AWARD NUMBER: W81XWH-12-1-0507

TITLE: SPANXB2 and Prostate Cancer Progression

PRINCIPAL INVESTIGATOR: Hangwen Li

CONTRACTING ORGANIZATION: Regents of the University of Michigan
Ann Arbor, MI 48109

REPORT DATE: December 2014

TYPE OF REPORT: Final Summary Report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
December 2014	Final Summary Report	15 Sep 2012 - 14 Sep 2014

4. TITLE AND SUBTITLE

SPANX-B2 and Prostate Cancer Progression

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-12-1-0507

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Hangwen Li

E-Mail: hangwen@umich.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Michigan
Ann Arbor, Michigan, 48109-5948

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

A critical problem in prostate cancer is our inability to reliably distinguish indolent from aggressive disease. Recent evidence has implicated a class of genes, termed Cancer Testis Antigens (CTA), in cancer progression. In preliminary studies, by crossing the CTA bank with a prostate cancer metastasis gene signature we attained from an orthotopical injection xenograft model, we postulated CTA SPANXB2 as a cancer metastasis related CTA. We observed that SPANXB2 is up-regulated in metastatic prostate cancer xenograft models and is induced upon exposure to stroma and stromal factors (i.e., TGF-β). We hypothesize that SPANX-B2 may be the key regulator of prostate cancer aggressive cell behavior and metastasis. In this report, for the first time, we illustrate that regulatory role of SPANXB2 in PC3 cells by using shRNA knockdown technique. Knockdown of SPANXB2 in PC3 cells significantly reduces prostate caner aggressiveness *in vitro* and *in vivo*. We confirm that stromal cells stimulate SPANXB2 expression and promote cell aggressiveness in prostate caner cells. We demonstrated that elevated TGF-β2 upon stromal cells co-culture induced SPANXB2 and enhance prostate cancer invasion and metastasis. Finally we find the SPANXB2 is associated with the reactive stromal accumulation in tumors and correlated to tumor metastasis and survival outcome in clinical patients. Our data may provide a potential therapeutic target to treat prostate cancer metastasis.

15. SUBJECT TERMS
SPANXB2, Prostate Cancer, Stromas, Metastasis

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	34	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

Page

1. Introduction……2

2. Keywords……2

3. Overall Project Summary……3

4. Key Research Accomplishments……4-27

5. Conclusion……28

6. Publications, Abstracts, and Presentations……29-30

7. References……31-32

Introduction

A critical problem in prostate cancer is our inability to reliably distinguish indolent from aggressive disease. Cancer metastasis gene, but not tumor initiating gene, might be the potential target to solve this problem. Recent evidence has implicated a class of genes, termed Cancer Testis Antigens (CTA), in cancer progression. In preliminary studies, we compared the CTA gene bank with a prostate cancer metastatic gene signature that we generated from a comparison between highly metastatic orthotopic tumors. We observed that SPANXB2 is up-regulated among these common up-ergulated genes and we postulated CTA SPANXB2 as a cancer metastasis related CTA. In addition, based on the unique tumor microenvironment of mouse prostate (vs mouse subcutaneous), we foretell that prostate stromal cells or stromal factors ((i.e., TGF-β). play critical role in regulating SPANBXB2 expression and tumor progression. We hypothesize that SPANX-B2 may be the key regulator of prostate cancer aggressive cell behavior and metastasis. We also hypothesize that prostate stroma promotes SPANX-B2 expression in prostate cancer cells *in vivo* and *in vitro,* and most likely, this process is modulated by TGFβ. Finally we seek to the link between SPANX-B2 expression and metastasis and clinical outcome, as well as reactive stromal markers. We expect our study will provide evidence to develop some potential clinical diagnosis and prognosis marker, and SPANXB2 and reactive stromal cells may be used as the therapeutic targets.

Key Words: Metastasis, Cancer Testis Antigen, SPANXB2, stromal cells, TGFβ

Overall Project Summary

We fully completed our specific Aim i and ii , and partially completed the aim iii. In aim i: Determine the association of SPANX-B2 and stroma with prostate cancer aggressiveness *in vitro*. In order to confirm this association, we initially show that reactive stromal cells promote SPANXB2 expression by using a modified *in vitro* 2-D stromal-epithelial model. We also demonstrate that reactive stromal cells co-culture increases prostate cancer cells proliferation, migration, and invasion. Our data implies that overexpression of SPANXB2 is associated with the enhanced cancer cells aggressiveness. Then we confirm this by SPANXB2 knocking down and we prove that blocking of SPANXB2 suppresses prostate cancer cells proliferation, migration and invasion. We also observe that SPANXB2 level is up-regulated upon TGF-β2 treatment. TGF-β2 level is also increased in these PC3 co-culture cells. Adding TGF-β2 or TGF-β inhibitor significantly alters the invasion ability of PC3 cells. These data fully supports that regulation of SPANXB2 and reactive stromal cells on prostate cancer aggressiveness *in vivo*. **ii**) Determine the association of SPANX-B2 and stroma with prostate cancer metastasis *in vivo* . We confirmed that these co-cultured cells exhibit more aggressive profiles in mouse dorsal prostate injection model. We also confirmed that SPANXB2 knock down attenuates tumor metastasis. In addition, TGF-β2 treatment augments tumor metastasis in PC3 subcutaneous model. These data fully supports that regulation of SPANXB2 and reactive stromal cells on tumor metastasis *in vivo*. **iii**) Test the association of SPANX-B2 expression with biochemical recurrence (PSA), lymph node metastasis, prostate cancer specific death and reactive stroma grade (RSG). Based on the relocation reason, we lost the cooperation with our previous partner and are no longer to access these human prostate tumor samples that linked with clinical outcome and RSG, we did not complete this part to seek the linking between SPANXB2 and RSG. But we still observed that a robust accumulation of myofibroblasts in HPS cells after co-culture. It suggests that SPANXB2 is associated with this accumulation of myofibroblasts. We still observed that SPANXB2 level is increased among the high Gleason patients samples. By using Oncomine and TCGA data, we found that SPANXB2 is overexpressed on metastasis sites and closed associated with clinical outcome. These data supports that overexpression of SPANXB2 is lined with metastasis and bad clinical outcome.

Key Research Accomplishments

Specific Aims of the Project as Proposed:

Aim 1: Determine the association of SPANX-B2 and stroma with prostate cancer aggressiveness *in vitro*.
1a) Assess the role of SPANX-B2 in regulating aggressiveness in *vitro*.

Although a few previous reports has shown the association between SPANXB and disease progression (1.2), the function of SPANXB2 in cancer progression, especially in prostate cancer is still an open question. Loss function study is the best way to verify the function of study gene. We purchased the knock down shRNA vector from Openbiosystem and establish three knock down clones in PC3 cells: SPANXB2-KD1, KD2, and KD3. Real time PCR and Western blotting analysis are applied to validate the knock down effect (Figure 1). Anti-SPANXB antibody is a gift from Dr. Kouprina from NIH, we also followed up the same protocol from their papers (2,3). Mel-938 is a melanoma line and used as a positive control. Except clone KD1, which only show knockdown effect on mRNA level, KD2 and KD3 clones show successful SPANXB2 knockdown effect on both mRNA level and protein level in PC3 cells (Figure 1).

We further analyze these aggressiveness markers including proliferation, migration, and invasion by comparing with PC3 NS cells and KD cells. Since PC3-KD1 clone did not show the protein level knock down effect, we more focus on PC3-KD2 and KD3 clones. In proliferation assay, we use the MTT method to check the proliferation rate of cells, basically, 5000 of each type of cells are plated in 96-well plate and incubate 72 hours, then MTT is added and proliferation rate is measured in the following day. The data clearly indicates that SPANXB2 knock down reduces the cell proliferation (Figure 2-A). Wound healing experiment is used to analyze the migration function. Cells are plated one day before and a scratch is made, then wound-healing images are taken after 48 hours. KD2 and KD3 cells show a deficiency of healing wound ability in these experiments (Figure 2-B).

B.D. invasion kit is one of the most common used methods to evaluate the cell invasion function. By using this kit, we put 25,000 of PC3 NS, PC3-KD2, PC3-KD3 in 500ul of non-serum contain medium on the top insert and 750 ul of 30% serum medium on the bottom well. After 22 hours, the invasion membrane is harvested and a dye staining is applied to facilitate count of these invaded cells. Control membranes are harvested and used to count for migrated cells (Figure 3). Our data demonstrates that migration and invasion ability are inhibited in both of PC3-KD2 and KD3 cells (Figure 3 A-B). A rescue experiment is also designed to check whether re-exposure or re-adding 19I cells to these KD2 and KD3 cells can rescue the phenotype. We generate the PC3-KD2-19I and PC3-KD3-19I cells according to the method we describe previously. And we also directly mix 19I cells with these KD2 and KD3 cells on the top insert. Unfortunately, these rescue experiments did not generate a fully clear result. It shows that a rescue phenotype on Migration assay and partially rescue on KD2-19I cells in PC3-KD2 clone. It did not show

significant rescue phenotype on PC3-KD3 clone (Figure 3). Considered that KD3 clone has more protein level knock down than that of KD2 clone (Figure 1-c). It implies that SPANXB2 protein level is critical for cells to maintain an aggressiveness phenotype, the re-exposure of 19I cells to these knock down cells seems is not sufficiency to re-generate enough SPANXB2 in these cells.

Summary of Key Research Accomplishments in this section

1) Successfully establishment of PC3-SPANXB2 knock down stable lines.
2) Validation of knock down effects by using real time PCR and western blotting.
3) SPANXB2 knock down inhibits the aggressiveness markers including proliferation, migration and invasion *in vitro*.

1b) Determine the influence of human prostate stroma (HPS) and TGF-β on modulating SPANX-B2 expression and aggressive cell behavior *in vitro*.

We initially get two HPS: 19I and 33F lines from my co-mentor Dr. Rowley's lab. HPS 19I and 33F are isolated from healthy donor prostate and cultured in a specific stromal medium without immortalization (4,6-7). Dr. Rowley's previous work has shown these two HPS lines promote development of normal prostate gland function and they stimulates paracrine TGF-β1 that make these HPS lines to become myofibroblasts or reactive stromal cells by using 3-D organoid co-culture model (7). Their further work includes generating a reactive stroma signature by using co-culture model and revealing that HPS-cancer cells co-culture contributes the Androgen receptors (AR) pathway through activating Stromal TGF-β signaling (6,8). There is still no report about how HPS 19I/33F promote prostate tumor progressive, and a 2-D model is not yet applied in HPS-epithelial cells co-culture. Therefore, our study majorly focuses on the influence of HPS on tumor cells aggressiveness by using a modified 2-D co-culture model.

One of unique characteristic of the metastatic gene signature that we use to identify SPANXB2 gene is tumor microenvironment. The distinct difference between the mouse dorsal prostate and subcutaneous environment makes us to believe that this might be the power to drive a different metastasis profile. Based on that, we design to use the human prostate stromal cells, 19I or 33F, to build the stromal-epithelial interaction model *in vitro* and validate the SPANXB2 expression level in this model. Currently, There are 2-D and 3-D models for studying the stromal-epithelial interaction in culture. Although 2-D is obviously defective in understanding of cells architecture, polarity and differentiation, it is one of the most valuable reductionist models to allow more straight study (5,9). We design to use a mixed culture model in that cancer cells and stromal cells have a physical contact instead of only using stromal cells conditional medium to treat cancer cells. In order to maximum the effect of co-culture, we modify this model by introducing a second round co-culture system. Briefly, after mixed co-culture 48 hours (usually it can not

continue to co-culture these cells due to the limited medium and oxygen), we do not terminate co-culture but we separate the stromal cells and cancer cells by using GFP based FACS sorting, then we put the isolated cancer cells back to a new stromal cells dish to re-start a co-culture (Figure 4). By using this modified model, we demonstrate that SPANXB2 level is increased in PC3 cells upon the human prostate stromal (HPS) cells 19I cells co-culture in both of mRNA level and protein level (Figure 5 A-B). Importantly, both of real time PCR data and Western blotting data show a greater level of SPANXB2 in PC3-19I-2R (second round PC3-19I co-culture cells) than the first round of PC3-19I. This result provides a strong support for our modified model in that the new model strengthens the power of stromal-epithelial interaction. In addition, we use immunofluorescence staining of SPANXB2 in serial PC3-19I co-culture cells as well as C-4-2B 19I co-culture cells (Figure 7, A-C). A similar result is obtained in this experiment. Except PC3 and 19I co-culture model, we also check a few of different prostate cancer cells lines with human prostate stromal cells 19I and 33F (Figure 6).

The functions of this PC3-19I serial co-culture are examined including proliferation, migration, and invasion in (figure 8, A-D). 33F, another HPS cells, is co-cultured with PC3 and DU145 cells and these co-cultures also indicate a increasing in migration and invasion (Figure 9). Put together, these results illustrate that co-culture with cancer cells and HPS is associated with the increased aggressiveness of cancer cells. Since directly mixed co-culture (physical contact) and conditional medium based (non-physical contact) co-culture have been widely reported, we examine the effect of both of models on the invasion ability of PC3 cells. Interestingly, we found that 19I conditional medium failed to promote PC3 invasion, the direct mixture of PC3 and 19I increased the invasion (Figure 8, E). This result implies that physical contact might be a prerequisite for HPS –cancer cells co-culture model. In addition of the 2D co-culture model, we also use a 3-D co-culture model: Organoids (7,9). In this model, cancer cells and HPS cells mixed together (1:2) and put the upper insert which is placed in a 24 well plates, medium will be put on the bottom well. We established PC3 cells and a few of other cell line organoids model. By immunohistochemistry, the organoids staining indicates that SPANXB2 positive cells are mostly located in epithelial layer but not stromal layer (Figure 10). Due to deficiency of technique to isolate cancer cells from Organoids at that time, we did not do further analysis of how much of SPANXB2 is elevated in these co-culture cancer cells in organoids.

It has been largely known that TGF-β is widely involved in modulating stromal-epithelial interaction (10-12). However, the role of TGF-β in regulating prostate cancer aggressiveness is still inconsistent (13, 14). More important, there is still unclear about the regulating mechanism of SPANXB2. We hypothesize that elevated TGF-β upon the stromal-epithelial interaction regulates SPANXB2 expression and further promote prostate cancer aggressiveness. We reported that TGF-β2 is most potent stimulator to induce SPANXB2 mRNA expression when I applied this grant, now we further confirm that TGF-β2 increase SPANXB2 protein expression in PC3 cells (Figure 11, B-C). Since we anticipate that TGF-β2 is up-regulated upon the

stromal-epithelial interaction, we use TGF-β2 ELISA to measure the TGF-β2 level in conditional medium from either serial PC3-19I co-culture cells or directly mixture of PC3 cells and 19I cells (Figure 11, A). The ELISA result is consistent with our prediction: stromal-epithelial interaction stimulates TGF-β2 production. Since both of PC3-19I and PC3-19I-2R demonstrate high level of TGF-β2 secreting, it suggests that PC3 co-culture cells be the main source of secreting TGF-β2 but not the stromal cells. As a support proof, mixed culture of PC3-19I with 19I cells does not produce dramatically higher level of TGF-β2 than that of PC3-19I only. In addition, because PC3-19I cells actually has isolated from the co-culture system after FACS sorting, it suggests that the effects of stromal-epithelial interaction may not be a transit influence and continue to impact cells after cells leaving the co-culture system. The function of TGF-β2 on prostate cancer invasion and metastasis is not completely understand, based on our invasion data (Figure 11, D) , it reveals that TGF-β2 significantly improve invasion assay in PC3 cells and TGF-β2 inhibitor SB431542 represses the invasion phenotype. Since IL-6 has been reported as a master regulator in tumor microenvironment and tumor metastasis (8,15,16), we examine the function of IL-6 in PC3 invasion assay. Similar with TGF-β2, IL-6 enhances the invasion ability of PC3. Take together, we conclude that cancer cells secrete TGF-β2 after exposure to stromal cells; elevated TGF-β2 increases SPANXB2 level and drives cancer cells to become more aggressive. It is interesting to know if SPANXB2 has reciprocal regulatory mechanism on TGF-β2. We examine the TGF-β2 level in SPANXB2 knock down cells by real time PCR, we find SPANXB2 knockdown inhibits TGF-β2 transcription (Figure 11, E), it reminds that SPANXB2 may have a regulatory role on TGF-β2 expression.

Summary of Key Research Accomplishments in this section

1) Develop the techniques of culture HPS cells and establish a modified 2-D stromal cells and epithelial co-culture model.
2) Validate that HPS co-culture induces SPANXB2 expression.
3) Illustrate that HPS co-culture promotes prostate cancer cells aggressiveness including proliferation, migration, and invasion.
4) Further confirm that TGF-β2 promotes SPANXB2 expression.
5) Demonstrate paracrine of TGF-β2 upon HPS co-culture.
6) Indicate that TGF-β2 promotes prostate cancer cells invasion ability.
7) Establish the correlation among SPANXB2, HPS co-culture and prostate cancer aggressiveness.
8) By using a 3-D Organoid model, confirm that SPANXB2 expression is mainly from cancer epithelial cells.

Aim 2: Determine the association of SPANX-B2 and stroma with prostate cancer metastasis *in vivo*.

2a) Assess the role of SPANX-B2 in regulating metastasis *in vivo*.

We use orthotopical (mouse dorsal prostate lobe injection, DP) prostate xenograft model as the *in vivo* model to demonstrate tumor metastasis. The advantage of DP model is obviously on its fidelity to full mimic human tumor metastasis. They initially form a prostate tumor and then metastasis to lung, liver, bone and even brain. In our previous study, DP exhibits a much stronger metastasis profiles than these mice with a subcutaneous injection. Considered the major point we care is the tumor metastasis but not the primary tumor initiation and growth, DP model might be the most appropriated model to meet our purpose.

By double transfection of PC3 cells with luciferase vector and GIPZ SPANXB2 knockdown vector, we establish the luciferase SPANXB2 knockdown cells. This allows us to the live bioluminescent images. We injected PC3-NS, PC3-KD2 and PC3-KD3 cells into mouse DP, meanwhile, we also set up a group that mixture of PC3-KD3 with 19I cells is injected into mice to determine whether or not the re-adding of 19I cells rescue the mouse phenotype of PC3-KD3 cells. The results indicate that SPANXB2 knockdown suppresses tumor metastasis (Figure 16, A-C). Adding 19I cells to PC3-KD3 cells partially rescue the metastasis phenotype. (Figure 16, A). The GPP whole mount lung images and IHC study confirm the inhibition of metastasis in live images.

Summary of Key Research Accomplishments in this section

1) Validate that SPANXB2 Knock down inhibits tumor metastasis in DP model.
2) Adding of 19I cells into the KD3 cells may rescue the inhibition of tumor metastasis phenotype in DP model.

2b) Determine the influence of HPS and TGF-β on modulating SPANX-B2 expression/metastasis *in vivo*

The role of 19I in promoting tumor progression is not very clear neither *in vitro* nor *in vivo*. By using these PC3-19I serial co-culture cells, we demonstrate that 19I promote tumor metastasis in PC3 DP model and PC3-19I-2R model exhibits more metastasis than that of PC3-19I model (Figure 13). We also observed the similar result in subcutaneous models (Figure 15, A). More importantly, western blotting analysis indicates that SPANXB2 level is up-regulated associated with adding of 19I cells in both of DP and subcutaneous model (Figure 15, B). It is clear that SPANXB2 is up-regulated upon PC3-19I co-culture and is associated with tumor metastasis.

In my previous DoD pre-Doc fellowship work, we found that TGF-β2 level is increased in DP tumor compared with the subcutaneous tumor, and TGF-β2 is critical in regulating tumor metastasis. Since we thought the insufficiency ability to form metastasis in subcutaneous tumor might be due to the lack of TGF-β2, we design an experiment by adding TGF-β2 into PC3 cells in subcutaneous tumor

model. As the result shown, supplement of TGF-β2 significantly increased the metastasis by comparing with the control group (Figure 14).

Summary of Key Research Accomplishments in this section

1) Validate that HSP 19I promotes tumor metastasis in both of DP and subcutaneous model.
2) Indicate that TGF-β2 improves tumor metastasis in a mouse subcutaneous model.
3) Demonstrate that SPANXB2 is increased upon PC3-19I co-culture.
4) Suggest the association between up-regulated SPANXB2 that is induced by 19I and TGF-β2 and tumor metastasis.

Aim 3: Test the association of SPANX-B2 expression with biochemical recurrence (PSA), lymph node metastasis, prostate cancer specific death and reactive stroma grade (RSG).

Due to moving and change of institution, we are no longer to access the BCM tissue microarray which is unique to provide the prostate cancer stromal cells grade, PSA level of patients, recording of lymph node metastasis and prostate cancer specific death (17). Hence, we did not complete this part of work.

Instead of stromal cells tissue microarray, we explore the association between SPANXB2 and reactive stromas by using real time PCR. We analyze 19I cells and 19I cells isolated from PC3-19I serial co-culture system. These results indicate a robust increase of reactive stromas markers in the 19I cells isolated from co-culture system (Figure 12, A). Moreover, our data show that the " reactive stromas gene signature" is also highly up-regulated in 19I cells after co-culture (Figure 12, B). FAP and α-SMA are markers of myofibroblasts and the increasing of these two markers strongly suggests that there is an accumulation of myofibroblasts in 19I cells after co-culture (5, 18-19). Since these myofibroblasts markers are also predictive markers for cancer malignance in multiple type of tumors (6,7), it predicts that elevated SPANXB2 level which is associated with these myofibroblasts markers is correlated to the rate of cancer progression.

We examine the SPANXB2 level in primary prostate tumors. SPANXB2 is overexpressed in high Gleason tumor samples (Figure 17, A). We further check the SPANXB2 level in Oncomine: in Grasso et al study, SPANXB2 is overexpressed at the metastatic lesion and patients with a worse clinical outcome (Figure 17, B-C). By using the TCGA database, we find the 13% of prostate cancer patients overexpress SPANXB2 and these overexpressed SPANXB2 patients have a worse survival curve (Figure 17 D). In summary, these data support that SPANXB2 is associated with prostate cancer metastasis and clinical outcome.

Summary of Key Research Accomplishments in this section

1) Demonstrate a robust increase of myofibroblasts markers and reactive stroma signature in 19I after PC3-19I co-culture.
2) Indicate that SPANXB2 level is up-regulated in high Gleason tumor samples
3) Demonstrate that SPANXB2 is correlated to metastasis and patient survival by using Oncomine and TCGA.

Figure 1

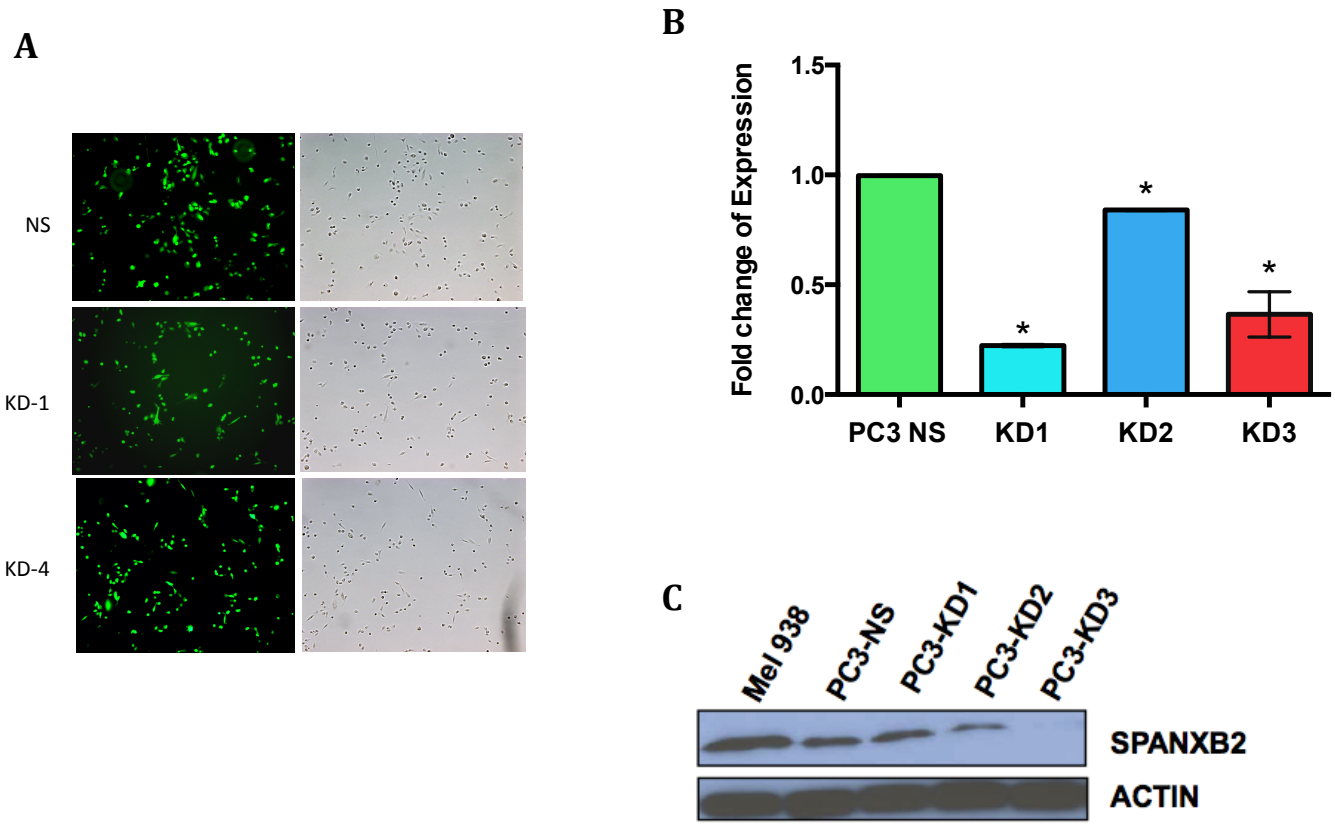
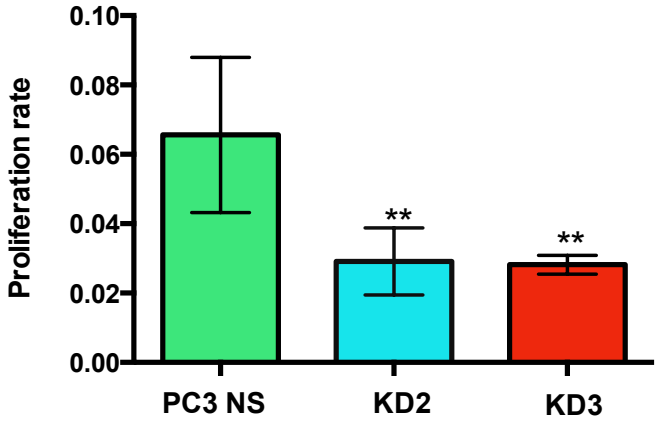


Figure 1. Establishment of PC3-SPANXB2 Knock down stable lines. **A** Images of SPANXB2 knockdown cell lines. GFP images indicate the transfection efficiency. GIPZ vector are purchased form Openbiosystems, NS is the non-silence backbone control, KD-1 and KD-4 are two different knockdown stable line; **B:** SPANXB2 knock down effect is checked by real time PCR; **C:** Western blot analyses of SPANXB2 levels in NS and KD cells. Clones of KD2 and KD3 demonstrate significantly protein level reduction, clone KD3 indicates more reduction of SPANXB2 protein level than that of clone KD3.

Figure 2

A



B

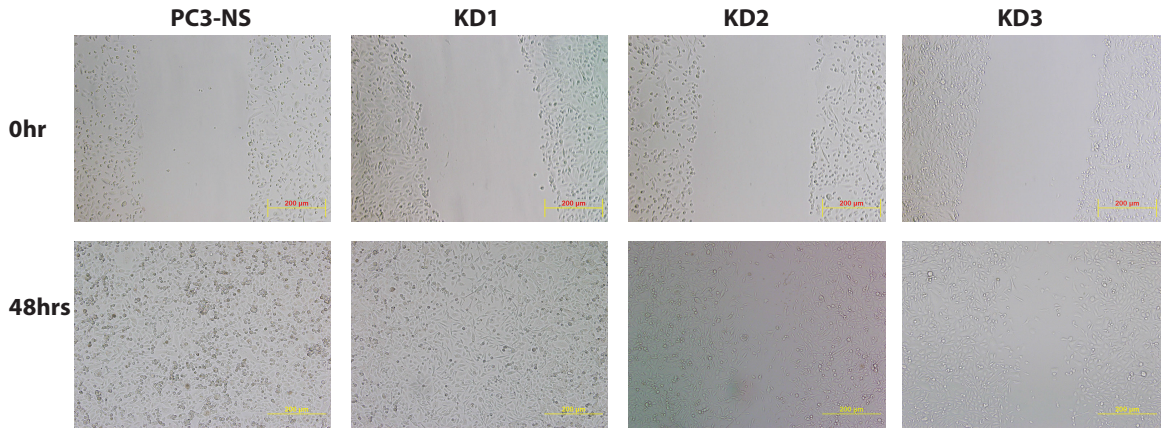
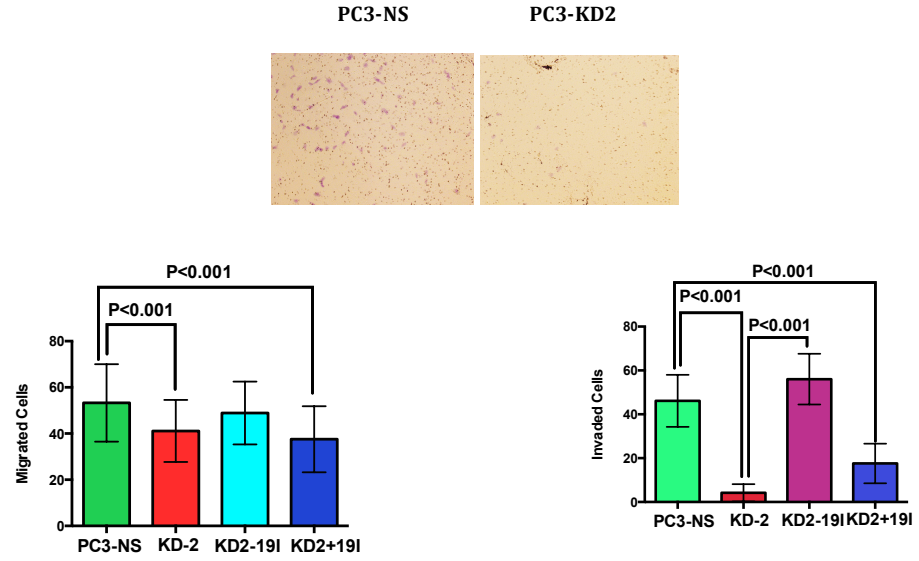


Figure 2: SPANXB2 Knock down inhibits PC3 cells proliferation and migration. **A**: Knocking down of SPANXB2 inhibits PC3 cells proliferation measured by using MTT kit; **B**: Blocking of SPANXB2 in PC3 cells impairs its wound healing ability in a 48 hours Scratch Assay.

Figure 3

A



B

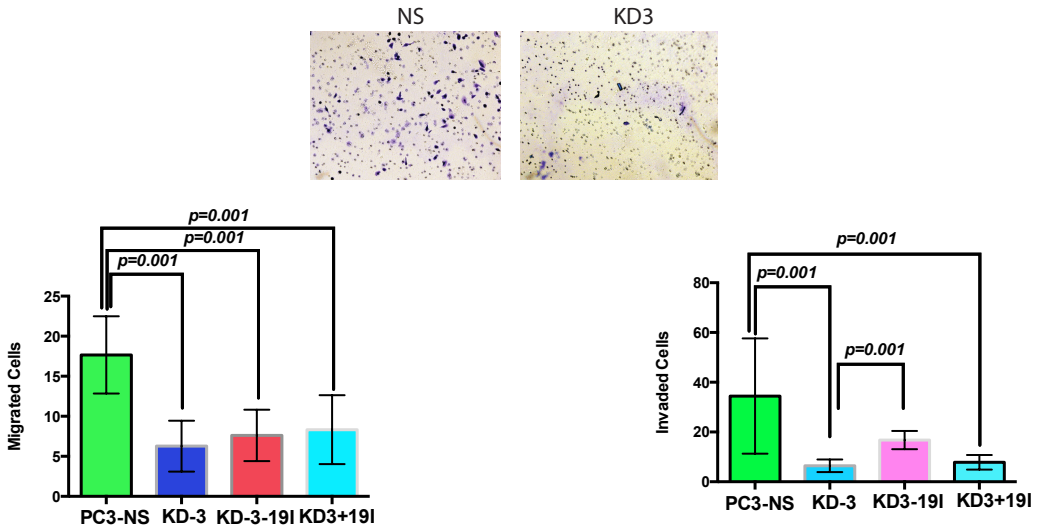


Figure 3: SPANXB2 Knock down suppresses PC3 cells invasion. **A**: SPANXB2 KD2 knock down cells indicated a reduction of migration and invasion, rescue this phenotype by co-culture of KD2 cells with 19I partially restore the migration and invasion. 25,000 of PC3 NS, KD2, KD2-19I(KD2 cells co-culture with 19I), and KD2+19I (KD2 and 19I mixture, 1:1 ratio) cells are plated into the top insert, migration and invasion are observed after 22 hours. Top image: Represented image of Invaded cells on membrane. Left bar graph: Migration assay of KD2 cells; right bar graph: Invasion assay of KD2 cells; **B:** SPANXB2 KD3 knock down cells demonstrate significantly decreasing of migration and invasion, rescue this phenotype by co-culture of KD3 cells with 19I fails to improve. Same procedure is done as describe in A. Top image: Represented image of Invaded cells on membrane. Left bar graph: Migration assay of KD3 cells; right bar graph: Invasion assay of KD3 cells

Figure 4

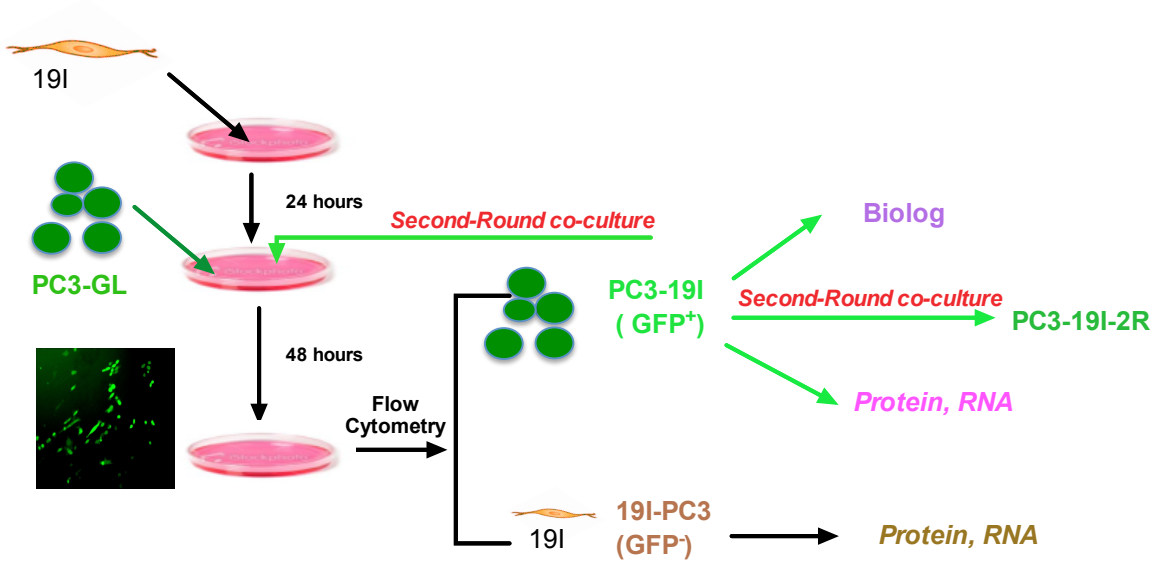
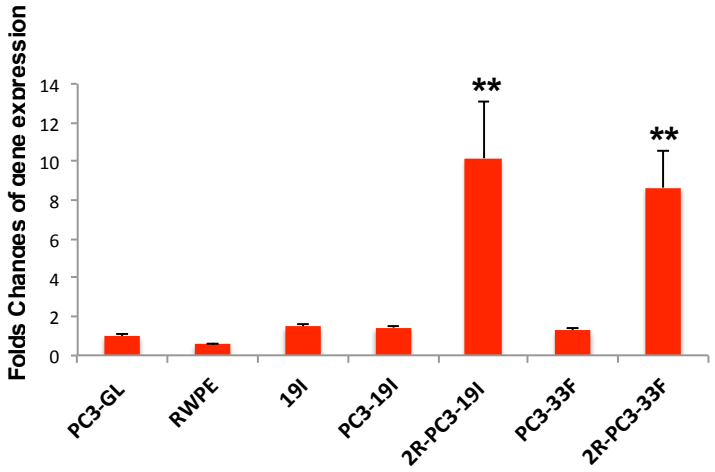


Figure 4: Schematic chart for a modified prostate cancer cells and stromal cells directly co-culture model. HPS 19I is pre-plated in dishes 24hrs earlier, then GFP labeled PC3 is plated on the top of 19I with a ratio of 1:1. After 48 hrs co-culture, mixture of cells will be harvested and immediately pass a FACS sorting. GFP^+ cancer cells and GFP^- stromal cells will be isolated. These GFP^+ cancer cells (PC3-19I) are either used for further experiment or re-plated on a pre-plated stroml cells to generate a second round PC3-19I co-culture cells (PC3-19I-2R). Protein and RNA are saved in each steps.

Figure 5

A



B

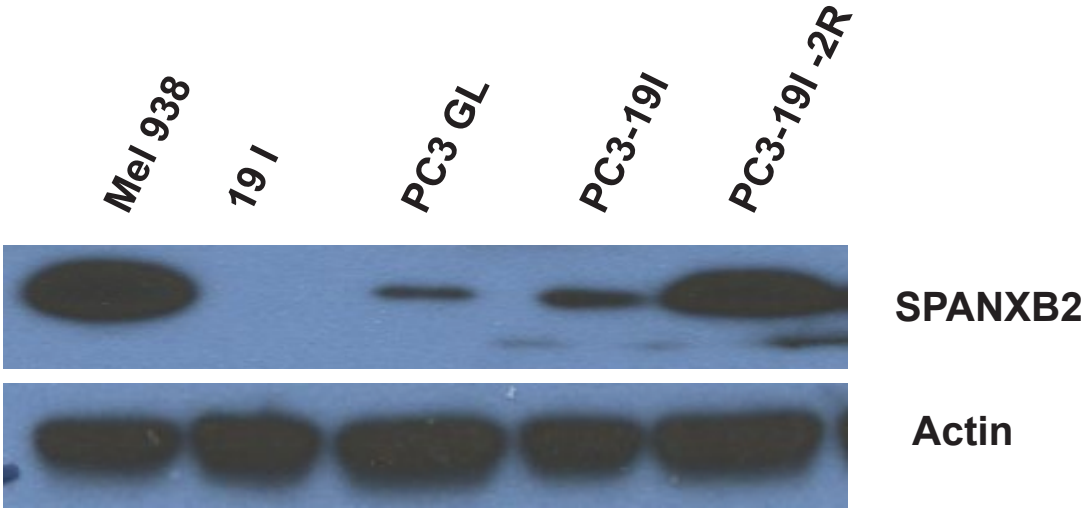
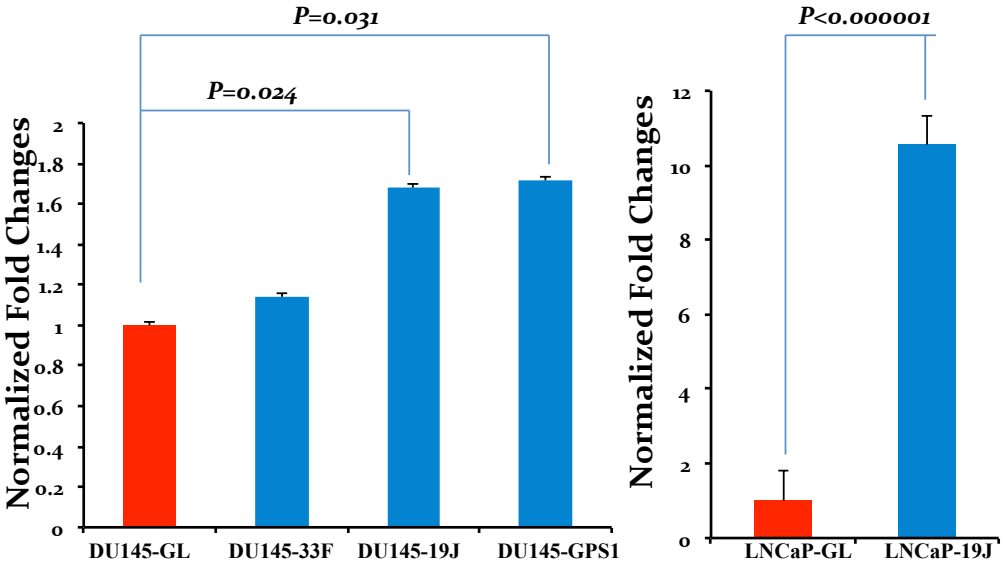


Figure 5: SPANXB2 is induced by reactive stromal cells co-culture in PC3 cells. A: Real time PCR demonstrates SPANXB2 mRNA levels are increased in PC3 cells after serial co-culture with 19I and 33F cells, ** P value < 0.01 as compared with endogenous levels in PC3 cells; **B:** Western blot analyses of SPANXB2 protein expression in Mel 938 cells (positive control), PC3 G/L (PC3 tagged with GFP and Luciferase vector, the parental cells of PC3-19I), PC3-19I and PC3-19I-2R.

Figure 6

A



B

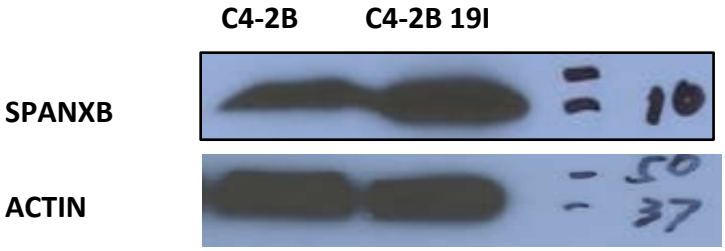


Figure 6: SPANXB2 is induced upon reactive stroma co-culture in different prostate cancer cells line. A: Real time PCR demonstrates SPANXB2 mRNA levels are increased in Du145 and LNCaP cells upon exposure to either 19I or 33F; **B:** Western blot analyses of SPANXB2 protein expression in C-4-2B and C-4-2B-19I.

Figure 7

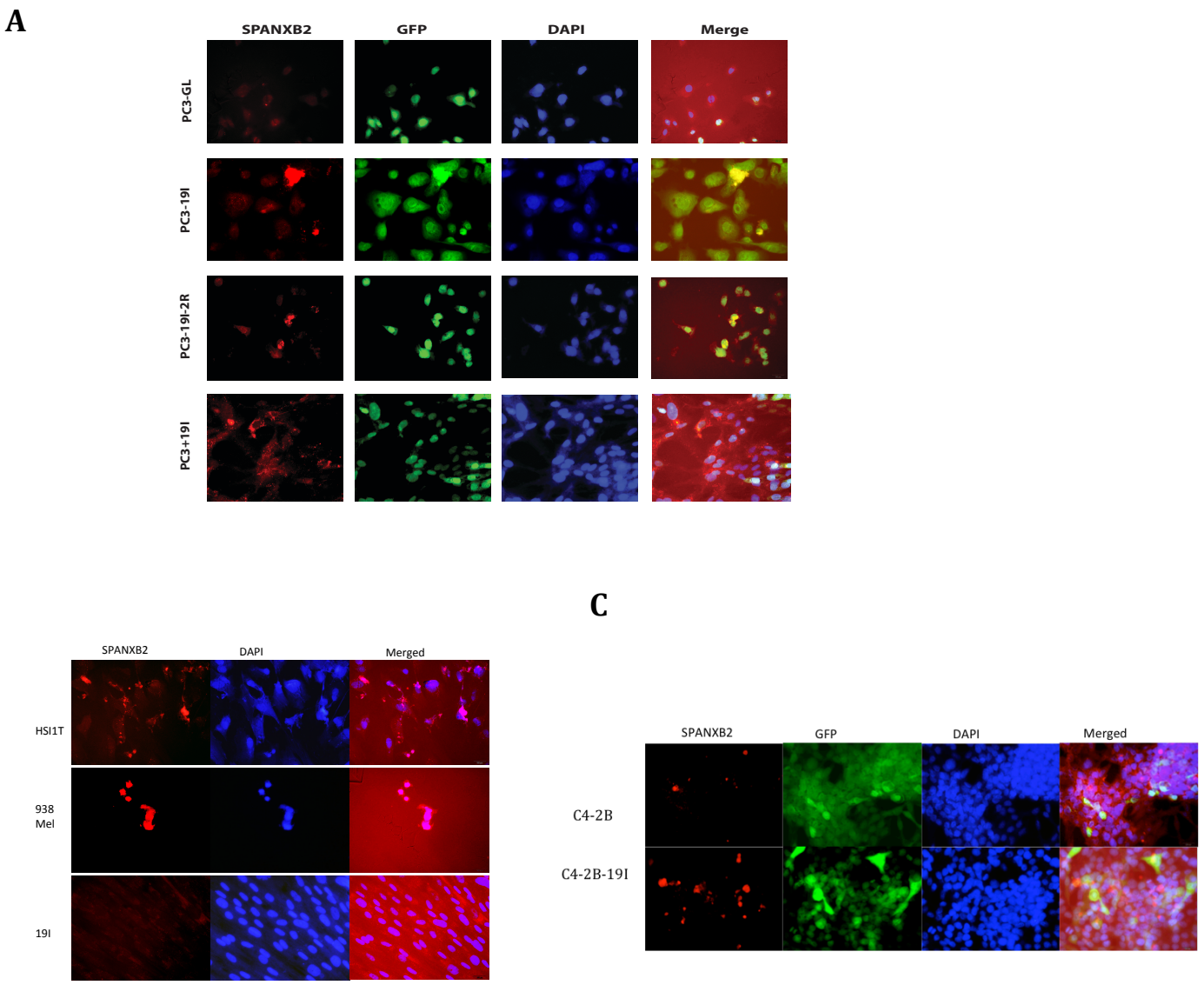


Figure 7: SPANXB2 level is increased upon reactive stroma co-culture in different prostate cancer cells line by immunofluorescence staining. A: Characterization of SPANXB2 expression in PC3, 19I and co-culture of cells using anti-SPANXB2 (red), GFP positive PC3 cells (green), Dapi (blue) staining of nucleus. Merged image shows SPANXB2 locates in both of nucleus and cytoplasm. **B:** SPANXB2 staining in two positive control cell lines : HSI1T and Mel 938, and 19I cells; **C:** immunofluorescence staining of SPANXB2 expression in C-4-2B and C-4-2B-19I.

Figure 8

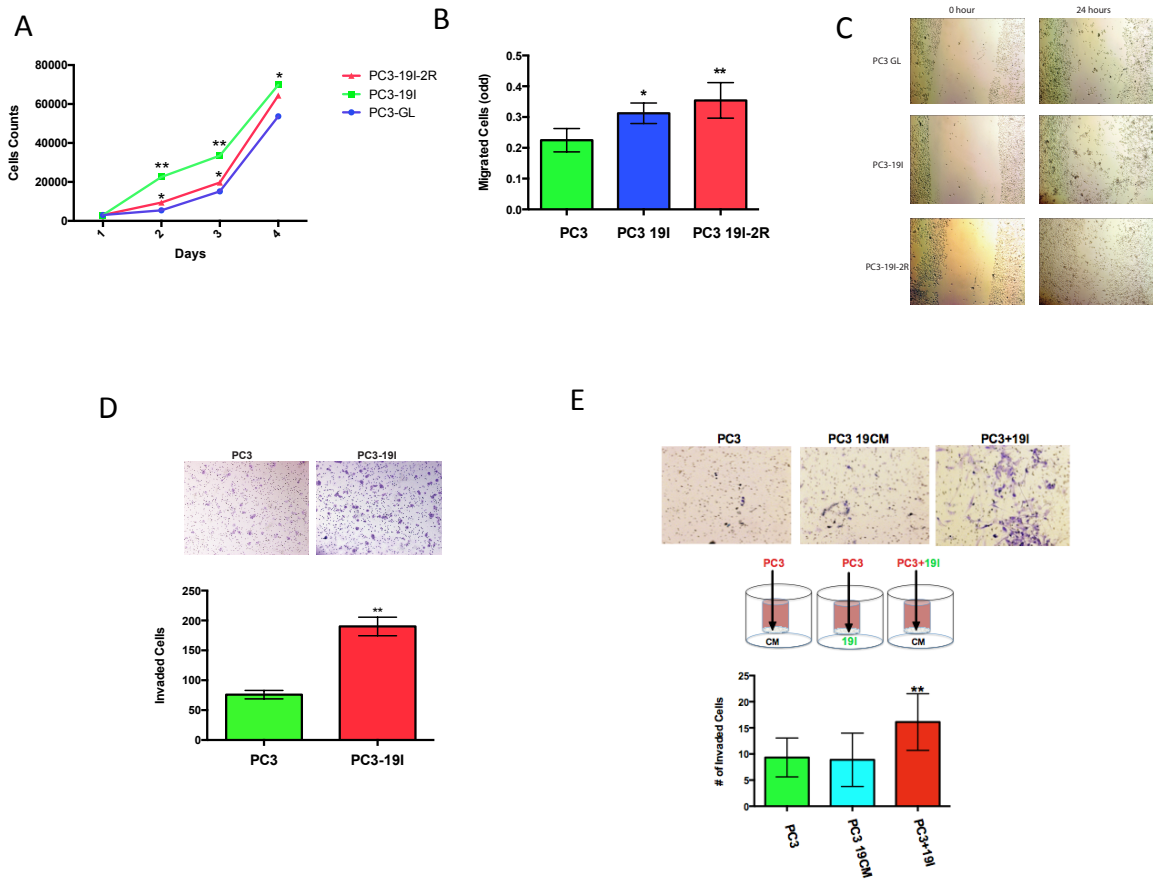
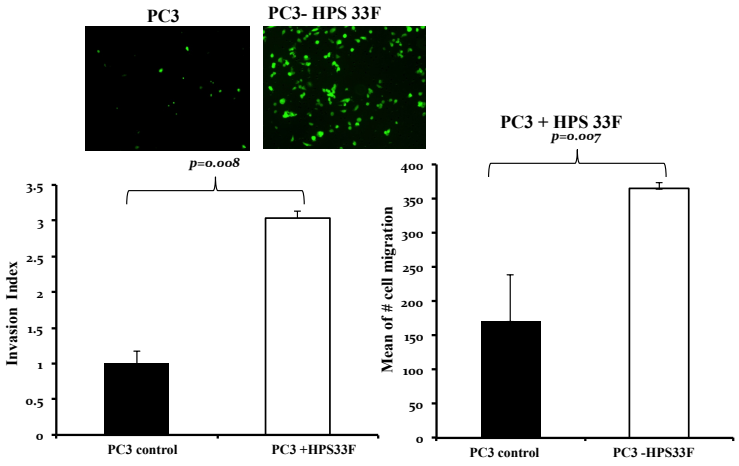


Figure 8: Reactive stroma 19I cells promote prostate cancer cells proliferation, migration, invasion *in vitro.* **A**: Serial PC3-19I co-culture cells indicate higher proliferation ability by using Cells population doubling counting ; **B**: Serial PC3-19I co-culture cells exhibit increased migration ability by using BD transfer well; **C**: : Serial PC3-19I co-culture cells demonstrate enhanced wound healing ability by using 24 hours Scratch Assay; **D**: PC3-19I cells show improved invasion ability by using BD invasion kit, the top images show representative invaded cells of PC3 and PC3-19I respectively; **E**: 19I conditional medium solely failed to promote invasion ability of PC3 cells compared with co-culture cells (19I and PC3 mixture). Experimental condition as from left to right: PC3 control group; 50,000 of PC3 are input in top well and plain medium (no serum) in the bottom well; middle, 50,000 of PC3 are input in top well and 19I conditional medium in the bottom well; right, 50,000 of PC3 and 50, 000 of 19I cells are mixed and input into the top well and plain medium in the bottom well, on this group, only the epithelial cells are counted as invaded cells (as the dark blue dot in the top images). The top images are reprehensive images of invaded cells on membrane from each groups (from left to right, PC3 control, 19I CM, and PC3-19I mixture). Only the co-culture group shows an increased invasion ability;

Figure 9

A



B

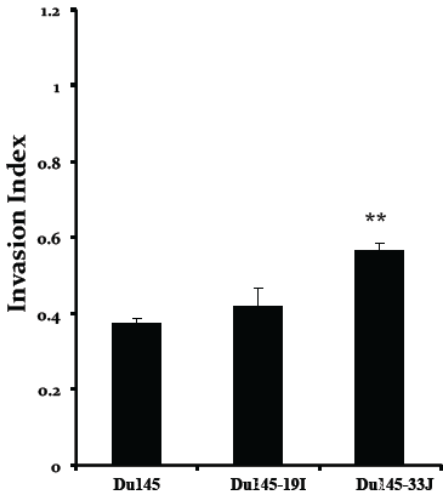


Figure 8: Reactive stroma 19I or 33F cells promote prostate cancer cells migration and invasion. **A:** PC3-33F co-culture cells exhibit an enhanced migration and invasion ability. These GFP images on the top left show the representative image of invasion membrane from PC3 wild type (left) and PC3-33F (right) respectively; **B)** Du145-33F co-culture cells demonstrate increased invasion ability.

Figure 10

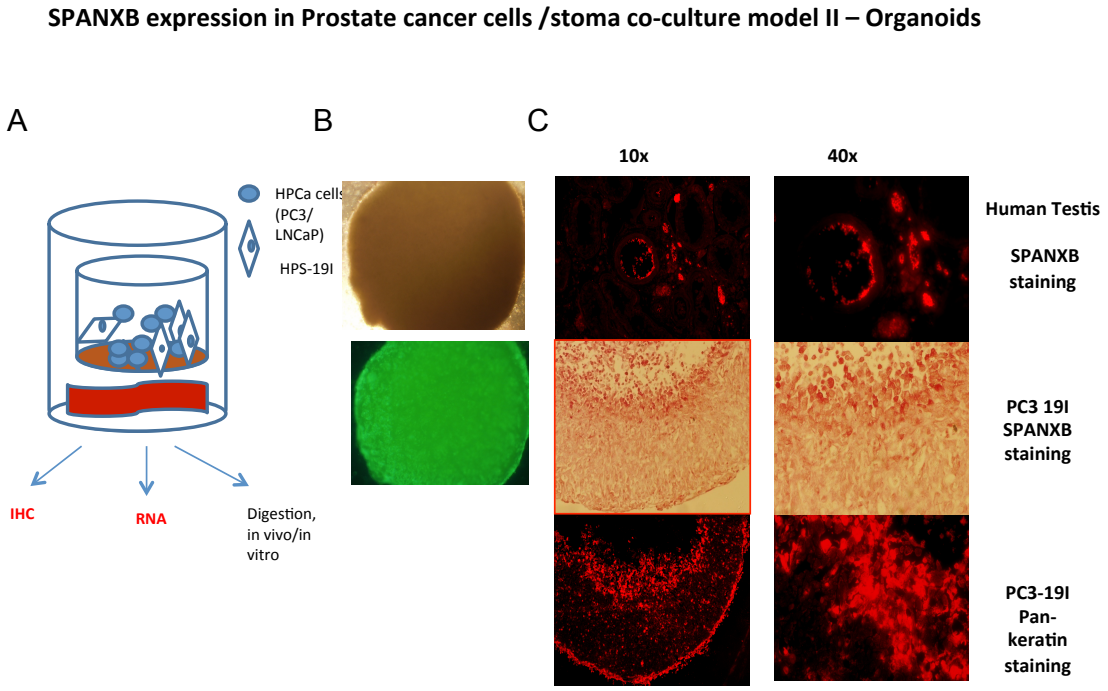


Figure 10: SPANXB expression in Prostate cancer cells /stoma co-culture model II – Organoids
A) is the cartoon model of the epithelial-stoma interaction Organoid. Prostate cancer cells are mixed with HSP cells (1:2 ratio) and plated into the top insert, the insert is immersed into the cup which is filled with medium. After 72 hours incubation, a 3-D organoid will be formed. Epithelial cells are located inside as the internal core and the stromal cells are surrounding the epithelial cells to form the outside layer; **B)** Phase and GFP Image of one Organoid formed by PC3-GFP cells with 19I cells. **C)** Immune staining for SPANXB in a PC3-19I Organoid model, the top images are SPANXB2 staining (red color) in human testis which is the positive control for SPANXB2, the middle images indicate that SPANXB positive cells are enriched in the internal layer, the bottom images reveal that epithelial cells are located in the inside layer by using a Pan-keratin staining. SPANXB2 positive cells may mostly come from epithelial cells.

Figure 11

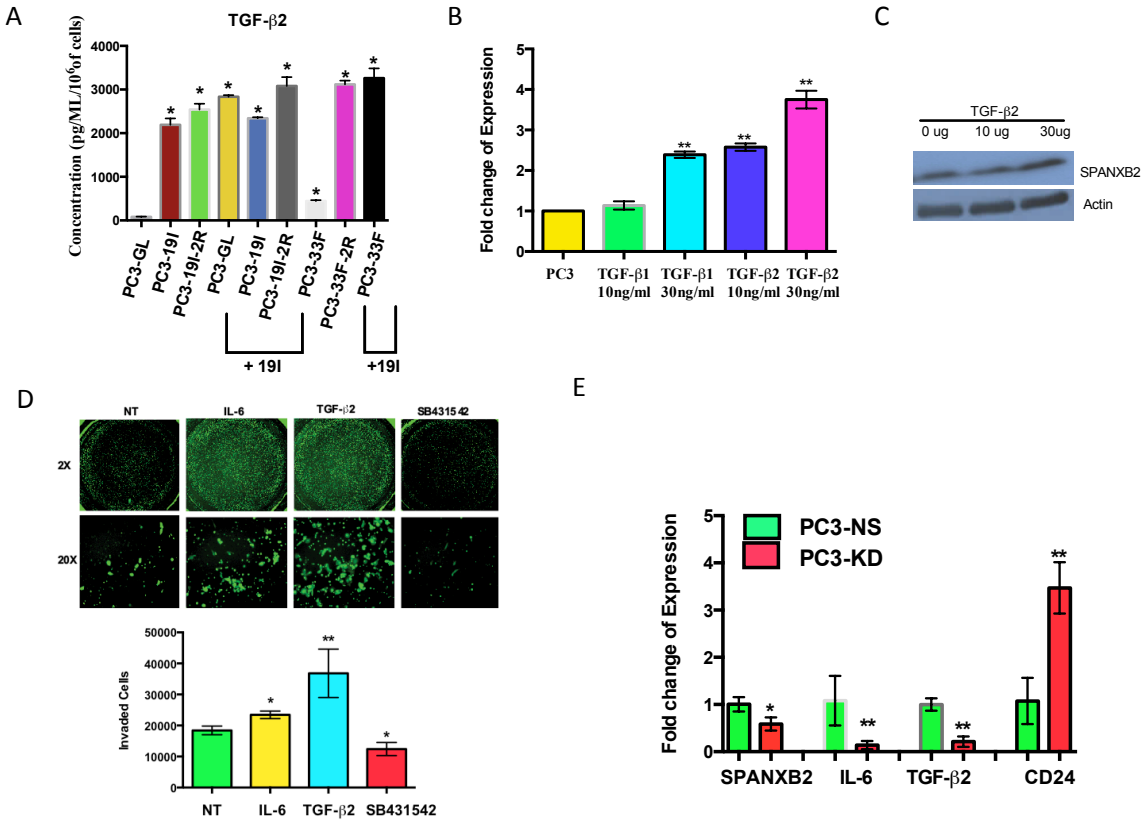


Figure 11: TGF-β2 is elevated in 19I co-culture system and contributes to stimulate SPANXB2 expression and promote invasion. A: ELISA results demonstrate that TGF-β2 level is significantly up-regulated in serial PC3-19I co-culture cells and mixed culture of PC3 and 19I cells. Two types of conditional medium are collected: conditional medium from PC3, PC3-19I serial co-culture cells; or conditional medium from mixed culture of PC3, PC3-19I serial co-culture cells with 19I cells (19I cells are pre-plated in dishes, then cancer cells are plated on the top of 19I cells, conditional medium is harvested after 48 hours. These CM have a underline marker " +19I" in figure); Another human prostate stromal cell line, 33F , is also applied in the same procedure as that of 19I to establish PC3-33F serial co-culture cells and mixed culture cells. Conditional medium also is collected from these cells; **B:** Exogenous TGF-β2 promotes SPANXB2 expression in PC3 cells by q-PCR. Two dose (10ng/ml and 30ng/ml) of TGF-β1 and TGF-β2 are added into PC3 cells culture respectively. TGF-β2 increases SPANXB2 level in both of low and high dose, while TGF-β1 only increases SPANXB2 expression in a higher does; **C:** Western blotting analysis shows SPANXB2 level is elevated upon a higher dose of TGF-β2 treatment in PC3 cells; **D**: Exogenous TGF-β2 and IL-6 facilitate PC3 cells invasion and TGF-β2 inhibitor suppresses PC3 invasion. By using invasion transwell, 50,000 of PC3 cells are put into top insert, vehicle control, IL-6 (10ug/ml), TGF-β2 (30ng/ml)are in the bottom well to attract cancer cells. SB431542, a TGF-β2 inhibitor, is added into the PC3 cells in the top insert. The top panel of images are the represented image (2x and 20X) of invaded cells on the membrane. Bottom is the bar graph of the invaded cells which is counted by GFP dots. **E**: Real time PCR reveals that SPANXB2 may regulate TGF-β2 and IL-6 expression in PC3 cells. Knocking down of SPANXB2 inhibits IL-6 and TGF-β2 mRNA expression but increase CD24 expression by using real time PCR. Note:; * p value < 0.05** ; P value < 0.01.

Figure 12

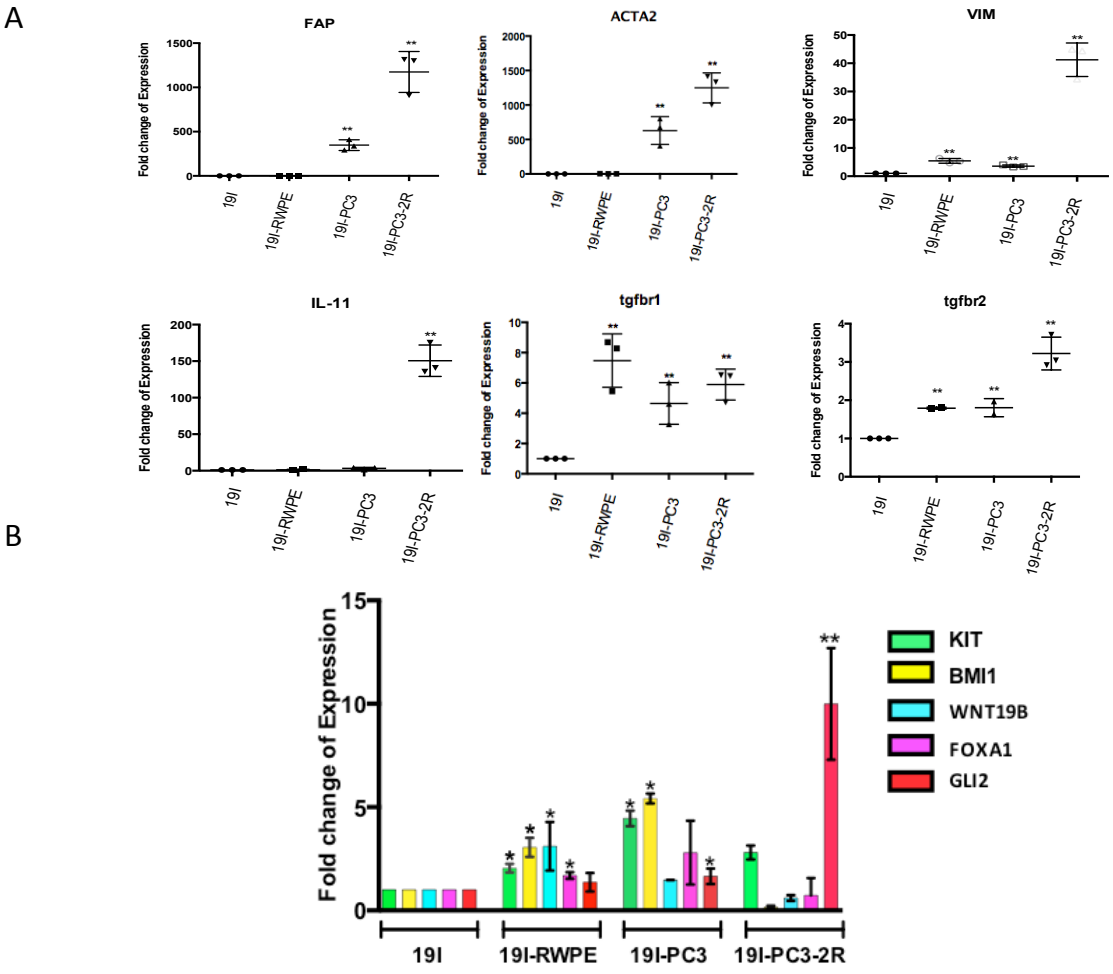


Figure 12: PC3-19I co-culture system promotes 19I cells become more " reactive" A : Markers of Reactive stromal genes including FAP, α-SMA (ACTA2), VIM, IL-11, tgfbr1 and tgfbr2 are significantly up-regulated in 19I cells isolated from PC3-19I serial co-culture. 19I-RWPE cells are used as another control since they are the stromal cells generated from co-culture of 19I with the benign prostate cells RWPE-1. 19I-PC3 and 19I-PC3-2R are 19I cells isolated from PC3-19I serial co-culture respectively. By using real time PCR, mRNA level of these markers are significantly increased in 19I isolated from PC3-19I serial co-culture compared with 19I wild type and 19I-RWPE; **B:** " Reactive Stromal Gene Signature" is consistently up-regulated in 19I isolated from PC3-19I serial co-culture. A " Reactive Stromal Gene Signature" has been identified in Dr. Rowley's lab from 19I stromal cells. By using real time PCR, 5 of genes from these signatures indicate a higher expression level in 19I-PC3 and 19I by compared with 19I and 19I-RWPE. Especially, GLI2 show the most prominent high expression level in these " reactive" stromal. . Note:; * p value < 0.05** ; P value < 0.01.

Figure 13

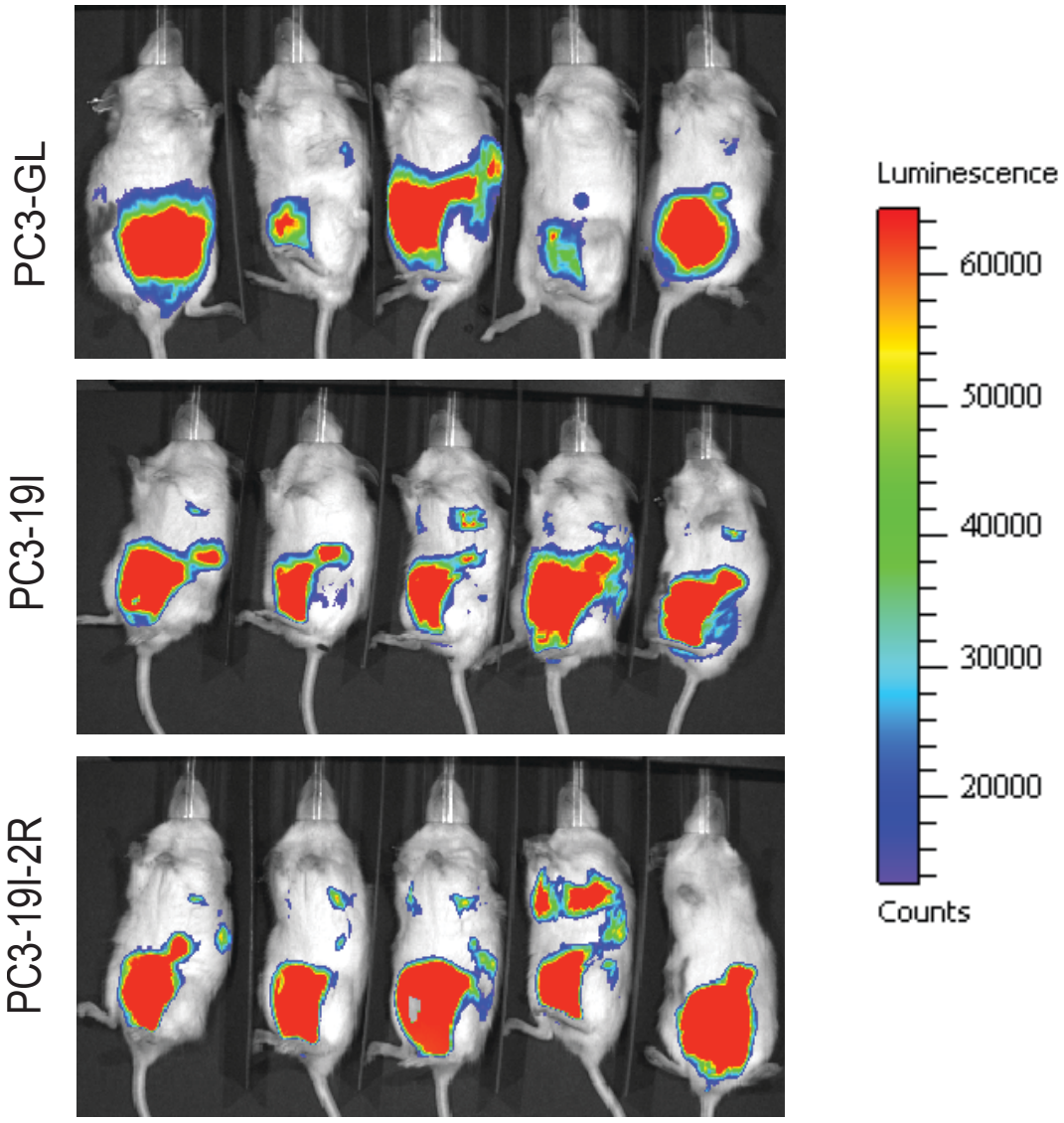
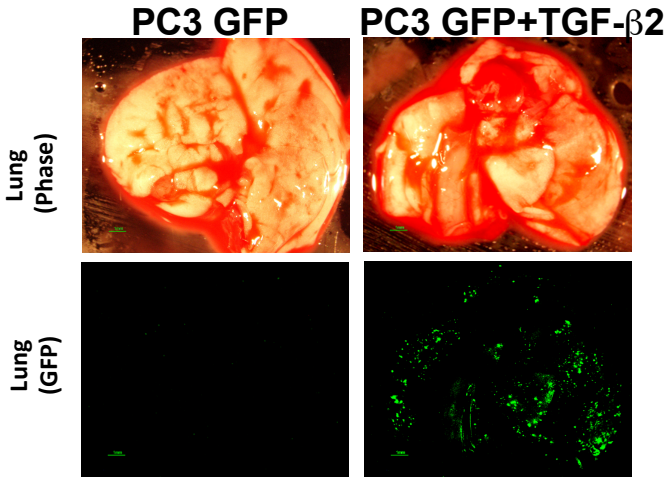


Figure 13: Reactive stroma 19I cells promote prostate cancer cells metastasis in orthotopical model. A: Serial PC3-19I co-culture cells show greater metastasis profiles in Orthotopical model (dorsal prostate injections): of serial PC3-19I co-culture cells indicates amPC3 serial co-culture cells exhibits more aggressive metastasis by using dorsal prostate injection model. 500,000 of PC3, PC3-19I and PC3-19I-2R (1:1 ratio mixed with Matrigel) were orthotopically injected and Bioluminescent images depicting lung metastasis after 6 weeks.

Figure 14

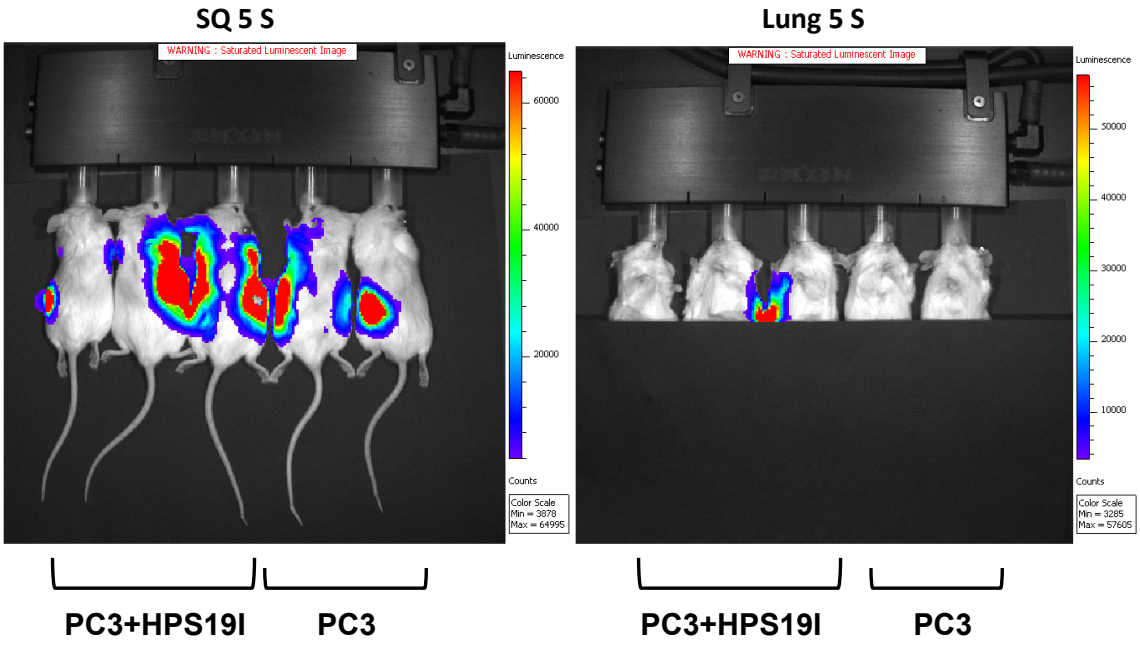


Tumor Group	PC3 GFP	PC3+TGF-β2
Lung Metastasis Rate	0/8	5/8

Figure 14: TGF-β2 stimulates prostate cancer cells metastasis in subcutaneous model. 25,000 of PC3-GFP cells are injected subcutaneous with Matrigel (1:1) (left group) or with Matrigel and TGF-β2 (right) into mouse flank of NOD/SCID mice. After 4 weeks of injection, Lung is harvested and green fluorescence whole mount image is taken. GFP dots represent PC3 GFP cells lung metastasis. The bottom table indicates the total lung metastasis rate in eight mice on each group.

Figure 15

A



B

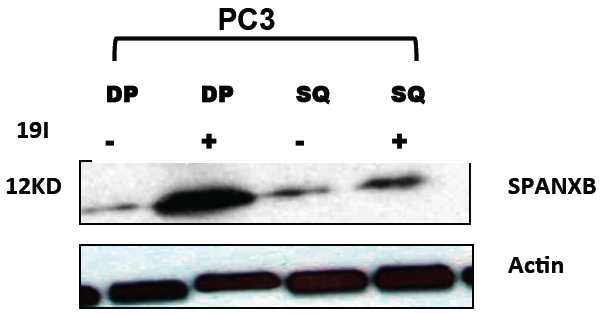


Figure 15: Reactive stroma 19I cells promote prostate cancer cells metastasis in subcutaneous model. **A** : Lucifierase vector) were subcutaneous co-injected with Matrigel (1:1) in a total 80 ul into mouse flank in NOD/SCID mice. PC3 G/L co-injected with Matrigel (1:1) in a total 80 ul into mouse flank was used as a control. After 4 weeks of injection, Live luciferase images were taken after post injection of substrate 15 mins , the exposure time is 5second. (Right) Metastasis from lung was imaged. Notice : same mice from the left side. Exposure time is 5 seconds. **B:** Boosting prostate cancer cells metastasis by reactive stroma 19I cells is associated with elevated SPANXB2 level. 500,000 of PC3 cells are injected into either mouse dorsal prostate (DP) or mouse flank subcutaneously (SQ) with/without adding 19I. Primary DP and subcutaneous tumors are harvest and western blotting analysis are perform by using represented tumor samples.

Figure 16

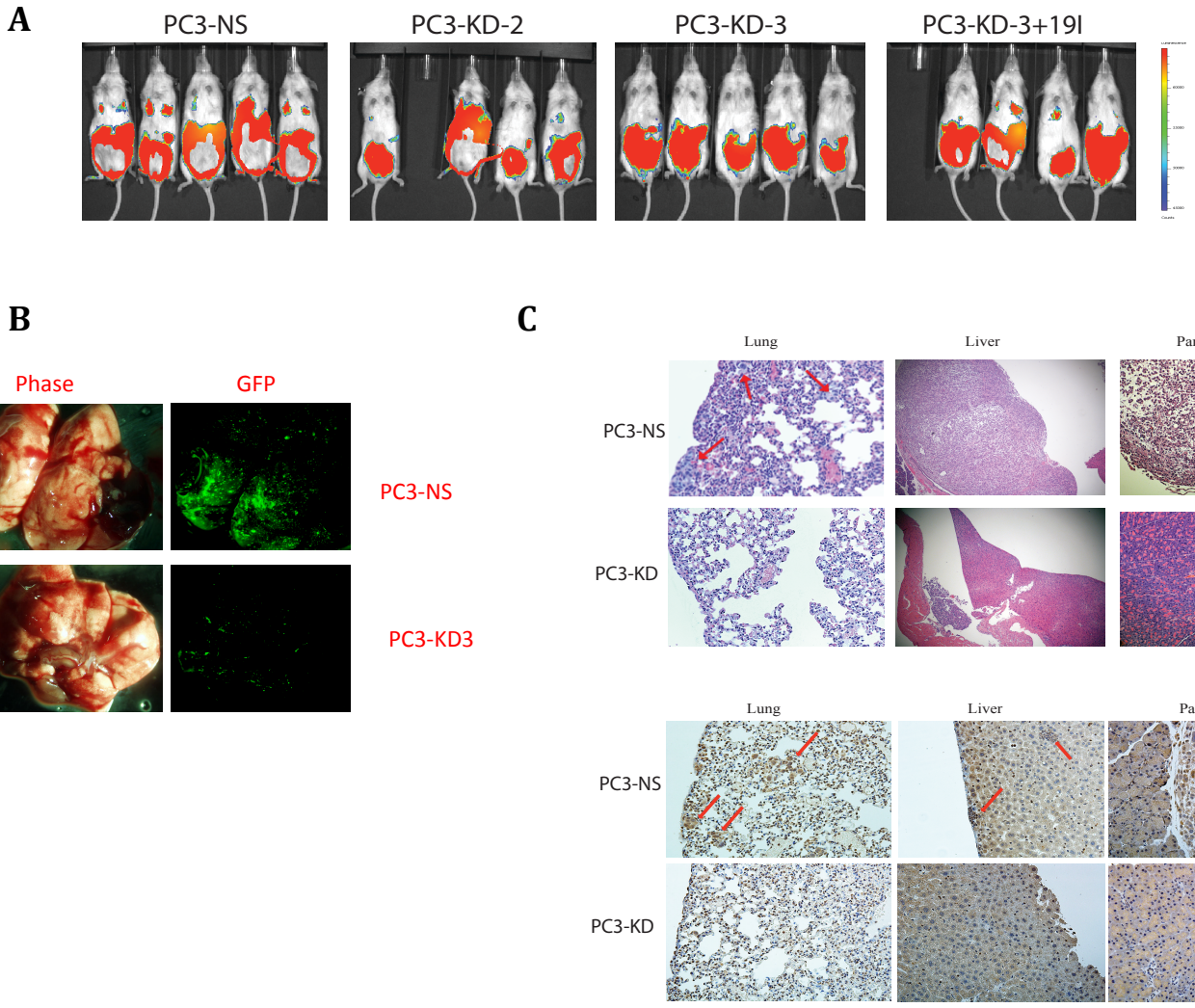


Figure 16: Knocking down SPANXB2 attenuates tumor metastasis. A: Two clones of SPANXB2 knock down cells indicate reduced level of metastasis in PC3 orthotopical model: 250,000 of PC3-NS, PC3-KD2, PC3-KD3, and PC3-KD3+19I (KD3 cells and 19I mixture, ratio 1:1) cells were injected into mouse dorsal prostate in NOD/SCID mice, Bioluminescent images indicate lung metastasis after 7 weeks; **B**: Green fluorescence whole mount images indicate that lung metastasis is suppressed in PC3-KD3 mouse. NS and KD3 mice are described as above and lungs are harvested after 8 weeks; **C**: Immunohistochemistry image of metastatic lesion in PC3 NS vs KD3 orthotopic models. Top: HE staining for metastasis lesion in Lung, Liver , Pancreas (left to right); bottom: GFP staining for metastasis lesion in Lung, Liver , Pancreas (left to right), arrows show the GFP staining of metastatic lesion.

Figure 17

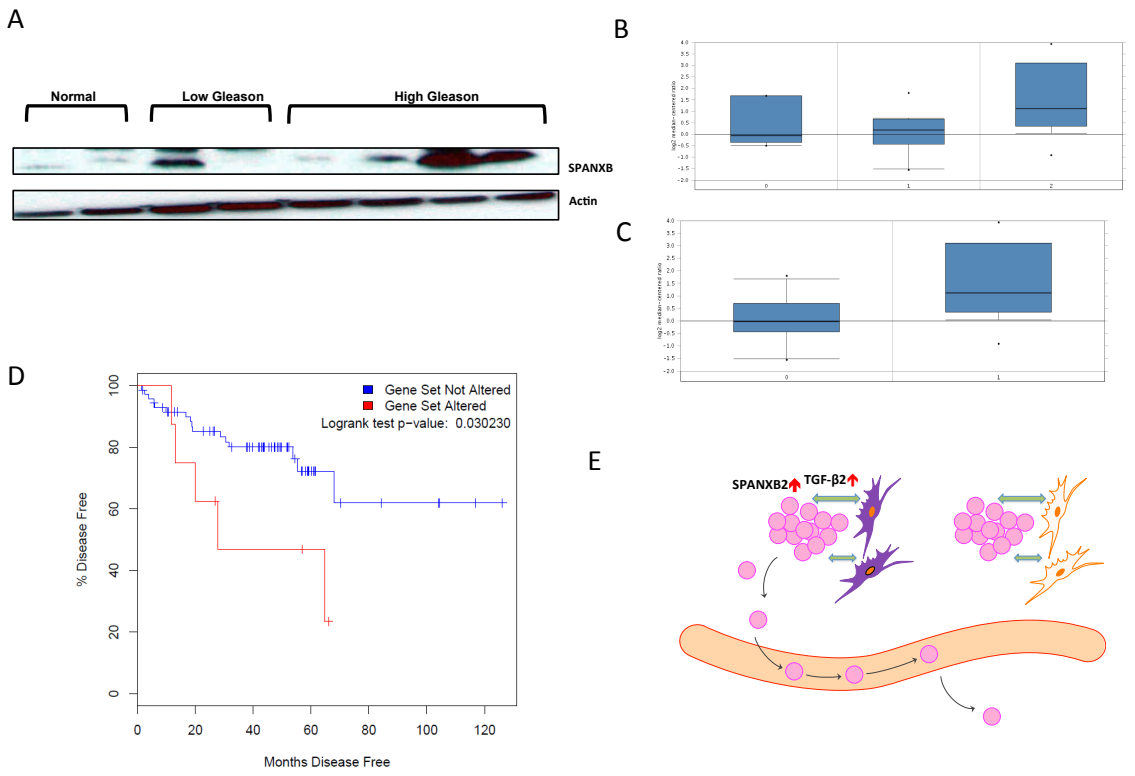


Figure 17: SPANXB2 level is up-regulated in malignant clinical prostate patient samples and associated with patient's survival. A: SPANXB2 protein level is higher in high Gleason patient samples than these of normal and low Gleason samples. These clinical samples were fresh samples directly from surgeon after a rapid pathological diagnosis. From left to right, normal, low Gleason (Gleason 3), high Gleason (Gleason 7); **B)** SPANXB2 Expression is increased more in metastasis sites than that of normal prostate and primary tumor site by using Grasso et al data from Oncomine. Prostate cancers are grouped by tumor sites: 0 represents benign patients (7 samples), 1 represents primary tumor site (12 samples); 2 represents metastasis sites (11 samples). SPANXB2 shows higher expression in metastasis sites; **C**: SPANXB2 Expression is increased in patients with a worse clinical outcome compared with that in patients with a better clinical outcome by using Grasso et al data from Oncomine. Prostate cancers are grouped by survival: 0 represent better survival outcome, 1 represent dead; SPANXB2 level is elevated in group 1: **D**: Patients with overexpression of SPANXB2 had a worse survival outcome compared with these patients with less expression of SPANXB2. By using TCGA data base, SPANXB2 is over expressed in 11% of prostate cancer samples and the survival-curve is representing as the group of patients with an overexpression SPANXB2 level vs groups of patients with normal SPANXB2 level ; **E**: Working model for SPANXB2 and activated stromas in prostate cancer progression. In this model, we hypothesis that prostate cancer cells interact with surrounding stromal cells, these interaction might be predispose to active SPANXB2 in prostate cancer cells. Increased level of SPANXB2 promotes cancer progression and metastasis. Meanwhile, the interaction between cancer cells and stromal cells also stimulates these " reactive stromal genes" expression in these stromal cells.

Conclusion

SPANXB2 is up-regulated upon stromal-epithelial interaction and plays critical role in regulating prostate cancer progression. Reactive human stromal cells promote SPANXB2 expression and are associated with the rate of prostate cancer aggressiveness. Elevated TGF-β2 is in prostate cancer cells after exposure to human stromal cells induces SPANXB2 expression and promotes cancer progression. Overexpression of SPANXB2 in clinical prostate patients is associated to the metastasis and survival outcome.

Publications

Zaslavsky A, Gloeckner-Kalousek A, Venghatakrishnan H, Putluri N, **Li H,** Morgan T, Sreekumar A, Palapattu G. Platelet synthesized testosterone in men with prostate cancer induces androgen receptor signaling. Submitted to Clinical Cancer Research

Panzitt K, Shojaie A, Kaushik A, Putluri N, Sonavane R, Venghatakrishnan H, Putluri V, Bhat V, Rajapakshe K, **Li H**, Samanta S , Zhang Y, Cao X, Tsouko E, Lloyd S, Zaslavsky A, Huang S, Mo Q, Coarfa C, Edwards D, Erho N, Vergara I, Jenkins R, Frigo D, Cao Q, Mitsiades N, Weigel N, Ittmann M, Chinnaiyan A, Palapattu G, Michailidis G, Sreekumar A. Integromics reveals importance of hexosamines in prostate cancer with therapeutic implications Submitted to Journal of Clinical Investigation

Li H, Diallo Krou E., Hernandez J, Ressler S., Sreekumar A., Ayala G., Rowley D., Pienta K, Palapattu G. SPANXB2 expression increases prostate cancer aggressiveness : A new view of stromal epithelial cell interaction. Manuscript in preparation.

Li H., Suraneni M., Chen X., John M., Liu X., Hu J., Huang J., Tang DG. Microenvironment-Regulated Manifestation of Metastatic Prostate Cancer Stem Cells. Manuscript in preparation

Li H, Suraneni M., John M ., Calhoun-Davis T ., Chen X., Liu X, Qin J., Tang DG. Tumor microenvironment promotes prostate cancer metastasis through TGF-β2. Manuscript in preparation

Wen J, **Li H**, Tao W, Savoldo B, Foglesong JA, King LC, Zu Y, Chang CC. High throughput quantitative reverse transcription PCR assays revealing over-expression of cancer testis antigen genes in multiple myeloma stem cell-like side population cells. Br J Haematol. 2014 Sep;166(5):711-9.

Roca H, Hernandez J, Weidner S, McEachin RC, Fuller D, Sud S, Schumann T, Wilkinson JE, Zaslavsky A, **Li H**, Maher CA, Daignault-Newton S, Healy PN, Pienta KJ. Transcription factors OVOL1 and OVOL2 induce the mesenchymal to epithelial transition in human cancer. PLoS One. 2013 Oct 4;8(10)

Qin J, Liu X, Laffin B, Chen X, Choy G, Jeter CR, Calhoun-Davis T, **Li H**, Palapattu GS, Pang S, Lin K, Huang J, Ivanov I, Li W, Suraneni MV, Tang DG. The PSA(-/lo) Prostate Cancer Cell Population Harbors Self-Renewing Long-Term Tumor-Propagating Cells that Resist Castration. Cell Stem Cell. 2012 May 4;10(5):556-69.

Abstracts, and Presentations

Li H, Diallo Krou E., Hernandez J, Ressler S., Sreekumar A., Ayala G., Rowley D., Pienta K, Palapattu G. A novel stromal induced cancer testis antigen SPANXB2 promote cancer metastasis. **Seventh Annual Prostate Cancer Program Retreat.** March 16-18, 2014, Fort Lauderdale, FL.

Li H, Diallo Krou E., Ressler S., Sreekumar A., Ayala G., Rowley D., Pienta K, Palapattu G. Cancer Testis antigen SPANXB2 and Prostate Cancer Progression. **AUA 2013 Annual Meeting** May 4-8, 2013, San Diego, CA.

Li H, Diallo Krou E., Hernandez J, Ressler S., Sreekumar A., Ayala G., Rowley D., Pienta K, Palapattu G. SPANXB2 expression increases prostate cancer aggressiveness : A new view of stromal epithelial cell interaction. **Sixth Annual Prostate Cancer Program Retreat.** March 18-20, 2013, Fort Lauderdale, FL.

Reference

1. Westbrook VA, Schoppee PD, Diekman AB et al. (2004) Genomic organization, incidence, and localization of the SPAN-x family of cancer-testis antigens in melanoma tumors and cell lines. *Clin Cancer Res*. 2004 Jan 1;10(1 Pt 1):101-12.
2. Almanzar G, Olkhanud PB, Bodogai M, et al. (2009) Sperm-derived SPANX-B is a clinically relevant tumor antigen that is expressed in human tumors and readily recognized by human CD4+ and CD8+ T cells. *Clin Cancer Res*. 2009 Mar 15;15(6):1954-63.
3. Kouprina N, Noskov VN, Pavlicek A, et al (2007) Evolutionary diversification of SPANX-N sperm protein gene structure and expression. *PLoS One.* 2007 Apr 4;2(4)
4. Barron DA, Rowley DR. (2012) The reactive stroma microenvironment and prostate cancer progression. Endocr Relat Cancer. Oct 30;19(6):R187-204.
5. Strand DW, Hayward SW. (2010) Modeling stromal-epithelial interactions in disease progression. *Discov Med*. 2010 Jun;9(49):504-11.
6. Dakhova O, Rowley D, Ittmann M. (2014) Genes upregulated in prostate cancer reactive stroma promote prostate cancer progression in vivo. *Clin Cancer Res*. 2014 Jan 1;20(1):100-9
7. Kim W, Barron DA, San Martin R, et, al,. (2014) RUNX1 is essential for mesenchymal stem cell proliferation and myofibroblast differentiation. *Proc Natl Acad Sci U S A*. 2014 Nov 18;111(46):
8. Yang F, Chen Y, Shen T, Guo D, et al,. (2014) Stromal TGF-β signaling induces AR activation in prostate cancer. *Oncotarget.* 2014 Nov 15;5(21):10854-69.
9. Yamada KM, Cukierman E. (2007) Modeling tissue morphogenesis and cancer in 3D. *Cell* 130(4):601-10,
10. Tuxhorn JA, McAlhany SJ, Yang F, et al,. (2002) Inhibition of transforming growth factor-beta activity decreases angiogenesis in a human prostate cancer-reactive stroma xenograft model. *Cancer Res.* Nov 1;62(21):6021-5.
11. Ao M, Williams K, Bhowmick NA, Hayward SW.(2006) Transforming growth factor-beta promotes invasion in tumorigenic but not in nontumorigenic human prostatic epithelial cells. *Cancer Res.* 2006 Aug 15;66(16):8007-16.
12. Basanta D, Strand DW, Lukner RB, et, al,. (2009) The role of transforming growth factor-beta-mediated tumor-stroma interactions in prostate cancer progression: an integrative approach. *Cancer Res.* Sep 1;69(17):7111-20
13. Franco OE, Jiang M, Strand DW, et al. (2011) Altered TGF-β signaling in a subpopulation of human stromal cells promotes prostatic carcinogenesis. *Cancer Res*. Feb 15;71(4):1272-81
14. Li X, Sterling JA, Fan KH, Vessella RL, et al ,. (2012) Loss of TGF-β responsiveness in prostate stromal cells alters chemokine levels and facilitates the

development of mixed osteoblastic/osteolytic bone lesions. *Mol Cancer Res*. Apr;10(4):494-503.
15. Jung HY, Fattet L, Yang J. (2014) Molecular Pathways: Linking Tumor Microenvironment to Epithelial-Mesenchymal Transition in Metastasis. *Clin Cancer Res*. 2014 Aug 8. pii: clincanres.3173.
16. Zheng Y, Basel D, Chow SO, et al,. (2014) Targeting IL-6 and RANKL signaling inhibits prostate cancer growth in bone. *Clin Exp Metastasis*. Dec;31(8):921-33.
17. Ayala GE, Muezzinoglu B, Hammerich KH, et al,. (2011) Determining prostate cancer-specific death through quantification of stromogenic carcinoma area in prostatectomy specimens. *Am J Pathol*. Jan;178(1):79-87
18. Brennen WN1, Rosen DM, Wang H, Isaacs JT, Denmeade SR. (2012) Targeting carcinoma-associated fibroblasts within the tumor stroma with a fibroblast activation protein-activated prodrug. *J Natl Cancer Inst*. Sep 5;104(17):1320-34.
19. Brennen WN1, Rosen DM, Chaux A, et al,. (2014) Pharmacokinetics and toxicology of a fibroblast activation protein (FAP)-activated prodrug in murine xenograft models of human cancer. *Prostate*. Sep;74(13):1308-19.
20. Grasso CS1, Wu YM, Robinson DR, et al,. (2012) The mutational landscape of lethal castration-resistant prostate cancer. *Nature*. Jul 12;487(7406):239-43.