Interaction of galectin-3 with MUC1 on cell surface promotes EGFR dimerization and activation in human epithelial cancer cells

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Running title: MUC1-galectin-3 interaction promotes EGFR dimerization

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Abbreviations: CRD, carbohydrate-recognition domain; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor/ErbB1; Gal-3, galectin-3; Gal-3C, galectin-3 C-terminal; MUC1ΔTR, MUC1 extracellular domain-depletion; MUC1ΔCT, MUC1 cytoplasmic domain-depletion; TF, Thomsen-Friedenreich Galβ1,3GalNAc-α

Abstract

EGFR is an important regulator of epithelial cell growth and survival in normal and cancerous tissues and is a principal therapeutic target for cancer treatment. EGFR is associated in epithelial cells with the heavily glycosylated transmembrane mucin protein MUC1, a natural ligand of galectin-3 that is overexpressed in cancer condition. This study reveals that the expression of cell surface MUC1 is a critical enhancer of EGF-induced EGFR activation in human breast and colon cancer cells. Both the MUC1 extracellular and intracellular domains are involved in EGFR activation but the predominant influence comes from its extracellular domain. Binding of galectin-3 to the MUC1 extracellular domain induces MUC1 cell surface polarization and increases MUC1-EGFR association. This leads to a rapid increase of EGFR homo-/hetero-dimerization and subsequently increased, and also prolonged, EGFR activation and signalling. This effect requires both the galectin-3 C-terminal carbohydrate recognition domain and its N-terminal ligand multi-merization domain. Thus, interaction of galectin-3 with MUC1 on cell surface promotes EGFR dimerization and activation in epithelial cancer cells. As MUC1 and galectin-3 are both commonly overexpressed in most types of epithelial cancers, their interaction and impact on EGFR activation likely makes important contribution to EGFR-associated tumorigenesis and cancer progression and may also influence the effectiveness of EGFR-targeted cancer therapy.

Key Words: galectin-3, MUC1, EGFR dimerization, cancer

Introduction

MUC1 is a large (>400kDa), heavily glycosylated transmembrane mucin protein and is expressed in a polarized manner on the apical side of all normal epithelial cells. MUC1 consists of a large extracellular domain, a transmembrane region and a short cytoplasmic domain/tail. The MUC1 extracellular domain contains various numbers of tandem repeats (VNTR) that are heavily glycosylated (up to 50% of the MUC1 molecular weight) with complex *O*-linked mucin type glycans[1]. The cytoplasmic tail of MUC1 contains 72 amino acids and harbours several phosphorylation sites and is able to interact with various growth factor receptors and intracellular signalling proteins [2, 3, 4]. In epithelial cancer cells, MUC1 is not only overexpressed up to 10-fold but also loses its apical membrane polarization to become expressed over the entire cell surface[5, 6]. In epithelial cancer cells, MUC1 also carries much reduced complex O-glycans with increased expression of shorter sugar chains such as the oncofetal oligosaccharides GalNAcα- (Tn antigen), sialylated-GalNAcα- (sialyl-Tn antigen) and Galβ1,3GalNAcα- (Thomsen-Friedenreich, T or TF antigen)[7]. MUC1 overexpression, its loss of apical polarization and increased expression of the oncofetal carbohydrate antigens have all, individually or in combination, been reported to be closely associated with high metastatic potential and poor prognosis in many types of cancers [8]. Immunological targeting of cancer-associated MUC1 has been under intensive investigation as a strategy for cancer treatment [9].

MUC1 is known to interact with various cellular proteins, through both its intracellular[10] and extracellular domains[11], and influences diverse signalling pathways that are important in cell proliferation, adhesion and immunomodulation[2, 4, 12, 13]. One of the proteins that have recently been reported to interact with MUC1 in epithelial cancer cells is the epidermal growth factor receptor (EGFR) [14 15 16].

EGFR is a member of the ErbB family of receptor tyrosine kinases that includes EGFR/ ErbB1 (Her1), ErbB2 (Her2/c-Neu), ErbB3 (Her3) and ErbB4 (Her4)[17]. EGFR is involved in the regulation of multiple cellular process including proliferation and survival and its activity is directly linked with tumorigenesis and metastasis[17]. EGFR exists normally in an inactive conformation. Binding to its extracellular domain by ligands such as EGF induces EGFR conformation change and enables its interaction with another member of ErbB family proteins to form homo- or hetero-dimers[17]. This leads to activation of EGFR tyrosine kinase domain and auto-phosphorylation of specific tyrosine residues at its cytoplasmic domain. These phosphorylated residues then serve as binding sites for proteins containing Src homology and phosphotyrosine binding domains, leading to activation of downstream signalling pathways such as the Ras/extracellular signal regulated kinase (ERK) pathway, the phosphatidylinositol 3-kinase (PI3) pathway, the Janus kinase/Signal transducer and activator of transcription (JAK/ STAT) pathway[17], crucial in cell proliferation, migration and survival.

In physiological conditions, EGFR activation is tightly regulated by its expression and by the availability of binding ligands to ensure that cell proliferation matches tissue requirement for homeostasis. In neoplasia, however, EGFR activation is often increased due to either increased EGFR expression, EGFR mutation or increased availability of the EGFR ligand produced by the same cells that express the ErbB receptors or by surrounding cells[18],[19]. Aberrant expression of EGFR by tumours typically also confers a more aggressive phenotype and has been shown to an indicator of poor prognosis in several types of epithelial cancer [20], [21, 22]. Not surprisingly, EGFR is currently a principal target for therapeutic intervention in cancer.

Galectin-3 is a β-galactoside-binding protein expressed by many types of human cells and particularly by epithelial and immune cells. Galectin-3 is distributed in the cytoplasm, nuclei, cell surface, extracellular space and in circulation. Overexpression of galectin-3 commonly occurs in most types of cancers such as colorectal, breast, lung, prostate, pancreatic, head and neck cancer and melanoma[23]. The level of circulating galectin-3 is also markedly elevated (up to 30-fold) in cancer patients and particularly in those with metastasis[24]. Overexpression of galectin-3 by cancer cells is increasingly shown to influence cancer cell-cell and cancer-microenvironment communication and contributes to cancer development, progression and metastasis as a result of galectin-3 interaction with various galactose-terminated glycans carried by glycoproteins and glycolipids on the cell surface as well as in the extracellular matrix[25].

Recently studies by us and others have revealed that galectin-3 is a natural ligand of MUC1 in epithelial cancer cells[11]. The interaction between galectin-3 and MUC1, via binding of galectin-3 to the oncofetal TF carbohydrate antigen on MUC1[11], induces MUC1 cell surface polarization and the exposure of underlying smaller cell surface molecules. This leads to increased cancer cell homotypic aggregation [26] and cancer cell heterotypic cell adhesion to vascular endothelium [27], two important steps in the cancer metastasis cascade. As MUC1 is also associated with EGFR in epithelial cancer cells, the effect of galectin-3-MUC1 interaction on MUC1 cell surface localization led us to examine the impact of their interaction on EGFR activity in epithelial cells.

We show here that both the MUC1 extracellular and intracellular domains contribute to EGF-induced EGFR activation in human colon and breast cancer cells with the predominate

contribution from the MUC1 external domain. Binding of galectin-3 to the MUC1 extracellular domain induces MUC1 cell surface polarization and increases MUC1-EGFR interaction, leading to increased EGFR homo-/hetero-dimerization and activation.

Result

MUC1 extra- and intra-cellular domains both contribute to EGFR activation

Interaction between MUC1 and EGFR has been shown to influence EGFR activity in breast cancer,[28] endometrial cancer,[29] and non-small cell lung cancer[30] cells. In this study we first tested the influence of MUC1 expression on EGFR activation in human breast epithelial and colon cancer cells and assessed the influence of MUC1 intra- and extra-cellular domains on this effect.

We transfected human colon cancer HCT116 cells with cDNA coding for full length MUC1, MUC1 with intra- or extra-cellular domain (VNTR region) depletion (Fig 1A). Immunoblotting with anti-MUC1 antibodies against the MUC1 extracellular (B27.29) and intracellular (CT2) domains showed neo expression of MUC1 in the parent (control transfected) cells (HCT116^{MUC1neo}), the expression of MUC1 extra- and intra-cellular domains in the full length MUC1 transfectants (HCT116^{MUC1Full}), the expression of MUC1 extracellular, but not intracellular, domain of MUC1 intracellular domain-depleted mutants (HCT116$^{MUC1\Delta CT}$) and the expression of MUC1 intracellular, but not extracellular, domain mutants (HCT116$^{MUC1\Delta TR}$) (Fig 1B). Immunoblotting also showed MUC1 expression in human breast epithelial cells transfected with full length MUC1 (HCA1.7+), neo MUC1 expression in the negative MUC1 revertants (HCA1.7-) and expression of the MUC1 extracellular, but not intracellular, domain of the MUC1 intracellular domain-depleted (HTD ΔCT) mutant cells (Fig 1C).

Armed with these transgenic mutant cells of two different cell types, we then assessed the effects of MUC1 expression and MUC1 intra- and extra-cellular domains on EGFR activity. In response to EGF binding, EGFR phosphorylation rapidly occurred in the full length

MUC1-expressing cells (HCT116^{MUC1Full} and HCA1.7+) of both cell types (Fig 2A and B, top panels) but very weak EGFR phosphorylation in the MUC1-negative cells (Fig 2A and B, second panels). In comparison to MUC1- negative cells, a 12-fold and 17 fold increase of EGFR phosphorylation were observed at 10 min and 60 min, respectively, of HCA1.7+ and HCT116^{MUC1Full} (Fig 2A-D). Depletion of the MUC1 extracellular domain markedly reduced EGFR activation in HCT116$^{MUC1\Delta TR}$ (Fig 2A and C) but depletion of the MUC1 intracellular domain resulted in less but still substantial inhibition of EGFR phosphorylation in HTD (ΔCT) and HCT116$^{MUC1\Delta CT}$. These results suggest that expression of MUC1 is critical to EGF-induced EGFR activation and that both the MUC1 intra- and extra-cellular domains contribute to the MUC1-associated increase of EGFR activity but with predominate influence from the MUC1 extracellular domain.

Galectin-3 interaction with cell surface MUC1 promotes EGFR activation

We next assessed the influence on EGFR activity of MUC1 cell surface interaction with galectin-3 at pathological galectin-3 concentrations observed in cancer patients[24, 31]. Without the presence of EGF, introduction of galectin-3 did not show any effect on EGFR activation in either MUC1-positive HCA1.7+ or MUC1-negative HCA1.7- cells of breast origin (Fig 3A-F). When EGF was introduced, galectin-3 presence caused more (e.g. by 106% at 5 min) EGFR activation in the MUC1-positive HCA1.7+ cells (Fig 3A and D) but had no effect in the MUC1-negative HCA1.7- cells (Fig 3B and E). In comparison to the cells treated with EGF alone, the presence of galectin-3 was shown to cause more (e.g. by 281% at 5 min) EGFR activation in the MUC1-cytoplasmic domain-depleted HTD(ΔCT) cells and a prolonged activation of EGFR was also observed in these cells (Fig 3C and F).

Similar results were observed with colon cancer HCT116 cells. Presence of galectin-3 alone did not show any effect on EGFR phosphorylation of either MUC1-positive HCT116^{MUC1Full} or -negative HCT116^{MUC1neo} cells (Fig 4A-D). When EGF and galectin-3 were both introduced, more (e.g. by 113% at 10 min) EGFR activation was observed in the MUC1-positive HCT116^{MUC1Full} than that treated with EGF alone. These results indicate that interaction of MUC1 with galectin-3 on the cell surface promotes EGFR activation.

We also assessed the contribution of endogenous cell surface galectin-3 to the effect of galectin-3-MUC1 cell surface interaction on EGFR activation in these cells. As the effect of galectin-3-MUC1 effect on EGFR activation shown in this study occurs on the cell surface, it is the potential effect of endogenous galectin-3 on the cell surface rather than the whole cellular galectin-3 expression that is relevant. A previous study has shown that treatment of HCT116 cell with 30mM lactose could abrogate cell surface galectin-3[32]. Using this strategy, we first removed the endogenous cell surface galectin-3 by pre-treatment of the cells with lactose before washing and introduction of EGF and galectin-3. Little difference in EGFR activation was observed between lactose pre-treated and untreated cells in both HCA1.7+ (Fig 4C and G) and HCT116MUC1full cells (Fig 4D and H) in cell response to EGF and galectin-3. This indicates that in this experimental setting the contribution of endogenous cell surface galectin-3 is minimal.

MUC1-galectin-3 interaction-induced EGFR activation increases downstream ERK1/2 signalling

It is known that EGFR activation on the cell membrane triggers an array of intracellular signalling pathways[17, 33]. One of the commonest signalling pathways triggered by EGFR activation is ERK signalling[17, 34] and galectin-3 in the MUC1- positive and -negative cells.

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Introduction of EGF to the cells induced rapid ERK1/2 phosphorylation in MUC1-positive HCA1.7+ and HCT116^{MUC1Full} cells (Fig 5A and C). The increase of ERK1/2 phosphorylation peaked at 10 min at which point a 3.6- and 10.3-fold increase of ERK1/2 phosphorylation was observed in HCA1.7+ (Fig 5A) and HCT116^{MUC1Full} (Fig 5C) cells respectively. Introduction of EGF also induced ERK1/2 phosphorylation of the MUC1 negative HCA1.7- and HCT116^{MUC1neo} cells but to a much lower level in comparison to the MUC1-positive cells (Fig 5 B and D), in consistence with the effect of MUC1 expression on EGFR activity shown in Figure 2-4. At 10 min, a 1.9- and 1.8-fold increase of ERK1/2 phosphorylation was observed in HCA1.7- and HCT116^{MUC1neo} cells.

When galectin-3 was also present, EGF induced a stronger (1.9- and 4.9-fold further increase at 10 min) and more prolonged ERK1/2 phosphorylation in the MUC1-positive HCA1.7+ (Fig 5A) and HCT116^{MUC1Full} (Fig 5C) cells, while ERK1/2 phosphorylation in the MUC1-negative HCA1.7- (Fig 5B) and HCT116^{MUC1neo} cells (Fig 5D) remained the same as for the cells treated with EGF alone. In contrast to the enhanced ERK1/2 activation by the full length galectin-3/EGF in the MUC1-positive cells, introduction of C-terminal galectin-3 form (galectin-3C) with EGF showed no further effect on ERK-1/2 phosphorylation in comparison to the cells treated with EGF alone. Moreover, without the presence of EGF, introduction of galectin-3 alone did not show any detectable influence on ERK1/2 phosphorylation.

These results suggest that, as predicted, EGFR activation on the cell surface induced by MUC1 and by MUC1-galectin-3 interaction effectively induces downstream signalling. The stronger and more prolonged ERK1/2 activation in the MUC1-positive cells seen with galectin-3 is in keeping with the stronger and more prolonged activation of EGFR (Fig 2-4).

In comparison to the marked effect on EGFR activation by full length galectin-3, the lack of effect by galectin-3C, in which its N-terminal ligand multimerization domain is depleted hence unable to crosslink MUC1 for cluster formation, suggests that MUC1 clustering is essential in galectin-3-MUC1 interaction-induced EGFR activation.

Activation of EGFR and ERK by galectin-3-MUC1 interaction is inhibited by the EGFR inhibitor lapatinib

To further determine whether the effect of galectin-3-MUC1 interaction on ERK activation was indeed the consequence of EGFR activation, we tested the effect of Lapatinib, an EGFR phosphorylation inhibitor[35] on activation of EGFR and ERK in these cells. As shown above, the presence of EGF induced EGFR phosphorylation and the introduction of galectin-3 further increased EGF-induced EGFR phosphorylation of HCT116^{MUC1Full} (Fig 6A) and HCA1.7 cells (Fig 6B). The presence of Lapatinib inhibited EGFR phosphorylation in response to EGF of HCT116^{MUC1Full} and HCA1.7 cells and also abolished EGFR activation in these cells associated with galectin-3. A similar effect was observed for ERK1/2 phosphorylation in those cells, Lapatinib prevented activation of ERK1/2 phosphorylation in HCT116^{MUC1Full} (Fig 6C) and HCA1.7 cells (Fig 6D). As shown in the earlier part of this study, the presence of galectin-3C also did not show any effect on phosphorylation of either EGFR (Fig 6A and B) or ERK1/2 (Fig 6C and D). These results suggest that the increased phosphorylation of ERK1/2 by MUC1 expression and by MUC1-galectin-3 interaction (Fig 5) is the consequence of EGFR activation. It also further confirms that the effect of MUC1 and galectin-3-MUC1 interaction on EGFR activation effectively enhances downstream EGFR signalling.

Galectin-3-MUC1 interaction increases EGFR homo-/hetero-dimerization

In EGF-induced EGFR activation, an immediate event upon EGF binding is EGFR dimerization, which is then followed by EGFR auto-phosphorylation and internalization [36, 37, 38]. As the MUC1 extracellular domain appears to play a critical role in EGFR activation and as galectin-3-MUC1 interaction occurs on the cell surface (Fig 2-5), we speculated that the effect of galectin-3-MUC1 interaction on EGFR activation might be associated with an effect on EGFR dimerization. To test this, we treated the cells without or with EGF or galectin-3 and then with non-cleavable crosslinker BS3 before EGFR analysis by immunoblotting.

It was found that, as expected, treatment of the cells with EGF induced EGFR dimerization in the MUC1-positive HCT116MUC1Full (Fig 7A) and HCA1.7+ (Fig 7C) cells. EGFR dimerization was seen predominately as homo-dimers in HCT116^{MUC1Full} but hetero-dimers in HCA1.7+ cells in response to EGF. The presence of galectin-3 further increased EGFR dimerization in both cell types. Interestingly, galectin-3-induced EGFR dimerization occurred both homo- and hetero-dimers in HCT116^{MUC1Full} cells (Fig 7A) but predominately as homo-dimers in HCA1.7+ cells (Fig 7C). Consistent with the lack of influence on activation of EGFR and ERK in the MUC1-negative cells (Fig 2-4), EGF alone, or with galectin-3, showed little effect on EGFR dimerization in the HCT116^{MUC1neo} (Fig 7B) and HCA1.7- (Fig 7D) cells. Moreover, although the presence of full length galectin-3 increased EGFR dimerization (Fig 7A and B) and EGFR phosphorylation (Fig 3-6), the presence of the truncated galectin-3C did not show any effect on EGFR dimerization and the levels of EGFR homo-/hetero-dimers remained the same as the EGF alone-treated HCT116^{MUC1Full} (Fig 7A) and HCA1.7+ (Fig 7C) cells.

These results suggest that EGFR activation induced by galectin-3-MUC1 interaction is associated with promotion of EGFR dimerization. The lack of effect of the truncated galectin-

3C on EGFR dimerization in comparison to the full length galectin-3 in MUC1-postive cells further supports an essential role of galectin-3-induced MUC1 clustering in EGFR activation.

Galectin-3 increases interaction of MUC1 with EGFR

To gain further insight into the action of galectin-3-MUC1 interaction-mediated EGFR activation, we assessed interaction of MUC1 with EGFR in cells in response to EGF and galectin-3. It was found treatment of the cells with EGF did not have any effect on MUC1-EGFR interaction in comparison to control untreated cells (Fig 7E, first and second lanes). However, treatment of the cells with galectin-3, regardless of the presence or absence of EGF, resulted in increased co-immunoprecipitation of EGFR with MUC1 (Fig 7E, third and fourth lane) in comparison to the control untreated or the EGF-alone treated cells (first and second lane). This suggests that galectin-3-MUC1 interaction promotes physical interaction of MUC1 with EGFR and this increased MUC1-EGFR interaction likely represents a key component of galectin-3-associated EGFR activation.

As galectin-3 has been previously reported to be able to interact directly with EGFR[16, 39], we also assessed whether direct interaction of galectin-3 with EGFR is involved in this action. Minimal galectin-3 was found to be co-immunoprecipitated with EGFR in these cells (Fig 7F). In comparison to EGF alone-treated cells (Fig 7F, second lane), introduction of galectin-3 and EGF did not increase galectin-3 presence in EGFR immunoprecipitates (Fig 7F, third lane), thus not supporting a role of galectin-3-EGFR interaction in this action of EGFR activation.

Galectin-3 increases EGFR internalization

Following EGFR dimerization and auto-phosphorylation in response to ligand binding, EGFR internalization is an essential next step in EGFR signalling. In both HCT116^MUC1Full and HCA1.7 cells, EGFR appeared both on the cell surface and inside the cells (Fig 8A and B). Addition of EGF resulted in substantial loss of EGFR from the cell surface and its intra-cellular accumulation in both HCT116^MUC1Full (Fig 8A) and HCA1.7+ (Fig 8B) cells. MUC1 localization was uniformly spread on the cell surface and was not affected by the absence or presence of EGF. Introduction of galectin-3, as reported previously[11], induced change of MUC1 cell surface localization (as illustrated by disruption of the uniform localization). The presence of galectin-3 with EGF also increased EGFR internalization in comparison to the cells treated with EGF alone. It is noted that the internalized EGFR induced by galectin-3/EGF interaction was seen to occur in a more clustered pattern inside the cells than that induced by EGF alone. Introduction of galectin-3 without addition of EGF did not show any effect on EGFR localization compared to the control cells. This, together with the lack of effect of full length galectin-3 on EGFR activation in MUC1-negative cells and the lack of effect of truncated galectin-3C on EGFR activation in the MUC1-positive cells, indicates that galectin-3-mediated EGFR activation is associated with its effect on alteration of MUC1 cell surface localization.

Discussion

This study shows that EGF-induced EGFR activation is substantially increased by expression of the transmembrane mucin protein MUC1 in human breast and colon epithelial cells. Both the MUC1 intracellular and extracellular domains contribute to the effect of MUC1 on EGFR activation but the predominant influence comes from the MUC1 extracellular domain. Interaction of cell surface MUC1 with galectin-3 induces changes of MUC1 cell surface localization and increases MUC1-EGFR interaction. This leads to an increase of EGFR homo-/hetero-dimerization and subsequently increased EGFR activation and downstream signalling. This effect of galectin-3 occurs only with the full length but not the truncated galectin-3 form that lacks its N-terminal domain responsible for galectin-3-mediated receptor clustering. Thus, expression of MUC1 promotes EGFR activation and its interaction with galectin-3 enhances EGFR dimerization and activation in epithelial cancer cells.

MUC1 is a type I transmembrane mucin protein and is ubiquitously expressed on the surface of epithelial cells. Over-expression of MUC1 is a common feature of epithelial cancer cells[40]. MUC1 is reported to be associated with EGFR in epithelial cancer cells such as breast[28, 41], pancreatic[16], endometrial[14] and lung cells[42]. Blocking MUC1-C terminal dimerization with a cell-penetrating peptide[43] or siRNA silencing MUC1-C expression[44] has been shown to suppress EGFR activation-associated cell signalling and survival in non-small cell lung cancer cells. Interaction of MUC1 with EGFR in the nucleus of breast epithelial cancer cells was shown to promote accumulation of chromatin-bound EGFR and co-localization of EGFR with phosphorylated RNA polymerase II[28]. The present study shows that MUC1 expression increases EGF-induced EGFR activation in human breast and colon cancer cells. Depletion of either the MUC1 intracellular or extracellular domain could only partly abolish MUC1-associated effect on EGFR activation. This suggests that while both the MUC1 cytoplasmic

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and extracellular domains contribute to EGFR activation, some of the effect mediated by the MUC1 intra- and extra-cellular domain on EGFR activation is also relatively independent. The fact that depletion of the MUC1 extracellular domain resulted in greater reduction of MUC1-associated EGFR activation than depletion of the MUC1 intracellular domain indicates that the predominate influence of MUC1 on EGFR activation derives from its extracellular domain. MUC1[13] and EGFR[45] have both been shown to be associated with lipid rafts on cell membrane. It is possible that the expression of MUC1 and its association with EGFR in the lipid raft on the cell surface might increase the proximity of EGFR molecules in the microdomains of lipid raft for them to be in a better position to form homo-/hetero-dimers in response to ligand banding.

Binding of galectin-3 to cell surface MUC1 is seen in this study to markedly increase EGFR activation and this effect requires not only the galectin-3 C-terminal CRD domain but also its N-terminal ligand polarization domain. Interaction between MUC1 and full length galectin-3 is known to induce MUC1 cell surface polarization[11, 26, 27]. The effect of galectin-3 on MUC1 cell surface localization was indeed visible in this study, irrespective of the presence or absence of EGF (Fig 8). However, galectin-3 presence enhances EGFR activation only when EGF is also present (Fig 5, 6). This indicates that galectin-3 cannot activate EGFR without the presence of an EGFR ligand. MUC1 cell surface polarization induced by MUC1-galectin-3 interaction has shown previously to expose underlying smaller cell surface molecules[11, 26, 27]. The discovery that EGF showed much weaker effect on EGFR activation in the MUC1-negative than in the positive cells (Fig 2-5), irrespective of the presence of galectin-3, indicates that exposure of cell surface EGFR for easy EGF access is unlikely a mechanism of the MUC1-galectin-3 interaction-associated EGFR activation.

MUC1 co-immunoprecipitation showed a weak presence of EGFR in MUC1 immunoprecipitates but a substantial increase after addition of galectin-3, with or without the presence of EGF (Fig 7E). This, together with the discovery that the presence of galectin-3 alone did not induce EGFR dimerization, suggesting that galectin-3-MUC1 interaction is essential for galectin-3-associated, EGF-induced EGFR activation. The importance of galectin-3-mediated change of MUC1 cell surface localization in EGFR activation is supported by the discovery that the presence of a truncated form of galectin-3 (galectin-3C), which lacks the N-terminal domain responsible for galectin-3-induced ligand clustering hence could not induce MUC1 polarization, did not show any effect on EGFR dimerization and activation or ERK signalling, in the presence of EGF in MUC1-positive cells (Fig 5-7).

An earlier study has proposed formation of a bridge formed by galectin-3 between MUC1 and EGFR in cancer cells[44]. In our study, very minimal galectin-3 was co-immunoprecipitated with EGFR and addition of exogenous galectin-3 also showed no effect on EGFR association with galectin-3 and in the cells (Fig 7F). Addition of galectin-3 also did not show any effect on EGFR phosphorylation, EGFR dimerization, or ERK activation in the MUC1-positive cells in the absence of EGF, nor did it show any effect on EGFR activation in the MUC1-negative cells even in the presence of EGF (Fig 2-7). These indicate that a direct binding of galectin-3 to EGFR, even if it occurs, does not contribute to galectin-3-MUC1-associated EGFR dimerization and activation in those cells. It is noted that a recent study has reported a role of galectin-3 in promoting spheroid formation of lung cancer cells through activation of EGFR[39]. Although that study did not identify the galectin-3 binding ligand related to the effect, their discovery of the requirement of the galectin-3 carbohydrate recognition domain in its effect is broadly in keeping with an effect of galectin-3-MUC1

interaction on EGFR activation, which requires galectin-3 CRD domain, shown in the present study.

In this study, EGF was seen to retain certain degree of its ability to induce EGFR activation, although to a less degree in comparison to full-length MUC1-expressing cells, in both MUC1-extracellular and -intracellular domain depleted cells (Fig 2 and 3). This suggests that the MUC1-extracellular and intracellular domains can have independent actions in MUC1-associated EGFR activity. This is in keeping with previous studies showing that the MUC1 extracellular[43] and cytoplasmic[44] domains can either interact with EGFR and affect EGFR activity. Interestingly, although our cell surface cross-linking experiments using BS3 revealed strong induction of EGFR dimerization in cell response to EGF and galectin-3, higher molecular weight MUC1-EGFR complex were not observed (Fig 7). MUC1 is a highly glycosylated protein with carbohydrates accountable for >50% of its molecular weight[1]. The long and complex sugar chains of MUC1 are highly likely involved in the cell surface MUC1-EGFR interaction hence the crosslinker used in this study, which crosslinks protein-protein, might not be able to effectively crosslink MUC1-EGFR on the cell surface.

It was found in this study that the contribution of endogenous cell surface galectin-3 to EGFR activation is minimal in this cell culture setting. The in vitro cell culture model in this study was very short term (one day culture in normal medium plus overnight culture in fresh serum-free medium). Our previous study has shown that galectin-3 secretion in these cells in such a short term cell culture condition is minimal[27]. In cancers, however, galectin-3 is typically constantly secreted and could reach higher levels which would impact on EGFR activation by interaction with cancer-associated MUC1. Moreover, the concentration of exogenous galectin-3 used in this study is close to the pathological level of circulating

galectin-3 in metastatic cancer patients shown in our previous study[24]. The impact of exogenous galectin-3 on EGFR activation via interaction with MUC1 reported in this study will therefore be particularly relevant to circulating tumour cells present during metastasis.

EGFR activation on the cell membrane is known to trigger activation of an array of intracellular signalling pathways[17] including commonly Ras/MEK/ERK signalling activation. EGFR activation induced by either MUC1 expression or by MUC1-galectin-3 interaction is shown in this study to be accompanied by an increase of ERK activation. It is noted that in addition to increase EGFR activation, galectin-3-MUC1 interaction also causes a prolonged activation of EGFR and ERK activation (Fig 5). It is generally believed that EGFR activation is terminated primarily through endocytosis of the receptor-ligand complex which are either degraded in the endosomes or recycled to the cell surface. It has been reported that if recycled EGFR is unable to reach the cell surface or to the lysosomal compartment but accumulates in the early endosomes, it will lead to prolonged signalling and increased activation of ERK[46]. This does seem to be supported in our study. We found that following EGFR activation, more EGFR was seen to be located in a clustered pattern inside the cells in the galectin-3/EGF treated cells than in EGF alone treated cells (Fig 8). There was a much weaker EGFR cell surface localization in the galectin-3/EGFR treated cells than in the other groups including EGF-alone treated cells. This indicates that the galectin-3/MUC1-mediated EGFR activation and subsequent EGFR endocytosis is associated with slower recycling of EGFR to the cell surface. This may provide an explanation for the prolonged activation of EGFR and ERK in those cells (Fig 5). This is also in keeping with an earlier study showing that MUC1 expression inhibits EGFR degradation in response to ligand binding but was accompanied by an increase of EGFR internalization in breast epithelial cells[47].

We noted that the presence of EGFR phosphorylation inhibitor lapatinib completely inhibited EGFR phosphorylation (Fig 6A and B) but detectable level, similar as the controls, of ERK activity remained in the cells irrespective of the presence or absence of EGF (Fig 6C and D). This indicates that endogenous, non-EGFR-related ERK activity exists in the cells. ERK is a one of the vital signalling pathways in cell proliferation and is known to be regulated by a variety of growth factors and molecules[48]. Expressions of either galectin-3[16, 32, 49] or MUC1[50] in cancer cells has been shown to induce ERK activation. It is most likely therefore the inability of lapatinib to completely inhibit ERK activity in the cells is due to the existence of non-EGFR-related actions of endogenous molecules such as galectin-3, MUC1 or other molecules expressed or secreted by the cells.

EGFR represents a key therapeutic target for cancer treatment. Development of anti-EGFR strategies is a crucial area of clinical study for the treatment of solid tumours. Currently, main strategies include monoclonal antibodies directed towards the extracellular domain of EGFR, small molecule tyrosine kinase inhibitors targeting the catalytic kinase domain of EGFR and strategies to disrupt receptor trafficking to the cell surface. The discovery in this study that the expression of MUC1 and its interaction with galectin-3 promotes ligand-dependent EGFR activation has implications in EGFR-targeted therapies in cancer treatment. MUC1 and galectin-3 are both well known to be commonly over-expressed by solid tumours. Over-expressions of MUC1 and galectin-3 and their increased interaction on EGFR activation may therefore not only have an influence on EGFR-mediated tumourigenesis and cancer progression, but may also have an impact on the effectiveness of EGFR-targeted therapy. For example, a closer localization of EGFR with MUC1 on the cell membrane induced by galectin-3-MUC1 interaction may limit the access of anti-EGFR antibodies to cell surface EGFR due to the massive size of MUC1 that easily protrudes over EGFR on the cell surface.

A slower recycling of EGFR to the cell surface induced by galectin-3-MUC1 interaction may also limit the treatment effectiveness of anti-EGFR antibody as well as kinase inhibitors. It is possible therefore that a combined therapy that targets EGFR as well as galectin-3 or MUC1 may improve treatment effectiveness in patients who have higher expressions of galectin-3 and MUC1.

Thus, MUC1 expression and its interaction with galectin-3 on cell surface both make important contribution to EGFR activation in epithelial cancer cells by promoting ligand-induced EGFR dimerization and activation. As over-expression of MUC1 and over-expression of galectin-3 are both common in epithelial cancer cells, the influence of MUC1 expression and cell surface MUC1-galectin-3 interaction on EGFR activation likely makes important contribution to EGFR-associated tumorigenesis and tumour progression and also to the treatment effectiveness of EGFR-targeted therapy.

Materials and methods

Materials

Antibodies against p-EGFR (SC-23420) EGFR (SC-03), p-ERK1/2(SC-7383) and ERK1/2 (SC-94), Protein A/G plus agarose beads were purchased from Santa Cruz Biotechnology, Santa Cruz, CA. Anti-EGFR antibody used in confocal microscopy and for immunoprecipitation (DB81) was purchased from New England Bio-Labs, Ipswich, MA. Anti-EGFR antibody (500-p306) and recombinant human EGF (AF-100-15) was from PeproTech rocky Hill, NJ. Bis(sulfosuccinimidyl) substrate (BS3) crosslinker was purchased from Life Technology Ltd, Waltham, MA. Lapatinib was purchased from Sigma-Aldrich. B27.29 anti-MUC1 antibody was kindly provided by Dr. Mark Reddish (Biomira, Edmonton, Canada) and CT2 anti-MUC1 antibody were kindly provided by Prof Sandra Gendler (Mayo Clinic, Scottsdale, AR).

Cell lines

Human colon cancer HCT116 cells were obtained from European Collection of Cell Cultures (Salisbury, UK) and were cultured in McCoy's 5a medium. MUC1-positive transfectants (HCA1.7+), MUC1 negative revertants (HCA1.7-) and MUC1 cytoplasmic domain-depleted MUC1 mutant (HTDΔCT) cells from human breast HBL-100 epithelial cells were described previously[5]. The cell lines were last authenticated by DNA profiling (DNA Diagnostics Centre, London, UK) in 2014.

MUC1 transfection

MUC1 expression vectors for full-length MUC1, the extracellular domain-depleted MUC1 (MUC1ΔTR), the cytoplasmic domain-depleted MUC1 (MUC1ΔCT) and control vector were kindly provided by Prof Tony Hollingsworth (University of Nebraska Medical Centre).

MUC1-expressing or control vector was pre-mixed with DNA Diluent and hydrated GenePOORTER-2 transfection reagent in serum-free medium for 10 min before addition to HCT116 cells in antibiotics-free and serum-containing DMEM in 24-well plates for 24 h at 37 °C. The culture medium was replaced with serum-containing medium for 48 h before the cells were cultured in normal medium containing 600 μg/ml G418 for 7–10 days at 37 °C. Single-cell clones were selected with Cell Cloning Cylinders, proliferated and analysed for MUC1 expression by immunoblotting with anti-MUC1 antibodies B27.29 and CT2.

Production of full length and truncated forms of recombinant galectin-3

The cDNA sequence encoding full length human galectin-3 (Gal-3F) and C-terminal carbohydrate recognition domain (CRD) of galectin-3 (Gal-3C) (residues 116-250) were cloned into pETm11 expression vector with a His-tag. The recombinant plasmids were transformed into Bl21(DE3) *E.coli* and the transformants were selected with kanamycin. The protein expression was induced using 1mM IPTG when the cell density (OD_{600}) reached approximately 0.6-0.85. Following induction, cells were incubated overnight at 18ºC before harvested by centrifugation. The cells were lysed in the presence of DNase using high pressure cell homogeniser. After centrifugation, the supernatant was applied onto a HisTrap FF 5ml column (GE Healthcare) and the His-tagged proteins were eluted with 150mM Imidazole. The collected fractions containing galectin-3 were incubated overnight with TEV protease to cleave the His tag and dialysed against His Trap buffer without Imidazole. After performing Reverse His Trap to remove the cleaved His tag and TEV protease from galectin-3 solution, the proteins were further purified by size exclusion chromatography using Superdex 75 26/60 column. The purified Gal-3C was eluted between 220 and 260 ml and the Gal-3F between 190 and 220 ml. Purify of the recombinant proteins was determined by SDS-PAGE to be >95%.

Immunoprecipitation

Sub-confluent cells were incubated in serum-free medium containing 0.5mg/ml BSA overnight. The cells were washed with TBS and then incubated with EGF (20ng/ml), or EGF (20ng/ml) and galectin-3 (2ug/ml), or galectin-3 (2µg/ml) or 20 ng/ml BSA (control) in serum free media for 10 min at 37^0 C. The cells were washed with ice cold PBS, scraped and lysed on ice in PBS containing 1% Triton-X-100 and protease inhibitors (Calbiochem) for 30 min before centrifugation at 10,000g at 4^0C for 15 minute. The supernatants were collected and pre-cleared by adding 20µl of the protein-A/G beads and incubating at 4^0C for 30 minutes with gentle agitation. One ml lysates (protein concentration 2mg/ml) were incubated with anti-MUC1 (B27.29, 1 µg/ml), anti-EGFR (DB81) (2 µg/ml) antibody or isotype-matched normal IgG at 4^0C with continuous agitation for 16 hours. Thirty µl of protein- A/G plus agarose beads were added for 4 hr and the beads were washed five times with ice cold PBS. Proteins were eluted from the beads by boiling in SDS-sample buffer for 10 minutes before application to SDS-PAGE and subsequent immunoblotting

Immunoblotting

Cellular proteins (cell lysate or immunoprecipitates) separated by SDS-PAGE were electro-transferred to nitrocellulose membrane. The membranes were first incubated with specific primary antibodies [anti-p-EGFR (SC-23420), EGFR (SC-03), anti-pERK (SC-7383) and ERK (SC-94) at a concentration of 1:500. Antibodies against MUC1 (B27.29, CT2) or actin at a concentration of 1:5000 were applied for 16 hr at 4^0C. The blots were washed 3 times with 0.05% Tween-20 in TBS before incubated with peroxidase-conjugated secondary antibody (1: 3000) for 1 hour. After 6 washes with 0.05% Tween-20 in TBS, the protein bands were developed using chemiluminescence Super Signal kit and visualized with

Molecular Imager® Gel Doc™ XR System (BioRad). The density of the protein bands was quantified using Imagelab version 3.0.1.

EGFR activation

Sub-confluent cells were incubated in serum-free medium containing 0.5mg/ml BSA overnight. The cells were washed with PBS before incubation with EGF (20ng/ml), EGF (20ng/ml) and galectin-3 (2ug/ml), galectin-3 (2 μg/ml), galectin-3C (2μg/ml) or BSA (2 μg/ml) (control) in the absence or presence of EGFR inhibitor lapatinib (2mM) for various time at 37^0C and 5% CO_2. In some experiments, the cells were first incubated with 100mM lactose or PBS for 30 min before washing and application of EGF(20ng/ml) or EGF (20ng/ml) plus galectin-3 (2ug/ml) for various time at 37^0C. The cells were washed immediately with ice cold TBS before lysed with SDS-sample buffer and analysed by immunoblotting.

Cell surface protein crosslinking

Sub-confluent cells were incubated in serum-free medium overnight. The cells were washed twice with Ca^{2+} and MG^{2+} free PBS and then treated with serum free media containing BSA 2μg/ml (control), EGF (20 ng/ml) without or with galectin-3 (2 μg/ml) or galectin-3C (2 μg/ml) for 10 minute at 37^0 C and 5% CO2. The cells were then washed with ice cold Ca^{2+} and MG^{2+} free PBS and incubated with 3mM BS3 crosslinker in Ca^{2+} and MG^{2+} free PBS on ice for 20 minute. Excess BS3 was quenched with 250mM glycine in PBS for 5 minutes at 4^0 C. The cells were washed three times with ice cold PBS, lysed in SDS- sample buffer and analysed by immunoblotting with antibodies against EGFR.

Confocal microscopy

Sub-confluent cells grown on glass coverslips in 24-well plates were incubated in serum-free at 37C overnight. The cells were treated with BSA (2 µg/ml) (control), EGF (20 ng/ml) without or with galectin-3 (2ug/ml) for 10 minute at 37^0 C. The cells were washed with ice cold PBS and fixed with 4% paraformaldehyde. The cells were then washed again with PBS and probed with anti-MUC1 B27.29 (1 µg /ml) or anti-EGFR (D38B1) (2 µg/ml) for 2 hours at room temperature. After two washes with PBS, FITC-conjugated anti-mouse or Alexa fluor 643 conjugated anti-rabbit antibodies were applied for 1 hour at room temperature. The cells were washed twice before being mounted using DAPI-containing fluorescent mounting media (Vector Laboratories, Burlingame,CA). The slides were analysed using a 3i confocal microscope (Marianas SDC, 3i Imaging) and Slidebook 6 Reader version 6.0.4 (Intelligent-imaging).

Conflict of interest: the authors declare no conflict of interest

<center>**Figure legends**</center>

Fig 1. Generation of MUC1-expressing and mutant cells. (A) Schematic diagram of MUC1 transfectants. MUC1 expression in the transfectants of human colon cancer HCT116 **(B)** the human breast epithelial HBL-100 **(C)** cells were analysed by immunoblotting with anti-MUC1 antibodies against the MUC1 extracellular domain (B27.27) and intracellular domain (CT2). The blots were also probed with anti-actin antibody for protein loading.

Fig 2. Both MUC1 extra- and intra-cellular domains influence EGF-induced EGFR activation. MUC1 transfectants of human colon (A) and breast **(B)** epithelial cells were treated with 20 ng/ml EGF for various times before EGF/EGFR phosphorylation were analysed by immunoblotting. The blots were also probed with anti-actin antibody for protein loading. Densitometry scanning of the bands from three independent experiments is shown in C and D and is expressed as ratio p-EGFR/EGF (mean ± SEM)**.** The cells transfected with full-length MUC1 showed rapid EGFR phosphorylation while the MUC1 negative cells showed little response. Depletion of the MUC1 extracellular domain largely reduced, while depletion of the MUC1 intracellular domain moderately reduced, EGFR phosphorylation in comparison to the cells express full-length MUC1. Representative blots are shown in A and B.

Fig 3. Galectin-3-MUC1 interaction promotes EGRF activation in human breast epithelial cells. HCA1.7+ **(A),** HCA1.7-**(B)** and HTD(ΔCT) **(C)** cells were treated with EGF in the presence or absence of galectin-3 for various time before analysed by immunoblotting with antibodies against p-EGFR, EGFR and actin. Galectin-3 treatment increased EGFR activation of the full-length MUC1 transfectants HCA1.7+ and the MUC1 cytoplasmic domain-depleted transfectants HTD(ΔCT), but not of the MUC1-negative revertants HCA1.7.

<center>27</center>

Densitometry scanning of the bands from three independent experiments is shown in **D-F** and is expressed as ratio p-EGFR/EGF (mean ± SEM)**.** Representative blots are shown in **A, B** and **C**.

Fig 4. Galectin-3-MUC1 interaction enhances EGRF activation in human colon cancer cells. MUC1-expressing HCT116^{MUC1Full} **(A)** and MUC1-negative HCT116^{MUC1neo} **(B)** transfectants were treated with EGF in the absence or presence of galectin-3 for various time before analysed by immunoblotting with antibodies against p-EGFR, EGFR and actin. Galectin-3 treatment increased EGFR activation only in the MUC1-expressing but not MUC1-negative cells. In C and D, HCA1.7+ and HCT116^{MUC1Full} cells were pre-treated with 100mM lactose or PBS before introduction of EGF 20ng/ml and 2µg/ml galectin-3 for various time and subsequent analysis of EGFR phosphorylation and EGFR expression by immunoblotting. Densitometry scanning of the bands from three independent experiments is shown in **E-H** and is expressed as ratio p-EGFR/EGF (mean ± SEM)**.** Representative blots are shown in A-D.

Fig 5. MUC1 expression- as well as MUC1-galectin-3 interaction-associated EGFR activation increases ERK activation. MUC1-expressing HCA1.7+ **(A)** and HCT116^{MUC1Full} **(C)**, and MUC1-negative HCA1.7**- (B)** and HCT116^{MUC1neo} **(D)** cells were treated with either 20ng/ml EGF, 20 ng/ml EGF and 2µg/ml galectin-3, 2 µg/ml galectin-3 or 2µg/ml galectin-3C for various times as in Fig3 and 4 before the expression of p-ERK1/2 and ERK1/2 were analysed by immunoblotting. EGF treatment increases ERK1/2 phosphorylation in the MUC1-expressing HCA1.7+ and HCT116^{MUC1Full} cells. Introduction of galectin-3, but not galectin-3C, further enhances ERK1/2 activation in the MUC1-expressing cells but not in the MUC1-negative cells. Representative blots from three independent experiments are shown.

Fig 6. Lapatinib inhibits EGFR and ERK activation induced by MUC1-galectin-3 interaction. HCT116^{MUC1Full} (A and C) and HCA1.7+ (B and D) cells were treated with and without EGF in the absence or presence of galectin-3, galectin-3C, EGFR inhibitor lapatinib for 10 min before analysed by immunoblotting with antibodies against p-EGFR, EGFR (A and B) or pERK1/2 and ERK1/2 (C and D). Densitometry analysis of the bands from two independent experiments was quantified and was presented as percentage changes of p-EGFR/EGF and p-ERK1/2/ERK1/2, respectively, in comparison to the controls.

Fig 7. Galectin-3-MUC1 interaction promotes EGFR dimerization and MUC1-EGFR interaction. HCT116 ^{MUC1Full} (A), HCA1.7+ (C), HCT116$^{MUC1 neo}$ (B) and HC1.7- (D) were treated with and without EGF in the absence or presence of galectin-3 or galectin-3C for 10 minutes before EGFR dimerization were analysed using BS3 cross linker and immunoblotting. The presence of galectin-3, but not galectin-3C, increased EGFR homo- and hetero-dimerization in the MUC1-expressing, but not MUC1-negative, cells. HCA1.7+ (E) or HCT116^{MUC1Full} (F) cells were treated with PBS (control), EGF with or without galectin-3 for 10 minute followed by immunoprecipitation of the cells with B27.29 anti-MUC1 antibody (E) or anti-EGFR antibody (F). The immunoprecipitates were analysed by immunoblotting with anti-EGFR, anti-MUC1 (B27.29) or anti-galectin-3 antibody. More EGFR was co-immunoprecipitated with MUC1 in cells treated with galectin-3 regardless of the presence of EGF (E). No difference of galectin-3 levels in the EGFR immunoprecipitates between cells treated with EGF and EGF plus galectin-3 (F).

Fig 8. Galectin-3-MUC1 interaction enhances EGFR internalization. HCT116^{MUC1Full} **(A)** and HCA1.7+ **(B)** cells were treated with PBS (control), EGF with or without galectin-3

for 10 minutes before localization of MUC1 (green) and EGFR (red) were determined by fluorescent immunohistochemistry and analysed by confocal microscopy. The cell nucleus was stained with DAPI (blue). Galectin-3 changes MUC1 cell surface localization (as illustrated by disruption of uniform MUC1 localization). More intense and clustered EGFR localization inside the cells were seen in the galectin-3 treated cells than in the EGF alone treated cells in both cell types. Representative images are shown.

References

1. Taylor-Papadimitriou J, Burchell J, Miles DW, Dalziel M. MUC1 and cancer. *Biochim Biophys Acta* 1999, **1455**(2-3)**:** 301-313.

2. Singh PK, Hollingsworth MA. Cell surface-associated mucins in signal transduction. *Trends Cell Biol* 2006, **16**(9)**:** 467-476.

3. Wei X, Xu H, Kufe D. Human MUC1 oncoprotein regulates p53-responsive gene transcription in the genotoxic stress response. *Cancer Cell* 2005, **7**(2)**:** 167-178.

4. Bafna S, Kaur S, Batra SK. Membrane-bound mucins: the mechanistic basis for alterations in the growth and survival of cancer cells. *Oncogene* 2010, **29**(20)**:** 2893-2904.

5. Wesseling J, van der Valk SW, Hilkens J. A mechanism for inhibition of E-cadherin-mediated cell-cell adhesion by the membrane-associated mucin episialin/MUC1. *Mol Biol Cell* 1996, **7**(4)**:** 565-577.

6. Hilkens J, Ligtenberg MJL, Vos HL, Litvinov SV. Cell membrane-associated mucins and their adhesion-modulating property. *Trend Biochem Sci* 1992, **17**(9)**:** 359-363.

7. Yu LG. The oncofetal Thomsen-Friedenreich carbohydrate antigen in cancer progression. *Glycoconj J* 2007, **24**(8)**:** 411-420.

8. Byrd JC, Bresalier RS. Mucins and mucin binding proteins in colorectal cancer. *Cancer Metastasis Rev* 2004, **23**(1-2)**:** 77-99.

9. Beatson RE, Taylor-Papadimitriou J, Burchell JM. MUC1 immunotherapy. *Immunotherapy* 2010, **2**(3)**:** 305-327.

10. Kufe DW. MUC1-C oncoprotein as a target in breast cancer: activation of signaling pathways and therapeutic approaches. *Oncogene* 2013, **32**(9)**:** 1073-1081.

11. Yu LG, Andrews N, Zhao Q, McKean D, Williams JF, Connor LJ*, et al.* Galectin-3 interaction with Thomsen-Friedenreich disaccharide on cancer-associated MUC1 causes increased cancer cell endothelial adhesion. *J Biol Chem* 2007, **282**(1)**:** 773-781.

12. Ahmad R, Raina D, Joshi MD, Kawano T, Ren J, Kharbanda S*, et al.* MUC1-C oncoprotein functions as a direct activator of the nuclear factor-kappaB p65 transcription factor. *Cancer Res* 2009, **69**(17)**:** 7013-7021.

13. Haddon L, Hugh J. MUC1-mediated motility in breast cancer: a review highlighting the role of the MUC1/ICAM-1/Src signaling triad. *Clin Exp Metastasis* 2015, **32**(4)**:** 393-403.

14. Engel BJ, Bowser JL, Broaddus RR, Carson DD. MUC1 stimulates EGFR expression and function in endometrial cancer. *Oncotarget* 2016.

15. Neeraja D, Engel BJ, Carson DD. Activated EGFR stimulates MUC1 expression in human uterine and pancreatic cancer cell lines. *J Cell Biochem* 2013, **114**(10)**:** 2314-2322.

16. Merlin J, Stechly L, de Beauce S, Monte D, Leteurtre E, van Seuningen I*, et al.* Galectin-3 regulates MUC1 and EGFR cellular distribution and EGFR downstream pathways in pancreatic cancer cells. *Oncogene* 2011, **30**(22)**:** 2514-2525.

17. Normanno N, De Luca A, Bianco C, Strizzi L, Mancino M, Maiello MR*, et al.* Epidermal growth factor receptor (EGFR) signaling in cancer. *Gene* 2006, **366**(1)**:** 2-16.

18. Hynes NE, Lane HA. ERBB receptors and cancer: the complexity of targeted inhibitors. *Nat Rev Cancer* 2005, **5**(5)**:** 341-354.

19. Mendelsohn J, Baselga J. The EGF receptor family as targets for cancer therapy. *Oncogene* 2000, **19**(56)**:** 6550-6565.

20. Umekita Y, Ohi Y, Sagara Y, Yoshida H. Co-expression of epidermal growth factor receptor and transforming growth factor-alpha predicts worse prognosis in breast-cancer patients. *Int J Cancer* 2000, **89**(6)**:** 484-487.

21. Swinson DE, Cox G, O'Byrne KJ. Coexpression of epidermal growth factor receptor with related factors is associated with a poor prognosis in non-small-cell lung cancer. *Br J Cancer* 2004, **91**(7)**:** 1301-1307.

22. Rubin Grandis J, Melhem MF, Gooding WE, Day R, Holst VA, Wagener MM*, et al.* Levels of TGF-alpha and EGFR protein in head and neck squamous cell carcinoma and patient survival. *J Natl Cancer Inst* 1998, **90**(11)**:** 824-832.

23. Liu FT, Rabinovich GA. Galectins as modulators of tumour progression. *Nat Rev Cancer* 2005, **5**(1)**:** 29-41.

24. Barrow H, Guo X, Wandall HH, Pedersen JW, Fu B, Zhao Q*, et al.* Serum galectin-2, -4, and -8 are greatly increased in colon and breast cancer patients and promote cancer cell adhesion to blood vascular endothelium. *Clin Cancer Res* 2011, **17**(22)**:** 7035-7046.

25. Newlaczyl AU, Yu LG. Galectin-3-A jack-of-all-trades in cancer. *Cancer Letters* 2011, **313**(2)**:** 123-128.

26.   Zhao Q, Barclay M, Hilkens J, Guo X, Barrow H, Rhodes JM*, et al.* Interaction between circulating galectin-3 and cancer-associated MUC1 enhances tumour cell homotypic aggregation and prevents anoikis. *Mol Cancer* 2010, **9:** 154.

27.   Zhao Q, Guo X, Nash GB, Stone PC, Hilkens J, Rhodes JM*, et al.* Circulating galectin-3 promotes metastasis by modifying MUC1 localization on cancer cell surface. *Cancer Res* 2009, **69**(17)**:** 6799-6806.

28.   Bitler BG, Goverdhan A, Schroeder JA. MUC1 regulates nuclear localization and function of the epidermal growth factor receptor. *J Cell Sci* 2010, **123**(Pt 10)**:** 1716-1723.

29.   Engel BJ, Bowser JL, Broaddus RR, Carson DD. MUC1 stimulates EGFR expression and function in endometrial cancer. *Oncotarget* 2016, **7**(22)**:** 32796-32809.

30.   Guddo F, Giatromanolaki A, Koukourakis MI, Reina C, Vignola AM, Chlouverakis G*, et al.* MUC1 (episialin) expression in non-small cell lung cancer is independent of EGFR and c-erbB-2 expression and correlates with poor survival in node positive patients. *J Clin Pathol* 1998, **51**(9)**:** 667-671.

31.   Iurisci I, Tinari N, Natoli C, Angelucci D, Cianchetti E, Iacobelli S. Concentrations of galectin-3 in the sera of normal controls and cancer patients. *Clin Cancer Res* 2000, **6**(4)**:** 1389-1393.

32.   Mori Y, Akita K, Yashiro M, Sawada T, Hirakawa K, Murata T*, et al.* Binding of Galectin-3, a beta-Galactoside-binding Lectin, to MUC1 Protein Enhances Phosphorylation of Extracellular Signal-regulated Kinase 1/2 (ERK1/2) and Akt, Promoting Tumor Cell Malignancy. *J Biol Chem* 2015, **290**(43)**:** 26125-26140.

33.   Oda K, Matsuoka Y, Funahashi A, Kitano H. A comprehensive pathway map of epidermal growth factor receptor signaling. *Mol Sys Biol* 2005, **1:** 2005.0010-2005.0010.

34.   Pierce KL, Tohgo A, Ahn S, Field ME, Luttrell LM, Lefkowitz RJ. Epidermal growth factor (EGF) receptor-dependent ERK activation by G protein-coupled receptors: a co-culture system for identifying intermediates upstream and downstream of heparin-binding EGF shedding. *J Biol Chem* 2001, **276**(25)**:** 23155-23160.

35.   Montemurro F, Valabrega G, Aglietta M. Lapatinib: a dual inhibitor of EGFR and HER2 tyrosine kinase activity. *Exp Opin Biol Ther* 2007, **7**(2)**:** 257-268.

36.   Bessman NJ, Bagchi A, Ferguson KM, Lemmon MA. Complex relationship between ligand binding and dimerization in the epidermal growth factor receptor. *Cell Rep* 2014, **9**(4)**:** 1306-1317.

37.   Blakely BT, Rossi FMV, Tillotson B, Palmer M, Estelles A, Blau HM. Epidermal growth factor receptor dimerization monitored in live cells. *Nat Biotech* 2000, **18**(2)**:** 218-222.

38.   Dawson JP, Berger MB, Lin CC, Schlessinger J, Lemmon MA, Ferguson KM. Epidermal growth factor receptor dimerization and activation require ligand-induced conformational changes in the dimer interface. *Mol Cell Biol* 2005, **25**(17)**:** 7734-7742.

39.   Kuo HY, Hsu HT, Chen YC, Chang YW, Liu FT, Wu CW. Galectin-3 modulates the EGFR signalling-mediated regulation of Sox2 expression via c-Myc in lung cancer. *Glycobiology* 2016, **26**(2)**:** 155-165.

40.   Gaemers IC, Vos HL, Volders HH, van der Valk SW, Hilkens J. A stat-responsive element in the promoter of the episialin/MUC1 gene is involved in its overexpression in carcinoma cells. *J Biol Chem* 2001, **276**(9)**:** 6191-6199.

41.   Schroeder JA, Thompson MC, Gardner MM, Gendler SJ. Transgenic MUC1 interacts with epidermal growth factor receptor and correlates with mitogen-activated protein kinase activation in the mouse mammary gland. *J Biol Chem* 2001, **276**(16)**:** 13057-13064.

42.   Kato K, Lillehoj EP, Kim KC. Pseudomonas aeruginosa stimulates tyrosine phosphorylation of and TLR5 association with the MUC1 cytoplasmic tail through EGFR activation. *Inflamm Res* 2016, **65**(3)**:** 225-233.

43.   Kharbanda A, Rajabi H, Jin C, Tchaicha J, Kikuchi E, Wong KK*, et al.* Targeting the oncogenic MUC1-C protein inhibits mutant EGFR-mediated signaling and survival in non-small cell lung cancer cells. *Clin Cancer Res* 2014, **20**(21)**:** 5423-5434.

44.   Ramasamy S, Duraisamy S, Barbashov S, Kawano T, Kharbanda S, Kufe D. The MUC1 and galectin-3 oncoproteins function in a microRNA-dependent regulatory loop. *Mol Cell* 2007, **27**(6)**:** 992-1004.

45.   Midgley AC, Rogers M, Hallett MB, Clayton A, Bowen T, Phillips AO*, et al.* Transforming growth factor-beta1 (TGF-beta1)-stimulated fibroblast to myofibroblast differentiation is mediated by hyaluronan (HA)-facilitated epidermal growth factor receptor (EGFR) and CD44 co-localization in lipid rafts. *J Biol Chem* 2013, **288**(21)**:** 14824-14838.

46.   Er EE, Mendoza MC, Mackey AM, Rameh LE, Blenis J. AKT facilitates EGFR trafficking and degradation by phosphorylating and activating PIKfyve. *Sci Signal* 2013, **6**(279)**:** ra45.

47.   Pochampalli MR, el Bejjani RM, Schroeder JA. MUC1 is a novel regulator of ErbB1 receptor trafficking. *Oncogene* 2007, **26**(12)**:** 1693-1701.

48.   Fang JY, Richardson BC. The MAPK signalling pathways and colorectal cancer. *Lancet Oncol* 2005, **6**(5)**:** 322-327.

49.   Song S, Ji B, Ramachandran V, Wang H, Hafley M, Logsdon C*, et al.* Overexpressed galectin-3 in pancreatic cancer induces cell proliferation and invasion by binding Ras and activating Ras signaling. *PloS One* 2012, **7**(8)**:** e42699.

50.   Alam M, Ahmad R, Rajabi H, Kharbanda A, Kufe D. MUC1-C oncoprotein activates ERK-->C/EBPbeta signaling and induction of aldehyde dehydrogenase 1A1 in breast cancer cells. *J Biol Chem* 2013, **288**(43)**:** 30892-30903.

Fig 1

A Extracellular domain | Cytoplasmic domain

VNTR region | Trans-membrane domain

MUC1Full (HCA1.7+, HCT116^MUC1Full)

MUC1ΔTR (HCT116^MUC1ΔTR)

MUC1ΔCT [HTD(ΔCT), HCT116^MUC1ΔCT]

B

HCT116^MUC1Neo
HCT116^MUC1Full
HCT116^MUC1ΔCT
HCT116^MUC1ΔTR

MW (KDa)

250

30

40

MUC1-N (B27.29)

MUC1-C (CT2)

Actin

C

HCA1.7+ (MUC1)
HCA1.7- (MUC1neo)
HTD^(MUC1ΔCT)

MW (KDa)

250

30

40

MUC1-N (B27.29)

MUC1-C (CT2)

Actin

Fig 2

A

HCT116^{MUC1Full} +EGF

150kDa- p-EGFR
         EGFR
37kDa-   Actin

HCT116^{MUC1NE0} +EGF

150kDa- p-EGFR
         EGFR
37kDa-   Actin

HCT116^{MUC1ΔCT} +EGF

150kDa- p-EGFR
         EGFR
37kDa-   Actin

HCT116^{MUC1ΔTR} +EGF

150kDa- p-EGFR
         EGFR
37kDa-   Actin

0   5   10   30   60   90   120 (min)

B

HCA1.7+ +EGF

150kDa- p-EGFR
         EGFR
37kDa-   Actin

HCA1.7- +EGF

150kDa- p-EGFR
         EGFR
37kDa-   Actin

HTD(ΔCT) +EGF

150kDa- p-EGFR
         EGFR
37kDa-   Actin

0   5   10   30   60   90   120 (min)

C

Ratio pEGFR:EGFR vs Minutes

- MUC1 Neo
- MUC1 Full
- MUC1 ΔCT
- MUC1 ΔTR

D

Ratio pEGFR:EGFR vs Minutes

- HCA1.7-
- HCA1.7+
- HTD ΔCT

Fig 3

A HCA1.7+

+EGF
150kDa- — p-EGFR
— EGFR
37kDa- — actin

+EGF+Gal3
150kDa- — p-EGFR
— EGFR
37kDa- — actin

+Gal3
150kDa- — p-EGFR
— EGFR
37kDa- — actin

0 5 10 30 60 90 120 150 (Min)

B HCA1.7-

+EGF
150kDa- — p-EGFR
— EGFR
37kDa- — actin

+EGF+Gal3
150kDa- — p-EGFR
— EGFR
37kDa- — actin

+Gal3
150kDa- — p-EGFR
— EGFR
37kDa- — actin

0 5 10 30 60 90 120 150 (Min)

C HTD (ΔCT)

+EGF
150kDa- — p-EGFR
— EGFR
37kDa- — actin

+EGF+Gal3
150kDa- — p-EGFR
— EGFR
37kDa- — actin

+Gal3
150kDa- — p-EGFR
— EGFR
37kDa- — actin

0 5 10 30 60 90 120 150 (Min)

D
Ratio pEGFR:EGFR
····◆···· HCA1.7+ + EGF
– ● – HCA1.7+ + Gal3
—■— HCA1.7+ + EGF + Gal3
Minutes

E
Ratio pEGFR:EGFR
····◆···· HCA1.7- + EGF
– ● – HCA1.7- + Gal3
—■— HCA1.7- + EGF + Gal-3
Minutes

F
Rato pEGFR:EGFR
····◆···· HTD ΔCT + EGF
– ● – HTD ΔCT + Gal3
—■— HTD ΔCT + EGF +Gal3
Minutes

Fig 4

A HCT116^MUC1FULL

+EGF
150kDa- .... p-EGFR
.... EGFR

+EGF +Gal3
150kDa- .... p-EGFR
.... EGFR

+Gal3
150kDa- .... p-EGFR
.... EGFR

0 10 30 60 90 120 150 (min)

B HCT116^MUC1NEO

+EGF
150- .... p-EGFR
.... EGFR

+EGF +Gal3
150- .... p-EGFR
.... EGFR

+Gal3
150- .... p-EGFR
.... EGFR

0 10 30 60 90 120 150 (min)

C HCA1.7+ +EGF+Gal3

No lactose pre-treatment
150- .... p-EGFR
.... EGFR

+ lactose pre-treatment
150- .... p-EGFR
.... EGFR

0 5 10 30 60 90 120 (min)

D HCT116^MUC1FULL+EGF+Gal3

No lactose pre-treatment
.... p-EGFR -150kDa
.... EGFR

+ lactose pre-treatment
.... p-EGFR -150kDa
.... EGFR

0 5 10 30 60 90 120 (min)

E
Ratio pEGFR:EGFR vs Minutes

····◆···· HCT116 MUC1 full + EGF
– –●– – HCT116 MUC1 full + Gal3
——■—— HCT116 MUC1 full + EGF + Gal3

F
Ratio pEGFR:EGFR vs Minutes

····◆···· HCT116 MUC1 neo + EGF
– –●– – HCT116 MUC1 neo + Gal3
——■—— HCT116 MUC1 neo + EGF + Gal3

G
Ratio pEGFR:EGFR vs Minutes

– –◆– – HCA1.7+ no lactose pre- treatment
······●······ HCA1.7+ lactose treatment

H
Ratio pEGFR:EGFR vs Minutes

– –◆– – HCT116 MUC1 full no lactose pre-treatment
······■······ HCT116 MUC1 full lactose pre-treatment

Fig 5

A HCA1.7+

+EGF — p-ERK1/2 / ERK1/2

+EGF+Gal3 — p-ERK1/2 / ERK1/2

+Gal3 — p-ERK1/2 / ERK1/2

0 10 30 60 90 120 150 (min)

B HCA1.7-

+EGF — p-ERK1/2 / ERK1/2

+EGF+Gal3 — p-ERK1/2 / ERK1/2

+Gal3 — p-ERK1/2 / ERK1/2

0 10 30 60 90 120 150 (min)

C HCT116 [MUC1Full]

+EGF — p-ERK1/2 / ERK1/2

+EGF+Gal3 — p-ERK1/2 / ERK1/2

+EGF+Gal3C — p-ERK1/2 / ERK1/2

+Gal3 — p-ERK1/2 / ERK1/2

0 10 30 60 90 120 150 (min)

D HCT116 [MUC1Neo]

p-ERK1/2 / ERK1/2

p-ERK1/2 / ERK1/2

p-ERK1/2 / ERK1/2

p-ERK1/2 / ERK1/2

0 10 30 60 90 120 150 (min)

Fig 6

A

HCT116^MUC1Full

p-EGFR/EGFR (% to control)

p-EGFR

150kDa-

150kDa- EGFR

EGF	-	+	+	+	+	-	-	+	+	-	-
Gal3	-	-	-	+	+	+	+	-	-	-	-
Gal3C	-	-	-	-	-	-	-	+	+	+	+
Laptinib	-	-	+	-	+	-	+	-	+	-	+

B

HCA1.7+

p-EGFR/EGFR (% to control)

p-EGFR

150kDa-

150kDa- EGFR

EGF	-	+	+	+	+	-	-	+	+	-	-
Gal3	-	-	-	+	+	+	+	-	-	-	-
Gal3C	-	-	-	-	-	-	-	+	+	+	+
Laptinib	-	-	+	-	+	-	+	-	+	-	+

C

HCT116^MUC1Full

p-ERK/ERK (% to control)

p-ERK1/2

37kDa-

37kDa- ERK1/2

EGF	-	+	+	+	+	-	-	+	+	-	-
Gal3	-	-	-	+	+	+	+	-	-	-	-
Gal3C	-	-	-	-	-	-	-	+	+	+	+
Laptinib	-	-	+	-	+	-	+	-	+	-	+

D

HCA1.7+

P-ERK/ERK (% to control)

p-ERK1/2

37kDa-

37kDa- ERK1/2

EGF	-	+	+	+	+	-	-	+	+	-	-
Gal3	-	-	-	+	+	+	+	-	-	-	-
Gal3C	-	-	-	-	-	-	-	+	+	+	+
Laptinib	-	-	+	-	+	-	+	-	+	-	+

Fig 7

A HCT116^MUC1Full

250kDa —
150kDa —

←EGFR homodimer
←EGFR heterodimer
←EGFR monomer

Control, EGF, EGF+Gal3, Gal3, EGF+Gal3C, Gal3C

B HCT116^MUC1neo

150kDa —

←EGFR homodimer
←EGFR heterodimer
←EGFR monomer

Control, EGF, EGF+Gal3, Gal3, EGF+Gal3C, Gal3C

E IP: MUC1 IgG

IB: MUC1
250kDa —

MUC1

IB: EGFR
100kDa —

←EGFR

Control, EGF, EGF+Gal3, Gal3, Control

C HCA1.7+

250kDa —
150kDa —

←EGFR homodimer
←EGFR heterodimer
←EGFR monomer

Control, EGF, EGF+Gal3, Gal3, EGF+Gal3C, Gal3C

D HCA1.7-

150kDa —

←EGFR homodimer
←EGFR heterodimer
←EGFR monomer

Control, EGF, EGF+Gal3, Gal3, EGF+Gal3C, Gal3C

F IP: EGFR IgG

IB: EGFR
100kDa —

←EGFR

IB: Gal3
25kDa —

←Gal3

Control, EGF, EGF+Gal3, Gal3, control

Fig 8

A   HCT116^{MUC1Full}

	MUC1	EGFR	MUC1/EGFR/DAPI

Control

+EGF

+EGF+Gal3

+Gal3

B   HCA1.7+

	MUC1	EGFR	MUC1/EGFR/DAPI