MUC1 extracellular domain confers resistance of epithelial cancer cells to anoikis

Qicheng Zhao[1], Tushar Piyush[1], Chen Chen[1], Michael A. Hollingsworth[2], John Hilkens[3], Jonathan M. Rhodes[1], Lu-Gang Yu[1*]

[1]Department of Gastroenterology, Institute of Translational Medicine, University of Liverpool, Liverpool L69 3GE, UK

[2]Department of Biochemistry and Molecular Biology, The Eppley Institute for Research in Cancer and Allied Diseases, University of Nebraska Medical Centre, Omaha, Nebraska 68198-6805, USA

[3]Division of Molecular Genetics, the Netherlands Cancer Institute, Amsterdam 1066 CX, Netherlands

Running title: MUC1 extracellular domain prevents anoikis

*Correspondence to: Dr. Lu-Gang Yu, Department of Gastroenterology, Institute of Translational Medicine, University of Liverpool, Liverpool L69 3GE, UK. Tel: +44 151 7946820; Fax +44 151 7946825; email: lgyu@liv.ac.uk

Abstract

Anoikis, a special apoptotic process occurring in response to loss of cell adhesion to the extracellular matrix, is a fundamental surveillance process for maintaining tissue homeostasis. Resistance to anoikis characterises cancer cells and is a pre-requisite for metastasis. This study shows that over-expression of the transmembrane mucin protein MUC1 prevents initiation of anoikis in epithelial cancer cells in response to loss of adhesion. We show that this effect is largely attributed to the elongated and heavily glycosylated extracellular domain of MUC1 that protrudes high above the cell membrane and hence prevents activation of the cell surface anoikis-initiating molecules such as integrins and death receptors by providing them a mechanically "homing" microenvironment. As over-expression of MUC1 is a common feature of epithelial cancers and as resistance to anoikis is a hallmark of both oncogenic epithelial-mesenchymal transition and metastasis, MUC1-mediated cell resistance to anoikis may represent one of the fundamental regulatory mechanisms in tumorigenesis and metastasis.

Key words: MUC1, anoikis, cell adhesion, cancer, epithelium

Abbreviations: CT, MUC1 cytoplasmic domain; ECM, extracellular matrix; FADD, Fas-associated protein with death domain; NECDS, non-enzymatic cell dissociation solution; poly-HEMA, poly-2-hydroxyethyl methacrylate; TF, Thomsen-Friedenreich antigen (Galβ1,3GalNAc-α); VNTR, various numbers of tandem repeats

Introduction

Anoikis, the apoptotic process which occurs in cells that have lost adhesion to the extracellular matrix[1, 2], is a fundamental process for maintaining tissue homeostasis. It removes displaced epithelial/endothelial cells and thus prevents them from seeding to inappropriate sites. Resistance to anoikis contributes prominently to tumourigenesis and, in particular, to metastasis by allowing survival of cancer cells that have invaded into the blood or lymphatic circulation and thus facilitating their metastatic spread to remote sites[3].

Initiation of anoikis starts from the cell surface through activation of the cell surface anoikis-initiating molecules, e.g. integrins, cadherins and death receptors, in response to loss of cell adhesion. Loss of the integrin-mediated cell-basement matrix contact[4], loss of the E-Cadherin-mediated cell-cell contact[5, 6] or ligation of the cell surface death receptors with their ligands[4, 7] all induce conformational changes or oligomerization of these cell surface anoikis-initiating molecules. This triggers a series of events leading to activation of either the caspase-8-mediated extrinsic apoptosis signalling pathway or the mitochondrion-mediated intrinsic apoptosis signalling pathway.

MUC1 is a large transmembrane mucin protein that is expressed exclusively on the apical side of normal epithelial and some other cell types. MUC1 consists of a large extracellular domain, a transmembrane region and a short cytoplasmic tail. The MUC1 extracellular domain contains a variable number of tandem repeats (VNTR) that are heavily glycosylated (up to 50% of the MUC1 molecular weight) with complex *O*-linked mucin type glycans[8] and flanked by a unique N terminal domain and an SEA domain. In the SEA domain autocleavage takes place resulting in a heterodimer but both moieties remain firmly attached. 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[9, 10, 11].

MUC1 is over-expressed up to at least 10-fold in epithelial cancers[12] and over-expression of MUC1 is closely associated with high metastatic potential and poor prognosis in many cancer types [13]. In epithelial cancer cells, MUC1 loses its apical membrane polarization and becomes expressed over the entire cell surface[14, 15]. In epithelial cancer cells, MUC1 also shows reduced expression of complex *O*-glycans and increased expression of short oncofetal oligosaccharides such as GalNAc-α (Tn antigen), sialylated GalNAc-α (sialyl-Tn antigen) and Galβ1,3GalNAc-α (Thomsen-Friedenreich, TF antigen)[16]. Immunological targeting of cancer-associated MUC1 has been under intensive investigation as a strategy for cancer treatment[17, 18] Our recent studies have shown that interaction of TF antigen on cancer-associated MUC1 with the galactoside-binding galectins promotes metastasis by enhancing tumour cell heterotypic adhesion to the vascular endothelium and also by increasing tumour cell homotypic aggregation for the potential formation of tumour emboli [19, 20, 21].

In this report, we describe a new role of MUC1 in anoikis. We show that over-expression of MUC1 in epithelial cells prevents initiation of anoikis in response to loss of cell adhesion, an effect that is found to be attributed substantially to the MUC1 extracellular domain.

Results:

Over-expression of MUC1 is associated with increased cell resistance to anoikis

MUC1-positive transfectants of human breast HBL-100 epithelial cells (HCA1.7+) showed marked resistance to anoikis in comparison to the MUC1-negative revertants (HCA1.7-) when released by ENCDS and cultured in suspension. After 24 hr culture in suspension, 6.1-fold more HCA1.7- cells became apoptotic compared with HCA1.7+ cells when assessed by Annexin-V cell surface binding (Fig 1A). When caspase3/7 activity was assessed, HCA1.7+ also showed substantially less casapse3/7 activity than HCA1.7- cells after culture of the cells either in serum free medium, in 10% FCS (Fig 1B) or in human serum (Fig 1C). Consistent with their increased ability to resist anoikis, HCA1.7+ cells also showed substantially higher survival rates than HCA1.7- cells when cultured in suspension (Fig 1D). Similar results were also observed with MUC1-transfected human melanoma cells (Fig 2). After 24hr culture in suspension, the MUC1-positive ACA19+ cells showed much lower caspase-3/7 activity (Fig 2A) and higher viability (Fig 2B) than the MUC1-negative ACA19- cells.

Trypsin- and NECDS-released MUC1-positive and MUC1-negative cells show different responses to anoikis initiation

To gain insight into the molecular mechanism of the MUC1-mediated cell resistance to anoikis, we investigated the impact of the use of NECDS and trypsin for cell release on anoikis initiation of MUC1-positive and –negative cells. NECDS releases the cells from culture plates but keeps the cell membrane proteins intact while trypsin releases the cells by proteolytic cleavage of lysine and arginine residues of extracellular domains of cell membrane proteins.

We found that detachment of the MUC1-positive HCA1.7+ cells with either NECDS or trypsin had no significant effect on resistance of the cells to anoikis in response to subsequent culture in suspension (Fig 3A). However, detachment of the MUC1-negative HCA1.7- cells with trypsin completely abolished the sensitivity of these cells to anoikis initiation in response to suspension culture while these MUC1 negative cells remained fully sensitive to anoikis when detached by NECDS. Detachment of the MUC1-positive HCA1.7+ cells with either trypsin or NECDS did not affect anti-MUC1 antibody accessibility to MUC1 (Fig 3B). This indicates that trypsin is unable to cleave the MUC1 extracellular domain, likely due to the large and heavy glycosylated extracellular domain that protrudes above the cell surface and prevents the access of trypsin to the protein backbone of MUC1. Accessibility of antibodies to cell surface antigens like E-cadherin and integrinβ1 on MUC1 positive HCA1.7+ cells released by trypsin or NECDS shows little difference, with the exception of CD44 which shows 23% less binding to trypsin-released than NECDS-released cells (Fig. 3C). On the other hand, the MUC1-negative HCA1.7- cells released by trypsin showed 29%, 23% and 85%, respectively, lower antibody accessibility to cell surface E-cadherin, Integrinβ1 and CD44 than those released by NECDS (Fig 3C). In the meantime, when the HCA1.7+ and HCA1.7- cells were compared, antibody accessibility to the cell surface domains of E-cadherin, integrinβ1 and CD44 were all substantially higher (53%, 20% and 83%, respectively) in the MUC1-negative HCA1.7- cells than the MUC1-positive HCA1.7+ when they were released by NECDS (Fig 3D, left panel). However, when the cells were detached by trypsin little differences of antibody accessibility were observed between these two cell types (Fig 3D, right panel).

Since Western blot analysis of the denatured cell lysate showed no difference in protein expression of these cell adhesion molecules between HCA1.7+ and HCA1.7- cells (Fig 3E)

(note an extra, slightly higher molecular weight CD44 band in HCA1.7- than in HCA1.7+ cells), the restricted access of trypsin to cell surface proteins on MUC1-positive cells likely explains the differences of the effect of trypsin on antibody accessibility to these cell surface molecules on both cell types. This is supported by the discovery that recombinant Fas-L showed 33.2% more binding to HCA1.7- cells than to HCA1.7+ cells when they were released by NECDS but such difference totally disappeared when the cells were released by trypsin (Fig 3F). Together, these findings indicate that MUC1 expression maintains the integrity of cell surface proteins and prevents activation of cell surface anoikis-initiating molecule(s) during the process of cell loss of adhesion. In support of this, an additional cell surface integrinβ1 population occurred in the MUC1-negative HCA1.7- cells released by NECDS, which subsequently underwent anoikis, but not in those released by trypsin, which did not undergo anoikis (Fig 3A). Also no difference of the cell surface integrinβ1 expression was seen in the MUC1-positive HCA1.7+ cells released by NECDS or trypsin (Fig 3C and 3D), which did not undergo anoikis. Thus, this additional integrin β1 population in HCA1.7- cells released by NECDS might represent the activated "open" form (see discussion below) of this molecule that is involved in anoikis activation.

MUC1 expression inhibits induction of anoikis induced by exogenous Fas-L

To further substantiate the association between MUC1 expression and anoikis resistance, we compared the ability of exogenous Fas-L to induce initiation of anoikis of MUC1-positive and –negative cells in suspension. Fas-L binds to cell surface Fas, resulting in activation of caspase-8 and the initiation of extrinsic apoptotic signalling in anoikis. It was found that the presence of 100ng/ml recombinant Fas-L induced 48% increase of caspase-8 activation of MUC1-negative HCA1.7- cells but had no effect on caspase-8 activation of the MUC1

positive HCA1.7+ cells (Fig 4). This supports the hypothesis that expression of MUC1 on the cell surface prevents activation of the cell surface anoikis-initiating molecules.

Effects of the MUC1 extracellular and intracellular domains on anoikis

To test the role of the extracellular domain of MUC1 in resistance to anoikis, MUC1-negative HCT116 cells were transfected with a construct containing full-length MUC1 (MUC1.Full) and a construct containing truncated MUC1 cDNA devoid of the tandem repeat domain (MUC1ΔTR) (Fig 5A). Immunoblotting experiments confirmed expression of the correct MUC1 mutants and provides a measure for the expression levels in these transfectants (Fig 5B). Suspension culture of the cells transfected with MUC1.Full resulted in 68% reduction of anoikis in comparison to transfection of the cells with the control vector (Fig 5C) whereas transfection of the cells with MUC1 without its tandem repeat domain gives significantly less resistance to anoikis. This provides strong support for a substantial role of the extracellular domain in MUC1-mediated resistance to anoikis. Interestingly, we observed that transfection of the cells with MUC1 without its extracellular domain also produced a smaller (31%) but significant reduction of cell anoikis in comparison to the control vector transfectants. This indicates that the MUC1 cytoplasmic domain may also contribute to the MUC1-mediated anoikis resistance.

To test this possibility, we compared A375 cells transfected with full length MUC1 and a truncated MUC1 construct without cytoplasmic domain for sensitivity to anoikis. A375 cells expressing full length MUC1 (ACA19+ cells) were highly resistant to anoikis in comparison to the MUC1 negative transfectants (ACA19-) when cultured in suspension (Figure 5 D and E), similar as demonstrated earlier (Figure 2A and B). MUC1 transfectants without the MUC1 cytoplasmic domain (ATD2) showed significantly higher anoikis (~50%) than the

ACA19+ cells expressing full-length MUC1 but significant less than the MUC1 negative ACA19- cells. This supports an independent anoikis resistance mechanism mediated by the cytoplasmic domain of MUC1 in addition to the anoikis resistance mediated by the extracellular domain of MUC1.

Effect of MUC1 on expression of apoptosis-related signalling proteins in cell response to culture in suspension

To gain further insight into the regulation of MUC1-mediated cell resistance to anoikis, we compare the expressions of 35 apoptosis-related signalling proteins in the MUC1 positive (HCA1.7+) and negative (HCA1.7-) cells in cell response to suspension culture. We found that among the 35 apoptosis-related proteins, four show a substantial increase in expression in the MUC1 positive transfectants in comparison to the MUC1 negative transfectants in response to 24h culture in suspension (Fig 6 A and B). These proteins are XIAP (inhibitor of apoptosis protein, 6-fold increase), Fas (51-fold), HSP27 (10-fold), pro-caspase-3 (115-fold). In addition, we found an increase of the phosphorylation of several p53 serine residues (S15, 44-fold; S46, 46-fold; S392 46-fold). As the MUC1-cytoplasmic domain is known to interact directly with p53 in apoptosis regulation[22], the observed change of p53 phosphorylation in MUC1 positive cells is in keeping with an impact of the MUC1 cytoplasmic domain in MUC1-mediated anoikis resistance shown in Fig 5E.

Discussion

These studies show that expression of the transmembrane mucin protein MUC1 confers resistance of epithelial cells to anoikis initiation in response to loss of cell adhesion. This effect is found to be substantially attributed to the extracellular domain of MUC1 which prevents the normal activation of cell surface anoikis-initiating molecules in response to loss of cell adhesion.

Anoikis is an important surveillance process for preventing cells from seeding to inappropriate sites. Anoikis is accomplished by a diversity of proteins in the extracellular matrix (ECM) and a range of the cell surface initiating molecules which initiate mitogenic signals in the normal cellular environment or apoptotic signals in the context of abnormal cell contact[7]. Key cell surface anoikis-initiating molecules include integrins, E-cadherin and death receptors.

Integrins are a family of heterodimers that mediate cation-dependent cell adhesion in a wide range of biological contexts. The integrin family is comprised of 18α and 8β subunits which on ligation give rise to 24 different types of integrins. Every cell has integrins that are specific to their ligands in the ECM. Integrins sense mechanical forces arising from contacts with the ECM and converting them into intracellular signals. Integrins have two alternative conformations, a closed, low-affinity ligand binding conformation and an open, high-affinity ligand binding conformation[23 24, 25]. The open conformation has >9000 fold higher affinity to its ligands than its closed conformation. In response to external signals, including loss of the cell surface integrin engagement with ECM, integrin undergoes rapid transition from the closed to the open conformation that triggers inactivation of the pro-survival signalling pathways such as those mediated by FAK, ERK and PI3K, leading to activation of the

mitochondrion-mediated apoptosis signalling and execution of apoptosis[7]. The conformational changes of integrins at the extracellular side can also mediate inside-out signalling[26].

It is shown here that detachment of the MUC1-negative cells with NECDS, which leads to anoikis (Fig 3A), is associated with the appearance of an additional cell surface integrinβ1 population (Fig 3C and 3D). Cells released by trypsin showed the expression of only one integrinβ1population and were resistant to anoikis (Fig 3A, C and D). Thus, the two different integrinβ1 populations in the MUC1-negative cells released with NECDS might represent the open (active) and closed (inactive) integrin conformations of integrinβ1 as a result of cell detachment and activation of integrinβ1. This is in keeping with the observation that detachment of the MUC1-positive cells with NECDS, to which the cells do not undergo subsequent anoikis (Fig 3A), showed the presence of one integrinβ1 population (Fig 3C). The presence of MUC1 therefore prevented the activation of integrinβ1 during cell detachment with NECSD and subsequent anoikis response.

Ligation of extracellular death receptor ligands to their transmembrane death receptors is also known to play an important role in initiation of anoikis in response to cell loss of adhesion. Binding of death receptor ligands (e.g. Fas-L) to the extracellular domain of death receptors (e.g. Fas) induces death receptor oligomerization that allows the recruitment to the death receptor cytoplasmic domain of several cytoplasmic proteins (e.g. FADD) and pro-caspase-8, leading to caspase-8 activation and eventual activation of executioner caspases3/7[4]. We found here that exogenous addition of Fas-L induced caspase-8 activation of the MUC1-negative cells but not the MUC1-positive cells in suspension culture (Fig 4) although these cells express similar levels of Fas (Fig 3E). This indicates that the expression of MUC1 not only

prevents integrin-mediated anoikis initiation by preventing integrin activation but also inhibits death receptor-mediated anoikis initiation by preventing ligation of the cell surface death receptors with their ligands in cell response to loss of adhesion.

Such a relatively broad influence of MUC1 expression on activation of the cell surface anoikis-initiating molecules is likely explained by its massive size since the heavily glycosylated MUC1 molecule protrudes up to 10 times higher above the cell surface than other typical cell surface molecules which do not reach farther than 30nm. The extracellular domain of MUC1 may thus provide the cell surface anoikis-initiating molecules with a "homing" microenvironment even after the cells are detached, preventing conformational changes of these molecules and thus inhibiting anoikis (Fig 7).

It was found in this study that depletion of the MUC1 extracellular domain abolishes ~61% of the MUC1-mediated cell resistance to anoikis (Fig 5C), suggesting a predominant role of the MUC1 extracellular domain in MUC1-mediated cell resistance to anoikis. We also found that MUC1 transfection without the extracellular domain still causes anoikis inhibition of the cells albeit much less efficiently. This indicates that the MUC1 cytoplasmic domain also makes significant contribution to the MUC1-mediated cell resistance to anoikis through different mechanisms. Both intrinsic and extrinsic apoptosis pathways are known to play an important role in anoikis and many of the intrinsic apoptosis signalling proteins (e.g. Bcl and p53 family members) are involved in regulation of anoikis process[27]. Several earlier studies have reported a role of the MUC1 cytoplasmic domain in regulation of apoptosis in cells growing under (anchored) adhesion conditions through interaction with a number of intracellular signalling proteins[9]. For example, interaction of the MUC1 cytoplasmic tail with mitochondrial membrane, p53 or β-catenin prevents mitochondrion-mediated apoptosis in

cell response to DNA damage[10]. Interaction of the MUC1 cytoplasmic tail with FADD (Fas-associated death domain) blocks caspase-8 recruitment to the death-inducing signalling complex in response to TNFα-induced apoptosis[28]. MUC1 transfection in rat 3Y1 fibroblast cells has been shown to increase the levels of phospho-Akt and phospho-Bad and increase the expression of anti-apoptotic Bcl-x(L) protein. This is accompanied by attenuation of the loss of mitochondrial transmembrane potential, mitochondrial cytochrome c release and activation of caspase-9[29]. Since many intracellular apoptotic signalling events that occur in response to stress also occur in anoikis [30], interactions of the MUC1 cytoplasmic domain with these intracellular signalling proteins seen in cell response to stress likely contribute to the lesser inhibition of anoikis that is shown to be associated with the MUC1 cytoplasmic domain (Fig 5C and E).

Substantial increases of the expression of XIAP (inhibitor of apoptosis protein) and p53 phosphorylation were observed in the MUC1 positive transfectants in comparison to the MUC1 negative transfectants in response to suspension culture. Moreover, MUC1 cytoplasmic domain is known to regulate p53 activity either directly[22] or indirectly[31] and it has been reported that phosphorylation of p53 at different residues may have impact on apoptosis[32]. In the light of these reports, the increase of p53 phosphorylation in MUC1 positive cells and the substantial increase in expression of XIAP[33] may, at least in part, provide an explanatory mechanism of the MUC1 cytoplasmic domain-associated anoikis resistance observed in this study. The marked accumulation of pro-caspase-3 observed in the MUC1 positive cells, which resist anoikis, in comparison to the MUC1 negative cells, which undergo anoikis, in response to suspension culture is very interesting. It indicates that one of the other possible mechanisms of the MUC1 cytoplasmic domain-mediated anoikis resistance

may be associated with its inhibition of pro-caspase-3 proteolytic cleavage hence caspase-3 activation.

Moreover, MUC1 cytoplasmic domain is known to regulate p53 activity either directly31 or indirectly32 and it has been reported that phosphorylation of p53 at different residues may have different impact on apoptosis33. In the light of these reports, the increase of p53 phosphorylation in MUC1 positive cells and the substantial increase in expression of XIAP34 may, at least in part, provide an explanatory mechanism of the MUC1 cytoplasmic domain-associated anoikis resistance observed in this study. The marked accumulation of pro-caspase-3 observed in MUC1 positive cells, which resist anoikis, in comparison to the negative cells, which undergo anoikis, in response to suspension culture is very interesting. It suggests that one of the other possible mechanisms of the MUC1 cytoplasmic domain-mediated anoikis resistance may be associated with its inhibition of pro-caspase-3 proteolytic cleavage hence caspase-3 activation.

Little was previously known of the influence of MUC1 on cellular anoikis. MUC1 transfection into ES-2 human ovarian tumour cells has been reported to decrease Annexin-V cell surface binding and increase chemo-resistance of the cells to anticancer drugs in suspension[34]. Transfection of MUC1 into rat 3Y1 fibroblasts was shown to increase the ability of the cells to grow in soft agar[35]. During preparation of this manuscript, a study has reported that depletion of the MUC1 extracellular domain by transfection in human renal cells increases viability of the cells in response to culture under suspension condition, implying a role of the MUC1 extracellular domain in anoikis, but without further information regarding molecular mechanisms[36].

The ability to grow under anchorage-independent conditions is one of the major hallmarks of transformed cells, key to this is the ability of the cancer cells to resist anoikis. Over-expression of MUC1 is a common feature in epithelial cancer cells. Over-expression of MUC1 has been shown to inhibit E-Cadherin-mediated cell-cell interactions and to increase the ability of the cancer cells to detach from adjacent cells at primary tumour sites and to promote tumourigenesis[15, 37]. Interaction of cancer-associated MUC1 with circulating galectin-3, a galactoside-binding protein whose concentration is markedly increased up to 30-fold in the bloodstream of cancer patients[21], via expression of the oncofetal TF antigen on MUC1[38], induces MUC1 cell surface polarization and exposure of the cell surface adhesion molecules. This consequently results in increased homotypic aggregation and heterotypic adhesion of circulating tumour cells to the blood vascular endothelium and tumour cell spread[19, 20]. Thus, over expression of MUC1 in epithelial cancer cells can influence several steps in tumorigenesis and metastasis and each of these is influenced not only by the MUC1 protein expression but also by the MUC1 localization/depolarization, its glycosylation patterns and the presence of its interacting proteins in the tumour microenvironment. For example, MUC1 over-expression prevents anoikis in suspended epithelial cancer cells as is shown here. But MUC1 overexpression also reduces adhesion of circulating tumour cells to the blood vascular endothelium, a metastatic step that is required for extravasation and establishment of a metastatic colony[20]. On the other hand, interaction of MUC1 on circulating tumour cells with galectins induces MUC1 cell surface polarization to reveal the smaller cell surface adhesion molecules, causing increased cancer cell – endothelial adhesion[20, 21] and allowing increased formation of tumour emboli[19].

Thus, expression of MUC1 prevents anoikis initiation of epithelial cells in response to loss of cell adhesion. The MUC1 extracellular domain makes a substantial contribution to this effect

by maintaining the integrity and preventing activation of the cell surface anoikis-initiating molecules. This likely represents one of the mechanisms by which cancer cells avoid anoikis and may have important implications for the development of new therapeutic agents for cancer treatment.

Conflicts of interest:

The authors declare no conflict of interest

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Author contributions:

Conception and design: LGY, JH

Development of methodology: QZ, JH, LGY

Acquisition of data: QZ, TP, CC

Analysis and interpretation of data: QZ, TP, CC, JH, JMR, LGY

Writing and review of the manuscript: QZ, MAH, JMR, JH, LGY

Study supervision: LGY

Materials and Methods

Materials

The Caspase3/7 Glo® kits, Caspase8 Glo® kits and CellTiter-Glo® Luminescent Cell Viability kit were obtained from Promega. Recombinant Fas-L was from PeProtech. Antibodies against CD44 (BBA10), integrinβ1 (MAB17782), E-cadherin (MAB1838), Fas (AF2267) and Fas-L (AF126) were from R&D Systems. GenePOORTER-2 transfection reagent was from AMS Biotechnology (Abingdon, UK). FITC-Annexin-V/PI apoptosis detection kit was from Cambridge Biosciences (Cambridge, UK). The Calcein AM cell labelling solution was from Invitrogen and the Non-Enzymatic Cell Dissociation Solution (NECDS) was from Sigma.

Cells

Human colon cancer HCT116 and SW620 cells were obtained from European Collection of Cell Cultures(Salisbury, UK) and were cultured in cultured in McCoy's5a medium (HCT116) or Dulbecco's modified Eagle's medium (DMEM) (SW620). MUC1 transfection of HBL-100 human breast epithelial cells and human melanoma A375 cells with full length cDNA encoding MUC1 and the subsequent selection of the MUC1 positive transfectant HCA1.7+ (from HBL-100) and ACA19+ (from A375), and the negative revertant HCA1.7- (from HBL-100) and ACA19- (from A375) was described previously[14]. The cell lines were last authenticated by DNA profiling (DNA Diagnostics Center, London) in May 2014. MUC1 transfection of A375 cells with cDNA encoding only the MUC1 extracellular and transmembrane domains and subsequent selection of the MUC1 positive transfectant ATD2 was described previously[39]

Assessments of cell anoikis and viability

These assessments were conducted in cell suspension culture in poly-2-hydroxyethyl methacrylate (poly-HEMA)-coated plates. Briefly, 96- or 6-well plates were coated twice with 10 mg/ml poly-HEMA in 95% ethanol overnight. Cells were released by NECDS from the culture flasks, washed with PBS, resuspended to $5x10^5$cells/ml with serum-free DMEM containing 0.5mg/ml BSA and applied to the poly-HEMA coated plates for various times at 37ºC. The cells were collected and the apoptotic (anoikis) cells were then measured either by FITC-Annexin-V/PI apoptosis detection kit with flow cytometry, or by the Caspase-Glo®3/7 Assay kit according to the manufacturer's instructions. The viability of the cells was determined by the ATP detection kit as described in our previous study[19].

Analysis of the expression of cell surface adhesion molecules

The cells were released from the culture plates with either trypsin or NECDS and fixed immediately with 2% paraformaldehyde for 15 min at room temperature. After washing with PBS, the cells were incubated with 5% goat serum in PBS for 30min. The cells were resuspended to $5X10^5$ cells/ml with 1% goat serum in PBS and incubated with antibodies (1μg/ml) against MUC1 extracellular repeat domain (B27.29), E-cadherin, CD44, integrinβ_1, FAS-L or control mouse IgG on a rotation platform for 1 hr at room temperature. After three washes with PBS, FITC-conjugated secondary antibody (1:500 in 1% goat serum in PBS) was applied for 1 hr. The cells were washed three times with PBS before flow cytometry analysis.

Generation of full-length and tandem-repeat-deleted MUC1 mutants

Generation of the MUC1 expression vectors for full length MUC1 (MUC1.full) and the extracellular domain-depleted MUC1 (MUC1FΔTR) were described previously[40, 41]. Each MUC1-expressing or control vector (1μg), pre-mixed with 25μl DNA Diluent and 5μl

hydrated GenePOORTER-2 transfection reagent in 20µl serum-free medium for 10 min at room temperature, was added to 70-80% confluent HCT116 cells in 250µl antibiotics-free and serum-containing DMEM in 24-well plates for 24 hr at 37°C. The culture medium was replaced with serum-containing medium for 48 hr before the cells were cultured in normal growth medium containing 600µg/ml G418 for 7-10 days at 37^0C. The cells were released and seeded at 30-50 cells/dish in 10cm culture dishes in normal growth medium containing 600µg/ml G418. Single cell clones were then selected with Cell Cloning Cylinders, proliferated and analysed for MUC1 expression by immunoblotting with B27.29 (0.5µg/ml) and CT-2 (0.25µg/ml) anti-MUC1 antibodies.

Effect of exogenous Fas-L on caspase-8 activation under anoikis conditions

HCA1.7+ or HCA1.7- cells were released by NECDS and diluted into $2x10^5$/ml in serum-free DMEM. The cells were introduced (100µl/well) to poly-HEMA-pre-coated 96-well plates with or without introduction of 100ng/ml Fas-L for 0 and 2 hrs followed by assessments of the cellular caspase-8 activity by Caspase-Glo® 8 Assay kit.

Protein array analysis of apoptosis-related proteins in cell response to anoikis culture

HCA1.7+/- cells were released with NECDS, washed and cultured at $1x10^5$ cells/ml in serum-free DMEM for 24 hr in poly-HEMA-coated plates at 37^oC. The cells were collected and lysed with lysis buffer (provided by the Human Apoptosis Array Kit, R&D Systems) at 4°C for 30 minutes. After centrifugation at 14,000rpm for 5 minutes, the supernatants were obtained and 500 µg proteins from each sample were applied to the Human Apoptosis Array as described by the array kit. Each array contains 35 apoptosis-related proteins, each in duplicate (Bad, bax, Bcl-2, Bcl-x, pro-caspase-3, cleaved caspase-3, catalase, cIAP-1, xICAP-2, claspin, clusterin, cytochrome c, TRAIL R1/DR4, TRAIL R2/DR5, FADD,

Fas/TNFSF6, HIF-1α, HO-1/HMOX1/HSP32, HO-2/HMOX2, HSP27, HSP60, HSP70, HTRA2/Omi, livin, PON2, p21/CDNK1A, phosphor-p53(S15), phosphor-p53(S46), phosphor-p53(S392), phosphor-Rad17(S635), AMAC/Diablo, surviving, TNF R1/TNFRSF1A, XIAP). The density of each apoptosis-related protein in the array was quantified by ChmiDoc XRS Imager (Bio-Rad).

Statistical analysis

Unpaired *t* test for single comparison, one-way analysis of variance (ANOVA) followed by Newman and Keuls test for multiple comparisons (StatsDirect for Windows, StatsDirect Ltd; Sale, UK) were used where appropriate. Differences were considered significant when $p<0.05$.

Figure legends

Fig 1. MUC1 transfection in human breast epithelial HBL-100 cells inhibits anoikis and increases cell survival

A: Representative flow cytometry plots showing Annexin-V cell surface binding of the MUC1-positive (HCA.17+) and -negative (HCA1.7-) transfectants, released by NECDS and cultured for 0 and 24 hr in suspension. Earlier apoptotic (Annexin-V positive and PI negative) cells show at the bottom right and late apoptotic (Annexin-V positive and PI positive) cells show at the top right in each of the correlation plots. **B** and **C**: Assessment of caspase3/-7 activity of HCA1.7+/- cells in cell response to 24 hr culture in suspension in serum-free medium, 10% FCS (B) or 10% human serum (C). The data are presented as mean ± SEM of triplicate determinations from 2 independent experiments. **D.** MUC1 expression increases cell viability in response to cell culture under suspension. The data are presented as mean ± SD of triplicate determinations. ***$p<0.001$.

Fig 2. MUC1 expression in human melanoma cells prevents anoikis and increases cell survival

A and **B**: MUC1 positive transfectants (ACA19+) show significantly less anoikis (A) and higher survival rate (B) than the MUC1 negative revertants (ACA19-) in cell response to 24 hr culture under suspension when assessed by caspase3/7 activity. The data are presented as mean ± SEM of triplicate determinations of 2 independent experiments. ***$p<0.001$.

Fig 3. Different effects of cell release by trypsin and NECDS on subsequent initiation of anoikis and on antibody accessibility to the cell surface anoikis-initiating molecules in MUC1- positive and -negative cells

A: HCA1.7+/- cells were released by NECDS or trypsin and cultured in suspension for 24 hr before the cell-associated caspase3/7 activities were assessed. The data are presented as mean ± SEM of triplicate determinations of 2 independent experiments. **B-D:** Representative flow cytometry plots show antibody access to cell surface MUC1 (**B**), E-cadherin, Integrinβ1, CD44 (**C** and **D**) and recombinant Fas-L access to cell surface Fas (**F**) in HCA1.7+/- cells released by trypsin or NECDS. Note, an additional integrinβ1 peak (arrowed) is seen in HCA1.7- cells released by NECDS in comparison to those released by trypsin. **E:** Immunoblotting of cell lysates shows total cellular expression of CD44, E-cadherin, integrinβ1, Fas, Fas-L and tubulin in HCA1.7+/- cells. ***p<0.001.

Fig 4. Differential effect of exogenous addition of Fas-L on anoikis of MUC1-positive and negative cells.

HCA1.7+/- cells were treated with 100ng/ml recombinant Fas-L under suspension for 0 and 2hr followed by assessment of cellular casapse-3/-7 activity. The data are presented as mean ±SEM of triplicate determinations of two independent experiments. ***p<0.001.

Fig 5. Depletion of MUC1 extracellular tandem repeat domain or cytoplasmic domain reduces MUC1-mediated cell resistance to anoikis

A: schematic diagram of MUC1 transfectants. **B:** MUC1 immunoblotting confirms transfection of MUC1 full length and extracellular tandem repeat domain depleted mutants. HCT116 cells transfected with control vecter (MUC1.neo), full length MUC1(MUC1.Full) or MUC1-tandem repeat domain depletion (MUC1.ΔTR) were separated by SDS-PAGE and immunoblotted with B27.29 (against the extracellular tandem repeat domain of MUC1), CT2 (against the cytoplasmic domain) or an anti-tubulin antibody. MUC1.Full cells show expression of MUC1 both extracellular and intracellular domains and MUC1.ΔTR cells show

expression of only the intracellular MUC1 domain. **C:** Depletion of MUC1 tandem repeat domain reduces MUC1-mediated cell resistance to anoikis when cellular caspase-3/-7 activity was assessed. **D**: MUC1 transfectants of A375 cells with control vector (ACA19-), full length MUC1 (ACA19+) or full length MUC1 without the cytoplasmic domain (ATD2) were immunoblotted with B27.29, CT2 or tubulin antibody. ACA19+ cells show expression of MUC1 extracellular and intracellular domains and ATD2 cells show expression of MUC1 extracellular but not cytoplasmic domain. E: The absence of MUC1 cytoplasmic domain reduces full MUC1-mediated cell resistance to anoikis when cellular caspase-3/-7 activity was assessed. The data are presented as mean ±SD of triplicate determinations of two (C) or three (E) experiments. $^{**}p<0.01$, $^{***}p<0.001$.

Fig 6. Analysis of the expression of 35 apoptosis-related proteins in HCA1.7+/- cells in response to suspension culture by protein array

HCA1.7+/- cells released by with NECDS and cultured at $1x10^5$ cells/ml in serum-free DMEM for 24 hr in poly-HEMA-coated plates at 37^oC. The cells were collected and lysed and applied to the Human Apoptosis Array. The array contains 35 apoptosis related proteins. In response to 24 hr culture in suspension, five proteins shows substantial increased expression (or phosphorylation) in HCA1.7+ cells in comparison to HCA1.7- cells (A, 1, pro-caspase-3; 2, Fas; 3, Hsp27; 4, phosphor-p53(S15); 5, phosphor-p53(S46); 6, phosphor-p53(S392); 7, (IAP). B: densitometry quantification of the expression (or phosphorylation) of the five proteins in A.

Fig 7. Proposed action of MUC1-extracellular domain in MUC1-mediated cell resistance to anoikis

A: In normal epithelia, MUC1 polarizes at the apical side and has no influence on activation of the cell surface anoikis-initiating molecules during loss of cell-matrix contacts. **B:** In epithelial cancer, MUC1 is over-expressed over the entire cell surface and thus able to interact with the cell surface anoikis-initiating molecules around the circumference of the cell thus preventing their activation during loss of cell-matrix contacts by providing them a mechanically "homing" microenvironment.

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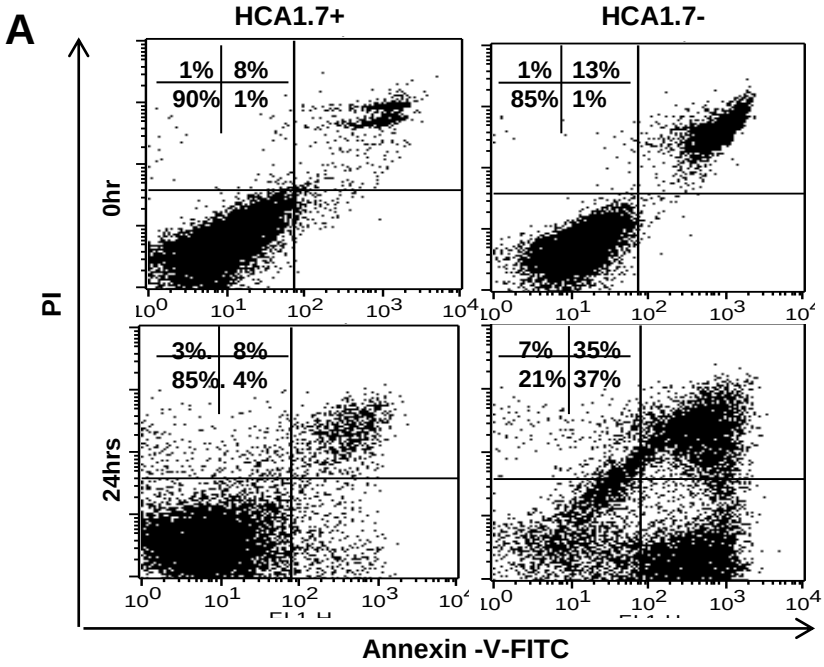
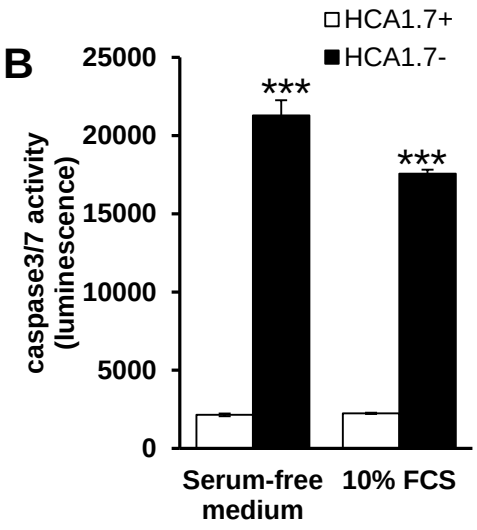
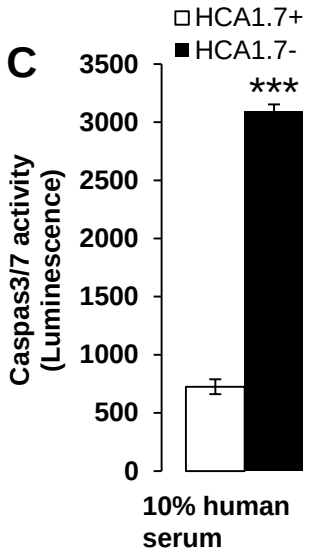
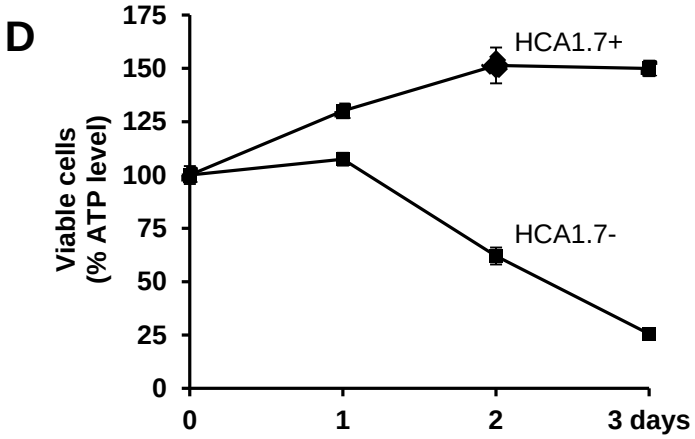
Zhao et al, Fig 1









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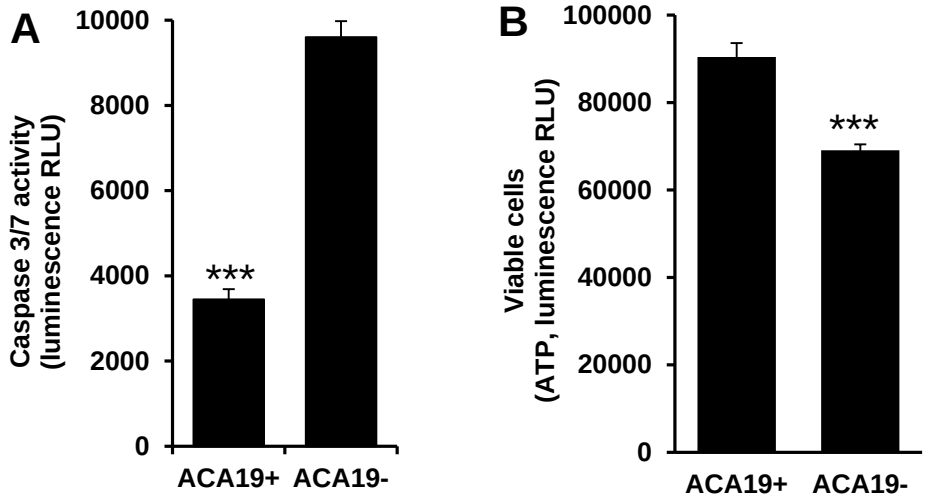

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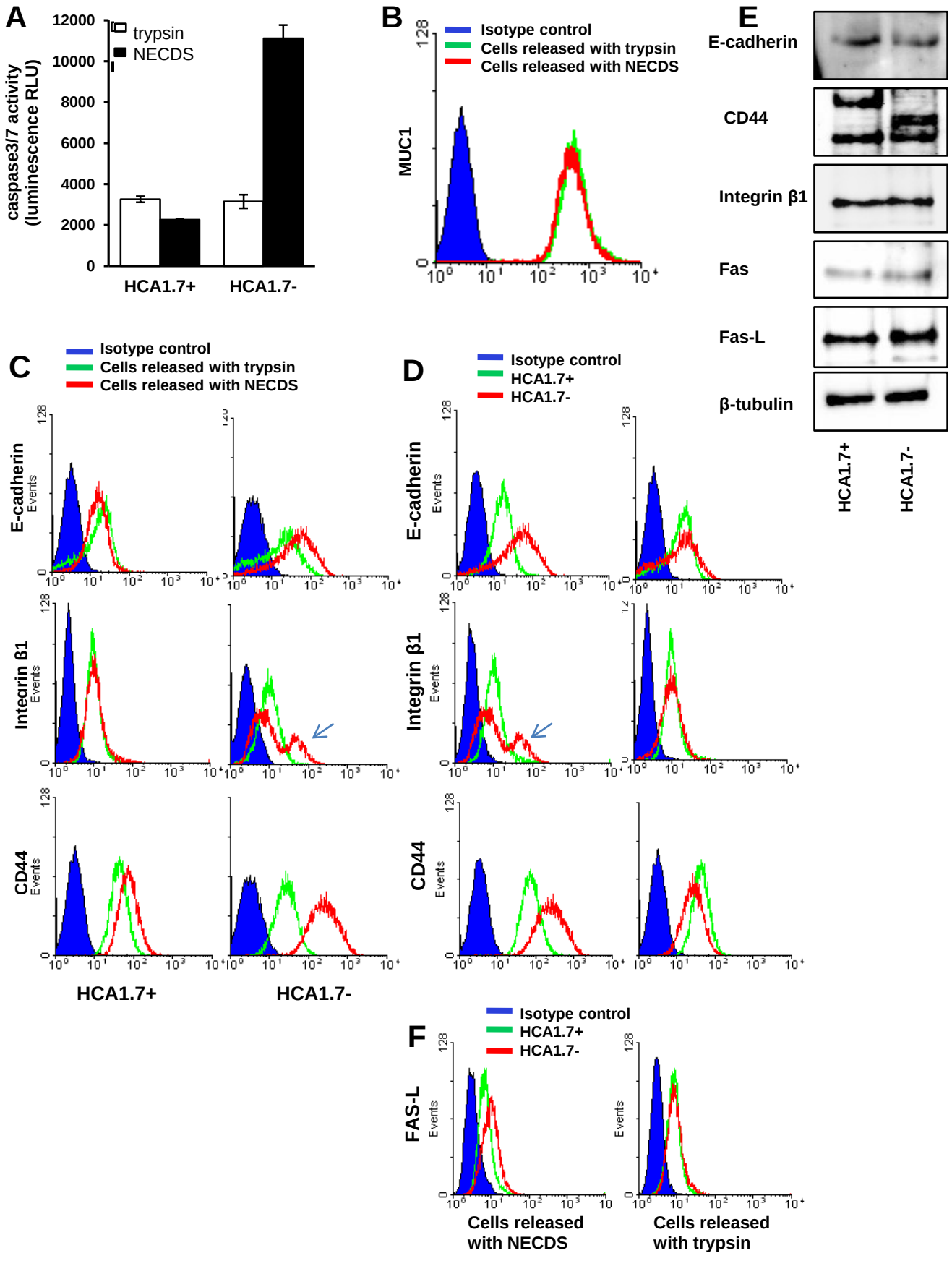

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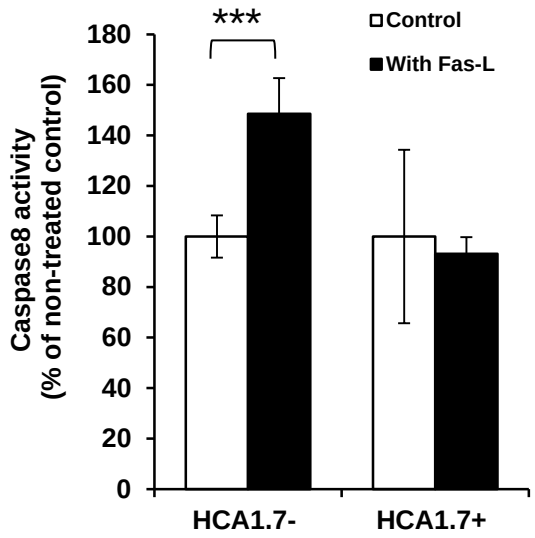

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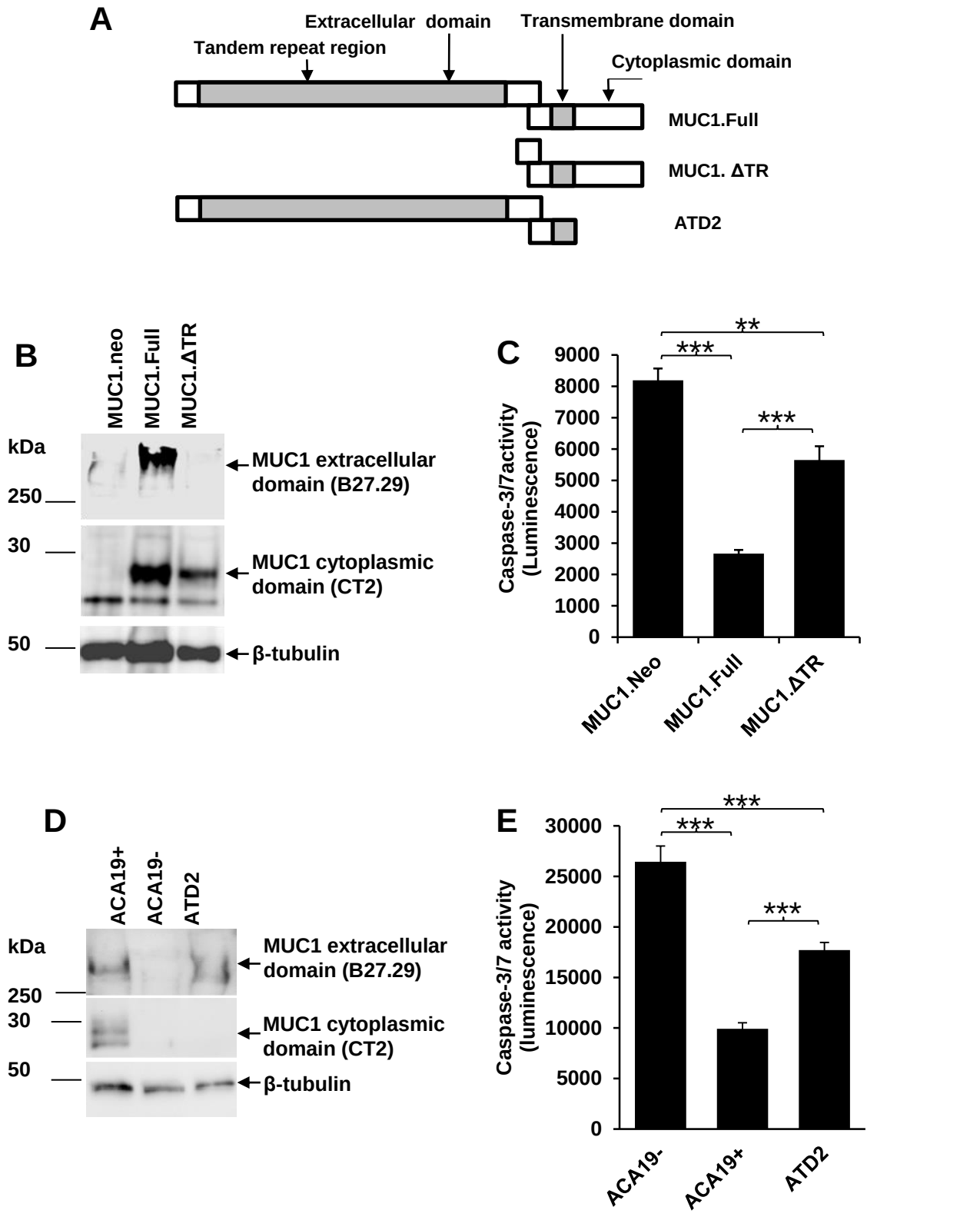

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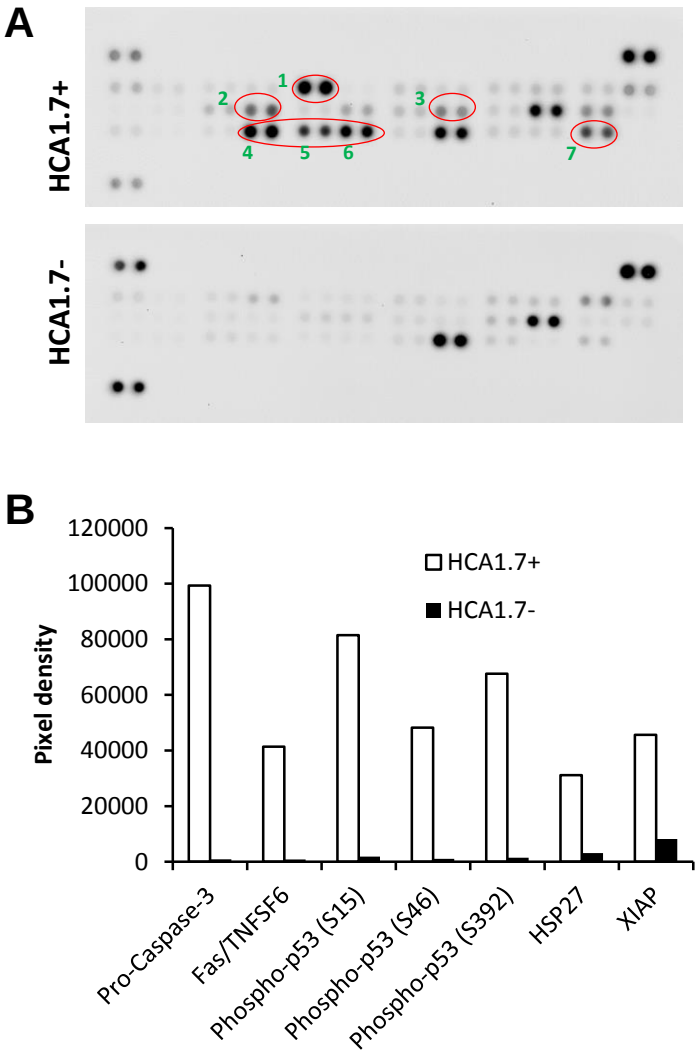

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