IDENTIFICATION OF PANCREATIC CANCER-SPECIFIC ANTIGENS USING A NOVEL QUANTITATIVE SEROPROTEOMICS APPROACH

by

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ABSTRACT

Pancreatic ductal adenocarcinoma (PDA) is among the most deadly cancers with less than 5% of the patients living beyond 5 years after diagnosis. Lack of early diagnostic biomarkers and resistance to current therapies help explain these disappointing outcomes. Thus, more effective and better-targeted therapies are quickly needed. Monoclonal antibodies offer an attractive alternative targeted therapy option for PDA. However, currently available monoclonal antibody therapies for PDA are still in its infancy with a low success rate of being approved and are facing several challenges. Discovery of novel PDA-specific antigen targets is needed. Predictive and response markers also need to be determined for PDA patient subgroups so that the most appropriate effective therapy can be delivered. Our group has developed a PDA vaccine that has recently completed phase II clinical trials. Here, we employed a novel quantitative seroproteomics approach, SASI, to identify proteins that elicit an increased antibody response post-vaccination. The SASI approach utilizes immunoprecipitation by serum antibodies, which is coupled to the quantitative SILAC. Using mass spectrometry analysis, regulatory subunit 8 of the 26S proteasome (PSMC5), regulatory subunit 12A of protein phosphatase 1 (MYPT1 or PPP1R12A) and transferrin receptor (TFRC) were shown to be PDA-associated antigens that were recognized by post-vaccination antibodies in the sera of patients that received the vaccine and have demonstrated a favorable disease free survival (DFS). We further analyzed PSMC5, MYPT1 and TFRC for tissue expression in normal and PDA specimens and found these proteins to increase in expression with tumor development. Most significantly, the antibody responses detected against these proteins in patients with
an improved DFS suggests an anti-tumor potential of targeting these proteins. Overall, our data demonstrates that the novel SASI approach can identify candidate proteins as possible new biomarkers for screening and as new targets for therapeutic intervention. Traditional and new serologic approaches, recombinant antibody producing technologies and advances in antibody engineering techniques will help identify additional predictive biomarkers and aid in the development of new therapeutic antibodies. A combinatorial approach simultaneously targeting antigens on the PDA cell, stroma and immunosuppressive cells should be employed to successfully treat PDA.

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CHAPTER 1: INTRODUCTION

Pancreatic cancer

Pancreatic ductal adenocarcinoma (PDA) is the fourth leading cause of death by cancer in the US. With its increasing incidence and lack of effective treatments, it is expected to overtake breast and colon cancers to become the second leading cause of cancer-related deaths by 2020\(^1\). It is notably the most aggressive and debilitating malignant disease with a median survival of less than 6 months. Less than 5% of patients have an overall survival of more than 5 years\(^2\). Inadequate early diagnosis, resistance to current chemoradiation therapies and ineffective treatment options account for these low survival statistics. Gemcitabine has been the established standard of care chemotherapy treatment for PDA since 1997\(^3\). The multidrug combination of FOLFIRINOX (oxaliplatin/irinotecan hydrochloride/5-fluorouracil/leucovorin) was recently shown to improve the overall survival over single drug gemcitabine\(^4\). However, it had an unfavorable safety profile and has not led to a significant impact on the overall treatment outcome for PDA. Nab-paclitaxel (Abraxane) given with gemcitabine is also an active combination and less toxic than FOLFIRINOX. This regimen was recently shown to improve survival by only a few months more than single agent gemcitabine\(^5\). Thus, alternative treatment approaches are urgently needed for PDA; this compelling need has propelled the development of new, better-targeted therapies, especially as we understand more about the intra- and inter- cellular pathways involved in the pathogenesis as well as the role of the immune system in PDA.
Monoclonal antibody therapies

Monoclonal antibodies are a promising class of targeted therapies for PDA. First, they are very specific in binding their targets on cell surfaces or in the extracellular matrix. This specific antibody-antigen binding results in a well-tolerated safety profile because the non-target tissues are spared. Second, antibody therapies are potent because they can directly and indirectly inhibit tumorigenesis and tumor progression. In addition to directly targeting the tumors, antibodies can mount an effective response against cancer cells through opsonizing, antigen presentation to T-cells, and mediating cell toxicity via natural killer cells or the complement system\(^6\).

Monoclonal antibody therapies are currently used either alone or in combination with other drugs for several other cancer types. Trastuzumab, a monoclonal antibody that targets human epidermal growth factor receptor 2 (HER2) has been used successfully in the treatment of HER2-positive early-stage and metastatic breast cancer\(^7\). Rituximab, the first US Food and Drug Administration (FDA) approved monoclonal antibody, has been used as a single agent for the treatment of B-cell non-Hodgkin’s lymphoma or in combination for the treatment of chronic lymphocytic leukemia. Rituximab is an anti-CD20 antibody that specifically binds to CD20, a B-cell surface antigen\(^8,9\). FDA has also approved cetuximab and panitumumab for the treatment of Kras wild-type colorectal cancers\(^10-12\). Cetuximab and panitumumab target the human epidermal growth factor receptor (EGFR), which is frequently overexpressed in colorectal cancers\(^13\). The high specificity of the antibodies allows the cancer patients to be screened for the presence of these antigens and then, receive the targeted antibody therapy with minimal side effects.
In addition to the targets present on the cancer cells, antibody therapies have also been developed for targeting antigens on immune cells to alter the immune milieu and hence, elicit an anti-tumor immune response. Ipilimumab blocks the inhibitory cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) receptor on T cells and allows activated T cells to attack cancer cells. Ipilimumab has been approved for the treatment of advanced melanoma\textsuperscript{14,15}.

**Current monoclonal antibody therapies for PDA treatment**

*Mononclonal antibody developments facing challenges*

Although tremendous efforts have been made in the clinical development of monoclonal antibody therapies to target neoplastic cells of PDA, the efficacy of these antibody therapies in treating PDA still needs to be demonstrated. Indeed, several monoclonal antibodies have already failed to prove their efficacy in phase III studies.

**EGFR: Cetuximab**

EGFR is part of the erbB receptor tyrosine kinase family. When a ligand, such as epidermal growth factor or transforming growth factor-\(\alpha\), binds to EGFR, EGFR dimerizes and a downstream signaling pathway is initiated via the phosphoinositide 3-kinase (PI3K)/Akt and mitogen-activated protein kinase (MAPK) pathways. Overexpression of EGFR has been observed in more than 70\% of PDA patients and has been associated with a decreased overall survival. Cetuximab is a chimeric monoclonal antibody that prevents the binding between EGFR and its ligands and eventually, leads to the internalization of the receptor from the cell surface. Cetuximab has been shown to
inhibit growth of cells in EGFR-expressing cell lines and tumor in xenograft models in preclinical studies\textsuperscript{16, 17}. A phase II clinical trial exhibited favorable outcomes in 76\% of the patients receiving gemcitabine plus cetuximab versus only gemcitabine\textsuperscript{18}. However, a subsequent phase III study showed no gain in overall survival with gemcitabine plus cetuximab versus only gemcitabine\textsuperscript{19}. Several phase II studies have shown that using a combination of cetuximab, gemcitabine and platinum-based drugs (cisplatin or oxaliplatin) has no effect on survival or response when compared to the drug combination without cetuximab\textsuperscript{20-22}. Currently, phase I and II studies combining cetuximab with other PDA therapies are being investigated in both the adjuvant as well as the neoadjuvant setting\textsuperscript{17}.

It is not surprising to see the low success with cetuximab, considering that more than 90\% of PDAs carry Kras mutations\textsuperscript{23, 24}. It has been shown that colorectal cancer patients whose tumors carry Kras mutations do not respond to the cetuximab treatment. A recent study has also correlated Kras mutation in codon 12 to decreased survival compared to patients with wild type Kras\textsuperscript{25}. In addition, several pathways utilized by PDA to gain resistance to cetuximab have been identified. Therefore, EGFR is not an ideal target for antibody therapies for PDA. Those resistance mechanisms for anti-EGFR therapies need to be targeted simultaneously to re-sensitize the patients to anti-EGFR therapies\textsuperscript{26, 27}.

**Insulin-like Growth Factor 1 Receptor (IGF-1R): Ganitumab**

IGF-1R belongs to the tyrosine kinase family of proteins. Binding of ligands, IGF-1 or IGF-2, leads to a conformational change and autophosphorylation of the receptor, which activates downstream signaling pathways such as the PI3K/Akt and the
Ras/Raf/MEK/ERK pathways. These pathways are shown to inhibit apoptosis, and improve cell survival, cell proliferation and invasion\textsuperscript{28, 29}. IGF-1R, IGF-1 and IGF-2 are overexpressed in PDAs and preclinical studies have shown that the IGF-1R signaling cascade plays a pro-oncogenic role\textsuperscript{30}. Ganitumab is a fully human anti-IGF-1R monoclonal antibody that disrupts the binding between IGF-1R and its ligands. Ganitumab has been shown to inhibit growth and survival \textit{in vitro} and \textit{in vivo}\textsuperscript{28}. Following favorable preclinical data and a phase I study\textsuperscript{31}, a phase II trial was carried out examining the effects of ganitumab and gemcitabine in metastatic PDA patients\textsuperscript{30}. Ganitumab in combination with gemcitabine showed improved 6-month survival, overall survival and tolerable toxicity. In this same study, the effect of conatumumab with gemcitabine was also examined. Conatumumab is a fully human monoclonal antibody agonist of human death receptor 5 that induces apoptosis and has been show to improve the activity of gemcitabine in preclinical and phase I studies\textsuperscript{30, 32}. Similar to ganitumab, conatumumab also showed improved 6-month survival in the phase II trial\textsuperscript{30}. However, Amgen terminated the phase III study involving ganitumab and gemcitabine prematurely because they perceived no significant results at the end of the study\textsuperscript{33}.

It is imperative to have predictive genetic and proteomic markers as well as methods to evaluate the IGF-1R structure to help clinicians deduce the sensitivity of different tumors to IGF-1R\textsuperscript{34-36}. Development of novel monoclonal antibodies that target receptors in this pathway other than IGF-1R are also needed\textsuperscript{37}. Finally, a better understanding of the alternate escape pathways upstream and downstream of IGF-1R will help us design better combination therapies\textsuperscript{38}.
Vascular Endothelial Growth Factor (VEGF): Bevacizumab

VEGF binds to and activates VEGF receptors, VEGFR-1 and VEGFR-2. Even though PDA is a hypovascular cancer, PDA overexpresses VEGF, VEGFR on not only the blood vessels but also on the cancer cells. An autocrine VEGF/VEGFR signaling pathway induces cancer cell growth and motility and plays a vital role in the invasion and migration of PDA\(^{39,40}\). Thus, VEGF is a potential therapeutic target. Bevacizumab is a recombinant humanized anti-human VEGF monoclonal antibody that disrupts the binding between VEGF and its ligand. Bevacizumab has been shown to have a synergistic effect in inhibiting tumor activity when combined with chemotherapies\(^{41,42}\). A phase II trial in advanced PDA patients showed that bevacizumab in combination with gemcitabine improved the response rate and the median survival time compared to gemcitabine alone\(^{40}\). However, the phase III trial of 535 patients showed no difference in survival between the two groups\(^{43}\). Phase II studies using bevacizumab plus capecitabine-based chemoradiotherapy, followed by gemcitabine showed no improvement in overall survival either\(^{44}\). Erlotinib, a small molecule inhibitor of EGFR, has been shown to improve survival in PDA patients when used in combination with gemcitabine in a phase III trial\(^{45}\). However, when bevacizumab was added to the mixture, there was no significant improvement in overall survival of the patients, even though the progression-free survival was longer with bevacizumab\(^{46}\). Along similar lines, cetuximab and bevacizumab used in combination with or without gemcitabine did not show favorable results\(^{47}\). The failed phase III studies infused doubt in the favorable results of another phase II study that combined gemcitabine, capecitabine and bevacizumab leading the investigators to not move forward to a phase III trial\(^{42}\). Trials involving bevacizumab as a second-line therapy
option have also failed to show much promise\textsuperscript{48, 49}. More recently, bevacizumab has been tested in combination with gemcitabine and other chemotherapies and radiation therapies in adjuvant settings and has continued to show no success\textsuperscript{50-52}.

Thus, the success witnessed in the preclinical PDA models is not being reflected in the clinical trials maybe due to a less complex tumor microenvironment system in the preclinical research settings\textsuperscript{43}. In addition, cancer cells also become resistant to anti-VEGF therapies by resorting to other escape pathways. Ongoing studies, using patient samples from the various discussed clinical trials, aim to elucidate predictive as well as resistance biomarkers to help screen for patients that will benefit from anti-VEGF based therapies as well as design an alternate treatment course once their cancer becomes resistant\textsuperscript{53-55}.

\textit{New antigenic targets of antibody therapies under clinical development}

There are several new monoclonal antibody therapies against promising PDA-specific antigens in preclinical and early clinical trials (\textbf{Table 1}). However, we may face the exact same challenges with these novel therapies as we did with cetuximab, ganitumab and bevacizumab. Thus, it is important for us to keep searching for new and better antibody therapies and targets.

\textbf{Mesothelin: SS1P and MORAb-009}

Mesothelin is a cell surface glycoprotein that is overexpressed in almost 100% of PDAs but not in normal pancreas. Mesothelin has recently been shown to play a role in cell proliferation and migration \textit{in vitro} and \textit{in vivo}\textsuperscript{56-58}. The specific overexpression of
mesothelin in PDA makes mesothelin an inviting therapeutic target. SS1P, a recombinant immunotoxin, comprises of an anti-mesothelin variable F region that is covalently linked to a truncated form of *Pseudomonas* exotoxin. SS1P is a type of an antibody-drug conjugate (discussed more in a following section). SS1P has been shown to kill mesothelin-expressing tumor in preclinical studies. Favorable anti-tumor activity and toxicity profile from recent phase I trials has warranted future trials in PDA patients expressing mesothelin. However, SS1P suffered from drawbacks such as immunogenicity, toxicity with repeated dosing, and short half-life. Yet, SS1P demonstrated efficacy alone and in combination with other drugs. Thus, in order to avoid the immunogenicity, an improved version was developed and has entered a phase I study. This study has shown significant tumor regression in mesothelioma patients.

MORAb-009 is a chimeric anti-mesothelin monoclonal antibody. Preclinical studies showed anti-tumor activity against mesothelin-expressing cancer when treated with MORAb-009 plus gemcitabine versus either treatment alone. These results were followed by a phase I trial that showed that MORAb-009 is relatively safe in PDA patients expressing mesothelin. These promising data has resulted in a recently completed phase II study, where MORAb-009 was tested in combination with gemcitabine in locally advanced and metastatic pancreatic patients. The results of this study are currently not available. Additionally, a pilot study testing radiolabeled MORAb-009 in various mesothelin-expressing cancers, including PDA, has recently been completed.

**Prostate Stem Cell Antigen (PSCA): AGS-1C4D4**
PSCA, a glycosylphosphatidylinositol (GPI)-linked plasma membrane protein, was first identified as being overexpressed in prostate cancer. PSCA is overexpressed in 60% to 80% of PDA\textsuperscript{68} but not expressed in normal pancreas\textsuperscript{69}; thus, PSCA is a potential therapeutic target. Targeting PSCA has yielded anti-tumor activity in preclinical studies. AGS-1C4D4, a fully human anti-PSCA monoclonal antibody, has favorably completed phase I trial in prostate cancer. A following phase II study has shown that AGS-1C4D4 plus gemcitabine improved 6-month survival rate compared to gemcitabine alone in previously untreated metastatic PDA patients\textsuperscript{68}.

**Gastrin 17: G17DT**

Gastrin is a peptide growth hormone that binds to the cholecystokinin-B (CCK-B)/gastrin receptor. Several studies have shown that CCK-B/gastrin receptors, progastrin, glycine-extended gastrin and amidated forms of gastrin are expressed in both PDA cells and resected PDA patient tissue. Gastrin has been shown to promote growth in PDA cell lines as well as tumor growth in mice via endocrine, autocrine and paracrine pathways\textsuperscript{70, 71}. Anti-gastrin antibodies have shown to inhibit PDA proliferation *in vitro* and *in vivo*\textsuperscript{72}. Administration of an immunogen, G17DT, induces the formation of anti-gastrin antibodies in humans\textsuperscript{73}. These antibodies neutralize the glycine-extended and amidated forms of gastrin and disrupt their binding to their receptor, and are thereby thought to exert anti-tumor activity. An uncontrolled phase II study showed that an anti-gastrin antibody response was induced in patients receiving G17DT\textsuperscript{73}. G17DT was well-tolerated and patients responding to the immunogen had improved survival when compared with patients who did not respond. A follow up controlled phase III study also showed
improved survival in patients developing an antibody response versus patients who did not or patients who received a placebo\textsuperscript{74}.

**Connective Tissue Growth Factor (CTFG): FG-3019**

CTFG plays an important role in the PDA stromal compartment through tumorigenic activities such as modulating cell adhesion, migration and cell growth. In addition, high levels of CTFG mRNAs have been observed in PDA tissue versus normal pancreas. Studies have shown that CTFG from cancer cells induce tumor growth and this pro-tumor effect and metastasis can be inhibited by FG-3019, a fully human anti-CTFG monoclonal antibody\textsuperscript{75, 76}. Very recently, a study also showed that CTFG in the tumor microenvironment confers resistance to gemcitabine in mouse PDA, which can be decreased by FG-3019 treatment\textsuperscript{77}. The results of a phase I study presented at 2013 ASCO Gastrointestinal Cancers Symposium involving a combination of FG-3019, gemcitabine and erlotinib showed that the drug combination is well-tolerated in previously untreated patients with advanced PDA\textsuperscript{78}.

**Lessons learned from current antibody therapy developments**

Multiple successful phase I and II trials have not resulted in any successful phase III trials. A thorough examination of the current monoclonal antibody PDA therapies sheds light on the challenges and drawbacks faced by these therapies. Understanding these issues will help design improved antibody drugs and trials going forward. At least five challenges stand out from this examination.
First, patients are not always screened for expression of the antibody targets; in some cases this is due to a lack of predictive and prognostic genetic and proteomic markers for the particular antibody therapy.

Second, preclinical data modeling antibody therapy does not appear to translate directly into the clinic setting as seen in the case of treatment of PDA using bevacizumab. The complexity of human PDA is not replicated in these preclinical models, which are often xenograft based immunocompromised mouse models, and provides a potential explanation for the failure seen in the case of VEGF inhibitors.

Third, some of the monoclonal antibody therapies are not cancer specific. The targeted proteins are not exclusively expressed by the PDA but can also be expressed on the normal pancreas or other tissues in the body. This non-specific expression often leads to an unfavorable safety profile and unacceptable toxicities in patients. Thus, it is vital to identify antibody targets that are highly specific to the tumor using the improved research tools that are now available for this purpose.

Fourth, there is also the issue of the tumor becoming resistant to a particular treatment as seen in the cases of cetuximab and ganitumab. When one pathway in PDA is targeted, the tumor learns to find alternate pathways to become resistant to the treatment and survive. Ongoing research is elucidating the various escape mechanisms utilized by PDA in response to these various monoclonal antibody therapies. By finding different targets in these other pathways that can be inhibited, a strong case for multiple targeting therapies can be established.
Fifth, in contrast to several other solid tumors, PDA is hypovascular. Additionally, the small number of functional blood vessels, complemented with a very dense stroma surrounding the tumor, hinders the different therapeutic agents from being delivered effectively to the cancer cells\textsuperscript{79,80}.

**Overcoming the challenges in antibody therapy development for PDA**

*Where to look for potential antigen targets*

A thorough genetic analysis of several PDAs revealed 12 cell signaling cascades that are frequently dysregulated in PDA\textsuperscript{23}. Proteins that are integral components of these commonly modified pathways provide a list of potential targets for PDA treatment\textsuperscript{24}. In addition, there has been growing evidence in the literature pointing towards the important role of the stromal compartment in PDA progression and metastasis\textsuperscript{81-83}. The majority of PDA consists of the stroma instead of the PDA cells and greater amounts of stroma have been linked with poor disease prognosis. The stromal compartment consists of fibroblasts, pancreatic stellate cells, blood vessels, immune cells as well as the extracellular matrix that is composed of collagen, hyaluronan, fibrin and fibrinogen. Preclinical and clinical studies have shown an increase in effectiveness of PDA treatment achieved by altering the stromal components\textsuperscript{80,84,85}. Immunotherapies also relieve the immunosuppressive PDA tumor microenvironment resulting in anti-tumor activity in the clinic. Thus, not only antigens expressed on PDA cells, but also those present in the different stromal compartments, should be considered as potential therapeutic targets. By developing monoclonal antibodies against these antigens, it will be possible not only to
disrupt intracellular pathways within the cancer cell but also the intercellular pathways that exist between the neoplastic cell and its stroma.

Several monoclonal antibody-based immunotherapy strategies aimed at creating a more anti-tumor immune environment are currently being tested in the clinic. In contrast to the view that PDA is a non-immunogenic cancer, several recent studies have shed light on the vital role of the tumor infiltrating immune cells in the PDA stromal compartment in immune suppression as well as anti-tumor activities. Immune suppression to various tumor-related antigens is associated with poor prognosis. A large number of immune suppressive cells such as tumor-associated macrophages, regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) are present early in the PDA progression. Through different mechanisms, these suppressive cells result in a lower number of functional effector cells such as CD8+ and CD4+ T cells and natural killer cells in the stromal compartment. Tregs suppress effector T cells by direct contact through its inhibitory receptors, CTLA-4 and programmed death-1 (PD-1). In addition, PDA cells also overexpress ligands such as programmed death receptor ligand 1 (PD-L1) that results in non-functional effector cells. Thus, immunotherapies aimed at creating an enhanced anti-tumor immune environment within PDA are currently being tested in the clinic (Table 1).

**CTLA-4: Ipilimumab**

Ipilimumab, a fully humanized anti-CTLA-4 monoclonal antibody, inhibits the inactivation of effector T cells. A functional CD8+ T cell can induce antigen-specific cancer cell cytotoxicity. Ipilimumab was shown to be effective as a single agent in
metastatic melanoma and renal cell carcinoma. However, it failed to be effective as a monotherapy when used in advanced PDA patients. Preclinical studies have shown that the effectiveness of anti-CTLA4 antibody is enhanced in the presence of granulocyte macrophage-colony stimulating factor (GM-CSF) cell-based vaccines. Several clinical trials involving an allogeneic, GM-CSF secreting PDA vaccine (GVAX) in resected PDA or metastatic PDA patients have shown the induction of a mesothelin-specific CD8+ T cell response that correlates with an improved disease free survival. A phase Ib trial testing ipilimumab with or without GVAX was carried out in PDA patients. The mesothelin-specific T cell responses again correlated with survival. It is important to note that there were significant immune-related adverse events with ipilimumab with or without GVAX. The results of this trial showed objective responses together with a better survival in the ipilimumab plus GVAX group when compared to the ipilimumab alone group, thereby reinforcing the enhanced effect of GVAX in the presence of ipilimumab as seen in earlier preclinical studies.

**PD-1/PD-L1: Nivolumab, BMS-936559, CT-011, lambrolizumab and MPDL3280A**

Nivolumab (BMS-936558), a fully human anti-PD-1 monoclonal antibody, inhibited the inhibitory receptor PD-1 and allowed T cells to remain functional and induce tumor cell cytotoxicity in preclinical studies. A follow up phase I study showed that nivolumab was well-tolerated and suggested a potential anti-tumor activity. BMS-936559, a fully human anti-PD-L1 monoclonal antibody, binds to PD-L1 on pancreatic tumor cells and prevents its association with its receptor PD-1 resulting in an anti-tumor immune environment *in vitro*. A clinical trial in advanced cancer patients showed favorable
clinical data\textsuperscript{96}. Another humanized anti-PD-1 antibody, CT-011, was shown to be safe in patients with advanced hematologic malignancies in a phase I trial\textsuperscript{97}. A following phase 2 trial testing CT-011 in diffuse large B-cell lymphoma patients has produced encouraging progression free survival data. CT-011 has undergone testing for various cancer patients, including PDA\textsuperscript{98, 99}. Very recently, a phase Ib trial testing lambrolizumab (formerly called MK-3475), Merck’s humanized anti-PD-1 monoclonal antibody, showed promising clinical response in advanced melanoma patients\textsuperscript{100}. Completed and ongoing clinical trials involving Genentech’s human anti-PD-L1 antibody, MPDL3280A, provides further ground for targeting this pathway\textsuperscript{101-104}. These results have paved the path for future trials using PD-1 and PD-L1 antibodies in treating various solid cancers, including PDA.

Nonetheless, it should be noted that PDA’s tumor microenvironment is dominated with immune suppressive mechanisms and infiltrated with few effector T cells. It was thus not surprising to see that single agent anti-PD-1 and anti-PD-L1 antibodies did not show objective response in the small number of PDA patients that were tested in the early phase clinical trials. It may be critical to combine PD-1/PD-L1 blockade therapies with those treatments that simultaneously induce the effective T cell infiltration or inflammatory cytokine responses. Therefore, it is intriguing to test the combination of vaccine-based immunotherapy and anti-PD-1/PD-L1 antibodies.

**CD40: CP-870,893**

CD40 is a costimulatory plasma membrane receptor present on antigen presenting cells (APCs). CD4\textsuperscript{+} T helper cells initiate a signaling pathway via CD40 that leads to
activation of APCs, which eventually results in functional effector CD8+ T cells. Activating CD40 antibodies have been shown to activate APCs without the need for CD4+ T helper cells\textsuperscript{105}. CP-870,893, is a fully human CD40 agonist monoclonal antibody. A phase I clinical trial using this CD40 agonist plus gemcitabine in previously untreated advanced PDA patients showed a favorable safety profile, an anti-tumor immune response as well as a preliminary therapeutic benefit\textsuperscript{106, 107}. It was surprising to see that CD40 agonist-activated macrophages, instead of the CD8+ T cells, were responsible for the anti-tumor activity via stromal depletion\textsuperscript{85}.

*What to look for in potential antigen targets*

Monoclonal antibodies offer a great therapeutic option because they are specific as well as potent\textsuperscript{6}. Several characteristics should be considered when identifying potential antigens for therapeutic antibody development: 1) the antigens should be overexpressed in PDA and have minimal expression in the normal pancreas; 2) the antigen should be present on the cell surface or in the extracellular matrix rather than inside the cell although it has been shown that antibodies can potentially target intracellular oncoproteins in mouse models\textsuperscript{108, 109}, 3) proteins that alter their cellular location from intracellular compartments to cell surface or extracellular compartments in PDA compared to normal pancreas would be excellent targets. For example, annexin A2 has been shown to translocate to the cell surface in PDA but is present inside the cell in the normal pancreas\textsuperscript{110, 111}. Thus, annexin A2 is a promising new target for PDA therapies.
<table>
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<tr>
<th>Antigenic PDA target</th>
<th>Monoclonal antibody therapy</th>
<th>Clinical trial phase</th>
<th>Targeted PDA patient group</th>
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<th>Up to date clinical trial results</th>
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<tr>
<td>Mesothelin</td>
<td>SS1P (Antibody-drug conjugate)</td>
<td>Phase I, single agent (bolus)</td>
<td>Mesothelin-positive PDA patients</td>
<td>MTD: 45μg/kg/dose i.v. QOD × 3 doses DLT: pleuritis 4 MR and 19 SD out of 33 patients No CR</td>
<td>Hassan, et al. (2007)⁶⁰</td>
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<tr>
<td>Mesothelin</td>
<td>SS1P (Antibody-drug conjugate)</td>
<td>Phase I, single agent (c.i.)</td>
<td>Mesothelin-positive PDA patients</td>
<td>MTD: 25 μg/kg/dose given as c.i. over 10 days 1 PR out of 24 patients No CR</td>
<td>Kreitman, et al. (2009)⁶¹</td>
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<tr>
<td>Mesothelin</td>
<td>MORAb-009</td>
<td>Phase I, single agent</td>
<td>Mesothelin-positive PDA patients</td>
<td>MTD: 200 mg/m² 11 SD out of 24 patients</td>
<td>Hassan, et al. (2010)⁶⁵</td>
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<td>Mesothelin</td>
<td>MORAb-009</td>
<td>Phase II, randomized, gemcitabine vs. gemcitabine and MORAb-009</td>
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<td>PSCA</td>
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<td>Phase II, randomized, gemcitabine vs. gemcitabine and AGS-1C4D4</td>
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<td>Gastrin 17</td>
<td>G17DT</td>
<td>Phase II, randomized, G17DT vs. placebo</td>
<td>Advanced PDA patients unsuitable for or unwilling to take</td>
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<tr>
<td>Treatment</td>
<td>Abbreviation</td>
<td>Phase</td>
<td>Description</td>
<td>Outcomes</td>
<td>Reference</td>
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<td>------------</td>
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<tr>
<td>CTFG</td>
<td>FG-3019</td>
<td>Phase I, combination of FG-3019, gemcitabine and erlotinib</td>
<td>Previously untreated, locally advanced or metastatic PDA patients</td>
<td>No serious adverse events, PFS: 4.3 months, OS: 9.4 months</td>
<td>Dimou, et al. (2013)</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>Ipilimumab</td>
<td>Phase Ib, ipilimumab vs. ipilimumab and GVAX</td>
<td>Locally advanced or metastatic PDA patients after failed chemotherapy</td>
<td>Median OS: 3.6 vs. 5.7 months (p = 0.072), 1 year OS: 7% vs. 27%</td>
<td>Le, et al. (2013)</td>
</tr>
<tr>
<td>PD-1</td>
<td>CT-011</td>
<td>Phase 2, gemcitabine and CT-011</td>
<td>Patients who have had surgically resected PDAs without any follow-up treatments</td>
<td>Trial currently recruiting participants</td>
<td>Khleif, et al. (2013)</td>
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<tr>
<td>PD-L1</td>
<td>BMS-936559</td>
<td>Phase I, single agent</td>
<td>Selected solid tumors including advanced PDA patients with or without previous therapy</td>
<td>No objective response seen in PDA patients</td>
<td>Brahmer, et al. (2012)</td>
</tr>
<tr>
<td>PD-L1</td>
<td>MPDL3280A</td>
<td>Phase I, single agent</td>
<td>Selected solid tumors including locally advanced or metastatic PDA patients</td>
<td>No objective response seen in PDA patients</td>
<td>Herbst, et al. (2013)</td>
</tr>
<tr>
<td>CD40</td>
<td>CP-870,893</td>
<td>Phase I, gemcitabine and CP-870893</td>
<td>Chemotherapy-naïve advanced PDA patients</td>
<td>MTD: 0.2 mg/kg dose level, 4 PR out of 22 patients, No CR</td>
<td>Beatty, et al. (2013)</td>
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<tr>
<td>Fibronectin ED-B</td>
<td>L19-IL2 (Antibody-cytokine fusion)</td>
<td>Phase I, gemcitabine and L19-IL2</td>
<td>Locally advanced or metastatic PDA patients not eligible for surgical resection</td>
<td>Trial currently recruiting participants</td>
<td>Philogen (2012)</td>
</tr>
</tbody>
</table>

Abbreviations: PSCA- Prostate Stem Cell Antigen, CTFG- Connective Tissue Growth Factor, CTLA-4- Cytotoxic T Lymphocyte-associated Antigen 4, PD-1- Programmed
Death 1, PD-L1- Programmed Death receptor Ligand 1, ED-B- Extradomain B, MTD-
Maximum Tolerated Dose, DLT- Dose-Limiting Toxicities, QOD- Every Other Day,
I.V.- Intra-Venous, C.I.- Continuous Infusion, SR- Survival Rate, OS- Overall Survival,
PR- Partial Response, CR- Complete Response, MR- Minor Response, SD- Stable
Disease, Unadj- Unadjusted, PFS- Progression Free Survival.
CHAPTER 2: MATERIALS AND METHODS

Patients, serum and tissue samples

Patients were enrolled in a phase II study of an allogeneic GM-CSF secreting whole cell pancreatic cancer vaccine in compliance with the Johns Hopkins Medical Institution Institutional Review Board (IRB)-approved J9988 protocol\textsuperscript{92}. Blood samples were collected pre-vaccination, 14 days after 1\textsuperscript{st} vaccination and 28 days after each subsequent vaccination. Sera was collected by centrifugation, aliquoted and stored at -80°C. Pancreatic tumor tissue samples were collect from patients at the time of pancreaticoduodenectomy and prior to vaccination. We also obtained tissue samples from patients enrolled in a neoadjuvant study, J0810, where they received the first vaccination prior to surgery.

Antibody purification

Antibodies were purified from pre- and post-3\textsuperscript{rd} vaccination sera using a protein G column (GE Healthcare, Piscataway, NJ, USA) as per manufacturer’s protocol. Quantification of purified antibodies was done using NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

SASI sample preparation

The human pancreatic cancer cell line, Panc 10.05, was grown as previously described. For the SILAC procedure, Panc 10.05 cells were grown in either light (\textsuperscript{12}C\textsubscript{6}-lys, \textsuperscript{12}C\textsubscript{6}-arg) or heavy (\textsuperscript{13}C\textsubscript{6}-lys, \textsuperscript{13}C\textsubscript{6}-arg) RPMI1640 media containing 10% fetal bovine serum and
antibiotics in a humidified incubator at 37°C with 5% CO₂. Stable isotope containing amino acids, ¹³C₆-arginine and ¹³C₆-lysine, were purchased from Cambridge Isotope Laboratories (Andover, MA, USA). Arginine and lysine-free RPMI1640 media, fetal bovine serum (FBS) and antibiotics (penicillin and streptomycin) were purchased from Invitrogen (Carlsbad, CA, USA). The light and heavy cells were washed with phosphate buffered saline and were harvested using M-PER buffer (Thermo Fisher Scientific) in the presence of cocktail protease inhibitors (Thermo Fisher Scientific). Protein was quantified using the Lowry method.

**Immunoprecipitation for mass spectrometry**

Equal amounts of light and heavy cell lysates were incubated overnight with purified pre- and post-vaccination antibodies, respectively. On the following day, the two sets of lysate: antibody mixture were each incubated with protein G beads (Invitrogen) and washed using M-PER buffer. The immunoprecipitates were eluted by boiling in NuPAGE® LDS sample buffer (Invitrogen). The light and heavy eluted lysates were mixed 1:1. The mixture was concentrated and resolved by 10% SDS-PAGE. The gel was stained using a coomassie dye staining kit (Invitrogen).

**Liquid chromatography tandem mass spectrometry and data analysis**

The stained gel was excised into 18 bands and each band was destained in 40mM ammonium bicarbonate/40% acetonitrile solution. The samples were reduced with 5mM dithiothreitol/20% acetonitrile solution, alkylated with 100mM iodoacetamide and digested with trypsin. Sequencing grade modified porcine trypsin was purchased from
Promega (Madison, WI, USA). The peptides were extracted, desalted, dried and reconstituted in 0.1% formic acid. The peptides were analyzed by reversed phase liquid chromatography tandem mass spectrometry (LC-MS/MS). Briefly, the peptides in solution were separated using an on-line reverse phase nano high-performance liquid chromatography using a C18 column and the Eksigent Nano 2D high-performance liquid chromatography (HPLC) pumping system (Eksigent). The nano-HPLC is interfaced directly with the LTQ-Orbitrap-XL (Thermo Electron) allowing for introduction of the separated peptide solution into the mass spectrometer for tandem mass spectrometric analysis. Isolated proteins from each band were identified using an automated database search algorithm, MASCOT, within the Proteome Discoverer software platform (Thermo Electron) and processed by MaxQuant. Our data was searched at a mass tolerance of 10 ppm for MS species and 1 Da for MS/MS with carbamidomethylation of cysteine as a fixed modification and oxidation of methionine as a variable modification. The proteolytic enzyme indicated was trypsin and we allowed up to two missed cleavage events.

Mass-spectrometry data validation

Panc 10.05 cells grown in light RPMI1640 media were lysed in M-PER buffer supplemented with protease inhibitor cocktail. The lysate was immunoprecipitated with either the pre- or post-vaccination purified antibodies using protein G beads. The immunoprecipitates were eluted by boiling in NUPAGE LDS sample buffer and resolved on a NuPAGE 4-12% Bis-Tris gel (Invitrogen). Proteins in the gel were transferred onto nitrocellulose membrane using a semi-dry apparatus (Invitrogen). The membrane was
blocked in 5% bovine serum albumin (BSA, Invitrogen) in 0.1% Tween 20-PBS (PBS-T) buffer for 1 hour at room temperature and probed with the relevant primary antibody overnight at 4 °C. Antibodies against galectin-3 (sc-19283), E3 ubiquitin protein ligase (sc-9561), Mesencephalic astrocyte-derived neurotrophic factor (sc-34560), Epidermal growth factor receptor kinase substrate 8-like protein 2 (sc-100722), Calpain-1 (sc-81171) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The membrane was incubated with the corresponding peroxidase conjugated secondary antibodies (A8419, Sigma) and then ECL Western Blotting Detection Reagents (GE Healthcare) was used for 1 minute at room temperature for developing.

**Western blot for detecting antibody responses in patients**

Purified recombinant proteins, PSMC5 (TP301251), MYPT1 (TP323540) and TFRC (TP300980) expressed in human HEK293 cells were purchased from Origene (Rockville, MD, USA). One microgram of purified protein was denatured by boiling in SDS-PAGE sample buffer and resolved on a NuPAGE 4-12% Bis-Tris gel (Invitrogen). Proteins in the gel were transferred onto nitrocellulose membrane using a semi-dry apparatus (Invitrogen). The membrane was cut into individual lanes and was blocked in 5% bovine serum albumin (BSA, Invitrogen) in 0.1% Tween 20-PBS (PBS-T) buffer for 1 hour at room temperature. After blocking, each individual lane was probed with either pre-vaccination or post-vaccination serum at 1:1000 dilution. A lane was used as a control and probed with mouse anti-FLAG antibody overnight at 4 °C. The membrane was incubated with the peroxidase conjugated secondary antibodies, mouse anti-human IgG antibody (Sigma) for patient serum lanes or rabbit anti-mouse IgG (Sigma) for control
lane. ECL Western Blotting Detection Reagents (GE Healthcare) was used for 1 minute at room temperature for developing.

**Immunohistochemistry**

PSMC5, MYPT1 and TFRC staining protocol was optimized using pancreatic cancer cell line Panc10.05 as positive control and pancreatic tissue as negative control. Immunohistochemistry was performed on formalin-fixed paraffin-embedded embedded 5µm thick sections of the available pancreatic tumor tissue samples and the tissue microarrays. The tissue samples of the patients enrolled in the study was obtained from the Department of Pathology at Johns Hopkins Medical Institutions tissue archive. The tissue microarrays were constructed from different types of malignant tumors and their companion normal tissues. The diagnoses were verified by evaluation of the histopathological and immunohistochemical stains by two reference pathologists.

Standard immunohistochemistry protocol was applied using Bond-Leica autostainer (Leica Microsystems, Bannockburn, IL). Briefly, tissue sections were baked for 20 minutes at 65°C followed by deparaffinization and primary antibody incubation at optimal conditions. Bond polymer detection system was applied to develop the reaction. 3’,3’ diaminobenzidin (DAB) chromogen-substrate was utilized for visualization of reaction as per manufacturer’s instructions (Leica Microsystems, Bannockburn, IL). All sections were then counterstained with hematoxylin, dehydrated and cover slipped. Antibody information is detailed in Table 2.

**Assessment of protein expression**
The percentages of malignant and normal cells in the pancreatic tissue and TMAs expressing each evaluated protein in the cytoplasm, nucleus and cell membrane were recorded. The staining intensity was graded as none (0), weak (1) and strong (2). Two pathologists, blinded to the study, independently scored the slides using the aggressive criteria set for positive/negative staining. A sample with more than 25% of the cancer or normal tissues expressing the marker with an intensity score of 1 or 2 were considered to be positive whereas a sample with less than or equal to 25% was considered negative.
### Table 2

Table 2. Information about the antibodies used for immunohistochemistry.

<table>
<thead>
<tr>
<th>Name</th>
<th>Clone/animal species</th>
<th>Dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-PSMC5</td>
<td>Rabbit</td>
<td>1:150</td>
<td>Sigma</td>
</tr>
<tr>
<td>Anti-MYPT1</td>
<td>Rabbit</td>
<td>1:500</td>
<td>Sigma</td>
</tr>
<tr>
<td>Anti-human TFRC</td>
<td>Mouse (Clone: H68.4)</td>
<td>1:2000</td>
<td>Invitrogen</td>
</tr>
</tbody>
</table>
CHAPTER 3: THE NEED FOR A NEW SEROPROTEOMICS APPROACH LED TO THE DEVELOPMENT OF SASI APPROACH

Introduction

Serologic approaches are gaining momentum in the identification of new cancer biomarkers. These cancer biomarkers are beneficial for early detection, predicting response to immunotherapies including monoclonal antibody therapies and the determination of new targets for the development of biologically relevant therapies. These proteins have high potential as future targets for effective PDA treatment. This translational approach will advance the development of new drugs, vaccines and antibody-based therapies that will halt the progression and metastasis of the disease.

Current serologic approaches to identify new biomarkers and therapeutic targets

As described below, common serologic approaches to identify immunogenic proteins are: serological screening of cDNA expression library (SEREX), protein arrays and seroproteomics/serological proteome analysis (SERPA). Although patient sera is a common component in these methods, the source of protein used can vary. It is imperative to validate antigens found using serologic approaches to prevent the issue of misidentification by mass spectrometry analysis. To confirm that the identified cancer-specific antigen is correct, one can employ western blot, immunohistochemistry or enzyme-linked immunosorbent assay (ELISA) analyses to validate their targets.

SEREX
SEREX involves expression of tumor cDNA phage library in bacteria\textsuperscript{120}. These expressed proteins are transferred to a membrane and are screened against patient sera. The most well known application of SEREX utilizes sera, from untreated cancer patients or individuals with known genetic susceptibilities for cancer, to screen for cancer-associated proteins that elicit an antibody response. NY-ESO-1 is a famous protein that was found from an esophageal cancer cDNA library using the SEREX technology\textsuperscript{119}. SEREX, however, utilizes proteins expressed in bacteria and thus, does not account for human posttranslational modifications.

**Protein Arrays**

Protein arrays come in many forms with more choices in the protein source that can be used and are comparatively more high-throughput\textsuperscript{114}. Some protein arrays use tumor cell lysate fractions, which identify proteins in their native conformation with the correct posttranslational modifications. However, these arrays do not identify which specific protein in the fraction instigates the immune response and thus, it would be hard to pinpoint the specific antibody-antigen. In contrast, other protein arrays are printed with recombinant human proteins expressed in bacteria or yeast, allowing the user to know the identity of each protein contained in a spot.

**SERPA/Seroproteomics**

Seroproteomic approaches utilize and thrive on the technological advances in the field of proteomics\textsuperscript{118, 121}. SERPA involves 2-dimensional electrophoresis (2-DE) followed by mass-spectrometry analysis\textsuperscript{114}. Either patient tumor or cell line lysates are subjected to 2-
DE to separate the proteins in the whole cell lysates. Using western blot analysis, the
differential antibody response is observed and finally, the proteins of interest are
identified by mass spectrometry.

**PDA vaccine (GVAX)**

Our group has developed an allogeneic, granulocyte-macrophage colony-stimulating
factor (GM-CSF)-secreting pancreatic cancer vaccine (GVAX), which has recently
completed phase II clinical trial, J9988\(^\text{92}\). Observation of favorable clinical and
immunological responses in patients has testified to the success of the vaccine\(^\text{92, 93, 122}\). This promising vaccine is used in conjunction with chemoradiation. In brief, 60
pancreatic cancer patients, who had their pancreas surgically removed, were involved in
the study (**Figure 1**). The patients received their first vaccination 2 months after surgery.
One month after the first vaccination, the patients underwent a 6-month course of chemoradiation. The second, third and fourth vaccines were administered at a one-month
interval from the time of chemotherapy completion. Finally, the fifth vaccination was
received 6 months after the fourth vaccination. Serum samples were obtained pre- and
post-vaccination for all five vaccinations.

The 60 vaccinated patients were divided into 3 groups based on length of disease free
survival (DFS)\(^\text{92}\). Group A was composed of 12 patients who received all of the
scheduled vaccinations and demonstrated a DFS > 3 years. The 21 patients in Group B
received at least 3 scheduled vaccinations, but had a DFS < 3 years. The 27 patients in
Group C relapsed before receiving their second scheduled vaccination.
GVAX-induced T cell responses

Our group originally evaluated CD8+ T cell responses to a new pancreatic cancer antigen, mesothelin\textsuperscript{92, 93}. Our group recently published data showing the induction of mesothelin-specific T cell responses only in patients with a DFS > 3 years, which suggests the vaccine induces immunologically relevant T cell responses\textsuperscript{92}. Functional genomic approaches were utilized to identify antigens recognized by T cells. However, finding T cell antigens is limited by the lack of reagents for each patient-specific HLA\textsuperscript{111}.

GVAX-induced B cell responses

GVAX has been shown to induce antibody responses in patients that responded as well as those that did not respond to the vaccine\textsuperscript{123}. Similar to T cell responses\textsuperscript{92}, most Group A patients demonstrated a post-induction or enhancement in antibody production against vaccine-associated proteins\textsuperscript{123}. On the contrary, very few Group B patients displayed an enhanced antibody response to vaccine-associated proteins.

Antibodies hold potential as a high throughput way of identifying antigens. Antibodies can directly attack tumors as well as indirectly by mounting an effective response against cancer cells through opsonizing, antigen presentation to T-cells, and mediating cell toxicity via natural killer cells or the complement system\textsuperscript{6}. Examining antibody responses can also aid in the identification of T cell antigens and T cell responses that be potentially used as a predictive marker for survival or response to therapy. Melanocyte differentiation antigen, RAB38/NY-MEL-1, was initially detected by studying antibody
responses in melanoma patients using the SEREX approach. Recent studies have shown that spontaneous CD8+ T cell responses are also detected against this antigen\textsuperscript{124}.

**Application of conventional seroproteomics to identify antibody-reactive proteins**

Our group developed a functional proteomic approach (Figure 2), which uses immunized sera from patients vaccinated with the GM-CSF vaccine (GVAX)\textsuperscript{123}. Briefly, pre- and post-vaccination sera from patients that showed favorable clinical and immune responses to GVAX in a phase II clinical trial were used\textsuperscript{92}. Cell lysates were obtained from the PDA cell lines (the proteome) that were used for the generation of the vaccine. The cell lysates were subjected to 2-DE, followed by western blot analysis, where the pre- and post-vaccination sera was compared. Proteins that showed an increased antibody response post-vaccination were identified via mass spectrometry.

In the initial screen employing this approach, our lab identified a number of cytoplasmic proteins that induce an immune response\textsuperscript{123}. Proteins identified in this study had a range of cellular functions such as cell growth and invasion (annexin A2 and rho GDP dissociation inhibitor α), metabolism (enolase α and pyruvate kinase), and regulation of T cell function (galectin-3). Most of these processes are found to be aberrant in pancreatic cancers\textsuperscript{23}.

Each of the identified proteins was subsequently validated. In order to do this, the genes for the identified proteins from the vaccine cancer cell lines were cloned into pcDNA3.3, a mammalian expression vector\textsuperscript{123}. The recombinant proteins were then expressed in HEK293 cells. Western blot analysis showed an increase in antibody response post-
vaccination to recombinant galectin-3 in 7 of the 12 patients that showed DFS > 3 years. Antibody response to recombinant galectin-3 pre- and post-vaccination in all 60 patients receiving the vaccine was studied using ELISA. 67% of the patients with DFS > 3 years (compared to only 9.5% of the patients with DFS < 3 years) showed a 2-fold increase in antibody response post-vaccination compared to pre-vaccination. These results imply that the vaccine-induced antibody response to galectin-3 strongly correlates clinically with an increased DFS.

Several of these new candidate proteins are involved in pancreatic cancer development and progression. For example, in patients who demonstrated prolonged survival, post-vaccination serologic responses were detected against rho GDP dissociation inhibitor α, annexin A2 and galectin-3. Ongoing research has already shown these proteins are promising targets involved in signaling pathways important to the biology of pancreatic cancer progression and metastases\textsuperscript{110,123}. The discovery of annexin A2 has not only led to a better understanding of PDA progression and metastasis but also helped identify a new monoclonal antibody therapy for PDA\textsuperscript{110,111}.

**Missing proteins: Membrane proteins**

Although our lab has elucidated cytoplasmic proteins that elicit an antibody response, membrane proteins remain uncharacterized. Several reasons account for their omission in the original study. First, the lysis buffer, consisting of weak detergents, was ineffective in stably removing the hydrophobic membrane proteins from their lipophilic environment for solubilization in an aqueous environment\textsuperscript{125}. These proteins must be well solubilized to prevent aggregation. Additionally, 2-DE is not a suitable approach for separating
membrane proteins\textsuperscript{125}. Membrane proteins tend to aggregate at their pI and transfer poorly from the hydrophobic gel matrices of immobilized pH gradient (IPG) strips to sodium dodecyl sulfate (SDS) gels.

Membrane proteins constitute about 30\% of all cellular proteins and are functionally key regulators\textsuperscript{126}. However, due to their hydrophobic nature and conformational instability during purification processes (when removed from their native lipid-rich environment)\textsuperscript{125}, working with membrane proteins remains a challenge to this day. Pharmacologically, membrane proteins are very important drug targets; around two-thirds of present-day drugs target membrane proteins\textsuperscript{126}. This highlights the importance of finding membrane proteins that induce an antibody response in vaccinated patients who show a favorable disease free survival. Not only will this study help bridge the informational gap obscuring these proteins, but it will also provide a thorough analysis of their role in the progression and metastatic process of this fatal disease. A better understanding of membrane proteins will also lead to improvements in the design of pharmacological drugs and immunotherapies. Specific and selective therapies targeting membrane proteins can be used to formulate a treatment plan that works in coordination with our vaccine therapy in order to enhance its efficacy in cancer treatment.

The identification of cancer associated membrane proteins that induce serologic responses and correlate with post-vaccination prolonged disease free survival will enrich the pool of potential proteins, thereby enhancing our efforts of identifying biologically relevant proteins. These proteins will serve as future targets for effective pancreatic cancer treatment, including antibody therapies.
**Identification of membrane proteins that induce antibody responses**

To address the complications of working with membrane proteins, we adopted a seroproteomic approach to include only the membrane proteome. Specifically, we first used a biotinylation approach from Pierce® (Cell Surface Protein Isolation Kit) that isolates only plasma membrane proteins from pancreatic cancer-vaccine cell lines, Panc10.05 and Panc6.03. In this method, after biotinylation of cellular plasma membrane proteins, the cells are lysed using a detergent rich buffer. The biotinylated proteins are isolated using a NeutrAvidin™ Resin, then eluted from the beads with SDS-PAGE buffer with a reducing agent (such as DTT).

Once the extracted membrane proteins are separated using SDS-PAGE, we used western blot analysis to compare post-vaccination to pre-vaccination antibody responses of Group A patients (DFS > 3 years) (Figure 3). Orbitrap, a LC-MS/MS mass spectrometer, was used to identify antibody-reactive membrane proteins. Lysates enriched in membrane proteins were separated by gel electrophoresis and stained with coomassie dye. To identify the membrane protein, we excised and destained gel bands corresponding to the proteins that show increased antibody response post-vaccination as seen by the western blot analysis. Proteins were trypsinized and extracted from the gel in order to prepare them for HPLC-MS/MS. The peptides in solution were separated using an on-line reverse phase nano high-performance liquid chromatography using a C18 column and the Eksigent Nano 2D high-performance liquid chromatography (HPLC) pumping system (Eksigent). The nano-HPLC is interfaced directly with the LTQ-Orbitrap-XL (Thermo Electron) allowing for introduction of the separated peptide solution into the mass
spectrometer for tandem mass spectrometric analysis. Isolated proteins from each band will be identified using an automated database search algorithm, MASCOT, within the Proteome Discoverer software platform (Thermo Electron). Our data was searched with a mass tolerance of 10 ppm for MS species and 1 Da for MS/MS. The proteolytic enzyme indicated was trypsin and we allowed up to two missed cleavage events. Proteins identified with a high confidence interval were analyzed using Protein Center, an online system that links every mascot identification with NCBI data.

Preliminary studies identified HLA class I histocompatibility antigen, RAS GTPase-activating like protein (IQGAP1), Tissue factor (F3) and ATP synthase (beta subunit, ATP5B) as potential membrane proteins that induce a biologically significant response. Many of these identified proteins are known to be associated with the membrane, which supported the rationale of our experimental approach.

One of the membrane proteins that was identified using this approach was ATP synthase (beta subunit, ATP5B). We used recombinant ATP5B expressed in HEK293 cells that we obtained from Origene as the antigen for the initial screen. We compared the post- and pre-vaccination sera from the GVAX-responders group to determine if ATP5B induces an antibody-response that correlates with improved DFS. In an effort to validate ATP synthase as a significant membrane protein, serum from 7 patients in Group A (DFS > 3 years) was screened using western blot analysis (Figure 4). Post-vaccination serum from 4 out of the 7 patients showed an increased antibody response to ATP synthase (beta subunit).

**Need for a new seroproteomic approach**
The traditional seroproteomic approaches has presented several obstacles in comprehensively identifying PDA-specific antigens that induce an antibody response in vaccinated PDA patients. In addition to the inability of the 2-DE seroproteomic approach to identify hydrophobic or membrane proteins, there are several other disadvantages of using these approaches.

First, the current membrane/plasma membrane isolation techniques are inefficient. We determined the extent of plasma-membrane protein enrichment in our samples by a thorough mass spectrometry analysis of the various bands seen in SDS-PAGE (Figure 5). We found that only 5% of the total identified proteins were cell-surface whereas 50% were membrane proteins. The rest were cytoplasmic contaminants. Thus, our ability to identify membrane proteins was 50% at best.

Second, only about 50% patients showed an increased antibody response to ATP synthase (beta subunit) post-vaccination. Thus, we need to find antigens with a more significant differential antibody response post-vaccination between the GVAX responders and non-responders. Additionally, there remain many unidentified membrane proteins that could potentially display an enhanced antibody response that did not feature through this initial screen.

Third, each visible gel band consists of several proteins that have similar molecular weight. It would be hard to conclusively determine which individual protein in the group of proteins is responsible for the increased antibody response. These similar molecular weight proteins also differ in their abundance and hydrophobicity. Mass spectrometry’s difficulty in identifying membrane proteins and low abundance proteins would further
create a bias towards the identification of the more hydrophilic proteins as well as the high abundance proteins and leaving out potentially important proteins. Thus, we need an approach where low abundance proteins (including membrane proteins) are not overshadowed by similar molecular weight higher abundance proteins.

Fourth, both SEREX and seroproteomic approaches identify linear epitopes and selects for antibody responses to denatured rather than the native proteins\textsuperscript{127}. The missed conformational epitopes may be better targets of the serologic response. These approaches are also relatively low throughput and semi-quantitative.

Thus, the development of a new high throughput quantitative comprehensive approach identifying native epitopes is needed. It is of the utmost importance to develop an effective method that will allow identification of a range of antibody-reactive proteins including under represented proteins due to their lower expression levels than the more abundant and common proteins. Incorporation of immunoprecipitation in this new approach would increase our specificity for the relevant proteins up front without loss of lesser-expressed but potentially more important proteins. This approach should also enhance our chances of identifying important serologically recognized proteins because the conformation of the proteins will be biologically relevant to the antibody response\textsuperscript{127}. An approach that allows us to know the antibody response status pre- and post-vaccination for each identified protein would be ideal. These considerations laid the foundation for the development of a new quantitative seroproteomics approach.

**Development of the SASI approach**
Serum-Antibodies based SILAC-Immunoprecipitation (SASI) couples serum-antibodies based immunoprecipitation and quantitative Stable Isotope Labeling with Amino acids in Cell culture (SILAC) (Figure 6). Thus, for the SASI approach to be successfully designed, we needed to optimize 2 components – cell labeling and high-throughput immunoprecipitation.

**Cell labeling**

We labeled the PDA cell line, Panc 10.05, that would provide the proteome for our approach. Panc 10.05 is one of the 2 cell lines used in GVAX\textsuperscript{122} and hence, would provide an ideal source of proteins that are human and presented with correct form of post translational modifications. SILAC is a labeling strategy where the same cell line is differentially labeled as light and heavy, thereby providing a method of distinguishing the proteins derived from the light and heavy cells by mass spectrometry\textsuperscript{128}. Specifically, the light Panc 10.05 cells were grown in the normal RPMI 1640 media (\textsuperscript{12}C\textsubscript{6}-lysine and \textsuperscript{12}C\textsubscript{6}-arginine) whereas the heavy cells were grown in a RPMI 1640 media that did not contain any lysines or arginines. This media was supplemented with \textsuperscript{13}C\textsubscript{6}-lysine and \textsuperscript{13}C\textsubscript{6}-arginine. The heavy cells were grown in the heavy media for about 10 passages because it took about 10 passages for > 98% heavy incorporation in this cell line as determined by mass spectrometry analysis.

**High-throughput immunoprecipitation**

While the cells were being grown and passaged to enable > 98% heavy media incorporation, we directed our efforts at optimizing the immunoprecipitation protocol. In
contrast to the traditional immunoprecipitation approach where antibodies against a single antigen are used, we wanted to develop a high-throughput immunoprecipitation protocol that could utilize the full range of antibodies present in a patient’s serum to identify their corresponding antigens.

For the purpose of optimizing the immunoprecipitation method, we used sera from a separate clinical trial, J0501 in an effort to conserve the precious patient sera from the J9988 study. 4 patient sera were analyzed by western blot to check for the patient that had the greatest difference in antibody response pre- and post-vaccination (Figure 7). Patient 5.034’s post-4th vaccination and pre-vaccination sera was chosen for the future optimization steps. Using this patient’s sera, we were able to determine the appropriate amount of beads to use (Figure 8), the optimum buffer system (Figure 9) and the elution method to employ (Figure 10) for the SASI approach.

In an effort to minimize background, we explored 3 different options – using magnetic beads, preclearing the lysate before immunoprecipitation and purifying the antibodies from the patient sera. The antibody binding capacity of the magnetic beads could not match the capacity of the agarose beads even when the amount of magnetic beads used was increased to more than 3 times the amount of agarose beads used (Figure 11). Therefore, we decided to use agarose beads so that we could capture and bind almost all the antibodies from the patient sera. Preclearing the lysate did not decrease the background significantly (Figure 12). In addition, SILAC labeling would allow us to know the heavy to light ratio of every protein identified. Thus, we did not deem it necessary to preclear the cell lysate prior to immunoprecipitation because the effect of
certain proteins sticking to the beads would be neutralized. Finally, we pursued the option of purifying the antibodies (IgGs) from the sera before immunoprecipitation using a protein G column. This step was most effective in eliminating the non-antibody components of the sera and hence, significantly decreasing the background. We further verified that the purified antibodies (IgGs) were still functional and were be able to recreate the differences in pre- and post-vaccination response that we observed using the sera for western blot analysis. A major post-vaccination increase observed with the sera of patient 5.034 continued to be seen when the purified antibodies (IgGs) were used instead (Figure 13). Based on these results, we opted to purify the antibodies from the sera and utilize the purified antibodies for the immunoprecipitation.

Thus, by utilizing a rigorous optimization process, we were able to determine the final conditions for a small scale immunoprecipitation reaction: agarose beads: 20µL, protein: 1mg, purified IgGs: 100µg, immunoprecipitation buffer: M-PER (obtained from Pierce®), elution by boiling. The beads and IgGs were mixed for 2 hours, after which the protein lysate was added to the mixture of beads and IgGs for an overnight incubation. Next day, following a series of washes, the bound proteins and IgGs were eluted by boiling. In order to improve the protein identification by mass spectrometry, we scaled the immunoprecipitation reaction by 5 fold. We carried out the large scale immunoprecipitation in 5 different tubes following the protocol described above with a slight modification – the protein and IgGs were incubated together overnight and the beads were added the following day. Finally, the eluent from the 5 tubes were combined after boiling.
We performed a large scale immunoprecipitation reaction using the pre- and post-vaccination IgGs of patient 5.034 separately and found a visible protein band that was present in the post- but not the pre-vaccination sera (Figure 14). The observation of this band supported our hypothesis that the SASI approach would be a useful way in identifying proteins that elicit an antibody response post-vaccination in vaccinated pancreatic cancer patients. Thus, we decided to extend our study by applying the SASI approach to the patient sera obtained from the J9988 study.
Figure 1. Vaccination schedule of completed phase II clinical trial, J9988. Vaccine is used in conjunction with chemoradiation. The patients received their first vaccination 2 months after surgery. One month after the first vaccination, the patients underwent a 6-month course of chemo-radiation. The second, third and fourth vaccines were administered at a one-month interval from the time of chemotherapy completion. Finally, the fifth vaccination was received 6 months after the fourth vaccination. Serum samples were obtained pre- and post-vaccination for all five vaccinations.
FIGURE 2

Figure 2. The application of seroproteomics for the discovery of PDA-specific antigens and novel monoclonal antibody therapies using the PDA-GVAX study as a representative example. 2-dimensional electrophoresis (2-DE) is performed using the cell lysate obtained from the PDA GVAX cell lines. Discovery patient sera is obtained from patients that have favorable clinical and immune responses to the vaccine. Pre- and post-vaccination discovery patient sera is used for the western blot analysis. Mass-spectrometry analysis is used to identify PDA-specific antigens that elicit a favorable antibody response in patients. These antigens are subsequently validated using recombinant protein technology and sera from other patients in the clinical trial (both responders and non-responders to the vaccine). Next, the PDA antigen is subjected to immunohistochemical and functional studies to establish it as a biomarker whereas a
monoclonal antibody, against the antigen, is tested as a potential antibody therapy. Sideway arrows indicate the flow of reagents into the process whereas vertical arrows represent the process flow.
Figure 3. Western blot comparing pre- and post-vaccination antibody responses to membrane proteins in two vaccinated patients. Arrows show serologic changes in protein detection pre- versus post-vaccination.
Figure 6. Western blot comparing pre- and post-vaccination antibody responses to ATP synthase (beta subunit) in vaccinated patients. Arrows show serologic changes in protein detection pre- versus post-vaccination.
Figure 5. Current membrane/plasma membrane isolation techniques are inefficient.

The pie chart shows the cellular compartments of the proteins obtained using a plasma-membrane protein enrichment protocol. 5% of the isolated proteins are cell-surface, 50% are membrane whereas the rest belong to other cellular (non-membrane) compartments.
Figure 6. Design of the SASI approach. Cells grown in light and heavy media were subjected to immunoprecipitation using purified pre- and post-vaccination antibodies, respectively. The light and heavy immunoprecipitates were eluted by boiling and mixed...
1:1. The immunoprecipitates were resolved by SDS-PAGE and the gel was stained. 18 protein bands were excised and digested with trypsin. The extracted peptides were analyzed by LTQ-Orbitrap mass spectrometer. The proteins were identified and quantified using Mascot™ and MaxQuant™, respectively.
Figure 7. Selection of patient sera for optimizing the SASI approach. Pre- and post-vaccination antibody responses for patients 5.020, 5.021, 5.034 and 5.039 were determined by western blot. Arrows show serologic changes in protein detection pre-versus post-vaccination. Primary antibody dilution: 1:1000 (*1:500); Secondary antibody dilution: 1:200,000. Asterisk indicates the patient sera that was selected for optimizing the SASI approach.
Figure 8. Selection of the amount of beads for SASI approach. Post-vaccination serum of patient 5.034 was incubated with varying amount of beads for 2 hours. The bound IgGs were eluted by boiling and separated by SDS-PAGE. The gel was stained using coomassie dye. Asterisk indicates the amount of beads selected to be used for the SASI approach.
**Figure 9. Selection of the buffer for SASI approach.** Post-vaccination serum of patient 5.034 was incubated with protein lysate in different buffers for immunoprecipitation. The bound IgGs and proteins were eluted by boiling and separated by SDS-PAGE. Post-vaccination serum was used for western blot analysis. Asterisk indicates the buffer selected to be used for the SASI approach.
Figure 10. Selection of the elution method for SASI approach. Post-vaccination serum of patient 5.034 was incubated with protein lysate in M-PER buffer for immunoprecipitation. The bound IgGs were eluted by various methods and separated by SDS-PAGE. The gel was stained using coomassie dye. Asterisk indicates the elution buffer selected to be used for the SASI approach.
Figure 11. Magnetic beads are not a great option for reducing background in the SASI approach. Post-vaccination serum of patient 5.034 was incubated with either agarose beads or varying amounts of magnetic beads for 2 hours. The bound IgGs were eluted by boiling and separated by SDS-PAGE. The gel was stained using coomassie dye. Asterisk indicates the type of beads selected to be used for the SASI approach.
Figure 12. Effect of preclearing protein lysate before immunoprecipitation. Post-vaccination serum of patient 5.034 was incubated with either precleared or not precleared protein lysate in M-PER buffer for immunoprecipitation. The bound IgGs and proteins were eluted by boiling and separated by SDS-PAGE. The gel was stained using coomassie dye. Asterisk indicates our choice of not clearing the protein lysate before immunoprecipitation.
Figure 13. Western blot showing purified antibodies from patient sera are still functional. Panc 10.05 cell lysate was separated by SDS-PAGE, followed by western blot. Purified pre- and post-vaccination antibodies obtained from sera of patient 5.034 were used to probe the blot. Primary antibody dilution: 1:500; Secondary antibody dilution: 1:200,000.
Figure 14. Large scale immunoprecipitation using pre- and post-vaccination IgGs from patient 5.034. A. Design of the large scale immunoprecipitation protocol to be used in the SASI approach. Briefly, purified IgGs and protein lysate were incubated overnight. Next day, agarose beads were added to the mixture for 2 hours. The bound proteins and IgGs were eluted by boiling. B. Eluents obtained using pre- and post-IgGs for immunoprecipitation were kept separate and resolved by SDS-PAGE. The gel was stained using coomassie dye. Red arrow indicates a protein band that is present in the post-vaccination eluent but not in the pre-vaccination eluent.
CHAPTER 4: APPLICATION OF THE SASI APPROACH
IDENTIFIED PROTEINS THAT INDUCED AN ANTIBODY
RESPONSE IN VACCINATED PDA PATIENTS WITH A
FAVORABLE CLINICAL OUTCOME

Source of purified antibody samples

Sera samples used were derived from a phase II, single institution study (J9988) of 60
PDA patients, who underwent pancreaticoduodenectomy with adjuvant GM-CSF
secreting cell vaccinations (GVAX) and 5-flourouracil based chemoradiation (Figure
1)\textsuperscript{92}. Patients were divided into 3 groups based on disease free survival (DFS) and
number of GVAX vaccinations: group A = DFS > 3 years, 5 GVAX (n=12); group B =
DFS < 3 years, 3 ≥ GVAX (n=21); group C = disease relapse before 2\textsuperscript{nd} GVAX (n=27).

The Serum Antibodies based SILAC-Immunoprecipitation (SASI) screen was designed
(Figure 6) to detect antibodies that are present in patient serum and to identify the
proteins targeted by the antibodies. Briefly, the proteome of the Panc 10.05 cell line (used
in GVAX) was differentially labeled using the SILAC technique with light (\textsuperscript{12}C) and
heavy (\textsuperscript{13}C) lysines and arginines\textsuperscript{128}. We applied the SASI approach to 3 Group A
patients (3.009, 3.027 and 3.052) because they had an excellent DFS status and were
alive at the end of the study. Total serum antibodies (IgGs) were purified from the pre-
and post-4\textsuperscript{th} vaccination sera of these 3 patients using a protein G affinity column (Figure
15).
Application and validation of the SASI approach

The pre-vaccination IgGs were mixed with the light cell lysate and the post-vaccination IgGs were mixed with the heavy cell lysate for immunoprecipitation. The light and heavy eluents were kept separate after boiling. Although we expected the heavy and light samples to have received equal amounts of the various reagents used during the SASI steps, sampling errors may have been introduced. In order to determine the extent of sampling errors, we mixed 10% light and heavy eluents in a 1:1 ratio by volume. This mixture was separated by SDS-PAGE and analyzed by mass spectrometry for the heavy to light profile for the various identified proteins. Assuming that most proteins were expected to have no change in antibody response post- versus pre-vaccination as determined by a corresponding 1:1 heavy to light ratio, we normalized the mixing of the remaining 90% heavy and light eluents accordingly. The observed heavy to light ratio for most proteins was 0.9:1, 1:3 and 1:2 for patients 3.009, 3.027 and 3.052, respectively. Thus, for the final mixing of the remaining 90% of the eluents, the heavy and light eluents were mixed in the ratio 1:0.9, 3:1 and 2:1 for patients 3.009, 3.027 and 3.052, respectively. This final normalized mixture was then subjected to SDS-PAGE. The stained gel was divided unequally into 18 bands and each of these bands were excised for further mass spectrometry analysis (Figure 16).

Immunoprecipitation with the isotope labeled cell lysates and purified patient antibodies coupled with mass-spectrometry analysis revealed antibody targets (proteins) present in pre- and post-vaccination patent serum. We validated the SILAC screen with western blot analysis. Three proteins, galectin-3, E3 ubiquitin-protein ligase UBR5 and mesencephalic
astrocyte-derived neurotrophic factor (MANF) had an increased antibody response post-vaccination by 15.3, 4.0 and 3.9 fold respectively while 2 proteins showed a decreased antibody response - calpain-1 (2 fold) and epidermal growth factor receptor kinase substrate 8-like protein 2, EPS8L2 (10.0 fold). Western blot analysis (Figure 17) shows dramatic increase in galectin-3 protein level in the post-vaccination blot, whereas E3 ubiquitin-protein ligase UBR5 and MANF showed a modest increase. Calpain-1 showed a dramatic decrease in detection whereas EPS8L2 showed a modest decrease in the blot containing the post-vaccination eluent. These results mirrored the trends we observed from the quantitative mass-spectrometry derived SILAC ratios.

Identification of proteins by the SASI approach

The pre- and post-vaccination serum samples from 3 group A patients (3.009, 3.027 and 3.052) were used for the SASI screen. We identified 2514 proteins (Figure 18) with a range of change from 16 fold increase to a 10 fold decrease in post-vaccination serum. Annexin A2 protein, previously identified as a biologically relevant PDA target, showed a median 1.3 fold increase. We narrowed our list of proteins from 2514 proteins to 31 proteins by employing a rigorous selection criteria (Figure 19). Of the 31 proteins (Table 3), 3 proteins, galectin-3, annexin A2 and pyruvate kinase were identified previously by a 2-DE proteomic approach and are currently under investigation for their role in PDA pathogenesis and progression. Identification of these 3 proteins further validated the capability of the SASI screen in uncovering biologically relevant PDA targets. Another identified protein was HLA class I histocompatibility antigen (HLA), which had also been previously identified. Antibody response to allogeneic non-
self HLA is expected and provided another internal positive control and validated the SASI approach once again. The remaining 27 proteins provided us with a list of proteins that warranted further study.

Through the SASI approach, we present a large scale study to identify and categorize proteins that are targeted by antibodies in the human body. The SASI approach comprehensively identified more than 2500 proteins of different cellular components, molecular functions and biological processes.

**Predictive vaccine response markers - PSMC5, MYPT1 and TFRC are antibody targets of immune response against PDA**

We decided to pursue 10 out of the 27 shortlisted proteins for convenience and financial reasons. Using purified recombinant FLAG-tagged proteins expressed in HEK293 cells (obtained from Origene) for western blot analysis, we examined the pre- and post-3rd vaccination antibody response of 7 (of the 12) Group A patients with favorable DFS for 10 proteins (Table 4). Anti-FLAG antibody served as a positive control to confirm the presence of the protein in the blot. 5 proteins (PSMC5, MYPT1, TFRC, HDGFRP2 and RDH11) out of the 10 proteins tested showed an increased antibody response post-vaccination in 5 or more patients. Therefore, these proteins were chosen for further analysis by western blot.

Next, we wanted to identify potential antibody targets of the vaccine by studying the antibody response pre- and post-vaccination to the 5 proteins by comparing all 12 Group A patients that responded to the vaccine against 12 (out of 21) Group B patients that did
not respond to the vaccine. The selection of the 12 out of the 21 patients in the group with DFS < 3 years was based on the number of vaccinations received. The 12 selected patients received at least 3 vaccinations, thereby allowing us to compare the pre- and post-3rd vaccination antibody response in both the responders and the non-responders.

Regulatory subunit 8 of the 26S proteasome (PSMC5) (Figure 20), regulatory subunit 12A of protein phosphatase 1 (MYPT1 or PPP1R12A) (Figure 21) and transferrin receptor (TFRC) (Figure 22) showed elevated antibody titers in patients with favorable DFS when compared to patients with an unfavorable DFS. PSMC5 and TFRC elicited an increased antibody response in 8 of 12 Group A patients (DFS > 3 years). MYPT1 showed an increased antibody response in 9 of 12 Group A patients. In contrast, an increase in antibody response post-vaccination to PSMC5 and TFRC was observed in only 2 of the 12 Group B patients (DFS < 3 years). Additionally, 5 of the 12 Group B patients had an increase in antibody response post-vaccination to MYPT1. Interestingly, 4 of the 12 Group B patients showed a decreased antibody response to MYPT1 post-vaccination compared to only 1 of the 12 Group A patients that showed a decreased response post-vaccination. Similarly, both PSMC5 and TFRC demonstrated a decreased antibody response in 2 out of the 12 Group B patients compared to only 1 Group A patient showing a decrease in antibody response. It is important to note that these 3 proteins also demonstrated an increased antibody response in each of the 3 patients tested by the SASI approach. Although we cannot correlate the quantitative SASI data with the qualitative western blot results, the overall trends were similar. Therefore, these western blot observations provided further validation of our SASI results.
In contrast, RDH11 (Figure 23) and HDGFRP2 (Figure 24) did not show any interesting difference in antibody response between the responders and non-responders. Therefore, RDH11 and HDGFRP2 were excluded from future immunohistochemical analysis to determine their expression levels in PDA tissues.

These results imply that the vaccine-induced antibody response to PSMC5, MYPT1 and TFRC have strong correlations to clinical benefit. An increased antibody response post-vaccination correlates to a longer and favorable DFS whereas a decreased response post-vaccination correlates to a shorter and unfavorable DFS. Thus, these proteins could serve as predictive markers for whether a patient will respond to GVAX in the long run depending on the patient’s ability to induce an antibody response to these proteins post-vaccination. The data also suggests that these proteins are antigenic targets of vaccine-induced humoral responses in PDA patients. Most significantly, the antibody responses detected against these proteins in patients with DFS > 3 years suggests an anti-tumor potential of targeting these proteins.
Figure 15. Purification of antibodies (IgGs) from patient serum pre- and post-vaccination. Pre- and post-vaccination antibodies were purified from patient 3.052 serum using a protein G affinity column. The serum (S) and purified antibodies (A) were resolved on SDS-PAGE. The gel was stained using coomassie dye.
**Figure 16.** **Gel band map for excision.** The final normalized mixture of heavy and light eluents was subjected to SDS-PAGE. The stained gel (left) was divided unequally into 18 bands (right) and each of these bands were excised for further mass spectrometry analysis.
Figure 17. Western blot validation of SILAC data. Pre- and post-vaccination antibodies of patient 3.052 were used for immunoprecipitation. The precipitate was separated by SDS-PAGE followed by western blot using antibodies against the indicated proteins. The fold-change as detected by mass spectrometry in the SASI screen for the same patient are shown to the right of each blot and correlates well with the qualitative results in the western blot.
Figure 18. Global changes in antibody recognition post-vaccination compared to pre-vaccination. Summary of the fold change (ratio of antibody response post-vaccination to pre-vaccination) in the proteins identified and quantified by the SASI screen. A total of 2,514 proteins were quantitatively identified following immunoprecipitation from the sera of 3 patients using the SASI approach.
Figure 19. Selection criteria narrows the list from 2514 proteins to 31 proteins.

Downward arrows indicate the number of proteins that meet the criteria. The operator box shows the selection criteria applied.
Figure 20. Increased antibody response to PSMC5 post-vaccination correlates with an improved disease free survival (DFS). Purified PSMC5 expressed in human HEK293 cells were resolved by SDS-PAGE and then transferred onto a nitrocellulose membrane. The membrane was cut into individual lanes. Each individual lane was probed with either pre-vaccination or post-vaccination serum of the various patients. Solid arrow indicates an increase post-vaccination whereas a dotted arrow shows a decrease post-vaccination in antibody response.
**Figure 21.** Increased antibody response to MYPT1 post-vaccination correlates with an improved disease free survival (DFS). Purified MYPT1 expressed in human HEK293 cells were resolved by SDS-PAGE and then transferred onto a nitrocellulose membrane. The membrane was cut into individual lanes. Each individual lane was probed with either pre-vaccination or post-vaccination serum of the various patients. Solid arrow indicates an increase post-vaccination whereas a dotted arrow shows a decrease post-vaccination in antibody response.
Figure 22. Increased antibody response to TFRC post-vaccination correlates with an improved disease free survival (DFS). Purified TFRC expressed in human HEK293 cells were resolved by SDS-PAGE and then transferred onto a nitrocellulose membrane. The membrane was cut into individual lanes. Each individual lane was probed with either pre-vaccination or post-vaccination serum of the various patients. Solid arrow indicates an increase post-vaccination whereas a dotted arrow shows a decrease post-vaccination in antibody response.
**Figure 23. Antibody response to RDH11 in patients with > 3 years and < 3 years DFS.** Purified RDH11 expressed in human HEK293 cells were resolved by SDS-PAGE and then transferred onto a nitrocellulose membrane. The membrane was cut into individual lanes. Each individual lane was probed with either pre-vaccination or post-vaccination serum of the various patients. Solid arrow indicates an increase post-vaccination whereas a dotted arrow shows a decrease post-vaccination in antibody response.
Figure 24. Antibody response to HDGFRP2 in patients with > 3 years and < 3 years DFS. Purified HDGFRP2 expressed in human HEK293 cells were resolved by SDS-PAGE and then transferred onto a nitrocellulose membrane. The membrane was cut into individual lanes. Each individual lane was probed with either pre-vaccination or post-vaccination serum of the various patients. Solid arrow indicates an increase post-vaccination whereas a dotted arrow shows a decrease post-vaccination in antibody response.
Table 3. Partial list of proteins identified by SASI screen that elicited an increased antibody response post-vaccination.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Gene symbol</th>
<th>Average fold change</th>
<th>Protein function‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Galectin 3*</td>
<td>LGALS3</td>
<td>11.0</td>
<td>Regulator of T-cell functions</td>
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<tr>
<td>26S proteasome, regulatory subunit 8</td>
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<tr>
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<td>CD9</td>
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<td>Cell adhesion and motility</td>
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<td>HDGF-2</td>
<td>HDGFRP2</td>
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<td>Function unknown</td>
</tr>
<tr>
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<td>3.1</td>
<td>Microtubule organization</td>
</tr>
<tr>
<td>Prohibitin-2</td>
<td>PHB2</td>
<td>2.4</td>
<td>Mediator of transcriptional repression via recruitment of histone deacetylases</td>
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<tr>
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<td>CDIPT</td>
<td>2.2</td>
<td>Phosphatidylinositol biosynthesis</td>
</tr>
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<td>RDH11</td>
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<td>Short-chain aldehyde metabolism</td>
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<td>GOT2</td>
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<td>Amino acid metabolism</td>
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<td>Regulator of protein phosphatase 1C and mediates binding to myosin</td>
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<td>Iron uptake via endocytosis of transferrin</td>
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<td>Annexin A2*</td>
<td>ANXA2</td>
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<td>Cell adhesion</td>
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*: Identified by previous approach
‡: The UniProt Consortium
Table 4. Summary of western blot analysis examining pre- and post-3\textsuperscript{rd} vaccination antibody response of 7 (of the 12) Group A patients with favorable DFS for 10 proteins. Positive sign denotes an increase in antibody response post-vaccination whereas a negative sign denotes an unchanged or a decrease in antibody response post-vaccination.

<table>
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<th>3.027</th>
<th>3.029</th>
<th>3.047</th>
<th>3.052</th>
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<th>Patients with increased antibody response</th>
</tr>
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<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>6/7</td>
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<tr>
<td>RDH11</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>6/7</td>
</tr>
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<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>6/7</td>
</tr>
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<td>PPP1R12A</td>
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<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
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CHAPTER 5: CHARACTERIZATION OF PSMC5, MYPT1 AND TFRC AS PDA-SPECIFIC ANTIGENIC BIOMARKERS

Expression of PSMC5, MYPT1 and TFRC in PDA and normal pancreas

Antibody responses are induced against oncoproteins due to changes in their expression levels, localization or post-translational modifications. Available serial analysis of gene expression (SAGE) data suggested that PSMC5, MYPT1 and TFRC maybe overexpressed in PDA compared to normal pancreas\(^2\). Therefore, we were interested in establishing the expression levels and tissue location of PSMC5 (Figure 25), MYPT1 (Figure 26) and TFRC (Figure 27) in PDA samples. We analyzed PDA tissue samples for 45 of the 60 patients from the phase II study\(^9\). We found 95% of PDA express PSMC5 in malignant compared to 24% non-malignant ductal epithelium. The PSMC5 protein is a part of the 26S proteasome typically located in the cytoplasm. There was significantly more nuclear PSMC5 in malignant (70%) cells compared to non-neoplastic ductal epithelium (8%) with an intermediate amount of expression in pre-neoplastic pancreatic intraepithelial neoplasia (PanIN) lesions. MYPT1, part of the Rho kinase pathway, was strongly expressed in cytoplasm of the PDA (93%) compared to normal ductal epithelium (8%). MYPT1 expression was also observed when the cancer was present in a nerve as well as a cancer metastasis in the lymph node. Membranous MYPT1 (48%) was only observed in PDA and not in normal ductal epithelium. Similarly, 90% of the PDA expressed cytoplasmic or membranous (21%) TFRC whereas none of the normal ducts showed any cytoplasmic or membranous TFRC expression which is
consistent with previous reports. The fact that TFRC is a well studied marker in not only PDA but other cancers further proves that the SASI approach is well suited in identifying biologically relevant oncoproteins such as PSMC5 and MYPT1, for which there is limited literature. We also noticed that the tissue expression of PSMC5, MYPT1 and TFRC correlated with PDA progression. In other words, their staining increased relatively as the disease progressed from normal pancreas to PanINs to PDA.

In order to validate our staining results, we used PDA tissue from a neoadjuvant GVAX study (J0810), where the PDA patients received the first GVAX prior to surgery. The expression levels of PSMC5 (Figure 28), MYPT1 (Figure 29) and TFRC (Figure 30) largely mirrored the data obtained from staining J9988 patient samples and thereby, reconfirmed our previous findings (Table 5). It is important to note that the expression data from the J9988 and J0810 studies varied in 2 areas. First, the nuclear staining was significantly higher in the PDA glands of the J9988 study (70%) compared to the J0810 study (27%). Second, the membrane staining observed in the PDA glands of the J9988 study (21%) was almost half when compared to the J0810 study (43%). Whether the observed variations in staining between the 2 trials is an effect of GVAX still remains to be studied.

**Expression of PSMC5, MYPT1 and TFRC in non-PDA cells**

Since we had the entire patient PDA section, we were able to observe PSMC5, MYPT1 and TFRC staining in various non-PDA components of the pancreas. We found that MYPT1 strongly stained goblet cells of the duodenum (Figure 31). Therefore, we propose that MYPT1 is a goblet cell marker, which may play an important role in the
diagnosis of certain esophageal cancers and autoimmune diseases that are characterized by goblet cells\textsuperscript{132}. We also observed MYPT1 expression in the goblet cells present in the colon cancer tissue microarrays.

We also observed that PSMC5 stains the endothelial cells of arteries whereas MYPT1 and TFRC are expressed in the smooth muscle of the arteries present in the tissue sample. Staining the J0810 patient tissue samples also yielded some interesting findings. Our group has found the presence of new lymphoid aggregates in PDA tissue of patients that received GVAX prior to surgery. Therefore, we were interested to see if our markers stained the lymphoid aggregates or the individual tumor infiltrating lymphocytes in these PDA tissues. PSMC5 frequently stained the lymphoid aggregates as well as some of the individual tumor infiltrating lymphocytes. However, we found MYPT1 and TFRC expression in only a few lymphoid aggregates but in almost none of the individual tumor infiltrating lymphocytes. In particular, we found PSMC5-positive lymphoid aggregate surrounding PSMC5-positive PDA in two J0810 patient tissues (Figure 32). However, the identity of the immune cells expressing PSMC5 in the lymphoid aggregates is unknown.

**Differential expression of nuclear PSMC5 and cytoplasmic and membrane MYPT1 in various tumors can help diagnose PDA**

Since PSMC5 and MYPT1 had not been previous reported as malignancy markers we sought to determine if these were unique to PDA. Tissue microarrays of biliary, lung, liver, colon and breast cancers were evaluated for MYPT1 (Figure 33) and PSMC5 (Figure 34) expression. We found cytoplasmic MYPT1 expression in biliary (54%)
(Figure 35) and colon (32%) (Figure 36) cancers whereas with very little to no staining in breast (19%) (Figure 37A), lung (5%) (Figure 38) and liver (0%) (Figure 39) cancers while cytoplasmic PSMC5 was expressed in breast (88%) (Figure 40A), lung (72%) (Figure 41), liver (59%) (Figure 42), biliary (22%) (Figure 43) and colon (48%) (Figure 44) cancers. In addition, we found membranous MYPT1 expression in colon (28%) cancers whereas with very little to no staining in breast (2%), biliary (1%), lung (0%) and liver (0%) cancers while nuclear PSMC5 was expressed in breast (35%), lung (21%), liver (14%), colon (4%) and biliary (4%) cancers. It was interesting to see that cytoplasmic MYPT1 staining was different in different breast cancer subtypes (Figure 37B) – Basal (triple-negative, 6%), HER2+ (13%) and ER+ (39%). However, the membrane MYPT1 staining was very little in ER+ (6%) and no membranous staining was observed in basal or HER2+ breast cancers. Cytoplasmic PSMC5 staining was similar between the different breast cancer subtypes (Figure 40B) - Basal (triple-negative, 94%), HER2+ (82%) and ER+ (88%). However, nuclear PSMC5 staining was most in basal (47%), followed by HER2+ (38%) and the least in ER+ (24%) breast cancers. Significantly, more pancreatic cancers express cytoplasmic and membranous MYPT1 when compared to the other tumors whereas only nuclear PSMC5 expression seems to be PDA-specific. Thus, by utilizing the differences in nuclear PSMC5, cytoplasmic and membranous MYPT1 staining among various cancers with the expression being specific for PDA, we can develop a differential diagnosis model for PDA in the clinic.

We also evaluated MYPT1 and PSMC5 expression in normal colon, lung, liver, biliary and breast tissues. These normal tissues were available either as control spots or as part of
the tissue microarray design. Cytoplasmic MYPT1 was expressed in normal colon (100%) and biliary (13%) tissues whereas was completely absent from normal lung, liver and breast tissues. Cytoplasmic PSMC5 was expressed in normal breast (38%), liver (34%) and colon (36%) tissues but had little to no expression in normal biliary (11%) and lung (0%) tissues. In addition, we found membranous MYPT1 expression in normal colon (100%) tissues whereas no staining in normal lung, breast, biliary and liver tissues while nuclear PSMC5 was expressed in normal biliary (22%) and liver (6%) tissues but no staining was observed in normal breast, lung and colon tissues. It must be noted that the number of normal spots available for scoring were considerably less than their corresponding cancer spots. Therefore, we need to validate our normal tissue expression data further.
Figure 25. Expression of PSMC5 parallels PDA progression. A. Representative tissue sections from the J9988 study stained for PSMC5 are shown. N and C denote regions with normal duct cells and cancer cells, respectively. B. PDA glands express both nuclear and cytoplasmic PSMC5. However, minimal staining is evident in non-neoplastic pancreatic ducts.
Figure 26. Expression of MYPT1 parallels PDA progression. A. Representative tissue sections from the J9988 study stained for MYPT1 are shown. N and C denote regions with normal duct cells and cancer cells, respectively. B. PDA glands express both membrane and cytoplasmic MYPT1. However, minimal cytoplasmic staining and no membrane staining is evident in non-neoplastic pancreatic ducts.
Figure 27. Expression of TFRC parallels PDA progression. A. Representative tissue sections from the J9988 study stained for TFRC are shown. N and C denote regions with normal duct cells and cancer cells, respectively. B. PDA glands express both membrane and cytoplasmic TFRC. However, no cytoplasmic or membrane staining is evident in non-neoplastic pancreatic ducts.
Figure 28. Validation study showing that PSMC5 expression parallels PDA progression. A. Representative tissue sections from the J0810 study stained for PSMC5 are shown. N and C denote regions with normal duct cells and cancer cells, respectively. B. PDA glands express both nuclear and cytoplasmic PSMC5. However, minimal cytoplasmic staining and no nuclear staining is evident in non-neoplastic pancreatic ducts.
Figure 29. Validation study showing that MYPT1 expression parallels PDA progression. A. Representative tissue sections from the J0810 study stained for MYPT1 are shown. N and C denote regions with normal duct cells and cancer cells, respectively. B. PDA glands express both membrane and cytoplasmic MYPT1. However, no cytoplasmic or membrane staining is evident in non-neoplastic pancreatic ducts.
Figure 30. Validation study showing that TFRC expression parallels PDA progression. A. Representative tissue sections from the J0810 study stained for TFRC are shown. N and C denote regions with normal duct cells and cancer cells, respectively. B. PDA glands express both membrane and cytoplasmic TFRC. However, no cytoplasmic or membrane staining is evident in non-neoplastic pancreatic ducts.
Figure 31. MYPT1 is a potential goblet cell marker. Representative tissue sections from the J9988 study stained for MYPT1 are shown. MYPT1 strongly stained goblet cells of the duodenum in the PDA tissue section.
Figure 32. PSMC5 staining in immune cells surrounding PSMC5-expressing PDA glands. Representative tissue sections from the J0810 study stained for PSMC5 are shown. C and LA denote regions with cancer cells and lymphoid aggregates, respectively.
Figure 33. Comparing cytoplasmic and membranous MYPT1 staining in PDA versus biliary, lung, liver, colon and breast cancers. Both cytoplasmic as well as membranous expression of MYPT1 seems to be PDA-specific.
Figure 34. Comparing cytoplasmic and nuclear PSMC5 staining in PDA versus biliary, lung, liver, colon and breast cancers. Nuclear expression of PSMC5 seems to be PDA-specific. However, cytoplasmic expression of PSMC5 is not PDA-specific and 72% lung, 88% breast, 59% liver and 48% colon cancers express PSMC5 in its cytoplasm.
Figure 35. Snapshot of MYPT1 expression in 2 biliary cancer tissue microarrays. 54\% of biliary cancers express cytoplasmic MYPT1 but only 1\% express membranous MYPT1.
Figure 36. Snapshot of MYPT1 expression in 4 colon cancer tissue microarrays. 32% of colon cancers express cytoplasmic MYPT1 and 28% express membranous MYPT1.
Figure 37. MYPT1 expression in 3 different subtypes (basal, HER-2 positive and ER positive) of breast cancer. A. Snapshot of MYPT1 expression in 3 breast cancer tissue microarrays. 19% of breast cancers express cytoplasmic MYPT1 but only 2% express membranous MYPT1. B. Cytoplasmic MYPT1 staining differs with different breast cancer subtypes – Basal (triple-negative, 6%), HER2+ (13%) and ER+ (39%). However, the membrane MYPT1 staining was very little in ER+ breast cancer (6%) and no membranous staining was observed in basal or HER2+ breast cancers.
Figure 38. Snapshot of MYPT1 expression in 1 lung cancer tissue microarray. Only 5% of lung cancers express cytoplasmic MYPT1 but none express membranous MYPT1.
Figure 39. Snapshot of MYPT1 expression in 4 liver cancer tissue microarrays. No cytoplasmic or membranous MYPT1 expression was observed in liver cancers.
Figure 40. PSMC5 expression in 3 different subtypes (basal, HER-2 positive and ER positive) of breast cancer. A. Snapshot of PSMC5 expression in 3 breast cancer tissue microarrays. 88% of breast cancers express cytoplasmic PSMC5 but only 35% express nuclear PSMC5. B. Cytoplasmic PSMC5 staining was similar between the different breast cancer subtypes - Basal (triple-negative, 94%), HER2+ (82%) and ER+ (88%). However, nuclear PSMC5 staining was most in basal (47%), followed by HER2+ (38%) and the least in ER+ (24%) breast cancers.
Figure 41. Snapshot of PSMC5 expression in 1 lung cancer tissue microarray. 72% of lung cancers express cytoplasmic PSMC5 but only 21% express nuclear PSMC5.
Figure 42. Snapshot of PSMC5 expression in 4 liver cancer tissue microarrays. 59% of liver cancers express cytoplasmic PSMC5 but only 14% express nuclear PSMC5.
Figure 43. Snapshot of PSMC5 expression in 2 biliary cancer tissue microarrays. 22% of biliary cancers express cytoplasmic PSMC5 but only 4% express nuclear PSMC5.
Figure 44. Snapshot of PSMC5 expression in 4 colon cancer tissue microarrays. 48% of colon cancers express cytoplasmic PSMC5 but only 4% express nuclear PSMC5.
Table 5. PSMC5, MYPT1 and TFRC expression data from 2 clinical trials, J9988 and J0810. Number of patient tissue samples evaluated is denoted by n.

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CHAPTER 6: CONCLUDING THOUGHTS

Conclusions

We have developed a novel quantitative proteomics approach, SASI. The application of the SASI approach has yielded in the identification of PSMC5, MYPT1 and TFRC that elicit antibody responses in vaccinated PDA patients. Furthermore, an increased antibody response to these proteins post-vaccination is strongly correlated with vaccinated patients with a favorable clinical outcome (improved DFS). Expression studies using patient tissue samples have elucidated that cytoplasmic PSMC5, MYPT1 and TFRC expression as well as nuclear PSMC5 and membranous MYPT1 and TFRC is preferentially expressed in PDA compared to normal pancreas. Tissue microarray analyses have revealed that expression of nuclear PSMC5 as well as cytoplasmic and membranous MYPT1 is highly specific for PDA compared to other cancers. Collectively this data shows that PDA express the PSMC5, MYPT1 and TFRC proteins that appear to be the targets of anti-tumor host response following GVAX therapy.

There are several important implications of our findings. First, the SASI approach can be used to identify proteins that have a differential sera-antibody response among different patients or pre- and post-treatment from the same patient. The SASI approach can help identify prognostic/predictive biomarkers and assist in the improved outcome of the various ongoing immunotherapy clinical trials. Second, the presence of an antibody response post-vaccination to PSMC5, MYPT1 and TFRC can help predict the long term outcome of the patients receiving GVAX. Third, PDA-specific staining of cytoplasmic
and membranous MYPT1 and nuclear PSMC5 can be coupled to the current PDA diagnosis protocol to improve specificity and sensitivity in successfully diagnosing PDA. Fourth, these markers also hold potential to serve as novel therapeutic targets for PDA treatment. Future studies need to test the efficacy of monoclonal antibody targeting these proteins in treating PDA.

**Future directions**

In addition to applying traditional or advanced seroproteomic approaches, there are several advancements in the field that can be employed to discover novel tumor-specific antigens. Furthermore, the therapeutic potential of antibody therapies targeting identified cancer-specific antigens (old or new) can be enhanced by utilizing the newer antibody engineering technologies.

**Novel approaches to identify native anti-tumor antibodies**

Based on recent advances, it is now possible to produce recombinant monoclonal antibodies from single B cells of human origin\textsuperscript{133-135}. These recombinant antibodies can potentially serve as new monoclonal antibody therapy options for PDA. Briefly, this method involves separating single B cells by flow cytometry, followed by amplification of the different immunoglobulin chains via reverse transcription polymerase chain reaction (RT-PCR). These amplified genes are cloned into an expression vector to produce human monoclonal antibodies \textit{in vitro}. One can imagine an application of this B cell technology to identify and produce monoclonal antibodies for therapeutic use in PDA. For example, single B cells, obtained from laser-capture microdissected PDA
tissue\textsuperscript{136} or from the tumor draining node followed by flow cytometry, could provide a rich source of diverse antibodies that are PDA-specific. However, similar to the validation steps for the seroproteomic approaches, this method necessitates thorough \textit{in vitro} and \textit{in vivo} studies to ensure that the recombinant antibodies produced indeed bind to cancer cells with high specificity and are effective anti-tumor agents. It is also important to note that the isotype of the antibody used should be carefully examined because a change in an antibody’s isotype can dramatically change its efficacy\textsuperscript{137}.

Lung cancer studies have also used a modified serologic approach where tumor-specific antibodies from tumor infiltrating B cells were used instead of the antibodies from the patient sera\textsuperscript{138}. It was shown that the change in antibody source resulted in fewer autoantigens being identified that were also expressed on normal cells and hence, an increased specificity towards identifying cancer-specific antigens was observed. A similar study in colorectal cancer has also shown that tumor infiltrating B cells are active and secrete tumor antigen-specific immunoglobulins\textsuperscript{139}. Thus, using antibodies from the tumor microenvironment, rather than the sera antibodies, may provide a more direct way of identifying biologically relevant PDA-specific antigens that could be targeted using monoclonal antibody therapies.

\textit{Advanced antibody engineering technologies}

In addition to discovering new PDA antigens that could be targeted, recent advancements in antibody engineering, including the technology developments for antibody-drug conjugates, antibody-cytokine fusions and bispecific/trispecific antibodies, could be utilized for PDA treatment.
**Antibody-drug conjugates (ADCs)**

The effectiveness of chemotherapies is thwarted by the ineffective drug delivery to the cancer site and the systemic toxicities observed due to the non-discriminatory nature of these therapies. ADCs help overcome both this hurdle. ADCs combine monoclonal antibody to a cytotoxic drug via a stable labile linker. Thus, when the antibody binds the cancer-specific antigen, it is internalized, where the attached drug is released only to specific antigen-expressing cells. Once released, the drug can hinder cell signaling pathways, leading to cytotoxicity. ADCs are thought to reduce the systemic side effects caused by the cytotoxic drugs by the increased specificity and thereby provide a greater therapeutic index. Preclinical and clinical trials using ADC alone have produced better anti-tumor outcomes compared to the trials involving the corresponding unconjugated monoclonal antibody.\(^{140-143}\).  

The monoclonal antibody can be attached to radioactive particles, chemotherapy drugs or cell toxins. Ibritumomab tiuxetan and iodine-tositumomab are radiolabeled monoclonal antibodies used for the treatment of B-cell non-Hodgkin’s lymphoma.\(^{143}\) FDA has approved brentuximab vedotin and trastuzumab emtansine for the treatments of lymphomas and HER2-positive breast cancers, respectively.\(^{144}\) Brentuximab vedotin links anti-CD30 monoclonal antibody to an anti-mitotic agent, monomethyl auristatin E. Trastuzumab emtansine connects anti-HER2 antibody to maytansine (DM-1), an anti-microtubule agent.\(^{145}\) Trastuzumab emtansine was shown to have greater efficacy in the clinic with reduced toxicities. The success of trastuzumab emtansine has paved the path
for the development of several ADCs in the treatment of blood and solid tumors, including PDA\textsuperscript{146}.

Preclinical studies have produced favorable results for mesothelin-targeting ADCs: MDX-1204 and BAY 94-9343\textsuperscript{58}. MDX-1204 is a human anti-mesothelin antibody, MDX-1382, that is linked to duocarmycin, a DNA damaging agent. BAY 94-9343 is a fully human anti-mesothelin antibody that is connected to DM4, an anti-tubulin agent. Both these ADCs are effective anti-tumor agents specific for mesothelin-expressing cells, including PDA, \textit{in vitro} and \textit{in vivo}. Thus, a phase I study testing the acceptable dosage and safety of BAY 94-9343 in advanced solid tumor patients is ongoing.

Cantuzumab mertansine is an ADC that links the humanized huC242 monoclonal antibody to DM-1\textsuperscript{147}. HuC242 binds specifically to CanAg, an antigen that is predominantly expressed on pancreatic, colon and biliary tumors but is expressed at very low levels on normal tissues. \textit{In vivo} studies have demonstrated the improved anti-tumor effects of cantuzumab mertansine over unconjugated huC242 alone or in combination with other chemotherapy options. Subsequent phase I trials have shown cantuzumab mertansine to be a well-tolerated treatment option, providing the rationale for future studies in PDA patients expressing CanAg\textsuperscript{147-149}.

Epithelial cell adhesion molecule (EpCAM) is highly expressed in several solid tumors, including PDA. It is also expressed by PDA stem cells. Although adecatumumab, an anti-EpCAM antibody, was shown to be safe in solid tumor patients, a phase II trial failed to show tumor regression in metastatic breast cancer patients. Thus, an ADC, chiHEA125-Ama, was developed\textsuperscript{140}. This ADC links the chimeric anti-EpCAM antibody,
chiHEA125, to amanitin, an inhibitor of DNA transcription. It was shown to be an effective anti-tumor agent *in vitro* and *in vivo* and thereby, warranting further evaluation through clinical trials.

**Antibody-cytokine fusions**

In addition to linking antibodies to drugs, strategies where antibodies are fused to anti-tumor cytokines, such as interleukin-2 (IL-2) or tumor necrosis factor-alpha (TNF-α), have also been tested. Systemic administration of IL-2 has serious toxicities in patients and reduced effectiveness due to its fast clearance from the body. However, tumor-specific administration, via antibodies, has shown to be clinically favorable. L19-IL2 is a fusion protein, where IL-2 is fused to a single-chain variable antibody fragment (L19) that binds specifically to extradomain B (ED-B) of fibronectin. ED-B is expressed on tumor vasculature and stroma whereas it is absent from the fibronectin in the plasma and normal tissues (except certain female reproductive organs). L19-IL2 is biodistributed in a tumor-specific manner *in vivo* and in patients. Enhanced anti-tumor effects and slow clearance rate was observed in preclinical studies. A phase I clinical trial showed that L19-IL2 was well-tolerated in patients with solid tumors. Subsequent animal and patient studies have further shown the efficacy of L19-IL2 in treating hematologic and solid cancers when used in combination with chemotherapies, immunotherapies and other monoclonal antibodies. The recruitment of anti-tumor immune cells following treatment of L19-IL2 in the PDA stroma coupled with the strong PDA-specific expression of ED-B provides a strong rationale for testing L19-IL2 in PDA patients (Table 1).
Bispecific/trispecific antibodies

Bispecific antibodies are recombinant antibodies that can bind two different antigens at the same time. These antibodies can activate/inhibit multiple pathways and increase avidity-based binding by involving two different receptors on cell surfaces. The most commonly used bispecific antibodies recruit effector T cells to the tumor cells by dually binding CD3 on T cells and cancer-specific overexpressed antigens such as EGFR and EpCAM\textsuperscript{156, 157}. Catumaxomab, a bispecific anti-EpCAM and anti-CD3 antibody, has shown to inhibit tumor growth and proliferation in hepatocellular carcinoma xenografts\textsuperscript{158}. Catumaxomab inhibited tumor growth of PDA xenografts and stem cells. It was also able to enhance effector cytokine production and extend the contact time between the T cells and the cancer cells \textit{in vivo} and \textit{in vitro}\textsuperscript{159, 160}. Catumaxomab has received approval in Europe for use in the treatment of malignant ascites in EpCAM-expressing cancer patients\textsuperscript{161, 162}. A multicenter, phase I/II trial testing catumaxomab for treatment of peritoneal carcinomatosis in cancer patients, including PDA, has produced favorable clinical outcomes and safety profile\textsuperscript{163}. Ongoing efforts aim to develop novel bispecific antibodies not only targeting other PDA-specific antigens, such as CD133 (a PDA stem cell marker)\textsuperscript{164} but also recruiting other T cell populations (such as γδ T-cells)\textsuperscript{165} to the site of action. Bispecific antibodies also provide an alternate solution to overcome the challenges seen with monoclonal antibody therapies such as cetuximab. Bispecific antibodies that are both anti-EGFR and anti-CD3 have shown promising anti-tumor activity at a reduced dosage when compared to cetuximab alone \textit{in vivo}\textsuperscript{166}. With advances in antibody engineering technologies, trispecific antibodies are also being developed and evaluated for use in different cancer treatments\textsuperscript{167-170}. 

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A path to cure PDA

Despite the numerous preclinical studies showing strong efficacy data for new monoclonal antibody therapies, similar successes have not been mirrored in clinical trials. Furthermore, favorable phase I and II clinical trials have not translated into successful phase III studies. PDA is a very complex cancer where not only the tumor cells, but also the tumor microenvironment, contribute substantively to PDA initiation, progression and metastasis. This complexity helps explain why single agent antibody therapies are not successful. In addition, the lack of predictive and response biomarkers prevent the selective treatment of PDA patients that are most likely to benefit from these therapies. Thus, further work needs to be done in two critical areas to help make monoclonal antibody therapies a success for PDA patients. First, combination therapies need to be developed. Using serologic approaches and new recombinant antibody producing methods, novel PDA-specific antigens need to be identified and monoclonal antibodies need to be developed. Advances in the production of antibody-drug conjugates, antibody-cytokine fusions and bispecific/trispecific antibodies will help evolve antibody therapy further. Combination therapy should involve multiple antibodies targeting both intracellular signaling pathways within the cancer cell as well as intercellular pathways between the stroma and the cancer cell. This approach can also be coupled to existing and new chemotherapies, radiation therapies and vaccines. Using this multipronged approach, antibodies against the stromal targets will degrade the stroma and the chemotherapy or the antibody targeting the cancer cell will be effectively delivered to the tumor cell, thereby killing it. At the same time, antibody therapies targeting the immune cells will make the immune environment less suppressive, thereby allowing the effector cells to
mount an effective immune response against the cancer. Second, predictive markers need to be developed for the various antibody therapies. Doing so will enable PDA patients to be screened for the marker or a panel of markers, and get directed to the most effective treatment or at least know what treatments they will not respond to without wasting time and hope.

We are experiencing a technological revolution that has allowed the therapeutic antibody field to make great progress in many cancers. Taking PDA’s unique biology into account when developing antibody approaches is key to the success of antibody therapy for this difficult cancer.
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Educational History
Ph.D. expected 2014 Pharmacology and Molecular Sciences
Mentor: Dr. Elizabeth M. Jaffee
Johns Hopkins School of Medicine, Baltimore, MD

B.S. 2008 Molecular and Cellular Biology
Minor in Chemistry
University of Illinois, Urbana-Champaign

Professional experience
Thesis project
2009 – 2014 Lab of Dr. Elizabeth M. Jaffee, Johns Hopkins School of Medicine

Research rotations
2009 – 2009 Lab of Dr. Heng Zhu, Johns Hopkins School of Medicine
2008 – 2009 Lab of Dr. Elizabeth M. Jaffee, Johns Hopkins School of Medicine
2008 – 2008 Lab of Dr. Jun O. Liu, Johns Hopkins School of Medicine

Undergraduate research
2004 – 2008 Lab of Dr. Martha U. Gillette, University of Illinois, Urbana-Champaign

Summer Internship
2007 – 2007 Department of Antimicrobial Product Development, Stepan Company

Awards and honors
Johns Hopkins School of Medicine, Baltimore, MD
2012 Scheinberg Travel Award
2012 Graduate Student Association Travel Award
2011 Student Recognition Award

University of Illinois, Urbana-Champaign
2008 Magna Cum Laude
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Posters


**Publications**
