone both inhibits EGFR-phosphorylation and enhances lysosomal degradation of EGFR leading to reduction of EGFR levels, attenuation of EGFR signaling and inhibition of EGFR-driven MUC1 expression.

downregulation, and degradation of EGFR is essential for attenuating its pro-oncogenic functions [Thien et al., 2001; Shtiegman and Yarden, 2003; Akinyeke and Stewart, 2011]. Interestingly, previous investigations demonstrated that overexpression of MUC1 reduces degradation of EGFR and enhances recycling [Pochampalli et al., 2007]. EGFR receptor degradation can occur either through proteosomal or lysosomal degradation pathways [Carpenter and Cohen, 1976; Katzmann et al., 2002; Sorkin and Von Zastrow, 2002]. $T_{1/2}$ of EGFR loss can vary from 6–24 h or longer depending on the expression levels of EGFR [Beguinot et al., 1984; Stoscheck and Carpenter, 1984a,b; Sorkin and Goh, 2008] and in good agreement with the kinetics of the rosiglitazone response described above. Our previous studies indicated that rosiglitazone triggered PRB degradation via the proteosomal pathway [Wang et al., 2010]. The proteasomal inhibitor, MG132, had no effect on rosiglitazone-stimulated EGFR loss (data not shown). In contrast, the lysosomal inhibitor, bafilomycin, stabilized EGFR levels in HPAF-II cells. Thus, it appears that rosiglitazone stimulates lysosome-mediated EGFR degradation (Supplementary Fig. 3). Our results demonstrate that rosiglitazone antagonizes EGF-stimulated MUC1 expression by stimulating lysosomal-mediated degradation of EGFR. How TZDs enhance degradation of cyclin D1 or the different classes of membrane (EGFR) and steroid hormone (PRB and ERα) receptors is unclear. Another TZD, troglitazone, was recently reported to enhance proteasome-mediated degradation of c-MYC in prostate cancer cells, although the mechanism has not been defined [Akinyeke and Stewart, 2011]. Similar to our results these authors found this action was not blocked by the PPARγ antagonist, GW9662. They also showed that this response was not affected by siRNA-mediated knockdown of PPARγ. While we were not successful in significantly

reducing PPARγ using siRNA, we nonetheless also conclude TZD actions on liganded EGFR levels are PPARγ independent based on the insensitivity of this response to GW9662.

Our previous studies of rosiglitazone antagonism of progesterone-stimulated MUC1 expression indicated that this response was partially PPARγ-dependent [Wang et al., 2010]. Rosiglitazone mediated inhibition of nicotinic acetylcholine receptor (α4 nAChR) expression is PPARγ independent and involves the p38-MAPK, ERK 1/2, and Akt pathways [Sun et al., 2009]. The TZD, troglitazone also inhibits the activation of the MAPK pathways in pancreatic cancer cells [Vitale et al., 2012]. Therefore, rosiglitazone's actions on EGFR degradation and EGF-stimulated MUC1 expression may involve these signaling pathways. Rosiglitazone treatment did not change levels of the stress response proteins, HSP70 or HSP90 indicating that the effects on EGFR and MUC1 expression were not drug-induced stress responses.

Rosiglitazone treatment inhibited EGFR phosphorylation at Tyr-1068. Previously, we found that rosiglitazone inhibited ligand induced PRB phosphorylation at S-294; however, EGFR-mediated phosphorylation of PRB was unaffected [Wang et al., 2010]. Thus, some TZD actions may involve kinase inhibition. In light of the accumulating evidence of PPARγ-independent actions of TZDs, protein kinase screening of these compounds is warranted. It is possible that different TZDs have wide spectra of actions in this regard and may reveal novel therapeutic uses for these drugs.

In summary, we have demonstrated that EGFR ligands stimulate MUC1 expression in certain cancer cell contexts. Thus, EGFR activation can be expected to enhance the protective functions of certain cancers by elevating MUC1 expression. EGFR activation also may enhance normal mucosal protective functions by increasing expression of apically-disposed mucins. The ability of rosiglitazone to antagonize EGFR responses provides a novel use for this TZD in modulating EGFR action and MUC1-dependent functions.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Fig. S1. Dose dependent rosiglitazone antagonism of EGF-stimulated MUC1 expression. HPAF-II cells were incubated with EGF (50 ng/ml) in the presence or absence of rosiglitazone (ROSI; 1, 5, 10, 50, and 100 μm) as indicated for 48 h. Cell lysates were subjected to SDS–PAGE and western blotting with the MUC1 antibody, CT-1 or β-actin as described by Materials and Methods Section. Combined treatments with EGF and rosiglitazone (50 and 100 μm) markedly reduced MUC1 expression.

Fig. S2. Erlotinib inhibits EGF-stimulated MUC1 expression. HPAF-II cells were treated with EGF (50 ng/ml) with or without Erlotinib (10 μM) for 48 h. Bar graphs represent densitometric analysis of MUC1 expression probed with CT-1 antibody from triplicate independently derived cell lysates. The bars indicate mean ± SD values representative of at least two experiments performed in triplicates in each case and are expressed relative to β-actin. $^{***}P < 0.001$ versus EGF only.

Fig. S3. Rosiglitazone stimulated reduction of EGFR is partially inhibited by Bafilomycin. HPAF-II cells were treated with EGF (50 ng/ml), rosiglitazone (ROSI; 50 μM) and the lysosomal inhibitor, bafilomysin (BAF; 10 nM) as indicated. Bafilomycin treatments were performed for 24 h. Cell lysates were probed with total EGFR and β-actin antibodies by western blotting as described in Materials and Methods Section. Bar graphs represent densitometric analysis of MUC1 expression probed with EGFR antibody from triplicate independently derived cell lysates. The bars indicate mean ± SD values representative of at least two experiments performed in triplicates in each case and are expressed relative to β-actin. Treatments with bafilomycin reversed loss of EGFR degradation in presence of EGF and rosiglitazone. ROSI, rosiglitazone; BAF, bafilomycin. $^{***}P < 0.01$ versus EGF and ROSI.