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Mucin 2 (MUC2) modulates the aggressiveness of breast cancer

Anna Astashchanka¹, Thomas M. Shroka¹, Britta M. Jacobsen^{1,2}

¹Department of Pathology, University of Colorado Anschutz Medical Campus, Aurora CO 80045

²Department of Medicine, Division of Endocrinology, Metabolism and Diabetes, University of Colorado Anschutz Medical Campus, Aurora CO 80045

Abstract

Purpose: Tumors that secrete large volumes of mucus are chemotherapy resistant, however, mechanisms underlying this resistance are unknown. One protein highly expressed in mucin secreting breast cancers is the secreted mucin, Mucin-2 (MUC2). While MUC2 is expressed in some breast cancers it is absent in normal breast tissue, implicating it in breast cancer. However, the effects of MUC2 on breast cancer are largely unknown. This study examined the role of MUC2 in modulating breast cancer proliferation, response to chemotherapy and metastasis.

Methods: Using patient derived xenografts we developed two novel cell lines, called BCK4 and PT12, which express high levels of MUC2. To modulate MUC2 levels, BCK4 and PT12 cells were engineered to express shRNA targeted to MUC2 (shMUC2, low MUC2) or a non-targeting control (shCONT, high MUC2) and proliferation and apoptosis were measured in vitro and in vivo. BCK4 cells with shCONT or shMUC2 were labeled with GFP-luciferase and examined in an experimental metastasis model; disease burden and site specific dissemination were monitored by intravital imaging and fluorescence guided dissection, respectively.

Results: Proliferation decreased in BCK4 and PT12 shMUC2 cells versus control cells both in vitro and in vivo. Chemotherapy induced minimal apoptosis in control cells expressing high MUC2 but increased apoptosis in shMUC2 cells containing low MUC2. An experimental metastasis model showed disease burden decreased when breast cancer cells contained low versus high MUC2. Treatment with Epidermal Growth Factor (EGF) increased MUC2 expression in BCK4 cells; this induction was abolished by the EGF-receptor inhibitor, Erlotinib.

Conclusions: MUC2 plays an important role in mediating proliferation, apoptosis and metastasis of breast cancer cells. MUC2 may be important in guiding treatment and predicting outcomes in breast cancer patients.

Correspondence: Britta Jacobsen, Ph.D., 12800 E. 19th Ave, Mail Stop 8104, Aurora CO 80045, Tel: 303 724-3939, Fax: 303 724-3712, britta.jacobsen@ucdenver.edu.

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Data Availability: Data generated during this study are included in this published article and its supplementary information files, available online.

Keywords

Breast Cancer; Mucin-2; Metastasis; Mucinous; Estrogen Receptor

Introduction:

Breast cancer is the most common non-skin cancer in women, accounting for 1/3 of all cancer diagnoses, and the second leading cause of cancer mortality in women worldwide [1]. The standard of care for breast cancer diagnosis and treatment includes determining the molecular subtypes of the cancer. Established molecular markers estrogen receptor (ER), progesterone receptor (PR) and Epidermal Growth Factor Receptor 2 (ERBB2/HER2) are used clinically to guide treatment and predict outcomes. Targeted therapies are available for women with ER⁺ and/or HER2⁺ breast tumors; women with tumors lacking these markers are commonly treated with chemotherapy. However, additional therapeutic targets are needed to treat women with breast tumors that are resistant to therapy, and to create more personalized therapy for breast cancer patients.

Mucins are heavily O-glycosylated macromolecules that can be membrane bound or secreted that are produced in healthy tissues and serve multiple functions including protective barriers, providing lubrication, communication between cells, regulating gene expression and maintaining the structure and function of the gastrointestinal, pulmonary, reproductive and urinary tracts [2]. Both transmembrane and secretory mucins can be upregulated in breast tumors versus normal breast tissue, implicating mucins in cancer progression [3]. Malignant transformation can also alter the glycosylation pattern of mucins and can significantly increase the expression of specific mucins [2]. The membrane bound mucin, Mucin-1, is the most frequently expressed mucin in breast tumors (>90%), is consequently the most studied mucin that has been used as a marker for prognosis and metastatic disease, and may be a promising therapeutic target (reviewed in [4]).

Secreted mucins protect and lubricate the luminal surfaces of epithelial ducts. Among the most abundant secreted mucins in breast cancer is Mucin-2 (MUC2), is a large glycoprotein that forms a gel when secreted. Normally, MUC2 is expressed in the gastrointestinal tract where it provides lubrication and protection from injury, and its expression is regulated by many factors including epidermal growth factor (EGF) and tumor necrosis factor alpha (TNFA, reviewed in [5]). MUC2 is not expressed in the normal breast, however it is expressed in ~1/3 of ductal and lobular in situ lesions (DCIS, LCIS) and in invasive breast cancer ([6,7] and reviewed in [3]) implicating MUC2 in breast cancer progression. While the majority of pure mucinous breast cancers (defined as tumors with >90% tumor volume secreted mucin) express MUC2, its expression among non-pure mucinous breast cancers (including invasive breast cancers with mucinous features where the tumor volume is <90% secreted mucin) ranges from 6-20% [8-14,7]. With regard to MUC2 expression and prognosis, one study found no correlation of MUC2 expression with patient outcome [9] while others show shorter overall survival for patients with MUC2⁺ breast cancer versus those that lack MUC2 [11,15].

The role of MUC2 in mediating response to chemotherapy has not been examined in breast cancer. However, two lines of evidence support a role of MUC2 in cancer therapeutic resistance. First, colon cancer cells resistant to chemotherapy express high levels of MUC2 [16] and second, pure mucinous breast tumors (which produce high levels of MUC2) are more resistant to chemotherapy [17] and patients have a worse outcomes with aromatase inhibitors than other special histotypes [18]. Furthermore, while pure mucinous breast tumors are usually considered indolent, the clinical assay Oncotype Dx shows patients with these tumors frequently have an intermediate risk for recurrence [19,20].

Whether MUC2 is involved in breast cancer metastasis is controversial. One study shows MUC2 expression in a tumor is positively associated with lymph node metastasis [12], while another shows MUC2 expression is inversely correlated with lymph node stage and vascular invasion, even when excluding mucinous carcinomas [9]. To test the contribution of MUC2 in breast cancer, we isolated two novel MUC2⁺ cell lines called PT12 and BCK4 [21] that model invasive breast cancer with mucinous features. These cell lines were modified to decrease MUC2 expression and studied in vitro, in vivo as solid tumors and in an experimental metastasis model to examine the effect of MUC2 on proliferation, chemosensitivity and metastasis of MUC2⁺ breast cancer.

Materials and Methods:

BCK4 and PT12 Cell Lines:

BCK4 and PT12 cell lines were created by us using the patient derived xenograft for BCK4 [21] and the UCD12 xenograft [22] respectively, as described [21]. Cell lines were grown in specified media [23] and authenticated by the University of Colorado Cancer Center Tissue culture core laboratory using Short Tandem Repeat Analysis as previously described [24]. Cells were screened regularly to ensure cultures were mycoplasma free. BCK4 cells were labeled with GFP-luciferase as previously described [23].

shRNA Lentiviral knockdown:

Lentiviral vectors encoding small hairpin RNA (shRNA) were used to stably inhibit MUC2. The empty shRNA parent vector was used as the negative control (shCONT). ShRNA vectors (Mission; Sigma) were from the University of Colorado Functional Genomics Facility. Two constructs (73551 and 73552) were used for further analysis and abbreviated shMUC2_51 and shMUC2_52, respectively (sequences and supplementary methods specified in Supplementary Information file available online). Cells were infected and selected as previously described [25]. MUC2 expression was measured using immunoblotting.

Immunoblotting:

Whole cell extracts of cells were prepared and resolved as previously described [26]. Primary antibody was MUC2 (Thermo MS-1729-P1) and loading control α -tubulin (Sigma-Aldrich, T5168). Secondary antibodies were Alexa-fluor 680 Goat-Anti-Mouse IgG and Alexa-fluor 680 Goat-Anti-Rabbit IgG (A21058 and A21109; Lifetechnologies,

ThermoFisher, USA). Immunoblots were imaged using the Odyssey Infrared Imaging System (Li-Cor Biosciences). Experiments were repeated at least twice.

Proliferation assays:

Proliferation assays on BCK4 cells were performed using the IncuCyte ZOOM live imaging system (Essen BioSciences) as previously described [25] using regular growth media. Briefly, 10,000 BCK4 cells (nuclear Red Fluorescent Protein) were used per well of a 96 well plate and quantified using IncuCyte analysis. For PT12 cells, cell counting was performed using trypan blue exclusion on a hemacytometer at 0, 3 and 6 days. Cells were plated into 6 well dishes using regular growth media.

Xenograft studies:

All animal studies were performed according to a protocol approved by the University of Colorado Institutional Animal Care and Use Committee and were conducted in accordance with the NIH Guidelines of Care and Use of Laboratory Animals. For BCK4 cells, 5×10^6 cells were injected bilaterally in Matrigel® (Corning) into the 4th mammary fat pad of female NOD/SCID gamma mice (NSG, Jackson Laboratory) supplemented with a pellet containing 2mg of estradiol (E2) as previously reported [25]. Briefly, cells containing shCONT were injected on one side, shMUC2_51 cells were injected in the bilateral #4 mammary glands. Tumor volume was measured weekly by digital calipers and was calculated using the formula $\text{volume} = (\text{length} \times \text{width}^2)/2$. For PT12 cells, NSG mice were injected as above using 1×10^6 shCONT and shMUC2_51 cells.

For the experimental metastasis studies, BCK4 cells expressing GFP-Luciferase (GFP-Luc) without shMUC2 (control cells) or expressing shMUC2 were injected intracardially into NSG mice treated with estradiol as above. Metastasis and disease burden were examined using IVIS live imaging as previously described [23]. IVIS analysis was confirmed using the Illumatool in vivo imaging system [27] and histological analysis of paraffin embedded tissue using H&E and immunohistochemistry.

Immunohistochemistry and microscopy:

Primary tumors and metastatic tissues were resected, formalin fixed and/or decalcified (bone) and sent to the University of Colorado Denver Biorepository Core where tissues were paraffin-embedded. Tumors were sectioned and stained using immunohistochemistry performed as previously described [21]. Antibodies used were: MUC2 (1:10000 Abcam ab134119) and MCM2 (Cell Signaling, 4007). Images were captured using the Aperio Digital Pathology system (Leica Biosystems) and positive staining was quantified via Imagescope software (Leica). Mucicarmin staining was performed as previously reported [21] and used to assess total secreted mucins and quantify necrotic regions. For mucicarmin staining, the Aperio imaging system was used followed by quantitation using the ImageJ software (FIJI).

Statistics:

Statistics were performed using Graphpad Prism 7.0. Two-tailed Student's t-test were used as indicated. Values $p < 0.05$ were considered significant. Tumor growth was analyzed using multiple comparisons ANOVA followed by Tukey post hoc multiple comparison tests.

Results:

MUC2 and overall survival of women with breast cancer

Whether MUC2 expression is correlated with survival of breast cancer patients is controversial. While pure mucinous breast tumors (typically express high levels of MUC2) are usually considered indolent, Oncotype Dx shows intermediate risk of recurrence [19]. Using the PROGgeneV2 prognostic database [28], we examined the Swedish breast cancer patient dataset [29] for correlation of MUC2 with overall survival. Patients whose breast tumors expressed high MUC2 (80 tumors) showed lower overall survival versus those that contained low MUC2 (79 tumors; Supplementary Figure 1, Hazard Ratio = 1.68, $p = 0.002961$ as calculated by the software [28]).

MUC2 is highly expressed in BCK4, PT12 cell lines and modulates proliferation

To identify the prevalence of MUC2 in breast cancer cell lines and select appropriate models for study, we evaluated MUC2 expression in seven ER⁺ breast cancer cell lines. Two novel breast cancer cell lines that we derived from patient derived xenografts, called BCK4 and PT12, had mucinous features. BCK4 cells are invasive lobular breast cancer (ILC) and ER⁺/PR⁺/HER2¹⁺ [21]; PT12 cells are invasive ductal breast cancer and ER⁺/PR⁻/low/HER2¹⁺. We examined BCK4 and PT12 cells with 5 additional ER⁺ breast cancer cell lines (MDA-MB-134VI, SUM44PE, BT474, ZR75-1 and MCF7) for MUC2 expression via immunoblotting (Supplementary Figure 2) and found BCK4 and PT12 cells expressed high levels of MUC2. Interestingly, MUC2 was also detected at low levels in two ER⁺ ILC cell lines, MDA-MB-134VI (MDA134) and SUM44PE, albeit at a lower level than the BCK4 ILC cells. Because of their high MUC2 expression, BCK4 and PT12 cells were used in subsequent studies. Next we decreased MUC2 in BCK4 and PT12 cells using two shRNA directed to MUC2, designated shMUC2_51 and shMUC2_52. Immunoblotting confirmed decreased MUC2 expression in shMUC2 cells versus the empty vector control (shCONT; Figure 1, left panels); quantification (Figure 1, middle panels) showed a 66% and 40% decrease in MUC2 in BCK4 cells, and 72% and 83% decrease of MUC2 in PT12 cells. Interestingly we were not able to get complete knockdown of MUC2 which likely indicates the dependence of these cell lines on MUC2. Next we examined the effects of decreasing MUC2 on proliferation in BCK4 and PT12 cells. Proliferation decreased 10-26% in BCK4 cells and 47-54% in PT12 cells expressing shMUC2 compared to control cells (shCONT; Figure 1, right panels). These data suggest that MUC2 enhances cell proliferation in vitro.

MUC2 modulates tumor volume, proliferation and necrosis in breast tumor xenografts

To assess the effects of MUC2 on solid tumor growth, BCK4 or PT12 control cells, or cells containing shMUC2 were implanted into immunocompromised mice in the mammary fat pad. Tumor growth was measured by calipers for the time indicated (Figure 2). Tumor

volume of BCK4 xenografts with shMUC2 was significantly decreased versus control tumors with higher MUC2 (Figure 2a). Tumor volume in PT12 cells with shMUC2 showed a trend in decreased volume versus control cells with high MUC2, however, the change was not statistically significant (Figure 2b). Decreased expression of MUC2 in shMUC2 vs. control BCK4 and PT12 tumors was confirmed by immunohistochemistry (Figures 3 and 4 respectively, top panels). Next, we examined the effects of MUC2 on proliferation using a sensitive marker for proliferation in ER⁺ breast cancer called Minichromosome Maintenance Complex Component 2 (MCM2) [30]. BCK4 tumors with shMUC2 showed decreased proliferation versus control tumors (Figure 3, middle panels). Proliferation was also decreased in PT12 tumors with shMUC2, however, this did not reach statistical significance (Figure 4, middle panels). Next we performed a special stain for total secreted mucin expression using mucicarmine staining of BCK4 and PT12 tumors (Figures 3 and 4 respectively, bottom panels). Total secreted mucin was decreased in both BCK4 and PT12 shMUC2 tumors versus controls as shown by mucicarmine and MUC2 staining (Figures 3 and 4 and Supplementary Figure 3). Mucicarmine staining was also used to assess necrosis more easily than H&E staining because it allows clear visualization of necrotic areas. Versus control tumors, necrosis did not significantly change in BCK4 tumors upon decreased MUC2 expression (Figure 3, bottom), however, necrosis was increased PT12 tumors with decreased MUC2 (Figure 4, bottom). Thus, while proliferation in PT12 tumors was slightly decreased by decreased MUC2 expression, necrosis was strongly increased. We also examined apoptosis using immunohistochemistry with an antibody to cleaved caspase-3, however, no changes in apoptosis were observed (data not shown).

Decreased MUC2 expression increases sensitivity of BCK4 cells to chemotherapy

To evaluate whether MUC2 expression affects chemosensitivity in breast cancer cells, shMUC2_51, shMUC2_52 and control cells were treated with the chemotherapeutic agent docetaxel and cleaved-caspase 3 activity was measured. Docetaxel treatment induced minimal apoptosis in BCK4 control cells (shCONT), however, it significantly increased apoptosis in BCK4 cells with reduced MUC2 (shMUC2_51 and shMUC2_52, Supplementary Figure 4).

ER increases in xenograft tumors with decreased MUC2

Previous reports examining the effects of secreted mucins on breast cancer showed an inverse correlation between ER and MUC2 [9] while others have shown a direct correlation [14] or no association [7]. We evaluated changes in ER levels in our MUC2 manipulated tumors. ER expression increased in shMUC2 vs. control cells in BCK4 and PT12 tumors although the later did not quite reach statistical significance (Supplementary Figure 5a and 5b).

MUC2 modulates metastatic seeding

Next we examined the role of MUC2 in metastasis of breast cancer cells using an experimental metastasis model where tumor cells were injected into the left ventricle of immunocompromised mice. We injected GFP-luciferase labeled BCK4 control cells containing wild type MUC2 levels and performed IVIS analysis to visualize metastatic disease (Figure 5a, left) at 11 weeks post injection. Cells expressing MUC2 seeded

predominantly in the jaw, adrenal gland and ovary, with occasional lesions seen in other sites. Next we examined MUC2 expression among these three metastatic sites (Figure 5a, right). Quantification of MUC2 staining shows MUC2 is expressed at higher levels in BCK4 cells that have seeded in the jaw (42% MUC2⁺) compared to cells seeded to the ovary (21% MUC2⁺) and adrenal gland (13% MUC2⁺). This indicates the metastatic microenvironment influences MUC2 expression.

To directly test the effects of MUC2 on breast cancer cell metastasis, luciferized BCK4 cells with decreased MUC2 (shMUC2) were injected intracardially into NSG mice and IVIS analysis was performed 13 weeks post injection. Decreased MUC2 levels in luciferase labeled cells was confirmed prior to injection using immunoblotting (Supplementary figure 6). Diminished MUC2 virtually eliminated metastatic disease (Figure 5b) as shown by IVIS analysis of mice in both the supine and prone position vs. control mice expressing higher levels of MUC2 (Figure 5a). Upon resection of tissues, minimal disease was observed in one jaw, one clavicle and one ovary by immunohistochemistry among the cohort of 13 mice (all three tissues of all mice were examined for presence of tumor cells; none were observed, data not shown). Viability of shMUC2 cells was confirmed by luciferase/GFP expression and trypan blue exclusion prior to and following intracardiac injection. These data suggest that MUC2 may be important for metastatic seeding and outgrowth of MUC2⁺ breast cancer metastases, and these metastatic cells may be dependent on MUC2.

Regulation of MUC2 expression in breast cancer cells

MUC2 expression is regulated in the gastrointestinal tract and pulmonary malignancies by cytokines including epidermal growth factor (EGF) and Tumor Necrosis Factor alpha (TNF, reviewed in [5]). Next we examined whether MUC2 expression was regulated by TNF or EGF in breast cancer cells by treating BCK4 cells with TNF, or EGF with or without the EGF receptor inhibitor Erlotinib (Supplementary Figure 7). MUC2 expression was measured by quantitative immunocytochemistry. Compared to vehicle treated control cells, MUC2 increased 1.8 fold in cells treated with EGF ($p=0.0003$) and 2.4 fold with TNF ($p<0.0001$); expression decreased 2.1 fold with Erlotinib ($p=0.0004$) and 1.9 fold when Erlotinib was added to EGF ($p<0.0001$).

Discussion:

Secreted proteins in the tumor microenvironment influence tumor biology and treatment. Secreted mucins like MUC2 can be intracellular, extracellular or both [31,32]. While MUC2 expression in breast tumors ranges from 6-20% [8-14,7], most of these studies used the anti-MUC2 antibody CCP58, which recognizes an immature (likely non-glycosylated) form of MUC2, but not secreted MUC2. Typically mucins are highly o-glycosylated in the tandem repeat regions commonly used as immunogens for antibodies, thus glycosylation can determine antibody recognition, however, glycosylation of mucins often decreases in cancer versus normal tissues [33]. Furthermore, several studies used tumor microarrays that allow high throughput analysis of multiple tumors, but use small tumor regions which might not accurately reflect the heterogeneity of the original tumor (i.e. mucinous versus non mucinous regions) [34]. MUC2 expression may be underestimated in published studies.

Importantly, the antibody used in our studies recognizes both cytoplasmic and secreted MUC2 with high affinity and detects even low levels of MUC2. Whether this antibody is more sensitive than other MUC2 antibodies on clinical samples remains to be determined.

Models to study the effects of secreted mucins on breast cancer are rare; to date only our models are ER⁺/MUC2⁺ [21,22]. Overexpression of secreted mucins using exogenous expression vectors is technically difficult due to their large size (550 kDa) and extensive glycosylation. While the function of MUC2 in breast cancer has not previously been reported, the secreted mucin 5B (MUC5B) is expressed in breast cancer [35] and has been studied in MCF7 cells which lack endogenous expression of secreted mucins (MUC2 expression is inducible by activated p53 [36]). Because MUC5B is over 500 kDa, MCF7 cells were engineered to express a “mini-mucin MUC5B”; expression promoted a proliferative and aggressive phenotype [37]. Importantly, MUC5B expression was observed in all mammary tumors derived from MMTV-ras mice [38]. MUC5B also modulates chemoresistance in breast cancer cells [39], implicating secreted mucins in the aggressiveness of breast cancers. Our two models of MUC2⁺ breast cancer allow, for the first time, the ability to study the effects of the full-length secreted mucin, MUC2, on breast cancer biology.

MUC2 and proliferation in cancer

MUC2 is the predominant mucin expressed in normal epithelial cells of the gastrointestinal tract where it functions as a tumor suppressor and regulates inflammatory and metabolic pathways [40]; diminished MUC2 expression increases proliferation of colon cancer cells in vitro and in vivo [41]. In contrast, MUC2 is a tumor promoter in breast cancer (in non-mucinous breast tumors with <90% mucin), perhaps a reflection of its aberrant expression in breast cancer versus normal breast. Similarly, MUC2 is not expressed in normal ovary however, it is a tumor promoter in ovarian cancer [42] where MUC2 levels are inversely correlated with patient survival and the M1/M2 macrophage ratio where tumor promoting M2 macrophages increase in tumors with high MUC2 versus those with low MUC2. Whether this also occurs in MUC2⁺ breast tumors remains to be determined.

The effects of MUC2 on proliferation of other cancer types is varied. MUC2 expression is often associated with noninvasive tumors with site restricted growth [43]. This could be for a multitude of reasons. Mucins may promote cell survival and proliferation by configuring the local microenvironment for cells to survive the hypoxic, acidic and protease-laden sites of tumor growth [44]. Kufe et al. theorized that MUC2 overexpression may, through the generation of the mucous barrier, protect cancer cells from recognition by anti-tumor immune effectors and contribute to the malignant phenotype [2]; while this may be the case for some tumor types (colon), it may not be true for others (ovarian).

MUC2 and metastasis

Metastasis of ER⁺ breast cancers most commonly occurs to the lungs, bone and brain [45]. In our metastasis model using ER⁺/MUC2⁺ breast cancer cells the predominant metastatic sites were bone and ovary; metastatic cells maintained MUC2 expression with highest expression in jaw metastases, suggesting MUC2 plays a role in breast cancer metastasis.

Furthermore, metastatic seeding of breast cancer cells with low MUC2 was dramatically decreased to all sites. This decrease in metastasis supports the data of Valque et al where overexpression of the secreted mucin MUC5B increased invasiveness of MCF7 breast cancer cells in vitro and in vivo [37].

In contrast, diminished MUC2 expression in a colon cancer model increases metastasis via increased expression of interleukin-6 [46], however, tumor cells were implanted directly into the liver thus metastatic outgrowth rather than seeding was measured. Our experimental model of breast cancer metastasis assesses both seeding and outgrowth and showed decreased MUC2 blocked both; this corroborates MUC2 expression correlating with lymph node metastasis [12] but contradicts a study using tumor microarrays where MUC2 inversely correlated with lymph node metastasis [9]. Analysis using larger tissue regions shows MUC2 expression correlates with higher aggressiveness in DCIS, and is associated with field effects [6]. It is also possible that MUC2⁺ tumors do not metastasize through the lymphatics but rather via hematogenous dissemination. Analyzing a larger breast tumor cohort using larger pieces of MUC2⁺ breast tumors may shed light on the role of MUC2 in breast cancer metastasis.

MUC2 regulation, therapeutics and clinical implications

Our studies indicate that high MUC2 predicts a poor prognosis for patients with breast cancer confirming other studies [11,15]. MUC2 has also been reported as an independent negative prognostic factor with significantly shorter disease free and survival time for patients with a variety of other cancers, including bladder [47], gastric [48], and ovarian [42]. This could be due to secreted mucins playing a role in chemoresistance (reviewed in [49]). MUC2 confers resistance to 5-fluorouracil in colon cancer [50] and to docetaxel in breast cancer cells in our study, suggesting alternative therapies are needed to effectively treat ER⁺/MUC2⁺ breast tumors. Interestingly, ER and MUC2 are inversely regulated in our study, confirming a previous report in MUC2⁺ breast tumors [9] suggesting crosstalk between ER and MUC2, however, the mechanism(s) underlying this is unknown. However, MUC2 is rarely expressed in ER-negative and triple negative breast tumors [7,14]. MUC2 is also regulated by natural factors including bile acids, EGF, TNF curcumin and resveratrol in colon and gastric cancer (reviewed in [5]). In our studies MUC2 expression increased with TNF and EGF, an effect that was abolished by inhibition of EGFR with Erlotinib. This has important clinical implications because Erlotinib could be used in MUC2⁺ tumors to decrease MUC2 expression and increase sensitivity to chemotherapy like docetaxel. Erlotinib is currently used clinically to treat non small cell lung cancer but could also be tested as neoadjuvant therapy for patients with MUC2⁺ breast tumors prior to resection. Further studies are required to address these possibilities.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1: Decreased expression of MUC2 attenuates proliferation of BCK4 and PT12 cells.

BCK4 (top panels) or PT12 (bottom panels) cells were engineered to express vector shRNA (shCONT) or shRNA directed to MUC2 (shMUC2). MUC2 levels were detected by immunoblotting of BCK4 and PT12 cells (left). Densitometry of MUC2 normalized to tubulin is shown graphically (middle). Proliferation was measured of cells expressing control shRNA (shCONT), shMUC2_51 or shMUC2_52 plated in a 96 well plate in triplicate and cell density was quantified using the IncuCyte live cell imaging system (BCK4) or trypan blue exclusion (PT12). Cells were plated and grown for times shown and proliferation was measured at times specified. Fold change is proliferation versus day zero.

* $p < 0.0001$ versus shCONT by student's t test

Figure 2: Decreased Muc2 inhibits tumor growth.

a) BCK4 cells or **b)** PT12 cells containing control (shCONT, black line) or shRNA targeting MUC2 (shMUC2, red line) were injected into the 4th mammary gland of immunocompromised mice supplemented with estrogen. Tumor volume was measured using digital calipers at the times specified. Growth curves are shown of shCONT tumors (black line) or tumors with decreased MUC2 (shMUC2, red line). p=0.0004 (left graph), p=0.4882 (right graph) by students t-test. For BCK4 tumors n=4 mice/group, for PT12 tumors n=8 mice/group.

Figure 3: Decreased MUC2 decreases proliferation in BCK4 tumors.

BCK4 xenograft tumors containing shRNA to MUC2 (shMUC2) versus wild type tumors (shCONT) (top panels) show decreased MUC2 expression by immunohistochemistry. Decreased Muc2 expression decreases proliferation by MCM2 staining (middle panels) and trends toward an increase in necrosis as examined by mucicarmine staining (mucin pink, nuclei green, bottom panels). N=4 tumors/group. Statistical significance determined using student's t-test. Quantitation for each stain of each tumor section in its entirety is shown on the right. 20x images shown for MUC2 and MCM2; mucicarmine images shown at 7.5x to illustrate necrotic areas.

Figure 4: Decreased MUC2 increases necrosis in PT12 tumors.

PT12 xenograft tumors containing shRNA to MUC2 (shMUC2) versus wild type tumors (shCONT) (top panels) show decreased MUC2 expression by immunohistochemistry. Decreased Muc2 expression decreases proliferation by MCM2 staining (middle panels) and increase in necrosis as examined by mucicarmine staining (mucin pink, nuclei green, bottom panels). N = 8 tumors/group. Statistical significance determined using student's t-test. Quantitation for each stain of the entire tumor is shown on the right. 20x images shown for MUC2 and MCM2; mucicarmine images shown at 7.5x to illustrate necrotic areas.

Figure 5: BCK4 cells metastasize to unusual sites and decreased MUC2 abolishes metastatic seeding.

a: **Left,** IVIS image of NOD-SCID gamma mice 11 weeks post injection in the supine position (top panel) and prone position (bottom panel). Mice were injected intracardially with luciferase tagged BCK4 cells ($n = 14$). Red denotes high signal, blue denotes low signal. **Right,** IHC showing MUC2 staining of BCK4 tumor cells seeded in different metastatic sites. Metastases were resected, paraffin embedded, sectioned and stained using an antibody to MUC2. Brown staining shows MUC2 expression, hematoxylin (blue) was used as a counterstain for nuclei. Green line denotes border of metastatic cells in ovary. Quantitation was performed of each metastasis section in its entirety using Aperio imaging at 20x and the ImageScope software. At least 3 tissues were stained per group: ovary ($n=4$), adrenal ($n=3$), jaw ($n=5$). Green line denotes boundary of tumor cells within the ovary. **b:** IVIS image of NOD-SCID gamma mice 13 weeks post injection in the supine position (top panel) and prone position (bottom panel). Mice were injected intracardially with luciferase tagged BCK4 cells with shMUC2 (low MUC2 levels) as in Figure 5a ($n = 13$). Compared to control mice with high MUC2 levels (Figure 5a) virtually no disease is detected.