SSX: Understanding an Antigen for Therapy in Prostate Cancer

by

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Abstract

Prostate cancer is the most commonly diagnosed malignancy for men in the United States. Fortunately, radiation and prostatectomy are curative in the majority of cases for this slow growing disease. However, metastatic prostate cancer, the lethal form of the disease, has a life expectancy of approximately five years. Identification of factors associated with this transition to metastatic disease are crucial for future therapies. One such factor is the SSX gene family, a family of cancer/testis antigens (CTA) transcription factors which have been shown to be aberrantly expressed in cancers and associated with the epithelial to mesenchymal transition (EMT). In order to target SSX in prostate cancer, we must first fully understand it. Thus, main aims of the following thesis are threefold: characterization of SSX expression, characterization of SSX function, and finally characterization of SSX specific CD8^{+} T cells. We have previously shown that SSX expression in prostate cancers was restricted to metastatic tissue and not primary tumors, however the specific SSX family members expressed was unknown. In this thesis, we have identified SSX2 as the predominant SSX family member expressed in prostate cancer, and found its expression in the peripheral blood of 19 of 54 (35%) prostate cancer patients, with expression restricted to circulating tumor cells, and in 7 of 15 (47%) metastatic cDNA samples. Further, we examined SSX2 function in prostate cancer through knockdown and overexpression in prostate cancer cell lines. While overexpression had little effect on morphology or gene transcript changes, knockdown of SSX2 resulted in an epithelial morphology, increased cell proliferation, increased expression of genes involved in focal adhesion, decreased anchorage independent growth, increased invasion, and increased tumorigenicity *in vivo*. We conclude from these findings that SSX2 expression in prostate cancer is not a driver of EMT, but is involved in processes associated with EMT including loss of focal adhesion that may related to tumor cell

dissemination. Finally, we previously identified the HLA-A2 restricted epitopes of SSX2 and determined SSX specific CD8 T cells were significantly more common in late state prostate cancer patients. We identified patients with SSX specific CD8$^+$ T cells n=15. Then, as before, assayed these patients for the presence of SSX expressing CTCs. We found patients with SSX expressing circulating tumor cells (CTCs) also possessed SSX specific CD8$^+$ T cells (6/15), while other patients possessed SSX specific CD8$^+$ T cells but not SSX CTCs (9/15). We sought to determine the cause of this duality, and further characterize SSX specific CD8$^+$ T cells. We determined that checkpoint molecule regulation was unlikely to be the cause of this duality. Further, we found SSX specific CD8+ T Cells in patients without SSX expressing CTCs expressed more T_h1 biased cytokines than those patients who possessed SSX CTCs. This finding indicated that a T_h1 biased immune response from SSX specific CD8$^+$ T cells eliminated SSX expressing CTCs.

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This thesis is dedicated to my family, my friends and my felines

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CHAPTER ONE: Introduction

Prostate Cancer and Therapy Thereof

Prostate Cancer (PCa) is an extremely common malignancy, which the CDC lists as most common cancer amongst men(1). In 2013, approximately 176,000 men were newly diagnosed, and 27,000 men died from PCa(1). Fortunately, the therapies for prostate cancer, radiation and prostatectomy are curative in the majority of cases. However, some patients relapse and develop metastatic castration resistant prostate cancer (mCRPC) which has a life expectancy of less than 3 years(2). Thus, as researchers we seek both better understanding and treatment for mCRPC.

To understand how to improve treatments for mCRPC we must first understand the course of treatment a patient may go through after the initial diagnosis of prostate cancer. According to the ACS(3), when a patient is newly diagnosed they will undergo what is known as "Watchful waiting or active surveillance", which means monitoring the patient and prostate for evidence of progression, this procedure is implemented due to prostate cancer's typical slow growing nature. If the physician monitoring the patient deems the disease is beginning to progress then the patient will receive radiation or prostatectomy depending on each individual case(3), as mentioned previously this treatments are curative in the majority of cases. Unfortunately, for some patients the cancer will return when initial radiation/prostatectomy are not successful. If this is the case, then the patient may be given hormone therapy, with agents such as Degarelix or Abiraterone(3). However, there are many side effects to hormone therapy including: loss of muscle mass, weight gain, depression, erectile dysfunction, anemia, and osteoporosis. Finally, if a patient has developed metastases and prior therapies have proven ineffective or failed, then chemotherapeutic agents such as Docetaxel are used to treat the disease. Like hormone therapy, chemotherapy has many side effects including: hair loss, nausea, vomiting, and increased chance of infections. Unfortunately, chemotherapy and hormone therapy

are unlikely to cure prostate cancer, and only will slow the growth of the cancer(3). Apart from chemotherapy there is one federally approved vaccine against prostate cancer, Sipuleucel-T(4). In this therapy, a patient's white blood cells (primarily dendritic cells) are extracted through leukapheresis. These cells are then co-incubated with peptides for prostatic acid phosphasetase (PAP) which is present in 95% of prostate cancer cells, and granulocyte-macrophage colony stimulating factor (GM-CSF). Presumably, these dendritic cells, will be primed to display PAP peptides to T and B cells of the patients' immune system, thus initiating a specific attack against cells expressing the PAP protein(4). Additionally, Sipuleucel-T offers less severe side effects than chemotherapy. Unfortunately, Sipuleucel-T has only been shown to extend patient survival on the order of months, while its cost is over $100,000. Due to the ultimately ineffective nature of mCRPC therapies (hormone therapy,chemotherapy, or Sipuleucel-T), it is obvious that as researchers we need to find more efficacious ways to treat mCRPC.

The McNeel Laboratory and DNA Vaccines as a cancer therapeutic

As I have briefly listed above, while prostate cancer is slow growing, and generally treatable with radiation and/or prostatectomy, treatments for advanced prostate cancer are ineffective, cause many side effects, and come at a high cost. One potential avenue to both reduce costs and side effects are DNA vaccines. Put simply, DNA vaccines are bacterial plasmids containing the coding nucleic acid sequence of a target antigen under the control of a eukaryotic promoter. The advantages to DNA Vaccines are numerous. DNA vaccines require no direct handling of potentially dangerous pathogen or toxin, easily modified to any potential vaccine targets, stable at room temperature, and cheap to mass manufacture (5-6). Due to these advantages, the McNeel laboratory is focused on the creation and utilization of DNA vaccines for the therapy of prostate cancer. Ongoing trials in the McNeel laboratory includes: a phase II

trial for a DNA vaccine encoding PAP, a phase II of a DNA encoding PAP with and without Sipuleucel-T, a phase II of a DNA encoding PAP with the anti-PD-1 antibody Pembrolizumab, and a phase I of a DNA vaccine encoding the androgen receptor (AR). In addition to investigations against PAP and AR, the McNeel group has also investigated the antigen SSX2. Dr. Heath Smith constructed a DNA vaccine encoding the antigen SSX2. Additionally, he performed preclinical work in mice and found an SSX encoding vaccine could both provide protection and therapy against a tumor that overexpressed SSX(5). Further, he determined the HLA-A2 specific regions of the gene(6), which will be discussed in greater detail later in this introduction.

Immunotherapy has great potential for cancer therapy

In addition to DNA vaccination, immunotherapy represents a highly attractive strategy for the treatment of cancers. Put simply, immunotherapy is the utilization of the host's own immune system for the treatment of cancer. This is in opposition to the use of chemotherapy or radiation, which aim to destroy all cells (including host cells) in the vicinity of the tumor. Cancer immunotherapy was declared as *Science*'s breakthrough of the year in 2013(7). Within immunotherapy there are several different approaches: cytokine therapy, vaccination, autologous cell infusion, CAR-T cells, bispecific T cell engager, checkpoint antibodies, and microenvironment disruption(8). I will detail each briefly below and their history of use in PCa if applicable(8):

Cytokine therapy: Briefly, cytokines are small proteins that cells use to communicate. One function of cytokines is the maturation and proliferation of immune cells. Thus, if patients receive cytokines responsible for T cell maturation and proliferation (e.g. IL-2) hypothetically the immune system becomes more "active" and better able to generate T and B cells specific for

a tumor. In practice there have been phase I trials investigating treatment with IL-2 alone(9) and in combination with IFN-alpha in prostate cancer(10). However, these trials demonstrated little efficacy.

Vaccination: Simply, this is the utilization of a vector to prime the immune system into targeting pathogens. There are several strategies of immunotherapy vaccination. GVAX-PCa, was a mixture of irradiated PC-3 and LNCaP prostate cancer cells line(11) which was ultimately investigated in two phase III trials that did not demonstrate more clinical efficiency over chemotherapy(12). PSA-TRICOM, is a vaccinia and fowlpox vector encoding prostate specific antigen (PSA)(13,14). Importantly, the phase I/II trials conducted through the McNeel lab investigating DNA vaccines are another example of immunotherapy vaccination. I have only mentioned three different vaccination strategies in this introduction, but there are potentially limitless possibilities.

Autologous cell infusion: An example of autologous cell infusion is Sipuleucel-T which was described earlier in this introduction(4). This strategy is the extraction of the host's own immune cells, and then expansion and/or priming the cells with a specific antigen.

CAR T Cells: These are T-cells where the TCR has been modified to recognize specific tumor antigens(15). This strategy has proven very successful in B-cell malignancies(16).

Bispecific T cell engagers: This strategy consists of the combination of binding domain of 2 antibodies. One binding domain is specific for T cells, while the other specific for the desired antigen of interest. The concept behind this strategy is to physically force the interaction between T cells and antigen(17). There is currently two phase I trials underway in prostate cancer. (ClinicalTrials.gov identifiers NCT01723475 and NCT00635596)

Checkpoint blockade therapies: Currently, there are two T-Cell checkpoint blockade therapies approved by the FDA for the treatment of cancer, with many more in phase I/II trials. The first FDA approved therapy is a humanized antibody against CTLA-4, ipilimumab, is approved for treatment of metatstatic melanoma(18). CTLA-4 is a checkpoint molecule that competes with CD28 to bind CD80/86. CD28 binding CD80/86 causes T cell proliferation, cytokine production, and T cell survival. However, CTLA-4 binding CD80/86 results in negative regulation of T cell mediated immune responses. Thus, the aim of anti CTLA-4 therapy and checkpoint blockade therapy is the disruption negative regulation of the immune response. The second FDA approved therapy are two different humanized antibodies against the checkpoint molecule PD1, pembrolizumab and nivolumab. Nivolumab has been approved for renal cell carcinoma(19), and Hodgkin lymphoma(20). Pembrolizumab is approved for advanced melanoma(21), non-small cell lung cancer(22), and head and neck squamous cell carcinoma(23). Similarly to CTLA-4, when PD-1 encounters its ligand PD-L1 the result is reduced T cell proliferation, and reduced IFNy secretion, thus blocking this interaction results in an increased immune response. With the success of these checkpoint blockade therapies, it is obvious that further understanding the regulation status of specific CD8 T cells could potentially open avenues for therapy in many different cancers.

Microenvironment disruption: Finally, microenvironment disruption consists of agents designed to disrupt or otherwise modify the immunosuppressive tumor microenvironment, making it more amenable to a cytolytic immune response. Many tumors are infiltrated by regulatory T cells and/or myeloid-derived suppressor cells (MDSCs), which have been shown to repress antitumor immune responses(24). Tumors also are known to have altered or disorganized vasculature, often not expressing the appropriate ligands necessary for immune cell trafficking.

Agents designed to disrupt the tumor vasculature and/or deplete tumor-infiltrating regulatory cells have been shown to have antitumor activity in many cancer types. An example is subitinib, which has been shown to inhibit tumor angiogenesis and deplete MDSCs from the tumor microenvironment(25). A phase III trial of sunitinib in prostate cancer demonstrated increased progression free survival, but did not affect overall survival compared to placebo(26).

SSX, Cancer Testis Antigens, and their potential as therapy targets

The SSX Family: Origins and Domains

SSX is a 10 member transcription factor family, with a very high degree of homology amongst them(27). SSX was originally discovery through cytogenetic studies of synovial sarcoma, in which 70% of both biphasic and monophasic synovial sarcomas had the same characteristic translocation event translocation event, *t*(X;18)(p11.2;q11.2)(28,29). This translocation event eventually was clarified to be between a gene now known as **SS**18 (**S**ynovial **S**arcoma Translocation) and SSX(**S**ynovial **S**arcoma **X** chromosome breakpoint) (30). Later, Tureci et al identified SSX as the cancer testis antigen HOM-Mel-40 through SEREX methodology(31). Sequencing additional synovial sarcoma cDNA clones of this fusion site, Crew et al. found that the C-terminal regions of two distinct genes, designated SSX1 and SSX2, can become fused to the N-terminus of SS1(32). A study by Chand, using pulse field analysis, revealed that there might be as many as 5 copies of the SSX gene(33). SSX3 was found by screen a testis cDNA lbrary(34). SSX4/5 were identified by southern blot analysis using PCR and restriction map analysis(35). SSX6 was identified through database searches(36). SSX7/8/9 were identified by Gure et al through screening and sequencing of a placenta genomic database(27), in addition 10 pseudogenes that mapped to the X chromosome were also discovered through the same methodology. Finally, SSX10 has been annotated in GenBank

(GeneID: 100128582), but to date no information about its expression in normal or malignant tissues has been described. The sequence homology of these family members is between 73%-92%(35)

SSX has two highly homologous domains, the Kruppel-associated box (KRAB) and SSX repression domain (SSXRD). The presence of the KRAB domain was identified in the initial screen of the SSX gene as the KRAB domain is a well-characterized domain. The KRAB domain has been shown to have transcriptional repression activity(32,37). Lim et al discovered the SSXRD by first examining the repressor activity of the SSX KRAB domain alone, compared with the repressor activity of the full length sequence. They determined the last 33 AAs of the C-terminal domain of SSX, was a highly conserved region they named the SSXRD(38). It has been suggested that the two domains may complement their activity. Taken together, the functions of these domains, and IHC staining demonstrating nuclear localization(6), indicates the SSX family are transcription factors.

Binding partners of the SSX family

Using yeast two-hybrid assays and glutathione-S-transferase (GST) pull-down assays, de Bruijn et al. identified the two proteins that directly associated with SSX2(39). The protein were identified as a human homologue of the *Rattus norvegicus Rabin3* gene, *Rabin3* is thought to potentially encode Ras-like GTPase-binding proteins, but this protein has not been fully characterized. The second protein SSX2IP is a 71 kDa protein 614 amino acids long, which has homology to the mouse and rat afadin DIL domain-interacting protein (ADIP) gene. There has been some evidence of a link between SSX2IP and cancer. It was identified as a leukemia antigen through a SEREX screen(40), and was found to be expressed in 33% of leukemic cells from acute myeloid leukemia patients(41).

Soulez et al. determined through double immunofluorescence studies, that SS18-SSX2 colocalized with members of the polycomb group complex proteins (PcG) specifically to RING1, and BMI-1(42). Further research demonstrated that the SSXRD domain interacts with PcG proteins(43). Although, most studies on PcG have been in *Drosophila melanogaster*, their function is to remodel chromatin structure to enable epigenetic silencing(44). De Bruijn and colleagues using yeast two-hybrid studies and found that one of the clones pulled out encoded the LIM homeobox protein LHX4(45). This protein was previously shown to be deregulated or translocated in multiple forms of leukemia(46,47). LHX4 is a 390 amino-acid protein containing two LIM domains (LIM1 and LIM2), a homeobox domain (HOX), two zinc fingers, and was found to have a C-terminal tail with a novel transcriptional activation domain.

Cancer Testis Antigens and their applicability to cancer therapy

The first cancer testis antigen (CTA) (later known as MAGE1) was discovered through autologous typing and the application of a newly developed DNA-cloning methodology for determining the targets of T Cells from a melanoma patient who had an unusually favorable outcome(48,49). Further CTAs were identified through the use of SEREX(serological analysis of cDNA expression libraries)(50) including SSX,NY-ESO-1, and SCP-1. As these tumor antigens were discovered it was found that many of these were normally expressed in and restricted to the testis(51) thus this growing list of antigens was termed cancer/testis antigens.

Before I get into what makes CTAs good targets for therapy, let us first think about what would be a perfect target for vaccine. A hypothetical perfect antigen against cancer would have the following characteristics: Overexpressed in cancer, expressed in all cancer cells, and expression specific to cancer and not host. If researchers could find such an antigen then immune

responses generated against said hypothetical antigen would be specific only for cancer, whilst targeting all malignant cells without damaging host cells.

Fortunately, for cancer researchers, CTAs fill many of these criteria. As their name implies, many CTAs (including SSX) has their expression restricted to testis tissue which is immune privileged tissue(52). Put simply, the testis tissue is protected from attack by the immune system. Specifically testis tissue is immune privileged to protect sperm production from destruction by the host immune system, to ensure the production of progeny. This immune privilege is achieved through both physical and immunological means(53). One specific immunological method of protection is the low expression HLA class I molecules on the surface of testis tissue(53). This is important because it means that the host's own immune system will not recognize these antigens as host. Typically, T cells specific for self-antigens are eliminated through thymic selection, or express many checkpoint molecules to greatly restrict their immune response, as immune responses against the host's own proteins are a bad thing. According to RT-PCR analysis CTAs are frequently expressed, bladder, lung, ovarian, hepatocellular carcinoma, and melanoma(54). CTAs are not only expressed but highly expressed relative to other genes, a microarray of lung cancer identified 20 CTAs highly overexpressed relative to normal tissue (54). SSX expression has been found in 24 different cancer malignancies, however the most commonly detected SSX family members are SSX1 and SSX2(55). In summation, vaccines elicited against CTAs will be specific for tumor cells due to the immune privileged nature of testis tissue, and many CTAs are highly expressed in tumors, these characteristics make CTAs highly desirable therapeutic targets.

Due to their highly attractive nature as cancer targets, several groups are investigating CTA targeting vaccination. The MAGE-A family (56–58) NY-ESO-1 (59) and SSX(5) are

currently being investigated as targets for cancer therapy. Vaccines targeting these antigens have demonstrated both cellular and antibody responses. Unfortunately, in spite of several phase III clinical trials, there are currently no approved therapies for cancer targeting CTAs. This indicates the further study on CTAs is needed for future therapies.

SSX and the Epithelial to Mesenchymal Transition

The epithelial to mesenchymal transition (EMT) is a normal cellular process by which cells will undergo a morphological change from polarized epithelial cell to motile mesenchymal. This process is critical for embryogenesis, wound healing, and stem cell behavior (60). EMT is driven by key transcription factors including: *SNAI1*(snail),*SNAI2*(slug),zinc finger E-box-box-binding homeobox (ZEB), and *Twist1*(60). Signaling pathways, such as transforming growth factor-B (TGF-B), frequently initiate these changes(60). One commonly used marker of a cell transitioning from epithelial to mesenchymal is the loss of expression of the surface molecule E-Cadherin, and the subsequent expression of N-Cadherin. Many cancers will utilize EMT to promote metastatic formation. Metastasis is a multistep process characterized by dissociation of tumor cells from the epithelial layer, penetration through the basement membrane into adjacent connective tissue, intravasion and survival in the bloodstream, extravasion at a distal site and finally growth at that distal site(61,62). As the first steps are dependent on increased invasion and motility, the utilization of EMT pathways seem like an obvious choice. Researchers have demonstrated that the transcription factor *Twist1* plays a critical role in the early steps of metastasis, down regulation of *Twist1* through siRNA reduced the number of circulating tumors cells(63)

There is some evidence that SSX is involved in the epithelial to mesenchymal transition. The Brodin group has reported that SSX co-localizes with EMT associated proteins matrix

metalloproteinase 2 (MMP2) and vimentin (64). Additionally they found, in a melanoma cell line, that knockdown of SSX also results in downregulation of MMP2 and vimentin, which also resulted in decreased migration(64). Further, they found through knockdown of SSX using RNAi in melanoma and osteosarcoma, they found reduced proliferation mediated through MAPK/Erk and Wnt signaling pathways(65). SSX was found to form transient interactions with B-catenin, which resulted in altered expression of EMT genes E-cadherin, *SNAI2*, and vimentin. In addition to the Brodin group's findings on SSX and EMT, Chen et al found overexpression of SSX induced cell growth and cell invasion in a breast cancer cell line(66). They also found E-cadherin expression repressed in this model, indicating a shift towards a mesenchymal state.

SSX and prostate cancer

The McNeel group has reported on the entirety of findings of the SSX family in the context of prostate cancer. We found that SSX2 was the most commonly expressed family member in prostate cancer lines, and found expression of SSX1 and SSX5 after the use of DNA demethylating agent 5-aza-2'-deoxycytidine. Through tissue microarray, Dr. Smith found SSX expression in patient tumor samples was restricted to metastatic lesions (5/22; 23%) and no expression was detected in primary prostate tumors examined (0/73; $P < 0.001$)(6). Further, we discovered the HLA-A2 restricted epitopes of SSX2 peptides: p41-49 (KASEKIFYV) and p103-111 (RLQGISPKI). It was also found that peptide p103-111 was immunodominant(5). Importantly, Dr. Smith reported that SSX103 specific CD8^{+} T cells were significantly more common in late state cancer patients.

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CHAPTER TWO: Characterization of SSX expression in Prostate Cancer

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JEB designed and performed the experiments, analyzed the data, and prepared the manuscript; DGM supervised the concept, experimental design, data analysis, and manuscript preparation.

Abstract

In this study, we sought to determine the pattern of SSX expression in prostate cancer. We previously identified that SSX expression is confined to metastases and not primary tumors. We found that SSX1 and SSX2 were detected in the metastatic samples at rates of 1 of 15 (6%) and 7 of 15 (47%) respectively. Further, we found SSX2 expression in 19 of 54 (35%) peripheral blood samples, but did not detect expression of any other SSX family. In addition, we isolated a circulating tumor cell population (CD45$^-$/EPCAM$^+$/CD63$^+$) which expressed SSX. Given these data, we concluded that SSX2 is the primary SSX member involved in prostate cancer, and future studies of SSX in prostate cancer should focus on SSX2.

Introduction

Prostate Cancer is an extremely common malignancy that affects approximately 170,000 men in the United States every year(1). Fortunately, prostate cancer is frequently slow growing and most men afflicted with the disease will die from alternate causes. In early stages of the disease, prostatectomy and radiation therapy are curative in the majority of cases. However, about 1/3 of patients who undergo therapy will ultimately relapse and develop what is known as castration resistant metastatic prostate cancer (mCRPC), which has a life expectancy of less than 3 years(2). In addition, chemotherapy, and hormone therapy used to treat disease progression present substantial side effects, and only serve to slow the disease without cure(3). Thus given the poor options for therapy, and the low survival time of mCRPC, as researchers we seek better understanding of mCRPC in order to develop treatments targeting disease progression.

One group of proteins with considerable interest as targets of cancer therapy are the cancer/testis antigens (CTAs). CTAs as their name implies are expressed primarily on immune privileged testis tissue(4). Immune privilege occurs in the central nervous system, eyes, placentas, and testes, as a means by which the body can protect vital organs from attack by the immune system(4). Immune privilege is achieved through both physiological and immunological means, but we are primarily interested in the lack of HLA class I molecule expression on the cell surface(4). This means is that host does not generate a class I CTL immune response to proteins expressed in testis tissue, and importantly the host will not recognize SSX specific CD8^{+} T cells as recognizing host. As researchers, we can generate CTL responses that can only target cancer cells expressing a CTA, and not host. Due to the lack of HLA class I expression, host tissue would be entirely untargeted by T cells specific for CTAs, which is important in respect to limiting side effects of cancer therapies. CTAs have also been shown to be upregulated in many

cancers(5). CTA's expression in immune privileged tissue and overexpression in cancer makes them excellent targets for the therapy.

The SSX family of proteins are family of CTAs of interest for prostate cancer therapeutics. SSX was originally discovered due to a pathognomonic translocation event with the protein SS18 in synovial sarcoma(6,7). Further research has demonstrated the SSX family consists of ten highly homologous family members(SSX1-SSX10)(8). The function of SSX is still largely unknown. Although its conserved domains KRAB and SSXRD point to transcriptional activity(9).Some groups have linked SSX's function with the epithelial to mesenchymal transition(10–12). The epithelial to mesenchymal transition is a typical cellular process with causes the transition of stationary, polar, epithelial cells to change to motile, non-stationary, amorphous mesenchymal cells(13). Researchers will frequently look to expression of the proteins E-Cadherin and N-Cadherin as markers if a cell is epithelial (E-cadherin) or mesenchymal (N-cadherin)(14). Unfortunately, cancers frequently utilize different proteins and cell signaling pathways of EMT to establish metastases(15).

In order to design therapies targeting SSX in prostate cancer, we must first understand which SSX members are expressed in prostate cancer. The McNeel laboratory first identified SSX2's potential as a therapeutic target in prostate cancer through the utilization of SEREX(16). In that study, SSX2 was identified as the 2nd most common CTA recognized in prostate cancer patient sera, and that SSX2 expression increases in patients with metastatic disease compared to localized prostate cancer. Additional research from the McNeel lab has demonstrated that treatment of human prostate cancer cell lines with epigenetic modifying agents (EMAs) can lead to expression of other SSX family members, including SSX3, SSX5, and SSX8 (17). In other diseases such as Hodgkin's lymphoma (18), multiple myeloma (19), and head and neck cancer

(20) many different SSX family members are expressed. SSX1, SSX2, and SSX4 are expressed in Hodgkin's lymphoma and head and neck cancer, while SSX1, SSX2, SSX4, and SSX5 are expressed in multiple myeloma. In the case of multiple myeloma, expression of multiple SSX family members leads to worse prognosis and survival time (19). Further we found in a tissue microarray that SSX expression in patient tumor samples was restricted to metastatic lesions (5/22; 23%) and no expression was detected in primary prostate tumors examined (0/73; P < 0.001). However, even with this mountain of research into SSX and prostate cancer, it is still unknown which SSX family members are expressed in prostate cancer metastases.

In the following study we sought to determine the identity of the SSX family members expressed in prostate cancer, and determine the frequency each family member is expressed. This information can then guide future therapies that target SSX.

<div align="center">**Results**</div>

SSX2 was the most frequently expressed SSX family member in prostate cancer metastases

The SSX family of proteins consists of 10 highly homologous members (21,22). Previous work has demonstrated through IHC of a tissue microarray that one or more SSX proteins were detectable in metastases but not primary prostate cancer tumors (17). Given the homology among the SSX family members, the precise family member(s) expressed could not be determined in those studies. Therefore, we first evaluated metastatic tissues for the expression of each SSX family member by PCR. Using primers specific for each of the ten SSX family members (17), we screened cDNA obtained from 15 different prostate cancer metastases from different individuals (Fig. 1C). SSX1(Fig. 1A) and SSX2 (Fig. 1B) were detected in the metastatic samples at rates of 1 of 15 (6%) and 7 of 15 (47%) respectively (Fig. 1D). Expression of the other SSX family members was not detected. The one patient with detectable SSX1 expression also had detectable SSX2 expression (Fig. 1A and B), but in a separate lung metastases there was no expression of either SSX1 or SSX2.

SSX2 was detected in a CD45$^-$/EpCAM$^+$/CD63$^+$ cell population in patient peripheral blood

Since SSX protein was not previously detected in primary tumors, and has been implicated in EMT, we next evaluated for the expression of SSX in cells in peripheral blood samples. SSX2 was the only family member detected in the peripheral blood, and overall detected in 19 of 54 (35%) patient blood samples (Fig. 2A). Importantly, SSX2 expression was only found in patients with recurrent disease; however there was no association between prevalence of SSX2 expression and stage of recurrent disease, or serum PSA level (data not shown). Given these findings, we concluded that SSX2 is the SSX family member most relevant to prostate cancer. Since we

detected SSX2 mRNA in the peripheral blood of prostate cancer patients but not healthy controls, we assumed that the detection was of circulating tumor cells expressing SSX2, rather than, for example, cell-free tumor-associated RNA. Using fluorescence activated cell sorting (FACS), we separated cells into distinct populations of interest, then performed qPCR to analyze those populations for SSX2 expression. We found SSX2 expression was highly enriched in the CD45$^-$ (non-hematopoietic) fraction, as compared to CD45$^+$ control (Fig. 2B). Furthermore, SSX2 was specifically enriched in the CD45$^-$/EpCAM$^+$/CD63$^+$ subpopulation, which marks prostate-specific circulating tumor cells (23) while differentiating from erythroid progenitor CD45$^-$/EpCAM$^+$ cells (Fig. 2C). Further, patients with late stage disease (Fig. 2d) displayed significantly more of the CD45$^-$/EpCAM$^+$/CD63$^+$ subpopulation than those with early stage disease (Fig. 2E).

Discussion

In this study, we have shown SSX2 is the primary member of the SSX family expressed in prostate cancer, expressed in metastases and in circulating tumor cells. This expression pattern in metastases and in circulating tumor cells suggests SSX2 could be functioning, or at least is expressed, in tumor cells with the capacity to disseminate.

Given the high amino acid similarity of the SSX family of proteins, our previous studies could not readily distinguish which SSX family members were expressed in metastatic prostate cancer using immunohistochemistry (17). By using rtPCR we were able to demonstrate more definitively that SSX2 is the most frequently expressed member, while SSX1 is less frequently expressed (Fig. 1A and B). Prior work from our lab has demonstrated that treatment of human prostate cancer cell lines with epigenetic modifying agents (EMAs) can lead to expression of other SSX family members, including SSX3, SSX5, and SSX8 (17). However, in this study, none of these were detected in untreated human tissue samples. In other diseases such as Hodgkin's lymphoma (18), multiple myeloma (19), and head and neck cancer (20) many different SSX family members are expressed. SSX1, SSX2, and SSX4 are expressed in Hodgkin's lymphoma and head and neck cancer, while SSX1, SSX2, SSX4, and SSX5 are expressed in multiple myeloma. In the case of multiple myeloma, expression of multiple SSX family members leads to worse prognosis and survival time (19). While we did determine that one patient had expression of both SSX1 and SSX2 in the same lymph node metastasis (Fig.1 A and B), we believe SSX expression prostate cancer is largely limited to SSX2, which may imply a difference in function from other cancers. Interestingly, there was no increase in the prevalence of SSX2 expression in PBMC from patients with more advanced stages of recurrent disease (Fig.

2A), which conflicts with earlier findings which demonstrated IgG responses to SSX2 increased

with disease stage(16). We initially hypothesized that late stage patients (D2, D3) would see an

increase in SSX expression in the blood, as tumor burden and CTC load are correlative. This

finding implies that even small tumors are capable of shedding circulating tumor cells.

Additionally, we found SSX2 is expressed in a CD45$^-$/EpCAM$^+$/CD63$^+$ cell subset in the blood

of patients with prostate cancer (Fig. 2C), a cell subset specifically representing prostate-specific

circulating tumor cells (23). Thus, this is the first report of SSX2 expression in human CTCs.

Figures and Figure Legends

Figure 1 <u>SSX1 and SS2 are expressed in metastases from patients with prostate cancer</u>

cDNA libraries from 15 metastatic prostate cancer samples were evaluated for SSX gene expression using primers specific for each SSX family member. A)Agarose gel of SSX2 responses in metastatic cDNA samples B) Agarose gel of SSX1 responses in metastatic cDNA samples C) Table summarizing the ordering of samples in panels A and B, as well as site of metastases. Samples highlighted in the same color represent samples from the same patient. D) Summary of findings for all SSX family members in cDNA from metastatic tissues.

Sample #	Site of Metastasis	Name
04-050T	Lymph Node	LN1
04-050V	Lymph Node	LN2
04-149E	Lymph Node	LN3
05-214L	Lymph Node	LN4
05-217F	Lymph Node	LN5
06-047H	Lymph Node	LN6
06-127E	Lymph Node	LN7
08-020N	Lymph Node	LN8
05-011F	Liver	LV1
05-148E	Liver	LV2
06-081E	Liver	LV3
05-165K	Lung	LG1
06-047K	Lung	LG2
06-081I	Lung	LG3
05-221F	Bone	B
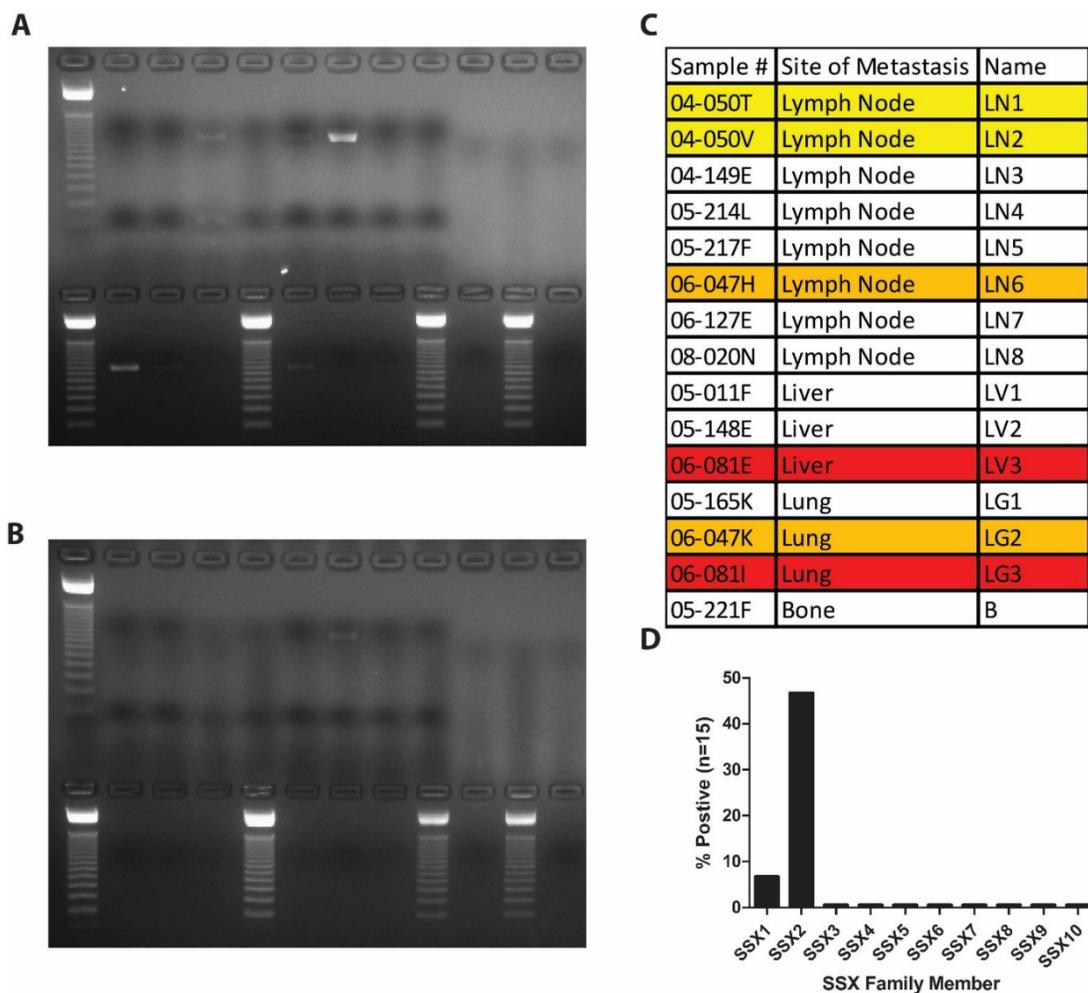
Figure 2. <u>A circulating tumor cell population expresses SSX2</u>

SSX2 mRNA was detected in the blood of patients with recurrent prostate cancer by PCR using primers specific for SSX2. Key: D0=non-castrate, non-metastatic; D0.5= castrate-resistant, non-metastatic; D2= castrate-sensitive, metastatic; D3= castrate-resistant, metastatic. PBMC previously found positive for SSX2 expression were FACS sorted based on expression of cell surface markers. Quantification of SSX2 expression was performed in CD45$^+$ or CD45$^-$ populations (A) and CD45$^+$ or CD45$^-$/EpCAM$^+$/CD63$^+$ (CTC) populations (B). *=P<0.05. Representative flow diagrams of isolated populations panel C is of a D3 patients and panel D is of a D0 patient.
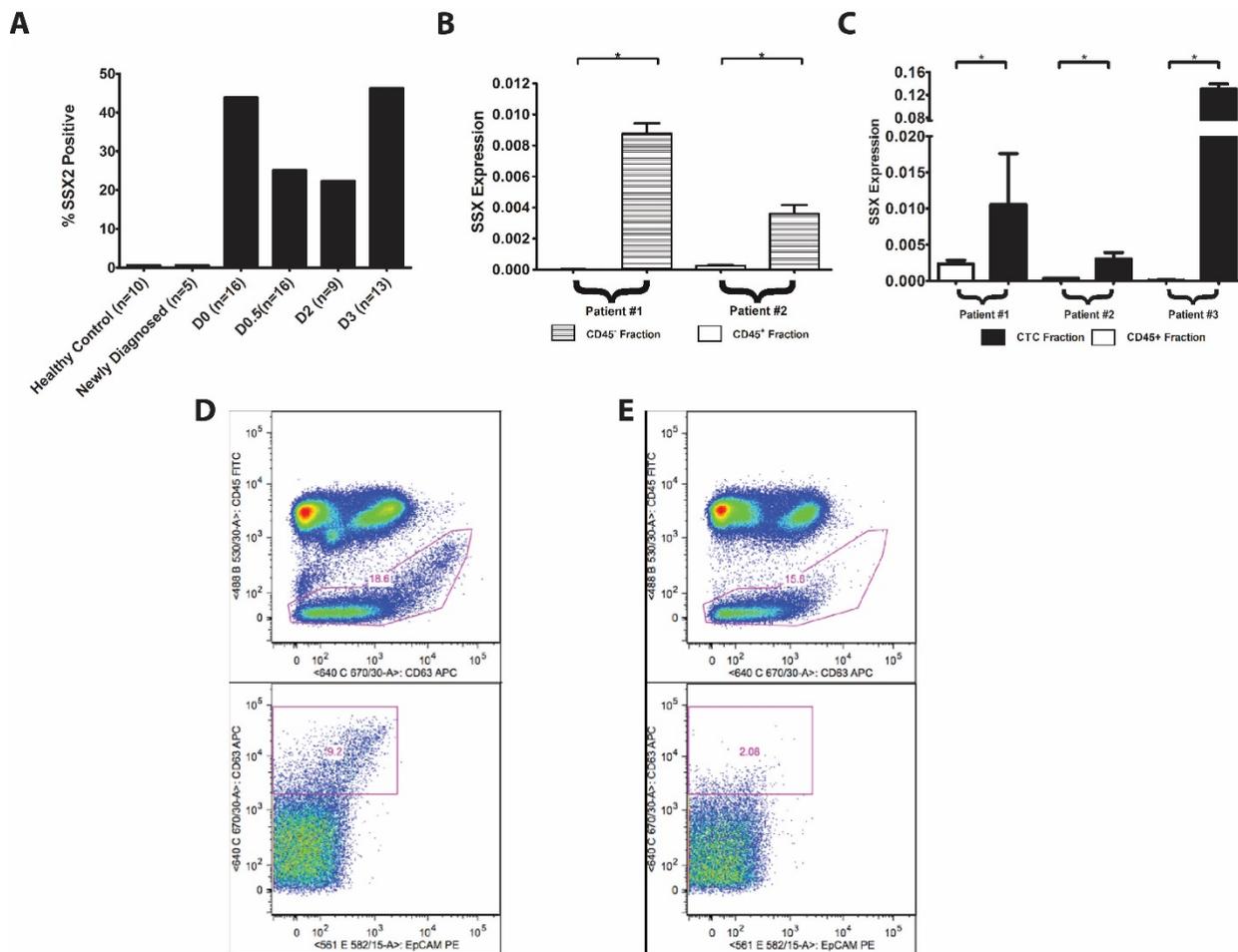
Materials and Methods

MATERIALS AND METHODS:

Reverse Transcription (RT)-PCR, and Quantitative PCR Analysis:

RT-PCR using the One-Step RT-PCR kit (Qiagen,Valencia,CA) was carried out on RNA collected using Reliaprep RNA cell miniprep system (Promega) from peripheral blood samples from patients with prostate cancer or healthy control donors, under the following PCR conditions: 50°C for 30min.,95°C for 15min., 35 cycles at 95°C for 1 min., 60°C for 1 min. and 72°C for 1 min. Final extension for 10 min. at 72°C was followed by 4°C incubation until amplified products were resolved on a 2% agarose gel. Primers specific for each individual SSX family member and actin were previously identified (17). cDNA generated from metastatic prostate tissue samples was provided by Dr. Robert Vessella (University of Washington, Seattle, WA). For quantitative PCR (qPCR), RNA was collected using Reliaprep RNA cell miniprep system (Promega) and reverse transcribed using iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. qPCR was performed using SsoFast™ EvaGreen® Supermix (Bio-Rad) in a MyiQ™2 Two-Color Real-Time PCR Detection System (Bio-Rad) with annealing temperatures specific for each primer pair. Primers specific for EMT associated genes and P0 are provided in Supplementary Table S1. All results were analyzed by the $2^{-\Delta Ct}$ method relative to P0 as a control gene(24). The following analysis was used to determine fold change: $2^{-\Delta Ct\,(Experimental)}/2^{-\Delta Ct\,(Control)}$. Primers used in this study are listed in Supplementary Table 1.

Patient Blood Samples:

With informed consent, peripheral blood or leukapheresis products were obtained from male subjects with (n=59) or without prostate cancer (n=10). Samples from patients with prostate cancer included men with newly diagnosed prostate cancer prior to prostatectomy (n=5), recurrent prostate cancer with rising PSA after definitive therapy (stage D0/M0, n=16), androgen-sensitive metastatic prostate cancer (stage D2, n=9), non-metastatic castration-resistant prostate cancer (stage D0.5, n=16), and metastatic castration-resistant prostate cancer (stage D3, n=13). Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Paque centrifugation (Pharmacia AB, Uppsala, Sweden) and cryopreserved in liquid nitrogen until use.

Flow Cytometry:

Patient PBMC which tested positive for SSX2 mRNA by RT-PCR were washed with Hank's buffered saline solution and then stained with antibodies specific for CD45-FITC(clone:30-F11 BD, Franklin Lakes, NJ), EpCAM-PE (clone: EBA-1 BD), CD63-APC (clone:MEM-259 Biolegend, San Diego, CA) and DAPI (Molecular probes/ThermoFisher, Waltham, MA). Stained cells were analyzed and sorted using a FACS Aria (BD, Franklin Lakes, NJ). Circulating tumor cells (gated as: $DAPI^-/Singlet^+/CD45^-/EpCAM^+/CD63^+$) or control lymphocytes ($DAPI^-/Singlet^+/CD45^+$) were sorted using a BD FACS Aria into microfuge tubes containing BL lysis buffer (Promega). RNA and subsequently cDNA was generated and then assayed for SSX2 using qPCR as described above. Flow cytometry data was analyzed using FlowJo software version 10.

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CHAPTER THREE: Characterization of SSX2 function in Prostate Cancer

Bloom JE, McNeel DG

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JEB designed and performed the experiments, analyzed the data, and prepared the manuscript;

DGM supervised the concept, experimental design, data analysis, and manuscript preparation.

Abstract

Prostate cancer is the most commonly diagnosed malignancy for men in the United States. Metastatic prostate cancer, the lethal form of the disease, has a life expectancy of approximately three years. Identification of factors associated with this transition to metastatic disease are crucial for future therapies. One such factor is the SSX gene family, a family of cancer/testis antigens (CTA) transcription factors which have been shown to be aberrantly expressed in other cancers and associated with the epithelial to mesenchymal transition (EMT). We have previously shown that SSX expression in prostate cancers was restricted to metastatic tissue and not primary tumors. In this study, we examined SSX2 function in prostate cancer through knockdown and overexpression in prostate cancer cell lines. While overexpression had little effect on morphology or gene transcript changes, knockdown of SSX2 resulted in an epithelial morphology, increased cell proliferation, increased expression of genes involved in focal adhesion, decreased anchorage independent growth, increased invasion, and increased tumorigenicity *in vivo*. We conclude from these findings that SSX2 expression in prostate cancer is not a driver of EMT, but is involved in processes associated with EMT including loss of focal adhesion that may related to tumor cell dissemination.

Introduction

Prostate cancer is the most commonly diagnosed malignancy of men in the United States [1]. The majority of newly diagnosed prostate cancer can be cured with radiation therapy or surgery [2,3]. However, approximately 1/3 of patients following treatment with surgery or radiation therapy will recur and eventually progress to metastasis. Metastatic prostate cancer, and metastatic disease progressing beyond initial androgen deprivation therapy is lethal, with a life expectancy of approximately five years [4]. This is despite the approval of several new therapies within the last few years that have on average each extended survival by a few months [5–8]. The identification of proteins involved in prostate cancer metastasis that might serve as targets for new therapies is consequently of high relevance to this disease.

One potential target of interest is the SSX gene family. The SSX family of proteins are cancer-testis antigens (CTAs), a group of proteins whose normal expression is restricted to immune-privileged testis germline cells, but display aberrant heightened expression in many different types of cancer [9,10]. These immune-privileged germ cell tissues lack HLA class I molecules [11]. Therefore in theory, any $CD8^+$ T cell-targeted therapy directed towards a CTA should be specific for cancer cells, effectively ignoring germ cells or other normal somatic cells, making these particularly interesting targets for immune-based therapies [12]. We previously screened the sera of prostate cancer patients against an expression library of 29 CTA family members, and identified SSX2 as one of the most commonly recognized CT antigens [13]. Importantly, we have shown SSX proteins are expressed solely in metastases and not in primary prostate tumors [14].

Despite the interest of our group and others in the SSX family as therapeutic targets, the function of the SSX family of proteins is largely unknown. An understanding of its function and

whether it has oncogenic activity given its expression in metastatic disease is therefore critical. SSX family members have been shown to possess two highly conserved domains: a Kruppel-associated box (KRAB) domain, and an SSX repression domain (SSXRD) [15]. Both regions have been shown to act as transcriptional repressors [16], however the targets of these domains remain unknown. SSX has two known binding partners, SSX2IP and RAB3IP [16], both of which are largely uncharacterized. However, SSX proteins have been found to be associated with the epithelial to mesenchymal transition (EMT) [17–19]. Cancer cells undergoing EMT have been shown to have greater invasive and proliferative capacity which can result in dissemination to distal organs, and to initiate metastases formation [20]. SSX knockdown in melanoma and osteosarcoma cell lines results in impaired cell migration, and down regulation of EMT associated genes [18]. Additionally, SSX was found to co-localize with EMT-associated proteins vimentin (*VIM*) and matrix metalloproteinase 2 (*MMP2*) [19]. SSX overexpression in a breast cancer cell line was shown to increase cell proliferation and repress the epithelial marker E-Cadherin (*CDH1*) [21]. Finally, SSX was found to be highly expressed in mesenchymal stem cells as compared to other CTAs [19]. However, there has been no evidence to date SSX is similarly involved with EMT in prostate cancer, and there has been no evaluation of its function in prostate cancer cells.

Our observation that SSX expression is confined to the metastases but not primary tumors suggested SSX may be similarly involved with EMT leading to metastasis formation in prostate cancer. This study aimed to further elucidate SSX's function and expression pattern in prostate cancer, and to specifically determine whether it is involved in prostate cancer EMT. We found through *in vitro* and *in vivo* studies that SSX2 is not a driver of EMT, however its loss leads to morphological changes and increases in proteins associated with focal adhesion.

Results

Changes in SSX2 expression level were associated with non-canonical changes in EMT-associated genes

Previous studies in other malignancies have suggested a role for SSX in EMT [17–19]. Given the prevalence of SSX2 in the peripheral blood of patients with prostate cancer, we next questioned whether SSX2 expression in prostate cancer cells was similarly associated with markers of EMT. For these studies we took advantage of prostate cell lines that were previously characterized with respect to SSX2 expression [14]: 22Rv1 (a prostate cancer cell line with high SSX2 expression), LNCaP (a prostate cancer cell line with low SSX2 expression), DU145 and PC3 (prostate cancer cell lines with no SSX2 expression), and RWPE-1 (a prostate epithelial cell line with no SSX2 expression). Of note, 22Rv1 is an epithelial cell line and PC3 has a more mesenchymal phenotype, suggesting that SSX2 expression itself was not necessarily associated with a mesenchymal phenotype. To study changes in EMT associated with changes in SSX2 expression, we knocked down expression of SSX2 in the 22Rv1 cell line using an shRNA plasmid specific for SSX2, and verified by qPCR and ELISA (Fig. 2A and B). Conversely, the non-SSX2 expressing prostate cell lines (DU145, PC3, RWPE-1) were transfected with a mini-intronic plasmid encoding SSX2 to generate cells which ectopically overexpressed SSX2, and confirmed by qPCR and ELISA (Fig. 2B and C). Upon transfection of the SSX knockdown plasmid, the 22Rv1 line began to exhibit a rounded morphology, less clumping, and "cobblestone" appearance (Fig. 2D and Supplementary Fig. 2). Upon overexpression of SSX2 in DU145, PC3, or RWPE lines, we observed no change in cell morphology (data not shown). We then evaluated genes with known EMT involvement by qPCR in the SSX2 knockdown and

ectopically overexpressed cell lines (Fig. 2E and F). SSX2 knockdown in the 22Rv1 line resulted in altered expression of EMT associated genes, but not in a canonical pattern typical of "drivers" of EMT (e.g. a "driver" would increase expression of Twist1 (*TWIST1*), Zeb2 (*ZEB2*), Vimentin (*VIM*), Snail (*SNAI1*), Slug (*SNAI2*) and N-Cadherin (*CDH2*), and decrease E-Cadherin (*CDH1*)). Rather, knockdown of SSX2 in the 22Rv1 line led to Twist1 being highly downregulated while Zeb2 and Vimentin were highly upregulated, and no change was seen in Snail or Slug (Fig. 2E). Overexpression of SSX2 in the SSX negative prostate cell lines resulted in no or ambiguous changes in EMT associated genes. However, canonical changes in EMT-associated genes were detected following SSX2 overexpression in the LNCaP line (Fig. 2F). Specifically, the transcription factor *ZEB2*, collagenase *MMP2*, and N-cadherin were upregulated in response to SSX2 overexpression (Fig. 2F). These gene expression changes in the SSX2 overexpressing LNCaP line were not inversely related to the gene expression changes following knockdown of SSX2 expression in the 22Rv1 cell line, or seen in PC3 or DU145 cell lines. Taken together, these results suggested that SSX2, while associated with changes that occur with EMT, is by itself not a driver of EMT in the context of prostate cancer. The finding that SSX2 overexpression led to gene changes in one cell line but not another further suggested SSX2 may require additional cofactors for function. Therefore, we hypothesized one or both of SSX's known two binding partners, SSX2IP and Rab3IP, may have been responsible for EMT associated gene changes in the LNCaP but not RWPE-1. However, expression of these binding partners was detected within an order of magnitude in examined cell lines and did not appear to account for the differences observed following SSX2 knockdown or overexpression (Fig. 2G and H).

Microarray analysis revealed SSX2 has an association with focal adhesion

To assess other, non-EMT-related, functions of SSX2 in an unbiased fashion, we assessed global gene expression changes in the 22Rv1 SSX2 knockdown line compared to scramble shRNA or wildtype controls by gene microarray. Of 67,528 transcripts analyzed, 2,213 showed a significant decrease and 2,025 showed a significant increase following SSX2 knockdown compared to scramble shRNA control. When comparing the control cell lines (scramble control and wildtype) only 107 genes were found to be significantly changed, as compared to 4,238 between KD and SCR (Table 1 and Fig. 3A). In particular, many genes related to focal adhesion were found to be upregulated including: *CADM1 ICAM1 COL12A1 EMP3 ITGA6* (Table 2 and Fig. 3B). We confirmed these findings through flow cytometry by examining the expression of highly regulated cell surface proteins: Annexin A2 (*ANXA2)*, Integrin α6 (*ITGA6*), as well as PSMA (*FOLH1*), on SSX2 knockdown and scramble control 22Rv1 lines. Interestingly, the expression of these proteins was not reciprocally changed on RWPE-1 or LNCaP lines overexpressing SSX2 (Fig. 3C).

Knockdown of SSX2 expression resulted in phenotypic and functional changes associated with cellular adhesion

The 22Rv1 cell line, and derivatives transfected with SSX2 or scramble shRNA, was next evaluated for differences in phenotype and tumorigenicity. We first examined the growth rate of the different cell lines, and found SSX2 knockdown line demonstrated the highest growth rate while scramble control and wildtype lines were not significantly different (Fig. 4A). We next investigated anchorage-independent growth of the SSX2 knockdown 22Rv1 line by colony formation in soft agar. The SSX2 knockdown line was less able to form colonies in the soft agar

matrix as compared to scramble shRNA or wildtype control (Fig. 4B). To phenotypically evaluate SSX2's effects on focal adhesion we investigated mechanisms reliant on focal adhesion [22–25]. Specifically, we found the SSX2 knockdown line both better able to fill new extracellular spaces (Fig. 4C) and invade (Fig. 4D) significantly better relative to shRNA scramble control. To specifically examine the effects of focal adhesion inhibition, we incubated SSX knockdown and scramble control with FAK inhibitor 14 and repeated the extracellular space filling assay. FAK inhibition abrogated the phenotype seen in the SSX2 knockdown line (Fig. 4C) and had no effect on shRNA scramble control cells (Fig. 4E). To ensure the observed migration was due to focal adhesion and not proliferation, migration studies (Fig. S3) were also conducted in the presence of mitomycin C [26]. Taken together, these data corroborate the finding that SSX2 knockdown increases focal adhesion and migration of a prostate cancer cell line.

Knockdown of SSX2 expression leads to increased tumorigenicity *in vivo*

Due to SSX2's association with focal adhesion and cell migration, we next investigated the effects of SSX2 knockdown on tumor growth *in vivo*. SCID mice were intravenously injected with 1×10^6 cells of either the SSX2 knockdown 22Rv1 cell line or scramble shRNA lines (Fig. 5). At six weeks post injection the lungs were collected, weighed, and tumor nodules were counted. Mice injected with the SSX2 knockdown line were found to have significantly increased lung weights and more tumor nodules (Fig. 5 A and B).

Discussion

Our data indicate that SSX2 is involved in processes related to EMT in prostate cancer, notably focal adhesion, potentially accounting for the prevalence of expression in circulating tumor cells, but is itself not a driver of EMT. 22Rv1 cells that express SSX2 underwent a morphological change from a spiked appearance to a rounded epithelial appearance following SSX2 shRNA knockdown (Fig. 2D and S2). SSX2 knockdown, in the 22Rv1 prostate cancer line, demonstrated a large fold change in many different EMT associated genes (*TWIST1, ZEB2,MMP2,VIM, CDH1, CDH2*) however these genes did not respond in an anticipated or canonical way (Fig. 2E). For example, both E-cadherin and N-Cadherin were down regulated, and the transcription factors Zeb2 and Twist1 were in opposition. Canonically, we expect EMT associated genes to move in the same direction when transitioning between epithelial to mesenchymal, and the cadherins to move in opposition (E-cadherin to decrease while N-cadherin increases, and vice versa). In fact, we found when knocking down SSX2 expression in the 22Rv1 line, N-cadherin expression was shut off while E-cadherin was down regulated. Curiously, while overexpression of SSX2 in the LNCaP cell line resulted in modest changes in EMT-associated genes as expected), we saw low or no change in EMT genes following overexpression of SSX2 in SSX2 negative prostate cancer cell lines (Fig. 2F). SSX's involvement with EMT was further supported by the microarray data of SSX knockdown in 22Rv1 cell line (Fig. 3 and Supplementary Table S1). EPH receptor A3 (*EPHA3*) was found to have the highest negative fold change in the microarray. EPH receptors have been shown to be involved in the EMT pathway, and may regulate E-cadherin and Snail expression, as well as regulate MMP-2 activity [27–30]. Three different annexins were the highest upregulated genes when SSX was knocked

down, and annexins have been shown to attenuate EMT in breast cancer [31] and regulate TGF-beta signaling [32].

Additionally, when we examined the effects of SSX2 knockdown on cellular proliferation (Fig. 4A), we found increased cell proliferation. This observation is in opposition of those seen by D'Arcy et al [18], who found SSX knockdown inhibited proliferation in the melanoma DFW cell line, but in agreement with Chen, who found SSX-expressing breast cancer MCF-7 cells grew slower than a non-SSX expressing MCF-7 line [17]. Further, D'Arcy *et al* found E-cadherin downregulated in both DFW and SAOS-2 SSX-expressing melanoma and osteosarcoma cell lines, in agreement with our findings, but saw opposite changes in the expression of slug and vimentin [18]. We suspect these discrepancies are due to context-dependent factors related to different diseases or possibly co-factors expressed by different cell lines. These context-dependent factors are likely not the known binding partners of the SSX family (*RAB3IP* and *SSX2IP*) given our findings (Fig. 2G and H). Hence, there is likely one or more undiscovered cofactor(s) that can function in tandem with SSX2. These data lead us to conclude SSX2 is associated with processes associated with EMT in prostate cancer, but SSX2 alone is not sufficient to drive EMT. SSX2 is known to associate with the polycomb-group complex proteins (PcG) [33] responsible for chromatin remodeling. Due to this association with PcG, methylation states between cell lines (or prostate cancer patients) may result in differential recruitment of needed cofactors, which could explain the observed context dependent differences. Further work should focus on identifying these context dependent factors.

Microarray analysis (Fig. 3 and Table 1) revealed, in response to SSX2 knockdown, focal adhesion molecules such as *CADM1* (cell adhesion molecule 1), *ICAM1* (intercellular adhesion molecule1), *COL12A1* (collagen type 12), *EMP3* (epithelial membrane protein3), and *ITGA6* (integrin), were upregulated (Fig. 3B and Table 1). The role of SSX2 in focal adhesion was further supported by studies demonstrating that loss of SSX2 led to increased invasion and extracellular space filling. In both assays the SSX knockdown line, with its heightened focal adhesion molecules, were able to invade or migrate more than the scramble shRNA or wildtype control lines (Fig. 4C and D). Conversely, we find SSX2 knockdown resulted in decreased anchorage independent growth (Fig. 4B). This suggests that when SSX2 is expressed in prostate cancer, focal adhesion molecules are downregulated. A decrease in focal adhesion could imply that factors leading to SSX2 expression aid in the extravasation from the primary tumor site into the blood stream and/or persistence of tumor cells in circulation.

Surprisingly, microarray analysis revealed knockdown of SSX2 also resulted in a knockdown of the androgen receptor (AR) and prostate specific membrane antigen (PSMA), both of which are intrinsically tied to prostate cancer disease progression [34,35]. This was confirmed by evaluating surface expression of PSMA on these cells by flow cytometry (Fig. 3C). Additionally, we examined the expression of the AR splice variants and similarly found them expressed at lower levels in response to SSX2 knockdown (Supplementary Fig. S1). Castrate-resistant prostate cancer cells that acquire a completely AR-independent phenotype, whether of neuroendocrine or non-neuroendocrine type, typically display a more aggressive phenotype with rapid disease progression [36,37]. Future research should investigate the relationship between SSX2 expression and AR-dependent growth.

Due to the high mortality of metastatic prostate cancer, therapeutic targets associated with advanced prostate cancer are needed. Because SSX2 is a CTA, and expressed in recurrent prostate cancer and not normal prostate cells, it is an attractive therapeutic target. The ability to specifically target metastatic tumor cells, and tumor cells in circulation without an immunosuppressive tumor microenvironment, could be advantageous. We have previously studied genetic vaccines targeting SSX2, and demonstrated that this is feasible [38]. Moreover, the absence of morphological or gene changes following overexpression in a normal epithelial cell line suggest this approach should not itself be oncogenic following expression of the gene in normal human cells. In fact, a therapeutic vaccine encoding SSX2 as one target antigen has recently opened to accrual for patients with advanced, metastatic prostate cancer (NCT02625857).

Figures and Figure Legends

Figure 1.

SSX2 modulated expression of EMT associated genes, but did not drive the EMT transition. A)
qPCR for SSX2 expression wild type (WT) and two different shRNA transfected and control
(shRNA scramble) 22Rv1 cell lines. B) SSX2 protein quantification using ELISA in SSX
shRNA and control cell lines. C) SSX2 expression by qPCR, relative to P0 housekeeping
protein, in RWPE-1 cells transfected with empty vector or to express SSX2. D) 30x Microscopy
images of Hoechst-stained shRNA transfected 22Rv1 cell lines or wildtype 22Rv1. E) Fold
change by qPCR of EMT associated genes (Twist1, Snail, Slug, Zeb1, Zeb2, MMP2, Vimentin,
E-Cadherin, N-Cadherin) in shRNA transfected 22Rv1 cell lines compared with scramble control
transfected 22Rv1 cells. F) Fold change of EMT associated genes in cell lines transiently
overexpressing SSX2 vs empty vector transfected control in different cell lines (MSC = human
mesenchymal stem cell line). Expression levels of RAB3IP (G) and SSX2IP (H) genes in 22Rv1,
LNCaP, and RWPE-1 cell lines by qPCR.

A SSX Expression Relative to Actin — 22Rv1 WT, Scramble shRNA, SSX shRNA #1, SSX shRNA #2

B SSX Protein (ng)/Total Protein (mg) — 22Rv1 WT, scramble shRNA, SSX shRNA #1, RWPE-SSX, RWPE-Control

C SSX Expression Relative to P0 — RWPE-SSX, RWPE-Control

D Wildtype | Control shRNA | SSX shRNA

E Fold Change — Twist1, Snail, Slug, Zeb1, Zeb2, MMP2, Vimentin, E-Cadherin, N-Cadherin

F Fold Change — Twist, Slug, Zeb2, MMP2, Vimentin, E-Cadherin, N-Cadherin

RWPE-1 (No SSX) PC3 (No SSX) DU145 (No SSX)
MSC (No SSX) LNCaP(Low SSX)

G SSX2IP Expression Level — Expression relative to P0
22Rv1 (High SSX), LNCaP (Low SSX), RWPE-1 (No SSX)

H Rab3IP Expression Level — Expression relative to P0
22Rv1 (High SSX), LNCaP (Low SSX), RWPE-1 (No SSX)

Figure 2.

<u>SSX knockdown showed changes in genes associated with focal adhesion</u>. A) Heat map of regulated genes in shRNA transfected (KD) and control (SCR) 22Rv1 cell lines (red=downregulated, green=upregulated), evaluated in triplicate samples each. B) Regulated genes by pathway in SSX shRNA vs scramble shRNA (red=downregulated, green=upregulated). C) Fold change of mean fluorescence intensity (MFI) of cell surface proteins found by microarray. For this analysis, change is evaluated with respect to mock transfection for 22Rv1 cells (knocked down for SSX2 expression) and RWPE-1 or LNCaP cells (overexpressing SSX2).

A

KD   SCR

B

Nuclear Receptors Meta-Pathway

miR targeted genes in lymphocytes   TarBase

miR-targeted genes in muscle cell - TarBase

Focal Adhesion

miR targeted genes in epithelium   TarBase

NRF2 pathway

EGF/EGFR Signaling Pathway

Integrated Pancreatic Cancer Pathway

MAPK Signaling Pathway

TGF-beta Signaling Pathway

Mesodermal Commitment Pathway

Histone Modifications

miR-targeted genes in squamous cell - TarBase

Integrin mediated Cell Adhesion

Spinal Cord Injury

Androgen receptor signaling pathway

Endoderm Differentiation

miR-targeted genes in leukocytes - TarBase

Integrated Breast Cancer Pathway

DNA Damage Response (only ATM dependent)

TGF-beta Receptor Signaling

Regulation of Actin Cytoskeleton

C

MFI Fold Change

375
325
275
15
5
-5
-30
-45
-60

Annexin A2   Integrin α6   PSMA

■ 22RV1
□ RWPE
▨ LNCaP

Figure 3.

SSX2 knockdown resulted in functional changes in prostate cancer cells associated with

adhesion and migration. A) Quantification of cell proliferation in shRHA transfected and control

22Rv1 cell lines. B) Equivalent numbers of SSX shRNA and scramble shRNA 22Rv1 cell lines

were plated in soft agar and colonies formed were counted after 7 days.   C) 10x images of SSX

shRNA and scramble shRNA 22Rv1 cell lines were grown to near confluence and a scratch

introduced. Cells migrating into the space were imaged at 1, 4, and 7 days later. D) Equivalent

numbers of SSX2 shRNA and scramble shRNA 22Rv1 cell lines were cultured in serum-free

medium in the upper chamber of a transwell plate. Migration into the lower chamber was

measured by OD_{560} after 48 hours. Positive and negative controls were as described in Methods.

E) 10x images of extracellular filling "scratch" assays were conducted as in panel C in the

presence or absence of FAK inhibitor 14. *=P<0.05

A

B

Soft Agar Colony Formation
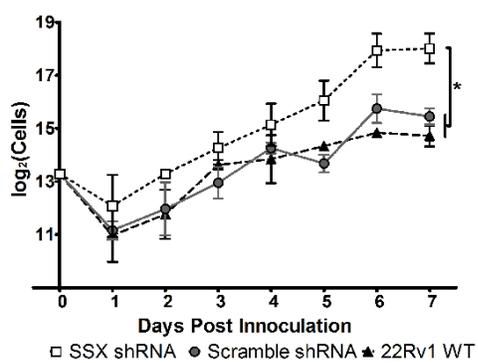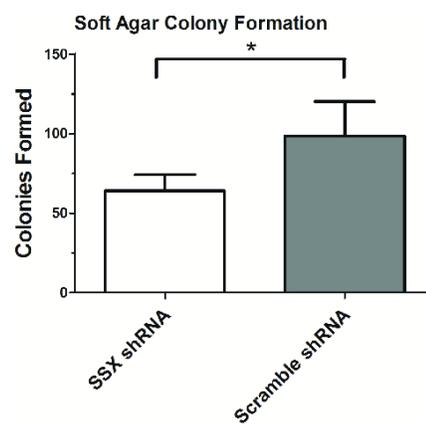
C
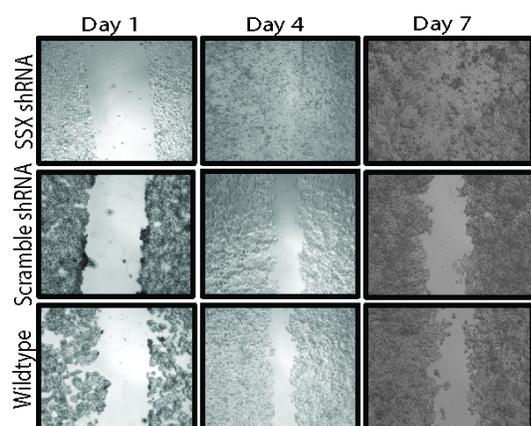
D
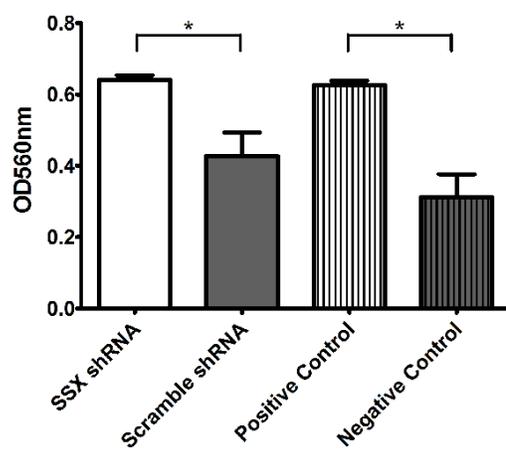
E
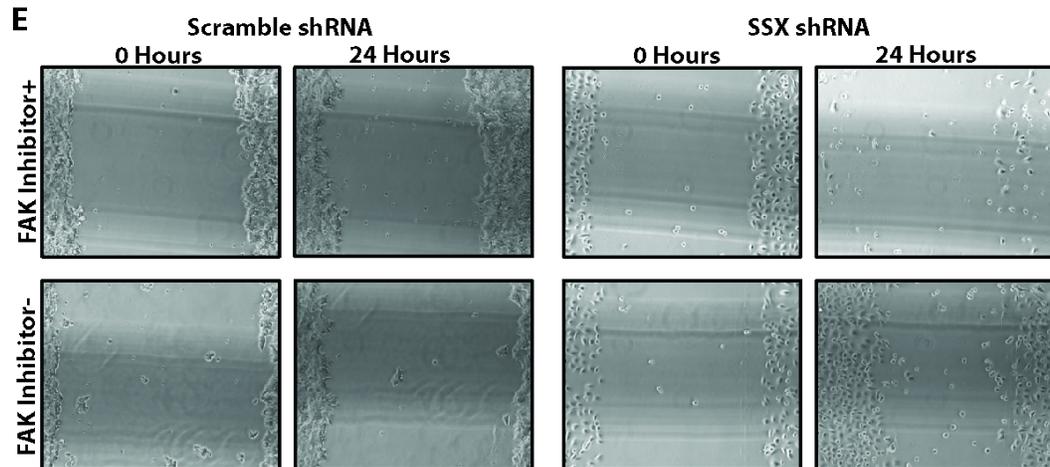
Figure 4.

<u>Knockdown of SSX increased tumor formation *in vivo*</u>. A) SCID mice were intravenously injected with equivalent numbers of SSX shRNA or scramble shRNA 22Rv1 cell lines and lungs harvested after 6 weeks. Shown are the lung weights (A), number of surface tumor lesions detected (B), and representative images (C) with arrows pointing to tumor nodules. *=P<0.05
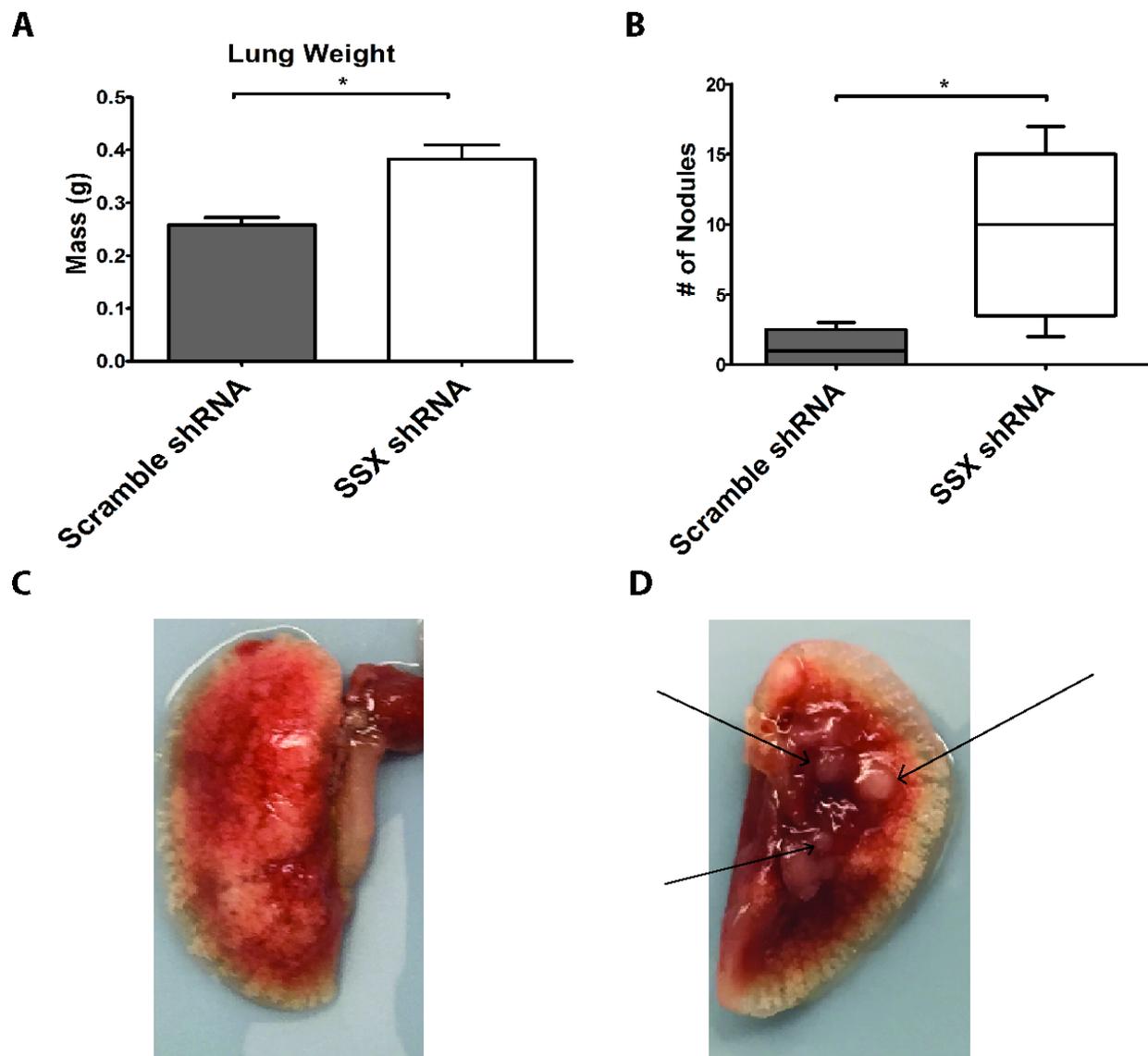
Table 1.
Table of genes, generated from the microarray, with fold changes ≥25 or ≤-25.

Fold Change (KD vs. SCR)	Gene Symbol	Description
81.65	ANXA2	annexin A2
66.37	ANXA1	annexin A1
65.07	MYOF	myoferlin
49.63	ANKRD1	ankyrin repeat domain 1 (cardiac muscle)
42	EDIL3	EGF-like repeats and discoidin I-like domains 3
33.01	ANXA3	annexin A3
32.26	UCHL1	ubiquitin thiolesterase
26.69	SLC36A4	solute carrier family 36 (proton/amino acid symporter), member 4
26.64	AXL	AXL receptor tyrosine kinase
25.6	RAB31	RAB31, member RAS oncogene family
-25.41	TPTE	transmembrane phosphatase with tensin homology
-25.43	SEMA6A	sema domain, transmembrane domain (TM), and cytoplasmic domain, (semaphorin) 6A
-26.17	FAR2	fatty acyl CoA reductase 2
-29.46	GPC6	glypican 6
-30.02	CKMT1A; CKMT1B	creatine kinase, mitochondrial 1A; creatine kinase, mitochondrial 1B
-31.48	SSX3	synovial sarcoma, X breakpoint 3
-36.78	HMGCS2	3-hydroxy-3-methylglutaryl-CoA synthase 2 (mitochondrial)
-37.59	SCG3	secretogranin III
-38.48	COLEC12	collectin sub-family member 12
-40.56	COLCA1	colorectal cancer associated 1
-42.29	POTEI	POTE ankyrin domain family, member I
-44.2	POTEI; POTEJ	POTE ankyrin domain family, member I; POTE ankyrin domain family, member J
-52.86	PTPRB	protein tyrosine phosphatase, receptor type, B
-54.29	POTEE	POTE ankyrin domain family, member E
-55.45	POTEF	POTE ankyrin domain family, member F
-63.35	AR	androgen receptor
-78.25	SLITRK6	SLIT and NTRK-like family, member 6
-78.75	FOLH1B; FOLH1	folate hydrolase 1B; folate hydrolase (prostate-specific membrane antigen) 1
-112.42	SSX2B; SSX2	synovial sarcoma, X breakpoint 2B; synovial sarcoma, X breakpoint 2
-150.18	EPHA3	EPH receptor A3

Table 2.
Table of regulated genes associated with focal adhesion, generated from the microarray, comparing SSX2 shRNA 22Rv1 and scramble shRNA 22Rv1.

Fold Change	Gene Symbol	Description
16.39	ITGA6	integrin, alpha 6
14.2	CAV1	caveolin 1, caveolae protein, 22kDa
11.13	THBS1	thrombospondin 1
9.74	MET	MET proto-oncogene
8.99	LAMB3; MIR4260	laminin, beta 3; microRNA 4260
7.75	AKT3	v-akt murine thymoma viral oncogene homolog 3
6.71	ITGA3	integrin, alpha 3
4.49	EGFR	epidermal growth factor receptor
4.43	FLNA	filamin A, alpha
4.15	SHC1	SHC transforming protein 1
3.99	LAMA3	laminin, alpha 3
2.28	VCL	vinculin
1.83	PAK1	p21 protein (Cdc42/Rac)-activated kinase 1
1.6	LAMA5; MIR4758	laminin, alpha 5; microRNA 4758
1.58	ZYX	zyxin

Supplementary Table 1.
Table of primers utilized in this study

Gene Target	Gene Symbol	Forward (5' to 3')	Reverse (5' to 3')
Twist1	TWIST1	GGAGTCCGCAGTCTTACGAG	TCTGGAGGACCTGGTAGAGG
Snail	SNAI1	GCTGCAGGACTCTAATCCAGA	ATCTCCGGAGGTGGGATG
Slug	SNAI2	TGGTTGCTTCAAGGACACAT	GTTGCAGTGAGGGCAAGAA
Zeb1	ZEB1	GATGATGAATGCGAGTCAGATGC	ACAGCAGTGTCTTGTTGTTGTAG
Zeb2	ZEB2	AACAACGAGATTCTACAAGCCTC	TCGCGTTCCTCCAGTTTTCTT
MMP2	MMP2	ATAACCTGGATGCCGTCGT	AGGCACCCTTGAAGAAGTAGC
Vimentin	VIM	AAAGTGTGGCTGCCAAGAAC	AGCCTCAGAGAGGTCAGCAA
E-Cadherin	CDH1	AGTGTCCCCCGGTATCTTCC	CAGCCGCTTTCAGATTTTCAT
N-Cadherin	CDH2	ACAGTGGCCACCTACAAAGG	CCGAGATGGGGTTGATAATG
P0	36B4	GACAATGGCAGCATCTACAAC	GCAGACAGACACTGGCAAC
SSX2qPCR	SSX2	ACGGTTGGTGCTCAAATACCAGAGAA	GGCATGATCTTCGGGGAGATTCC
AR full length	AR	ACATCAAGGAACTCGATCGTATCATTGC	AGCTTCTGGGTTGTCTCCTCAGTGG
AR-V1	AR	CCATCTTGTCGTCTTCGGAAATGTTATGAAGC	CTGTTGTGGATGAGCAGCTGAGAGTCT
AR-V5	AR	CCAAGGCCTTGCCTGATTGC	TTGGGCACTTGCACAGAGAT

Supplementary Figure 1.

Expression levels of the androgen receptor (AR) and two of its known splice variants AR-V1 and

AR-V7, in SSX2 shRNA and Scramble shRNA 22Rv1 cell lines.
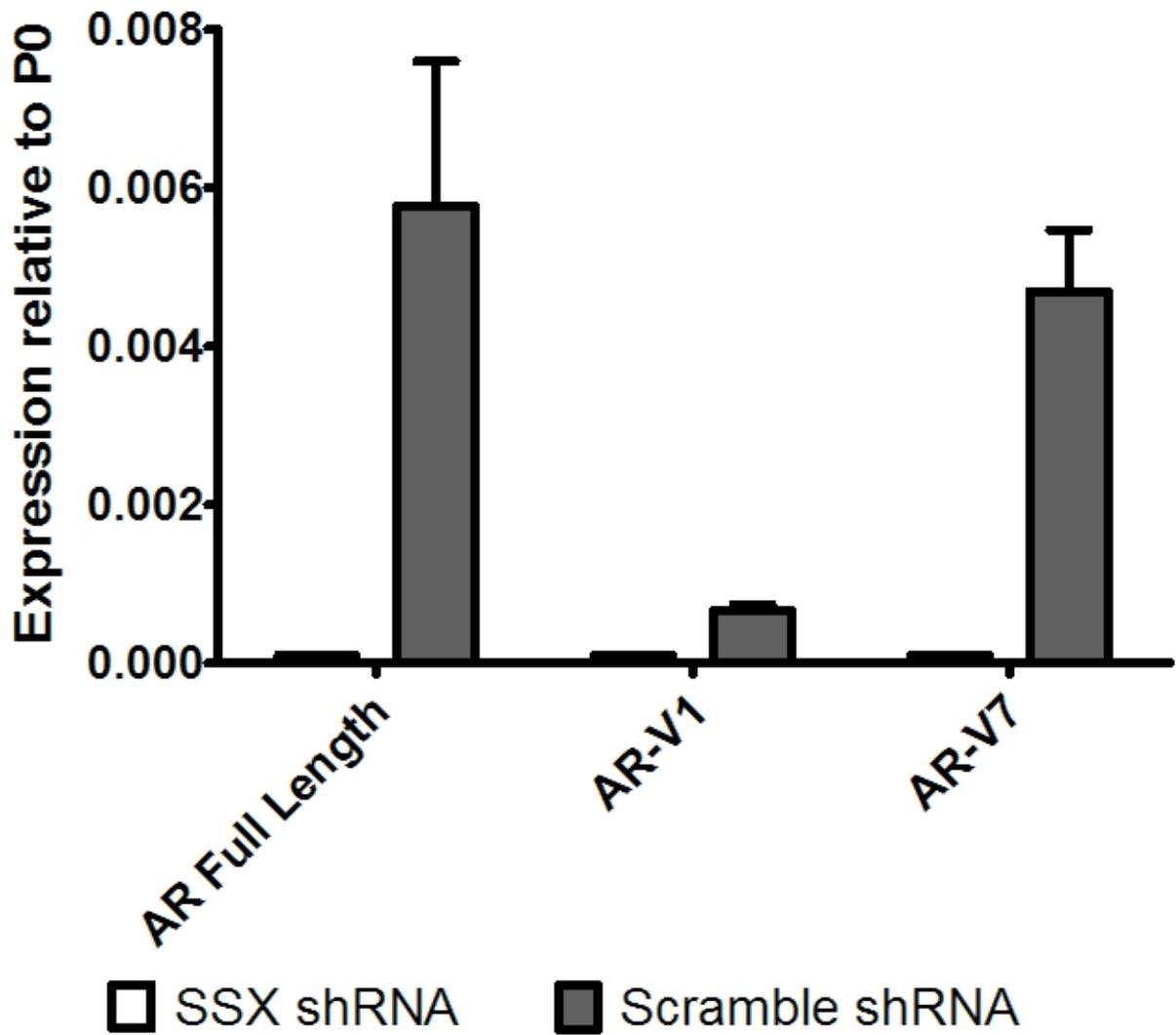
Supplementary Figure 2.
10x view of 22Rv1 lines investigated in this study to demonstrate morphology.

SSX shRNA
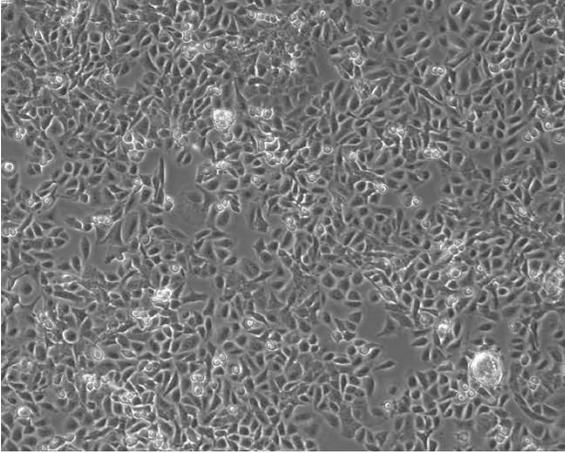
Scramble shRNA
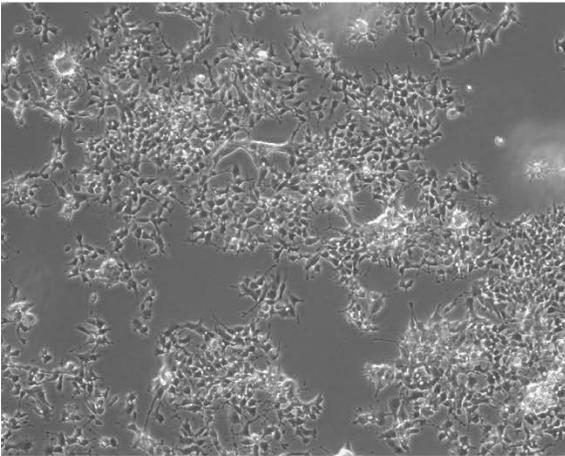
WT 22Rv1
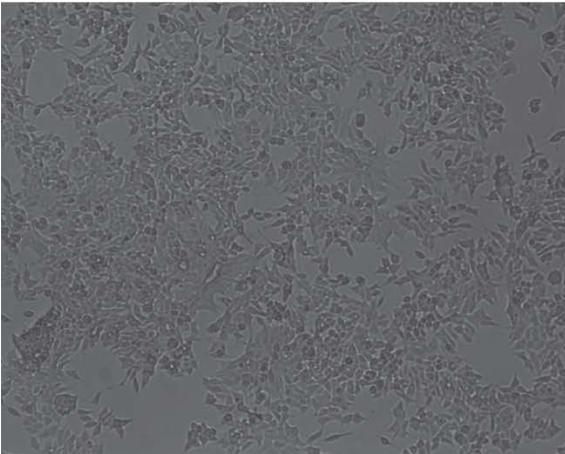
Supplementary Figure 3.
Migration studies were repeated in the presence of Mitomycin C (MMC). A. Scratch migration

assay on SSX shRNA and scramble shRNA 22Rv1 lines in the presence or absence of MMC

after 24 hours. B. Boyden chamber migration on SSX shRNA and scramble shRNA 22Rv1 lines

in the presence or absence of MMC
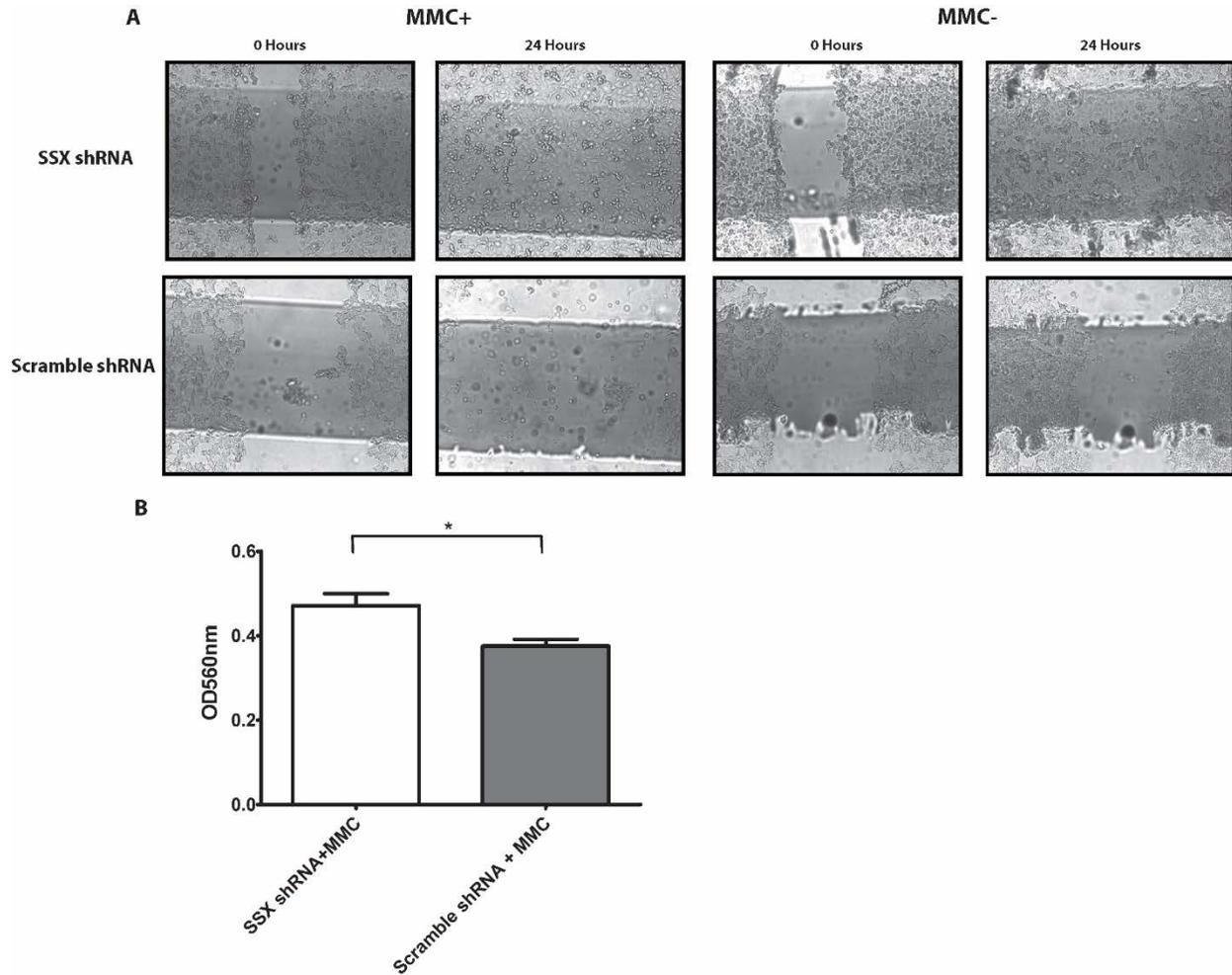
Materials and Methods

Cell Culture:

Prostate cancer cell lines (LNCaP, 22Rv1, DU145, PC3) and an immortalized prostate epithelial cell line (RWPE-1) were grown in DMEM medium (CellGro/Mediatech, Manassas, VA) supplemented with 10% fetal calf serum (FCS)(Hyclone/GE,Logan,UT), 200U/ml penicillin/streptomycin (Thermofisher, Waltham, MA), 1 mM sodium pyruvate (Thermofisher, Waltham, MA), and 0.1µM β-mercaptoethanol at 37°C/5% CO_2. All cell lines were validated by DDC Medical (Fairfield, OH), for identity and mycoplasma contamination.

Cell line plasmid DNA transfections:

Cell lines of interest were plated in triplicate, and cultured to 80% confluence. Cells were then transfected with mini-intronic plasmid encoding SSX2, or empty vector control, using Lipofectamine® LTX with Plus™ Reagent (Invitrogen, Carlsbad, CA). Cells were harvested after 3 days for RNA using Reliaprep RNA cell miniprep system (Promega, Fitchburg, WI). cDNA was generated using iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. SureSilencing™ shRNA plasmids against SSX2 were constructed by SAbiosciences (Frederick, MD). Transfected cells were then placed under neomycin selection, and SSX2 expression was verified using qPCR and ELISA as described below.

Reverse Transcription (RT)-PCR, and Quantitative PCR Analysis:

RT-PCR using the One-Step RT-PCR kit (Qiagen,Valencia,CA) was carried out on RNA collected using Reliaprep RNA cell miniprep system (Promega) from peripheral blood samples

from patients with prostate cancer or healthy control donors, under the following PCR

conditions: 50°C for 30min.,95°C for 15min., 35 cycles at 95°C for 1 min., 60°C for 1 min. and

72°C for 1 min. Final extension for 10 min. at 72°C was followed by 4°C incubation until

amplified products were resolved on a 2% agarose gel. Primers specific for each individual SSX

family member and actin were previously identified [14]. cDNA generated from metastatic

prostate tissue samples was provided by Dr. Robert Vessella (University of Washington, Seattle,

WA). For quantitative PCR (qPCR), RNA was collected using Reliaprep RNA cell miniprep

system (Promega) and reverse transcribed using iScript™ cDNA Synthesis Kit (Bio-Rad,

Hercules, CA) according to the manufacturer's instructions. qPCR was performed using

SsoFast™ EvaGreen® Supermix (Bio-Rad) in a MyiQ™2 Two-Color Real-Time PCR Detection

System (Bio-Rad) with annealing temperatures specific for each primer pair. Primers specific for

EMT associated genes and P0 are provided in Supplementary Table S1. All results were

analyzed by the $2^{-\Delta Ct}$ method relative to P0 as a control gene[39]. The following analysis was

used to determine fold change: $2^{-\Delta Ct\,(\text{Experimental})}/2^{-\Delta Ct\,(\text{Control})}$. Primers used in this study are listed

in Supplementary Table 1.

Patient Blood Samples:

With informed consent, peripheral blood or leukapheresis products were obtained from male

subjects with (n=59) or without prostate cancer (n=10). Samples from patients with prostate cancer

included men with newly diagnosed prostate cancer prior to prostatectomy (n=5), recurrent

prostate cancer with rising PSA after definitive therapy (stage D0/M0, n=16), androgen-sensitive

metastatic prostate cancer (stage D2, n=9), non-metastatic castration-resistant prostate cancer

(stage D0.5, n=16), and metastatic castration-resistant prostate cancer (stage D3, n=13). Peripheral

blood mononuclear cells (PBMC) were isolated by Ficoll-Paque centrifugation (Pharmacia AB,

Uppsala, Sweden) and cryopreserved in liquid nitrogen until use.

ELISA:

Detection of SSX2 in cell lysates was performed using a standard molecular biology sandwich

ELISA with a capture monoclonal antibody specific for SSX2 (Abnova,Walnut, CA,

Clone:1A4), and an SSX2 polyclonal detection antibody (Abnova,Walnut, CA).

Soft Agar Colony formation:

22Rv1 cells transfected with SSX2 shRNA or shRNA control, with SSX2 expression or

knockdown confirmed via RT-PCR, were suspended in media with 0.343% agar

(RPMI+10%FCS,2% Pen/Strep) and were then plated on a 0.6% agar bottom layer

(RPMI+10%FCS,2% Pen/Strep, 1mg/ml G418). Initial cell inoculums were 1000 cells, and were

plated in triplicate wells. After 2 weeks, wells were examined for colony formation, and colonies

in a 1cm^2 area were enumerated.

Extracellular Space-Filling ("Scratch") Assay:

Cells were plated in triplicate and grown in a 6 well dish. Scratches were made using a P200

pipette tip and imaged at 3 and 7 days after. Images are representative of the entire length of the

scratch. To inhibit focal adhesion in this assay, 10µm of FAK Inhibitor 14 (Abcam

Cambridge,MA) was added to the media at time of the scratch. To inhibit proliferation

mitomycin C (Fisher) 10µg/ml was added to the media at the time of scratch.

Cell Proliferation:

$1x10^4$ 22Rv1 cells, or derivatives transfected with SSX2 shRNA or scramble shRNA control, with confirmed SSX2 expression, were plated in triplicate into a 24-well dish. Cell counts were made each day for 7 days using trypan blue staining to assess cell viability.

Cell Migration:

A Boyden chamber assay (Cell Biolabs INC, San Diego,CA) was performed as per manufacturer's instructions using an FBS gradient. A positive control used cells seeded into the lower chamber, and the negative control was without the FBS gradient (no FBS in lower or upper chamber). To inhibit proliferation 10 µg/ml mitomycin C was added to the upper chamber.

Microscopy:

All Images were taken using a Nikon Eclipse Ti microscope and NIS-Elements D3.0 software. Magnification for each image is listed in the figure legend.

Flow Cytometry:

Patient PBMC which tested positive for SSX2 mRNA by RT-PCR were washed with Hank's buffered saline solution and then stained with antibodies specific for CD45-FITC(clone:30-F11 BD, Franklin Lakes, NJ), EpCAM-PE (clone: EBA-1 BD), CD63-APC (clone:MEM-259 Biolegend, San Diego, CA) and DAPI (Molecular probes/ThermoFisher, Waltham, MA). Stained cells were analyzed and sorted using a FACS Aria (BD, Franklin Lakes, NJ). Circulating tumor cells (gated as: DAPI$^-$/Singlet$^+$/CD45$^-$/EpCAM$^+$/CD63$^+$) or control lymphocytes (DAPI$^-$/Singlet$^+$/CD45$^+$) were sorted using a BD FACS Aria into microfuge tubes containing BL lysis

buffer (Promega). RNA and subsequently cDNA was generated and then assayed for SSX2 using qPCR as described above. Flow cytometry data was analyzed using FlowJo software version 10.

To measure levels of relevant surfaces markers found by the gene array, cells of interest were stained with Annexin A2-PE(clone: D11g2, Cell Signaling Technologies, Danvers,MA), Integrin alpha6-FITC(Clone:GoH3,Biolegend), and PSMA-PE-Cy7(clone:LNF-17,Biolegend). MFI was determined by comparison with IgG control using the FlowJo software. Fold change was then calculated by: $MFI_{experimental}/MFI_{control}$ and done in triplicate.

Gene Array:

cDNA was generated and hybridized to an Affymetrix Human Transcriptome Array 2.0 (Affymetrix Santa Cruz,CA). Samples were normalized by RMA algorithm to a log2 intensity value and were analyzed by Transcriptome Analysis Console v3.0. Genes showed at least fivefold increased or decreased expression are shown after analysis using the Bonferonni correction. A one-way ANOVA test was used to determine significance between SSX knockdown expression profile to the scramble or wildtype control. These data have been deposited in NCBI's Gene Expression Omnibus [40] and are accessible through GEO Series accession number GSE77811 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE 77811).

***In vivo* metastases formation in SCID mice:**

The protocol used was adapted from Mohanty and Xu[41]. Briefly, 10^6 SSX knockdown or scramble control 22Rv1 cells were injected intravenously into CB17-Prkscid mice (Jackson

Laboratories, Bar Harbor, ME). Mice (n=5 per group) were euthanized after 6 weeks, with

collection of lungs for weights and histological analysis. The harvested lungs were collected into

10% formalin and metastatic nodules on the surfaces of each lung were counted.

Statistical Analysis:
The Student's T Test was used for comparisons, unless otherwise noted, with $p < 0.05$

considered as statistically significant.

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Chapter 4: Characterization of SSX2 Specific CD8$^+$ T Cells in Prostate Cancer

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This work is undergoing preparation for submission

JEB designed and performed the experiments, analyzed the data, and prepared the manuscript;

DGM supervised the concept, experimental design, data analysis, and manuscript preparation.

Abstract

Prostate cancer is the most commonly diagnosed malignancy for men in the United States. Fortunately, radiation and prostatectomy are curative in the majority of cases for this slow growing disease. However, metastatic prostate cancer, the lethal form of the disease, has a life expectancy of approximately three years. Identification of factors associated with this transition to metastatic disease is crucial for future therapies. One such factor is the SSX gene family, a family of cancer/testis antigens (CTA) transcription factors which have been shown to be aberrantly expressed in cancers. We previously identified the HLA-A2 restricted epitopes of SSX2 and determined SSX specific CD8 T cells were significantly more common in late state prostate cancer patients. In this study, we identified late stage prostate cancer patients with SSX specific CD8$^+$ T cells (n=15). Then, assayed these patients for the presence of SSX expressing CTCs. We found patients with SSX expressing circulating tumor cells (CTCs) also possessed SSX specific CD8$^+$ T cells (6/15), while other patients possessed SSX specific CD8$^+$ T cells but not SSX CTCs (9/15). We sought to determine the cause of this duality, and further characterize SSX specific CD8$^+$ T cells. We determined that checkpoint molecule regulation was unlikely to be the cause of this duality. Further, we found patients who lacked SSX CTCs but had SSX specific CD8$^+$ T cells expressed more T_h1 biased cytokines than those patients who possessed both SSX specific CD8 T cells and SSX CTCs.

Introduction

The CDC has listed prostate cancer as the most widespread cancer amongst men in the United States and the western world, as well as the second leading cause of cancer-associated death[1]. Worldwide, it is the fourth most commonly diagnosed cancer. Fortunately, the treatment for localized prostate cancer, radiation and/or prostatectomy, is curative for the majority of instances of the disease. However, approximately one third of patients will relapse and most of these will develop metastases. Metastatic prostate cancer is treated with androgen deprivation; however, castration resistance develops in most of these individuals. Despite many new therapies approved over the last decade, metastatic, castration-resistant prostate cancer (mCRPC) still has a life expectancy less than 3 years. Thus, there remains an urgent need to obtain a better understanding of, and develop new avenues of therapy for, mCRPC.

The scope of antigens targeted current vaccination strategies against prostate cancer has been limited, with most therapies targeting tissue specific antigens such as prostate specific antigen (PSA), or prostatic acid phosphatase (PAP). PSA is a peptidase that is highly expressed in the prostate[2], but also in other tissues[3]. One strategy at targeting PSA is, PSA-TRICOM. PSA-TRICOM is a vaccine of vaccinia and fowlpox vectors expressing PSA[4]. Phase II studies reported an increase in overall survival[5], and a phase III approval trial is currently underway in patients with mCRPC (ClinicalTrials.gov identifier NCT01322490). There are also phase I and II trials investigating DNA plasmid (ClinicalTrials.gov identifier NCT01341652) and listeria vectors (ClinicalTrials.gov identifiers NCT02625857 and NCT02325557) which target PSA. PAP was the first characterized serum marker for prostate cancer[6], and has increased activity and prevalence in metastatic prostate cancer[7]. The only FDA approved vaccination treatment for prostate cancer Sipuleucel-T, targets PAP[8]. Sipuleucel-T utilizes autologous cell transfer for

vaccination. Briefly, Sipuleucel-T is the leukapheresis and subsequent incubation the host's dendritic cells with PAP and granulocyte macrophage colony-stimulating factor. This process hypothetically, matures and specifies the host dendritic cells to PAP. These now matured PAP specific dendritic cells are then re-infused to the host. Unfortunately, on average Sipuleucel-T only extends life expectancy of prostate cancer patients on the order of months. Although targeting of PSA and PAP is very rational due to their high expression in the prostate, the limited responses in mCRPC trials indicate a need for the study of antigens associated with the progression of disease or specific to mCRPC.

A group of proteins that has great potential as targets of therapy are the cancer testis antigens (CTAs). CTAs are expressed by immune privileged testis tissue. Immune privileged tissue lacks HLA class I molecules on their surfaces, thus the immune system is unable to direct cytotoxic lymphocyte (CTL) responses to these cells[9]. Additionally, CTAs can be aberrantly expressed in different cancers, including prostate[10]. Thus, a CD8 T cell immune response directed towards CTAs should hypothetically target CTA- expressing cancer cells and not normal tissues. The SSX family is a group of ten highly homologous transcription factors[11], which are CTAs. Although the specific function of SSX is still obfuscated, our group and others have investigated the function of SSX. We previously identified SSX2 as the most commonly expressed SSX gene in prostate cancer[12], thus we focus on SSX2 in this study. As a transcription factor, SSX may interact with pathways for the epithelial to mesenchymal transition[13,14] or focal adhesion[12]. Both pathways are potentially critical in establishing metastatic niches. Further, we had identified two HLA-A2 restricted epitopes of SSX2[15]. These domains were identified as SSX_{41-49} and $SSX_{103-111}$; we demonstrated that the $SSX_{103-111}$ domain was immunodominant. Additionally, we demonstrated that late stage prostate cancer patients were more likely to

possess SSX specific CD8 T cells than early stage patients[15]. Finally, we identified patients with SSX2 expressing circulating tumor cells (CTCs)[12]. SSX represents an excellent potential target for future prostate cancer therapies, due to its function in metastatic development and CTA status.

Given we have identified prostate cancer patients with SSX2 specific CD8 T cells, and patients with SSX expressing CTCs; in this study we sought to sought to characterize the nature and effector function of SSX2-specific CD8 T cells in patients with advanced prostate cancer. We hypothesized that patients with SSX specific CD8s would not possess SSX expressing CTCs, as the presence of SSX specific CD8s represresents the generation of an immune response. We also sought to examine the effector function of these SSX specfic CD8 T cells.

Results

Detection of Antigen Specific CD8+ T Cells in peripheral blood of late stage prostate cancer patients and delineation by detection of SSX2 mRNA

Utilizing flow cytometry and tetramer staining we examined the prevalence CD8 T cells specific for $SSX_{103-111}$, $NYESO_{157-165}$, $CMV_{495-503}$, $EBV_{356-364}$ in the peripheral blood of patients with prostate cancer. We sought to compare HLA-A2 restricted epitopes in SSX to NY-ESO-1 to determine the similarities of CD8 T cells targeting CTAs. We utilized $CMV_{495-503}$, $EBV_{356-364}$, as antigens that are common in the human population, and as antigens of chronic exposure. We found human samples positive for each of the antigens of interest (Figure 1). CMV specific CD8 T cells were detected in the highest percentage (1.0-6.0% of CD8+ T cells) relative to the other antigens of interest, which were detected by CD8+ T cells between 0.2-1.0% of all CD8+ T cells.

We next sought to categorize these patients based on the presence of SSX expressing CTCs. As previously demonstrated[12] using RT-PCR and qPCR we identified patients that had detectable SSX2 mRNA in the peripheral blood, attributed to SSX2 expressing circulating tumor cells (Figure 2 A and B). In this study, we assayed n=22 late stage prostate cancer patients and found 8/22 patients with SSX2 mRNA expression. Further we found 15/22 patients to have SSX2 specific CD8 T cells. Of the fifteen patients with SSX specific CD8 T cells, 6 patients expressed SSX2 mRNA in the blood, while 9 patients did no express SSX2 mRNA (Figure 2C). We detected SSX specific CD8 T cells both in patients with and without the presence of SSX2 mRNA, indicating that presence of SSX specific CD8 T cell alone did not dictate presence of SSX specific CTCs.

Presence of SSX expressing CTCs does not reveal differentiation in checkpoint expression

We next sought to determine if presence/absence of SSX expressing CTCs might be associated with impaired functionality of SSX2 specific CD8 T cells. We first examined T cell checkpoint molecule expression. Using flow cytometry (Supplemental Figure 4) we assessed the checkpoint molecule expression on CD8 T Cells. We compared the percent positive expression of checkpoint molecules on antigen specific CD8 T Cells to that of the whole population of CD8 T Cells. Using this strategy, we determined if the antigen specific cells were actively expressing the checkpoint molecule and to correct for patient to patient variability). We found that CMV specific CD8 T cells had the highest percent expression of CD160 and PD1, and significantly higher than those of whole CD8s in the same patients (Figure 3C). Interestingly, both viral antigens, CMV and EBV, expressed significantly less TIM3 in the antigen specific CD8 T Cells than the whole CD8 population (Figure 3C and D). Across all samples regardless of antigen specificity, we found very low expression levels of CTLA4 and LAG3. The CD8 T cells specific for the CTAs, SSX2 and NY-ESO-1, were found to express checkpoint molecules lower than the viral specific CD8s. 40% of viral specific CD8s expressed checkpoint molecules, while 20% of CTA specific CD8s expressed checkpoint molecules. Further, none of the checkpoint molecules examined on CTA specific CD8+ T cells were significantly higher than those of whole CD8s (Figure 3A and B). In fact, we found NY-ESO-1 specific CD8 T Cells expressed significantly lower CD160 and TIM3 compared to the whole CD8 population. Using the delineation of SSX2 expressing CTCs in the peripheral blood, we again compared the expression of checkpoint molecules on SSX2 specific CD8 T cells to global CD8s (Figure 3E and F). We found that there was no significant difference between the expression of checkpoint molecules on SSX2-specific CD8 T cells in patients with detectable SSX2 mRNA expression in the peripheral blood or not.

PMA/Ionomycin stimulation reveals cytokine complement of SSX specific CD8 T cells

We next sought to characterize the functionality of SSX2 specific CD8+ T cells, with respect to cytokine expression, in patients with and without evidence of SSX2 mRNA expression in the peripheral blood. PBMC were stimulated with PMA and Ionomycin and cytokine profiles of tetramer+ CD8+ T cells were then analyzed by flow cytometry. We found both SSX and CMV specific CD8 T cells were capable of expressing the T_h1 biased cytokines IFNγ, IL-2, TNFα, and Granzyme B, and the T_h2 biased cytokine Il-4,IL-6, and Il-10. We found that CMV specific CD8 T cells expressed significantly higher IFNγ and TNFα compared to SSX specific CD8 T cells (figure 4A), while SSX2 specific CD8 T cells expressed higher IL-4 (figure 4B). We further examined the multifunctionality of the antigen specific CD8 T cells. We found multifunctional CD8 T cells specific for CMV and SSX, however significantly more SSX specific CD8 T cells expressed only one cytokine compared to CMV specific CD8 T cells (Figure 4C and D. As before, we divided patients based on the presence of SSX expressing CTCs, and examined T_h1 cytokine expression of SSX specific CD8 T cells. We found that CD8 T cells from patients without SSX CTCs expressed significantly more TNFα than patients without SSX CTCs .Further, IFNγ and IL2 expression trended higher in patients without cytokines than those patients where there was still SSX mRNA present (Figure 5E).

Discussion

In this study, we wanted to characterize the function of SSX2-specific CD8, and determine if the immune response generated by these CD8s explain why some patients do not have detectable SSX2 mRNA in the peripheral blood, suggesting that SSX2 expressing CTCs are eliminated in these individuals. In this paper, we characterized SSX+ CD8 T Cells by analyzing their cytokine and checkpoint phenotypes. We determined that presence of SSX specific CD8s nor their expression of checkpoint molecules had bearing on whether patients possess SSX expressing CTCs. However, patients without SSX expressing CTCs had SSX specific CD8 T cells that expressed significantly more T_h1 biased cytokines. This report is the first evaluation of function of ex-vivo SSX specific CD8s, and the first direct comparison of two different CTA specific CD8 T cells. Further, we found checkpoint molecule expression is different on CTA specific CD8s when compared to viral antigen specific CD8s.

In this study, we sought to determine if the presence or absence of SSX expressing CTCs was due to immunological elimination or repression. We previously identified patients with SSX specific CD8s[15], and SSX expression in the blood is due to SSX expressing CTCs[12]. Using this methodology, we were able to group patients with SSX specific CD8 samples by the presence of SSX expressing cells in the blood. Of the 15 patients we found with SSX specific CD8 T cells, 6/15 possessed SSX expressing CTCs while 9/15 did not (Figure 2C). This demonstrated that even patients with SSX specific CD8s, could have SSX expressing CTCs, and lead us to conclude presence of SSX specific CD8s alone was not sufficient to eliminate SSX expressing CTCs. Further, we saw no difference in expression of checkpoint molecules between SSX specific and total CD8s in patients with and without SSX expressing CTCs. This finding implies

that checkpoint molecule expression is not related to presence of SSX expressing CTCs. However, if we use this distinction and then look at cytokine expression we find that there is an increase in T_h1 biased cytokines in patients with SSX expressing CTCs, indicating that these CTCs were eliminated from the host, due to a T_h1 biased response. This increase was significant in TNFα, while other T_h1 biased cytokines (IFNγ,Granzyme B, IL-2) trended in the same direction (figure 4E). We believe this data may indicate that a heightened T_h1 response may contribute to elimination of SSX expressing CTCs.

In this study, we report the first comparison of phenotype and prevalence of CTA specific CD8 T cells. Through the utilization of flow cytometry and tetramer staining, we are able to obtain the prevalence of CD8 T cells specific for SSX2 and NY-ESO-1 (Figure 1 and Supplemental Figure 1 and 2). We detected specific CD8s for both CTAs at a rate of 0.1-0.2% of total CD8s, with some patients as high as 0.8% of total CD8s. We also found similar rates of checkpoint molecule expression in both CTA specific CD8s. Roughly 20% of CTA specific CD8s expressed CD160, while PD1 was detected at rates of 10% and 30% for SSX and NY-ESO-1 specific CD8 T cells respectively (Figure 3A and B). We found that relative to all CD8s CTA specific CD8s did not express heightened checkpoint molecule expression, leading us to conclude that the activity of these CD8s were not being inactivated through checkpoint molecule pathways.

Using flow cytometric analysis (Figure 3 and Supplemental Figure 4), we assessed checkpoint molecule expression on CD8 specific T cells for several antigens of interest. We found significant differences in checkpoint molecule expression for each antigen. We found the

highest percent of checkpoint expression on the viral antigens CMV and EBV; roughly 50% of CMV specific CD8s express CD160 or PD1, and ~40% of EBV specific CD8s have PD1, though lower expression CD160 at ~20% (Figure 3C and D). Both expression of CD160 and PD1 was higher in specific CD8s than whole CD8s implying a mechanism of regulation. CD160 and PD1 expression was significantly higher in CMV specific CD8s than whole CD8s, and PD1 expression was higher in EBV specific CD8s relative to whole CD8s. Surprisingly, we found TIM3 expression was lower in viral specific CD8s relative to control. This could represent that viral antigens are more likely to be regulated by C160/HVEM or PD1/PDL1 pathways as opposed to TIM3/Galectin 9. When comparing these viral specific CD8s to those of CTAs, we found lower expression of checkpoint molecules. 20% of SSX and NY-ESO-1 specific CD8s expressed CD160, and between 10-30% expressed PD1. We also found that CTA specific CD8s did not express checkpoint molecules more than the whole CD8 population, implying these CD8 T cells are not being regulated through checkpoint molecules. We expect checkpoint regulated CD8 T cells to significantly express checkpoint molecules more than the baseline CD8 T cells, as we see in CMV and EBV specific CD8s. Interestingly, the NY-ESO-1 specific CD8 T cells had significantly lower CD160 and TIM3 expression than whole CD8s. These differences in checkpoint molecule expression can be explained due to differences in immune synapse contact time, or peptide affinity to the MHC, or is due to the context in which the antigens are expressed. Interestingly, the CMV antigen we investigated had the highest estimated MHC affinity based on the SYFPEITHI algorithm relative to the other antigens investigated in this report. Researchers have shown that increased contact time leads to heightened expression of checkpoint molecules (zahm2017). We found SSX specific CD8 T cells express IFNγ, TNFα, and Granzyme B, but significantly less so than CMV specific CD8s (Figure 4A and B). Similarly, SSX specific CD8s

are less often multifunctional than CMV specific CD8s, and expressed checkpoint molecules less frequently. Taken together, these observations, of CMV specific CD8s, could represent a mechanism by which the immune system can exert increased control over high T_h1 cytokine expressing CMV specific CD8.

In this study, we sought to understand different facets of SSX specific CD8 T cells in prostate cancer. Successfully, we were able to determine the checkpoint molecule phenotype as well as the cytokine profile of these SSX targeting CD8s. This report is the first evaluation of function of ex-vivo SSX specific CD8s, and the first direct comparison of two different CTA specific CD8 T cells. Finally, we found checkpoint molecule expression is different on CTA specific CD8s relative to viral antigens, and does not contribute to the presence of SSX expressing CTCs.

Figures and Figure Legends

Figure 1: <u>Antigen Specific CD8+ T Cells of interest were detected in peripheral blood of late stage prostate cancer patients</u>
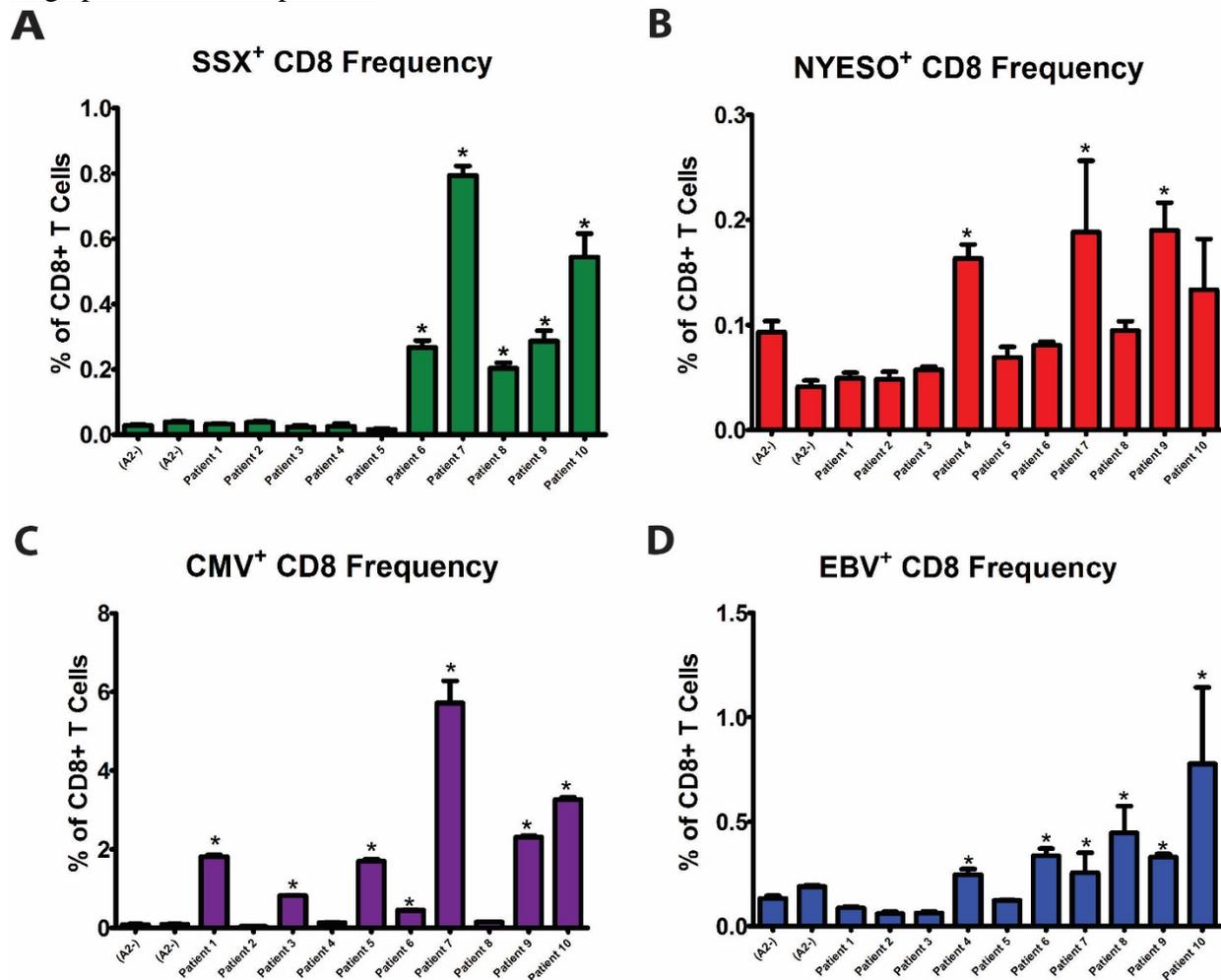
Whole peripheral blood was stained and analyzed by flow cytometry for the presence of CD8+ T cells specific for antigens of interest A) SSX2 B) NYESO-1 C) CMV D)EBV. Significance was determined by comparing each sample to HLA-A2- controls using the student's T test.

Figure 2: Delineation of Human Sampels by detection of SSX2 mRNA

SSX mRNA was detected using RT-PCR (A) (L:Ladder S:SSX specific lanes A:Actin Specific lanes) B) Chart represents the aggregation of all patients examined for SSX2 mRNA in the peripheral blood and the presence of SSX2 specific CD8 T cells. Green spaces represent SSX

mRNA in the peripheral blood or presence of SSX specific CD8+ T cells. Gray spaces represent no SSX mRNA in the peripheral blood or no presence of SSX specific CD8+ T cells.

Figure 3: <u>Presence of SSX expressing CTCs does not reveal differentiation in checkpoint expression</u>

SSX mRNA was detected using RT-PCR (A) (L:Ladder S:SSX specific lanes A:Actin Specific lanes) B) Chart represents the aggregation of all patients examined for SSX2 mRNA in the peripheral blood and the presence of SSX2 specific CD8 T cells. Green spaces represent SSX mRNA in the peripheral blood or presence of SSX specific CD8+ T cells. Gray spaces represent no SSX mRNA in the peripheral blood or no presence of SSX specific CD8+ T cells.

Figure 4: <u>PMA/Ionomycin stimulation reveals cytokine complement of SSX and CMV specific CD8 T cells</u>
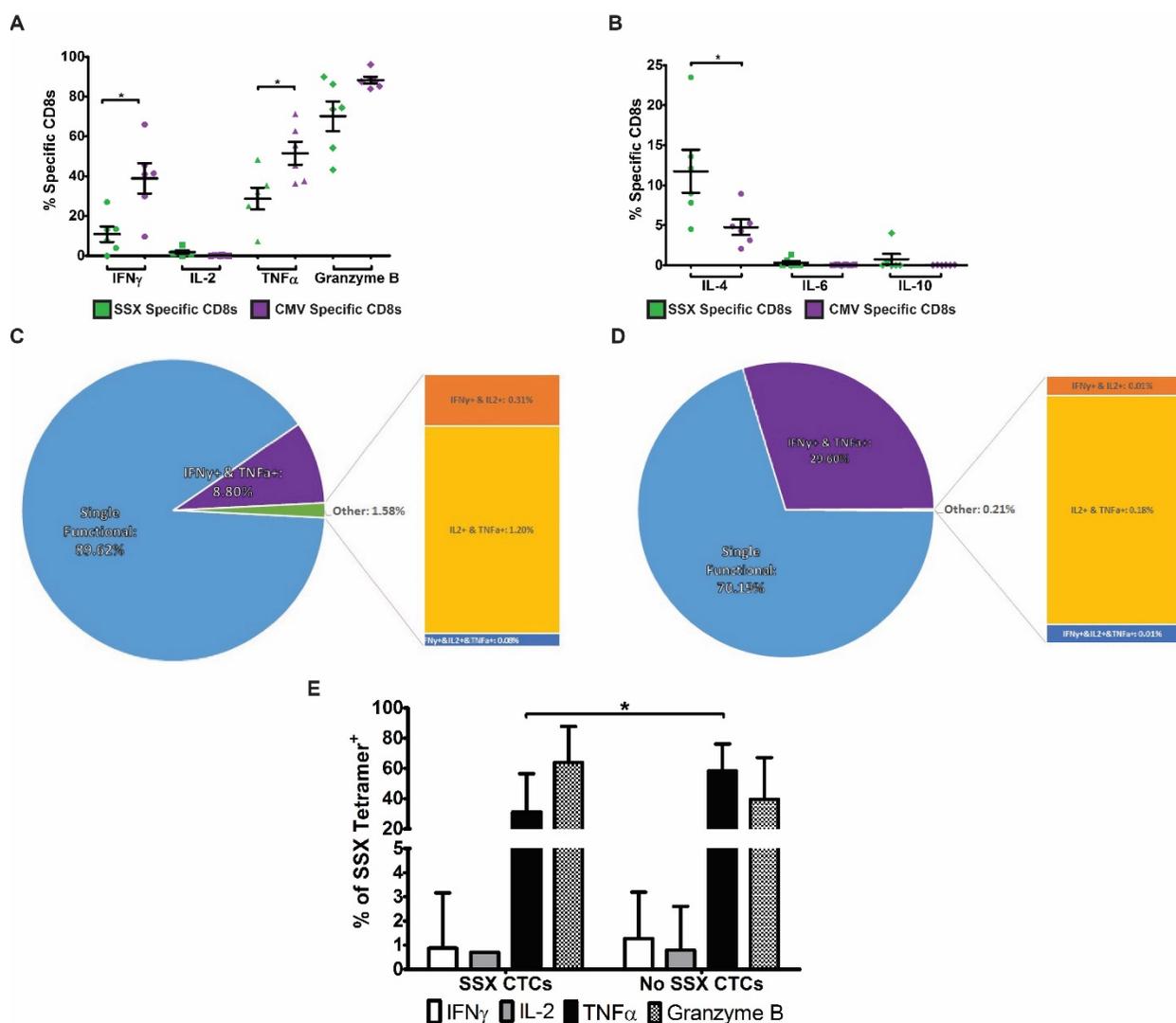
Human samples previously shown to possess SSX2 or CMV specific CD8+ T cells were stimulated with PMA and Ionamycin, and then intracellularly stained for cytokine expression. The cells were then analyzed by flow cytometry. A)Th1 biased cytokine expression in PMA stimulated SSX specific or CMV specific CD8+ T Cells B)Th2 biased cytokine expression in PMA stimulated SSX specific or CMV specific CD8+ T Cells C)Prevalence of single and multifunctional Th1 biased SSX specific CD8 T cells D) Prevalence of single and multifunctional Th1 biased CMV specific CD8+ T cells E) Expression of Th1 Cytokines in patients with and without SSX expressing CTCs

Supplement Figure 1: Gating Strategy for CD8^{+} T cells
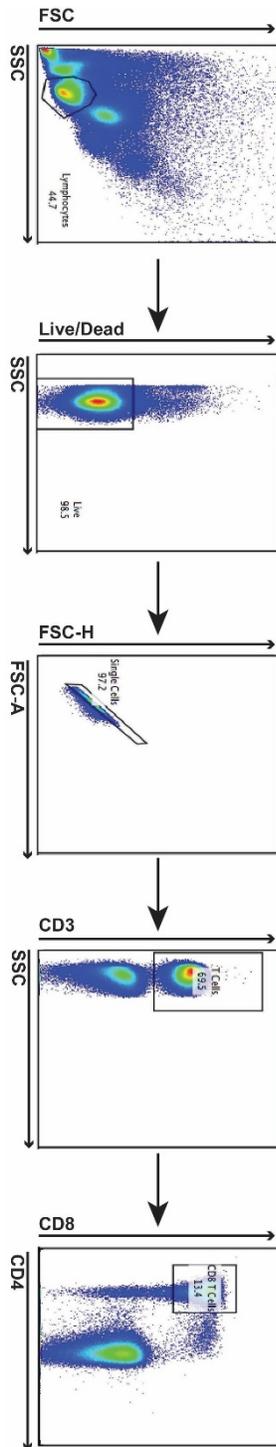
Supplemental Figure 2: Representative Flow Cytometry of Tetramer+ Events
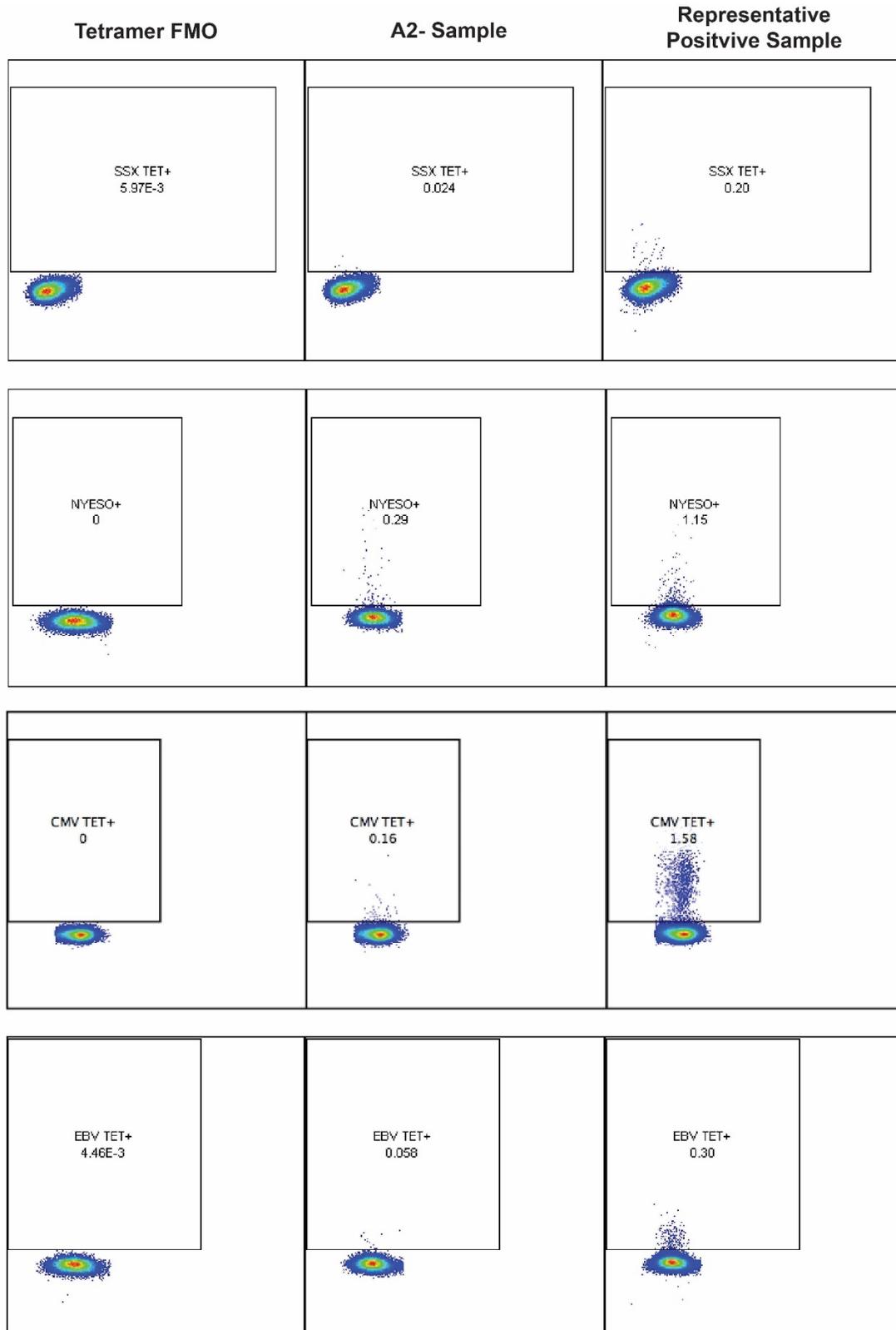
Supplemental Figure 3: Flow Cytometry Gating Strategy for Cytokine Expression
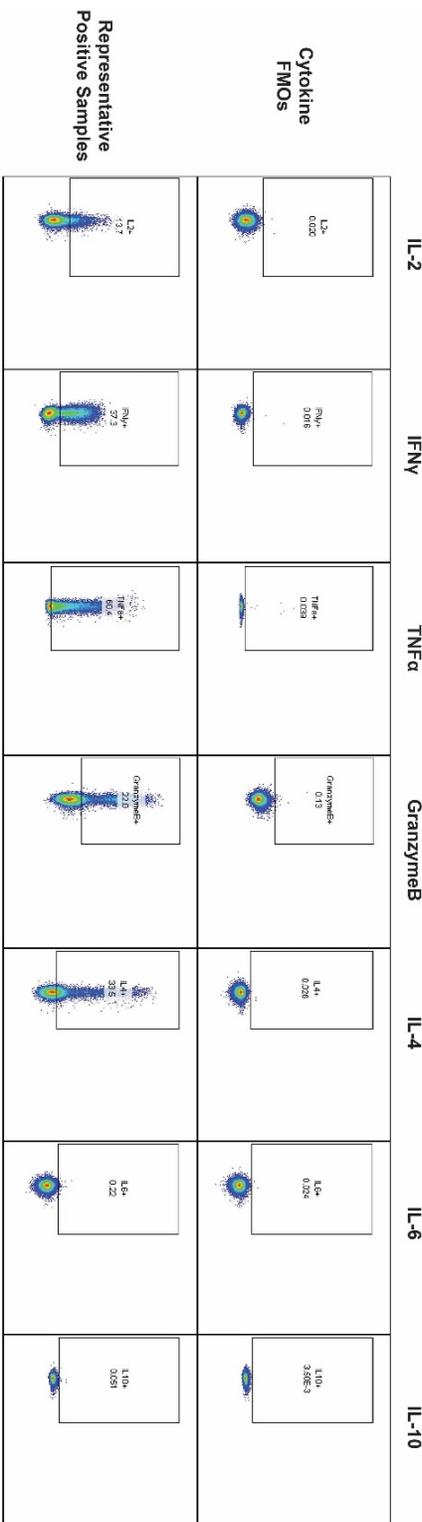
Supplemental Figure 4: Flow Cytometry Gating Strategy for Checkpoint Expression
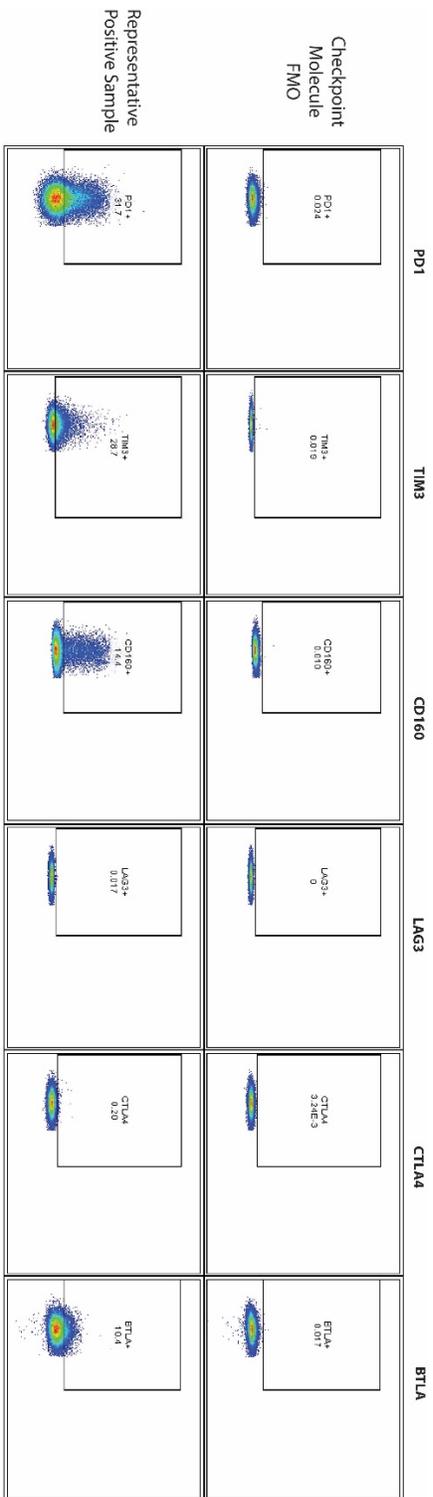
Materials and Methods

Patient Samples

With informed consent, peripheral blood or leukapheresis products were obtained from male

subjects (n=33) with late stage prostate cancer. Peripheral blood mononuclear cells (PBMC)

were isolated by Ficoll-Paque centrifugation (Pharmacia AB, Uppsala, Sweden) and

cryopreserved in liquid nitrogen until use.

Flow Cytometry Phenotypic Analysis

For analyzing antigen-specific T cells, tetramers specific for HLA-A2 restricted epitopes

$CMV_{495-503}$ PP65[16], $EBV_{356-364}$ LMP2[17], $SSX2_{103-111}$[15] to NY-ESO-$1_{157-165}$[18] were obtained from

the NIH Tetramer Core Facility (Atlanta, GA). PBMC were thawed, washed 2 times in HBSS,

and then stained for (SSX/CMV 20min at 37C, EBV/NYESO 30 min at 22C) FACS buffer (PBS

+3%FCS+50mM EDTA) containing a 1:X (X=500 for SSX, ,CMV X=1000 EBV/NYESO)

dilution of tetramer. Cells were then washed with FACS buffer and stained with CD3-BUV395

(Clone: UCHt1,BD), CD8-BV786 (Clone: RPA-T8,BD), CD4-BUV805 (Clone: SK3,BD) at a

concentration of 1:100 total staining volume and with Ghost Dye Red-780 (Tonbo Biosciences)

viability marker. Cells were analyzed on an LSR Fortessa and antigen-specific T cells were gated

as Lymphocytes/Live/Singlets/CD3+/CD4-/CD8+/Tetramer+ using FMO and HLA-A2$^-$ samples

as controls (Supplemental Figure 1 and 2). To analyze the presence of checkpoint molecules the

following antibodies were added to the antibody staining cocktail in addition to those above,

TIM3-PECF594 (Clone 7D3,BD), BTLA-BV421 (Clone MIH26, BioLegend), PD1-BV711

(Clone EH12.2H7,BioLegend), CTLA4-PE (Clone 14D3, eBiosciences), CD160-AF488 (Clone

BY55, eBioscience), LAG3-PECy7 (Clone 3DS223H, eBioscience). The gating strategy of

Lymphocytes/Live/Singlets/CD3+/CD4-/CD8+/Tetramer+/Checkpoint+ was used with FMOs to serve as controls, and then analyzed using FlowJo software (version 10.2).

Flow Cytometry Intracellular Analysis

PBMC from patients with SSX2-specific or CMV-specific CD8 T cells were stimulated with 40 ng/mL phorbol 12-myristate 13-acetate (PMA, Sigma), 2.6 μg/mL ionomycin (MP Biomedicals, Santa Ana, CA) 1.5 μM monensin in RPMI medium, for 4 hours. Following the 4 hour of treatment stimulation, cells were stained for tetramers as before, washed with FACS buffer and then stained for the surface markers CD4-BUV805 (Clone: SK3,BD), CD3-BUV395 (Clone: UCHt1,BD), CD8-BV605 (Clone:RPA-T8, BioLegend), as before. Cells were then fixed using Cytofix (BD) per manufacter instructions. Intracellular cytokine staining was performed as per the manufacturer's protocol (Cytofix/Cytoperm Kit, BD Biosciences). Antibodies included IFNγ-BV786 (Clone: 45.B3, BD), IL-2-PerCP-Cy5.5 (Clone: Mq1-17H12,BD), TNFα-PE-Cy7 (Clone:Mab11,BioLegend), GranzymeB-BV510 (Clone:GB11,BD), IL-4-PE (Clone: 8D4-8, BioLegend), IL-6-PE-CF594 (Clone:MQ2-13A5,BD), IL-10-AF488 (Clone: JES3-9D7,BioLegend). Cells were analyzed on a BD Fortesssa using the gating strategy Lymphocytes/Live/Singlets/CD3+/CD4+/CD8+/Tetramer+/Cytokine+ (Supplemental Figure 3) using FlowJo software (version 10.2).

Reverse Transcription (RT)-PCR Analysis:
RT-PCR using the One-Step RT-PCR kit (Qiagen,Valencia,CA) was carried out on RNA collected using Reliaprep RNA cell miniprep system (Promega) from peripheral blood samples from patients with prostate cancer or healthy control donors, under the following PCR conditions: 50°C for 30min.,95°C for 15min., 35 cycles at 95°C for 1 min., 60°C for 1 min. and 72°C for 1 min. Final extension for 10 min. at 72°C was followed by 4°C incubation until

amplified products were resolved on a 2% agarose gel. Primers utilized were: SSX2 RT-PCR:

forward: GTGCTCAAATACCAGAGAAGATC reverse: TTTTGGGTCCAGATCTCTCGTG,

Actin forward: TCATGAAGTGTGACGTTGACATCCGT reverse:

CTTAGAAGCATTTGCGGTGCACGATG

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CHAPTER FIVE: Summative Discussion

The McNeel laboratory has been investigating SSX for more than ten years. This thesis is a continuation of that work. As we have primarily been interested in SSX as a vaccine target, past work in the McNeel lab focused on the immunological recognition of SSX. Namely: identifying SSX as a highly recognized CTA due to SEREX(1), identifying the HLA-A2 regions of the SSX2 gene(2), using an SSX encoding DNA vaccine to protect against SSX encoding tumors(3), and identifying SSX specific CD8+ T cells in late stage patients(2). While these works were crucial in understanding immune responses to SSX, in order to target SSX for future therapeutics, and ensure therapeutic safety, we needed additional information on SSX's function and expression pattern.

In order to properly target SSX, we first need to understand 'where' and 'who'. The 'where' is SSX being expressed: primary tumors, in the blood, or in metastases. The 'who' being which SSX family members are expressed during prostate cancer. Through a variety of methods and experimentation, we identified both the 'where' and 'who' of SSX in prostate cancer. Fortunately, prior work in the McNeel lab identified SSX expression confined to metastases, and not primary tumors answering most of the 'where' question. Unfortunately, this work was not able to distinguish between SSX family members leaving 'who' to be discovered. My first major finding was that SSX2 is the canonical member of SSX in prostate cancer. Although, we did see the low level expression of SSX1; and use of epigenetic modifying agents on prostate cancer cell lines will cause expression of other SSX family members. We identified SSX2 as the canonical member in prostate cancer due to the prevalence of SSX2 expression in PBMC and metastatic tissue. Second, we defined a circulating tumor cell population (CD45-/EPCAM+/CD63+) that expresses SSX. In summation, we can now answer the 'where': SSX expression is confined to the blood and metastases, and the 'who': SSX2 is the canonical family member expressed in

prostate cancer. Given these pieces of information, future therapeutic studies should target SSX2 in prostate cancer in patients with recurrent disease.

Many groups have investigated the function of the SSX family, but much about its function remains unknown. Additional data about SSX's function could inform vaccine design or vaccination strategy. Groups have identified SSX as a transcription factor[4], with two conserved domains[5,6], and several binding partners[7]. Further evidence also pointed to SSX's involvement in the epithelial to mesenchymal transition (EMT)[8–10], which is important for metastases formation. In addition, the expression pattern of SSX (expressed in metastases and not primary tumors[2]) suggested that it might be involved in disease progression. Given these data, we sought to investigate SSX2's function in prostate cancer particularly in respect to EMT. I found that SSX does not drive the epithelial to mesenchymal transition in prostate cancer. Although, modulation of SSX expression did result in changes in EMT associated genes, the changes were not canonical to a driver of EMT. Further, we found that SSX may influence genes associated with focal adhesion. Focal adhesion represents a portion of a greater EMT network of genes. The exact mechanism of SSX's interaction with focal adhesion, is still unknown. Interestingly, we also identified a link between SSX expression and expression of the androgen receptor. We found the knockdown of SSX lead to knockdown of the androgen receptor and androgen receptor splice-variants.

Finally, we sought to characterize SSX specific CD8 T cells. Prior work from the McNeel group had identified the HLA-A2 immunodominant epitope ($SSX_{103-111}$) and demonstrated that SSX specific CD8s were more prevalent in late stage prostate cancer patients[2]. Because we had the methods both to identify SSX specific $CD8^+$ T cells, and patients with SSX expressing CTCs, we asked if theses SSX specific CD8s provided protection and eliminated SSX expressing CTCs.

However, we found that patients with SSX specific CD8s could still possess SSX expressing CTCs. This demonstrates that the possession of SSX specific CD8 T Cells alone was insufficient to eliminate SSX expressing CTCs from the host. We next asked if patients with SSX specific CD8s and SSX expressing CTCs, was due to downregulation of T cell activity caused by expression of checkpoint molecules. We found that relative to viral specific CD8 T cells, SSX specific CD8$^+$ T cells (and NY-ESO-1 specific CD8$^+$ T cells) expressed low levels of several checkpoint molecules. Additionally, SSX specific and whole CD8s did not significantly differ in checkpoint expression. Nor was there a difference in checkpoint expression on SSX+ CD8s between patient groups with and without CTCs. These studies represent the first investigation of checkpoint molecule expression on CTA specific CD8s. In summation, my studies have identified the 'who' and further clarified the 'where' in regards to SSX and prostate cancer.

As these studies were not completely exhaustive into all aspects of SSX, there are several interesting lines of investigation for future studies of SSX in prostate cancer. Future work may investigate how the functional domains (KRAB and SSXRD) of SSX affect disease progression or SSX function. Although others groups (ie groups specializing in molecular biology) may be better suited to do this. There are a few interesting pieces of data in regards to SSX function, that I feel could warrant future research. First, there seems to be some connection between SSX, SSX2IP (a known binding partner), and cell cycle regulation. Bertha Brodin's group had observed that knockdown of SSX in osteosarcoma and melanoma cell lines resulted cell cycle arrest in the G1 phase(9). Further, in acute myeloid leukemia SSX2IP is associated with the translocation event t(15;17), which resulted in heightened expression of cyclins D2,D3,E2,and B2(11). Given these two pieces of data, it is possible one mechanism of SSX is regulation of cell cycle, mediated through its binding of SSX2IP. Second, is my finding of the relationship

between SSX and AR. I demonstrated that knockdown of SSX2 in the 22Rv1 cell line also resulted in shut down of AR expression, and expression of AR splice variants AR-V1 and AR-V7. This is an entirely novel finding, which I discovered through the gene microarray, and then validated with qPCR, so I have high confidence that this finding is 'real'. In my opinion, there are several obvious questions to follow up on this finding. The first, why is this relationship between SSX and AR happening/what is the mechanism that causes this change. Second, is this finding unique to the 22Rv1 cell line or could we see similar changes in other cells lines. Third, does this cause a change in disease progression. While I believe these are interesting questions, they are basic science questions, and thus funding opportunities are dubious in the current funding climate.

The focus of this thesis has been on SSX and how it affects disease progression, and if it makes a suitable vaccine target. I believe SSX represents a good target to vaccinate against in prostate cancer due to its expression pattern and status as a CTA. The major issue with SSX as a vaccine target is its lack of expression in primary tumors and low expression in metastases(2). Roughly, 30-40% of patients will have some SSX expression in the blood or metastases at very low levels. I cannot overstate how low the level of expression of SSX can be in these patients, and I believe this to be the primary factor keeping SSX from being an excellent therapeutic target. The low percent of patients expressing SSX and low levels of SSX expression put a severe restriction on the number of patients that may react to an SSX targeted vaccine. I am still unsure if SSX is involved with disease progression, I believe it is equally possible that the loss of SSX expression will cause prostate cancer to worsen. As I demonstrated, knockdown of SSX in the 22RV1 cell line resulted in phenotypes consistent with worse disease. However, other research seemingly has demonstrated the opposite(8–10). These differences in findings could be

explained due to the differences in specific cancers examined, and some functional aspects of SSX may be context dependent. Given these conflicting reports I conclude, that SSX likely does not drive disease progression.

Much of the reasoning that lead us to study SSX was its high degree of recognition in late state cancer patients(1,2). Then we take this to mean SSX is important in disease progression because the host's immune system is mounting a response. However, I believe there could be an alternative explanation: SSX recognition in late stage patients is simply a casualty of antigen spread. Antigen spread simply is this, immune cells responding to a tumor will lyse open tumor cells, a milieu of contents will spill out and then be phagocytosed by APCs (antigen presenting cells), who then subsequently present these antigens to T and B cells. I believe SSX is released in the milieu of lysed tumor cells and then presented by APCs. Then, given SSX's nature as a CTA, it had previously never been seen by the immune system, which would then generate an immune response to this 'foreign' antigen. If this is true then SSX likely has nothing to do with disease progression, but this does not detract from its attractiveness as a target for future prostate cancer therapies. It has been a hell of a ride.

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End of Thesis