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The role of membrane mucin MUC4 in breast cancer metastasis

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Abstract

A major barrier to the emergence of distant metastases is the survival of circulating tumor cells (CTCs) within the vasculature. Lethal stressors including shear forces from blood flow, anoikis arising from cellular detachment, and exposure to natural killer cells, combine to subvert the ability of primary tumor cells to survive and ultimately seed distant lesions. Further attenuation of this rate-limiting process via therapeutic intervention offers a very attractive opportunity for improving cancer patient outcomes, in turn prompting the need for a deeper understanding of the molecular and cellular mechanisms underlying CTC viability. MUC4 is a very large and heavily glycosylated protein expressed at the apical surfaces of the epithelia of a variety of tissues, is involved in cellular growth signaling and adhesiveness, and contributes to the protection and lubrication of cellular linings. Analysis of patient-matched breast tumor specimens has demonstrated that MUC4 protein levels are upregulated in metastatic lesions relative to primary tumor among all breast tumor subtypes, pointing to a possible selective advantage for MUC4 overexpression in metastasis. Analysis of a genetically engineered mouse model of HER2-positive breast cancer has demonstrated that metastatic efficiency is markedly suppressed with *MUC4* deletion, and MUC4-knockout tumor cells poorly associate with platelets and white blood cells known to support CTC viability. In this review we discuss the diverse roles of MUC4 in tumor progression and metastasis, and propose that intervening in MUC4 intercellular interactions with binding partners on blood-borne aggregating cells could potentially thwart breast cancer metastatic efficiency.

Keywords

breast; metastasis; circulating tumor cell; mucin; Muc4

Introduction

Breast cancer metastasis.

Despite significant advances in the detection and treatment of breast tumors over the last few decades, metastatic disease remains the primary cause of breast cancer-related

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Declaration of interest

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deaths (Weigelt et al., 2005). Metastasis is a multi-step process involving primary tumor cell invasion into the stromal compartment, intravasation into the blood or lymphatic systems, extravasation from capillaries at secondary sites, and colonization and outgrowth of metastatic lesions (Talmadge and Fidler, 2010, Lambert et al., 2017). Metastasizing tumor cells exhibit unique characteristics including altered cellular adhesion, evasion of programmed cell death, induction of the epithelial-to-mesenchymal transition (EMT), and association with cells in circulation (Lambert et al., 2017). Important intermediaries of the distant metastatic cascade are circulating tumor cells (CTCs), which exit primary tumors as single cells or clusters and travel to distant sites to seed metastatic lesions (Lambert et al., 2017, Micalizzi et al., 2017). It has been estimated that only ~0.01% of cells that enter circulation survive to seed metastatic lesions (Reymond et al., 2013), prompting questions concerning the mechanisms by which CTCs are able to shield themselves from the harsh conditions of transit.

While our understanding of the molecular underpinnings governing breast cancer metastasis have improved in recent years, our ability to successfully treat metastatic disease lags far behind. Novel methods and reliable markers to predict the probability of metastasis together with new approaches to therapeutically intervene could provide significant clinical benefit to the patients. Currently employed anti-metastatic therapeutic strategies closely align with those employed in the treatment of primary breast tumors, and typically involve a combination of chemotherapeutics, targeted therapeutics, and immunotherapies (Scully et al., 2012, Ganesh and Massagué, 2021). A significant challenge to this approach is that metastatic lesions often accumulate genetic alterations that are molecularly distinct from primary tumors, rendering such therapeutic avenues ineffective (Fidler and Kripke, 1977, Ganesh and Massagué, 2021). Thus, increasing effort has been dedicated toward identifying new prognostic markers for metastasis and developing strategies to target processes that contribute to aggressive disease, such as angiogenesis, cell motility, the metastatic microenvironment, metastatic dormancy, and circulating tumor cells (Steeg, 2016; Fontebasso and Dubinett, 2015).

Mucin proteins.

Mucins are large, heavily glycosylated cell surface proteins that normally function to lubricate and protect epithelial and vascular surfaces. The human mucin family is comprised of at least twenty distinct members that fall into two categories, secreted and membrane-bound. Both classes are characterized by the presence of a highly O-glycosylated variable number of tandem repeat (VNTR) domain, which contributes to cell protection by forming a large, protruding and negatively-charged structure that helps physically shield the cell surface from external assaults (Van Klinken et al., 1995). However, each class is also distinguished by its own distinct domains and functions. The secreted mucins (including MUC2, MUC5AC, MUC5B, MUC6, MUC7, and MUC19) contain trypsin inhibitor-like (TIL), von Willebrand factor type D (vWD), and C-terminal cysteine knot domains (Perez-Vilar and Hill, 1999) (Figure 1A). Importantly, secreted mucins oligomerize through their cysteine-rich vWD and C-terminal cysteine-knot domains (Perez-Vilar and Hill, 1999), directly contributing to their ability to coat epithelial and vasculature structures, and protect against outside infection and physical or chemical damage.

Membrane-bound mucins (including, MUC1, MUC3, MUC4, MUC12, MUC13, MUC15, MUC16, MUC17, and MUC20) also contain cysteine-rich regions similar to vWD domains and C-terminal cysteine knot domains (Bansil and Turner, 2018), but are distinguished from secreted mucins by the presence of a single transmembrane domain that tethers them to the cell surface. All membrane-bound mucins contain a sea urchin sperm protein, enterokinase, and agrin (SEA) domain, with the exception of MUC4, which instead contains a nidogen domain and an adhesion-associated (AMOP) domain (Kufe, 2009). Many of the transmembrane mucins also contain EGF-like domains important for interactions with other proteins (Figure 1B). While transmembrane mucins also contribute to coating and protection of epithelial surfaces, they also appear to play additional roles in cell signaling (Carraway et al., 2003). Importantly, numerous studies have demonstrated that aberrant expression of membrane-bound mucins, most notably MUC1 and MUC4, confer aggressive characteristics to tumors by promoting cellular proliferation, motility, and survival (the contributions of MUC1 to malignancy are reviewed in Nath and Mukherjee, 2014; Chen et al., 2021). MUC4 has been widely implicated as a prominent contributor to breast cancer progression and metastasis (Workman et al., 2009a, Rowson-Hodel et al., 2018) and thus will be the focus of this review.

Membrane mucin MUC4.

Mucin-4 (MUC4), a membrane-bound mucin, has been extensively studied in a diverse collection of normal tissues and solid tumors. Many of the foundational studies on MUC4 structure and expression patterns were performed in rat tissues over two decades ago, and key observations have been subsequently confirmed in human tissues following the cloning of the human gene (Moniaux et al., 1999). While MUC4 has been assigned diverse functions across many cell and tumor types, and function may be context-dependent in some cases, its central role as a mediator of metastasis is beginning to emerge. In this regard, MUC4 has been implicated as a regulator of several processes critical to metastasis, including cell adhesion (Komatsu et al., 1997), EMT and invasion (Moniaux et al., 2007), tumor cell docking (Senapati et al., 2012), therapeutic resistance (Price-Schiavi et al., 2002), and cell survival (Carraway et al., 2001; Komatsu et al., 2001). Because of its recent identification as an important regulator of metastasis by aiding CTC survival in circulation by associating with blood cells (Rowson-Hodel et al., 2018), we will provide an overview of MUC4 characteristics, emphasizing its role as a mediator of CTC-blood cell associations, and discuss the implications of these observations in cancer progression and the development of anti-metastatic therapeutic strategies.

Regulation of MUC4 expression

MUC4 expression is regulated through a collection of diverse mechanisms throughout both transcription and translation. The *MUC4* 5’ promoter region contains tissue-specific as well as positive and negative regulatory elements, underscoring the complex regulation of *MUC4* by a plethora of signaling pathways (Price-Schiavi et al., 2000a, Perrais et al., 2001). For example, in rat mammary adenocarcinoma cells, the transcription factor PEA3 directly binds the *MUC4* promoter and mediates *MUC4* transcription via Erk and SAPK/JNK signaling (Perez et al., 2003). Others have reported that, in human epithelial cancer cells,

MUC4 expression is spatiotemporally regulated by various transcription factors including HNF-1/-4, FOXA1/A2, GATA-4/-5/-6 and CDX-1/-2 (Jonckheere et al., 2007). Similarly, β-catenin can directly bind to the promoter of *MUC4* and alter transcript and protein levels in colorectal and pancreatic cancer cells in a Wnt-dependent manner (Pai et al., 2016a, Pai et al., 2016b). *MUC4* is also regulated by insulin and IGF-1 in an Erk-dependent manner in rat mammary epithelial cells (Zhu et al., 2000), and the pro-inflammatory cytokine IL-6 engages the gp130/STAT3 signaling pathway to stimulate the direct binding of phosphorylated-STAT3 to the *MUC4* promoter in gastric cancer cells (Mejías-Luque et al., 2008).

Evidence suggests that *MUC4* is also regulated epigenetically. In pancreatic cancer cells, the *MUC4* 5'UTR is methylated at CpG sites, and inhibition of histone deacetylation alters *MUC4* expression (Vincent et al., 2008). A similar study confirmed that methylation in the *MUC4* promoter region regulates *MUC4* expression, and that the pattern of DNA methylation correlates with *MUC4* expression across a number of solid tumor types (Yamada et al., 2009).

MUC4 is transcriptionally induced in both normal and cancer tissues via hormonal and cellular transformation inputs, respectively. For example, in the rat uterine epithelium, *MUC4* is transcriptionally regulated by ovarian hormones, such as estrogen and progesterone, where *MUC4* expression patterns are important for proper blastocyst implantation (McNeer et al., 1998). On the other hand, *MUC4* expression is elevated during pancreatic carcinogenesis by K-ras to engage MAPK, NF-κB, and RalB signaling pathways (Vasseur et al., 2015), and by the FXR/FAK/c-Jun signaling axis to promote pancreatic tumor progression and metastasis (Joshi et al., 2016).

It has also been observed that *MUC4* expression is altered through the direct binding of multiple regulators to *MUC4* mRNA in the 3' UTR. In mouse epithelial tissues, galectin-3 regulates *MUC4* mRNA stability and expression through the intermediate hbRNP-L (Coppin et al., 2017). Moreover, Srivastava et al. (Srivastava et al., 2011) demonstrated that miRNA-150 directly binds to the 3' UTR of *MUC4* mRNA to reduce MUC4 expression, resulting in decreased growth, clonogenicity, migration and invasion, but increased cell-cell adhesion of pancreatic cancer cells. Similarly, miR-219-1-3p negatively regulates *MUC4* expression in pancreatic cancer cells by binding to the *MUC4* 3'UTR to decrease cell proliferation and downstream activation of Akt and Erk (Lahdaoui et al., 2015). In cervical cancer cells, miR-211 directly binds the *MUC4* 3' UTR to decrease *MUC4* expression and cancer cell invasion, likely via reversal of EMT phenotypic properties (Xu et al., 2017).

TGFβ has been identified as a major regulator of MUC4 at multiple levels. Price-Shiavi et al. (Price-Schiavi et al., 2000b) found that in the rat mammary epithelial cells, but not in tumor cells, MUC4 is post-transcriptionally upregulated by TGFβ during pregnancy (Price-Schiavi et al., 1998), while in rat uterine luminal epithelial cells, regulation of MUC4 protein is controlled by TGFβ expressed in the stroma and may be antagonized by estrogen (Idris and Carraway, 2000). Mechanistically, it has been observed that TGFβ targets the MUC4 precursor protein for proteasomal degradation (Lomako et al., 2009, Price-Schiavi et al., 1998). In pancreatic tumor cells, retinoic acid alters MUC4 through direct regulation

of TGFβ expression (Choudhury et al., 2000). *MUC4* was also found to be transcriptionally upregulated by TGFβ in pancreatic cancer cells, and Smad2/Smad4 activation at the *MUC4* promoter was negatively regulated by Smad7 and c-Ski (Jonckheere et al., 2004). Similarly, SMAD2 regulates TGFβ-induced *MUC4* expression in mammary epithelial cells, whereas IFN-gamma, through induction of STAT1 activation and upregulation of expression of the inhibitory Smad7, inhibits the effect of TGF-β on MUC4 expression (Soto et al., 2003). Together, these studies underscore the remarkably complex regulatory mechanisms governing *MUC4* expression in both normal and cancer contexts.

MUC4 structure and function

MUC4 structure.

MUC4 was identified as sialomucin complex (SMC) in a highly metastatic rat adenocarcinoma (Sherblom et al., 1980a; Sherblom et al., 1980b). Human *MUC4* is located on chromosome 3q29 (Porchet et al., 1991; Gross et al., 1992), varies in length from 4468 to 8468 amino acid residues, and is highly similar in structure to rat MUC4 (Moniaux et al., 1999). The MUC4 precursor is synthesized in the endoplasmic reticulum where it is *N*-glycosylated, folds, and is then proteolytically cleaved to produce the two non-covalently associated subunits, MUC4α and MUC4β (Sheng et al., 1990; Sherblom and Carraway, 1980; Helm and Carraway, 1981; Soto et al., 2006). MUC4α contains the variable tandem repeat domain, which is subject to extensive *O*-glycosylation following cleavage of the precursor (Spielman et al., 1987, Nollet et al., 1998). *O*-glycans primarily contribute to the bulky and protruding structure of MUC4, which is important for the protection and lubrication functions of the complex. While the structure of the alpha subunit is quite similar between rat and human, human MUC4 contains a much more extensive variable tandem repeat domain, making the molecule significantly larger (Moniaux et al., 1999). The membrane-associated subunit MUC4β shares a 60-70% amino acid sequence identity between rat and human (Moniaux et al., 1999). MUC4β consists of a ~120 kDa extracellular region, a single hydrophobic transmembrane domain and a very short (~20 residue) cytoplasmic tail. The extracellular region contains *N*-glycosylation sites and two epidermal growth factor (EGF)-like domains (Sheng et al., 1992, Moniaux et al., 1999) that confer important roles to the MUC4β subunit in cell signaling (Carraway et al., 1999; Wu et al., 1994).

MUC4 expression patterns.

MUC4 is expressed in numerous normal epithelial tissues during development and in adult (Price-Schiavi et al., 2000b; Rossi et al., 1996; Idris and Carraway, 1999; Zhang et al., 2005). In adult tissues, MUC4 is expressed in endothelial cells lining the vasculature (Zhang et al., 2005) and on the apical surfaces of epithelial cells, where it may serve as a marker of differentiation (Li et al., 2001). While early observations with a rat mammary adenocarcinoma model suggested that MUC4 may be overexpressed in tumors relative to normal tissues (Rossi et al., 1996), subsequent findings with patient samples indicate that MUC4 is aberrantly expressed across diverse solid tumor types, and is often, but not always, correlated with worsened prognosis or advanced disease (Table 1). MUC4 expression is increased in the primary tumors of various breast tumor subtypes (Komatsu et al., 1999;

Rakha et al., 2005; Mukhopadhyay et al., 2013; Shet et al., 2013; Mercogliano et al., 2017b), upper aerodigestive tract squamous cell carcinomas (Weed et al., 2001), ovarian tumors (Chauhan et al., 2006), pancreatic adenocarcinomas (Mimeault et al., 2010; Kaur et al., 2014), lung adenocarcinomas (Gao et al., 2014), the “columnar type” of mucin-producing bile duct tumors (Shibahara et al., 2004b), glioblastomas (Li et al., 2014b), cervical tumors (Xu et al., 2017), and intrahepatic cholangiocarcinomas (Shibahara et al., 2004a) compared to normal tissues. In TCGA datasets, high MUC4 expression is correlated with poor patient survival in pancreatic cancer, bladder cancer, colon cancer, lung adenocarcinoma, lung squamous carcinoma, ovarian cancer, skin cancer, and stomach cancer. In these tumors, MUC4 expression is linked to genes involved in cell adhesion, cell-cell junctions, glycosylation, and cell signaling (Jonckheere and Van Seuningen, 2018).

In human breast tumors, MUC4 appears to undergo significant changes in expression throughout the tumor progression process. We previously assessed MUC4 protein abundance using tissue microarrays containing patient-matched normal breast tissue, primary breast tumor tissue, and lymph node metastases. In contrast to other studies, we observed decreased MUC4 expression in the primary tumors compared to matched normal tissues, followed by a recovery of MUC4 expression in lymph node metastases (Workman et al., 2009a). Low MUC4 expression in the primary tumor is consistent with the role of MUC4 as a marker of differentiation in epithelial cells (Li et al., 2001); as tumors develop, the cellular transition from a differentiated to a de-differentiated state decreases MUC4 expression (Gabbert et al., 1985). However, the increased expression of MUC4 in lymph node metastases suggests that MUC4 may confer an advantage to cells attempting to metastasize. These MUC4 expression trends were independent of ER/PR and HER2 status, pointing to a possible universal role of MUC4 in promoting breast cancer malignancy. Collectively, MUC4 expression in various cancer types and stages of disease is highly diverse, suggesting that MUC4 function is likely dependent on the biological context.

MUC4 as a mediator of tumor cell adhesion and cell-cell interactions.

A major function of MUC4 is the promotion of anti-adhesiveness. The extensively modification of MUC4α by *O*-linked glycans creates a bulky and rigid structure that contributes to steric hindrance and the approach of foreign pathogens, bodies and molecules (Komatsu et al., 1997). Early studies in rat tissues suggest that overexpression of MUC4 disrupts both cell-cell and cell-substrate interactions, the degree to which is dependent on the number of repeats in the VNTR region and extent of O-glycosylation of the rat MUC4α subunit (Komatsu et al., 1997). *N*-glycosylation of the MUC4β extracellular domain can also contribute to the steric hindrance properties of MUC4, enabling the protein to mask the cell surface and prevent the binding of molecules to their substrates (Komatsu et al., 1997). In normal polarized human mammary epithelial cells, overexpression relocalizes MUC4 from the apical surface to the lateral surfaces of epithelial cells, resulting in disruption of adherens junctions and impairing cell-cell attachments (Pino et al., 2006). Additionally, MUC4 overexpression in pancreatic tumor cells disrupts tumor cell-extracellular matrix (ECM) interactions by inhibiting integrin-mediated cell adhesion (Chaturvedi et al., 2007), and inhibits cell adhesion and aggregation by preventing interactions between MUC4-expressing tumor cells (Singh et al., 2004). Importantly, loss of MUC4 in melanoma cells results

in a rapid reversal from a non-adherent to an adherent state (Komatsu et al., 1997), a process critical to metastasis. In rat mammary adenocarcinoma, MUC4 expression on the cell surface aids in tumor cell evasion of immune killing by masking antigens for immune cell recognition through steric disruption of interactions between tumor cells and cytotoxic immune cells (Sherblom and Moody, 1986, Komatsu et al., 1999). While MUC4 is an important mediator of anti-adhesiveness, the ability of MUC4 to directly engage molecules on the surface of other cells appears to promote adhesion in some biological contexts. In human pancreatic cancer cells, surface MUC4 interacts with galectin-3 on endothelial cells to support the attachment and docking of CTCs to the endothelium, a process essential for extravasation and seeding of metastatic colonies (Senapati et al., 2012). Together, these studies demonstrate that MUC4 mediates a diverse array of cell-cell adhesion and cellular interactions that promote metastasis.

MUC4 contribution to cellular survival mechanisms.

MUC4 also contributes to anti-apoptotic signals in cancer cells to promote tumor growth and metastasis through its membrane-associated beta subunit. Overexpression of rat MUC4 in human melanoma cells promotes xenografted tumor growth through suppression of cell death rather than alteration of cellular proliferation rate by directly regulating cell survival signals (Carraway et al., 2001, Komatsu et al., 2001). In human breast cancer cells, MUC4 suppresses apoptosis through mechanisms both dependent and independent of the HER2 (ErbB2) receptor tyrosine kinase, in some contexts signaling through the PI3K-Akt pathway (Workman et al., 2009b). Additionally, increased MUC4 expression in melanoma cells decreases the expression of the cell cycle inhibitor $p27^{kip}$, inactivates the proapoptotic protein Bad, and increases expression of the prosurvival protein Bcl-xL (Jepson et al., 2002, Workman et al., 2009b). Similar effects were observed in pancreatic tumor cells, where upregulation of MUC4 expression leads to increased cell proliferation and decreased cell death (Chaturvedi et al., 2007). Importantly, regulation of cell survival signals by MUC4 was observed in response to a variety of insults, including chemotherapeutic agents, lack of serum factors, and loss of adhesion (Workman et al., 2009b). In contrast, loss of MUC4 reduces ErbB2/HER2-mediated signaling, induces FOXO1 transcription, and promotes caspase 3-mediated apoptosis in ovarian cancer cells (Bae et al., 2017). These combined observations support a role for MUC4 as a potent regulator of programmed cell death through diverse signals, a feature critical for successful metastatic dissemination and survival of CTCs in circulation.

MUC4 contribution to therapeutic resistance.

Consistent with the anti-adhesive and cell survival properties ascribed to the MUC4α and MUC4β subunits, MUC4 has also been implicated in mediating therapeutic resistance through steric hindrance and regulation of programmed cell death. Human ER+/HER+ breast cancer cells orthotopically transplanted into mice exhibit upregulated MUC4 expression after therapeutic intervention with tamoxifen, an ER-targeted therapy (Chen et al., 2012). Additionally, ErbB2/HER2 receptor tyrosine kinase expression and signaling are elevated post tamoxifen treatment, implying that MUC4 contributes to a phenotypic switch to ErbB2/HER2 dependence and renders ER-targeted therapeutics ineffective (Chen et al., 2012). Moreover, it appears that MUC4 directly blocks therapeutic efficacy through steric

hindrance at the cell surface. Indeed, cell surface MUC4 in melanoma and breast cancer cells reduced binding of anti-HER2 antibodies through steric hindrance and the formation of MUC4-ErbB2/HER2 complexes, which prevents binding of the anti-HER2 antibody Herceptin to its target (Price-Schiavi et al., 2002). Consistent with these findings, MUC4 expression is significantly higher in the Herceptin-resistant breast cancer cell line JIMT-1 compared with Herceptin-sensitive lines, and correlates with less binding of Herceptin to HER2. Importantly, RNAi-mediated MUC4 suppression restores Herceptin binding (Nagy et al., 2005). The mechanisms by which MUC4 is upregulated to promote therapeutic resistance appear varied and highly context-dependent. Studies by Mercogliano et al. suggest that the upregulation of MUC4 and reduction of Herceptin binding and efficacy in HER2-positive breast cancers may be TNFα-dependent (Mercogliano et al., 2017a). However, others have observed that breast and gastric cancer cells treated with Herceptin exhibit STAT3-dependent upregulation of MUC1 and MUC4, blocking Herceptin binding to HER2 to mediate therapy resistance (Li et al., 2014a).

The ability of MUC4 to regulate programmed cell death and cell survival provides yet another mechanism by which MUC4 mediates therapeutic resistance. In pancreatic tumor cells, MUC4 promotes both cell survival and resistance to apoptosis, rendering the tumor cells resistant to gemcitabine, a commonly employed chemotherapeutic, and shRNA-mediated MUC4 knockdown re-sensitizes tumor cells to the cytotoxicity (Mimeault et al., 2010). Mechanistically, overexpression of MUC4 in pancreatic tumor cells appears to engage an ErbB2/HER2-Erk signaling axis that results in the inactivation of Bad and resistance to apoptosis (Bafna et al., 2009). Together, these studies demonstrate that MUC4 engages multiple signaling pathways and mechanisms to mediate therapeutic resistance to both HER2-targeted and other therapeutics, enabling tumor cells to adapt and progress to malignancy.

MUC4 Signaling

Signaling through ErbB2/HER2.

Over two decades of studies point to a role for MUC4 as an intramembrane binding partner for ErbB2 (human form referred to as HER2). MUC4β was shown to directly interact with the extracellular domain of ErbB2/HER2 via one of its EGF-like domains (Carraway et al., 1999). Subsequent studies demonstrated that MUC4 induces ErbB2/HER2 phosphorylation on tyrosine residues 1139 and 1248 (Jepson et al., 2002; Ramsauer et al., 2003; Ramsauer et al., 2006), and that ectopic MUC4 potentiates neuregulin-1-mediated ErbB2/HER2 signaling (Carraway et al., 1999). Investigation of the complex revealed that MUC4 alters the subcellular localization of ErbB2/HER2 in diverse cellular contexts. For example, in the developing rat mammary gland, MUC4 and ErbB2/HER2 are localized to the apical and basolateral membranes, respectively, and expression of ErbB2/HER2 and MUC4 is controlled through distinct regulatory mechanisms. However, during late pregnancy and lactation, MUC4 and ErbB2/HER2 form a complex that is localized to apical cell surfaces in the rat mammary gland epithelia. Similarly, MUC4 promotes the re-localization of phosphorylated ErbB2/HER2 from the basolateral surface to the apical surface of epithelial cells without altering cell polarity (Ramsauer et al., 2003). The

apical localization of ErbB2/HER2 may serve to segregate it from its dimerization partner ErbB3/HER3 and activating ligands to alter signaling and downstream outcomes. Despite segregation, ErbB2/HER2 signals through p38-MAPK and Akt, providing evidence that MUC4 independently stimulates ErbB2/HER2 signaling (Ramsauer et al., 2006).

In melanoma and breast cancer cells, MUC4 expression promotes PI3K recruitment to ErbB3/HER3 at the plasma membrane and potentiates ErbB2/HER2-ErbB3/HER3-PI3K signaling (Funes et al., 2006). However, MUC4 expression significantly increases Neuregulin-1 ligand binding to ErbB2/HER2 and ErbB3/HER3 in these cells but does not impact receptor protein levels (Funes et al., 2006). These findings suggest that MUC4 potentiates ErbB2/HER2 signaling by trafficking both receptors to the plasma membrane from intracellular compartments and by suppressing receptor internalization (Funes et al., 2006). In ovarian cancer cells, MUC4 increases both ErbB2/HER2 expression and signaling through FAK-Akt-Erk (Ponnusamy et al., 2008).

In pancreatic and ovarian cancer cells MUC4 appears to stabilize ErbB2/HER2 and drive signaling through the FAK, MAPK, and JNK pathways, contributing to cancer cell proliferation, migration, and invasion (Chaturvedi et al., 2008; Ponnusamy et al., 2011; Jonckheere et al., 2012). Consistently, stabilization of ErbB2/HER2 by MUC4 in pancreatic cancer cells results in PI3K-dependent activation of Akt, increased signaling through NF-κB, and elevation of the expression of Lipocalin2, a multifunctional glycoprotein that may serve as a potential biomarker during tumor progression (Kaur et al., 2014). Additionally, MUC4 increases ErbB2/HER2-FAK-Src signaling, which results in the lysosome-dependent degradation of E-Cadherin and β-Catenin-mediated Wnt-signaling, driving pancreatic cancer cell proliferation and metastasis, as well as angiogenesis (Zhi et al., 2014).

MUC4 signaling through other axes.

MUC4 can also signal through other EGFR family proteins, including EGFR and ErbB3/HER3. In triple-negative breast cancer, MUC4 increases expression of EGFR and ErbB3/HER3, activating Erk1/2, PKC, and FAK to drive cell proliferation, motility, and invasiveness *in vitro*, as well as increased tumor growth and metastasis *in vivo* (Mukhopadhyay et al., 2013). In pancreatic cancer, MUC4 interacts with ErbB3/HER3 in the absence of ErbB2/HER2 to mediate signaling through PI3K/Erk/c-Myc to increase cell proliferation, and signaling through FAK/Src to mediate cell motility (Lakshmanan et al., 2015). In glioblastoma, MUC4 mediates higher cell proliferation and invasiveness through regulation of EGFR expression (Li et al., 2014b). In pancreatic cancer cells, overexpression of MUC4/Y, a splice variant that lacks much of the alpha subunit, enhances both angiogenic and metastatic properties of cells *in vitro* and *in vivo* through activation of Notch3 signaling, and correlates with a decreased overall survival *in vivo* (Tang et al., 2016).

MUC4 is a mediator of cell motility events critical to metastasis in a variety of solid tumor types (Moniaux et al., 2007; Chaturvedi et al., 2007; Ponnusamy et al., 2010; Ponnusamy et al., 2008). Mechanistically, MUC4-mediated motility is driven by reorganization of the actin cytoskeleton, including the formation of microspikes, lamellipodia, and filopodia-like structures (Chaturvedi et al., 2007, Ponnusamy et al., 2008). Further support for a role of MUC4 in promoting metastatic behavior comes from studies demonstrating that MUC4

may be a critical regulator of EMT in multiple tumor types. In ovarian cancer cells, MUC4 overexpression was observed to drive a phenotypic shift from an epithelial-like cellular morphology to a mesenchymal-like cellular morphology, decreased expression of epithelial markers such as E-cadherin and cytokeratin-18, and increased expression of mesenchymal markers including N-cadherin and vimentin (Ponnusamy et al., 2008). Further investigation demonstrated that these expression changes were mediated by the EMT-inducing transcription factors Twist1, Twist2 and Snail (Ponnusamy et al., 2010). MUC4-mediated induction of EMT was also observed in pancreatic cancer and lung adenocarcinoma cells via an FGFR1-β-catenin dependent mechanism (Rachagani et al., 2012, Gao et al., 2014).

MUC4 Involvement in Circulating Tumor Cell Survival

Circulating Tumor Cells.

CTCs are a heterogenous population of cells that leave the primary tumor and seed metastases through the acquisition of characteristics that support their exit from primary lesions, entry into the vasculature, survival in circulation, and ability to colonize and proliferate at distant tissues. CTCs can access the vasculature through either passive shedding from the primary tumor or through local invasion and intravasation. Tumors cells are able to invade from the primary tumor through multiple modes of migration including single cell invasion, where single cells undergo EMT and acquire a mesenchymal and invasive phenotype (Pantel and Speicher, 2016), and collective invasion, where cells enter circulation collectively and transit the vasculature as clusters (VanderVorst et al., 2019). While CTCs largely exist as single cells, Aceto et al. reported that in mouse models of breast cancer, CTCs form clusters of 2-50 cells in circulation. While rare compared to single CTCs, these clusters are 20 to 50-fold more metastatic than single CTCs (Aceto et al., 2014). Indeed, the presence of CTC clusters in the bloodstream of breast cancer patients is correlated with poor clinical outcomes (Wang et al., 2017).

CTCs and CTC clusters must also survive the harsh environment of circulation by withstanding shear forces, evading immune attack, and suppressing anoikis. Through acquired mesenchymal and stem-like features, often driven by stemness-associated gene upregulation via transcription factor binding site hypomethylation, breast CTC clusters achieve enhanced anchorage-independent growth and metastasize more effectively than single CTCs (Gkountela et al., 2019). Furthermore, studies in lung and breast cancer suggest that association with stromal cells (e.g., cancer-associated fibroblasts (CAFs), tumor-associated macrophages (TAMs), and endothelial cells) from primary tumors increases CTC viability both in the bloodstream and at metastatic sites by conferring shear stress resistance (Duda et al., 2010), and by stimulating angiogenesis and EMT (Matsumura et al., 2019), respectively.

While the characterization of CTCs is crucial to our understanding of metastasis, their very low abundance in the bloodstream and heterogeneity makes both identification and isolation difficult. Thus, much effort has been put into improving the detection and enrichment of CTCs in circulation through selection techniques based on biological and physical properties (reviewed in (Zhu et al., 2018)). Emerging advancements in CTC technologies and analysis

will further our understanding of their involvement in metastatic disease and hopefully augment their potential as biomarkers of disease progression and therapeutic response.

MUC4 involvement in CTC viability.

Initial observations that MUC4 protein is more abundant in metastatic lesions compared to patient-matched primary tumors led to speculation that MUC4 may aid in successful metastatic dissemination (Workman et al., 2009a). Our studies employing MUC4-deficient mice crossed into the NDL (Neu DeLetion mutant) murine model of ErbB2/HER2-induced mammary tumorigenesis revealed that MUC4 ablation significantly reduces the number of metastatic lesions in the lungs (Rowson-Hodel et al., 2018). Importantly, no differences in primary tumor growth rates or tumor burden are observed between $MUC4^{WT}$/NDL and $MUC4^{KO}$/NDL animals, nor is any effect of MUC4 knockout on NDL tumor cell migration *in vitro*. A tail-vein mouse model of metastasis using $MUC4^{WT}$/NDL and $MUC4^{KO}$/NDL tumor-derived cells recapitulated MUC4 impact on lung metastatic colonization, demonstrating that differences could not be attributed to unequal access to the vasculature. Combined with the observation that MUC4 ablated tumor-derived cells are less viable in suspension *in vitro*, these findings support a role for MUC4 in promoting cell survival in circulation (Rowson-Hodel et al., 2018).

CTCs are known to form aggregates in circulation with other tumor cells, immune cells and platelets (Gay and Felding-Habermann, 2011). Loss of MUC4 in NDL tumor-derived CTCs exhibited significantly impaired abilities to interact with platelets compared to their MUC4-expressing counterparts both *in vitro* and *in vivo*, suggesting that MUC4 may aid in the metastatic process through this mechanism. In this model, MUC4-expressing cells promote cell-cell aggregates with platelets and immune cells in circulation, aiding CTC survival in the vasculature and more efficiently seeding metastatic lesions. On the other hand, MUC4-negative cells are less able to form CTC-blood cell aggregates, thus rendering them unable to survive non-adherent conditions, and impairing their successful colonization of metastatic lesions (Figure 2).

CTCs have a short survival time in circulation, necessitating the acquisition of survival advantages during transit. Association of tumor cells with blood cells is a long-recognized means by which CTCs are bolstered against harsh bloodstream conditions (Gasic et al., 1968). Since Gasic and colleagues first observed metastatic suppression via thrombocytopenia more than 50 years ago (Gasic et al., 1968), researchers have identified essential roles for platelets in CTC immune evasion, cell survival, and invasiveness (Nieswandt et al., 1999). While aggregation of CTCs with platelets is variable and largely dependent on the tumor type, this process is generally thought to enhance metastatic capabilities. Mechanistically, enhanced metastatic potential is mediated by diverse drivers including the induction of tumor cell proliferation, the facilitation of tumor cell extravasation, and the enhancement of tumor cell interactions with the extracellular matrix (Tsuruo et al., 1986). Importantly, CTC-platelet aggregation occurs within minutes of entry into the bloodstream, and plays a critical role in evasion of immune surveillance by “cloaking” CTCs from natural killer (NK) cells and in promoting survival in the face of harsh environmental factors (Nieswandt et al., 1999; Palumbo et al., 2005). CTCs have also

been observed to interact with circulating immune cells such as neutrophils, which mediate cell cycle progression and increased CTC adhesion at secondary sites to facilitate metastatic seeding (Huh et al., 2010).

Given the contribution of CTC-platelet interaction to metastatic dissemination, significant effort has been put into identifying mechanisms to intervene in this association to clinically address metastasis. Currently, platelet inhibitors (Lou et al., 2015), platelet-mimicking drug delivery nanovehicles (Hu et al., 2015), and genetic engineering of platelets (Li et al., 2016) are being investigated to determine if disruption of CTC-platelet interactions might provide significant clinical benefit to cancer patients. Findings by Rowson-Hodel et al. that MUC4 is an essential component of platelet-CTC interactions suggest that MUC4 may be a viable therapeutic target for eliminating CTCs in the vasculature (Rowson-Hodel et al., 2018). Further evidence supporting the targeting of mucins during CTC transit in the vasculature comes from observations that sialic acid-containing glycans mediate tumor cell-platelet aggregation (Bastida et al., 1987), and a portion of mucin glycan side chains terminate in negatively-charged sialic acids (Linden et al., 2008). These observations implicate transmembrane mucins, such as MUC4, as important mediators of CTC-blood cell interactions critical to successful metastatic dissemination and highlight the need for a better understanding of the molecular mechanisms underlying CTC-blood cell interactions to identify druggable targets for clinically addressing metastasis.

Potential Therapeutic Strategies

Because CTC viability is a remarkably stringent barrier to metastatic efficiency; squeezing that bottleneck tighter by suppressing MUC4-mediated interactions of CTCs with other blood-borne cells offers a tremendous opportunity to intervene in metastatic efficiency. A MUC4/CTC-based strategy would necessarily involve the systemic delivery of an anti-MUC4 agent to CTCs via the vasculature, and such an agent would likely be a component of maintenance therapy following primary treatment. However, development of such a strategy will likely be hampered by high MUC4 expression in intestinal epithelial and vascular endothelial cells, each of which could serve as a competing sink for small molecule or biologics in delivery to tumors. Targeting transcriptional regulators of MUC4 expression is another possible path to therapeutic intervention. However, because such transcriptional regulators control broad expression programs in multiple tissue types, the advantage of tumor-specific MUC4 targeting is lost with this approach. Likewise, strategies to provoke cytokine-induced MUC4 degradation in CTCs also suffer from specificity issues, and strategies to deliver MUC4-targeting knockdown or knockout agents via CTC-directed nanoparticles could be rendered ineffective by the reported ability of highly-expressed MUC4 to interfere with the binding of large molecules and complexes to the cell surface (Nagy et al., 2005, Komatsu et al., 1997, Price-Schiavi et al., 2002). Thus, the most promising avenue for therapeutic intervention will likely involve the identification of the MUC4 binding components on blood-borne cells, and the inhibition of those contacts to reduce CTC viability. In this regard, it is reasonable to suspect that MUC4 is simply a major presenter of tumor cell-enriched glycan motifs recognized by lectins and other carbohydrate-binding proteins, and that glycomic studies with CTCs could provide key insight into the

nature of intravascular cell-cell interactions as well as inform the development of novel therapeutic approaches.

Conclusions and Future Directions

Based on its structure, expression patterns, and function, MUC4 is beginning to emerge as an attractive target for therapeutic intervention into metastatic cancer. A large and growing body of evidence points to roles for aberrant MUC4 expression in the progression of a variety of solid tumor types. At the same time, studies of the full knockout mouse demonstrate that MUC4 is not essential for development, viability or fertility, and that its ablation has minimal impact on adult tissue morphology and function, strongly suggesting that systemic approaches for targeting MUC4 could elicit minimal side effects. While MUC4 appears to harbor an array of functions that might benefit the progression of a growing tumor, its role in promoting CTC viability is particularly intriguing. For example, observations from the study of patient-matched breast cancer patient samples and a genetically engineered mouse model of breast cancer indicate that the re-expression of MUC4 during the transition from primary to metastatic tumor drives metastatic efficiency at least in part by promoting CTC viability in circulation. This conclusion then raises the possibility that the process of MUC4-mediated CTC survival may be targeted for the benefit of breast cancer patients whose tumors are at risk of distant metastasis.

While the role of MUC4 in CTC-mediated breast cancer metastasis is most well-characterized, it is likely that MUC4 plays roles in CTC stabilization of other carcinomas as well. The immediate availability of a MUC4 knockout mouse with few discernable developmental phenotypes will facilitate analogous studies with genetically engineered mouse models of other tumor types. As with breast cancer, such studies will be most impactful when coupled with MUC4 expression surveys of patient-matched primary and metastatic lesions. For these studies it will be essential to employ immunohistochemical or other protein-based methods, as it has been demonstrated that MUC4 protein stability is highly dependent on factors produced by the microenvironment (Price-Schiavi et al., 1998; Price-Schiavi et al., 2000b; Lomako et al., 2009). Finally, a particularly interesting question concerns the role of MUC4 in CTC self-association, an issue not previously addressed in the MUC4 knockout study (Rowson-Hodel et al., 2018). Because clusters are 1-2 orders of magnitude more potent in seeding metastases than single CTCs, the identification of homotypic cell-cell contacts is an issue of immediate interest.

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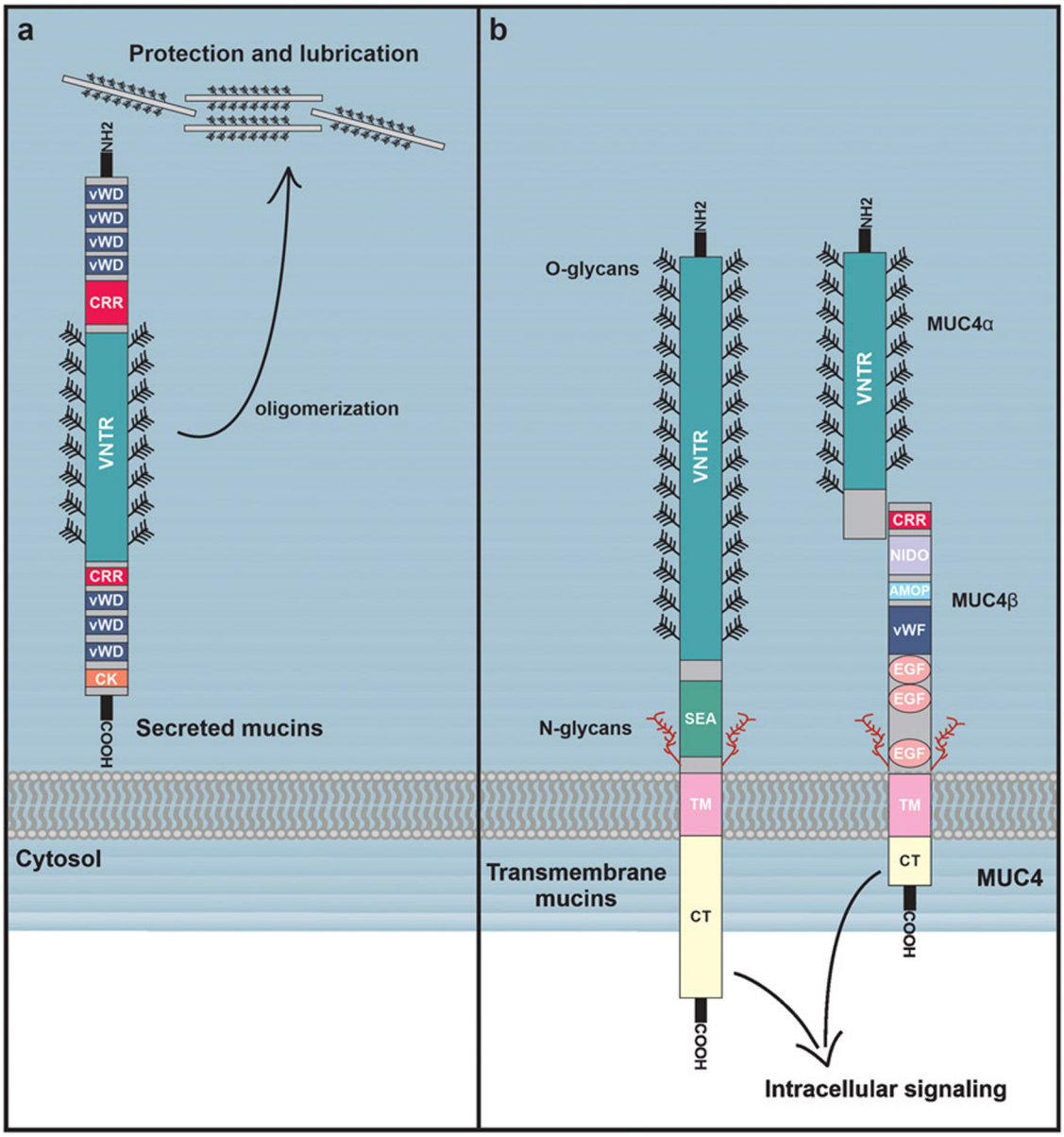

Figure 1.

Structures of secreted and membrane-bound mucins. (A) Secreted mucins contain multiple vWD (von Willebrand factor D) domains, cysteine-rich regions (CRRs), as well as a single C-terminal cysteine-knot (CK) domain. The middle regions of the secreted mucins consist of a highly *O*-glycosylated VNTR domain which contains cysteine-rich regions dispersed throughout. Secreted mucins oligomerize through their CRR and CK domains, forming large highly viscous aggregates that contribute to the lubrication and protection of epithelial surfaces. (B) Membrane-bound mucins are structurally similar to secreted mucins, and contain vWD domains, CRRs, and *O*-glycosylated VNTR domains, but are distinguished by a single transmembrane domain that tethers them to the cell surface. Membrane-bound mucins also contain SEA domains as well as some *N*-glycosylated regions. Notably, in MUC4 the SEA domain is replaced with NIDO and AMOP domains. MUC4 also contains

three EGF-like domains, and is proteolytically cleaved to produce noncovalently-linked MUC4α and MUC4β subunits. While membrane-bound mucins are also critical to the protection and lubrication epithelial surfaces, they can simultaneously play extensive roles in cellular signaling, often through their cytoplasmic tails.

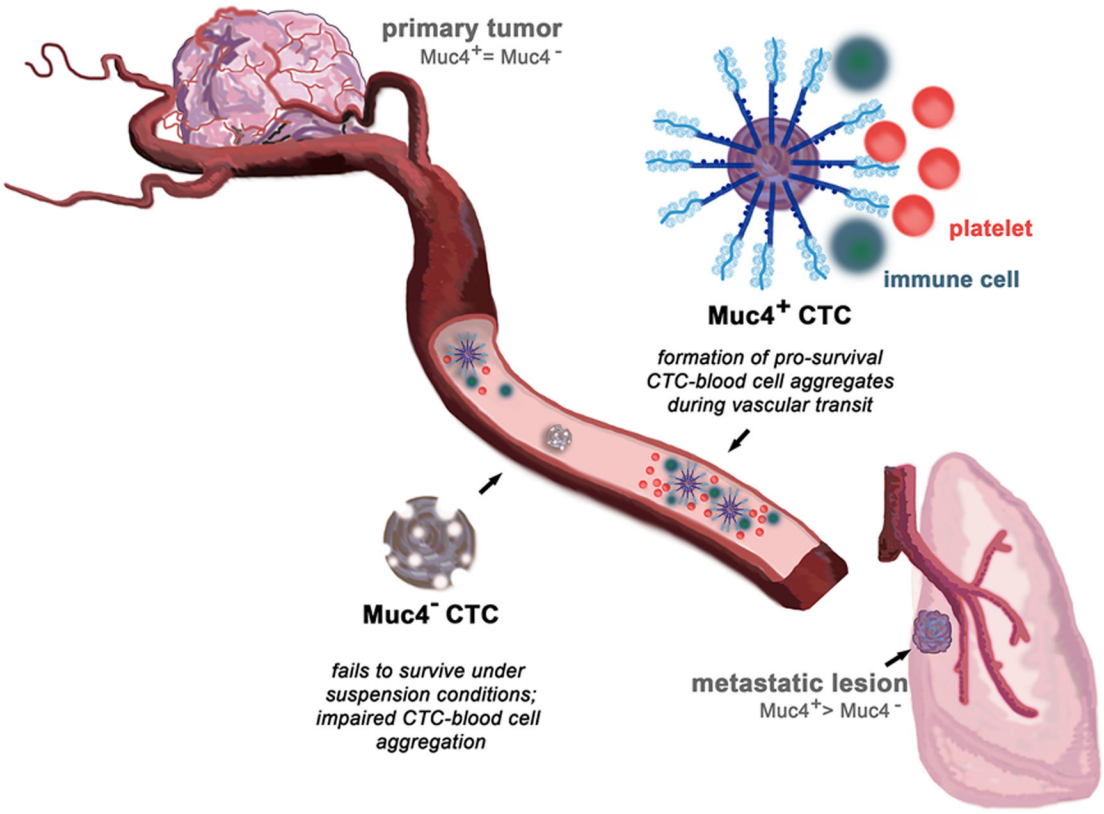

Figure 2.

Muc4 mediates CTC survival in circulation. Muc4-positive CTCs form pro-survival cell aggregates with blood-borne cells during vascular transit, enabling them to more efficiently seed metastatic lesions at distant organs. On the other hand, Muc4-negative CTCs fail to survive the harsh conditions of transit, succumbing to shear forces and undergoing anoikis. These cells are unable to form CTC-blood cell aggregates, impairing their ability to successfully colonize metastatic lesions (Rowson-Hodel et al., 2018).

Table 1.

Muc4 expression in primary tumors and metastatic lesions. NR, not reported; ICC, immunocytochemistry; IHC, immunohistochemistry; WB, western blotting.

Cancer Type	Muc4 Expression Patterns (Primary Tumor)	Muc4 Expression Patterns (Metastases)	Correlation with Prognostic Factor or Disease Stage	Method of Detection	Reference
Breast Cancer	Expressed in 95% of primary breast tumors	NR	Muc4 expression correlates with tumor grade	IHC	(Rakha 2005)
	Overexpressed in primary invasive triple-negative tumors compared to normal	NR	NR	IHC	(Mukhopadhyay 2013)
	Expressed in the majority of secretory carcinomas of the breast	No increased expression in metastatic lesions	NR	IHC	(Shet 2013)
	Decreased expression in primary tumor compared to patient-matched normal	Increased expression in patient-matched lymphnode metastases	NR	WB/IHC	(Workman 2009)
	Expressed in majority of patient breast cancer samples	NR	High frequency of Muc4 expression in malignant cells in body fluids	ICC	(Komatsu 1999)
	Highly expressed in invasive micropapillary carcinoma of the breast	NR	Muc4 expression correlates with shorter death free survival and poor response to standard of care	IHC	(Mercogliano 2017)
Aerodigestive Tract Squamous Cell Cancer	Expressed in primary tumors	Expression persists in metastatic lesions	No correlation with patient prognosis	ICC/WB	(Weed 2001)
Ovarian Cancer	Overexpressed in primary tumor compared to normal tissue	NR	No correlation with patient survival	IHC	(Chauhan 2006)
Pancreatic Cancer	Overexpressed in primary tumor compared to normal tissue	NR	NR	IHC	(Mimeault 2013)
	Expressed in primary tumors	NR	Muc4 expression correlates with disease progression	IHC	(Kaur 2014)
Lung Cancer	Highly expressed in primary tumor	Decreased expression in lymph node metastases	Correlates with risk of lymph node metastasis	WB	(Gao 2014)
Glioblastoma	Upregulated in tumor compared to normal tissue	NR	NR	WB/IHC	(Li 2014)
Cervical Cancer	Overexpressed in primary tumor compared to normal tissue	NR	Strongly associated with lymph node metastases	IHC/gene microarray	(Xu 2017)
Cholangiocarcinoma	Expressed in "columnar type" primary tumors	NR	No correlation with patient prognosis	IHC	(Shibahara 2004b)
	Overexpressed in intrahepatic type compared to normal tissue	NR	Correlates with patient prognosis	IHC	(Shibahara 2004a)