Anti-MUC4β antibodies as a novel therapeutic modality for the treatment of pancreatic cancer

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Anti-MUC4β antibodies as a novel therapeutic modality for the treatment of pancreatic cancer

By

Catherine Orzechowski

A DISSERTATION

Presented to the Faculty of
The Graduate College in the University of Nebraska
In Partial Fulfillment of Requirements
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Under the Supervision of Professor Maneesh Jain
University of Nebraska Medical Center
Omaha, Nebraska
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Anti-MUC4β antibodies as a novel therapeutic modality for the treatment of pancreatic cancer

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University of Nebraska Medical Center, 2020

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The deregulation of cell surface glycoproteins, such as mucins, is a hallmark of many tumors of epithelial origin. The transmembrane mucin MUC4, is differentially overexpressed in several malignancies, including pancreatic, breast, ovarian, lung, cervical, and head and neck cancers. Of all the aforementioned cancers, the role of MUC4 has been the most thoroughly in pancreatic cancer (PC), which is the 4th leading cause of cancer-related deaths in the United States. While MUC4 is undetectable in the normal or inflamed pancreas (pancreatitis), its expression progressively increases during PC progression and its higher expression correlates with poor survival. The role of MUC4 in neoplastic transformation, enhanced motility, invasiveness, and drug resistance of cancer cells in vitro, and in tumorigenicity and metastasis in vivo has been conclusively established. Due to the differential overexpression of MUC4 in cancer cells and its functional involvement in disease pathobiology, it is an attractive therapeutic target. Of specific interest is the MUC4β-subunit of the MUC4 glycoprotein. The α-domain of MUC4 can be shed from the surface of the cell thus rendering it a feckless target. Thus, we propose to target the MUC4β-domain, a growth factor-like subunit that remains attached to the cell surface. To date no MUC4-targeted therapeutics have been developed. In the first part of the dissertation research, I sought to develop monoclonal antibodies (mAb) against the MUC4β subunit. MAb-based therapeutics have emerged as a promising cancer treatment modality due to their low toxicity and high specificity. Antibodies used in the treatment of solid tumors may inhibit oncogenic signaling, block
cell-cell interactions, or engage immune effector cells to attack the tumor by antibody-dependent cellular cytotoxicity (ADCC) in a target expression-specific manner. Alternatively, mAbs can serve as vectors to deliver cytotoxic cargo (drugs, radionuclides, or toxins) to cancer cells in antigen-specific manner. The development of MUC4 specific mAbs was achieved through the use of hybridoma technology. The immunization of BALB/c mice with recombinant MUC4β protein purified from E.coli allowed for the development of antibodies specific to our protein of interest. Through the fusion of mouse splenocytes and myeloma cells we were able to create hybridoma cells that had the properties a B-cell allowing for the production and secretion antibodies of and had the immortality conferred by the myeloma cell partner.

The antibodies produced from this hybridoma fusion were screened for reactivity against MUCβ recombinant protein and against cell lysates from MUC4-expressing cells to identify antibody clones with high binding affinity. These clones were further purified and taken through specificity testing. We began testing for specificity by Western blotting cell lysates from MUC4-expressing and non-expressing cells lines. Determination of MUC4 molecular weights occurred through the use of both horizontal agarose gels for the detection of full length MUC4 and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) to detect the smaller MUC4β fragment. The specificity testing further extended to testing on the native conformation of the MUC4 protein in flow cytometry and confocal microscopy. The specific detection of MUC4 in PC patient samples was also performed using immunohistochemistry (IHC). The binding affinity of the mAbs was determined in surface plasmon resonance (SPR), which demonstrate binding with nanomolar affinity. Overall, the mAbs were validated for MUC4β specificity and could be used for further cell-based functional testing.
To determine the therapeutic potential of MUC4β antibodies, we propose to study their applications for inhibiting MUC4-mediated cell-based functions. Cell-based growth and motility assays were studied as part of the functional application of the MUC4β mAbs. Each assay was performed across several MUC4-expressing and non-expressing cell lines. Growth inhibition was only found to occur to a significant degree for the MUC4-expressing cell line CD18. The inhibition of growth was not found to occur through the induction of apoptosis. The inhibition of motility was only observed to occur to a significant degree for the cell lines engineered to express MUC4. Additional testing included use of the anti-MUC4β mAbs in combination with the chemotherapy gemcitabine against the cell line CD18. This combination demonstrated and additive effect at least equal to each of the therapeutics alone. Together these results demonstrate that the anti-MUC4β mAbs may have the capacity to be used as therapeutic agents and warrant further study in vivo.
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List of Abbreviations

ADC: Antibody-drug conjugate
ADCC: Antibody-dependent cellular cytotoxicity
ADCP: Antibody-dependent cellular phagocytosis
AMOP: Adhesion-associated domain in mucin MUC4 and other proteins
CDC: Complement-dependent cytotoxicity
CRISPR: Clustered regularly interspaced short palindromic repeats
CT: Cytoplasmic tail
ECM: Extracellular matrix
EGF: Epidermal growth factor
EGFR: Epidermal growth factor receptor
Fab: Antigen binding fragment
Fc: Fragment crystallizable region
hCNT: Human concentrative nucleoside transporter
HER: Human epidermal growth factor
IgG: Immunoglobulin G
IHC: Immunohistochemistry
IPTG: Isopropyl β-d-1-thiogalactopyranoside
KD: Knockdown
mAb: Monoclonal antibody
NGS: Next generation sequencing
NIDO: Nidogen-like
NK: Natural killer cell
PanIN: Pancreatic intraepithelial neoplasia
PC: Pancreatic cancer
PD-1: Programmed cell death protein 1
PD-L1: Programmed cell death protein 1 ligand
PDAC: Pancreatic ductal adenocarcinoma
RIPA: Radioimmunoprecipitation assay buffer
SEA: Sea urchin sperm protein, Enterokinase and Agrin module
scFv: Single-chain variable fragment
SDS PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SPR: Surface plasmon resonance
TAA: Tumor associated antigen
TM: Transmembrane
TME: Tumor microenvironment
TR: Tandem repeat
VEGF: Vascular endothelial growth factor
V_H: Variable heavy chain
V_L: Variable light chain
VNTR: Variable number of tandem repeats
vWD: Von Willebrand factor
Chapter 1: Introduction
1.1 Pancreatic Cancer Overview

Pancreatic cancer (PC) is one of the most lethal, treatment-refractory malignancies and it has emerged as the third leading cause of cancer-related deaths in the United States (1). Existing therapies for PC include chemotherapy, radiotherapy, and radical surgery. However, the failure to diagnose PC at an early stage makes the treatment options ineffective in almost 80% of the patients (2). Even after surgery, recurrence occurs in 80% of the patients. Until recently, only five FDA-approved drugs and one combination therapy has existed for PC patients (Table 1.1). Chemotherapy combined with radiation has not shown much success in the patients. In addition, PC patients have a high recurrence rate, develop drug resistance, and are refractory to systemic therapies due to the desmoplastic and immunosuppressive tumor microenvironment (TME) (2). Extensive research is now focusing on the development and testing of more effective and safer treatment modalities including anticancer vaccines, immune checkpoint inhibitors, adoptive T-cell transfer, and antibody-targeted therapies. These regimens utilize overexpressed tumor associated antigens (TAAs), neoantigens, or checkpoint molecules. Among these overexpressed proteins, mucins have been explored as biomarkers and therapeutic targets (3, 4).
**Table 1.1. Current FDA-approved treatments target the mechanisms of cell growth in pancreatic ductal adenocarcinoma (PDAC).**

<table>
<thead>
<tr>
<th>Therapeutic</th>
<th>Mechanism of Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erlotinib Hydrochloride</td>
<td>Tyrosine kinase inhibitor. Reversibly binds EGFR blocking signaling pathways for cancer growth, invasion, and metastasis</td>
</tr>
<tr>
<td>5-FU (Fluorouracil Injection)</td>
<td>Nucleoside analog</td>
</tr>
<tr>
<td>Gemcitabine Hydrochloride</td>
<td>Nucleoside analog</td>
</tr>
<tr>
<td>Irinotecan Hydrochloride Liposome</td>
<td>Topoisomerase I inhibitor</td>
</tr>
<tr>
<td>Mitomycin C</td>
<td>Inhibits the synthesis of DNA produces interstrand DNA cross-links</td>
</tr>
<tr>
<td>Olaparib</td>
<td>PARP inhibitor</td>
</tr>
<tr>
<td>Paclitaxel Albumin-stabilized Nanoparticle Formulation</td>
<td>Spindle assembly checkpoint</td>
</tr>
</tbody>
</table>

**Combination Chemotherapy Regimens**
- FOLFIRINOX (Folinic Acid, 5-FU, Irinotecan, Oxaliplatin)
- GEMCITABINE-CISPLATIN
- GEMCITABINE-OXALIPLATIN
- OFF (Oxaliplatin, Folinic Acid, 5-FU)
1.2 Mucin overview

Mucins are a family of 20 distinct glycoproteins. They are divided up into two categories: transmembrane and secreted. Mucins have serine and threonine rich domains, which allow for O-linked glycosylation. Mucins are expressed on the surface of most epithelial cells in addition to being found in a variety of bodily secretions. A major role of mucins is to maintain a barrier between cells and the external environment. In the lungs, mucins provide protection to epithelial cells by forming a mucus raft. This barrier is not inert but rather provides a dynamic microenvironment, contributing a defense against pathogens and other external insults (5). Similarly, the mucins in the mucosal layer of the intestines act as a dynamic layer mediating bacterial and host interactions (6). Mucins are not limited to forming a protective barrier, since they also have signal transduction capabilities. While mucins contribute to the normally functioning signal transduction capabilities for proliferation, differentiation, and apoptosis, they can also become aberrantly expressed and contribute to oncogenic signaling (7). Mucins have long been used as biomarkers for diagnosis in many cancers and have been tested in advanced trials for targeted therapies like radioimmunotherapy and cancer vaccines (8-12) (Table 1.2). Most of these efforts have centered on MUC1, a relatively ubiquitous mucin, which is expressed on various epithelial surfaces. Unlike MUC1, MUC4 is undetectable in normal pancreas or pancreatitis and exhibits de novo expression in PC (11).
<table>
<thead>
<tr>
<th>Mucin Target</th>
<th>Therapeutic Type</th>
<th>Therapeutic Name</th>
<th>Disease Indications Tested in Clinical Trials</th>
</tr>
</thead>
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<tr>
<td>MUC1</td>
<td>Vaccine</td>
<td>TG4010</td>
<td>NSCLC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tecemotide (L-BLP25)</td>
<td>Multiple Myeloma, Lung, Breast, Prostate, Colorectal Cancer</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PANVAC</td>
<td>Breast Cancer</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CV9202</td>
<td>NSCLC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ImMucin</td>
<td>Multiple Myeloma</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PankoMab-GEX (Gatipotuzumab)</td>
<td>Ovarian Cancer</td>
</tr>
<tr>
<td></td>
<td>Antibodies</td>
<td>AS1402</td>
<td>Breast Cancer</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BTH1704</td>
<td>Pancreatic Cancer</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Anti-MUC1 Anti-CD3 bispecific</td>
<td>Liver, Gastric, Kidney, Breast, Colorectal, Pancreatic Cancer</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Yttrium (90Y) clivatuzumab tetraxetan-MUC1 target (IMMU-107):</td>
<td>Pancreatic Cancer</td>
</tr>
<tr>
<td></td>
<td>Peptides</td>
<td>GO-2-03-2C- Peptide</td>
<td>Acute Myeloid Leukemia, Solid Tumors, Multiple Myeloma</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MUC1-peptide-pulsed dendritic cells (DCs)</td>
<td>SCLC, NSCLC, Gastric, Esophageal, Prostate, Pancreatic Cancer</td>
</tr>
<tr>
<td>MUC16</td>
<td>Vaccine</td>
<td>Abagovomab (MUC16 mimic anti-idiotype vaccine)</td>
<td>Ovarian Cancer</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Oregovomab</td>
<td>Pancreatic and Ovarian Cancer</td>
</tr>
<tr>
<td></td>
<td>Antibody Drug Conjugate</td>
<td>Sofituzumab vedotin or DMUC5754A (anti-MUC16 ADC)</td>
<td>Pancreatic and Ovarian Cancer</td>
</tr>
<tr>
<td>MUC17</td>
<td>Bispecific antibody</td>
<td>AMG199</td>
<td>Gastric and Gastroesophageal Junction Cancer</td>
</tr>
<tr>
<td>MUC2</td>
<td>Vaccine</td>
<td>MUC-2-KLH</td>
<td>Prostate Cancer</td>
</tr>
<tr>
<td>MUC5AC</td>
<td>Antibody</td>
<td>NEO-102</td>
<td>Colon and Pancreatic Cancer</td>
</tr>
</tbody>
</table>
1.3 MUC4 and Pancreatic Cancer

The transmembrane mucin MUC4 was first discovered from analysis of a cDNA library of human tracheo-bronchial mucosa and mapped to chromosome 3q29 (13). The expression of MUC4 at the mRNA level was absent in normal pancreatic tissue; however, it was expressed in pancreatic cancer (14). A complete coding sequence of MUC4 was further elucidated, along with 23 alternative splice site that have been identified, which are grouped into secretory, membrane-bound with tandem repeat (TR) and membrane-bound lacking a tandem repeat, MUC4/X and MUC4/Y (15, 16). MUC4 was also determined to be absent in pancreatitis, making it a tumor-specific biomarker in the pancreas (11). MUC4 as a biomarker was further studied in clinical samples where its expression was found to rise as pancreatic intraepithelial neoplasia (PanINs) increase in grade ultimately resulting in an invasive carcinoma (17). Although MUC4 is differentially overexpressed in several malignancies, including pancreatic, breast, ovarian, lung, cervical, and head and neck cancer the role of MUC4 has been the most well studied in pancreatic cancer.

MUC4 is a ~550-930KDa protein with unique domains including nidogen-like (NIDO), the adhesion-associated domain in mucin MUC4 and other proteins (AMOP), von Willebrand factor (vWD), variable number of tandem repeats (VNTR), three EGF domains, transmembrane (TM) and cytoplasmic tail (CT) (Figure 1.1). Based on the presence of Gly-Asp-Pro-His (GDPH) cleavage site in rat MUC4 and its homology with human MUC4, a putative cleavage site in human MUC4 results in the generation of heavily glycosylated MUC4α (3000-7300 aa) and MUC4β subunits (1156 aa) (18). Due to the presence of EGF domains, MUC4 is hypothesized to interact with EGFR family members and trigger downstream signaling associated with cell proliferation, invasion, and migration of pancreatic cancer cells (19-21). In addition, the AMOP domain of MUC4 results in
angiogenesis via increased production of VEGF-A (22). The NIDO domain of MUC4 has been associated with metastasis of tumor cells (23).

Figure 1.1 MUC4 Schematic

MUC4 schematic detailing α and β- subunits separated at cleavage site GDPH. MUC4α-subunit can be cleaved and putatively shed while the membrane-bound β-subunit will remain attached to the surface of the cell.
Functional roles of MUC4 in pancreatic cancer have been elucidated both \textit{in vitro} and \textit{in vivo}. A comparison study of \textit{in vitro} and \textit{in vivo} functional effects utilized cells with MUC4 compared to MUC4-siRNA knockdown (KD) cells that expressed 80-90\% less MUC4. Increased tumorigenicity and pancreatic cancer motility have been correlated with MUC4 expression both \textit{in vitro} and \textit{in vivo} (24, 25). Mice with MUC4-expressing tumors had larger tumors and exhibited increased metastasis compared to MUC4KD counterparts. The presence of MUC4 increased the ability of cells to migrate and decreased cellular adhesion and the ability of cells to aggregate. Additionally, MUC4KD cells had lower expression of HER2 at the protein level (24).

Clinically, MUC4 has been established as a prognostic factor for PDAC that is associated with poor survival (26). Additional \textit{in vitro} studies of MUC4-mediated mechanisms in PDAC were conducted using the CD18/HPAF cell line and transfection with shRNA to create a stable MUC4KD cell line. This study replicated findings of previous work by demonstrating an increase in tumorigenicity and motility in MUC4-expressing cells (24, 25). Other findings associated with MUC4 expression included increased cellular growth rate, reduced apoptosis and cell cycle arrest, increased invasiveness, and actin reorganization. Changes to cellular adhesions were further studied. MUC4 acts sterically to inhibit binding of both the basement membrane component complex and the ECM components: laminin, collagen, and fibronectin. However, this can be mitigated by a capping phenomenon, wherein MUC4, upon binding to an anti-MUC4 tandem repeat antibody, clustered into distinct polarized regions rather than being expressed evenly across the cell surface. Capping was found to expose cell surface integrins that restored cellular adhesion. Thus, the data from this report establishes MUC4 as a novel target in pancreatic cancer.
Additional work to determine the role of MUC4 in pancreatic cancer was performed by transfecting MUC4-negative cell lines with a truncated construct of MUC4 described as miniMUC4 (27). This truncated miniMUC4 sequence contains only 10% of the tandem repeat region of a wild-type MUC4 sequence and was stably transfected into the MiaPaCa and Panc-1 cell lines. Cells expressing miniMUC4 displayed increased growth, motility, and invasiveness in vitro. In vivo work to determine tumorigenicity and metastasis was also performed. While the miniMUC4 tumors were significantly larger compared to controls, they did not have a greater frequency of metastasis. This observation suggests the tumorigenicity associated with MUC4 may be attributed to the β-subunit of MUC4, while metastasis may be associated with the tandem repeat sequence in the α-subunit that was significantly truncated in the miniMUC4 construct. The MUC4-NIDO domain in the α-subunit was later the subject of study with respect to the metastatic properties of MUC4 (23). While the MUC4-NIDO domain did not affect cellular motility in vitro it was found to enhance invasion and extravasation. In vivo studies of the MUC4-NIDO domain demonstrated that it did not affect tumor size but was associated with an increase in metastasis.

MUC4 has been confirmed to have interacting partners that have also been linked to cellular proliferation, migration, and adhesion: HER2, HER3, and galectin-3 (19, 21, 28). Recently, microscale thermophoresis has been used to determine binding affinity between HER2 and the EGF-like domain of MUC4β (29). This research further showed an additional lower affinity interaction of HER2 within the MUC4β region, suggesting another domain of MUC4β may interact with HER2. This finding further highlights targeting the MUC4β subunit as a means of targeting pancreatic cancer.
1.4 MUC4 involvement in drug resistance

MUC4 has been linked to resistance of gemcitabine a first-line therapeutic in the treatment of PDAC. A main mechanism of gemcitabine resistance in PDAC is an altered apoptotic threshold (30). MUC4 expression increases phosphorylation of Bad protein, suppressing its ability to translocate to the mitochondria. This leads to a decrease in cytochrome c release to the cytosol, which decreases intrinsic apoptosis (31). Additionally, MUC4 has been found to regulate the human Concentrative Nucleoside Transporter 1 (hCNT1) as a mechanism of gemcitabine resistance (32). This may impact the efficacy of other nucleoside analogs when treating PDAC. Additional clinical implications for MUC4 and drug resistance have been studied in HER2-positive breast cancer. Analysis of a cohort of patients found an association between MUC4 expression and poor outcomes to the anti-HER2 therapeutic trastuzumab (33).

To date no MUC4 based therapeutics are in clinical trials to treat epithelial cancer (Table 1.2).

1.5 PC and therapeutic approach

PC is the 3rd leading cause of cancer deaths in the United States (1). Currently, fewer than 20% of patients with PDAC are candidates for surgical resection with 80% of these patients relapsing after surgery. For the majority of patients with PC, surgical resection may not be an option due to the location of the tumor; and many develop resistance to conventional therapy. Additionally, current therapeutics only target actively proliferating cells and do not address metastasis, which is found in 80% of all diagnosed cases (2). Recent incremental advances in PC therapeutics have done little to improve clinical outcomes, thus underscoring an unmet need for better therapeutics (34). Current therapeutics focus on inhibiting DNA synthesis (Table 1.1). However, to make further progress to treat pancreatic cancer new therapeutic targets and modalities need to be studied.
1.6 Antibody-based therapy in cancer

Monoclonal antibody (mAb) based therapeutics have emerged as a promising cancer treatment modality due to their low toxicity and high specificity (35). Antibodies used in the treatment of solid tumors may inhibit oncogenic signaling, block cell-cell interactions, or engage immune effector cells to attack the tumor by antibody-dependent cellular cytotoxicity (ADCC) in a target expression-specific manner (35). Alternatively, mAbs can serve as vectors to deliver cytotoxic cargo (drugs, radionuclides, or toxins) to cancer cells in antigen-specific manner (36). Advances in molecular and protein engineering technologies have enabled the development of therapeutic mAbs with lower immunogenicity, increased specificity and affinity, and efficient distribution through the vasculature to the tumor mass. In recent years, new antibody-based formats allow for combinatorial targeting approaches, including, bispecific antibodies (bsAbs), antibody-drug conjugates (ADC), and chimeric antigen receptor (CAR) T cells (37).

1.7 Advancements and bottlenecks of antibody technologies

Antibodies that were initially developed through hybridoma production have undergone a revolutionary transformation with the advances in genetic and protein engineering technologies. Antibody discovery has transitioned from mouse to chimeric, humanized and the latest generation of fully human antibodies. The details of success and failures of each type of technology being used for antibody discovery and development in a clinical format for biomarker and/or therapeutic applications are covered in this section.
1.8 Hybridoma technologies

Hybridoma technology involves the fusion between antibody-secreting B-cell and myeloma cell, for the continuous generation of antibodies (38). This technology has allowed the field of monoclonal antibody therapeutics to advance to translational applications. Initially, this technology was limited by low throughput in terms of speed and number of clones being screened, but innovations in chemistry and physical sciences in the last decades have led to the development of tools for high throughput screening which include robotic liquid handling, multiplex immunization, and microarray screening (39). Screening of antibody-secreting single cells using droplet-based microfluidic platform or cell surface-fluorescence immunosorbent assay have facilitated the selection of hybridomas at greater speed (40-42). Of note, the microfluidic platform has allowed the generation of single cells with great variation in production levels from a single hybridoma cell line. Recent use of the Celigro imaging cytometer for high-throughput cell-based hybridoma screening might improve the efficiency and speed of antibody discovery (43, 44).

Drawbacks to the use of hybridomas still exist, which include additional V\_H or V\_L chains present in the hybridoma lines due to continuous chromosomal rearrangement and point mutations that may impact antibody specificity and efficacy (45, 46). Analysis of 185 unique commercially available and proprietary hybridomas revealed that more than 30% of the hybridomas expressed an additional V gene (47). This creates a hindrance to therapeutic antibody development and highlights the need to sequence and identify the correct V\_H/V\_L functional pairs. A solution to this problem increases throughput of hybridoma sequencing through the use of next-generation sequencing (NGS) followed by bioinformatics analysis to ultimately report unique sequences per well (48). Other drawbacks to hybridoma technology are the requisite immunization of animals to produce antibodies and grafting variable regions onto the human Fc portion to prevent an immune
response against the therapeutic (anti-drug antibodies). These steps can be low throughput, time intensive methods. Other antibody technologies developed have sought to circumvent these obstacles.

New CRISPR-Cas9 gene editing techniques have also begun to be incorporated into hybridoma technology. A plug-and-(dis)play hybridoma platform has been used to produce antibody libraries which can both display and secrete full-length IgG, an advantage for translational applications (49-51). Other applications of CRISPR-Cas9 allow for incorporation of chemical moieties, thus providing sites specific for bioconjugation to produce antibody-drug conjugates without sacrificing antibody affinity (52).

**1.9 Phage display technology**

Phage display technology originally developed for peptide display was adapted to display antibody scFv and Fab regions (53-55). Phage display technology allows the development of antibody libraries with great genetic diversity (reported up to $10^{10}$ unique combinations) and generation of fully human monoclonal antibodies (56). While developing antibodies from a phage display, format maintenance of the $\text{V_H}/\text{V_L}$ pairings is crucial to binding affinity and requires careful handling for conversion of a full-length IgG format. A high-throughput platform which reformats scFvs from phage-display pools to full-length IgG ‘Screening in Production Format’ (SiPF) has demonstrated efficacy with both scFv and Fab phage display libraries (57). To determine if potent functional antibodies were derived from the SiPF format full-length IgG antibodies were compared to traditionally obtained scFv and scFv-Fc fusions. The full-length IgG from SiPF were found to have better inhibitory activity than scFv-Fc fusions and were found to be more potent than scFvs. Other display methods followed the development of phage display, including display on yeast, ribosomes, and mammalian cells (Figure 1.2) (58-60).
Figure 1.2 Antibody Display Technology Advancements.

The first antibody display technology began with phage display which was soon followed by yeast display and later ribosome and mammalian cell surface display. Each of these technologies allows for the creation of a diverse synthetic antibody library without the need for animal immunization. However, only yeast and mammalian cell displays are capable of full-length IgG display. Antibody engineering techniques are required for grafting onto scFv or Fab fragments onto a framework. Additionally, display antibodies can require affinity maturation to improve antibody stability and differences in glycosylation in yeast display can contribute to immunogenicity.
1.10 Coupling display technology and *in silico* technology

Phage display technology allows the discovery of various antibodies for a particular antigen through high throughput screening. However, *in silico* technologies are now being combined with phage display technologies for increased accuracy and speed in the process of high throughput screening (61). NGS has several advantages over the traditional technique of Sanger sequencing. NGS allows for the validation of more diverse antibody libraries and identification of low abundance antibodies that may have been overlooked with classical screening methods (62, 63). Improvements in the selectivity of difficult to drug targets such as MMPs have been demonstrated with NGS (64-66). NGS also allows for screening of antibodies without repetitive screening rounds. A drawback to NGS sequencing is the number of base pairs that it is able to read. The scFv antibody format is greater than 800 bp long and thus full-length reads are not compatible with all NGS technologies. The application of NGS as an antibody development tool is highly dependent on bioinformatics software which provides an *in silico* solution to collate \( V_H \) and \( V_L \) sequences. Pairing strategies are used on the basis that \( V_H \) has higher diversity that contributes more to antibody binding and affinity than \( V_L \) (67).

Another *in silico* strategy allows for the sequencing of both the \( V_H \) and \( V_L \) genes (68). Through the utilization of single-molecule real-time sequencing (SMRT) technology by Pacific Biosciences in combination with the IMGT/V-QUEST software the researchers were able to use *in vivo* phage display to fully sequence VH/VL pairs with high fidelity on par with the more time intensive Sanger sequencing. The end result of the experiment was double-stranded DNA from scFv clones analyzed using the SMRT technology to determine their sequences. This information was then analyzed by the IMGT/V-QUEST software that can analyze two V-domain sequences. These data serve as a proof-of-concept to demonstrate how antibody selection from phage-display can be used in combination with NGS and *in silico* techniques to determine the sequence of \( V_H/V_L \) pairs.
The NGS technology also requires further corrections for error-prone readings in addition to pairing $V_H/V_L$ sequences. An error correction tool, computational program ABOS, relies on amino acid sequences (specifically conserved cysteine residues) to determine structural viability of the antibody sequence (69).

1.11 Transgenic animal models

To develop fully functional humanized antibodies researchers have turned to the development of transgenic animals with a humanized immune system. Several proprietary transgenic mouse models have been developed on this principle all of which have been used to produce FDA approved antibodies: HuMAb, Xenomouse, and VelcolImmune (70-73). Since these models are proprietary the published research on the discovery of antibodies using this technology is limited.

1.12 Mechanisms of anti-tumor activity of therapeutic antibodies

Therapeutic mAbs induce anti-tumor effects via diverse mechanisms (Figure 1.3), which depend on the nature of target antigen, target cell, and the nature of interactions between Fab and Fc regions of mAbs with target antigen and effector cells respectively. The mechanism of action of therapeutic mAbs guides the clinical applications in terms of selecting patients, disease setting, and combination therapies.
The mechanism of action of mAb therapeutics can occur through inhibition of oncogenic signaling pathways by either binding cell surface receptors or the binding of soluble ligands. These mechanisms can either inhibit oncogenic pathways to initiate cell cycle arrest or apoptosis, or inhibit immune checkpoints allowing for the recruitment of immune effector cells to target and kill cancer cells. Antibody-drug conjugates can also be constructed as delivery vehicles for cytotoxic payload delivery which ameliorates the toxicity which can be found in systemic delivery.
1.13 Functional neutralization of target antigen
Therapeutic mAbs directly targeting tumor cells or the non-tumor cells in the TME recognize: a) growth factor receptors or their ligands; b) angiogenic receptors present on tumor vasculature, or their ligands; c) immune checkpoint molecules. Anti-tumor activity of these mAbs depends on the expression levels and persistence of target antigen on tumor cells. These mAbs induce conformational changes, cause steric hindrance or facilitate internalization and downregulation of cell surface receptors, and abrogate downstream signaling (Fig 1.3 a & b). In the last decade, therapeutic antibodies directly targeting immune checkpoint molecules [referred to as immune checkpoint blockade (ICB)] (Fig 1.3 c) have captured the center-stage for cancer immunotherapy (74). Since the approval of the anti-CTLA4 mAb ipilimumab for treatment of advanced unresectable melanoma in 2010, antibodies targeting immune checkpoint molecules have been approved for several malignancies (75). MAbs to another checkpoint molecule, PD-1, or its ligand (PD-L1) showed significant success in reversing immune exhaustion and demonstrated better survival in various cancers, based on which two anti-PD-1 antibodies, pembrolizumab (Keytruda) and nivolumab (Opdivo), were approved by FDA for cancer treatment in 2014. However, pembrolizumab performed better in PD-L1-positive non-small-cell lung carcinoma (NSCLC) patients. Other mAbs targeting checkpoint molecules such as LAG3 or CD223, BTLA (B and T lymphocyte attenuator), VISTA, and TIM3 are also being investigated to reduce immunosuppression, and a few are in early phase clinical trials for various malignancies (76).

1.14 Facilitation of engagement with effector cells
Therapeutic mAbs crosslink tumor cells and the effector arm of the immune system via their antigen-binding domain (Fab) and Fc domain, respectively, and elicit a range of effector functions. These include induction of antibody-dependent cell cytotoxicity (ADCC) through neutrophils and natural killer (NK) cells, antibody-dependent cell phagocytosis
(ADCP) via macrophages, and complement-dependent cytotoxicity (CDC) by the complement pathway of the immune system (Fig 1.3d).

1.15 Antibody-dependent cellular cytotoxicity (ADCC)
Therapeutic mAbs can induce ADCC activity by binding to various Fcγ receptors (FcγR) present on immune effector cells, including FcγRIIa (CD32) and FcαR1 (CD89) expressed exclusively on neutrophils and FcγRIIIa (CD16) on NK cells. Neutrophils exert cytotoxic activity by degranulation resulting in the release of proteases, cytokines, and tumor necrosis factor alpha (TNFα) upon binding to opsonized target cells (77). The presence of high affinity receptor FcαR1(CD89) on neutrophils makes them potent effector cells for IgA antibodies (78). Therefore, an isotype exchanged IgA version of IgG1 cetuximab, exhibited potent in vivo ADCC activity against EGFR transfected Ba/F3 target cells (79). Similarly drastic tumor reductions were observed due to the engagement of neutrophils with tumor cells in the presence of anti-EGFR, Her2, CD30, and Ep-CAM antibodies of IgA isotype, possibly through the release of chemoattractant Leukotriene B4 (LTB4), which causes increased neutrophil accumulation in the tumor microenvironment (77, 80, 81). Another study showed an increase in neutrophil extracellular trap (NET) formation by engagement of FcαR with IgA antibodies (82). However, chronic granulomatous disease patients with no NET formation, showed effective antibody-mediated tumor cell killing, suggesting the involvement of multiple mechanisms for mAb-mediated tumor killing by neutrophils that need to be further explored. Interestingly, a recent study has identified trogoptosis as a potential mechanism for neutrophil-mediated ADCC, which occurs by the formation of synapse between mAb-opsonized tumor cells and neutrophils via CD11b/CD18, resulting in the disruption of target cell membranes (83). This killing is enhanced by the targeting CD47-SIRPα axis, which is an innate immune checkpoint pathway.
NK cells serve as critical components in tumor cell killing through ADCC. Interaction of target-bound mAb constant region (F<sub>c</sub>) with FcγRIIIA (CD16), and/or FcγRIIC (CD32c) present on NK cells induces ITAM (immune tyrosine based activation motif) phosphorylation, ZAP-70 and Syk kinase-dependent activation of PI3K, NF-κB, and ERK pathways, which leads to both NK cell degranulation and signaling, causing target cell killing (Fig 1.3d) (84). In addition, IFNγ secretion from NK cells facilitates the recruitment of cytotoxic T cells to lyse target cells (85, 86).

1.16 Antibody-dependent cellular phagocytosis (ADCP)
Antibody-mediated phagocytosis by macrophages has been best studied in hematological malignancies. Unlike NK cells, which express only FcγRIIIa, macrophages express all classes of Fc receptors (FcγRI, FcγRII, and FcγRIIIa) and are thus believed to be the major effectors of antibody-mediated therapy (87). The intricate relationship between macrophages and tumors complicates the understanding of macrophage’s role as effectors for therapeutic mAbs. While macrophages have been traditionally viewed as vital cells for cancer immunotherapy, a high number of tumor-associated macrophages has been associated with adverse patient outcomes. The critical role of macrophages in antibody-mediated therapy became evident when their depletion resulted in decreased efficacy of anti-CD142 mAbs in preventing breast cancer growth and metastasis (88). Attempts are warranted to enhance macrophage response to mAb therapies. In fact, agonistic anti-CD40 mAb displayed an effective clinical response in pancreatic cancer mainly through the reactivation of macrophage effector activity, and subsequently several anti-CD40 mAbs have been developed and are in clinical trials as monotherapy or combinations with chemotherapy, radiations or immunotherapy for various malignancies (89, 90).
1.17 Complement-dependent cytotoxicity (CDC)

Therapeutic mAbs mediate complement-dependent cytotoxicity (CDC) via their interaction with soluble complement protein C1q through the Fc domain. This interaction activates the complement cascade culminating in the formation of a membrane attack complex (MAC) on the tumor cell surface and leading to cell lysis. Although tumor killing by antibodies through CDC in a clinical setting is debatable, complement activation predominantly promotes the elimination of mAb-opsonized cells via ADCC and ADCP (91). Importantly, these diverse immune effector mechanisms are interlinked and can thus augment tumor cell killing in a synergistic manner (92).

Most therapeutic mAbs exert anti-tumor effects by multiple mechanisms of action that are interdependent on each other and therefore might influence their anti-tumor activity. For example, extensive research on trastuzumab and cetuximab in non-clinical studies suggested mAb action beyond signaling inhibition which include receptor downregulation, degradation (in case of cetuximab), decrease in angiogenic factors, reduced production of active truncated Her2 (p95) fragments via inhibition of Her2 cleavage (in the case of trastuzumab) and ADCC-dependent killing of tumor cells (93-96). It can be argued that the induction of signaling and receptor downregulation on target cells might alter ADCC activity of antibodies. Nonetheless, preclinical studies have shown enhancement of T cell response via effective DC presentation of mAb-lysed target cells. Over the past decades, technological innovations in antibody engineering have allowed the generation of bispecific antibodies (bsAb) with novel functions where two binding specificities are linked in one mAb for encompassing their functional properties. Currently, 25 bsAbs targeting tumor heterogeneity, growth factor receptors, multiple checkpoints, and angiogenesis are in various phases of clinical development for solid tumors (97). Though the bsAb catumaxomab targeting EpCAM and CD3 received approval based on better response in a subset of cancer patients, it was recently discontinued due to toxicity. Nevertheless,
clinical data obtained from bsAbs trials would guide further optimization for better drug safety and efficacy and might open new avenues for cancer treatment.

1.18 Antibody-drug conjugates

The ability of mAbs to demonstrate specific targeting and stability has fueled the development of antibody conjugates for the delivery of cytotoxic payloads to the tumor site and overcome off-site toxicity issues associated with chemotherapies (98, 99). ADCs exert their activity by specific binding to tumor cells, internalization via receptor mediated endocytosis, endosome formation, and fusion with lysosome thus creating acidic and protease (cathepsin, plasmin etc.) rich environments. This results in ADC cleavage and cytotoxic payload release in the cytoplasm that in turn abolishes cell proliferation via hindering replication machinery or microtubule assembly and subsequent cell death (Fig 1e). While, there exist challenges with the successful development of ADC (including target antigen selection, mAb specificity and affinity, the payload potency and stability in circulation, and linker selection), more than 60 ADCs are in different phases of clinical development with the latest approval of fam-trastuzumab deruxtecan-nxki for metastatic breast cancer patients (99).

The field of mAb therapeutic agents has grown significantly from the early experiments of Köhler and Milstein. Antibody can now be produced as either fully humanized from specialized mouse models or be produced without animal immunization through display technologies. Additionally, antibodies are no longer bound to a traditional IgG format. Antibodies can be bound to small molecule drugs for targeted delivery or engineered for binding of two separate antigens. Although there are currently no FDA approved mAb therapeutics for the treatment of pancreatic cancer it remains only a matter of time before a mAb therapeutic is clinically successful.
References


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2 Chapter 2: Materials & Methods
2.1 Materials & Methods

Generation of MUC4β recombinant protein: Standard PCR and molecular cloning techniques were used to make a His<sub>6</sub>-tagged pET-28a-MUC4β construct by inserting a 2199 bp fragment of MUC4 into a pET28a (+) vector (Novagen). Specific primers were designed using MUC4 sequence NM_018406.6 to amplify the fragment from nucleotides 14278 to 16395, which represents the region immediately downstream of the degenerate repeats found C-terminal to the TR domain. Amplification was done using the Expand Long Template PCR system (Roche) using JER103 and JER109 as templates. NdeI and an XhoI restriction sites were added in the forward (pET28a-MUC4b_Ndel_FP, GTTATCATATGATCACCACCTTGGATGGTGCAG) and reverse primers (pET28a_MUC4b_XhoI_RP, CTTATCTCGAGTCAAGGCAAGGCCTCAGC), respectively, allowing in-frame cloning.

The construct was sequenced to confirm the proper reading frame and was maintained in Rosetta-2 (DE3) competent cells (Sigma-Aldrich Corp., St. Louis, MO). A 5 mL overnight pre-culture was used to inoculate 4 L of LB medium containing 50 µg/mL kanamycin and 40 µg/mL of chloramphenicol and the culture was grown under agitation at 37 °C for 3 to 4 h until absorbance measured 0.5-0.6 at 600nm. Expression of MUC4β was induced with 1 mM of IPTG, and cells were subsequently allowed to grow for 4 h at 37 °C.

Cells were harvested by centrifugation (6000 rpm, for 30 min, at 4 °C), washed once with ice cold PBS, and weighed. The bacteria pellets were suspended in ice cold buffer containing 50 mM Tris-HCl, 2 mM MgCl<sub>2</sub>, 10 mM NaCl, protease inhibitors cocktail (250 µL/10 g of cell pellet) and treated with Benzonase nuclease 60 units/mL. Cells were lysed by three passages through an EmulsiFlex-C3 at 15,000 psi. Cell debris was removed by centrifugation (9000 rpm, for 30 min, at 4 °C) and the supernatant was incubated overnight at 4 °C for digestion of oligonucleotides. Pellets containing insoluble inclusion
bodies were collected by centrifugation (18,000 rpm for 60 min at 4 °C) using a Sorvall RC 5Cplus with an SS-34 rotor. The recovered pellets were re-suspended in 40 mL buffer A (pH 8.0, containing 6 M Urea, 1% Triton X-100, 1 mM imidazole, and 2 mM β-mercaptoethanol in PBS) and placed on rotating rocker overnight at 4 °C. The dissolved pellet was then treated with a second cycle of Benzonase nuclease (30 units/mL) for 1 h at 4 °C. The suspension was collected by centrifugation (18,000 rpm for 60 min at 4 °C).

Protein supernatant was passed through a 0.22 µm filter and passed over a HisTrap HP affinity column using an AKTA FPLC (GE Healthcare). The column was washed with Buffer A using a flow rate of 1 mL/min for 25 mins prior to elution. Protein fractions were eluted using a gradient consisting of 5%, 10%, 25%, 50%, 75% and 100% buffer B (pH 8.8, containing 6 M Urea, 1% Triton X-100, 1 mM imidazole, and 2 mM β-mercaptoethanol in PBS).

Eluted protein fractions were assessed using 10% SDS-PAGE, Coomassie staining, and western blot analysis with anti-His tag antibody. Fractions containing protein were pooled and concentrated to a volume of 10 mL using Amicon Ultra centrifugal filters (30 kDa, Merck Millipore 2015-08, Cork Ireland) before dialysis at 4 °C using a 10-12 mL, 10 kDa Slide-A-lyzer® dialysis cassette (Thermo Scientific, Pierce Product 66810).

Dialysis was performed against PBS containing decreasing concentrations of urea (6 M to 0 M); followed by PBS (3 cycles of 6 h) and ultra-purified (> 18 Ω) water (3 cycles of 6 h), respectively. Bacterial endotoxins (lipopolysaccharides) were removed using high-capacity endotoxin removal spin columns (Thermo Scientific, Pierce Product 88273). Purified MUC4β recombinant protein was aliquoted and stored at -80 °C. Recombinant protein was quantified using a BCA Protein Assay kit (Thermo Scientific, Pierce Product 23225). The final purity of the protein was confirmed using 10% SDS-PAGE, Coomassie
staining, and a 1% agarose gel containing SYBER® GREEN II to assess the presence of DNA contamination.

**Mouse immunization:** The immunization and selection of mAbs were carried out using established procedures at the UNMC.Briefly, three BALB/c mice were immunized by IP injection of recombinant MUC4β His-tagged fusion protein at three-week intervals with Incomplete Freund’s adjuvant. As a final immunization, the animals were injected with the lysate of MUC4-positive HPAC human pancreatic cancer cells. Sera from these mice were evaluated in direct binding assays for antibody reactivity with the recombinant MUC4 fusion protein, and the MUC- negative cell line MIA PaCa-2 as a negative control. Once an appropriate antibody response was observed in ELISA, the animals were given a final booster injection with the recombinant protein four days prior to exsanguination and splenectomy.

**Hybridoma fusion:** Splenocytes were isolated and fused with P3X63Ag8.653 myeloma cells. Briefly, myeloma cells were washed 2 times with prewarmed RPMI media. The spleens were resected from immunized mice using sterile techniques and crushed in between sterile frosted slides to release the splenocytes. The splenocytes were then washed once with RPMI along with an additional 3rd wash of myeloma cells. A total of 108 splenocytes were combined with 107 myeloma cells and washed once again with RPMI. The supernatant from the combined pellet was removed completely and pellet gently dislodged. In a 37 °C water bath, 1 mL of prewarmed 50% PEG3350 was added dropwise for 30 s to the pellet to begin cellular fusion. Next, the pellet was gently stirred with the end of a sterile tip for 30 s. Then, dropwise additions of prewarmed RPMI were added beginning with 1 mL which was added for 15 s, 2 mL for the next 30 s, and finally 4 mL over the next 45 seconds. Additional RPMI was added for a final volume of 35 mL. The fused cells were then centrifuged at low speed. Cells were resuspended in 15% advanced
RPMI, containing HCF (hybridoma cloning supplement) and plated in 96-well plates at a
100 µL/well volumes. The next day, in each well add equal volume of 15% advanced RPMI
containing 2X HAT (hypoxanthine-aminopterin-thymidine) selection media. Cells were
incubated additional HAT selection media was added after 4 days. Hybridoma growth was
observed in every well with almost all the wells having multiple colonies of growing cells.
Supernatant were taken for screening after 10-14 days. Hybridomas producing the
antibodies of interest were selected by screening for specific antibody binding to the
immunogen of interest (recombinant protein and MUC4-expressing cellular lysate) and a
lack of binding to irrelevant control antigens (a His6x-tag containing MUC16 recombinant
protein and negative cell line MIA PaCa-2). Positive hybridomas were expanded and
immediately cloned by the limiting dilution method. This allowed for the production of a
single cell per well to produce a monoclonal antibody. At least 2 rounds of cloning were
performed to ensure the monoclonalinity of each hybridoma.

**MUC4β Hybridoma Screen**: ELISA using Immulon plates were coated with 50 µL of
the antigenic preparation (MUC4β recombinant protein or MUC16(HIS) protein; or protein
lysates from MUC4 positive/negative cell lines) at a concentration of 2.5 mg/well in
bicarbonate buffer (pH 9.6). The plates were incubated overnight at 4 °C. The plates were
washed in PBST and the free binding sites of the wells were saturated to eliminate
nonspecific binding of the immunoglobulins by incubating with 200 µL/well of 5% non-fat
skim milk in PBS for 2 h at 37 °C. The plates were then washed in PBST. A 100 µL volume
of the culture supernatant was transferred from wells of culture plates into corresponding
wells in ELISA plates. Mouse pre-immune serum was used as a negative control in each
assay. The plates were incubated for 1 h at 37 °C and washed again in PBST. A
100 µL/well volume of the peroxidase conjugated antibody (anti-mouse HRP, Amersham
Biosciences, 1:5000 dilution in 0.1% non-fat skim milk in PBS) was added and incubated
for 1 h at 37°C. The plates were washed in PBST and 100 µL of TMB substrate (Dako Substrate) was added to each well and incubated at 37 °C. The reaction was stopped by adding 100 µL of 2 M sulfuric acid solution. The absorbance was measured at 450 nm using a Spectramax 190 microplate reader. Further ELISA based tittering of anti-MUC4β mAbs was performed on different concentrations (0.125-1 mg/mL) of purified MUC4β recombinant protein and MUC4-positive lysate of pancreatic COLO 357 cells.

**SDS-PAGE Immunoblotting:** Hybridomas were also selected for their ability to react with the MUC4 positive cell line HPAF-II. Preparatory SDS-PAGE was conducted to screen multiple antibodies with membrane strips cut from transferred protein run on 8% gels. Total protein lysates were prepared from cell lines on ice using RIPA buffer containing [10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM NaF, 1 mM sodium orthovanadate (Na₃VO₄), supplemented with Complete™ Protease Inhibitor Cocktail tablets (Antipain-dihydrochloride, Aprotinin, Bestatin, Chymostatin, E-64, EDTA-Na₂, Leupeptin, Pefabloc SC, Pepstatin, Phosphoramidon; (Roche Applied Science, Basel, Switzerland)].

Cell lysates were passed through a 22-gauge needle with syringe 10 times to facilitate disruption of the cell membranes. Cell lysates were centrifuged at 14,000 rpm for 30 min at 4 °C, and supernatants were collected. Protein samples were quantified using a modified Lowry method (Protein Assay, Bio-Rad, Hercules, CA). For preparatory SDS-PAGE, 150 µg HPAF-II total protein lysate was loaded into the single preparatory well (BIO RAD Mini-PROTEAN® Tetra System). For standard SDS-PAGE, 10 or 15 µg total protein lysate was used to prepare samples.

Samples were denatured for 5 min at 95°C in the presence of 1× denaturing sample buffer (4× loading dye: 40% glycerol, 250 mM Tris-HCl, 8% SDS, 0.04% bromophenol blue, pH 6.8). Electrophoresis was performed at 100V in SDS/Tris-glycine
migration buffer (0.1% SDS, 25 mM Tris base, 115 mM glycine). Proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon P, Millipore, Boston, MA) at 200mA for 1.5 h.

For MUC4 immunodetection, anti-MUC4 mouse monoclonal antibody 8G7 (1 mg/mL) positive control, while anti-MUC4β mouse monoclonal antibody, from hybridoma supernatant, were diluted in antibody diluent (PBS, 0.05% Tween 20, 3% nonfat skim milk) at either 1:10 / 1:100 (for neat, before clonal selection) or either 1:400 / 1:1000 (following clonal selection). The primary antibodies were incubated with membranes overnight at 4 °C with gentle agitation (~15 h). Anti-human beta actin (1:10,000, Sigma AC-15) was used or the protein loading control. Washes were carried out in phosphate-buffered saline, containing 0.05% Tween 20 (PBST) for a total of 45 min and consisted of one quick rinse followed by three 15 min washes with agitation.

Secondary antibody (goat anti-mouse IgG (H+L), Thermo Fisher Scientific, Pierce Product 31430) was used at a dilution of 1:3000 in antibody diluent (PBST, containing 3% nonfat skim milk) and was incubated with membranes for 1 h. Washing to remove secondary antibody was performed as previously mentioned with a final PBS rinse. The blots were processed with ECL (Thermo Fisher Scientific, Pierce Biotechnology, Rockford, IL) incubation for 1 min, and the signal was detected by exposing the processed blots to X-ray films (Biomax Films, Kodak, NY).

Agarose Immunoblot Analysis: For horizontal agarose western blot, samples consisting of 30 µg (for 8G7 primary antibody detection) or 60 µg (for anti-MUC4β primary antibody detection) total protein cell line lysate were denatured for 5 min at 95°C in the presence of 1× denaturing sample buffer in a 30 µL volume. Horizontal agarose western blots were performed on 2% agarose Tris-glycine gels containing 0.1% sodium dodecyl sulfate (SDS) in SDS/Tris-glycine migration buffer at 100V for 7-8 h. Proteins were transferred to
polyvinylidene difluoride (PVDF) membranes (by passive diffusion overnight using Tris-glycine transfer buffer containing 20% methanol. Membranes were blocked for 2 h in PBST containing 5% non-fat skim milk. Primary antibodies (MUC4 TR Ab 8G7, and MUC4β Ab clones E9 and 6E8) were prepared in antibody diluent as stated above. Washes, 2° Ab incubation, and ECL development were performed following the same procedures used for SDS-PAGE immunoblotting.

**Flow Cytometry:** Cells were harvested non-enzymatically using Cellstripper™ (Mediatech, VA), washed with flow cytometry buffer (PBS, 1% BSA, 0.1% azide, 2mM EDTA). Cells were incubated in flow cytometry buffer on ice for 45-60 minutes with mAbs (10 µg/mL). All subsequent steps were performed with incubations on ice. Cells were washed three times with ice cold flow cytometry buffer and incubated with Alexa 488 conjugated anti-mouse antibody (1:100) (Invitrogen, Carlsbad, CA) for 45 minutes. Cells were washed three times with ice cold flow cytometry buffer and then analyzed using a BD FACSCalibur™ flow cytometer. Propidium iodide (2 µg/mL) was added to the samples just before acquisition to eliminate interference from dead cells. Flow cytometry analysis was used to assess binding of anti-MUC4β antibodies native MUC4 expressing cells (NCI-H3122, T3M4, and CD18-HPAF).

**Immunoblot and Immunoprecipitation:** Total protein lysates from CD18 cells were prepared in RIPA buffer containing protease inhibitors [10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2mM phenylmethylsulfonyl fluoride (PMSF), 10 mM NaF, 1 mM sodium orthovanadate (Na₃VO₄), supplemented with Complete™ Protease Inhibitor Cocktail tablets (Antipain-dihydrochloride, Aprotinin, Bestatin, Chymostatin, E-64, EDTA-Na₂, Leupeptin, Pefabloc SC, Pepstatin, Phosphoramidon; (Roche Applied Science, Basel, Switzerland)]. Cell lysates were passed through a 22-gauge needle with syringe 10 times to facilitate disruption of the cell membranes. Cell lysates were centrifuged at 14,000 rpm
for 30 min at 4°C, and supernatants were collected. Protein samples were quantified using a modified Lowry method (Protein Assay, Bio-Rad, Hercules, CA).

For standard SDS-PAGE, 80 μg total protein lysate was used to prepare samples. Samples were denatured for 5 min at 95°C in the presence of 1× denaturing sample buffer (4× loading dye: 40% glycerol, 250 mM Tris HCl, 8% SDS, 0.04% bromophenol blue, pH 6.8). Electrophoresis was performed at 100V in SDS/Tris-glycine migration buffer (0.1% SDS, 25 mM Tris base, 115 mM glycine). Proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon P, Millipore, Boston, MA) at 200mA for 1.5 h.

For MUC4 immunodetection, anti-MUC4 mouse monoclonal antibody 8G7 (1 μg/mL) positive control and anti-MUC4β mouse mAbs (2 μg/mL) were diluted in antibody diluent (PBS, 1% BSA). The primary antibodies were incubated with membranes overnight at 4°C with gentle agitation (~15 h). Anti-human beta actin (1:10000, Sigma AC-15) was used or the protein loading control. Washes were carried out in PBST for a total of 45 min and consisted of one quick rinse followed by three 15-min washes with agitation.

Secondary antibody (goat anti-mouse IgG (H+L), Thermo Fisher Scientific, Pierce Product 31430) was used at a dilution of 1:3000 in antibody diluent (PBST, containing 3% nonfat skim milk) and was incubated with membranes for 1 h. Washing to remove secondary antibody was performed as previously mentioned with a final PBS rinse. The blots were processed with ECL (Thermo Fisher Scientific, Pierce Biotechnology, Rockford, IL) incubation for 1 min, and the signal was detected by exposing the processed blots to X-ray films (Biomax Films, Kodak, NY).

For horizontal agarose western blotting, samples consisting of 30 μg (8G7 detection) or 60 μg (anti-MUC4β detection) total protein cell line lysate were denatured for 5 min at 95°C in the presence of 1× denaturing sample buffer in a 30 μL volume. Horizontal
Agarose western blotting was performed on 2% agarose tris-glycine gels containing 0.1% sodium dodecyl sulfate (SDS) in SDS/Tris-glycine migration buffer (at 100V for 6 h). Proteins were transferred to polyvinylidene difluoride (PVDF) membranes (by passive diffusion overnight using Tris-glycine transfer buffer containing 20% methanol. Membranes were blocked for 2 h in PBST containing 5% Carnation instant nonfat dry milk. Primary antibodies were prepared in antibody diluent as stated above. Washes, 2\(^\circ\) Ab incubation, and ECL development were performed following the same procedures used for SDS-PAGE immunoblotting.

For immunoprecipitation, protein A/G PLUS-agarose CL-6B Sepharose beads (Santa Cruz Biotechnology, Santa Cruz, SC-2003) were equilibrated with purified anti-MUC4 antibodies (8G7, E9, 6E8, 1C7, and 3B4) against MUC4\(\beta\), for 1 hour on ice. A nonspecific IgG isotype control (K2G6) was used at a concentration of 5 \(\mu\)g/mL. In order to bind the IgA 3B4 antibody, Protein A/G beads were first incubated with a rabbit polyclonal anti-mouse IgG antibody followed by binding of anti-MUC4 mAb 3B4. Antibody-bound beads were rinsed twice with RIPA buffer. The beads were incubated overnight with 1 mL (~1 mg) of total protein lysate, at 4\(^\circ\)C with rotation. The samples were then centrifuged, and unbound supernatants were collected as flow through control samples. The beads were washed 3× with RIPA buffer and antibody-antigen complexes were eluted by boiling in denaturing SDS sample buffer containing 2-mercaptoethanol for five mins at 95 \(^\circ\)C before resolving on 2% agarose gels.

**Epitope mapping:** MIA PaCa-2 cells were transiently transfected with constructs representing deletions of the EGF-like domains within the MUC4\(\beta\) subunit using Lipofectamine 2000 (Invitrogen). Total cell lysates were prepared and used for western blotting assays.
Immunofluorescence/Confocal Immunofluorescence Microscopy: NCI-H3122, CD18, MUC4/X MIA PaCa-2, Capan-1, and COLO357 cells were seeded at 0.5 x 10^5 cells per well on sterilized glass cover slides and allowed to grow until they reached 60-70% confluency. Following cell growth, the media was aspirated, and the cells were washed with ice cold PBS twice, then cells were fixed in ice-cold methanol at −20°C for 10-15 min. After methanol fixation the cells were washed twice with ice cold PBS and a blocking solution of 1% BSA/PBS was added to the cells. Blocking media was aspirated from each well and replaced with a 1 µg/mL concentration of antibody or and allowed to incubate at room temperature for 2 h. Polyclonal serum, preimmune serum, and 8G7 antibody were used as controls. The anti-MUC4β antibody supernatants tested included 6E8, 3B4, and E9. The cells were washed 4X with blocking buffer, followed by incubation with Alexa 488-conjugated goat anti-mouse secondary antibodies for 1 hour at RT. Cells were washed three times with blocking buffer, and mounted on glass slides in anti-fade Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Immunostaining was observed and captured digitally using a Zeiss LSM 800 with Airyscan confocal laser-scanning microscope.

Immunohistochemistry: Formalin fixed pancreatic cancer sections were deparaffinized in xylene and rehydrated in a decreasing ethanol series. Endogenous peroxidase activity was quenched by incubating sections in 3% H_2O_2 in methanol for 15 min. Antigen retrieval was carried out by boiling the samples in citrate buffer (10 mM sodium citrate, 0.05% Tween-20, pH 6.0). The tissue microarrays were blocked by incubating the sections with normal horse serum for 60 min at RT. Sections were then incubated with anti-MUC4 mAbs (6E8 and E9 at 5 µg/mL; 8G7 at 1 µg/mL) diluted in PBS overnight at 4 °C. The slides were washed with PBST (3x washes for 5 min each) followed by incubation with secondary antibody for 30 min. Slides were washed with PBST (3x
washes for 5 min each) and were incubated with DAB (Vector Laboratories, Burlingame, CA) until color development was evident. The slides were washed in water and counterstained with hematoxylin for 30 s. Slides were washed in water and dehydrated in an increasing ethanol series, followed by soaking in xylene and mounting with Permount permanent mounting media (Fisher Scientific, Fair Lawn, NJ). All stained slides were scored by a certified pathologist.

**SPR Characterization:** All SPR analysis was performed on a BiOptix 404pi. Anti-MUC4β antibodies (6E8, E9, and 3B4) or control anti-MUC16 CT antibody were immobilized at a 100 nM concentration on a BiOptix CMD200m chip using EDC/NHS coupling chemistry. MUC4β protein or control MUC16CT protein were passed over the chip at 1000 to 0.1 ng/mL. Data was analysis was performed using the Biologic Software program Scrubber.

**In vitro Functional Assays**

**Cytotoxicity assay using MTT:** Cytotoxicity of antibodies or antibodies in combination with gemcitabine was determined using the MTT assay. Cells were seeded at a density of $5 \times 10^3$ cells per well in a 96-well plate and allowed to incubate overnight (24 h) at 37 °C. Following incubation, the cells were treated with fresh media containing antibody dilution or antibody dilution in combination with gemcitabine for 48 and 72 h timepoints. Following incubation, a 5 mg/mL solution of thiazolyl blue tetrazolium bromide (MTT) reagent in DPBS was added to each of the wells at a 10 μL volume. The microtiter plate was allowed to incubate for 4 h. The media was then replaced with 100 μL of DMSO and the plate was read using a SpectraMax 190 microtiter plate read at an absorbance wavelength of 540 nm.
**Annexin-V staining and flow cytometry for measurement of apoptosis:** The cells were seeded at 0.5 × 10^6 cells/60 mm in a cell culture dish and grown overnight (~24 hours). The following day, the media was replaced by aspirating the old media, rinsing the plate 2X with PBS, and adding media containing diluted antibody, and then the plates were incubated for another 48 or 72 h at 37 °C. On the day of analysis, the old media and PBS rinses were collected, the cells were detached from the plate non-enzymatically using CellStripper, and the cells were centrifuged together with media and PBS rinses. The supernatants were aspirated, and the pellets were rinsed and additional 2X with PBS. The cells were resuspended with PBS and stained with Annexin V-Cy5 dye and propidium iodide. A 15 min incubation was allowed before the addition of binding buffer and the cells were analyzed using flow cytometry.

**Transwell migration:** The migratory potential of cells when treated with anti-MUC4β antibodies was also tested using 6-well inserts of 8-µm pore size (BD Biosciences). Cells were harvested non-enzymatically using CellStripper and rinsed using DPBS prior to diluting to a final concentration of 1 × 10^6 cells in serum-free DMEM. Antibodies were added at a 10 µg/mL concentration to separate aliquots of cells. Cells were seeded onto the top chamber of the membrane and DMEM + 10% FBS was added to the bottom chamber as a chemoattractant. Cells were then incubated for 24 h at 37 °C. Following the incubation, the top chamber of the membrane was wiped with a cotton swab to remove any cells that had not migrated. Cells which had migrated to the lower side of the membrane were stained with the Diff-Quick cell stain kit and photographed in eight random fields of view at 10X magnification for each replicate.

**Scratch assay:** The ability of cells in a monolayer to proliferated across a wound when treated with anti-MUC4β antibodies was tested using live imaging. For monitoring the migration rate via live cell imaging, a 200 µL pipet tip was used to make a horizontal and
vertical intersecting scratches in a confluent monolayer of cells grown in 12-well plates. Following the scratches, the cells were washed with DPBS to remove any loose cells and media with 10 µg/mL of antibody was added to each well. Live images of cells were captured at 0 and 24 h timepoints. The speed of migration was calculated by a ratio of distance travelled, measured in µm, over time.

**Statistical Methods:** Statistical analysis was performed using GraphPad Prism version 8.4.3. software (GraphPad Software San Diego, CA) for analyzing *in vitro* data. Statistical significance of comparisons of differences between groups was evaluated using one way ANOVA with a Dunnett’s multiple comparison test; differences were considered to be significant for values of P <0.05.
Chapter 3: Results
3.1 Synopsis

Pancreatic cancer (PC), is currently the 3rd leading cause of cancer-related deaths in the United States (1). To improve these survival rates of patients new therapeutic targets are needed. One such target is the transmembrane mucin, MUC4. The transmembrane mucin MUC4 is differentially overexpressed in pancreatic cancer as well as several other cancers of epithelial origin (11, 17, 100-105). While MUC4 is undetectable in the normal or inflamed pancreas (pancreatitis), its expression progressively increases during PC progression and its higher expression correlates with poor survival (11, 17, 106). The role of MUC4 in neoplastic transformation, enhanced motility, invasiveness, and drug resistance of cancer cells in vitro, and in tumorigenicity and metastasis in vivo has been conclusively established (19, 21, 24, 25, 27, 31, 107-109). Due to the differential overexpression of MUC4 in cancer cells and its functional involvement in disease pathobiology, it is an attractive therapeutic target (9). However, to date no MUC4-targeted therapeutics have been developed. Monoclonal antibody (mAb)-based therapeutics have emerged as a promising cancer treatment modality due to their low toxicity and high specificity. Antibodies used in the treatment of solid tumors may inhibit oncogenic signaling, block cell-cell interactions, or engage immune effector cells to attack the tumor by antibody-dependent cellular cytotoxicity (ADCC) in a target expression-specific manner (110). Alternatively, mAbs can serve as vectors to deliver cytotoxic cargo (drugs, radionuclides, or toxins) to cancer cells in an antigen-specific manner (111). MAbs have been successfully developed against the α-domain of MUC4 however, this domain can be putatively shed from the surface of the cell thus rendering it a feckless target (112, 113). We propose to target the MUC4β-domain, a growth factor-like subunit responsible for intra-cellular signaling that remains attached to the cell surface (8). Here we demonstrate the therapeutic potential of MUC4β antibodies and study their applications for inhibiting
MUC4-mediated cell-based functions, determining the mechanism of action for the antibody by identifying how the MUC4β epitope contributes to MUC4 oncogenic function.

3.2 Background and Rationale

MUC4 has been found to interact with, and stabilize, EGFR family members, and to activate downstream PI3K/Akt, MAPK/ERK, and Fak/Src signaling pathways that result in increased cell proliferation, survival, invasion/metastasis and chemotherapeutic resistance (19, 21, 24, 25, 31, 107, 108, 114, 115). In addition, MUC4 has been shown to modulate cancer cell interaction with various components of the extracellular matrix (ECM) for enhanced metastasis (25, 114). These studies suggest that MUC4 in metastasizing cells may be maintaining integrin-mediated downstream signaling, while at the same time modulating the cells’ interaction with the ECM (25). By targeting the MUC4β portion of MUC4, which remains attached to the cell surface, we hypothesize we will be able to disrupt MUC4 functions of growth and migration in MUC4-expressing cancer cells. An antibody-based approach offers the unique opportunity to specifically target MUC4, while lowering off-target toxicity due to its exclusive expression in PC cells. Our mAbs against the MUC4β subunit have been developed using mouse hybridoma technology. A library of mAbs was cloned and lead candidates were characterized for specificity. These mAbs were tested in a series of in vitro functional assays to determine if any of them are capable of disruption of MUC4 functions.

Previous studies of MUC4 function will be used as a benchmark for assessing the results of the mAb functional experiments. There are currently no therapeutics which inhibit MUC4 function however, these mAbs have the potential to act as MUC4 inhibitors that can be evaluated alone or in combination with other therapeutics.
3.2.1 Expression and purification of recombinant MUC4β protein.

The schematic of the cloning strategy used in the construction of the pET28a-MUC4β expression vector (plasmid) is mentioned in Fig 3.1A. The recombinant MUC4β protein was efficiently expressed using the pET28 vector in E. coli, as shown by the selective expression of a 72 kDa protein upon IPTG treatment (Fig.3.1B). The observed molecular mass is in agreement with the predicted size of ~80 kDa for MUC4β, derived from gene sequence analysis. However, the overexpression resulted in the formation of inclusion bodies as the protein was predominantly found in the insoluble pellet after cell-lysis and very little protein could be recovered from soluble fraction. The protein was recovered from inclusion bodies by solubilizing the pellet in a buffer containing 6M Urea, 0.5% CHAPS and 2 mM β-mercapoethanol and passing through a Ni-NTA column. The purified protein was refolded back into PBS by slow, gradual removal of urea by stepwise dialysis at 4°C. It was further dialyzed into endotoxin-free water and passed through an endotoxin removal column. The purified protein was finally assessed through Coomassie staining and western blotting with anti-histidine antibody (Fig. 3.1C).
Figure 3.1 Expression and purification of recombinant MUC4β protein

(A) The bacterial expression vector pET28a was created to encode for the MUC4β subunit of the MUC4 glycoprotein. (B) The expression vector was transformed into *E. coli* and the protein was induced by different concentrations of IPTG treatment. The resulting protein was purified on a Ni-NTA column. (C) A Coomassie stain was performed to verify the molecular weight and purity of the protein.
**Generation of mAbs reactive to MUC4β domain**

BALB/c mice were immunized with intra-peritoneal injection of 20 µg of purified MUC4β protein along with Freund’s adjuvant every 3-4 weeks. The immune response was frequently monitored by measuring antibody levels in the serum by ELISA (Fig 3.2). Once a sufficient response was obtained (3-4 boosters), mice were sacrificed, splenocytes were isolated and fused with P3X63Ag8.653 myeloma cell line. The fused hybridomas were plated into 96 well plates and first HAT selection was given on day 1. On day 4, fused colonies could be observed by microscopic examination. On day 5, 2nd HAT selection was given. After a further 3 more days, supernatants from each well was collected and tested for antibody binding activity by ELISA on MUC4β coated wells and positive hybrids were isolated and cloned. To determine specificity, the positive clones were again checked by ELISA using both MUC4β coated plates and an irrelevant his-tagged purified protein (MUC16 carboxy terminus). Those reacting to the irrelevant protein were discarded (Table 3.1). Thirty-four hybridomas were positive in the first screen that selectively reacted with MUC4β protein. Out of these, eight stable secreting hybridomas were finally isolated. (Fig 3.3).
Figure 3.2 Titer determination for antisera generated against MUC4β from different Balb/C mice

Antisera was collected 4-5 days after 1st booster with MUC4 β protein and checked at different dilutions from 1:100 to 1:100,000 on recombinant MUC4β protein in ELISA.
### Table 3.1 Relative reactivities of anti-MUC4 mAbs in the first round of screening

Third and fourth column indicate results from ELISA measuring the relative reactivities of anti-MUC4β mAbs with immobilized MUC4β and MUC16 carboxy terminus recombinant proteins.

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<th>Sr. No.</th>
<th>Hybrid well</th>
<th>MUC4β A&lt;sub&gt;280&lt;/sub&gt;</th>
<th>MUC16CTD A&lt;sub&gt;280&lt;/sub&gt;</th>
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Figure 3.3 Reactivity of anti-MUC4β mAb supernatants in ELISA

Different anti-MUC4β hybridoma supernatants were screened for their ability to bind both recombinant MUC4β protein as well as endogenous forms of MUC4 in COLO357 cell line lysate.
Figure 3.4 Reactivity of MUC4β protein with anti-MUC4 β mAbs

Left Panel. Coomasie stained gel of uninduced (UN) and IPTG induced (IN) E. coli extracts transformed with MUC4β plasmid and purified MUC4β protein. Right Panel. The same samples were transferred to PVDF membranes and probed with the indicated mAbs (1 µg/mL). Signal was detected with goat anti-mouse IgG HRP.
The antibodies were tested by western blot on MUC4β expressing *E. coli* lysates and purified MUC4β protein. All the tested anti-MUC4β mAbs specifically reacted with IPTG induced *E. coli* extracts and purified MUC4β but did not bind to uninduced *E.coli* extract thus demonstrating that these mAbs are reactive with MUC4β protein (**Fig 3.4**). Two of the mAbs 6E8 and E9 were also reactive by western blots on human MUC4 expressing lysates and were taken forward for further characterization (**Fig. 3.4**). The reactivity of anti-MUC4 mAbs was tested in western blot analysis against lysates of MUC4-expressing pancreatic cancer cell lines in 2% agarose gel electrophoresis system, which is a traditional way for observing very high molecular weight proteins that do not normally enter the conventional SDS PAGE gel system. MAbs 6E8 and E9 recognized high molecular weight protein bands in the lysates of the MUC4 positive cells (**Fig. 3.5**) and the reactivity pattern was similar to that of anti-TR MAb 8G7. Each of the MUC4-positive cell lines exhibited a characteristically distinct band size that is consistent with our previous reports of VNTR polymorphisms in MUC4 (113). No reactivity was observed for any antibody with the lysate of the MUC4 negative cell line MIA PaCa-2.

Since MUC4 is expected to be cleaved at the GDPH cleavage site, it was desired to compare the reactivity of mAbs to different molecular weight forms of MUC4 as seen when MUC4-containing cellular lysates were run in 2% SDS agarose (for detecting full length high molecular weight form) and 10% SDS PAGE (for detecting cleaved low molecular weight MUC4β subunit). It was expected that owing to its very high molecular weight, the full-length forms would be stuck up at the stacking gel interface and would not interfere with detection of MUC4β subunit in 10% SDS PAGE. Both of the anti-MUC4β mAbs retained their ability to bind to cellular lysates transferred to PVDF membrane in both 2% SDS agarose and 10% SDS PAGE gel systems.
Figure 3.5 Relative reactivity of anti-MUC4 mAbs to endogenous forms of MUC4 in different pancreatic cancer cell lines

Western blotting analysis of protein lysates (80 µg) from 5 cell lines resolved either on (top) 2% agarose/SDS gels or (bottom) 10% SDS PAGE gels and passively transferred to the PVDF membrane and probed with the indicated mAbs. For the 2% SDS/agarose panel probed with 8G7, 20 µg lysates were loaded. The arrow indicates the interface of stacking and resolving gels.
Figure 3.6 Cleaved MUC4β is N-glycosylated

Lysates of NCI-H3122 cells which express human MUC4 (20µg each) were incubated in the presence or absence of PNGase F for 16 hours at 37 °C. SDS sample buffer was added to each tube and the samples were electrophoresed on SDS PAGE. After transfer of the proteins to the PVDF membrane the membrane was incubated with the indicated antibodies. 20 ng of recombinant MUC4β (rMUC4β) was loaded in the 4th lane.
As expected, anti-MUC4β mAbs recognized the full-length MUC4 completely mirroring 8G7 reactivity on 2% SDS agarose gel western blotting even though the intensity of staining was lower as compared to reactivity shown by mAb 8G7 (Fig. 3.5 upper panel). Both anti-MUC4β mAbs detected a ~180 kDa band selectively in MUC4 expressing lysates as seen in 10% SDS PAGE western blotting whereas mAb 8G7 reactivity was limited to the stacking gel interface (Fig. 3.5 lower panel). The intensity of the 180 kDa band was highest in NCI-H3122 (concomitant with its high expression in 2% SDS agarose western blotting) and intermediate in T3M4 and low in CD18/HPAF. No binding was observed in MIA PaCa-2 and AsPC-1 lysates. Low level recognition of low molecular weight bands (at ~100 kDa and 40 kDa) was seen selectively in MUC4-expressing lysates. However, the latter observation was not reproducible between experiments and might represent non-specific degradation. Upon deglycosylation of lysates with PNGase F, the ~180 kDa band shifted to ~90 kDa, the putative molecular weight of MUC4β subunit (Fig 3.6).

Previous reports have indicated formation of heterodimers between the MUC4α and MUC4β subunit (15). We tested this hypothesis by immunoprecipitation. A MUC4/X construct (entire MUC4 sequence but lacks the tandem repeat (TR) domain), with FLAG-tag at the N-terminus and hemagglutinin (HA-tag) at the C-terminus, was transfected into the human pancreatic adenocarcinoma cell line MIA PaCa-2. Lysates from transfected cells were immunoprecipitated with the anti-MUC4β antibodies. Antibody-antigen complexes were resolved on SDS-PAGE gels under reducing conditions and were immunoblotted with either anti-FLAG or anti-HA antibody. This assay showed that mAbs 8D9, 8C6 and 7G5 only weakly immunoprecipitated MUC4/X protein showing them to be low affinity binders to human MUC4. MAb 3B2 was of IgM isotype and was not selected for further analysis. Three hybridoma clones (6E8 (IgG2b), E9 (IgG2b) and 3B4 (IgA))
showed strong immunoprecipitation of MUC4/X protein from detergent-extracted lysates (Fig. 3.7).

MUC4/X MIA PaCa-2 cells were grown to 80-90% confluency in 145-mm culture dishes. Triton X-100 lysates were prepared and divided into 200 µL (200 µg) fractions. One µg of individual mAbs was added and allowed to bind at 4 °C for 12 h. The immune complexes were collected with 20 µL of protein A/G agarose beads. Rabbit anti-mouse IgA (2 µg) was also added in case of mAbs 3B4 and control IgA to improve the binding of these mAbs to the beads. The beads were then washed 3 times with cold PBS containing 0.5% Triton X-100 and immune complexes were eluted by boiling in reducing sample buffer. The eluates were separated on an SDS-12% polyacrylamide gel and transferred to nitrocellulose membrane. Signal was detected by probing the membranes with anti-FLAG mAb followed by goat anti-mouse IgG-HRP.
Detergent-extracted lysates from NCI-H3122 cells were immunoprecipitated by mAbs against MUC4α subunit (anti-TR mAb 8G7, anti-N terminal subunit mAbs 2175 and 2214) and anti-MUC4β mAbs (6E8 and E9). The immunoprecipitated material was simultaneously electrophoresed on 2% SDS agarose (for full length MUC4) and 10% SDS PAGE (for cleaved MUC4) and probed either with mAbs 8G7 or 6E8 (Fig. 3.8). Hypothetically, the presence of cleaved 180 kDa band in the immunoprecipitates from anti-MUC4α mAbs would unequivocally prove active heterodimer formation. Such a strategy has been successful in identifying heterodimerization in the cases of MUC1 and MUC16. Both anti-MUC4α (8G7, 2175, 2214) and anti-MUC4β (6E8, E9) mAbs immunoprecipitated full length MUC4. Interestingly, both anti-MUC4β mAbs preferentially precipitated lower molecular weight fragments as compared to the high molecular weight protein that was preferentially precipitated by anti-MUC4α mAbs in 2% SDS-agarose western blotting. MAb 8G7 expectedly showed the strongest ability to precipitate its antigen owing to its binding to multiple repeated epitopes. The pattern of immunoprecipitation shown by mAbs 2175 and 2214 was the same as has been shown before. When mAb 6E8 was used as a probing agent for 2% SDS-agarose immunoprecipitates, the pattern was the same as that of mAb 8G7 except that the intensity of staining was lower. This showed that material resolved in 2% SDS agarose was intact, full-length MUC4 harboring both MUC4α and MUC4β epitopes. When the immunoprecipitates were simultaneously run on 10% SDS PAGE all the mAb 8G7-reactive material was stuck at the stacking gel interface and did not separate in the resolving gel section. In stark contrast, mAb 6E8-reactive material resolved as a ~180 kDa band that was immunoprecipitated by anti-MUC4β mAbs (6E8 and E9) but not by anti-MUC4α mAbs (8G7, 2175 and 2214). This was despite the intense immunoprecipitation of full-length, intact MUC4 by 8G7. This reveals that heterodimerization between subunits might be taking place at low levels. Interestingly, mAb 6E8 recognized a lower molecular
weight band in 8G7 immunoprecipitate (~150 kDa). This band was not present in any of the other immunoprecipitates. The exact reasons for these observations are presently unclear.

Figure 3.8 Relative abilities of anti-MUC4β mAbs to immunoprecipitate full-length MUC4 and cleaved/variant smaller forms

Detergent extracted lysates from NCI-H3122 cells were immunoprecipitated with indicated antibodies and run simultaneously on 2% SDS agarose gels (A) and 10% SDS PAGE gels (B) and probed with the indicated mAbs. The arrow indicates the interface of stacking and resolving gels. The two bands at 50 and 25 kDa are the immunoglobulin heavy and light chains respectively.
Indirect immunofluorescence by flow cytometry was used to compare the binding properties of selected anti-MUC4β mAbs on MUC4-expressing cell lines keeping anti-TR mAb 8G7 as a control (Fig. 3.9). The panel of cell line chosen includes cells which express MUC4 on their surface to varying degrees. The lung cancer cell line NCI-H3122 expresses a high level of MUC4 on the cell surface, while the pancreatic cell lines CD18/HPAF and T3M4 were chosen as mid-level expression cells, and the pancreatic cancer AsPC-1 expresses a low level of MUC4. The two anti-MUC4β mAbs (6E8 and E9) and anti-tandem repeat (TR) mAb 8G7 bound strongly to NCI-H3122 cells but did not bind to AsPC-1 cells that express low levels of MUC4. However, mAb 8G7 showed heterogeneous staining on H3122 cells with a small fraction of cells displaying bright staining while anti-MUC4β mAbs displayed uniform staining on the entire cell population. Interestingly, mAb 8G7 staining was low or absent on all the pancreatic cancer cell lines as per the conditions tested. In contrast, mAb 6E8 stained all the MUC4-expressing pancreatic cancer cells with highest staining seen in CD18/HPAF followed by the T3M4 cell line. MAb E9 stained the NCI-H3122 cell line brightly but had weak staining on CD18/HPAF and T3M4 cells.
Figure 3.9 Fluorescence-activated cell sorter histograms of human tumor cells binding anti-MUC4 mAbs

The antibodies were first allowed to react with the cell surface at 4°C. After 3 wash steps, bound antibody was labeled by addition of Alexa Fluor-488 labeled goat anti-mouse IgG. Forward and side scatter thresholds were set up to exclude debris. Propidium iodide staining was used to exclude dead cells.
These results were further confirmed by directly observing the stained cells under a confocal microscope (Fig. 3.10). The binding of the 8G7 anti-TR antibody was only observed in the highest MUC4-expressing cell line NCI-H3122. While the staining of anti-MUC4β mAb 6E8 was observed across the cell lines NCI-H3122, CD18/HPAF, and T3M4. The other anti-MUC4β mAb E9 has observable binding in NCI-H3122 but, low binding in CD18/HPAF, and T3M4. There was no observable binding of any of the mAbs in the AsPC-1 cell line.

Further analysis in confocal microscopy on live and fixed NCI-H3122 cells showed that anti-MUC4β mAb reacted in a similar manner as compared to mAb 8G7 except that the staining intensity was lower (Fig 3.11). The reactivity was found to be on both membrane and cytoplasm. Internalization of anti-MUC4 mAbs was tested on NCI-H3122 and AsPC-1 cells by incubating anti-MUC4 mAbs for different time intervals at 37 °C (Fig. 3.12 and 3.13). Another set of cells was treated to acid stripping after the antibody incubation to shave off the surface bound antibody to measure internalized antibody. Using these conditions, we could observe slow internalization of anti-MUC4 mAbs with time starting from 3 h and peaking at 16 h. Interestingly, mAb 8G7 demonstrated capping of surface MUC4 at 1 h post incubation as has been demonstrated before (25).
Figure 3.10 Confocal microscopy of live human tumor cells stained in solution

The antibodies were mixed with cells at 4°C for 60 min in 100µl FACS buffer. After 3 wash steps, bound antibody was labeled by addition of Alexa Fluor-488 labeled goat anti-mouse IgG. After the final wash, cell pellets were fixed with 2% paraformaldehyde in PBS and stored at 4°C. A drop of cell suspension was mixed with a drop of DAPI mounting medium on a glass slide and a coverslip was put on the mixture and sealed. The slides were viewed on a ZEISS LSM 810 microscope.
Cells were grown on sterilized coverslips until a 70-80% confluency was reached. The cells were fixed with 4% paraformaldehyde in PBS and permeabilized with 0.1% Triton X-100 PBS. Cells were then blocked with 10% goat serum to reduce non-specific binding. Cell lines were incubated with mAbs at a concentration of 10 μg/mL (for 8G7, 1 μg/mL) for 2 hours at RT. Coverslips were washed 4 times with PBST to remove any unbound antibody. Cells were incubated with goat anti-mouse IgG Alexa Fluor-488 2° antibody. Coverslips were mounted using DAPI mounting medium and analyzed by confocal microscopy.
Internalization of MUC4 mAbs was performed by incubating the cells with the antibody at 4 °C to allow for cell surface binding, but not internalization. Cells were then moved to a 37 °C incubator and allowed to incubate with the antibody for 1, 3, 4, 8, and 16-h intervals to determine the rate of internalization. Cells were processed by acid stripping (Lower panel) to remove any antibody bound to the surface of the membrane so, only antibody that had internalized would remain.

Figure 3.12 Internalization of anti-MUC4 mAbs into NCI-H3122 cells.
Internalization of anti-MUC4 mAbs into AsPC-1 cells.

Internalization of MUC4 mAbs was performed by incubating the cells with the antibody at 4 °C to allow for cell surface binding, but not internalization. Cells were then moved to a 37 °C incubator and allowed to incubate with the antibody for 1, 3, 4, 8, and 16-h intervals to determine the rate of internalization. Cells were processed by acid stripping (Lower panel) to remove any antibody bound to the surface of the membrane so, only antibody that had internalized would remain.
To further study the ability of the anti-MUC4β mAbs to binding to MUC4β protein a panel of binding affinity experiments were performed. An antibody binding curve was established in two separate systems which either expressed the native form of the protein or the denatured form of the protein. The binding was specific and saturable at approximately 10 nM concentrations when native MUC4 expressing cells were taken as binding target (Fig. 3.14B). In the case of purified protein coated on ELISA wells, saturable binding was achieved at approximately 1 nM concentrations (Fig. 3.14A). Surface plasmon resonance (SPR) was used to analyze the kinetics of binding of selected mAbs to MUC4β (Fig. 3.15). SPR data demonstrated that mAb 6E8 and E9 had high affinities of KD 25.2 nM and 5.1 nM respectively.

Further binding experiments were performed in order to determine where the antibodies are specifically binding. An epitope mapping experiment was performed using various FLAG tagged deletion constructs of MUC4β domain. Each of the constructs tested was progressively truncated, removing a domain with each iteration. The constructs were transfected in the MIA PaCa-2 cells and detergent extracted lysates were analyzed in a Western blot format. The analysis of each of the clones, 6E8 and E9, indicated that the full structure of MUC4β subunit was required for recognition by anti-MUC4β mAbs that is encompassed by the AMOP and vWD deletion construct. None of the other deletion constructs were reactive with anti-MUC4β mAbs but were fully recognized by the anti-FLAG mAb (Fig. 3.11).
Figure 3.14 Saturable binding of anti-MUC4β mAbs to MUC4β protein either as a recombinant protein coated on ELISA wells (A) or as native structure present on the surface of the NCI-H3122 cell line (B)

(A) ELISA was used for measuring the dose dependent reactivity of anti-MUC4β mAbs with recombinant MUC4β protein coated wells. 1 µg/mL MUC4β recombinant protein was coated in ELISA wells. After blocking dilutions of mAbs were added as indicated. Specifically, bound antibody was detected with goat anti-mouse IgG-HRP followed by TMB substrate. (B) NCI-H3122 cells were mixed with various dilutions of mAbs and incubated on ice for 60 minutes. After 3 wash steps, bound antibody was labeled by addition of Alexa Fluor-488 labeled goat anti-mouse IgG. Forward and side scatter thresholds were set up to exclude debris. Propidium iodide staining was used to exclude dead cells.
Figure 3.15 SPR to demonstrate the binding affinity of anti-MUC4β mAbs

Determination of binding affinity of the anti-MUC4β mAbs to the MUC4β protein by SPR. Antibodies were coupled to a CMD sensor chip using EDC/NHS chemistry. The MUC4β protein analyte was passed over the chip at a concentration range of 1000-0.1 ng/mL. To determine the binding affinity a wash step with an acidic 10mM glycine-HCl solution was performed to remove any unbound analyte. Biologic Scrubber software was used to determine the on/off rates of ligand-analyte binding
Figure 3.16 Epitope mapping using deletion constructs transfected in MIA PaCa-2 cells

FLAG-tagged MUC4 deletion constructs were transfected into MIA PaCa-2 cells. Lysates (60 µg) were separated on an SDS-12% polyacrylamide gel and transferred to nitrocellulose membrane. Signal was detected by probing the membranes with indicated mAbs followed by goat anti-mouse IgG-HRP. Extract of NCI-H3122 (that endogenously expresses MUC4) served as the positive control.
The final piece of characterization involved the use of human pancreatic cancer sections. A critical piece of knowledge in the pursuit of using the anti-MUC4β mAbs as a therapeutic is their ability to specifically target pancreatic cancer cells while eschewing normal pancreatic cells. To that end the anti-MUC4β mAbs were further tested for their ability to bind formalin-fixed pancreatic cancer sections by immunohistochemistry (Fig. 3.17 and Fig. 3.18). MAb 8G7 that has been extensively used previously in multiple immunohistochemistry applications was used as a control (17, 116-120). Both anti-MUC4β mAbs reacted faintly with PDAC sections, probably indicating that the epitopes are sensitive to formalin fixation. However, the reactivity was seen in the same regions where mAb 8G7 was positive. Normal pancreas sections were uniformly negative for all the mAbs tested. Comparative staining revealed that anti-MUC4β mAbs mainly stained the intra-luminal and membrane areas of PDAC cells while mAb 8G7 stained the cytoplasm of PDAC cells.
Figure 3.17 Immunohistochemical reactivity of anti-MUC4 mAbs on paraffin embedded pancreatic cancer sections

Peroxidase immunohistochemical results are shown from the same area of the same case of PDAC. Formaldehyde-fixed, paraffin-embedded tissue sections were treated with 5 µg/mL of anti-MUC4 mAbs and bound antibody was detected with HRP-linked secondary antibody and DAB Vector universal kit (magnification, 200X). One positive section and one negative section are shown. Adjoining normal pancreas sections are shown in the lower images.
Figure 3.18 Comparative immunohistochemistry on human PDAC sections stained with MUC4 mAbs

Peroxidase immunohistochemical results are shown from the same area of the same case of PDAC. Formaldehyde-fixed, paraffin-embedded tissue sections were treated with 5 µg/mL of anti-MUC4 mAbs and bound antibody was detected with HRP-linked secondary antibody and DAB Vector universal kit (magnification, 200X). MUC4β mAbs stain the luminal apical membranes of cancer cells whereas 8G7 stains mainly the cytoplasm.
3.2.2 Evaluating the therapeutic efficacy of anti-MUC4 mAbs.

Several *in vitro* and *in vivo* studies have demonstrated the association of MUC4 with oncogenic potential, including cell proliferation, survival, invasion/metastasis, and chemotherapeutic resistance in pancreatic cancer and other malignancies (10, 24, 28, 31, 32). With this in mind, a series of *in vitro* experiments were performed to determine the therapeutic potential of these mAbs. Determination of functional effects of the anti-MUC4 antibodies began with determining their impacts on cellular growth. An anti-KLH antibody (K2G6) was used as a negative control. Growth inhibition was tested across three MUC4-expressing cell lines. Two of the cell lines tested, CD18/HPAF and T3M4, endogenously express MUC4 while the MiniMUC4 MIA PaCa-2 is based on a MUC4-negative cell line that has been engineered to express MUC4. The MIA PaCa-2 Psectag vector control cell line was tested as a negative control for MUC4 expression. Cell growth was measured indirectly by measuring the metabolic activity of the cells through a tetrazolium dye MTT. Colorimetric analysis correlating with the presence of metabolically active cells was measured on a spectrophotometer. The only cell line in which a statistically significant inhibition of growth was measured was the CD18/HPAF cell line. Growth of the cells was inhibited to a statistically significant degree by the anti-TR mAb 8G7 (26%) and both of the anti-MUC4β mAbs 6E8 (37%) and E9 (48%), compared to the IgG isotype control antibody K2G6 (1.4%) (Fig. 3.19). While only partial growth inhibitions were observed in both the T3M4 and MiniMUC4 MIA PaCa-2 cell line by the anti-MUC4β mAb E9. These differences in growth reduction can be attributed to varying MUC4 expression across the different cell lines.
Figure 3.19 Growth measurements of various pancreatic cancer cell lines when treated with anti-MUC4 mAbs

Pancreatic cancer cells were plated on 96-well plates and allowed to incubate for 24 hours. Following the incubation period, a 10 µg/mL antibody solution was added to the cells. Cells were allowed to proliferate in the presence of the antibodies and measured for the degree of growth using a tetrazolium dye reduction. *P < 0.05.
To determine the mechanism of growth inhibition, the phosphatidyl serine residues on the cell surface were quantified as a measurement of apoptosis. The CD18/HPAF cell line was treated with the anti-MUC4 mAbs or negative control antibody and then screened by flow cytometry for cell surface expression of phosphatidyl serine by a fluorescently tagged Annexin V (Fig. 3.20). No significant difference in the population of apoptotic cells was observed between the cells treated with the anti-KLH control antibody and the cells treated with anti-MUC4 mAbs. This indicates growth inhibition is occurring through another mechanism.
Figure 3.20 Analysis of apoptotic and necrotic indices of the CD18/HPAF cell line following antibody treatment

The cell line CD18/HPAF was treated with a 10 µg/mL antibody solution. Following incubation with the antibodies the cells were harvested and a fluorotagged Annexin V was used to bind to any phosphatidyl serine residues present on the cell surface. Propidium iodide was used to identify the cell population that had undergone cell death through necrosis. Cells were analyzed by flow cytometry to quantify apoptotic and necrotic populations.
Another MUC4-associated function that can be tested in vitro is cellular migration. Previous data has shown that when MUC4 expression is knocked down, the cells have decreased migration compared to MUC4-expressing cells (25). A transwell migration chamber was used to test the ability of anti-MUC4 mAbs to inhibit migration across a barrier towards a stimulus. Cells were incubated with a 10 µg/mL solution of antibody in serum free media on the top chamber of the well, while a 10% FBS solution was added to the bottom chamber. After a 24 h incubation period, the membrane of the migration chamber was stained for cells that had migrated across the barrier. The membranes were examined using bright-field microscopy and ten random fields of view were chosen to take an average of the number of migratory cells. The cell lines used to test migration were MiniMUC4 MIA PaCa-2 and the Psectag vector control. Additionally, a mouse pancreatic cancer cell line KCT3248 transfected with a MiniMUC4 construct and the Psectag vector control (Fig 3.21). The KCT3248 mouse cell line that can be used in a syngeneic mouse model and is directly applicable to translating the experiment into an in vivo model. The results migration assay show that both of the MiniMUC4-transfected cell lines displayed a significant reduction in the number of migratory cells when treated with the anti-MUC4 mAbs while the vector control cell lines did not have any significant difference. The MiniMUC4 MIA PaCa-2 cell line had a significant reduction in the migratory cell count when treated with the anti-MUC4ß mAbs 6E8 and E9, as well as the anti-TR mAb 8G7. The KCT3248 MiniMUC4 cell line had significant reductions in the number of migratory cells when treated with the mAbs E9 and 8G7.
Figure 3.21 Effect of anti-MUC4 mAbs on cell motility in pancreatic cancer cell lines

Cells in a 10 µg/mL antibody solution were added to the top chamber of a migration chamber. Media containing 10% FBS was added to the bottom chamber as a chemoattractant. The cells that did not migrate to the bottom chamber were removed, while cells that migrated were stained and observed under bright-field microscopy. The number of cells that migrated through the membrane was determined by averaging 10 random fields of view. *P < 0.05.
The motility of the pancreatic cancer cell line, T3M4 was further studied in a wound healing assay. The T3M4 cell line was chosen for its surface expression of MUC4 as well as its ability to form a monolayer of cells which was necessary for the successful application of this assay. The cells were grown until they had reached a confluent monolayer and a scratch was made in order to measure the rate at which the cells can grow and migrate across a barrier (Fig 3.22). While there was a change in the percentage of wound closure, in the mAb treatment groups this was not to a statistically significant degree.

The expression of MUC4 is associated with resistance to the first-line pancreatic cancer therapeutic gemcitabine. When MUC4 expression is knocked down in cell lines endogenously expressing MUC4 they have an increased response to gemcitabine (31, 32). The cell line CD18/HPAF was used to test the ability of the MUC4 antibodies to affect MUC4-related gemcitabine resistance (Fig. 3.23). A 10 µg/mL antibody solution was added to cells in combination with a 10 nM gemcitabine solution. Cell growth was indirectly measured through cellular metabolic activity through the use of MTT. The addition of the combination of anti-MUC4β mAbs and gemcitabine resulted in greater inhibition than either of the treatments alone.
Cells were grown in a monolayer and a wound was administered by scratching a T3M4 culture monolayer, followed by treatment with MUC4β mAbs. Images of the wound healing assay at 0 and 24-h timepoints are shown above. Quantitation of the percentage of wound closure is shown in the bar graph below. While the mAb E9 had the highest degree of inhibition of wound closure it was not statistically significant.
Figure 3.23 Combination treatment of CD18/HPAF cells with anti-MUC4β mAbs and gemcitabine

CD18/HPAF cells were plated on 96-well plates and allowed to incubate for 24 hours. Following the incubation period, a 10 µg/mL antibody solution, a 10 mM gemcitabine solution, or a combination of both was added to the cells. Cells were allowed to proliferate and measured for the degree of growth using a tetrazolium dye reduction.
Overall, the data presented in this thesis demonstrates that the anti-MUC4β mAbs 6E8 and E9 are specifically binding to MUC4. Additionally, mAbs 6E8 and E9 have demonstrated the detection of full-length MUC4 and the MUC4β subunit in both native and denatured conformations. Although the majority of MUC4 remains intact immunoprecipitation data has definitively proven the cleavage of the MUC4α and MUC4β subunit. Which allows these antibodies to be further used not only as a therapeutic targeting or diagnostic agent but, also a research tool for further studying the MUC4β subunit. While the internalization of the mAb 6E8 demonstrates the potential of this mAb to be used as an antibody drug conjugate. The anti-MUC4β antibodies bind with high affinity to their target, which has been measured in the nanomolar range. Epitope mapping has narrowed the binding region of the anti-MUC4β mAbs to the vWD domain of MUC4β. These antibodies have been further used to specifically stain MUC4 in human pancreatic cancer tissue sections while eschewing normal pancreatic tissue.

*In vitro* analysis of the mAbs 8G7, 6E8, and E9 demonstrates their utility in ameliorating MUC4-related functions, specifically cell proliferation and motility. Although, these mAbs were not inducing apoptosis in their target cell lines they were slowing the cellular growth to a significant degree compared to an IgG isotype control. This mechanism of slowing cellular growth still requires further study. The pathways PI3K/Akt, MAPK/ERK, and Fak/Src which have been effected by the knockdown of MUC4 would be a starting point. Furthermore, the anti-MUC4β mAbs 6E8 and E9 have demonstrated an additive effect on growth inhibition in the presence of gemcitabine. Further experimentation can be performed to determine if a synergistic effect can be attained using the right combination of concentrations. Overall, the results presented here warrant further study of the mAbs in an *in vivo* system.
References


Chapter 4: Discussion, Conclusion, and Future Directions
MUC4, a transmembrane mucin, has roles in both normal physiology and pathological conditions, including pancreatic, breast, ovarian, lung, cervical, and head and neck cancers (8, 10-12, 20, 103, 121, 122). Of all of the aforementioned cancers, MUC4 has been the most studied in pancreatic cancer models, and it has the potential to be used as a novel therapeutic target. Clinically, MUC4 expression has been found in precancerous PanIN lesions with expression increasing until a carcinoma stage has been reached (17). Expression of MUC4 has also been found across all stages of pancreatic cancer. Furthermore, patients exhibiting high levels of MUC4 have been associated with a poor prognosis (26).

A promising treatment modality for specifically targeting MUC4 is the monoclonal antibody. While several antibodies against the MUC4α domain have been characterized, prior to this study no antibodies had been developed against the MUC4β-subunit (123, 124). The MUC4β-subunit is an attractive possible target for pancreatic cancer due to its proximity to the cell surface where binding will not be hindered by cleavage events, and the potential to disrupt functions of the growth factor domains and domains responsible for adhesion located on the β-subunit (9). To this end, we generated additional mAbs against MUC4 that target the β subunit domain that lies C-terminal to the proposed cleavage site. The purpose of this was multifold. The anti-MUC4β antibodies may be used as tools to study MUC4 cleavage events and MUC4 splice forms that lack TR epitopes, and as targeted therapies that are less impacted by MUC4 glycosylation and MUC4 cleavage than previously generated anti-MUC4 antibodies.

The mice were first evaluated whether they respond to MUC4β immunization. It was found that all mice responded vigorously to the antigen as evaluated by ELISA on sera collected 4-5 days after immunization. However, the titer of the sera varied from mouse to mouse. The first fusion that was performed on the mouse exhibiting the best titer after 3-
4 boosters. However, the fusion was a failure, and we could not obtain any antigen reactive hybrids even though there was hybrid growth in every well. In the meantime, second mouse was given a booster dose and a second fusion was performed. This time not only was there hybrid growth in every well, but more than 30-40% of the wells demonstrated very high reactivity as demonstrated by ELISA. More than 80% of the wells had a signal above background. This was because each well contained multiple hybridoma colonies as visualized by microscopy. Hence, the positive clones were quickly identified and cloned by limiting dilution before the quick growing non-producer the cells overpower the slow growing secretory cells. By this method, 34 hybridomas were isolated that selectively reacted with MUC4β and not with an irrelevant his tagged protein. Out of these, 7 hybridomas could be successfully cloned by limiting dilution method (Table 3.1). Other clones either stopped secreting or were overpowered by non-secretory clones. Out of these 7 clones,

The antibody clones 6E8 and E9 demonstrated strong reactivity in an ELISA against the immunizing protein, and in addition these mAbs strongly reacted with native human MUC4. These mAbs were also able to bind to denatured or modified MUC4, as seen by reactivity in western blots, immunohistochemistry, and fixed cell immunofluorescence thus validating their efficacy across multiple assays. However, a complete and intact MUC4β sequence was essential for binding (Figure 3.11). The affinities of the new MUC4β mAbs were evaluated by SPR and found to be high, with KD values of 5.1 nM for mAb 6E8 and 25.2 nM for mAb E9 (Figure 3.10). Both of these anti-MUC4β mAbs developed by our lab recognize the cell-surface associated MUC4 as seen by flow cytometry analysis on live MUC4-expressing cells (Figure 3.7). These binding studies indicate the existence of multiple variables, namely the avidities of each antibody, the epitopes recognized, physiochemical behavior of MUC4 and the isotype of the antibodies. The latter is of lesser
concern, since the fluorescently labeled secondary antibody raised against heavy and light chains would be expected to cover all IgG isotypes. Furthermore, all antibodies exhibited negative binding activity on the low MUC4 expressing cell line AsPC-1. A clear difference in the binding was observed upon comparing binding of anti-MUC4TR mAb (8G7) and anti-MUC4β mAbs (6E8 and E9), most probably due to cleavage and glycosylation limiting binding of mAb 8G7 to native exposed epitopes. Nevertheless, mAb 8G7 could still bind to NCI-H3122 cells even though the binding was highly heterogeneous. The very high staining at the cell surface by mAb 8G7 demonstrates the binding to repeated epitopes (Figure 3.8). Differences between the binding of anti-MUC4β mAbs 6E8 and E9 were also seen in the live cell surface-binding experiments. The mAb 6E8 bound strongly to all MUC4-expressing cell lines, while mAb E9 bound strongly to NCI-H3122 cells but weakly to CD18/HPAF and T3M4 cells. One conclusion could be that the antibodies react to different antigenic determinants. Also, it should be noted that apart from cellular binding assays, the binding behavior of mAbs 6E8 and E9 in other assays (like immunoprecipitation and western blot) was almost exactly identical. These observations also hint to different localization behaviors of MUC4 in various cell lines. In NCI-H3122 cells, MUC4 may be acting as a true membrane associated protein whereas in other cell lines (CD18/HPAF and T3M4), MUC4 may be secretory in nature or might have a transient cell surface localization prior to shedding. Since mAbs 8G7 and anti-MUC4β bind at non-overlapping sites, these mAbs can potentially be used together in experiments such as pair labeled biodistribution.

In western blotting assays, mAbs 6E8 and E9 recognized the full-length MUC4 with a high molecular weight band size similar to that recognized by anti-TR MAb 8G7 (Figure 3.5). Like 8G7, the reactivity of these mAbs fully mirrored the allelic variation in the sizes of MUC4 as seen in various cell lines. The intensity of bands was expectedly highest for
8G7 since it can bind multiple times to a single molecule, while 6E8 and E9 can bind only once or twice (for divalent binding). MUC4 contains a characteristic GDPH cleavage sequence that also occurs in other gel-forming and membrane-bound mucin members including: MUC2, MUC5AC, MUC5B and the rat orthologue of MUC4 (sialomucin complex) (125-128). Previous reports have indicated that MUC4 is cleaved at the GDPH site and after the cleavage the two subunits heterodimerize. To ascertain whether cleavage is taking place in the native MUC4 molecule, western blots were run simultaneously on 2% SDS agarose (for full-length MUC4) and on 10% SDS PAGE gels to detect the smaller cleaved fragments (Figure 3.5). In SDS-PAGE western blots, anti-TR mAb 8G7 did not detect any smaller fragments but only detected a high molecular weight smear that was caught at the stacking gel interface. MUC4β mAbs (6E8 and E9), in contrast, were able to detect a ~180 kDa fragment in MUC4-expressing cell lines with the highest expression seen in the NCI-H3122 lysate. No variation in the 180 kDa band size was observed between cell lines, as seen for the full-length MUC4, due to allelic variation. Interestingly, anti-MUC4β mAbs strongly recognized full-length MUC4 (as seen on 2% SDS-agarose western blot) but comparatively had weaker recognition of the ~180 kDa band on 10% SDS-PAGE western blot. Considering that transfer efficiency in 2% SDS-agarose gel system is much lower than wet electroblotting, this observation indicates that cleavage of MUC4 may be happening at low levels and most of MUC4 within the cell exists as a non-cleaved full-length state. The 180 kDa fragment migrated at approximately the 90 kDa position after being treated with PNGase F, almost matching the migration of non-glycosylated bacterially expressed MUC4β recombinant protein (Figure 3.6). This indicates that MUC4 is cleaved (possibly at the GDPH site) to generate a MUC4β fragment that is heavily N-glycosylated at asparagine residues. Such heavy N-glycosylation of cell surface MUC4 molecules has been shown before, but its exact relevance still needs to be deciphered (128). To determine whether the MUC4α and MUC4β subunit heterodimerize
after cleavage an immunoprecipitation assay was performed with all anti-MUC4 mAbs available in our laboratory (Figure 3.13). If cleavage and heterodimerization have occurred, the precipitates from anti-MUC4α mAbs should contain the cleaved MUC4β fragment. If heterodimerization has not occurred, then immunoprecipitates from anti-MUC4α mAbs should not contain the ~180 kDa cleaved MUC4β fragment. This is assuming that any pre-formed heterodimer would be separated by boiling in SDS and β-mercaptoethanol-containing sample buffer, as shown previously for other mucins such as MUC1 and MUC16. All the anti-MUC4 mAbs successfully immunoprecipitated full-length MUC4 as seen in 2% SDS agarose western blots done with mAbs 8G7 and 6E8. Anti-MUC4β mAbs preferentially immunoprecipitated lower molecular weight bands in contrast to high molecular weight bands precipitated by anti-MUC4α mAbs. These were still full length MUC4 since they were recognized by both mAbs 8G7 and 6E8. One explanation for these lower molecular weight fragments might be differentially glycosylated fragments that are more preferentially recognized by anti-MUC4β mAbs as compared to anti-MUC4α mAbs. In contrast to full-length MUC4, the cleaved MUC4β fragment (~180 kDa fragment seen on SDS PAGE western blot) was immunoprecipitated by anti-MUC4β mAbs but, not by anti-MUC4α mAbs. The anti-TR mAb 8G7 faintly immunoprecipitated a lower molecular weight band at ~150 kDa that was not apparently immunoprecipitated by other mAbs. This might represent some low levels of altered glycosylated MUC4β that associates with the MUC4α chain. It might also represent some nonspecific degradation of full-length MUC4 after it has been immunoprecipitated from detergent-extracted lysates. Hence our observations are in stark contrast to previous studies in which rat Muc4 was shown to be predominantly cleaved and exist as a heterodimer with α chain (127). One reason for the discrepancy could be different processing of MUC4 in humans as compared to rat since we have done our experiments on human cells. Our observations also demonstrate that human MUC4 may be processed in a different manner as compared to other mucins (such
as MUC1 and MUC16), hinting that it might be playing a unique role as compared to conventional mucins. It is interesting that MUC4 is the only membrane-bound mucin that lacks SEA domain (in stark contrast to MUC1 and MUC16 that harbor at least one SEA domain). This might possibly be one of the reasons for less efficient cleavage of the whole MUC4 backbone.

Confocal analysis indicated membranous and cytoplasmic staining in NCI-H3122 cells with all anti-MUC4β mAbs showing the same pattern as that of mAb 8G7, albeit with lower intensity (Figure 3.14). The anti-MUC4β mAbs 6E8 and E9 also seemed to work in immunohistochemistry on formalin-fixed pancreatic tumor sections giving reactivity similar to that of anti-TR mAb 8G7 (Figure 3.17 & 3.18). However, the intensity of staining was very low, possibly indicating the epitopes to be significantly modified upon formalin fixation. The results of the internalization experiments (Figure 3.12 & 3.13) demonstrate the mAb 6E8 has the potential to be used for conjugation to either small molecule drugs or radionuclides for targeted delivery.

In addition to the mAbs developed in this project being used as tools for the study of MUC4 they also have the potential to be used as novel therapeutics, particularly in the treatment of pancreatic cancer. The role of MUC4 in neoplastic transformation, enhanced motility, invasiveness, and drug resistance of cancer cells in vitro, and in tumorigenicity and metastasis in vivo has been conclusively established (19, 21, 24, 25, 31, 32, 129, 130). Clinically, MUC4 is undetectable in the normal or inflamed pancreas (pancreatitis) and its expression progressively increases during PC progression and its higher expression correlates with poor survival (11, 122, 131).

The anti-MUC4 mAbs were further tested for their efficacy at inhibiting oncogenic functions attributed to MUC4 expression. We began with testing the antibodies ability to inhibit proliferation. An inhibition of proliferation can occur through the induction of cell
death or the arrest of cell growth. Previously, when the MUC4 knock down cell line of CD18/HPAF (which has a >70% decrease in MUC4 expression), was tested for apoptosis, approximately 20% of the cell population experienced apoptosis compared to the 10% in the endogenous MUC4-expressing cells (25). The same cells analyzed for cell cycle phase exhibited a 25% decrease in the number of cells entering the S phase and a 33% increase in the number of cells at the G0/G1 phase. In our initial experiment, growth inhibition was measured indirectly through a tetrazolium dye, MTT, which distinguishes between metabolically active and inactive cells. The cell line CD18/HPAF was the only cell line for which a statistically significant difference was measured between the anti-MUC4 antibody treated group and the negative control antibody (Figure 3.19). Although the cell lines T3M4 and MiniMUC4 MIA PaCa-2 exhibited growth inhibition in the anti-MUC4 mAb treated groups, compared to the negative control antibody the results were not statistically significant (Figure 3.19).

To determine if the mechanism of growth inhibition was occurring through an induction of programmed cell death, the CD18/HPAF cell line was chosen for further testing. The results of this experiment did not establish a significant difference between apoptosis in the negative control group and the anti-MUC4 antibodies (Figure 3.20). This leads us to conclude that the diminished proliferation of cells when treated with MUC4 antibodies could be the result of cell cycle arrest, leading to fewer actively proliferating cells and thus affecting the overall number of metabolically active cells. Other mechanism, at play could include the phosphorylation status of MUC4-interacting partner Her2. In vitro data from MUC4 knockdown studies correlated a decrease in MUC4 expression with a decrease in phosphorylated Her2 (19, 24). The anti-MUC4 mAbs may be blocking the interaction between MUC4 and Her2 leading to a decrease in the phosphorylation of Her2 thus, affecting downstream signaling pathways that regulate cellular growth.
Since monoclonal antibodies have the potential to be a targeted therapy for pancreatic cancer, we next test the efficacy of the antibodies in combination with gemcitabine. MUC4 expression contributes to pancreatic cancer resistance to gemcitabine. A main mechanism of pancreatic cancer gemcitabine resistance is an altered apoptotic threshold (30). By stabilizing HER2 increasing phosphorylation of Erk, MUC4 expression increases phosphorylation of Bad protein, thereby suppressing its ability to translocate to the mitochondria. This leads to a decrease in cytochrome c release to the cytosol, which decreases intrinsic apoptosis (31). Additionally, a decrease in MUC4 expression has been correlated with an increase in the human Concentrative Nucleoside Transporter 1 (hCNT1). An additional mechanism has been proposed in which MUC4, by contributing the increased phosphorylation of Erk, decreases the expression of hCNT1 via the NF-κB pathway (32). Translational evidence of MUC4 contributing to gemcitabine resistance has been tested through the use of apicidin, a histone deacetylase inhibitor. Treatment of Capan-1 cells with apicidin resulted in a decrease in MUC4 expression and synergistic inhibition of growth when combined with gemcitabine (132).

To test our anti-MUC4β mAbs in combination with gemcitabine a concentration of gemcitabine which inhibits cellular growth by ~20% was chosen. Cells treated with gemcitabine alone had inhibited growth by 22.4%, while cells treated with either the anti-MUC4β mAb 6E8 or E9 were shown to have growth inhibition of 48.6% and 29.8%, respectively. The combination of gemcitabine with the anti-MUC4β 6E8 resulted in a 60.5% inhibition of growth, and the E9 combination resulted in a 52.3% inhibition of growth (Figure 3.21). While there is an increase in the growth inhibition through combination treatment we have not yet determined if this is occurring synergistically. By identifying the mechanism by which the anti-MUC4 mAbs are inhibiting MUC4-mediated growth we can
further determine whether this mechanism is influencing one of the predicted pathways of MUC4-mediated gemcitabine resistance.

Lastly, our panel of antibodies was tested for that ability to affect MUC4-mediated cell migration. MUC4 has been previously found to affect cell migration in vitro, as well as influence metastasis in vivo (24, 25). For this experiment we tested two separate cell lines both of which had been transfected with a MiniMUC4 construct. The MiniMUC4 MIA PaCa-2 cell line and the pSecTag negative control have been previously tested for differences in migration and found to have significantly different numbers of migratory cells (27). The KPC3248 cell line is a murine cell line that can be used for in vivo efficacy experiments. Both of the MiniMUC4-transfected cell lines demonstrated a significant change in the number of migratory cells when treated with the anti-MUC4β mAb E9 and the anti-TR mAb 8G7 (Figure 3.22). However, treatment with the anti-MUC4β mAb 6E8 demonstrated a significant change in migratory cells only for the MiniMUC4 MIA PaCa-2 cell line (Figure 3.22). Neither of the negative control pSecTag cell lines demonstrated any significant difference in the number of migratory cells when treated with any of the anti-MUC4 mAbs.

In conclusion, these mAbs are excellent reagents for studying the distribution of MUC4 on various cell types and deciphering information on MUC4 cleavage and tracking the fate of the MUC4β subunit after cleavage. The internalization and nuclear translocation of the MUC1 cytoplasmic tail has been previously studied and found to affect the expression of oncogenes (133). These mAbs have the ability to be used as tool in further MUC4 studying including potential nuclear translocation. These mAbs can also be used to study additional interacting partners of MUC4. Previous studies of MUC4 interacting partners have been performed exclusively with the mAb 8G7 (19, 21, 114). In the event of MUC4 cleavage, there may be interactions at the cell surface that cannot be determined with the 8G7 antibody. The MUC4β mAbs as well as the anti-TR mAb 8G7, have also
demonstrated efficacy in mitigating MUC4-mediated growth and cellular motility. In addition, they may be able to be used synergistically with the chemotherapeutic gemcitabine to control the growth of pancreatic cancer cells.

Future directions of this work can include a further study of the mechanism by which these antibodies are inhibiting growth. While the mAbs studied here were not determined to induce apoptosis several other MUC4-associated pathways can be studied as potential mechanisms for the inhibition of cellular growth. The data presented in this dissertation warrants further study of the effect of these antibodies in an \textit{in vivo} system. For the study to these antibodies \textit{in vivo} a specialized syngeneic murine cell line containing a MUC4 construct has been developed, that can be employed to evaluate the efficacy of anti-MUC4 therapies \textit{in vivo} in mouse models in the presence of an intact immune system. These antibodies have been proven to be vital tools for the study of MUC4. The use of the anti-MUC4 mAbs as therapeutic tools has been proven \textit{in vitro} and can now be translated to an applicable preclinical model system.
References


