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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/cancer 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 therapies targeted at the prostate fibroblast microenvironment and the development of new biomarkers that reflect stromal protein production.*

Keywords

Cancer associate fibroblasts (CAFs)
Myofibroblasts
Pim protein kinase
Exosomes
Prostate stem cells
Pim inhibitors, AZD1208 and LGH447

Accomplishments

Major goals of the project

1- Compare the secretome of immortalized prostate fibroblasts with freshly isolated human CAFs: develop a data set of proteins that are regulated by the Pim protein kinase in CAFs.
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: document whether induced changes are reflected in the EPS-urine of different Gleason grades of cancer.

Accomplishments under these goals

Major Task 1

Subtask 2 and 4. An essential element in the Major Task 1 is to be able to isolate purified exosomes with confidence that these vesicles are pure and not contaminated by cellular debris. These purified vesicles will allow an analysis of the ability of Pim protein kinase to 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. 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 × 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 exosome marker proteins TSG101, CD63, and Flotillin1 known exosome proteins, and the absence of golgi vesicl and demonstrated by the absence of golgi protein GM13(*Figure 1* shows an example of such a preparation with nc contamination seen. The left portion of the Figure demonstrates a western blot of exosomes while the right the cell lysate. The golgi marker GM130 protein is posit in cells lysate while not in Exosomes. This figure also sl exosomes isolated from doxycyclin treated and Pim inhil (LGH) treated cells as well. Interestingly the Pim induc cells exhibited an increased level of the exosome marker TSG101 compared with untreated controls suggesting th number of exosomes was increased by Pim induction in stromal cells. To compare the number of exosomes secre by a fixed number of cells an Elisa kit measuring the acetylcholine esterase activity was used to quantify the number of exosomes per group. *Figure 2* demonstrates 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). This data demonstrates that the team is ready to attempt to identify proteins that are excreted 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.

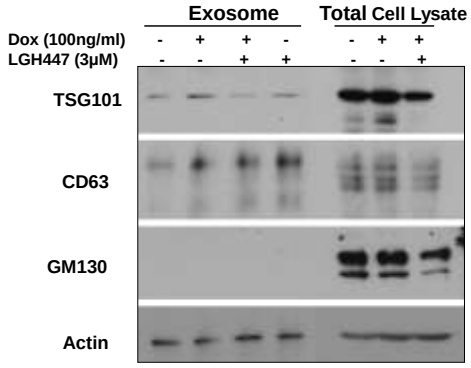

Figure 1. **Characterization of exosome preparations by Western blot.** Western blot analysis of common exosome markers (TSG101 and CD63) and golgi marker (GM130). 10 μg of exosomes isolated by ultrafiltration and serial centrifugation methods. Total cell lysates from BHPrS1-Tripz-Pim1 with or without Doxycyclin and Pim inhibitor LGH447 treatments was loaded as positive control.

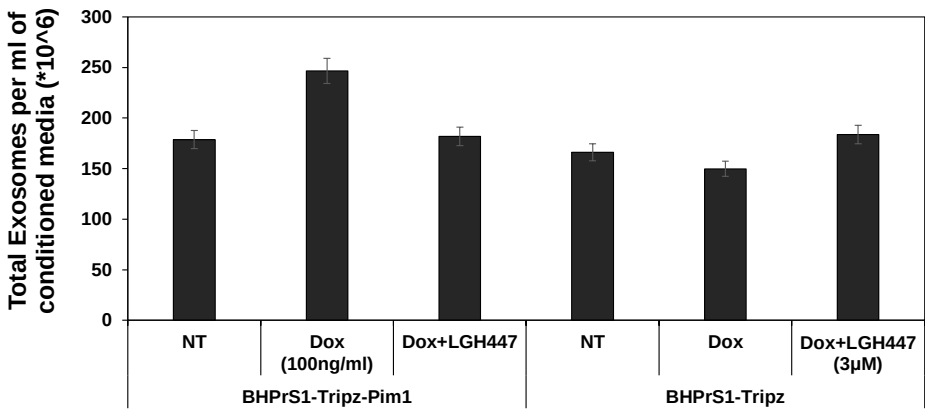

Figure 2. **Quantification of exosomal production by Prostate stromal cells**. Equal numbers of BHPrS1-Tripz and BHPrS1-Tripz-Pim1 cells were treated with and without Doxycyclin (100ng/ml) and LGH447 (3μM) for 72h and exosomes were collected. The total amount of exosomes was determined by an ELISA assay measuring acetylcholine esterase activity in exosomal protein lysates. Results are presented as bar graph +/- the standard deviation of results in triplicate.

Subtask 2, 4, and 6. As shown in Figure 1 the team was able to produce "pure" exosomes. To examine the ability of Pim to stimulate the production of proteins that enter the exosome the BHPrS1-Tripz-Pim1 cells were treated with Doxycyclin (100ng/ml) to induce Pim1 overexpression. As proof-of-principle that Pim inhibitors could block the transfer of specific proteins into exosomes the ability of a Pim inhibitor, LGH447 (3uM) to block the increase in specific proteins into the exosomes cells was tested. Cells were incubated with Doxycyclin along with a Pim inhibitor or with Pim inhibitor alone. These incubations were carried out for 72h prior to exosome isolation. To compare protein levels by Western blotting of the exosomes an Elisa kit measuring the acetylcholine esterase activity was used to quantify the number of exosomes per group. Although it was not the goal of these experiments to identify all of the protein differences induced, we did demonstrate that specific changes of importance did occur. Briefly, 5μl of exosomal protein and BHPrS1 cell lysate protein were added to SDS sample loading buffer and heated at 95C for 5 mins. Samples were loaded on SDS-PAGE and western blots were performed.

The proteins analysed were:

- Translational regulators EIF4b AND 4EBP1.
- Metastasis markers NOTCH1, DKK1
- Differentiation protein NKX3.1
- Receptor tyrosine kinase FAK

An example of such results is shown in *Figure 3* 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 and as shown in the right figure levels may or may not reflect what is occurring in the cytoplasm. Also, importantly to the ability to use Pim inhibitors to treat prostate cancer, 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 4*, the expression of

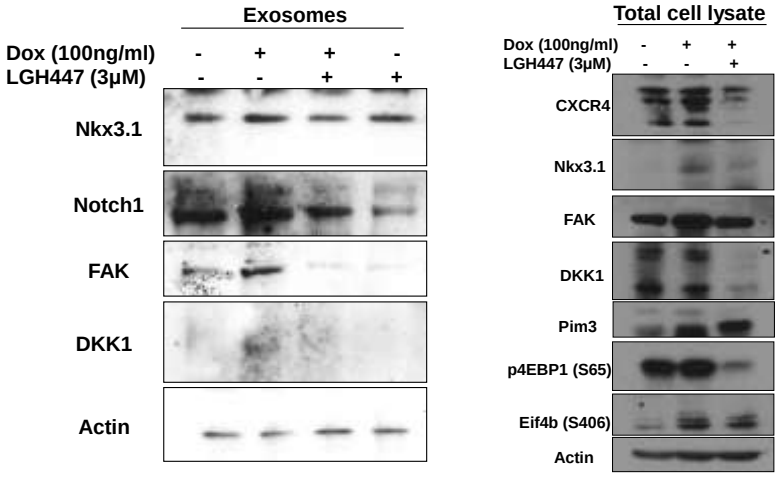

Figure 3. Exosomal protein content after PIM1 induction in BHPrS1-Tripz-Pim1 cells: Western blot analysis of translational regulators (eIF4b, 4EBP1), metastasis markers (NOTCH1, DKK1), differentiation protein (NKX3.1) and tyrosine kinase (FAK) in 10 µg of exosome protein lysate and total cell lysate from prostate stromal BHPrS1-Tripz-Pim1 treated with Doxycyclin alone or in combination with the Pim inhibitor LGH447.

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. This work also identifies the procedures to be used with human fresh CAF samples in the second year of this proposal.

Major Task 3.

Subtask 1. 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

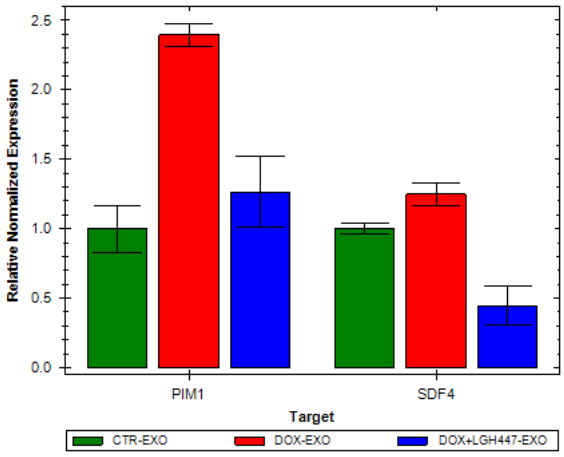

Figure 4. Prostate stromal fibroblast upregulate and secrete Pim1 and stromal derived factor 4 (CXCR4) into the exosomes. qPCR: Exosomes secreted from BHPrS1-Tripz-Pim1 treated with DMSO (CTR-EXO), Doxycyclin (Dox-EXO), Doxycyclin+LGH447 (DOX+LGH447-EXO) were collected and total exosomal RNA was isolated. 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

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 5*, the green flourescent PKH67 dye transfer visualized is the transfer of membrane vesicles from BHPrS1 cells to epithelial cells through the transwell to the bottom BPH1 cells.

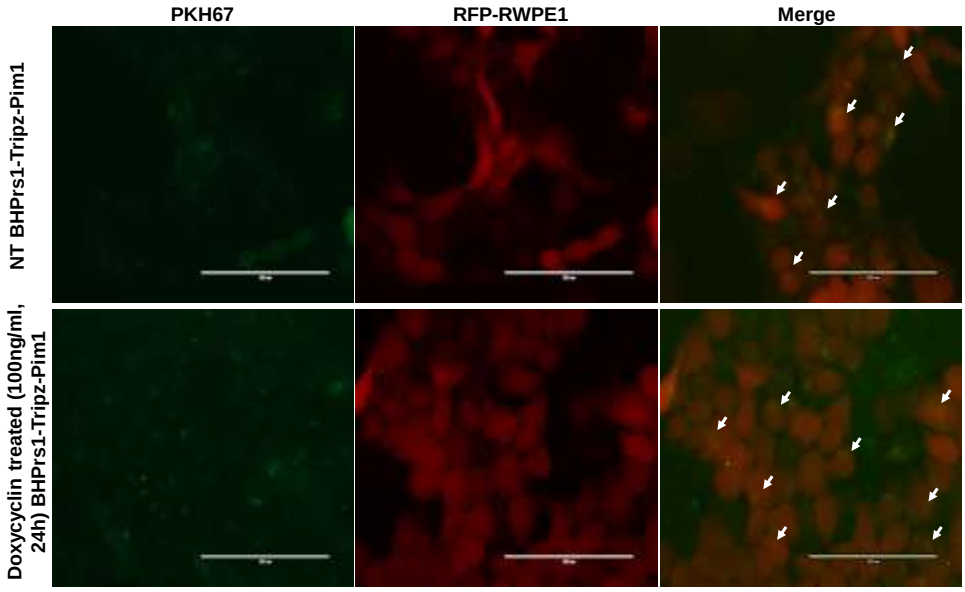

Figure 5. Exosomal Transfer (Paracrine) from stromal BHPrS1 to RWPE1 cells: Representative images of exosomal transfer from green fluorescent PKH67 labelled stromal BHPrS1-Tripz-Pim1 (plated in transwell with 0.4micron membrane cutoff) to RFP tagged RWPE1 cells (plated on coverslips in 6 well plates) co-cultured for 24h under untreated (NT) or Doxycyclin treatment. Arrows in merged images represent exosomal intake by RWPE1 cells. 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. As seen in *Figure 6* this transfer is easily visible when cells are incubated together (blue

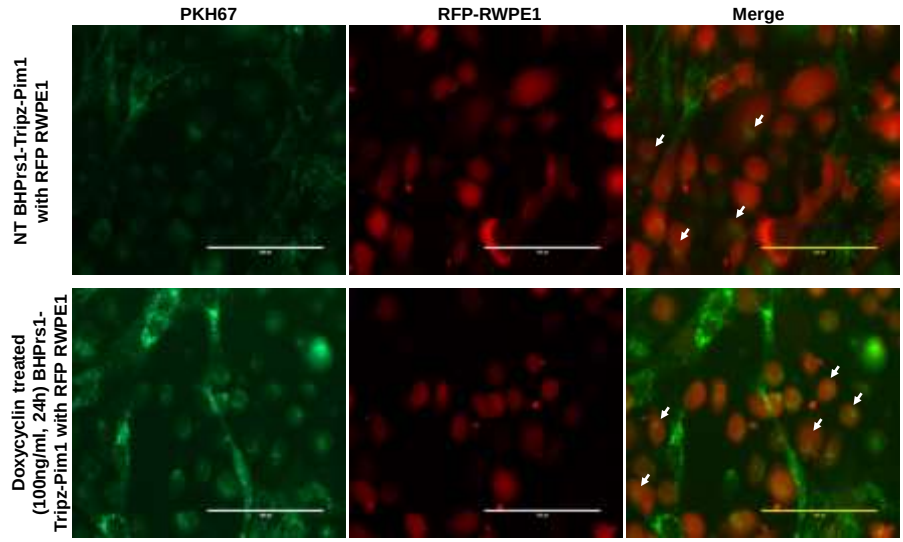

Figure 6. **Exosomal Transfer (Juxtacrine) from stromal BHPrS1 to RWPE1 cells**: Representative images of exosomal transfer from green fluorescent PKH67 labelled stromal BHPrS1-Tripz-Pim1 to RFP tagged RWPE1 cells co-cultured together for 24h in co-culture untreated (NT) or Doxycyclin treatment. Arrows represent exosomal uptake by RWPE1 cells. Scalebar: 100µm.

arrows). It is also apparent that the BHPrS1 cells with doxycyclin 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 7*. 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.

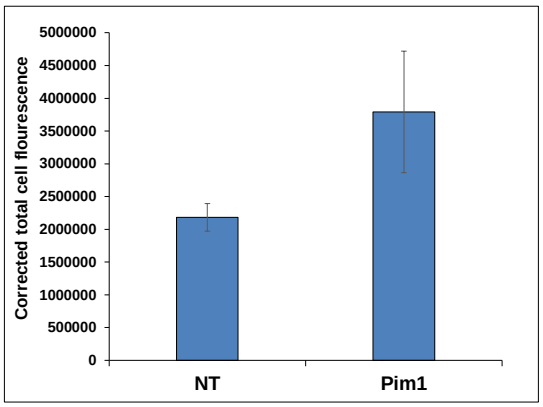

Figure 7. **Pim1 enhances the exosomal production by stromal cells**. Juxtacrine and transwell paracrine exosome production from BHPrS1-Tripz-Pim1 stromal cells with no treatment (NT) and Doxycyclin (100ng/ml, 72h) (Pim1) was analyzed by labelling the stromal BHPrS1-Tripz-Pim1 cells with lipophilic dye 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.

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 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 8.* These normal stem cells can be plated on plastic or incubated with exosomes for further study.

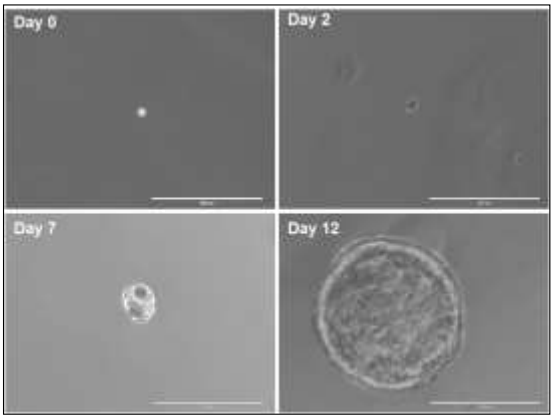

Figure 8. **Progression of prostate tissue organoid culture:** Representative image of prostate epithelial cell organoid culture in matrigel. Epithelial cells were dissociated from 8 week old mice prostate and digested before plating them in growth factor reduced matrigel in ENR (minimal) media for 12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

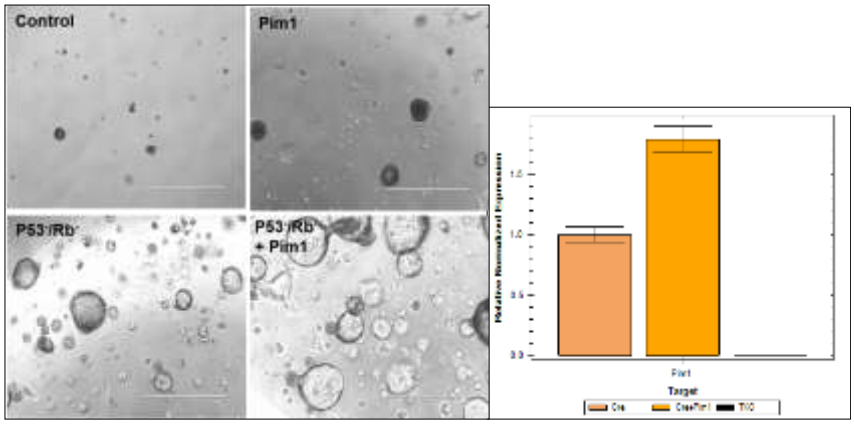

Figure 9. **Prostate Organoid formation efficiency:** Representative images from organoid cultures of $p53^{flox}/Rb^{flox}$ mice transduced with Cre for the knockout of p53/Rb gene ($p53^{-}/Rb^{-}$). Pim1 overexpression in these organoids was achieved by lentiviral transduction ($p53^{-}/Rb^{-}$ + Pim1). Scalebar: 200µm. Right panel demonstrates result from qPCR validating the overexpression of Pim1 in these organoid culture models.

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 organoids into smaller cell clusters and spinoculating them with lentivirus containing the 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 9*). 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.

Training and professional development has the project provided

This project has provided training for Dr. Neha 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 Ph.D. in North Dakota State University and has been working on this project. Prior to joining this experimental team she had not had knowledge of the issues related to the progression and outcomes of prostate cancer. In addition, she had not worked on myofibroblast/CAFs and studied their impact on cancer. This project has afforded her the opportunity to develop technical knowledge allowing her to isolate and interrogate exosomes from CAFs, investigate the protein makeup of exosomes, isolate and purify prostate stem cells, and examine the outcome of expression of Pim protein kinase in CAFs. 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 exosomes. She will plan on attending the American Association of Cancer Research during the coming project period.

Dissemination of results to the community – *Nothing to Report*

Plans during the next reporting period to accomplish goals

With the recruitment of Dr. Singh to this project and given that she has accomplished initial experiments that have enabled her to learn the biologic system and the goals of the project, she is ready for a more complete analysis of Major Task 1 as defined in the Statement of Work (SOW). Because she has now has a good understanding of how to use Pim inhibitors to treat cells in culture she will be able to experiments defined in Specific Aim 2 (SOW Major Task 3). Additionally, the samples to be used in Specific Aim 3 (Major Task 2) will be collected during this project period.

Although she has carried out Subtasks in Major Task 1 she will now begin a more comprehensive analysis of exosomal proteins from both freshly isolated CAFs and compare them to immortalized CAF cell lines. Preliminary experiments have suggested that exosomal proteins cannot be quantitated without specific methodologies to allow comparison from one cell line to the next. This will involve SILAC labeling to improve the quantitation of measured changes in protein secretion. This technique will require six passages in SILAC media suggesting that this could cause changes in cell secretion. If quantitation is a problem, cells will be maintained in full (dialyzed) serum SILAC media for the duration of experiments. For double metabolic labeling experiments, 1×10^6 SILAC-labeled cells are seeded to 10 cm plates and grown for 48 hours, followed by incubation with 40μm tetraacetyl-N-acetylmannosamine-azide (ManNAcAz) for 48 hours. Media is changed

following sugar incorporation Four biological replicates, including a SILAC label swap, were prepared for cell lysates and two biological replicates for exosomes. The applicant team is expecting to establish the strong correlation between Pim1 levels and myofibroblast phenotype in CAFs isolated from human prostate tissues, however, because of the heterogeneity of this cell type additional samples may be needed to analyze CAFs with high Pim levels. If cell heterogeneity in individual samples is a problem, then cell surface markers, *i.e., PDGFR,* that are expressed highly in Pim containing cells may be used to FACS separate Pim over expressor cells.

This method will be used to quantify proteins secreted from WPMY-1 and BHPrS1 cells as well as freshly isolated CAFs. This data collection will allow bioinformatics and biostatistical analysis of changes in secreted proteins with CAFs from patients with different Gleason grades. This data can also be used to identify specific proteins in exosomes that could have significant impact on tumor biology. From the accomplishments in year 1, she has identified exosomal proteins that could have impact on cancer cell growth and the initiation of precancerous cell growth, including FAK which transfers signals from the environment to internal protein kinases, Nkx3.1 which is an important in controlling the induction of genes that regulate prostate differentiation and DKK1 which plays a role in WNT signaling. It will be important to focus not only on global changes but on specific proteins that can regulate pathways within tumor cells that can modulate growth.

In Specific Aim 2 (SOW Major Task 3) of the application the goal is to examine whether myofibroblast/CAFs with Pim expression enhance tumor growth and the transformation of an immortalized BPH1 cell line. In these experiments the ability of Pim inhibitors to block these changes will be evaluated. As a corollary to these studies we will evaluate a clinically important question and examine whether myofibroblast/CAFs with Pim expression when incubated with tumor cells will inhibit the ability of taxotere to kill prostate cancer. The laboratory can then attempt to overcome this resistance with Pim inhibitors.

Impact

Impact on the development of the principal disciplines of the project – *Nothing to Report*
Impact on other disciplines- *Nothing to Report*
Impact on the technology transfer –*Nothing to Report*
Impact on society beyond science and technology –*Nothing to Report*

Changes/problems

Changes in approach and reasons for change *Nothing to Report*

Actual or anticipated problems or delays and actions or plans to resolve them
To successfully be able to isolate CAFs from human prostate tumors it takes a strong Department of Urology and an active and attentive tumor bank. Since taking over the University of Arizona Cancer Center, in my position as Cancer Center Director I have worked to enhance the activities of both of these entities with the goal of increasing the number and quality of samples available to this project and to all DOD sponsored prostate research at the University of Arizona. To this end, I have helped to recruit Dr. Lee from Louisiana to become the Chief of Urology, and Dr. Agnes Witkiewicz to head our tumor bank. Dr. Witkiewicz has been the former tissue bank director at UT Southwestern Medical Center in Dallas Texas. These two individuals should enhance the ability to carry out this proposal.

Changes that had a significant delay on actions or plans to resolve them *Nothing to Report*

Changes in the use or care of human subjects, vertebrate animals, biohazards and/or select change of agents *Nothing to Report*

Products

Publications, conference papers, and presentations – *Nothing to Report*

Participants & Other Collaborating Organizations

Individuals who have worked at least one person month per year during reporting period

Andrew Kraft, MD	Principal Investigator	No Change
Jin Song, PhD	Co-Investigator	No Change
Neha Singh, PhD	Postdoctoral Fellow	New
Richard Drake, PhD	Co-Investigator	No Change
Elizabeth. Garret-Mayer	Biostatistician	No Change

Change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period

Nothing to Report

Other organizations involved as partners

Nothing to Report