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14. ABSTRACT

There is an urgent need to develop both new approaches to the treatment of prostate cancer. Analysis of human prostate samples demonstrates that a specific signaling pathway, the Pim kinase pathway is elevated in the fibroblasts from human prostate tumors. To understand the role of myofibroblast/cancer associated fibroblasts (CAFs) in transformation, the laboratory proposes (1) to examine in detail the proteins secreted by the stroma that can modulate epithelial growth, (2) to evaluate the ability of Pim inhibitors to block this activity, and (3) to investigate whether exosomes can potentially be used as a biomarker of Pim kinase inhibitor activity. Results to date demonstrate that Pim increases in prostate stromal cells enhances protein synthesis, the levels of important transcription factors, long non-coding RNAs, and tyrosine kinases associated with signal transduction as well increased exosomal transfer both in cells co-cultured and when conditioned media is placed on prostate epithelial cells. These changes are blocked by the addition of Pim inhibitors. These results suggest that the Pim protein kinase can regulate stromal cell biology to modulate epithelial growth and that inhibitors of this protein kinase have the potential to block this process and thus inhibit tumor growth.

15. SUBJECT TERMS

Cancer associate fibroblasts (CAFs); Myofibroblasts; Pim protein kinase; Exosomes; Prostate stem cells, Pim inhibitors, AZD 1208 and LGH447

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Introduction

Prostate cancer (PCa) stromal cells, also known as myofibroblasts associated fibroblasts (CAFs) have a critically important interdependent interaction with the surrounding epithelial cells. Proteins secreted from CAFs stimulate PCa progression and metastasis. Interrupting this interdependency by targeting signal transduction pathways that mediate the production and secretion of these proteins is a novel approach to blocking PCa growth. In this proposal, the applicant research team demonstrates that protein kinases can induce normal prostate stromal fibroblasts to produce proteins that enhance PCa growth, thus mimicking a CAF-like phenotype. The addition of kinase inhibitory drugs that have entered Phase I clinical trials reverses this secretion. New results also demonstrate that changes in the proteins secreted by myofibroblasts can be detected in the urine of cancer patients, suggesting that these proteins can be used as a marker of the activity of drugs targeted at CAFs. *The knowledge gained through the analysis of CAFs from cancer patients proposed in this application is essential for the further development of new biomarkers that reflect stromal protein production*

Keywords

Cancer associated fibroblasts (CAFs)
Myofibroblasts
Pim Protein Kinases
Exosomes
Prostate Stem Cells
Pim Inhibitors, AZD1208 and LGH447
Long non-coding RNA H19
Small interfering RNAs

Accomplishments

Major goals of the project

1- Compare the secretome of normal prostate fibroblasts with inducible Pim1 with freshly isolated human CAFs: develop a data set of proteins that are regulated by the Pim1 protein kinase in prostate fibroblasts.

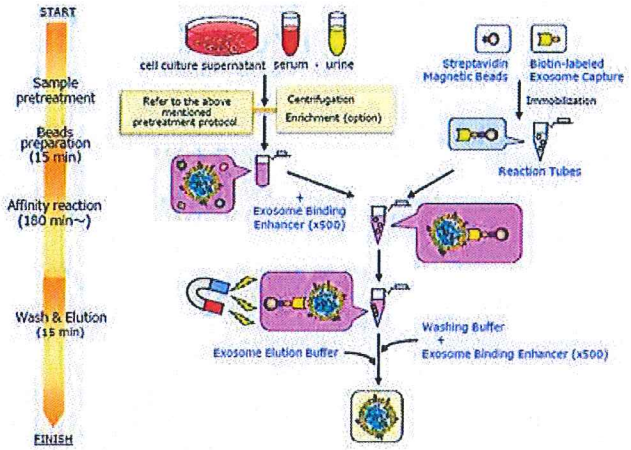
2- Examine whether inhibitors of PIM protein kinase block the tumor stimulating activity of myofibroblast/CAF-induced, including migration, invasion, and growth of epithelial tumor cells.

3- Investigate whether exosomes can potentially be used as a biomarker of Pim kinase inhibitor activity in myofibroblast/CAFs: and whether exosomes can be used to deliver anti-cancer genes to tumor cells.

Accomplishments

Task 1- Compare the secretome of normal prostate fibroblasts with inducible Pim1 with freshly isolated human CAFs: develop a data set of proteins that are regulated by the Pim1 protein kinase in prostate fibroblasts. To be able to isolate purified exosomes with confidence that these vesicles are pure and not contaminated by cellular debris, we have developed special techniques. These purified vesicles will allow an analysis of the ability of Pim protein kinase to

Figure 1. Exosome isolation protocol from Magcapture



change and regulate the secretory pattern. Although some laboratories simply do a high-speed spin to pellet vesicles, experiments by this team have found that these exosomes are not pure. To tackle this problem the following procedure has been developed. First, a prostate stromal cell line was derived that contained a Doxycyclin-inducible Pim 1 gene, BHPrS1-Tripz-Pim1. BHPrS1-Tripz-Pim1 stromal cell lines, typically, 20-40 $\times 10^6$ cells, were cultured in 25 mL DMEM medium with 5% exosome depleted FBS (Thermofisher). Using this FBS is critical to the success of this procedure. Culture supernatants were then harvested, and serially centrifuged to remove cells and debris (10 minutes at 300g, followed by 30 minutes at 3000g), and filtered (0.22 μm) to remove small debris and microvesicles. Importantly experiments demonstrated that this media contained vesicles of multiple sizes and types. To enhance the purity of this preparation 150 mL of filtered media was concentrated to 1ml with the Centricon Plus-70 Centrifugal Filter (Ultracel-PL Membrane, 100 kDa) device using an Allegra® X-15R centrifuge at 3,500 g at 4°C. The concentrate was then recovered with a reverse spin at 1,000 g for 2 minutes. Exosome pellets were then isolated and concentrated using ultracentrifugation (3h at 110 000g, 4°C). Exosome pellets were suspended in PBS to a desired concentration and filtered (0.45 μm). The presence of exosomes was validated using western blots to demonstrate enrichment of exosome marker proteins TSG101, CD63, and Flotillin1 known exosome proteins, and the absence of golgi vesicles and demonstrated by the absence of golgi protein GM130.

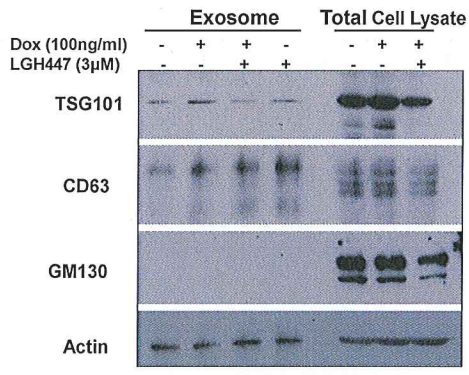
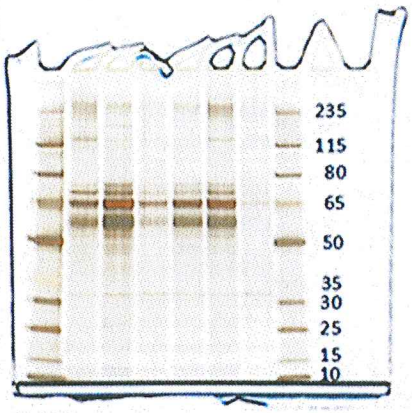


Figure 2. Characterization of exosome preparations by Western blot. Western blot analysis of common exosome markers (TSG101 and CD63) and golgi marker (GM130). 10 μg of exosomes isolated by ultrafiltration and serial centrifugation methods. Total cell lysates from BHPrS1-Tripz-Pim1 with or without Doxycyclin and Pim inhibitor LGH447 treatments was loaded as positive control.

Figure 2 demonstrates that exosomes isolated from doxycycline-treated stromal cells in which Pim is induced contain well known proteins associated with exosomes. Interestingly, the Pim induced cells exhibited an increased level of the exosome marker CD63 compared with untreated controls suggesting that the number of exosomes was increased by Pim induction in stromal cells. Figure 3 shows that the overall loading of these proteins was similar.

Figure 3: Gel image of 1 ug protein of each sample loaded for Mass spec

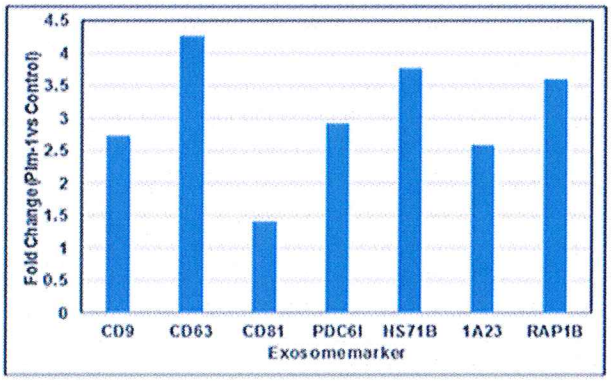
Lane	sample	ug
1	MW marker	
2	Control BHPrS1-XO-1	1
3	Control BHPrS1-XO-2	1
4	Control BHPrS1-XO-3	1
5	Pim BHPrS1-XO-1	1
6	Pim BHPrS1-XO-2	1
7	Pim BHPrS1-XO-3	1
8	MW marker	
9	LB	
10	LB	



Exosomes were isolated from control BHPrS1prostate stromal cells or Dox-induced Pim expressing BHPrS1 cells and 1 ug of each prep was loaded SDS gels which were Comassie blue stained.

Our results in Figure 4 illustrate that the number of exosomes produced by Pim expressing cells was increased and that this increase was inhibited by treating BHPrS1 cells with a Pim inhibitor (LGH447 3uM) (data not shown). This data demonstrates that the team can identify proteins that are in exosomes as a result of Pim kinase overexpression. Importantly, it also shows that Pim kinase expression in stromal cells is increasing the quantity of exosomes released by these stromal cells. This finding is consistent with the hypothesis that Pim is playing an important role in stromal biology. In Figure 4, the number of exosomes secreted by a fixed number of cells was measured by an Elisa kit that quantitates acetylcholine esterase activity to measure the number of exosomes per group.

Figure 4: Enrichment of exosomes in BHPrS1 with Pim induction



The level of markers found in exosomes isolated from Pim expressing BHPrS1 prostate stromal cells were compared to wild type cells.

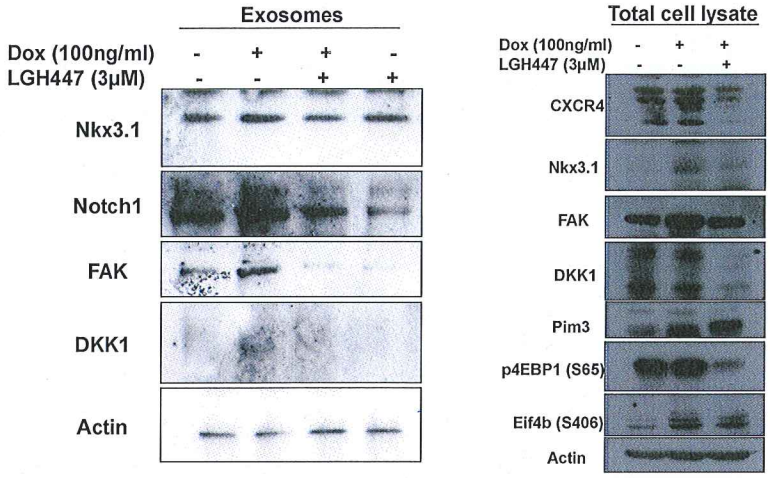


Figure 5. Exosomal protein content after PIM1 induction in BHPrS1-Tripz-Pim1 cells: Western blot analysis of translational regulators (eIF4b, 4EBP1), metastasis markers (NOTCH1, DKK1), differentiation protein (NKX3.1) and tyrosine kinase (FAK) in 10 μg of exosome protein lysate and total cell lysate from prostate stromal BHPrS1-Tripz-Pim1 treated with Doxycyclin alone or in combination with the Pim inhibitor LGH447.

An example of such results is shown in Figure 5 and demonstrates increases in the exosomal proteomic profile of these genes on Pim1 overexpression. Importantly, addition of Pim Kinase inhibitor (LGH447, 3μM) showed a reversal of the Pim kinase induction of the level of a number of these proteins in exosomes. These results demonstrate that the level of Pim protein kinase controls the expression of specific proteins found in exosomes. As shown in the right figure levels may or may not reflect what is occurring in the cytoplasm. Also, importantly the addition of Pim inhibitors to these stromal cells can decrease the level of specific proteins in secreted exosomes. It is well known that exosomes carry mRNA. We have analyzed two mRNAs that could be transferred to tumor cells and modulate their behavior, Pim1 and CXCR4 (SDF4). As can be seen in Figure 6, the expression of Pim in BHPrS1 cells increases the level of both Pim1 and SDF4 (CXCR4) mRNA and this increase is inhibited by the addition of the Pim inhibitor LGH447 (3uM). These results clearly set the stage for a broader examination of the differences in exosome content stimulated by Pim1 using mass spectrometry and SILAC as described in Major task 1.

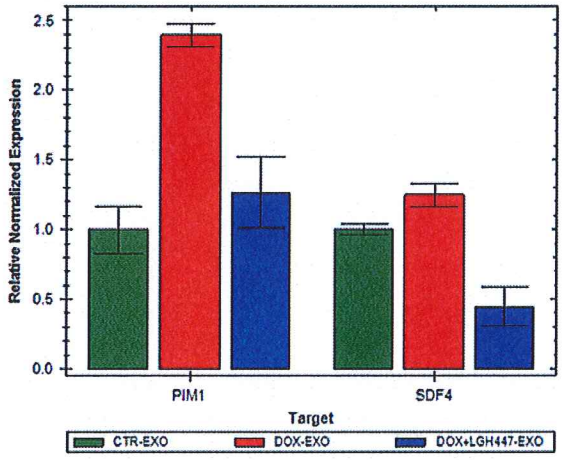


Figure 6. Prostate stromal fibroblast upregulate and secrete Pim1 and stromal derived factor 4 (CXCR4) into the exosomes. qPCR: Exosomes secreted from BHPrS1-Tripz-Pim1 treated with DMSO (CTR-EXO), Doxycyclin (Dox-EXO), Doxycyclin+LGH447 (DOX+LGH447-EXO) were collected and total exosomal RNA was isolated. Pim1 and SDF4 mRNA levels within the fibroblast exosomes were quantified by qPCR using relative delta Ct values. Doxycyclin (100ng/ml) was used for Pim1 induction in stromal cells and Pim kinase inhibitor LGH447 (3μM) was applied to the cells

Mass spec description: Samples were sent for digestion and Sequential window acquisition of all theoretical mass spectra (SWATH) quantitation

Sample description: Exosomes isolated by WAKO MagCapture Kit from Human Prostate Stromal Cells

Purpose: Digestion and nanoLC-MS-SWATH quantitation of proteins across the triplicates samples

Protocol: Based on the protein concentrations provided, 10 ug was removed from each sample, concentrated by speedVac, reconstituted in Laemmli sample buffer and run on a mini gel with prestained markers until the dye front reached about 2 cm. The entire section of gel for each samples was excised and subjected to in gel digestion with trypsin after DDT reduction and iodoacetamide alkylation. The recovered peptide were reconstituted to 400 ng/uL in 0.1% formic acid such that 5 uL injected would equate to about 2 ug (the maximum amount for the nanoLC-MSMS trap column). The spectral libraries were generated from 3 sample combinations as follows: 1) equal mix of the 3 control samples, 2) equal mix of the 3 Pim samples, 3) equal mix of all samples. Subsequently, each control and each Pim sample was collected independently using a SWATH acquisition on a Sciex 5600 + quadrupole-tof system. The SWATH range was 350-750m/z with 8Da windows with a 1Da overlap. Data were collected at 50 ms with a cycle time of 3.15 sec. Gradient elution was from 5% ACN to 40% in 70min (0.5%/min) from a 75um X 150 cm Thermo Acclaim c18 column. The spectral library and the SWATH analysis were done using Sciex Peakview software with the SWATH module. Protein areas were used to normalize each samples using Total Area Normalization in Excel and the significance of protein changes between the control and Pim groups was done by T-Test.

2. SWATH Spectra Library--122 proteins were identified at a 1% protein FDR
a. The low number of protein may be related to a lower than expected protein load as judged by TIC of the TOF-MS which appears to be more consistent with about 0.5 ug load than the expected 2 ug.
b. The variability in protein load among the samples is also evident from the TIC profiles of both the control 3 and Pim 3 samples.
c. This low number of protein and variability in protein load has implications for sample normalization.

3. SWATH protein identification and comparative quantitation

a) There were a number of exosome marker protein detected (Figure 2), suggesting a good enrichment of exosomes.
b) The upregulated proteins on Pim-1 induction in Dox inducible BHPrS1 stromal cells are categorized in Table 1 and Figure 3.

Next Steps: The low amount analyzed and the variable levels of protein likely limited the number of protein detected and the ability to obtain relative quantitation data. One option might be to boast the amount of the control 3 and Pim 3 samples to get a closer load to the other 4 samples, but this may not add much since the spectral library is only 122 proteins and many of these are based on only one peptide detected. As such, the only real option is to expand the spectral library by first running about 4X more for each of the protein mixtures. This unfortunately would require preparing more samples for digestion. Since sample Pim-3 was only reported to 15 ug total (and we used 10 already) this may require more sample.

Results from First analysis:

Protein	Description	T-test	Fold change (PIM/C)
K2C1	Keratin, type II cytoskeletal 1 Present in intermediate filament	0.043395	3.285346
K1C9	Cytokeratin-9	0.087222	5.308301
K1C14	Cytokeratin-14	0.305837	1.594855
FINC	This gene encodes fibronectin, a glycoprotein present in a soluble dimeric form in plasma, and in a dimeric or multimeric form at the cell surface and in extracellular matrix. The encoded preproprotein is proteolytically processed to generate the mature protein. Fibronectin is involved in cell adhesion and migration processes including embryogenesis, wound healing, blood coagulation, host defense, and metastasis.	0.590513	1.493957
LAMA4	Laminin alpha 4: Laminins, a family of extracellular matrix glycoproteins, are the major noncollagenous constituent of basement membranes. They have been implicated in a wide variety of biological processes including cell adhesion, differentiation, migration, signaling, neurite outgrowth and metastasis.	0.109969	1.61176
LAMB1	laminin subunit beta 1	0.176344	1.532355
SVEP1	Sushi, Von Willebrand Factor Type A, EGF And Pentraxin Domain Containing 1, May play a role in the cell attachment process.	0.575089	1.792747
ITGA2	This gene encodes the alpha subunit of a transmembrane receptor for collagens and related proteins. The encoded protein forms a heterodimer with a beta subunit and mediates the adhesion of platelets and other cell types to the extracellular matrix. Has a role in Bone metastasis	0.274516	1.750415
ITGB1	Integrins are heterodimeric proteins made up of alpha and beta subunits. At least 18 alpha and 8 beta subunits have been described in mammals. Integrin family members are membrane receptors involved in cell adhesion and recognition in a variety of processes including embryogenesis, hemostasis, tissue repair, immune response and metastatic diffusion of tumor cells. This gene encodes a beta subunit.	0.078302	1.421418
CD44	The protein encoded by this gene is a cell-surface glycoprotein involved in cell-cell interactions, cell adhesion and migration. It is a receptor for hyaluronic acid (HA) and can also interact with other ligands, such as osteopontin, collagens, and matrix metalloproteinases (MMPs). This protein participates in a wide variety of cellular functions including tumor metastasis.	0.00626	1.842827

RAP1B	member of RAS oncogene family: This gene encodes a member of the RAS-like small GTP-binding protein superfamily. Members of this family regulate multiple cellular processes including cell adhesion and growth and differentiation. This protein localizes to cellular membranes and has been shown to regulate integrin-mediated cell signaling.	0.001455	1.707018
RAB10	member RAS oncogene family: RAB10 belongs to the RAS superfamily of small GTPases. RAB proteins localize to exocytic and endocytic compartments and regulate intracellular vesicle trafficking	0.012159	1.772537
CO1A1	Collagen type 1a1 and 1a2 are significantly higher in CAFs	0.572933	1.468351
CO1A2		0.635968	1.538359
EHD2	EH domain containing protein 2: The encoded protein interacts with the actin cytoskeleton through an N-terminal domain and also binds to an EH domain-binding protein through the C-terminal EH domain. This interaction appears to connect clathrin-dependent endocytosis to actin, suggesting that this gene product participates in the endocytic pathway.	0.064491	1.500703
Microtubule-associated protein 1 light chain 3 alpha	MAP1A are microtubule-associated proteins which mediate the physical interactions between microtubules and components of the cytoskeleton. It may be involved in carcinogenesis.	0.019645	1.667629
STOM	Erythrocyte band 7 integral membrane protein: Data indicate that a stomatin-specific, raft-based process is involved in storage-associated vesiculation.	0.038822	2.000507
1433Z	14-3-3 protein zeta/delta : Adapter protein implicated in the regulation of a large spectrum of both general and specialized signaling pathways. Binds to a large number of partners, usually by recognition of a phosphoserine or phosphothreonine motif. Binding generally results in the modulation of the activity of the binding partner.	0.064844	1.905192
MMP1	This gene encodes a member of the peptidase M10 family of matrix metalloproteinases (MMPs). Proteins in this family are involved in the breakdown of extracellular matrix in normal physiological processes, such as tissue remodeling, as well as in disease processes, such as metastasis.	0.372926	2.082898
AAAT	solute carrier family 1 member 5: High expression of AAAT correlates with metastasis and invasion and plays an important role in tumor cell growth.	0.080108	2.808409

WDFY3	WD repeat and FYVE domain containing 3: This gene encodes a phosphatidylinositol 3-phosphate-binding protein that functions as a master conductor for aggregate clearance by autophagy. This protein shuttles from the nuclear membrane to colocalize with aggregated proteins, where it complexes with other autophagic components to achieve macroautophagy-mediated clearance of these aggregated proteins. However, it is not necessary for starvation-induced macroautophagy.	0.197735	2.067479
VEGF165R	This gene encodes one of two neuropilins, which contain specific protein domains which allow them to participate in several different types of signaling pathways that control cell migration. Neuropilins bind many ligands and various types of co-receptors; they affect cell survival, migration, and attraction. Some of the ligands and co-receptors bound by neuropilins are vascular endothelial growth factor (VEGF) and semaphorin family members.	0.089005	1.430977

Next, in order to get higher number of protein hits, we pooled the exosomal proteins from 2 samples and ran in Mass spec. As expected we got many more proteins by loading more sample (366 vs the 122 previously), but the high levels of BSA and a few other proteins as detected on the gel, resulted in a significant dynamic range issue to go after lower abundance proteins from the exosomes. In the table below, some selected proteins which have been implicated in Prostate cancer such as ARIP4, 1433Z, BMP-1 and HOOK3 are downregulated and SLC2A1, RHOC, RAC1, MMP1 are upregulated in Pim1 overexpressing cells. This comparative proteomic analysis could serve as a basis for studying the underlying mechanisms of Pim1 kinase regulation of prostate cancer through crosstalk by stromal cells.

Results from second analysis:

Downregulated proteins		**Description**	**Log10 Pim/average control**
1433Z	**14-3-3 protein zeta/delta**	The protein has been implicated in many cancers, including lung cancer, breast cancer, lymphoma, and head and neck cancer, through pathways such as mTOR, Akt, and glucose receptor trafficking. Notably, it has been associated with chemo resistance and, thus, is a promising therapeutic target for cancer treatment.	-1.094853751
LAMP1	**Lysosomal-associated membrane protein 1**	LAMP1 expression on the surface of tumor cells has been observed for a number of different cancer types, particularly in highly metastatic cancers	-0.681448428
ARIP4	**Androgen receptor-interacting protein 4**	DNA helicase that modulates androgen receptor (AR)-dependent transactivation in a promoter-dependent manner.	-0.170460751

FBLN1	**Fibulin 1**	Down regulated in most of the cancers, Fibulin 1 is a secreted glycoprotein that becomes incorporated into a fibrillar extracellular matrix. Calcium-binding is apparently required to mediate its binding to laminin and nidogen. It mediates platelet adhesion via binding fibrinogen.	-0.099417834
BMP1	**bone morphogenetic protein 1**	Loss of bone morphogenetic protein is associated with prostate cancer.	0.080634233
BGH3	**Transforming Growth Factor Beta Induced**	This gene encodes an RGD-containing protein that binds to type I, II and IV collagens. The RGD motif is found in many extracellular matrix proteins modulating cell adhesion and serves as a ligand recognition sequence for several integrins. This protein plays a role in cell-collagen interactions and may be involved in endochondrial bone formation in cartilage. The protein is induced by transforming growth factor-beta and acts to inhibit cell adhesion	0.103631873
MVP	**Major vault protein**	Required for normal vault structure. Vaults are multi-subunit structures that may act as scaffolds for proteins involved in signal transduction. Vaults may also play a role in nucleo-cytoplasmic transport. Down-regulates IFNG-mediated STAT1 signaling and subsequent activation of JAK. Down-regulates SRC activity and signaling through MAP kinases.	0.127755212
PLS3	**Plastin 3**	Plastins are a family of actin-binding proteins.Higher expression is found in breast cancer cells.	0.111443837
SEM5A	**Semaphorin 5A**	May promote angiogenesis by increasing endothelial cell proliferation and migration and inhibiting apoptosis.	0.114645551
HOOK 3	**Hook microtubule-tethering protein 3**	HOOK3 is an adaptor protein for microtubule-dependent intracellular vesicle and protein trafficking. High-Level HOOK3 Expression Is an Independent Predictor of Poor Prognosis Associated with Genomic Instability in Prostate Cancer.	0.085634187
Upregulated Proteins		**Description**	**Log10 Pim/average control**
GTR1	**Solute Carrier Family 2 Member 1, Slc2A1**	SLC2A1 is expressed in breast cancer cells and is likely responsible for avid glucose uptake observed in established tumors.	0.496779212
ARF5	**ADP ribosylation factor 5**	play a role in vesicular trafficking and as activators of phospholipase D.	0.516700394

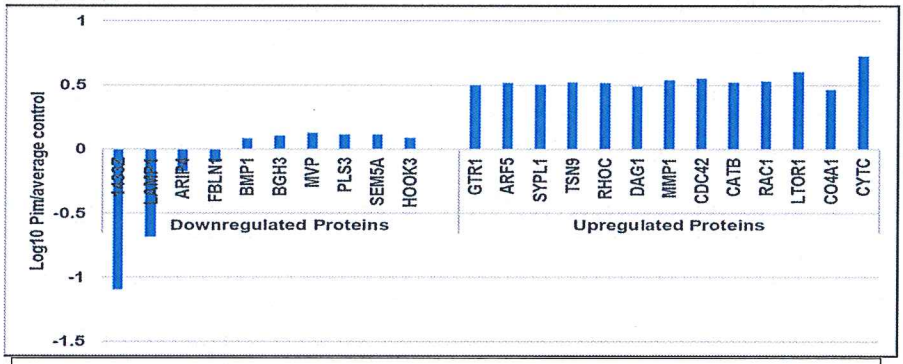
SYPL1	**Synaptophysin-like 1**	SYPL1 overexpression predicts poor prognosis of hepatocellular carcinoma and associates with epithelial-mesenchymal transition.	0.503239197
TSN9	**Tetraspanin-9**	The proteins mediate signal transduction events that play a role in the regulation of cell development, activation, growth and motility.	0.520730987
RHOC	**Ras homolog gene family, member C**	Overexpression of RhoC is associated with cell proliferation and causing tumors to become malignant. It has also been found to enhance the creation of angiogenic factors such as VEGF, which is necessary for a tumor to become malignant.	0.515580357
DAG1	**Dystroglycan**	The dystroglycan complex is involved in a number of processes including laminin and basement membrane assembly, sarcolemmal stability, cell survival, peripheral nerve myelination, nodal structure, cell migration, and epithelial polarization.	0.487696737
MMP1	**Matrix metalloproteinase-1**	MMPs are involved in the breakdown of extracellular matrix in normal physiological processes such as tissue remodeling, as well as in disease processes, such as metastasis.	0.537646122
CDC42	**Cell division control protein 42 homolog**	Activated Cdc42 activates by conformational changes p21-activated kinases PAK1 and PAK2, which in turn initiate actin reorganization and regulate cell adhesion, migration, and invasion.	0.551815364
CATB	**Cathepsin B**	Overexpression of the encoded protein has been associated with esophageal adenocarcinoma and other tumors.	0.520186274
RAC1	**Ras-related C3 botulinum toxin substrate 1**	Activating or gain-of-function mutations of Rac1 are shown to play active roles in promoting mesenchymal-type of cell movement assisted by NEDD9 and DOCK3 protein complex. Such abnormal cell motility may result in epithelial mesenchymal transition (EMT) – a driving mechanism for tumor metastasis as well as drug-resistant tumor relapse.	0.52862404
LTOR1	**Late Endosomal/Lysosomal Adaptor, MAPK And MTOR Activator 1**	LTOR1 is directly responsible for anchoring the Ragulator complex to membranes. Also required for late endosomes/lysosomes biogenesis it may regulate both the recycling of receptors through endosomes and the MAPK signaling pathway through recruitment of some of its components to late endosomes. May also play a role in RHOA activation.	0.603161464

CO4A1	**Collagen Type IV Alpha 1 Chain**	It functions as part of a heterotrimer and interacts with other extracellular matrix components such as perlecans, proteoglycans, and laminins.	0.46311414
CYTC	**Cytochrome C**		0.726349597

The proteins that were upregulated and downregulated by the induction of Pim in stromal cells are listed in this graph.

In conclusion, our results suggest that increasing Pim in stromal cells increases the number of exosomes that are being secreted from these stromal cells. It also shows that Pim induces increases in specific proteins in the exosomes. Many of these proteins are associated with rapid growth and increased motility including CDC42, RAC1, MMP1, RHOC and GTR1. Further investigations will be needed to see if these proteins impact on malignant epithelial cells.

Figure 7: Comparative proteomic analysis of proteins secreted in the exosomes of Pim1 overexpressing BHPrS1 cells versus control cells. (Pooled samples)



Proteomic analysis demonstrating proteins that were increased by Pim1 expression and those that were downregulated by this increase

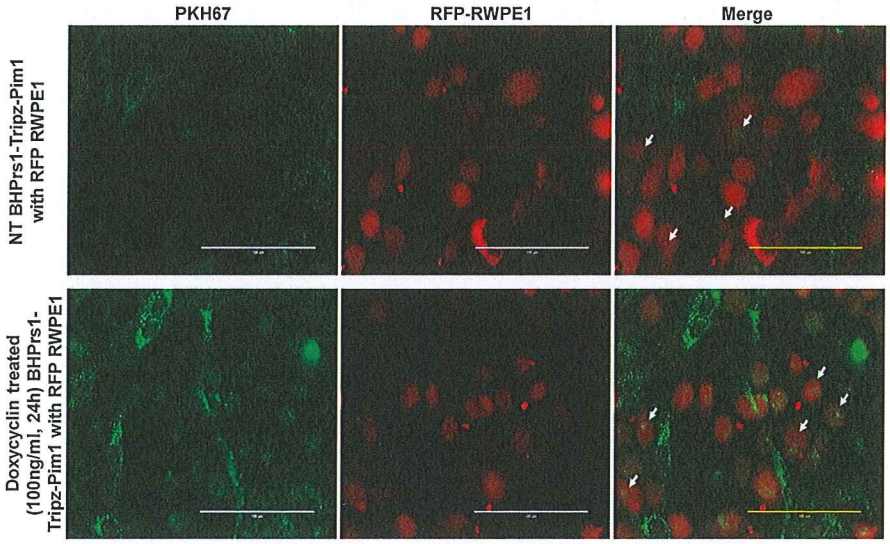


Figure 8. Exosomal Transfer (Juxtacrine) from stromal BHPrS1 to RWPE1 cells: Representative images of exosomal transfer from green fluorescent PKH67 labelled stromal BHPrS1-Tripz-Pim1 to RFP tagged RWPE1 cells co-cultured together for 24h in co-culture untreated (NT) or Doxycyclin treatment. Arrows represent exosomal uptake by RWPE1 cells. Scalebar: 100µm.

Major Task 2 and 3. A major goal of this subtask is to demonstrate that myofibroblast/CAFs overexpressing Pim produce exosomes that can induce biologic changes in known prostate tumor cells and that Pim inhibitors could reverse these changes. As a first step in being able to carry out these experiments, the experimental team needed to demonstrate that exosomes produced by prostate myofibroblast /CAFs were actually being taken up by prostate epithelial cells. Two approaches were used in these experiments. First, a transwell approach in which myofibroblast/CAFs are in the upper well and prostate epithelial cells are in the lower well. Second, a co-cultivation in which myofibroblast/CAFs are incubated with epithelial cells directly. The epithelial cells that have been analyzed include BPH1 and RWPE1 which are both immortalized but not transformed prostate epithelial cells A unique approach to membrane/exosome labeling technology was utilized for monitoring the transfer of exosomes by myofibroblast/CAFs cells into epithelial cells. Briefly, PKH67 Fluorescent Cell Linker Kits were used to stably incorporate a green fluorescent dye with long aliphatic tails (PKH67) into lipid regions of the cell membrane of BHPrS1 cells labeling the exosomes. For 24 hours PKH67-labelled BHPrS1-Tripz or BHPrS1-Tripz-Pim1 cells were co-cultured with the stromal cells grown in inserts with 4 micron pours with prostate epithelial cells (BPH1 and RWPE1) at the base of dish. As seen in Figure 9, the green flourescent PKH67 dye transfer visualized is the movement of membrane vesicles from BHPrS1 cells to epithelial cells through the transwell to the bottom cells.

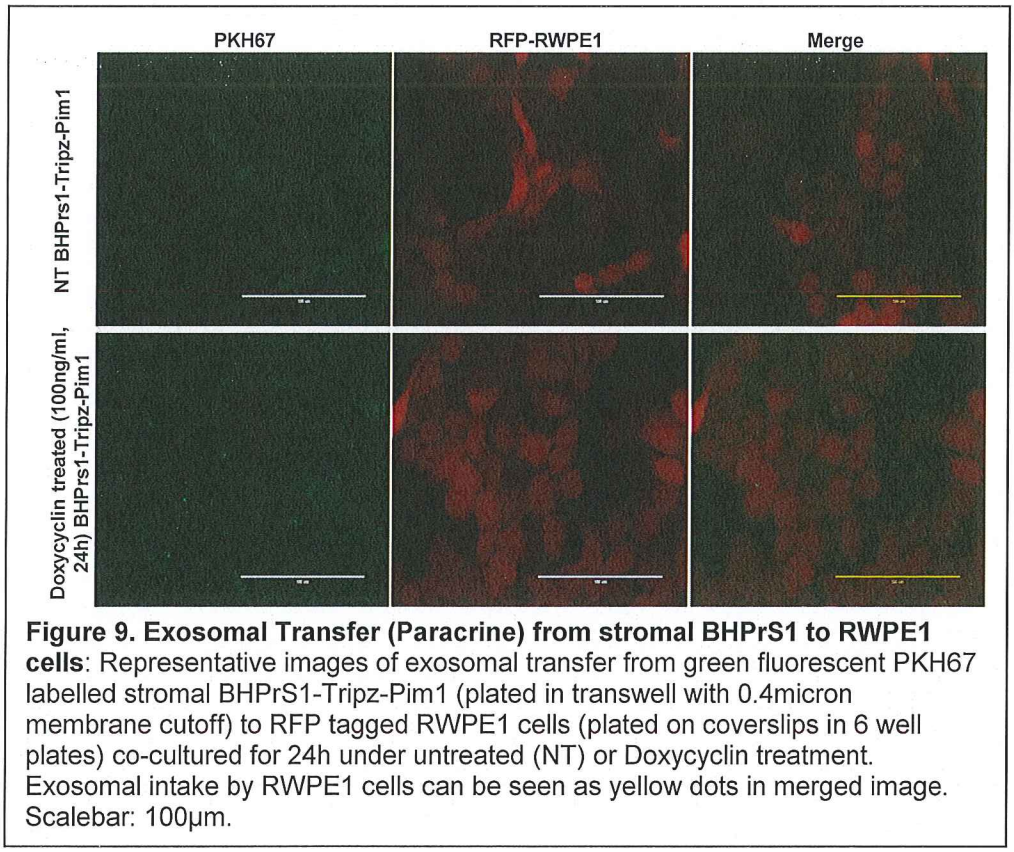


Figure 9. Exosomal Transfer (Paracrine) from stromal BHPrS1 to RWPE1 cells: Representative images of exosomal transfer from green fluorescent PKH67 labelled stromal BHPrS1-Tripz-Pim1 (plated in transwell with 0.4micron membrane cutoff) to RFP tagged RWPE1 cells (plated on coverslips in 6 well plates) co-cultured for 24h under untreated (NT) or Doxycyclin treatment. Exosomal intake by RWPE1 cells can be seen as yellow dots in merged image. Scalebar: 100µm.

Juxtacrine stromal-epithelial communication was also assessed using a mixed co-culture ratio of one epithelial cells to two PKH67-labelled-BHPrS1 cell. Cells were plated together in culture dishes and after 24h the microvesicle transfer demonstrated using the PKH67 transfer from CAF to epithelial cells. The immortalized prostate epithelial cells RWPE1 are RFP positive, the BHPrS1 cells are green, and the cells receiving the transfer are red with green dots, Figure 9. It is also apparent that the BHPrS1 cells with doxycycline induced Pim-1 cells are making many more exosomes and this is seen in the receiving cells. Counting ten fields under the microscope in triplicate demonstrates that the stromal cells expressing Pim-1 shed more exosomes that are picked up by RWPE1 cells Figure 10 (next page). These results are important because they demonstrate that exosomes that are being produced by these myofibroblast/CAFs can be transferred to epithelial cells and potentially have biologic effects. This data also shows that those cells expressing Pim kinase produce many more exosomes.

Subtask 3 - in this subtask, the goal is to examine the ability of Pim expressing myofibroblast /CAFs to induce the transformation of immortalized , such as BPH1 or tumor epithelial cells (PC3). However, a potentially important alternative target is the prostate cancer stem cell. These stem cells (Figure 11) can be used to examine the ability of exosomes to modulate the biology of precancerous and cancerous stem cells. We have perfected the technique of growing prostate stem cells from mice. Briefly, we have purified stem cells from prostate tissue. Prostates from 8-week-old mice are collected and dissociated with collagenase (2h, 37C) and trypsin (5min, 37C). The cells are dissociated into single cells by Pasteur pipette and then plated in growth factor reduced Matrigel. The media constituting of EGF, noggin and R-Spondin (ENR) is added to the set Matrigel drops containing prostate cells. The cells are then allowed to grow for 10-14 days and then fully developed organoids can be subcultured and used for genetic and pharmacological studies. An example of these growing stem cells is shown Figure 11. These normal stem cells can be plated on plastic or incubated with exosomes for further study.

These stem cells can be transduced with specific genes to allow us to design experiment to test the impact of exosomes on stem cells containing, for example, an overexpressed PTEN gene, an important driver of prostate cancer. Lentiviral transduction of the organoids is achieved by trypsinizing and breaking the

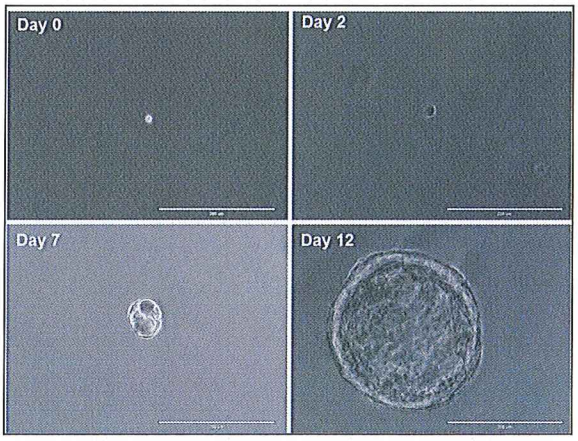


Figure 11. Prostate tissue organoid culture by Day. Representative image of prostate epithelial cell organoid culture in matrigel. Epithelial cells were dissociated from 8 week old mice prostate and digested before plating them in growth factor reduced matrigel in ENR (minimal) media for 12 days. Scalebar 200µm

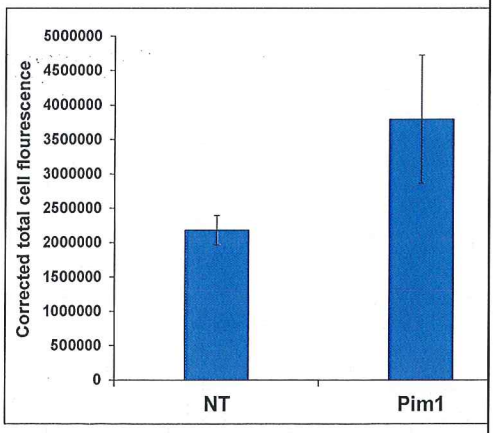


Figure 10. Pim1 enhances the exosomal production by stromal cells. Juxtacrine and transwell paracrine exosome production from BHPrS1-Tripz-Pim1 stromal cells with no treatment (NT) and Doxycyclin (100ng/ml, 72h) (Pim1) was analyzed by labelling the stromal BHPrS1-Tripz-Pim1 cells with lipophilic dye PKH67 and coculturing labelled cells with epithelial RWPE1 cells either together (Juxtacrine) or in a Transwell with 0.4 micron membrane cutoff for 24h. Fluorescent images were captured (10 images per group in triplicates) and were analyzed for quantification via ImageJ for corrected total cell fluorescence. Results represented as bar graph.

the organoids into smaller cell clusters and spinoculating them with lentivirus containing gene of interest for 1h at 600g, 32 degree C. The cells are kept in the 37C incubator for 3h and then replated in growth factor reduced Matrigel and ENR media.

To test our ability to create prostate stem cells from mice that contain mutations that lead to prostate cancer and to transduce these stem cells, we have made stem cell from $P53^{fl}/Rb^{fl}$ mice (Figure 12). These stem cells were first transduced with Cre+ to delete these genes and as can be seen (lower left panel) this enhances stem cell growth. The lentiviral transduction of Pim-1 further enhanced the growth of these stem cells (lower right panel). This proof-of-principle study demonstrates the laboratory can isolate prostate stem cells, manipulate them in culture and then use them for further studies. The goal will be to use this technology to isolate human normal and malignant prostate stem cells and use these cells for the experiments outlined in Subtask 3. These cells can come from the identical tissue samples used for CAF production.

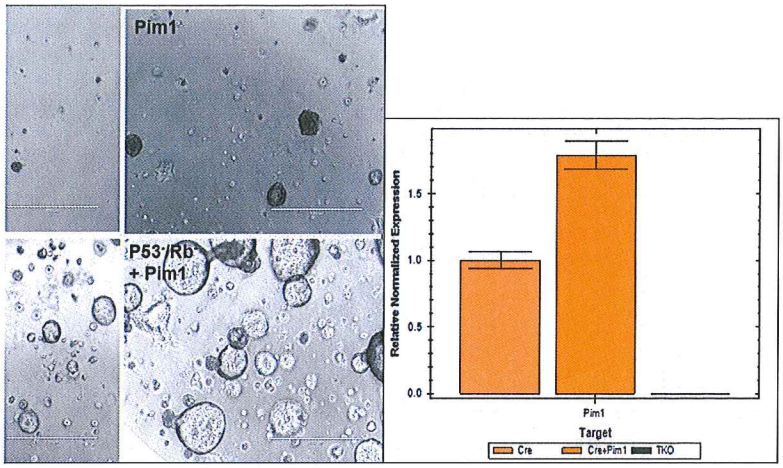
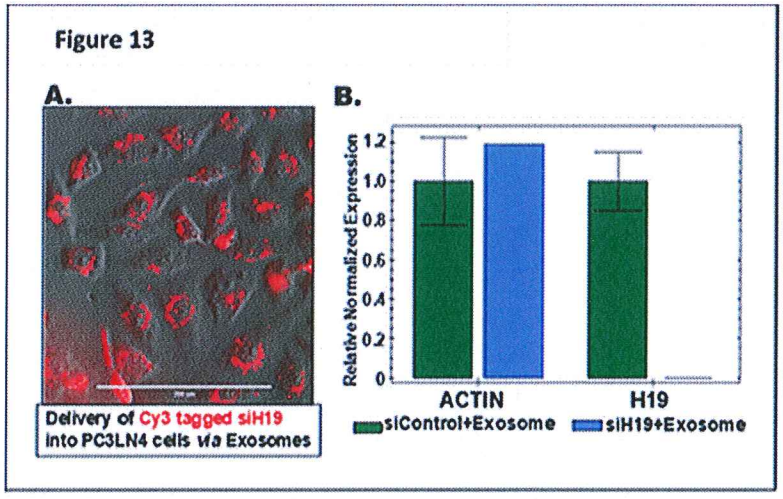


Figure 12. Prostate Organoid formation efficiency: Representative images from organoid cultures of $p53^{flox}/Rb^{flox}$ mice transduced with Cre for the knockout of p53/Rb gene ($p53^{-}/Rb^{-}$). Pim1 overexpression in these organoids was achieved by lentiviral transduction ($p53^{-}/Rb^{-}$ + Pim1). Scalebar: 200µm. Right panel demonstrates result from qPCR validating the overexpression of Pim1 in these organoid culture models.

Therapeutic targeting using exosomes is possible. Exosomes were isolated using magnetic apture (Wako, Japan) and were quantified by Fluorcet analysis kit (System Biosciences). Next these exosomes were engineered to carry short interfering RNA specific to a long non-coding RNA, H19. H19 has been implicated by our laboratory in driving neuroendocrine prostate cancer. The siRNA was labelled with Cy3 to aid the visualization of its cellular uptake. As demonstrated (Figure 13), we have successfully used exosomes derived from BHPrS1 cells to deliver an siRNA to the prostate cancer cell line PC3-LN4. The left figure demonstrates that the Cy3 has been taken up by the prostate cancer cells, while the right figure is a qRT-PCR that shows that exosomes alone are not capable of knocking down the long non-coding H19, while the modified exosomes containing the siRNA are able to knock down this gene. This demonstrates that exosomes from stromal cells can be used to deliver a specific payload to prostate cancer cells and knockdown genes driving the cancer phenotype, e.g. H19. This result suggests a future direction in which patient fibroblasts from prostates that are removed at the time of surgery can be used to produce exosomes that can be manipulated to act a treatment tool for prostate cancer.



Training

Based on the initial funding and training from this project, we are very excited to announce that Dr. Neha Singh, a fellow on this project, has been awarded a *DOD Post-Doctoral Training Grant*. This research has provided training for Dr. Singh who was recruited specifically to carry out this work. She graduated with a BS in Chemistry from Banaras Hindu University, Varanasi, INDIA. She completed her PhD at North Dakota State University and has been working on this project. Prior to joining this experimental team, she had no knowledge of the issues related to the progression and outcomes of prostate cancer. This project enabled her to gain this knowledge and understand the nature of the progression of this disease. As part of the training on this project, she has worked on myofibroblast/CAFs and prostate organoids and studied their impact on cancer. This project has afforded her the opportunity to develop technical knowledge allowing her to isolate and interrogate exosomes investigate the protein makeup of exosomes, isolate and purify prostate stem cells, and examine the outcome of expression of Pim protein kinase on prostate cancer growth. This project has enabled her to learn about the Pim protein kinase as a target for prostate cancer therapy and to use in her research inhibitors of this enzyme obtained from industry that are being investigated in human clinical trials. As part of her training she attends Cancer Center grand rounds, Cancer Biology seminars and Therapeutic Development Conferences. She has interacted with invited speakers with expertise in prostate cancer. She attended the American Association of Cancer Research during the project period, and has submitted an abstract for their April 2019 meeting.

Dissemination

1. Abstract control No: Abstract Number: 19-A-6167-AACR
   Submitting Author: Neha Singh
   Mentor: Dr. Andrew S. Kraft, MD, Professor and Director, University of Arizona Cancer Center
   Role of long noncoding RNA H19 in driving enzalutamide resistant neuroendocrine prostate cancer

2. Manuscript in preparation.

Next Reporting Period - nothing to report

4- IMPACT

We have demonstrated that it is possible to isolate and purify exosomes from prostate stromal cells. This has been accomplished by adapting a number of procedures and refining these to using the stromal cells to produce purified exosomes. We have been able to quantitate these exosomes and send these exosomes for mass spectrometry analysis. We next analyzed the effect of overexpressing the Pim kinase on the exosome secretion. *Our results demonstrate that increasing Pim in stromal cells increases the number of exosomes that are being secreted from these stromal cells.* Analysis of these exosomes demonstrates that Pim induces increases in specific proteins found in prostate cancer cells. Many of these proteins are associated with rapid growth and increased motility including CDC42, RAC1, MMP1, RHOC and GTR1. Further investigations will be needed to see if these proteins secreted in exosomes impact on malignant epithelial cells.

We have shown that it is possible for stromal cells to secrete exosomes that can then be taken up by prostate cancer cells. In addition, we have demonstrated that expression of the Pim protein kinase in organoids markedly enhances their growth. It is possible that Pim kinase increases in stromal cells and then is transferred by exosomes to prostate stem cells. Using exosomes from BHPrS1 cells we have demonstrated that they can be used to deliver small interfering RNAs to prostate cancer cells and thus downregulate specific genes in prostate cancer cells. As mentioned above, we have used exosomes from prostate stromal cells to deliver small interfering RNAs to knockdown long non-coding RNA H19 which has been implicated in neuroendocrine prostate cancer. This result suggests that normal fibroblasts isolated from patients, possibly from prostectomy specimens, could be used to produce exosomes that then act as a delivery vehicle to control the level of specific RNAs in prostate cancer. This hypothesis suggests the possibility of new technologies to affect the growth of prostate cancer.

Changes/Problems – none to report

Products – none to report

Participants & Other Collaborating Organizations – none to report