Downregulation of Mucin1 in Cancer Cells is Associated with Modulation of Calcium Signalling Pathways and Alteration in Procoagulant Related Activity

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Abstract

Background Intracellular calcium ions play an essential role in regulating numerous physiological functions by acting as a second messenger in signal transduction pathways. Alterations in calcium signaling are also implicated in several pathological conditions including cancer.

Aim The purpose of this study is to investigate whether MUC1, a well-established tumor marker, plays a role in modulating calcium signalling in cancer cells.

Methods MUC1 knockdown breast cancer MCF-7 cells were used to investigate whether the overexpression of MUC1 by cancer cells has an influence on calcium signalling pathways, by determining the level of a range of calcium signalling proteins and the dynamics of intracellular calcium mobilization as well as the cellular effects induced by calcium treatment. The investigation was also extended to normal human mammary epithelial, human breast and pancreatic cancer cell lines, which express different levels of MUC1.

Result MUC1 downregulation altered the aberrant expression of several calcium transporter proteins in MCF-7 cancer cells. The level of each of these calcium signalling pathways proteins was correlated to MUC1 level in normal human mammary epithelial, human breast and pancreatic cancer cell lines. Consequently, a number of proteins whose activities are calcium dependent such as p-CaMKII, and procoagulant proteins Va, Xa and thrombin were found to be reduced in MUC1 downregulated MCF-7 cells. In addition, the effect of thrombin on calcium mobilization and the cleavage of the thrombin receptors was also reduced in MUC1 downregulated MCF-7 cells.

Conclusion The downregulation of MUC1 is capable of modulating calcium cellular effects via alteration in the expression of calcium signalling proteins and by impacting calcium induced initiation of procoagulant activity, which is capable of influencing the effect of thrombin on cancer cells. Such properties contribute to another crucial role for MUC1 within the signalling network associated with tumorigenesis.

Keywords: Mucin1, calcium signalling, coagulation, breast and pancreatic cancer
Background
Calcium is one of the most important elements in the human body, and its regulation plays a vital role in many physiological processes. Intracellular calcium ions (Ca2+) act as a second messenger in regulating gene transcription, cell proliferation and death. The calcium signal arises from the coordinated activity of a suite of calcium channel pumps which are comprised of exchangers and binding proteins. Calcium signaling, in addition to its numerous physiological roles, is also implicated in several pathological conditions including cancer. Accumulating evidence has demonstrated that intracellular Ca2+ homeostasis is altered in cancer cells. Tumorigenic pathways are often associated with abnormal degrees of activation of Ca2+ channels, Ca2+ transporters, Ca2+-ATPases [1, 2] and a number of known molecular players in cellular Ca2+ homeostasis, such as calcium release-activated calcium channel protein 1 (ORAI1), stromal interaction molecule 1 (STIM1) and transient receptor potential (TRP) channels [3]. Such dysregulation of calcium signaling has been linked to each of the cellular hallmarks of cancer including tumour initiation, angiogenesis, progression and metastasis [1-5]. Correction of these derailed Ca2+ signals provides an alternative approach towards the treatment of cancer [2]. However, although specific aspects of the Ca2+ signal have been shown to be altered in cancer cells, the overall basis of the altered Ca2+ signaling in cancer is not completely understood [2, 4]. In particular, tumorigenic related differences in gene expression and cell signalling in respect of the dysregulation of Ca2+ signaling in cancer remain largely unknown.
Membrane proteins are known to be involved in intra and inter cellular signalling, in the coupling of extracellular events with intracellular responses, and for maintaining intracellular calcium homeostasis and they thereby contribute to varying extents to the pathophysiological features of individual types of cancer [4,5]. Mucin 1 (MUC1), a large transmembrane protein, is one of the mucin family members that form protective mucus barriers on epithelial surfaces [6]. Tumor associated MUC1 differs from the MUC1 expressed in normal cells with regard to its biochemical features, cellular distribution, and function. In cancer cells, MUC1 regulates diverse cellular functions that promote the aggressive and metastatic phenotypes of cancer cells through an intricate interplay of the MUC1-C subunit with various signaling effectors thereby regulating the expression of its target genes at both the transcriptional and the post-transcriptional level and at different subcellular localities [6-8].

It has been reported previously that MUC1 initiates a calcium signal after ligation by intercellular adhesion molecule-1 (ICAM-1), since blocking of the ICAM-1 binding site on the MUC1 molecule inhibited the calcium-based oscillatory response [9]. MUC1 also interacts with calcium-modulating cyclophilin ligand (CAML) and transfection of cells with plasmids encoding MUC1 and CAML, respectively, increased intracellular calcium levels compared with cells transfected with either plasmid alone, suggesting a possible biological significance of a MUC1-CAML interaction [10]. Mucin1 (MUC1) is well established as a tumor marker and overexpressed in variety of epithelial cancers. To date, however, there have been no reports about the effect of the level of MUC1 on the expression of proteins of the calcium signalling pathways, as well any study to determine whether MUC1 is linked with calcium induced cellular functions. The hypercoagulable state is often a pathogenic characteristic of cancer in patients and contributes to tumour aggressiveness [11]. Our previous work found that tumour-associated Mucin1 correlates with the procoagulant properties of cancer cells. MUC1 gene downregulation could affect the level of expression as well as the activity of procoagulant proteins in cancer cells following calcium stimulation [12]. As calcium ions are required for the promotion or acceleration of blood clotting pathways, we have considered whether calcium signalling pathways could also be impacted by the level of MUC1 in cancer cells. Our investigation has observed that MUC1 downregulation modulates the level of proteins of the calcium signalling pathways in human breast cancer cells as well as calcium mobilization and calcium induced cellular function. The level of MUC1 was also found to be coincident to the level of calcium signal pathways proteins in pancreatic and breast cancer cell lines. All of these findings suggest that MUC1 contributes to the calcium signalling pathways of human cancer cells. Mucin1 downregulation is able to modulate calcium signalling pathways, and could be an early contributory factor to tumor pathology.

Materials and Methods
Cell Culture Human breast cancer cell lines, MCF-7 and MDA-MB-231 were obtained from the Health Protection Agency culture collection (UK). MCF-7 cells were maintained in DMEM medium supplemented with 15% fetal bovine serum (FCS, Life Technology, UK), 100 U/ml penicillin, and 100 mg/ml streptomycin, and cultured in a humidified atmosphere of 5% CO2 in air. MDA-MB-231 cells were cultured in Leibovitz’s L-15 medium with the above same supplements and conditions but in the
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absence of CO2. Human pancreatic carcinoma cells lines, PANC-1, Capan-1 and BxPC-3 and human normal mammary cell lines, MCF-12A and MK16C were purchased from ATCC (US). Following each of the respective ATCC protocols, ANC-1 cells were maintained in DMEM medium supplemented with 10% FCS. Capan-1 cells were cultured in IMDM with 20% FCS. BxPC-3 cells cultured in RPMI1640 with 10% FCS. MCF-12A and MK16C cells were cultured in DMEM medium supplemented with endothelial cell growth media MV (Promo Cell, Germany).

**sh-MUC1 Knockdown Transfection Procedure**

MCF-7 cells were transfected with MUC1 MISSION shRNA Lentiviral Transduction Particles (Sigma) following the manufacturer’s protocol. The properties of stable MUC1 gene knock down cell line (shMUC1-MCF-7) were compared to those of MCF-7 cells transfected with the MISSION pLKO-1 puro non-mammalian shRNA control plasmid (Sigma) (supplement Figure 1). Puromycin (Sigma) was used to select each of the stably transduced cell lines prior to the investigations.

**Western Blot**

The cells were cultured until 90% confluence in medium containing 10% FBS, which was then replaced with serum free medium containing 0.2% Lactalbumin Hydrolysate (LAH, Sigma-Aldrich UK) overnight. Lysates of harvested cells were analysed by Western Blot [13]. In time course experiments, the cells were cultured in Keratinocyte-SFM (serum-free medium) (Thermofisher, UK) in the absence of calcium chloride. In experiments to investigate the effect of (Ca+2) treatment, a low concentration (4mM) of calcium chloride was added, which has been shown in previous reports to be functionally active as an agonist without cytotoxicity (14, 15) and samples taken at timed intervals from 5 to 90 mins. Cell lysates were prepared and assayed by Western blot for protein expression and kinase activities using antibodies for MUC1, phospholipase Cα1 (PLC α1), phospholipase Cβ3(PLCβ3), N CKX (SLC24A1), Orai 1, PMCA ATPase, SERCA2 ATPase, STIM1, Calcium/calmodulin-activated protein kinase II (CaMKII), Phospho-CaMKII, Calmodulin, CAML (all were obtained from Abcam UK). Primary antibodies for p-ERK, p-Akt, ERK, and Akt and the appropriate HRP-conjugated secondary antibodies were obtained from Cell Signaling (UK).

**Intracellular calcium flux assay**

To detect the effect of MUC1 level on the intracellular calcium mobilization in breast cancer cells and follow the release of Ca^{2+} into the cytoplasm, a Flu-4 direct calcium assay kit (Thermos Fisher Scientific, UK) which provides a homogeneous fluorescence-based calcium flux assay was used, according to the manufacturer’s protocol, cells were plated in a poly-d-lysine coated 96-well plate and incubated overnight. The following day, cells were assayed for a calcium response following the addition of agonists including thapsigargin (TG), phorbol 12-myristate 13-acetate (PMA) and ionomycin and thrombin, respectively. A water-soluble probenecid supplied with the Fluo-4 Direct™ Calcium Assay Kits, was used to inhibit extrusion of the calcium indicator out of the cell by organic anion transporters. Measurements are given in relative fluorescent units as calculated according to value observed as the maximum response minus the minimum response divided by the minimum response.

The assay of blood coagulation activity induced by calcium blood clotting assays measure the time required for recalcified citrated plasma to clot in response to activators of either the extrinsic pathway (PT) or the intrinsic coagulation pathway (aPTT) of the blood coagulation cascade. The extrinsic pathway is activated by the addition of complete thromboplastin, a mixture of tissue factor (TF), phospholipids and CaCl2, to the plasma samples [16]. In this investigation, the clotting activity assay was determined by the recalification of pooled citrated normal human plasma in the presence of cell extracts as a source of Tissue Factor (TF) and phospholipid. 30 µl of the cell lysate sample prepared with octyl-β-D-glucopyranoside were added to 40 µl of 40mM CaCl2 in TBSN buffer (50 mM Tris-HCl, pH7.5, 150 mM NaCl, and 0.02 % Na Azide) before mixing with 50 µl normal pooled plasma (NPP, TCS Biosciences UK) in microwell plates incubated at 37°C in a SpectraMax plate reader. The clotting time was determined from the timeline plots of the progressive increase in absorbance at 405 nm by extrapolation to the x-axis (time) of the slope of the steep increase in absorbance, which is observed as the clot is formed.

**Results**

The level of expression of calcium signalling proteins corresponded with the MUC1 level in normal and cancer cells. The levels of calcium signalling proteins selected from their involvement in Ca2+ influx pathways and aberrant expression in cancer [1, 2, 17-28] were assayed by Western Blot using cell samples prepared at timed intervals after treatment with 4mM CaCl2 (Figure 1A). Compared with shCtrl-MCF-7 cell, the expression of phospholipase Cα1 (PLC α1), an isotype of PLC, capable of triggering an increase in the cytoplasmic Ca2+ level by opening channels in the endoplasmic
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The level of PMCA ATPase, which has a role in maintaining cytoplasmic levels of Ca2+ and exhibits a lower expression in a variety of cancer types [24], was gradually increased during the Ca2+ stimulated time course in shMUC1-MCF-7 cells. However, one of the calcium signalling proteins which is highly expressed in cancer cells, SERCA2 ATPase (a member of the SERCA pumps responsible for constantly moving Ca2+ uphill into the endoplasmic reticulum (ER)), was reduced (Figure 1A). The expression of stromal interaction molecule (STIM 1, the endoplasmic reticulum (ER) Ca(2+)-sensor) as well as Orai 1 which mediates store-operated Ca2+ entry (SOCE) was each reduced in shMUC1-MCF-7 cells. The activity of calcium/calmodulin-dependent protein kinase II (CaMKII), which is calcium dependent is extremely low in its basal state [29]. Our work showed that the level of CaMKII at basal state (time 0) was lower in shMUC1-MCF-7 cells compared to shCtrl-MCF-7 cells, but following calcium treatment its level in shMUC1-MCF-7 cells was increased at the 30 min time point. Moreover, the level/activity of phospho-calcium/calmodulin- dependent protein kinase II (p-CaMKII) in response to Ca2+ treatment, was only slightly enhanced in shMUC1-MCF-7 cells compared to a large increase in shCtrl-MCF-7 cells, even though the levels of Calmodulin did not show significant difference between each of the cell lines (Figure 1A). The results clearly indicated that the calcium dependent cellular reaction was reduced by MUC1 downregulation in this breast cancer cell line.

To investigate further the relationship between the MUC1 level and these calcium signalling proteins, Western blot assay was also performed with the lysed samples of human pancreatic epithelial cancer cell lines, PANC-1, Capan-1 and BxPC-3; human breast cancer epithelial cell lines, MCF-7 and MDA-MB231; and normal human mammary epithelial cell lines MCF-12A and MK16C, since different levels of MUC1 exist between these cell lines. It was observed that a high level of expression of SERCA2, Orai 1 and CaMKII occurred in pancreatic cancer cells, PANC-1 and Capan-1 and breast cancer cells MCF-7 and MDA-MB231 (Figure 1B), these cells have a relatively higher expression of MUC1 than that of the pancreatic cancer cells, BxPC-3 and the normal mammary cell lines MCF-12a and MK16C. STIM1 was also highly expressed in PANC and MCF-7 and MDA-MB231 cells. On the other hand, a lower level of PMCA ATPase was observed in both pancreatic cancer cell lines, PANC and Capan-1 as well as the breast cancer cell line, MCF-7 compared with the two normal mammary
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It was also noted that the level of p-CaMKII corresponded with MUC1 level in these cells, being relatively low in pancreatic cancer cells BxPC-3 and the normal mammary cell lines MCF-12a and MK16c (Figure 1B).

Figure 1. The expression level and activity of calcium signalling proteins corresponded with MUC1 level in normal and cancer cells. A) The levels of selected calcium signalling proteins, which are involved in Ca2+ influx pathways and are known to be modulated in cancer cells, were analysed with Western Blot in the cell lysates of the Ca++ treatment time course samples. B) Western blot assay of the expression level of calcium signalling proteins in human mammary normal (MCF12a and MK16c) and pancreatic cancer (PANC-1, Capan-1 and BxPC-3) and breast cancer (MCF-7 and MDA-MB-231) epithelial cell lines also showed correspondence with the level of MUC1. Full blots are presented in Supplement file.

Downregulation of MUC1 altered the ability of calcium treatment to induce the coagulation activities of cancer cells. Most of the reactions in blood coagulation factors require Ca2+ for catalysis and/or complex assembly [30], for example, the activation of coagulation factors, F-X, and prothrombin are dependent on calcium ion. To investigate the effect of MUC1 downregulation on the influence of calcium treatment on the procoagulant properties of cancer cells, cells were treated with 4mM CaCl2 and sampled at timed intervals for analysis of cell lysates by Western blot. Compared with shCtrl-MCF-7 cells, the protein level of F-X, and prothrombin did not show significant change in calcium treated shMUC1-MCF-7 cells, but the levels of F-Xa, FVa and thrombin were significantly lower throughout the Ca++ treatment time course, and levels of F-V were also reduced (Figure 2A).
The coagulation activity was also observed in the cell lysates/protein extracts of the treated shCtrl-MCF-7 and shMUC1-MCF-7 cells. The activity is measured by a plasma clotting assay, in which cells were added to citrated plasma before initiating the reaction by the addition of CaCl2. It was observed that the rate of formation of the clot was significantly reduced in shMUC1-MCF-7 cells (Figure 2B), both in the time taken for an initial clot to form (Ts) and the time taken for clot formation to reach a maximum (Tmax) [31].

Downregulation of MUC1 strikingly reduced the effect of thrombin on calcium mobilization and the cleavage of the thrombin receptors (PARs) in cancer cells. As one of major coagulation players, the multiple effects of thrombin depend on calcium. Since the level of thrombin was reduced in shMUC1-MCF-7 cells, it was decided to determine whether the effect of thrombin treatment of the cells was also impacted. The majority of the effect of serine protease, thrombin on cells is mediated by the binding to and cleavage of its cellular receptors (the proteinase activated receptor family, PARs) [32, 33]. Using Western blot, it was observed that there were differences between shCtrl-MCF-7 and shMUC1-MCF-7 cells in the response of the two major thrombin receptors, PAR1 and PAR4 to calcium treatment. The level of expression of both receptors was shown to be slightly reduced in the MUC1 knockdown cells. In addition, both of the protein bands associated with thrombin cleavage were significantly weaker in shMUC1-MCF-7 cells compared with shCtrl-MCF-7 cells (Figure 2A).

It is well known that thrombin treatment induces cytosolic Ca2+ mobilization in a variety of cell types via cleavage of the cell receptor. As an example, thrombin-induced Ca2+ mobilization in human gingival fibroblasts is mediated by PAR-1 [34]. The time course of intracellular calcium mobilization was therefore determined using the Fluo-4 direct calcium assay kit, while cells were stimulated with increasing concentrations of each of the agonists tested with a lower response in each case in the shMUC1-MCF-7 cells compared to the shCtrl-MCF-7 cells (Figure 3). However, the difference between the response of shMUC1-MCF-7 and shCtrl-MCF-7 cells was relatively minor using the PMA and ionomycin mixture or TG, but was strikingly different when using thrombin as an agonist such that the potency of thrombin was reduced by 7- to 8-fold in the MUC1 knockdown
cells. These results suggest that the interaction of thrombin with the thrombin receptors is impacted by MUC1 either through facilitation of the binding to the receptor or to the cleavage and activity of the PAR receptor.

Figure 3. Downregulation of MUC1 reduced the effect of thrombin on calcium mobilization in cancer cells. Intracellular calcium mobilization activity analysis using a Fluo-4 direct calcium assay kit. Cells were stimulated with increasing concentrations of each of, phorbol 12-myristate 13-acetate (PMA) & ionomycin, thapsigargin (TG), and thrombin, respectively. Data represented by relative fluorescent units (RFU) calculated as the maximum response minus the minimum response divided by the minimum response.

Discussion
Intracellular Ca2+ homeostasis is governed by a network comprised of various Ca2+ channels and transporters proteins. Alterations in calcium signaling and/or the expression of calcium pumps and channels are an increasingly recognized property of certain cancer cells, which have resulted in calcium levels that exceed the typical threshold of normal cells. These elevated calcium levels allow the cells to proliferate and become malignant [35]. This present investigation now shows that the expression of several calcium signalling proteins, whose levels had been found to be abnormally modulated in tumor cells [17-28], was altered in the opposite direction following MUC1 gene knock down of breast cancer cells. The correspondence between the levels of this group of proteins and the level of MUC1 was also observed upon testing of human normal, pancreatic and breast cancer cells. The altered calcium transporter proteins consequently impacted calcium mobilization and thereby may be capable of interfering with prompt cell signalling.

It had been reported previously that MUC1 interacts with calcium-modulating cyclophilin ligand (CAML) and transfection of cells with plasmids...
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encoding MUC1 and CAML, respectively, increased intracellular calcium levels [10]. Our work found the expression level of CAML was not significantly changed in MUC1 downregulated cancer cells (data did not show). This indicates that MUC1 impacted calcium signalling did not depend on CAML as a transmembrane protein. Instead MUC1 may directly or indirectly, via various adhesion/receptor molecules impact the calcium dependent cellular functions.

Several observations regarding the effect of MUC1 on calcium induced protein and cellular function have been provided in this investigation. One of them is calcium/calmodulin-dependent protein kinase II (CaMKII). High levels of CaMKII are expressed in a variety of malignant diseases and the role of CaMKII in the diagnosis of different kinds of cancer has led to the development of CaMKII inhibitors as a novel cancer therapeutic target [36]. In particular, phosphorylation of CaMKII at T286, is involved in the control of breast cancer metastasis. The overexpression of T286D leads to phosphorylation of FAK, STAT5a, and Akt and offers a promising target for the development of therapeutics to prevent breast cancer metastasis [17]. Our investigation demonstrated that the activity of p-CaMKII (T286), as assessed by anti p-CaMKII (Phospho T286) antibody, was reduced in MUC1 downregulated cells. This correspondence between the activity of p-CaMKII and the MUC1 level was also observed in the tested human normal, pancreatic and breast cancer cell lines.

Another phenotypic feature of cancer cells is their procoagulant activity (PCA) and the activation of a number of the coagulation factors, requires calcium ions as a cofactor. TF expressed on cell surface is maintained in a cryptic state, which does not exhibit any measurable coagulant activity. A large calcium influx triggers both the exposure of phosphatidylserine and the expression of TF procoagulant activity on the cell surface [37]. The clotting activity assay used in this investigation is based on the use of cell extracts as a source of TF and phospholipid. Our results showed that the clotting activity, as well as the levels of other procoagulant proteins F-Va, F-Xa as well the thrombin induced increase in intracellular calcium which occurs following cleavage of PAR receptors, were reduced by MUC1 down regulation.

In conclusion, the downregulation of MUC1 is capable of modulating calcium cellular effects via the aberrant expression of calcium signalling proteins, which consequently impact calcium mobilization and thereby lead to interference in prompt cell signalling with a most striking effect on the properties of the breast cancer cells. These properties could play a crucial contributory role to MUC1 mediated effects on numerous signalling network within tumorigenesis. The identification of this novel property of MUC1 may help to the understand the effect of the altered Ca2+ signaling in cancer cells and guide the development of therapeutic targeting of MUC1.

Abbreviations:

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<tr>
<td>CaMKII</td>
<td>calcium/calmodulin-activated protein kinase II</td>
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<td>CAML</td>
<td>calcium-modulating cyclophilin ligand</td>
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<td>ICAM-1</td>
<td>intercellular adhesion molecule-1</td>
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<td>MUC</td>
<td>Mucin</td>
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<td>PCA</td>
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<td>PMCA</td>
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<td>PMA</td>
<td>phorbol 12-myristate 13-acetate</td>
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<td>SEACA2</td>
<td>sarcoplasmic or endoplasmic reticulum calcium ATPase2</td>
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Declarations

Ethics approval and consent to participate: Not applicable
Consent for publication: Not applicable
Availability of data and materials: The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request
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Authors’ contributions: Y. Chen designed and performed experiments, analysed the data, wrote the manuscript. M. Scully discussed and reviewed the data, and helped to write and edit the manuscript.
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SUPPLEMENTARY MATERIAL

Supplement Figure 1. Western blot shown the level of MUC1 was reduced after MUC1 gene knock down in shMUC1-MCF-7 cells in comparison with shCtrl-MCF-7 cells.