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14. ABSTRACT Prostate cancer (PCa) is the most common non-skin cancer in American men and contributes to a leading number of cancer-related mortalities. With the advent of anti-androgen drugs for instance enzalutamide (Enza), most tumors that advance to metastatic castration-resistant PCa develop resistance to this therapy which is generally incurable. There is an urgent need to come up with therapeutic interventions to overcome such resistance. The mechanism of Enza resistance leading to aggressive PCa disease is not very well understood. Recently, the resistance to Enza due to deletions/mutations in the TP53 (p53) and retinoblastoma (Rb) genes have been linked to the overexpression of SOX2 gene - a reprogramming transcription factor; SOX2 knockdown in these cells restores the sensitivity to Enza. In this proposal, we identified a novel signaling cascade showing that Pim kinase, a serine threonine protein kinase that is overexpressed in PCa, control a long noncoding RNA H19 (H19), which in turn regulates the expression of SOX2 along with other stem cell genes, Oct4, Klf4, and Nanog. The knowledge gained through the studies proposed in this application during the first year have unravel mechanistic details of Pim/H19 mediated p53-/Rb- induction of stem cell genes that is responsible for the ADT. Additionally, the rational and novel therapeutic strategies proposed to overcome resistance to Enza by inhibiting the Pim/H19 axis were demonstrated to be effective in PCa cells. As a young investigator award, the career development plan and research support outlined in this proposal helped strengthen my expertise and my long-term goal of becoming an independent investigator with focus on PCa research.					
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1. Introduction

Prostate cancer (PCa) continues to cause leading number of cancer related mortalities in American men. Enzalutamide (Enza), is a chemotherapy drug for the treating PCa, that functions as androgen receptor (AR) inhibitor (1). Although, clinical trials with Enza on CRPC patients have demonstrated antitumor activity, more cases of tumors eventually develop resistance (2). This leads to an aggressive subset of the disease in the form of neuroendocrine prostate cancer (NEPC). There is an urgent need to come up with therapeutic interventions to overcome resistance that develops to androgen deprivation therapy (ADT). Enza resistance was shown to occur after cells accumulate deletions/mutations in TP53 (p53) and retinoblastoma (Rb) gene leading to overexpression of Sox2, a gene reprogramming transcription factor, and consequently making them Enza resistant (3,4). Sox2 silencing restores sensitivity to this agent (4). Since direct targeting of Sox2 is not currently clinically feasible, novel drugable targets need to be identified to restore sensitivity to Enza in P53/Rb deleted PCa. Our preliminary results identified a novel signaling cascade that mediates Sox2 overexpression in the form of Pim1→H19→Sox2. The goal of this research proposal is to overcome Enza resistance by understanding and targeting the Pim1/H19 pathway. This goal is based on the hypothesis that Pim kinase overexpression in prostate cancer induces H19, which in turn increases Sox2/Oct4/Klf4/Nanog, making these tumor cells basal-like and ADT resistant. Based on this hypothesis, we propose that targeting this pathway would restore the sensitivity to ADT, providing a novel therapeutic approach. The focus of the research carried out in the first year of this grant has been to provide novel insights into the PIM dependent signaling of H19 induced stem cell gene expression in murine prostate organoids with loss of P53/Rb, due to the importance of this pathway in development of resistance to ADT and development of NEPC phenotype. During the second year, further investigation into this pathway is hinged on finding the mechanistic underpinning of the Pim1/H19 pathway functions in P53/Rb null background to promote ADT resistance. This focus was built based on preliminary data collected during the time of application. The results obtained aim to solidify these mechanisms of PIM/H19/stem cell gene pathway regulation that mediates ADT and testing the therapeutic combination of inhibiting the PIM/H19 axis under ADT. Together, the results delineate a unique signaling cascade (year 1) with additional mechanistic insights (year 2) driven by the PIM kinases that involves a lncRNA and stem cell genes to regulate aggressive PCa growth.

2. Keywords

Prostate cancer, SOX2, PIM1, long noncoding RNA H19, Stem cells, P53/Rb loss, Resistance mechanisms, Androgen deprivation therapy.

3. Accomplishments

The cumulative accomplishments during the total period of this grant has been divided for research specific tasks and training accomplishments during this period.

Research specific tasks:

The tasks are outlined and the results have been discussed. Additionally, the importance of major findings under each task has been discussed after each observation. Due to Covid-19 and consequent delay in the timeline, the tasks outlined in Specific Aim 2 Major tasks 2 and 3 were impacted. The order of analysis has been tried to keep consistent with the statement of work to enhance the efficacy and validity of the results within the scope and direction of the project. Major tasks completed during year one and two have been described in detail. The experiments have been performed in a logical sequence and have led to identifying a *novel pathway controlled and mediated by the PIM kinases and long non-coding RNA H19 whose inhibition could impact clinical outcomes in aggressive prostate cancer disease. PIM kinase induction increases the lncRNA H19 and this in turn regulates stem cell genes, including the transcription factor SOX2 which plays a role in controlling the response to anti-androgen therapy. Additionally, we demonstrate that the combination of a PIM inhibitor and H19 knockdown can sensitize prostate cancer cells to Enza while also reducing the tumor forming capacity of the highly aggressive NEPC. In the second year, our results uncovered the mechanistic aspects of this PIM kinase/H19 mediated effect. Results suggested microRNA 675 (miR-675), a miRNA encoded within the H19 sequence and shown to be causative of some of its phenotypic effects, is not responsible to drive the ADT resistance mechanism. Additionally, we uncovered a link between H19 and DNA methylation on specific gene promoters that then modulate the expression of genes responsible for aggressive subset of prostate cancer, NEPC, that is resistant to ADT.* Overall, the research accomplishments during the award period have uncovered a novel pathway regulated by PIM kinase/long noncoding RNA H19- in controlling the highly aggressive NEPC phenotype exploring which could lead to potential therapeutic strategies to counter this lethal disease.

Specific Aim 1: Major task 1: Culture genetically manipulated prostate organoids from GEM mouse models

The task was designed to generate models to test the hypothesis that PIM1/H19 mediates the $P53^{-/-}/Rb^{-/-}$ induction of stem cell genes. During the award period, I was able to successfully isolate the prostate from Wild type, $P53^{flox/flox}/Rb^{flox/flox}$, $P53^{flox/flox}/Rb^{flox/flox}$ x probasin cre mice (**Figure 1a**). The prostate tissues were then individually dissociated using collagenase and trypsin via previously established protocols (5) and then cultured in the EGF/Noggin/R-Spondin media in

growth factor reduced Matrigel for growth of organoids (Subtask 1) (**Figure 1b**). The organoids were then genetically manipulated with the lentiviral system (6) to cause knockdown/loss of p53 and Rb gene expression as well as knockdown of H19. All studies involving the use of animals were approved by and conducted in accordance with the guidelines of the Institutional Animal Care and Use Committees at the University of Arizona Cancer Center. The murine organoids were replenished with fresh media every 3–4 days during organoid growth. Dense cultures with organoids ranging in size from 200 to 500µm were passaged weekly. Organoid cultures were cryopreserved using Bambanker (Gibco) at –80°C.

Lentiviral particle production and infection was performed as described previously (Tiscornia et al., 2006). The plasmids used for knockdown of human H19 in LNCaP was performed using the lentiviral plasmids pLenti-siH19-GFP (Abm, #i009382) and pLenti-scrambled siRNA-GFP (Abm, #LV015-G) as a control. These siH19 plasmids allow for direct non-viral plasmid transfection for immediate expression (siH19) and also package into lentiviral particles for high efficiency transduction and stably integrated expression (shH19). Overexpression of human H19 in LNCaP cells was performed using pLenti-GIII-CMV-H19-GFP-2A-Puro (Abm, # LV178008). For H19 knockdown in mouse organoids, plasmid GIPZ Mouse H19 shRNA purchased from Dharmacon (RMM4431), and for Cre recombinase expression, the plasmid FUGW-Cre (Addgene) was used. After the lentivirus of the indicated gene manipulation was produced, we proceeded with the infection/transduction of either adherent PCa cells-LNCaP and/or murine prostate organoids. infection of adherent PCa cells- LNCaP, 10⁶ cells/well were seeded in six-well plates and infected with concentrated lentiviral particles 1 day after seeding. For lentiviral transduction, organoids were pre-incubated for at least 48h with regular organoid media supplemented with Wnt-3a and Rho kinase inhibitor (6). After 48h, organoids were dissociated with TrypLE and spinoculated with lentiviral particles along with polybrene (8µM final concentration) at 600g for 1h at 32°C. After incubating organoid cells at 37°C for 3h, the cells were replated in Matrigel in ENR media without lentivirus and allowed to grow for several days. Infected organoids were monitored by fluorescence microscope and successful transductions were indicated by the green fluorescence (for CRE and shH19 conditions) and red fluorescence (for Control and PIM1 conditions) as shown in **Figure 2a** and **Figure 2b** respectively.

To confirm the genetic manipulations caused due to the lentiviral transductions, quantitative PCR was performed. Briefly, total RNA was isolated from organoids using TRIzol reagent (Invitrogen, 15596–018). 1µg total RNA were reverse transcribed by using i-Script cDNA Synthesis System kit (Biorad, 1708891). To measure gene expression, Real time PCR was performed using SsoAdvanced™ Universal SYBR® Green Supermix (Biorad, 1725271), following the

manufacturer's protocol. Expression level of each transcript was quantified by using Bio-Rad CFX96 Real-Time PCR Detection System. Quantitative real-time PCR (qPCR) assay were performed using the primers for H19, PIM1, KLF4, SOX2 and OCT4. 18S and HPRT were used as endogenous controls. The qPCR results (**Figure 2c-f**) demonstrated the desired genetic manipulations.

Combined deletion and consequent knockout of p53 and Rb gene expression induced by Cre-recombinase infection (p53/Rb double knockout or DKO) results in a neuroendocrine (NE) like phenotype that has been shown to be responsible for developing ADT resistance. To confirm that the DKO organoids are indeed NE like we performed Western blots on protein extracted from organoids (Control, Cre and Cre+shH19) (**Figure 3**). Results of the western blot indicates that while the DKO organoids do have a higher protein expression of NE markers Neuron specific enolase (Nse), Ezh2, Synaptophysin (Syn) and Sox2, the knockdown of H19 in these DKO organoids reduced these NE marker levels to return to normal control level. These results indicate that H19 is responsible for maintaining the aggressive variant of PCa that is ADT resistant and could potentially play a role in NEPC development.

These organoids were further validated using Hematoxylin and eosin (H&e) staining and Ki67 antibody staining. Immunohistochemistry (IHC) for Ki67 was performed using Leica Bond RXM system (Leica). Formalin fixed mouse organoid samples were deparaffinized with xylene/ethanol then antigen retrieval performed using Citrate Retrieval Buffer (AR9961, Leica), heat induced at pH 6.0 at 98°C for 20 mins. After washing, slides were incubated with primary antibody 15 mins at RT. Post primary antibody was applied as needed (HRP conjugated IgG, DS9800, Leica) for 8 mins. A post polymer signal was amplified. DAB staining kit (DS9800, Leica) was used for final detection, counterstained with hematoxylin (5 mins), and mounted in non-aqueous solution (Leica micromount, 3801730). The images were captured using binocular Leica light microscope (Leica™ DM2500) at bright field and CCD color video camera (Leica DFC320) attached to a computer system.

Results (**Figure 4**) indicated that control organoid cultures grow slowly in predominant luminal glandular structure (A). P53/Rb DKO organoids showed solid not cystic growth pattern with abundant cells (arrows) positive for Ki67staining. Inhibition of H19 by shH19 moves the organoid back to predominantly luminal glandular structure with fewer cells (arrows) positive for Ki67.

*Thus, through Task 1 in Year 1 and 2, we extensively **validated** (via qPCR, Western blots and IHC) the successfully established organoid cultures from mouse prostate, which serve as better cell culture models that closely recapitulate prostate histology. We also induced the desired*

genetic manipulations and confirmed those changes and these organoid models will help us dissect the role of PIM1/H19 axis in Enzalutamide resistance via the loss of P53/Rb. Moreover, these DKO organoids (P53^{flox/flox} /Rb^{flox/flox} + Cre) are models of NEPC like culture system and could potentially be used for studies of NEPC biology.

Specific Aim 1: Major task 2: Mycoplasma testing of mouse organoid cultures

Mycoplasma testing- Regular testing of mycoplasma contamination was performed in the cell lines and organoids using MycoAlert™ Mycoplasma Detection Kit (LT07-118, Lonza) and only mycoplasma free cells were used for experimentation.

Specific Aim 1: Major task 3: Cell line authentication of mouse organoid cultures and human cell lines

To maintain the authenticity and purity of cell lines used for a long time in culture conditions, STR cell line authentication was performed. Additionally, we are not using a cell line either human or murine for more than 3 months continuously to mix up the cell identity. We take out cryopreserved early passage cell lines for use after that time period. Keeping the cell lines for shorter span of times does not affect the purity and authenticity of the cell lines.

Specific Aim 1: Major task 4: Test whether Pim/H19 mediates the effect of p53/RB loss on increasing Sox2 in prostate stem cells

Subtasks 1 and 2: This task was designed to test whether PIM/H19 mediate the effect of P53/Rb loss on increasing SOX2 in prostate stem cells.

To test this, organoids generated in Specific aim 1, Task 1 were utilized. To evaluate whether changes in the levels of P53 and Rb1 modulate H19 expression, organoids derived from Trp53^{flox/flox}/Rb1^{flox/flox} mouse prostates were transduced with lentivirus expressing Cre recombinase (Cre-GFP) to generate double-knockout organoids. Organoids transduced with control lentivirus (EV-GFP) were used as control. As demonstrated by qPCR results these DKO organoids demonstrate markedly enhanced expression of H19, as well as the induction of stem cell transcripts (Klf4, Oct4 and Sox2) (**Figure 5a**). To test whether the expression of stem cell genes is through H19 in P53/Rb knockdown cells, we decided to knockdown H19 using shRNA in the CRE organoids. qPCR results demonstrated that the knockdown of H19 in CRE organoids abrogated the upregulation of stem cell genes (SOX2/OCT4/KLF4/Nanog) (**Figure 5a**) indicating that the lineage plasticity marker SOX2 is indeed controlled by H19 in these P53/Rb double knockout organoids. These findings were confirmed at the protein expression level (**Figure 5b**)

with the help of western blots of the proteins extracted from the same organoids used for RNA extraction.

We next tested the relevance of these findings at the phenotypic level by testing the effects of the H19 knockdown on proliferation and invasion of CRE organoids. Aggressive prostate tumors progressing by escaping androgen deprivation therapy (ADT) are characterized by highly proliferative cells with increased metastatic potential (7). To assess the effect of H19 knockdown on the growth of these aggressive prostate tumors, murine organoid (CRE) with or without stable knockdown of H19 was evaluated for organoid growth. While CRE organoids exhibited enhanced proliferation, H19 knockdown significantly inhibited the growth of these ($p=7 \times 10^{-9}$) (**Figure 5c**), demonstrating the essential role of H19 in controlling proliferation in aggressive tumors with P53/Rb loss that could potentially lead to ADT resistance. To examine the ability of H19 to modulate tumor cell invasion, dissociated mouse Trp53/Rb1 DKO organoid cells were placed in a transwell with Fluoroblock inserts and fluorescence on the bottom of transwell was measured after 5 days (**Figure 5d**). In this model, while the loss of P53/Rb enhanced migration/invasion of murine organoids, knockdown of H19 was able to inhibit their invasive capabilities.

Together this data suggests that a decrease in H19 affects the growth and invasive potential that might be responsible for ADT resistance upon the loss of P53/Rb which are shown to be mediated at least in part by H19 regulation of SOX2 and other stem cell genes.

To evaluate the effect of PIM1 overexpression on growth of normal stem cells, we isolated wild type mouse prostate organoids that have been shown to possess stem cell like characteristics (5). We utilized the PIM1 overexpressing murine organoids as developed in Task1. First, we wanted to observe the phenotypic effects of PIM1 overexpression on organoid proliferation. PIM1 overexpressing and EV control murine prostate organoids were dissociated with TrypLE (Invitrogen) into tiny cell clusters, plated (5000 cell clusters/well) for 4-6 days. A real-time imaging system (Incucyte™, Essen Bio) was used to measure organoid growth. Images were captured every 12h and results were plotted as the percent average organoid growth versus time. PIM1 overexpression in these organoids caused a significant increase in proliferation of these cells (**Figure 6a**; PIM1-RFP vs EV-RFP, $p<0.001$). Next, we evaluated the gene expression of stem cell genes and H19 with PIM1 overexpression in these mouse organoids. qPCR results demonstrated that PIM1 overexpression induced increases in H19 as well as the stem cell genes Klf4, Oct-4, and Sox2 (**Figure 6b**).

Next, we evaluated the role of miR-675 in Pim1/H19 mediated regulation of stem cell gene expression.

MiR-675 has been implicated in causing the phenotypic effects of long non-coding RNA H19 in embryonic stem cell systems and cancer models. This task was designed to elucidate the involvement of miR-675 in H19 mediated upregulation of stem cell genes particularly Sox2.

To test this, we overexpressed miR-675 encoding fragment of H19 in P53/Rb – Cre murine organoids and investigated the Sox2 expression levels.

For expression of H19 fragments in mouse organoids we used a retroviral vector expressing full length and Middle H19 fragment (741-1407) encoding miR-675 fragments of H19 (8). pMSCV retro was used as a control. Retrovirus encoding these fragments was produced and murine prostate organoids were transduced with the control and H19 fragments. After, stable transduction of these organoids post 2 passages, we extracted the RNA and qPCR was performed.

For Taqman based assays for miR-675, RNA was extracted and 1µg total RNA were reverse transcribed by using Taqman MicroRNA reverse transcription kit (Life technologies, Invitrogen) following the manufacturer's protocol. Taqman primers for murine miR-675-6p, miR-675-3p and endogenous control U6 snRNA were used. qPCR was performed using Taqman Fast advanced master mix (Life technologies, Invitrogen) following the manufacturers protocol.

qPCR results (**Figure 7c**) demonstrated that while miR-675 was indeed overexpressed in the organoids with middle H19 fragment, there was no change in Sox2 expression (**Figure 7b**).

RNU6 used as an endogenous control demonstrated no change in any condition (**Figure 7a**).

These results indicate that there is no potential involvement of miR-675 in H19 mediated overexpression of stem cell genes to cause ADT resistance. Hence, there could be other mediators responsible for this mechanism.

Thus, experiments conducted in Specific Aim 1 Task 4, help to establish a novel signaling cascade connecting PIM1, H19, and stem cell genes, suggesting that the PIM1 induction of stem cell genes may occur with the activity of H19. We also prove that the phenotypic effects of increased proliferation and invasion is mediated at least in part by the regulation of SOX2 by H19 (with no potential involvement of miR-675) in the P53/Rb double knockout organoids and PIM1 in normal organoids.

Specific Aim 1: Major task 5: Mechanism by which p53/Rb and Pim-1 protein kinases regulate H19 levels

H19 is encoded by the IGF2/H19-imprinted gene cluster located on human chromosome 11p15 (9), and its transcription is controlled by differentially methylated regions (DMRs) of the upstream DNA called 'imprinting control regions' (ICR). When hypomethylated, four specific DMRs

upstream of H19 start site bind the transcription factor CCCTC-binding factor, which acts as insulator preventing IGF2 promoter activation and enhancing H19 transcription (10). The goal of this task is to investigate whether PIM1 induces epigenetic changes in DNA methylation in the control regions of H19, suggesting a mechanism by which it regulates the transcription of this lncRNA.

A possible mechanism by which PIM regulates H19 levels is by modulation of the H19 DMR methylation. There is a striking difference in the methylation state of H19 between PCa and benign prostate hyperplasia (BPH). Eight percent of the CpGs in the DMRs and in the ICRs are methylated in BPH, while only 41% of CpGs are methylated in PCa (11). For bisulphite sequencing, Genomic DNA from informative samples were treated with bisulfite (EpiTect Plus DNA Bisulfite Kit; Qiagen, Hilden, Germany; Cat No./ID: 59124) to convert unmethylated cytosines to uracils, whereas methylated cytosines are unaffected according to the manufacturer's protocol. Bisulfite-treated DNA was subsequently amplified using the forward 5'-TGGGTATTTTTGGAGGTTTTTTT-3' and reverse 5'-TCCCATAAATATCCTATTCCCA 3' primers.

Bisulfite sequencing demonstrates that while the DMR is methylated in 100% of the SUP-T1 clones, PIM1-overexpressing SUP-T1 cells demonstrated a demethylation of the DMR in 30% of the clones analyzed, reflecting the loss of imprinting of H19 (**Figure 8**). This result suggests a potential mechanism for the suppression of H19 expression in SUP-T1 cells and its potential regulation by PIM1 kinase.

The goal of subtask 2 was to examine changes in global methylation pattern on H19 knockdown upon the loss of P53/Rb in organoids by Reduced Representation Bisulphite sequencing. This is an extensive test and requires rigorous bioinformatic analysis of the DNA samples. Keeping the sensitivity of this experiment in mind and the large amount of data that is generated from such a gene analysis on global genomic level, we decided to perform this eRRBS analysis on DNA extracted from NEPC organoid line, OWCM155 (12). This is also a better choice than using the p53/Rb DKO organoids since we have already demonstrated that the p53/Rb DKO organoids recapitulate NE biology due to which they are resistant to ADT. Hence, to make the study more useful to the scientific community in the study of ADT resistance in PCa, we decided to perform this eRRBS analysis on DNA extracted from NEPC organoid line, OWCM155, with and without H19 knockdown (shH19). We collected the organoids and extracted DNA from the 2 conditions (control and shH19). The eRRBS was performed on the DNA samples as follows. Briefly, bisulfite

reads were aligned to the bisulfite-converted hg19 reference genome using Bismark. All samples had bisulfite conversion rates of >99.7%. Percent methylation scores of bisulfite converted cytosines (T's, unmethylated C's) and non-converted Cs (methylated C's) for CpG cytosine methylation were analyzed further using MethylKit (v. 1.11) and R (v. 3.4). Methylation changes for each organoid (OWCM-155-shH19 versus OWCM-155-Scr) was analyzed separately. Genome wide differential methylation was calculated between control and shH19 samples. Differentially methylated scores with a cutoff ± 25 were chosen as significant. GrCH37/hg19 genome and hg19 CpG islands bed files were downloaded from UCSC and were used as reference to annotate the differentially methylated regions. ± 2 -kb upstream and downstream of transcription sites was searched for methylated regions. Custom R scripts were used for file processing and summarizing the output. Bed files were visualized and analyzed using Integrated Genome Viewer (IGV v3.5). Functional association analysis was done utilizing David and networks were generated using enrich map in Cytoscape.

Results from the eRRBS (**Figure 9**) indicated that H19 knockdown induces alteration in genome wide DNA methylation resulting in corresponding gene expression changes. Epigenomic modifications have been shown to play a critical role in development of NEPC phenotype. We investigated the effect of H19 knockdown on genome wide methylation status in NEPC organoids OWCM-155 by Enhanced Reduced Representation Bisulfite Sequencing (eRBBS) to detect quantitative base pair changes. Differential methylation genome wide analysis for shH19 versus control OWCM-155 showed methylation changes for 8540 genes (59982 sites; adjusted p-value<0.01) with 3061 genes (12,766 sites) hyper-methylated and 5479 genes (47,216 sites) hypo-methylated (**Figure 9a**). 52% of the promoters were hypomethylated versus 27% hypermethylated (**Figure 9b**). We found 2592 protein coding genes hypermethylated while 3963 hypomethylated. Functional association analysis with gene ontology of these changes in methylation status reveals that these genes are enriched in clusters (p-value <0.001) belonging to cell migration and generation of neurons are enriched in hypermethylation; while those with pattern specification processes (**Figure 9c**). Further, gene expression analysis was performed on RNA derived from the same organoid conditions evaluated for Errbs, confirmed that changes in methylation corresponded with changes in gene expression with decreased expression for newly hypermethylated genes e.g. P2RY6, SOCS3, or increased expression for induced hypomethylated genes RGS7, ERG, CCND2, CDH4 (**Figure 9d**). These results strongly point to the potential role of H19 in regulating the methylation of genes associated with the aggressive and ADT resistant prostate cancer phenotype.

The goal of Subtask 3 was to determine changes in CTCF binding to H19 in Pim-overexpressing organoids or p53/Rb deletion. Studies have shown that H19 is encoded by the IGF2/H19-imprinted gene cluster located on human chromosome 11p15 (9), and its transcription is controlled by differentially methylated regions (DMRs) of the upstream DNA called 'imprinting control regions' (ICR). When hypomethylated, four specific DMRs upstream of H19 start site bind the transcription factor CCCTC-binding factor (CTCF binding factor), which acts as insulator preventing IGF2 promoter activation and enhancing H19 transcription (10). So, changes in CTCF binding on H19 promoter upon Pim1 Overexpression will potentially lead to H19 and IGF2 expression being reciprocally changing with Pim1 overexpression. To test this, we extracted RNA from PC-3 cells with and without constitutive Pim1 overexpression. qPCR analysis in prostate cancer cells, PC-3, demonstrated no changes in IGF2 RNA expression when PIM1 kinase was transfected in PC3 cells (**Figure 10**), even though concomitant changes in H19 expression were observed. Results from this task suggests that Pim1 overexpression does not modulate CTCF binding otherwise IGF2 expression would have decreased while upregulating H19 expression.

Taken together, results from the Major task 5 of specific aim 1 indicated the following:

- *Pim1 overexpression potentially hypomethylates the H19 differentially methylated region (DMR) thereby increasing the expression of the later.*
- *H19 modulates the methylation of CpG bases located on promoter sites of specific genes responsible of conferring resistance to ADT and thus causing aggressive PCa disease.*
- *CTCF binding potentially remains the same upon Pim1 overexpression since IGF2 levels remain unchanged in Pim1 overexpression condition.*

Specific Aim 2: Major task 1: Evaluate whether Pim inhibitors can overcome Enza resistance in stem cell derived tumors in vitro

In the tasks outlined under specific aim 2, we majorly wanted to investigate whether inhibition of PIM/H19 axis was sufficient to overcome Enza resistance.

To further this investigation, it was imperative that we establish that H19 is modulated by Androgen receptor (AR) signaling. We performed a simple experiment where in normal murine organoids, which grow under dihydrotestosterone (DHT- an AR agonist that upregulated androgen signaling), a removal of DHT was performed and qPCR was done on the murine organoids cultured with or without DHT. qPCR results demonstrated that removal of DHT from the organoid media induced a 7 to 10-fold upregulation of H19 expression (**Figure 11a**).

This data suggest that androgen signaling plays a role in regulating H19 expression, and that the blockade

of AR induces H19, potentially enhancing the stem cells genes inducing lineage plasticity. Patients diagnosed with advanced PCa initially respond to ADT but ultimately develop hormone-refractory disease that leads to death. Recent evidence suggests that increased expression of the reprogramming stem cell transcription factor SOX2 makes PCa cells resistant to hormone blockade by the anti-androgen Enza (4). To evaluate if PIM inhibitors could overcome Enza resistance in vitro, we used the organoids as developed in Specific aim1, Task1. Control (EV), P53/Rb knockout (Cre) organoids with or without H19 knockdown (shH19) were dissociated with TrypLE (Invitrogen) into tiny cell clusters, plated (5000 cell clusters/well) and treated under desired conditions for 4-6 days. The drug treatment conditions were as follows, Enza (5 μ M, 10 μ M) and PIM inhibitor (PIM447, 3 μ M). DMSO was used for control drug treatment. A real-time imaging system (Incucyte) which is more accurate than the MTS assays, was used to measure organoid growth. Images were captured every 12h. The images shown in Figure 5b are a compilation of Pim inhibitor and Enza treatment either individually or in combination (as seen on day 5) when added to Control EV and CRE organoids with or without H19 knockdown (shH19). As observed from the images of multiple replicates of the each treatment condition- Enza treatment did not appear to modulate organoid growth in this system. Although the organoids exhibited the previously established phenotypic effects of the genetic manipulations (for instance, CRE organoids exhibited increased proliferation than control and shH19 organoids had reduced proliferation). The negative effect of Enzalutamide on murine organoids (**Figure 11b**) was surprising since we showed previously that H19 is modulated by AR signaling. We concluded that since murine organoids require DHT this system is not an ideal one to show the treatment with Enza. Alternatively, we decided to check for drug cytotoxicity in LNCaP cells.

LNCaP cells are androgen responsive and after TP53/RB1 knockdown become less sensitive to growth inhibition by AR antagonist ENZA than the parental cell line (3). However, when transduced with shH19 these prostate tumor cells regain sensitivity to hormone blockade (**Figure 11c**) and demonstrate growth inhibition by ENZA. Conversely, ENZA (2 μ M or 5 μ M) treatment for 5 days inhibited the growth of control LNCaP cells. *Together this data highlights the importance of H19 in regulating the sensitivity of prostate cancer cells to ADT.*

To investigate the role of PIM kinases in ADT, we analyzed the effect of Enza treatment on PIM1 overexpressing androgen-responsive PCa cell line LNCaP cells. For this, LNCaP cells were seeded into 96-well plates at a density of 5,000 cells per well and were allowed to grow under desired conditions. At the end of each experiment, cell viability was measured using XTT cell proliferation assay (Trevigen, Cat # 4891-025-K) following the manufacturer's protocol. Briefly, the XTT reagent was added to cell culture (1:2 dilution) and incubated for 4h at 37°C and 5%

CO2. The absorbance of the colored formazan product was measured at 450 nm. The results of this cell viability assay demonstrated that while Enza treatment (48h, 10 μ M) was cytotoxic to the empty vector transduced control LNCaP cells (EV), overexpression of PIM1 caused resistance to Enza treatment (**Figure 12a**). This result is consistent with our finding that PIM kinase activity stimulates increases in SOX2 (**Figure 6a**). To explore this further, we examined the growth of LNCaP with control vector transduction (LNCaP-EV) and H19 overexpression (LNCaP-H19), followed by treatment with increasing concentrations of PIM447 with or without Enza (2 μ M, 5 μ M) treatment for 72h (**Figure 12b-c**). The PIM inhibitor treatment significantly sensitized the control LNCaP cells to growth inhibition by Enza (**Figure 12b**). Overexpression of H19 in LNCaP cells blocked the effect of PIM kinase inhibitor treatment (**Figure 12c**). Additionally, treatment of LNCaP cells (**Figure 12d**) with PIM inhibitor decreased H19 levels indicating that Pim inhibitor works through suppressing H19 to overcome Enza resistance. *These results suggest that the PIM-mediated control of H19 levels modulates Enza sensitivity in LNCaP cells. Overall, the experiments conducted for Specific Aim 2 Task 1 Year 1 demonstrate in vitro that the level of lncRNA H19 is responsible for Androgen deprivation therapy in cell culture and murine organoid models and inhibiting this axis will restore the sensitivity to Enza thereby treating this highly aggressive prostate tumor.*

Specific Aim 2: Major task 2 and 3: Through these major tasks we sought to determine the efficacy of H19 knockdown and PIM kinase inhibition in overcoming Enza resistance *in vivo*. During the pursuit of these major tasks, we hit several roadblocks: Firstly, the number of organoids was not enough to generate tumors *in vivo* so we had to repeat the experiment a few times. Secondly, the tumors if developed took longer to form and were uneven in size, to test the various inhibitors. Finally, due to the onset of Covid-19 we could not find enough time to complete the experiment in its proposed form and decided to reapproach this issue in a slightly modified way.

P53/Rb DKO organoids are models of NEPC and injecting the number of organoids in FVB mice with similar background was either not generating tumors at our hands or resulted in smaller tumors. Of the one time the experiment demonstrated positive result (**Figure 13**), we found reduction in tumor growth when the DKO organoids had H19 knockdown, as demonstrated by GFP imaging post necropsy.

To have more clinical relevance and to understand the role of H19 in tumor growth further, we decided to inject OWCM155 organoids that are NEPC organoids and Enza resistant. We injected the OWCM-155- with and without H19 knockdown (shH19) into NSG mice. Organoids transduced with scramble shRNA were used as control (shSCR). We observed reduced tumor weight post

experiment (**Figure 14b**). Additionally, the mRNA levels of NEPC and stem cell gene markers were significantly reduced in the H19 group as compared to the control injected group (**Figure 14b**). We have demonstrated through results published in the Molecular Oncology that PIM kinase inhibition is synergistic to ENZA in reducing the proliferation of these organoids in vitro. Hence, we anticipate that these results will hold in vivo as well.

Thus through these studies for Specific aim 2, Major task 2 and 3, we find that *H19 is critical for growth of metastatic prostate cancer and knockdown can overcome Enza resistant in NEPC organoids*.

Specific Aim 2: Major task 4: Writing the manuscript(s) derived from results of the project

Under this task of writing manuscripts derived from the results of the project generated through this grant.

Through the experiments conducted in year 1 and 2 of the research supported by this grant, I have been able to prepare 2 manuscripts as the lead first author presenting the results of the findings under the guidance with my mentor Dr. Andrew Kraft. Out of the 2 manuscripts, one is published after rigorous peer review in the open access journal “Molecular Oncology”. The results presented in this publication are all conducted under in vitro conditions and the manuscript is titled “PIM protein kinases regulate the level of the long noncoding RNA H19 to control stem cell gene transcription and modulate tumor growth”.

The second manuscript with more extensive mechanistic details was accepted “in principle” (pending formatting requests) by the editor after rigorous peer review for publication in the journal “Nature Communications”. Please see Appendix B for a copy of the Molecular oncology article for further details. We will not be able to share the Nature Communications manuscript until published, however a copy of the accepted manuscript will be available until the time of publication for the scientific community upon request.

Training specific tasks:

Major task 1: Training and educational development in prostate cancer research

I am continuing to further my training and educational development in the field of prostate cancer research and have accomplished this task through the following.

Oral/Poster presentation:

- Invited Oral presentation at **Prostate Cancer Working Group Annual Meeting: New Strategies for Lethal Disease** held at University of Arizona Cancer center on June 19, 2019. Title of presentation: “Role of lncRNA H19 in Neuroendocrine Prostate Cancer”.
- Presented this research from this project at **Association of American Cancer research (AACR) Annual meeting 2019**, Atlanta, Georgia. The presentation was titled “Role of long noncoding RNA H19 in driving enzalutamide resistant neuroendocrine prostate cancer” and was published in Cancer Research as a conference paper (DOI: 10.1158/1538-7445.AM2019-3017 Published July 2019).
- Presented this research from this project at **27th Annual Prostate Cancer Foundation Virtual Scientific Retreat, 20th -23rd October 2020**. This conference is one of the most important scientific conferences in the world on the biology and treatment of prostate cancer. The presentation was titled “The long noncoding RNA H19 is an important epigenetic regulator of tumor plasticity in neuroendocrine prostate cancer”. Proceeding from the PCF retreat are not published in any journal but are available on their website (www.pcf.org).
- Submitted abstract for poster presentation in AACR- Advances in prostate cancer research TBH March 2020 in Denver, Colorado. The conference was later postponed/cancelled due to COVID-19. The abstract was titled “The long noncoding RNA H19 is an important epigenetic regulator of tumor plasticity in neuroendocrine prostate cancer”.

Workshops:

- ☐ Attended the Organoid workshop at AACR Annual meeting 2019.
- ☐ Attended Personal development workshop at AACR annual meeting 2019.

While attending the conference enhance my skills and understanding in the field of prostate cancer, I continue to attend weekly lab meetings and Journal club from our lab (Subtask 3) where I provide updates on my progress in Research. Also, I attend monthly PCa group meeting at the UACC to understand and be abreast of the PCa research going on at the department.

Plan for the next reporting period to accomplish the goals: *Nothing to report*

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Supporting data

Figures 1-14

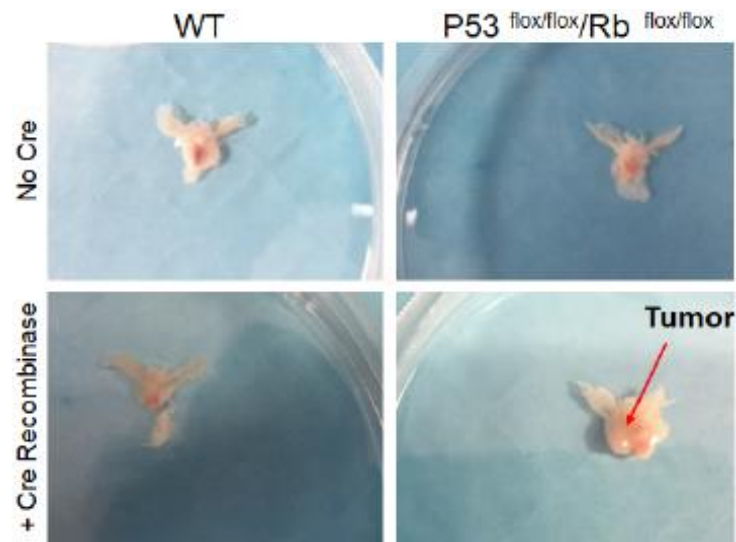


Figure 1a: Representative images of prostate tissue isolated from different mice as indicated. Note the Tumor development in the P53/Rb mouse induced to express Cre recombinase thus leading to a loss of P53/Rb. These prostate tissues were further dissociated to develop murine prostate organoids.

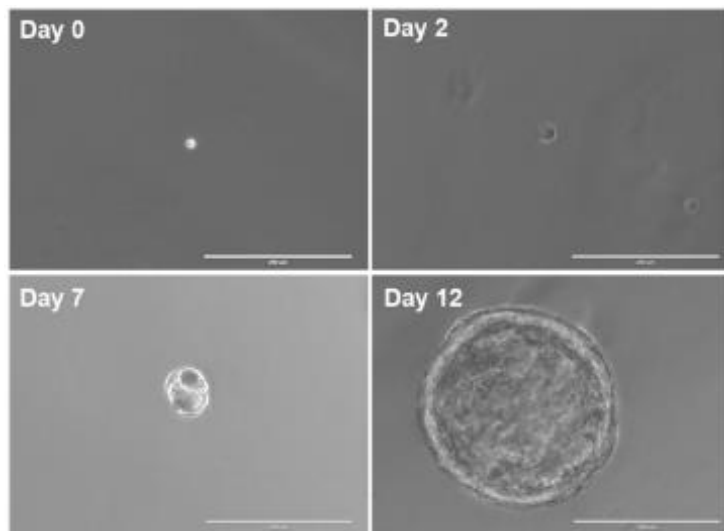


Figure 1b: Phase contrast images demonstrating progression of growth of a representative murine prostate organoid from a single cell. Scale bar: 200 μ m.

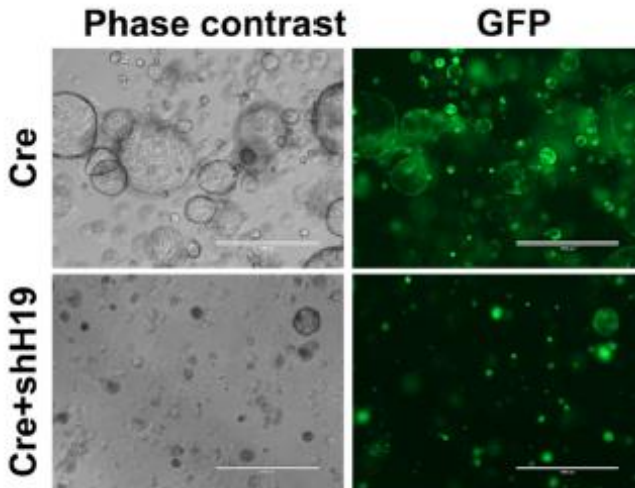


Figure 2a: Representative images of murine prostate organoids derived from P53 flox/flox/RB flox/flox. The organoid were then transduced with lentivirus expressing Cre recombinase (Cre) and another lentivirus expressing shRNA to H19 (shH19). Successful transductions are indicated by the GFP fluorescence due to lentiviral transduction. Scale bar: 1000 μ m.

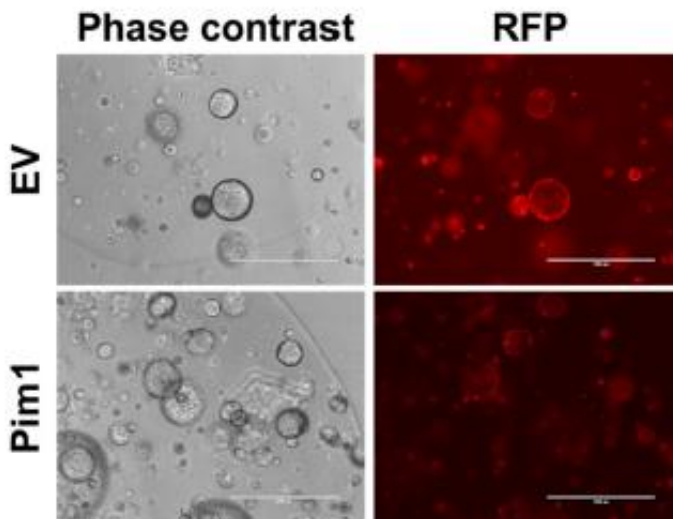


Figure 2b: Representative images of murine prostate organoids derived from Wild type mice. The organoid were then transduced with lentivirus overexpressing PIM1 (PIM1) and another lentivirus expressing empty vector used as a control (EV). Successful transductions are indicated by the RFP fluorescence due to lentiviral transduction. Scale bar: 1000 μ m.

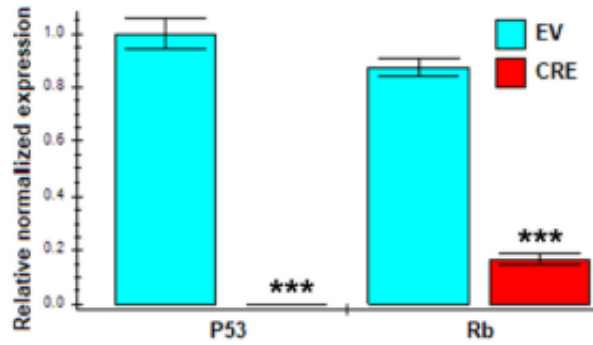


Figure 2c: Relative RNA expression indicating the loss of P53 and Rb in P53 flox/flox/RB flox/flox murine prostate organoids transduced with lentivirus expressing Cre recombinase (CRE). The empty vector (EV) transduced organoid was used as a control. Values are mean $\pm$ S.D. n=3, *** indicates p values<0.001.

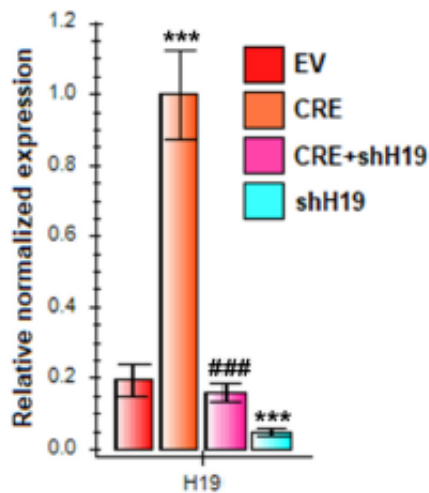


Figure 2d: Relative RNA expression indicating the knockdown of H19 in P53 flox/flox/RB flox/flox murine prostate organoids both in control organoids (shH19) and CRE organoids (CRE+shH19) after lentiviral transduction with shH19. Positive controls include Cre recombinase (CRE) expressing organoids that show enhanced expression of H19.. The empty vector (EV) transduced organoid was used as a control. Values are mean $\pm$ S.D. n=3, *** indicates p values<0.001 vs EV, ### indicates p values<0.001 vs CRE.

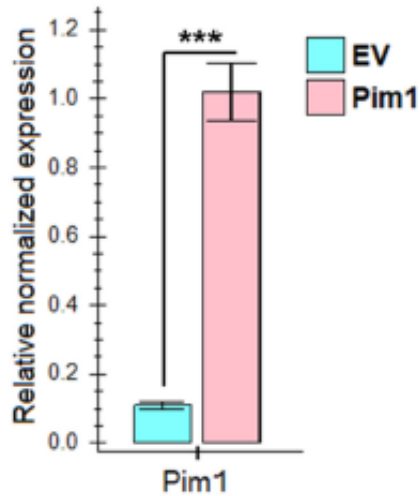


Figure 2e: Relative RNA expression indicating the overexpression of Pim1 in Wild type murine prostate organoids transduced with lentivirus expressing Pim1 (Pim1). The empty vector (EV) transduced organoid was used as a control. Values are mean $\pm$ S.D. n=3, *** indicates p values<0.001.

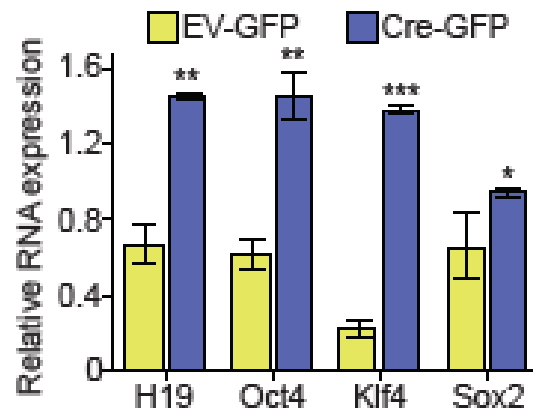


Figure 2f: Relative RNA expression of H19 and stem cell genes (Sox2, Oct4 and Klf4) after *Trp53/Rb1* DKO induced by lentiviral transduction of Cre-recombinase in *Trp53^{fllox/fllox}/Rb1^{fllox/fllox}* mouse prostate organoids (Cre-GFP) vs control lentiviral transduced mouse organoids (EV-GFP). Data are the mean $\pm$ SEM, n=3. Data with statistical significance are as indicated: *, p<0.05; **, p<0.01; ***, p<0.001 as compared to respective controls.

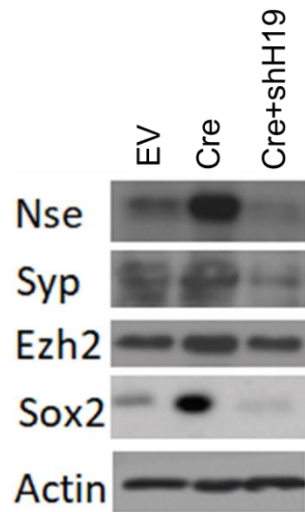


Figure 3: Western blot of representative neuroendocrine genes in the $\text{Trp53}^{\text{lox/lox}}/\text{Rb1}^{\text{lox/lox}}$ mouse prostate organoids transduced with EV, Cre, Cre+shH19.

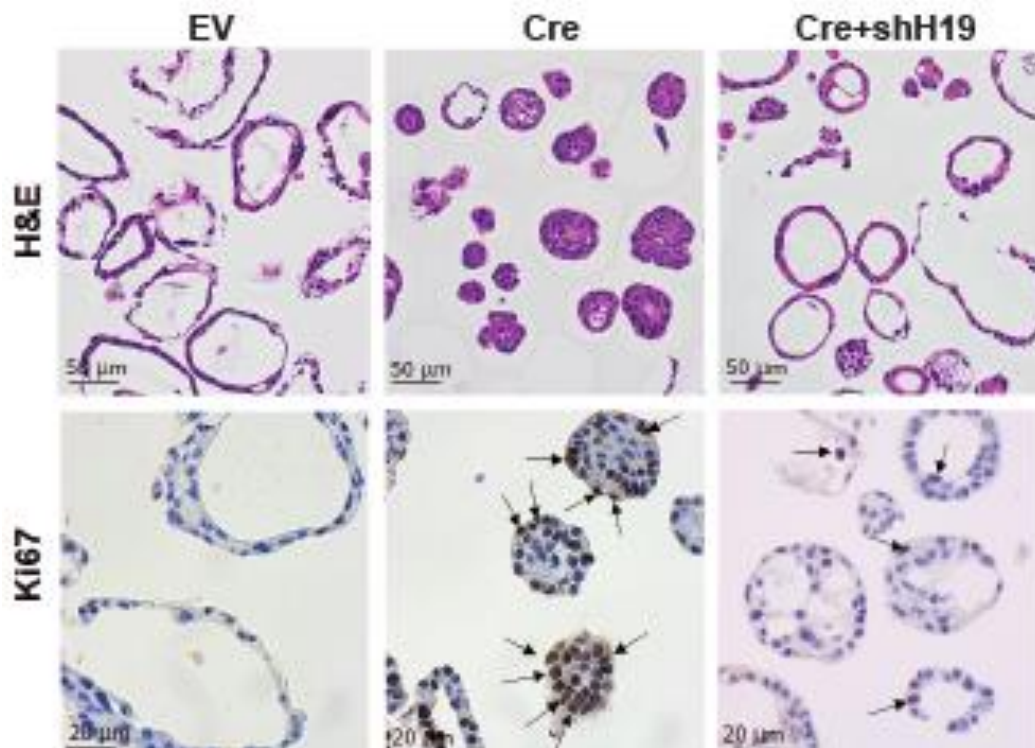


Figure 4: Representative images showing H&E staining (scalebar 50 μm) and immunostaining (scalebar 20 μm) for Ki67 in the same organoids from J. Arrows represent areas of intensive staining indicative of high protein expression.

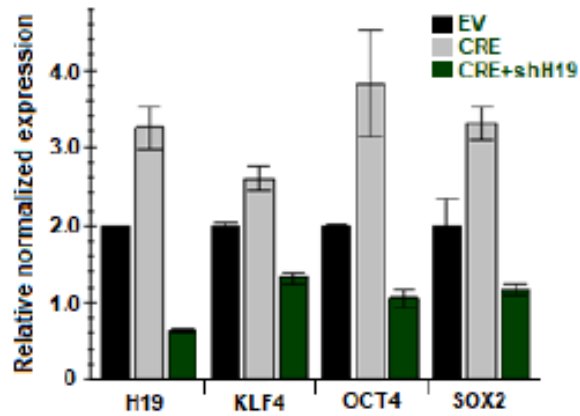


Figure 5a: Relative RNA expression of stem cell genes in mouse prostate organoids after P53/Rb1 double knockdown induced by lentiviral transduction of Cre-recombinase in P53^{flox/flox}/Rb1^{flox/flox} mouse prostate organoids (Cre) and H19 knockdown (Cre+shH19). EV transduced organoids (EV) were used as controls. Values are mean $\pm$ S.D. from 3 independent experiments.

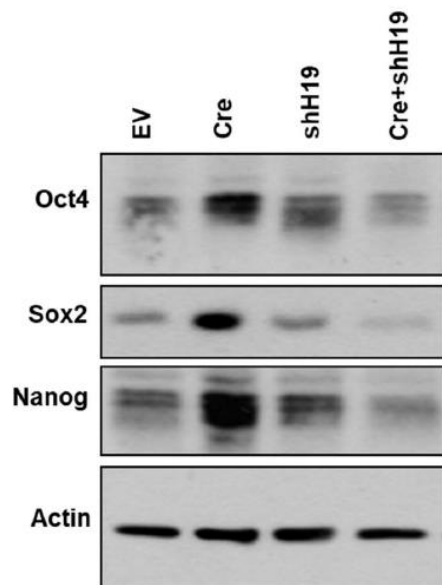


Figure 5b: WB of representative stem cell genes in the Trp53^{flox/flox}/Rb1^{flox/flox} mouse prostate organoids transduced with EV, Cre, Cre+shH19 or shH19.

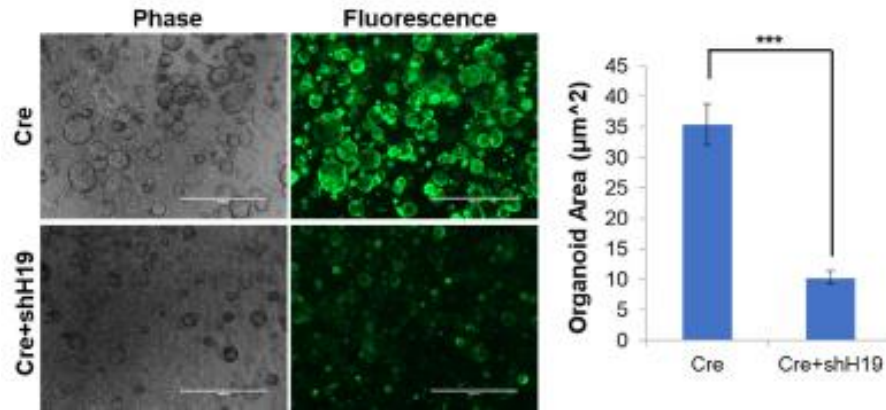


Figure 5c: Mouse organoid cells (5000/well/condition) were plated for organoid growth analysis. Right: Representative fluorescence images. Magnification: 4x, Scale bar: 1000 μm , left: Organoid area quantification (n=10). Values are mean $\pm$ S.D., n=3, ***p value < 0.001.

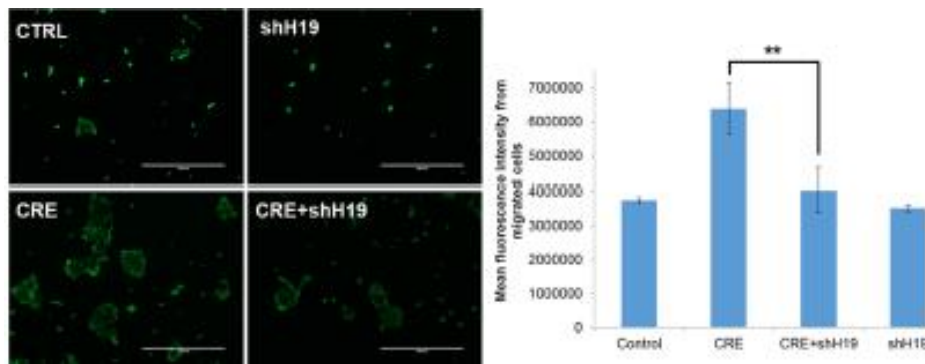


Figure 5d: Organoid invasion assay. Transwell assay using FluoroBlock inserts with 50,000 cells/well of DKO organoids transduced with a Control (CTRL), CRE and CRE+shH19 (n=3). fluorescence intensity of migrated cells on the bottom of the transwell was quantified 5 days post-plating. Ctrl-GFP was used as control. Values are mean $\pm$ S.D., n=3, ***p value < 0.001.

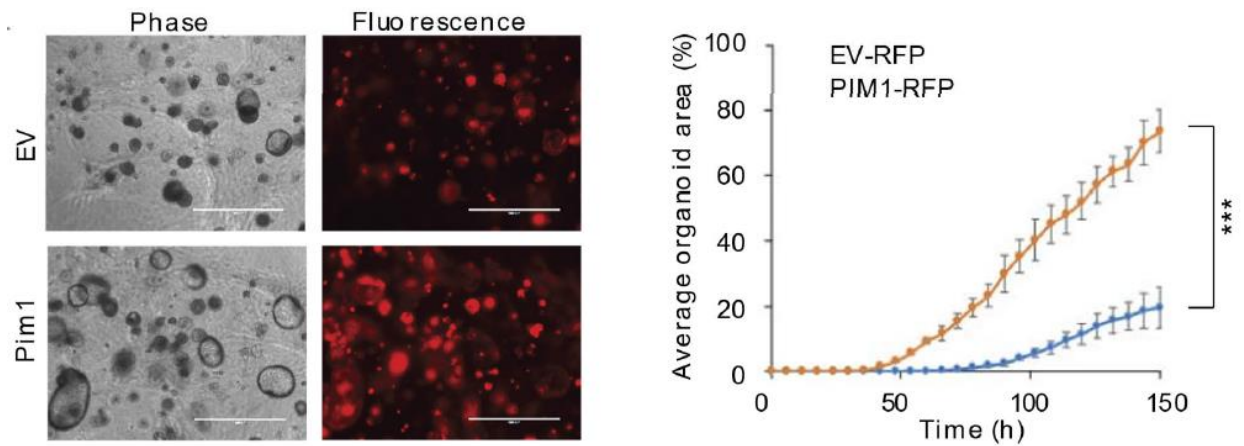


Figure 6a: Mouse prostate organoid cells transduced with empty vector (EV-RFP) or FUCRW-PIM1 plasmid (PIM1-RFP) (5000/well) were plated for organoid growth analysis. Representative images of the growth analysis (left panel), scale bar: 1000 μm. Quantification of the organoid growth (right panel). Values are mean $\pm$ SEM; n=6; ***p<0.001.

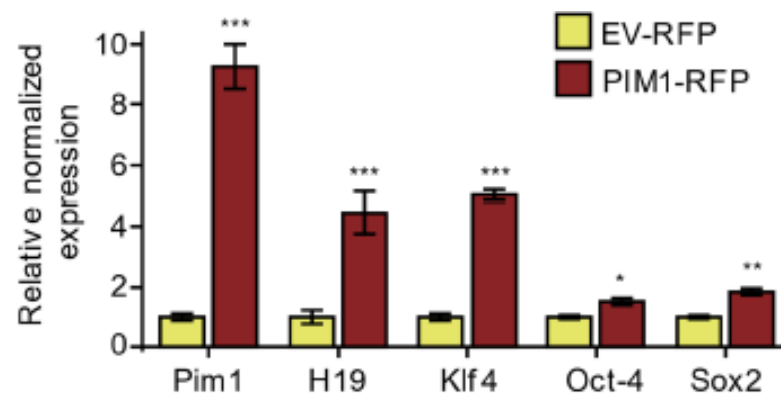


Figure 6b: Relative RNA levels of Pim1, Klf4, Oct-4, Sox2 in mouse prostate organoids described in A. RNA expression was normalized to Hprt RNA. Values are mean $\pm$ SEM; n=3; *p<0.05, **p<0.01, ***p<0.001.

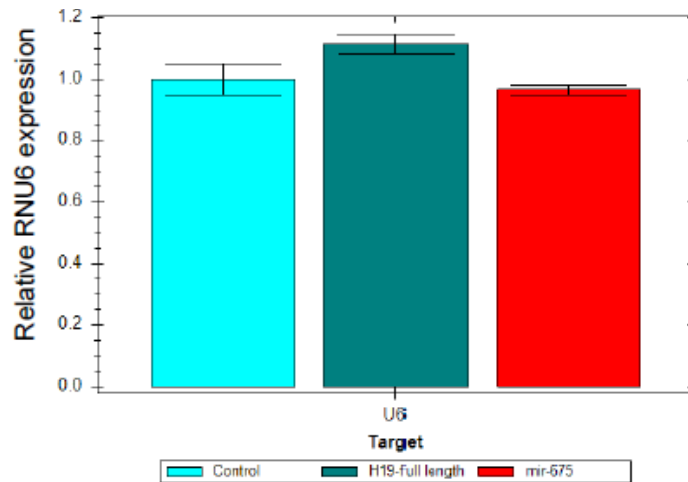


Figure 7a: Relative RNA expression of endogenous control RNU6 in *Trp53/Rb1* DKO prostate organoids stably overexpressing full length H19 (H19 full length) or middle fragment of H19 encoding miR-675 (mir-675). pMSCV backbone transduced DKO organoids were used as Control. Data are the mean $\pm$ SD, n=3.

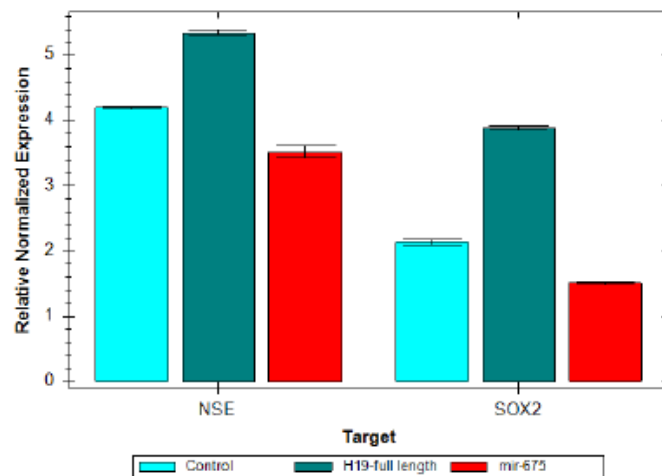
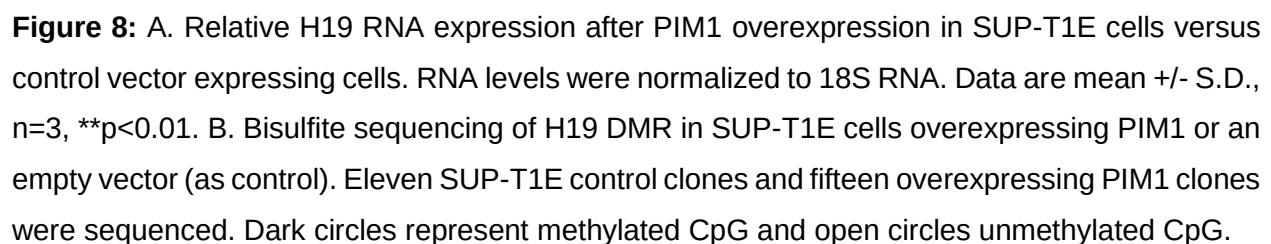
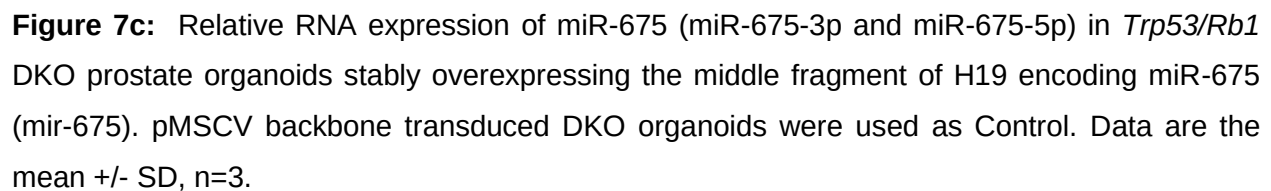


Figure 7b: Relative RNA expression of Nse and Sox2 in *Trp53/Rb1* DKO prostate organoids stably overexpressing full length H19 (H19 full length) or middle fragment of H19 encoding miR-675 (mir-675). pMSCV backbone transduced DKO organoids were used as Control. Data are the mean $\pm$ SD, n=3.



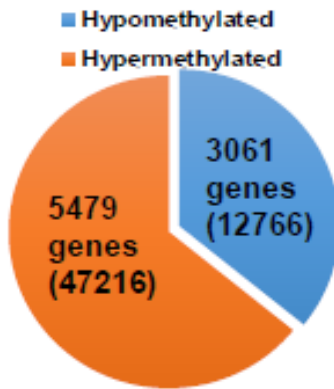


Figure 9a: eRRBS analysis depicted by Pie chart showing the number of differentially methylated genes (shH19 vs control OWCM-155, n=3), identified by annotating hyper- and hypomethylated loci (number is reported in parentheses) (p-adj. <0.01).

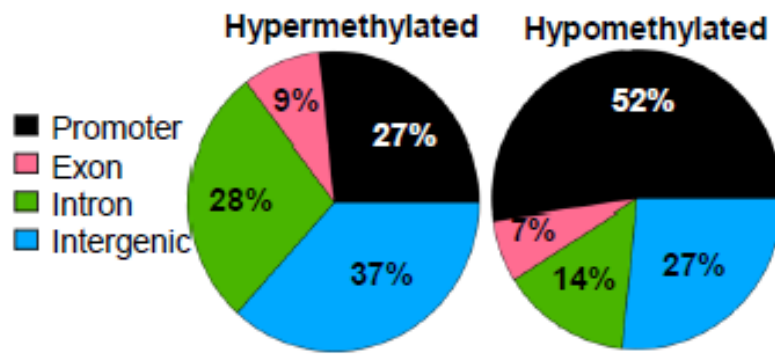


Figure 4b: Pie chart depicting the percent DNA methylation based on indicated gene regions.

Gene ontology		
Hypermethylated	Term	P Value
	GO:0016477~Cell migration	2.06E-04
	GO:0048699~Generation of neurons	2.40E-04
	GO:0000902~Cell morphogenesis	1.56E-04
	GO:0007399~Nervous system development	3.00E-05
Fold Enrichment		
		2.32
		2.20
		2.31
		2.01

Hypomethylated	Term	P Value
	GO:0007389~Pattern specification process	1.52E-10
	GO:0043565~Sequence-specific DNA binding	4.64E-16
	GO:0048598~Embryonic morphogenesis	1.66E-11
	GO:0051960~Regulation of nervous system development	1.02E-08
	GO:0045596~Negative regulation of cell differentiation	5.44E-07
Fold Enrichment		
		3.74
		3.05
		3.44
		2.68
		2.62

Figure 9c: Gene ontology analysis of eRRBS data and selection of functional categories enriched after analysis of differentially methylated genes (p value < 0.001).

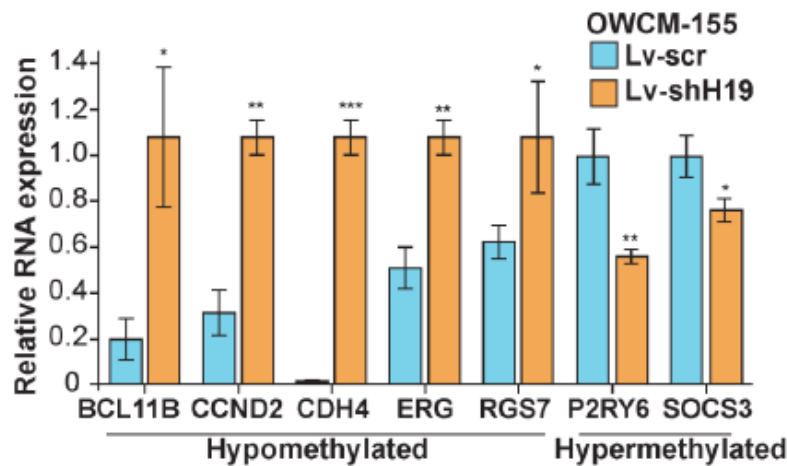


Figure 9d: Gene expression analysis of differentially methylated genes in OWCM-155 NEPC organoid with shH19 (Lv-shH19) as compared to control (Lv-Scr). Data are mean $\pm$ SEM, n=3. Data with statistical significance are as indicated: *, p<0.05; **, p<0.01; ***, p<0.001.

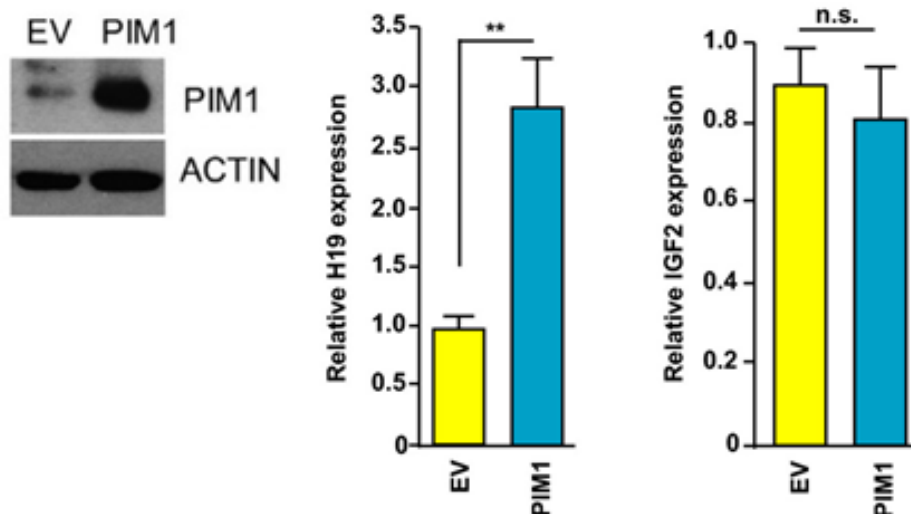


Figure 10: Relative H19 and IGF2 RNA expression (middle and center respectively) in PC-3 cells transfected with EV as control or FUCRW-PIM1 (PIM1). 18S was used as endogenous. PIM1 protein expression was validated by Western blot. Values are mean $\pm$ SEM; $n = 3$; n.s., not significant, ** $P < 0.01$.

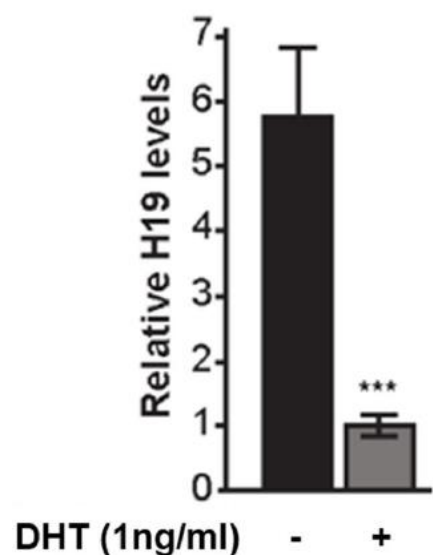


Figure 11a: Relative H19 expression in murine prostate organoids upon DHT removal from media (-) as compared to culture with regular media containing DHT. Data are Mean $\pm$ SEM, $n=3$. ***, $p<0.001$.

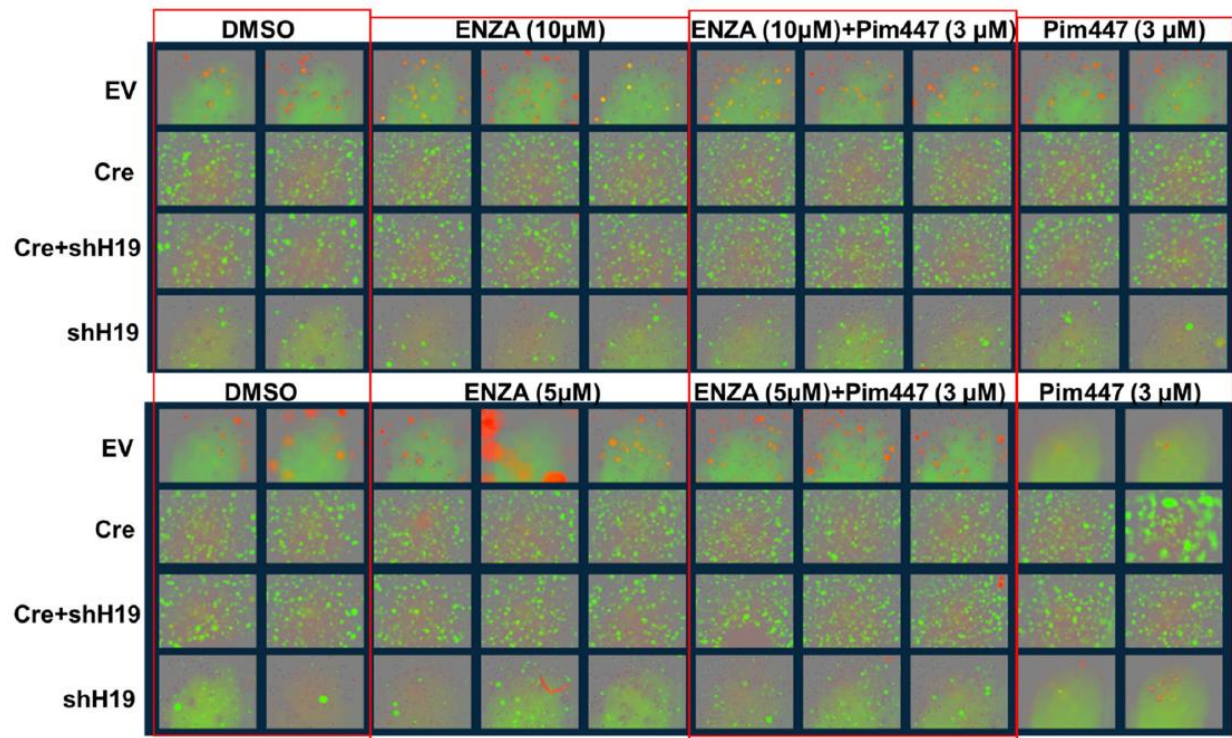


Figure 11b: Organoid growth assay as measured by a live cell imaging system. Organoids were dissociated with TrypLE (Invitrogen) into tiny cell clusters, plated (5000 cell clusters/well) and treated under desired conditions for 4-6 days. A real-time imaging system (Incucyte) was used to measure organoid growth. Images were captured every 12h. The images shown above are a compilation of Pim inhibitor and Enza treatment either individually or in combination (as seen on day 5) when added to Control EV and CRE organoids with or without H19 knockdown (shH19). As observed from the images of multiple replicates of the each treatment condition- Enza treatment did not appear to modulate organoid growth in this system.

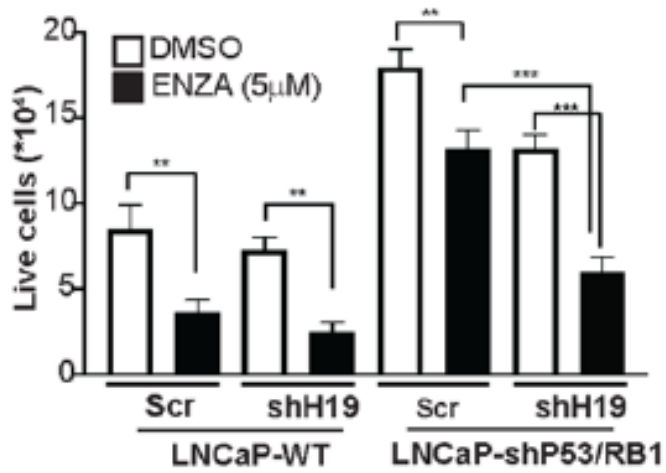


Figure 11c: Growth response of TP53/RB1 knockdown (shTP53/RB1) LNCaP with and without knockdown of H19 (shH19 vs Scr) exposed to 5 μmol/L of ENZA for 5 days. DMSO was used for control treatment.

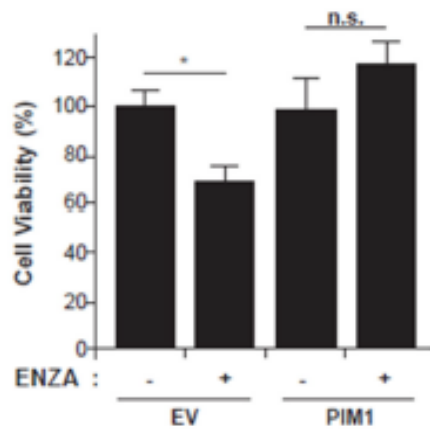


Figure 12a: Percent cell viability of LNCaP cells stably expressing empty vector (EV) or FUCRW-PIM1 plasmid (PIM1), treated with Enza (MDV3100, 10 μM, 48h). DMSO treatment was used as control.

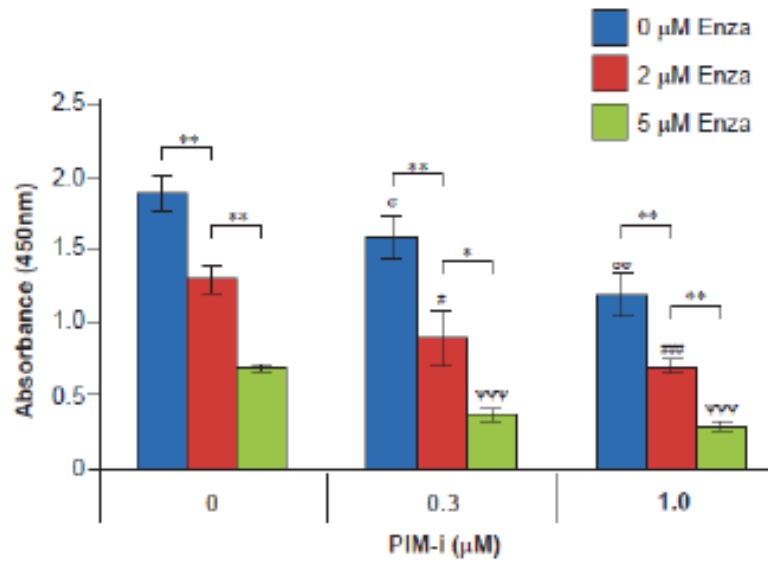


Figure 12b: Cell viability of LNCaP cells stably expressing empty vector (LNCaP-EV) treated with either Enza (2μM and 5μM) or PIM-i (0.01μM, 0.03μM, 0.1μM, 0.3μM and 1μM, 72h) and combination, measured by XTT assay. Treatment with 0μM denotes DMSO treatment in lieu of the inhibitor. Values are mean +/- SEM; n=3; *p<0.05, **p<0.01, ***p<0.001.

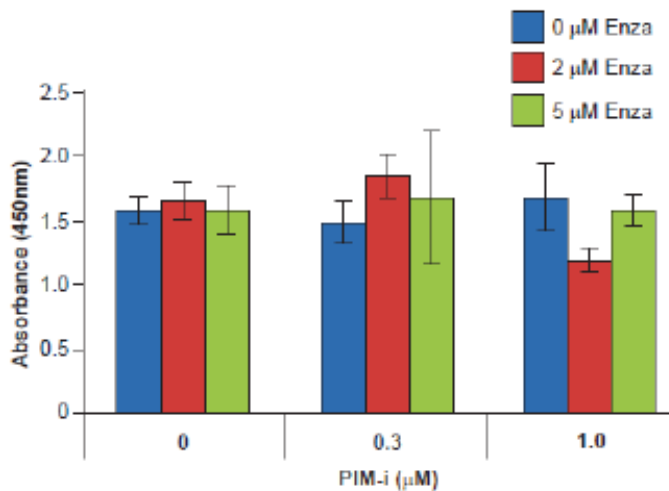


Figure 12c: Cell viability of LNCaP cells stably overexpressing H19 (LNCaP-H19) treated with Enza or PIM-i and combination, as stated in Fig. 6b. XTT assay was carried out 72h after the start of drug treatment and absorbance was measured at 450nm. Results from cell viability in Fig. 6b and 6c were represented as absorbance at 450nm. Values are mean +/- SEM; n=3.

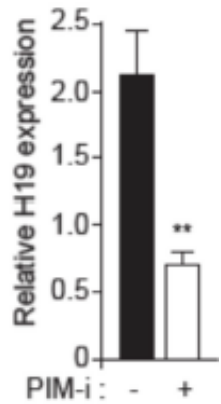


Figure 12d: Relative H19 RNA expression in LNCaP cells treated with PIM-i (PIM447, 1 μ M, 24h). DMSO was used for control treatment. Relative H19 expression was normalized to 18S RNA. Values are mean $\pm$ SEM; n=3; **p<0.01.

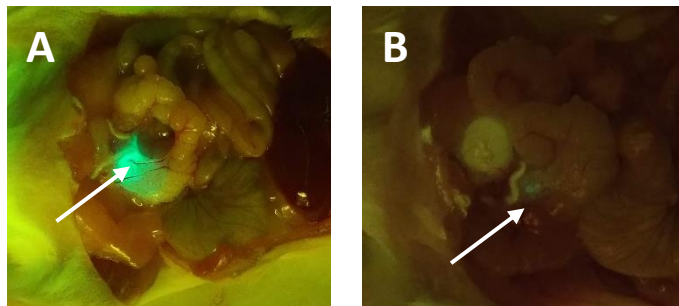


Figure 13: GFP fluorescence (white arrows) from tumors developed from control Trp53/Rb DKO murine organoids (A) and DKO +shH19 (B) injected into mice. Smaller tumor is detected in the mice that received DKO organoids with H19 knockdown (B) than control (A).

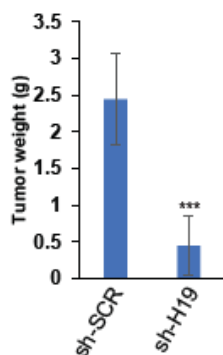


Figure 14a: Mean tumor weight of the tumor xenografts harvested at 11-week post-injection. Data is mean $\pm$ SEM, n=4 per group.

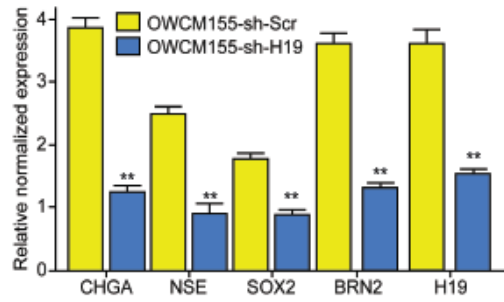


Figure 14b: F, Relative mRNA expression of H19 and NE markers in OWCM 155 shH19 vs shScr xenografts. Graph represents pooled data from 3 tumors per group. Data is mean +/- SEM.

4. Impact

Prostate cancer remains the second largest killer of men with cancer in the United States. The commonly administered antiandrogen drug- Enzalutamide has been shown to be very effective in PCa patients. But, more and more patients are escaping or became unresponsive to this androgen deprivation therapy (ADT). The proposed research project investigates mechanistic details into the resistance to ADT specifically by enzalutamide and aims to identify the role of upregulated Pim1/H19/ Sox2 pathway in driving such resistance which could ultimately lead to ADT treatment induced neuroendocrine prostate cancer. Results from first year of investigation identified Pim kinases and H19 as key players in ADT resistance. The important findings during year 2 from this study are the mechanistic underpinnings of the Pim/H19 axis in mediating antiandrogen resistance (Published Manuscript in “Molecular Oncology” journal and another manuscript under review in Nature Communications- Appendix B). The mechanistic details include the control of H19 promoter methylation by Pim1 and most importantly promoter methylation of genes associated with ADT and consequently neuroendocrine markers regulated by H19, suggesting that in PCa, H19 has some epigenetic function in the form of DNA methylation. These results will help introducing and detecting novel pathways which have the potential to be targeted against resistance to ADT. The studies conducted throughout the award period experiments helped solidify the above pathway which could lead to the potential development of various therapeutic combinations in future to effectively target advanced metastatic prostate cancer in general and enzalutamide resistant neuroendocrine prostate cancer in particular. The proposed therapeutic combination would be helpful in targeting patients with advanced stage of prostate cancer and stop them to progress the cancer in an event they develop resistance to an already clinically effective second generation drug.

Overall, the impactful outcomes of this study are as follows:

- Identification of novel resistance mechanism pathway in the form of PIM/H19/SOX2 for ADT.
- Novel epigenetic mechanism of H19 in the form of promoter DNA methylation leading to gene expression changes in aggressive PCa.
- Novel therapeutic combinations of PIM inhibitor and anti-androgen Enzalutamide to overcome the associated resistance in vitro.
- For training related goals – I was able to present my research at national and international meetings such as AACR and virtual Prostate cancer foundation annual retreat (Appendix A) and was able to prepare 2 manuscripts as a first author highlighting the results from this

projects- one of which has been published in Molecular Oncology and the second one is under a second review in Nature Communications.(Appendix B).

5. Changes/Problems

A minor issue in Specific aim 2 -Major task 1: The murine organoids treated with PIM1 inhibitor and Enza did not show any changes under in vitro conditions (Figure 5b). As I attended the organoid workshop at AACR 2019, I gained insights into this negative result where the organoid experts claimed that it has been shown that in-vitro treatment of organoids with various drugs especially Enzalutamide will not show any changes mainly because the organoids require DHT which interferes with our experiment. This was not known at the time of designing the experiments. Alternatively, we performed the experiments in LNCaP cells, which are commonly used prostate cancer cell line that are androgen responsive. We note that the switch from murine organoids to LNCaP cells in some experiments is a minor change and does not alter either the scope or direction of the project.

We decided to perform the enhanced reduced representation bisulfite sequencing (eRRBS) on NEPC organoid line OWCM-155 instead of p53/Rb DKO. It is a minor change in the sense that it **does not alter either the scope or the direction of the project**. We made the decision considering that eRRBS sequencing would uncover changes in DNA methylation at base pair level and such extensive genome wide data analysis should be performed on accurate models, so that the data generated could be properly represented and used by the scientific community. The OWCM-155 organoid line is better than the p53/Rb organoid model and has been shown to contain high levels of long noncoding RNA H19 by us (Nature Communications manuscript). These were the reasons to use this culture system to knockdown H19 and perform the eRRBS. During the pursuit of major task 2 and 3 for specific aim 2, we had few minor issues: Firstly, the number of organoids was not enough to generate tumors in vivo so we had to repeat the experiment a few times. Secondly, the tumors if developed took longer to form and were uneven in size, to test the various inhibitors. Finally, due to the onset of Covid-19 we could not find enough time to complete the experiment in its proposed form and decided to reapproach this issue in a slightly modified way.

6. Products

- Journal publication:

Singh N, Padi SKR, Bearss JJ, Pandey R, Okumura K, Beltran H, Song JH, Kraft AS, Olive V. PIM protein kinases regulate the level of the long noncoding RNA H19 to control stem cell gene

transcription and modulate tumor growth. **Molecular Oncology**. <https://doi.org/10.1002/1878-0261.12662>

Status of publication – Published in March 2020

Acknowledgement of federal support – Yes

Singh N*, Ramnarine VR*, Song JH, Pandey R, Padi SKR, Nouri M, Kobelev M, Okumura K, McCarthy D, Hanna MM, Mukherjee P, Sun B, Lee BR, Parker JB, Chakravarti D, Zhou M, Bearss JJ, Gibb EA, Alshalalfa M, Karnes RJ, Small EJ, Aggarwal R, Feng F, Wang Y, Buttyan R, Zoubeydi A, Rubin M, Gleave M, Davicioni E, Beltran H, Olive V, Collins C, Kraft AS. The long noncoding RNA H19 regulates tumor plasticity in neuroendocrine prostate cancer. **Nature Communications**. Manuscript **NCOMMS-19-36759A**

*co-first author

Status of publication - Under review- Minor revision submitted July 1ST 2021).

Acknowledgement of federal support – Yes

Manuscript available upon request until publication.

- Conference paper:

Abstract of research presentation during the AACR Annual meeting, 2019 as published in Cancer Research 2019.

Neha Singh, Virginie Olive, Ritu Pandey, Jin Song, Jeremiah Bearrs, Sathish K. Padi, Koichi Okumura, Andrew S. Kraft. Role of long noncoding RNA H19 in driving enzalutamide resistant neuroendocrine prostate cancer [abstract]. In: Proceedings of the American Association for Cancer Research Annual Meeting 2019; 2019 Mar 29-Apr 3; Atlanta, GA. Philadelphia (PA): AACR; Cancer Res 2019;79(13 Suppl):Abstract nr 3017.

Abstract of research presentation during the 27th Annual Prostate Cancer Foundation Virtual Scientific Retreat, 20th -23rd October 2020. The abstracts to this meeting are not published but are present on their webpage (www.pcf.org)

Neha Singh, Varune R. Ramnarine, Jin H. Song, Ritu Pandey, Amina Zoubeydi, Elai Davicioni, Himisha Beltran, Colin Collins, Andrew S. Kraft. 2020. The long noncoding RNA H19 is an important epigenetic regulator of tumor plasticity in neuroendocrine prostate cancer. 27th Annual Prostate Cancer Foundation Virtual Scientific Retreat, 20th -23rd October 2020. <https://doi.org/10.26226/morressier.5f69edb69b74b699bf38c66b>

7. Participants & Other Collaborating Organizations

N/A

8. Special Reporting Requirements

N/A

9. Appendices

Attachments

- Appendix A: Abstract of research presentation during the 27th Annual Prostate Cancer Foundation Virtual Scientific Retreat, 20th -23rd October 2020. The abstracts to this meeting are not published but are present on their webpage (www.pcf.org)

Neha Singh, Varune R. Ramnarine, Jin H. Song, Ritu Pandey, Amina Zoubeydi, Elai Davicioni, Himisha Beltran, Colin Collins, Andrew S. Kraft. 2020. The long noncoding RNA H19 is an important epigenetic regulator of tumor plasticity in neuroendocrine prostate cancer. 27th Annual Prostate Cancer Foundation Virtual Scientific Retreat, 20th -23rd October 2020.

<https://doi.org/10.26226/morressier.5f69edb69b74b699bf38c66b>

- Appendix B: Abstract of research presentation during the AACR Annual meeting, 2019 as published in Cancer Research 2019.

Neha Singh, Virginie Olive, Ritu Pandey, Jin Song, Jeremiah Bearrs, Sathish K. Padi, Koichi Okumura, Andrew S. Kraft. Role of long noncoding RNA H19 in driving enzalutamide resistant neuroendocrine prostate cancer [abstract]. In: Proceedings of the American Association for Cancer Research Annual Meeting 2019; 2019 Mar 29-Apr 3; Atlanta, GA. Philadelphia (PA): AACR; Cancer Res 2019;79(13 Suppl):Abstract nr 3017.

- Appendix C: First author publication in the journal “Molecular Oncology” generated through the efforts conducted with the help of this grant.

Singh N, Padi SKR, Bearss JJ, Pandey R, Okumura K, Beltran H, Song JH, Kraft AS, Olive V. PIM protein kinases regulate the level of the long noncoding RNA H19 to control stem cell gene transcription and modulate tumor growth. **Molecular Oncology**. <https://doi.org/10.1002/1878-0261.12662>

Appendix A

Abstract presented at 27th Annual PCF Scientific Retreat Virtual Poster Session

Abstract presented at 27th Annual PCF Scientific Retreat Virtual Poster Session

Oct 20-23, 2020

Authors:

Neha Singh¹, Varune R. Ramnarine², Jin H. Song¹, Ritu Pandey¹, Amina Zoubeidi², Elai Davicioni³, Himisha Beltran⁴, Colin Collins², Andrew S. Kraft¹

Affiliations:

1. University of Arizona Cancer Center, University of Arizona, 1515 N. Campbell Ave. Tucson, AZ, USA 85724; 2. Vancouver Prostate Centre & Department of Urologic Sciences, University of British Columbia, Vancouver, BC, Canada; 3. Decipher Biosciences Inc., Vancouver, BC, Canada; 4. Department of Medical Oncology, Dana-Farber Cancer Institute, 44 Binney St, Boston MA 02115

Abstract Body:

"Background: Neuroendocrine prostate cancer (NEPC) is a lethal subtype of castration resistant prostate cancer that is primarily treatment-induced driven by androgen deprivation therapy (ADT) arising from transdifferentiated prostate adenocarcinoma (AdPC) cells. Key mechanism includes the lineage switching of epithelial cells to neuroendocrine (NE) phenotype making them independent of androgen receptor (AR) pathways. There is an urgent need to identify drivers/regulators of this transdifferentiation to gain deeper understanding of this lethal subset that could be helpful in devising therapeutic strategies. Multiple long non-coding RNAs (lncRNA) have been identified to be dysregulated and investigated in NEPC, and in the current study we have focused on the role of lncRNA H19 as a potential driver of this phenotype.

Methodology and Result: Bioinformatic analysis using a large collection of clinical samples (benign, n=14; AdPC, n=111; NEPC, n=66), high fidelity models, and the prostate cancer clinical database (Decipher GRID, n=26245), identified the lncRNA H19 as increasingly associated with increasing Gleason grade of prostate cancer, ADT, and the induction of NEPC. Gene expression analysis across various pre-clinical models of NEPC demonstrated H19 to be significantly elevated in patient derived NEPC organoids (OWCM154, OWCM155 and OWCM1262) and NE cell lines (NCI-H660, LASCPC-01, 42DENZR and 42FENZr) as compared to AdPC derived organoids and cell lines. Additionally, N-Myc overexpression, deletion of TP53 and Rb1 or Rb1 and PTEN in both murine prostate organoids and LNCaP cells which leads to the NE phenotype caused H19 expression to be markedly induced. Stable knockdown of H19 in these NEPC models demonstrated that this lncRNA is capable of controlling prostate cancer plasticity by decreasing both stem cell genes and NE markers while upregulating luminal markers associated with prostate cancer. Knockdown of H19 in murine organoids with TP53/Rb1 knockout led to a regression of tumor growth and invasiveness and importantly, sensitized cells to growth inhibition by the anti-androgen- enzalutamide. Mechanistically, H19 was demonstrated to regulate specific epigenetic changes. In prostate cancer cells, RNA immunoprecipitation analysis revealed that H19 physically bound to the PRC2 complex members and increased cellular levels of H3K27me3. Perturbations in H19 levels in NEPC patient derived organoids caused alterations in genome wide DNA

methylation as demonstrated by the enhanced reduced representation bisulphite sequencing (eRRBS) analysis, resulting in changes in the expression patterns of multiple NE associated genes, e.g. RGS7, CCND1, SPDEF, GATA2. Importantly, Kaplan-Meier survival analysis of clinical samples showed that patients on ADT had poor survival related outcomes if their tumor expresses elevated H19 levels, demonstrating the role of H19 in determining the outcome of prostate cancer therapy.

Conclusion: Our data demonstrate that lncRNA H19 is an important epigenetic controller of prostate cancer plasticity, regulates the induction of NEPC and is associated with poor clinical outcomes in patients receiving ADT."

Funding Acknowledgments:

This work was supported by the Department of Defense award (PCRP W81XWH-18-1-0533 to NS), Mitacs Accelerate PhD Fellowship Program (IT04310 to VRR) in collaboration with Decipher Biosciences, Prostate Cancer Foundation (HB), National Cancer Institute (SPORE P50-CA211024 to HB), Department of Defense (PCRP W81XWH-13-1 to HB), Terry Fox Foundation (201012TFF to CC), Prostate Cancer Canada Team Grant (T2013-01 to CC), Canadian Foundation of Innovation – Innovation Fund (33440 to CC), National Institute of Health award (R01CA173200 to ASK), and Department of Defense award (W81XWH-12-1-0560 ASK).

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Conflict of Interest:

Nothing to Disclose

Appendix B

Abstract of research presentation during the AACR Annual meeting, 2019 as published in Cancer Research 2019.

Abstract 3017: Role of long noncoding RNA H19 in driving enzalutamide resistant neuroendocrine prostate cancer

Neha Singh, Virginie Olive, Ritu Pandey, Jin Song, Jeremiah Bearrs, Sathish K. Padi, Koichi Okumura and Andrew S. Kraft

DOI: 10.1158/1538-7445.AM2019-3017 Published July 2019

Proceedings: AACR Annual Meeting 2019; March 29-April 3, 2019; Atlanta, GA

Abstract

INTRODUCTION: Approximately one-fourth of the prostate cancer (PCa) patients relapsing androgen deprivation therapy (ADT) have been shown to develop neuroendocrine prostate cancer (NEPC). One of the primary mechanisms attributed to this resistance is the lineage switching of epithelial cells to neuroendocrine (NE) phenotype making them independent of androgen receptor (AR) pathways for survival. Molecular basis essential to this lineage switch requires further elucidation. Thus, we aim to understand key mechanistic pathways that drive the development and maintenance of NEPC, and to investigate if the inhibition of pathway can re-sensitize to ADT in NEPC.

METHODS: Bioinformatics analysis was performed with existing databases to compare prostate adenocarcinoma (PCA) vs NEPC. Molecular features of NEPC have been shown to depict RB1 loss in 70-90% and TP53 loss in 56-67% of the cases. Thus, we established prostate organoid cultures from P53^{fl/fl}/Rb^{fl/fl} mouse and infected them with Cre-lentivirus for combined loss of TP53/Rb1. Various PCA and NEPC cell lines and patient derived organoids were used to study the role of the long non-coding RNA H19 in NEPC. Organoid growth were assessed with Incucyte and invasive capacity was measured by Matrigel invasion assay.

RESULTS: Bioinformatic analysis of existing human PCa data sets demonstrate that H19 is one of the most highly expressed genes in NEPC and parallels the expression of NE markers. Indeed, we confirmed that H19 expression was high in various NEPC cell lines and NE patient derived organoids. H19 was also found to be markedly induced in mouse prostate organoids and LNCaP cells after TP53/Rb1 loss. Importantly, overexpression of H19 induced NE genes while suppressing the genes involved in AR signaling in LNCaP and primary PCa patient derived organoids and inversely, knockdown (KD) of H19 in LNCaP and mouse prostate organoids with TP53/Rb1 loss reduced the expression of NE gene. Interestingly, the KD of H19 in these cells re-induced the luminal phenotype, consequently restoring their sensitivity to ADT. These findings strongly implicate H19 in driving the NE lineage switch. The KD of H19 in these organoids led to a regression of tumor growth and invasiveness, suggesting H19 is required in malignant states. H19 KD decreases stem cell genes (SOX2, OCT4) via methylation of their promoters, and levels of EZH2, which methylates 'Lys-9' (H3K9me) and 'Lys-27' (H3K27me) of histone H3 and represses transcription of the target genes. H3K27me3 levels, which are high in NEPC, was reduced in the H19 KD, suggesting H19 works in an epigenetic regulation to induce NEPC phenotype.

CONCLUSION: These data highlight H19 as a novel regulator in controlling the lineage switch to NEPC, tumor growth, and the sensitivity to hormone blockade, suggesting that H19 regulation will be important for developing treatments specifically aimed for this highly aggressive form of prostate cancer.

Appendix C

PIM protein kinases regulate the level of the long noncoding RNA H19 to control stem cell gene transcription and modulate tumor growth. **Molecular Oncology.** <https://doi.org/10.1002/1878-0261.12662>

PIM protein kinases regulate the level of the long noncoding RNA H19 to control stem cell gene transcription and modulate tumor growth

Neha Singh¹, Sathish K. R. Padi¹, Jeremiah J. Bearss¹, Ritu Pandey^{1,2}, Koichi Okumura^{1,3}, Himisha Beltran⁴, Jin H. Song^{1,2} , Andrew S. Kraft^{1,5}  and Virginie Olive¹

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Keywords

androgen deprivation therapy resistance; H19; Pim kinase; prostate cancer; SOX2; T-ALL

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The proviral integration site for Moloney murine leukemia virus (PIM) serine/threonine kinases have an oncogenic and prosurvival role in hematological and solid cancers. However, the mechanism by which these kinases drive tumor growth has not been completely elucidated. To determine the genes controlled by these protein kinases, we carried out a microarray analysis in T-cell acute lymphoblastic leukemia (T-ALL) comparing early progenitor (ETP-ALL) cell lines whose growth is driven by PIM kinases to more mature T-ALL cells that have low PIM levels. This analysis demonstrated that the long noncoding RNA (lncRNA) H19 was associated with increased PIM levels in ETP-ALL. Overexpression or knockdown of PIM in these T-ALL cell lines controlled the level of H19 and regulated the methylation of the H19 promoter, suggesting a mechanism by which PIM controls H19 transcription. In these T-ALL cells, the expression of PIM1 induced stem cell gene expression (SOX2, OCT-4, and NANOG) through H19. Identical results were found in prostate cancer (PCa) cell lines where PIM kinases drive cancer growth, and both H19 and stem cell gene levels. Small molecule pan-PIM inhibitors (PIM-i) currently in clinical trials reduced H19 expression in both of these tumor types. Importantly, the knockdown of H19 blocked the ability of PIM to induce stem cell genes in T-ALL cells, suggesting a novel signal transduction cascade. In PCa, increases in SOX2 levels have been shown to cause both resistance to the androgen deprivation therapy (ADT) and the induction of neuroendocrine PCa, a highly metastatic form of this disease. Treatment of PCa cells with a small molecule pan-PIM-i reduced stem cell gene transcription and enhanced ADT, while overexpression of H19 suppressed the ability of pan-PIM-i to regulate hormone blockade. Together, these results demonstrate

Abbreviations

ADT, androgen deprivation therapy; ALL, acute lymphoblastic leukemia; AR, androgen receptor; BPH, benign prostate hyperplasia; DMR, differentially methylated region; Dox, doxycycline; Enza, enzalutamide; ES cells, embryonic stem cells; ETP-ALL, early progenitor-type acute lymphoblastic leukemia; HP1, heterochromatin protein 1 γ ; ICR, imprinting control region; IRS1, insulin receptor substrate 1; KD, knockdown; KiD, kinase-dead; KLF4, Krüppel-like factor 4; lncRNA, long noncoding RNA; NEPC, neuroendocrine prostate cancer; OCT-4, octamer-binding transcription factor 4; PCa, prostate cancer; PIM, proviral integration site for Moloney murine leukemia virus; PIM-i, PIM inhibitor; PRC2, polycomb repressor complex 2; qPCR, quantitative PCR; SEM, standard error of the mean; SOX2, SRY (sex-determining region Y)-box 2; T-ALL, T-cell acute lymphoblastic leukemia; WB, western blot; WT, wild-type.

that the PIM kinases control the level of lncRNA H19, which in turn modifies stem cell gene transcription regulating tumor growth.

1. Introduction

The proviral integration site for Moloney murine leukemia virus (PIM) protein kinase family, comprising three serine/threonine protein kinases, PIM1, PIM2, and PIM3, has been implicated in cancer initiation and progression (Brault *et al.*, 2010; Cuypers *et al.*, 1984). PIM kinases are elevated in hematopoietic neoplasms including myeloid leukemia, myeloma, and lymphoma (Alizadeh *et al.*, 2000; Cohen *et al.*, 2004; Wingett *et al.*, 1996), and play an important role in prostate tumorigenesis (Chen *et al.*, 2005; Shah *et al.*, 2008). These enzymes have been associated with aggressive prostate tumor growth (Cibull *et al.*, 2006). PIM kinases control tumor cellular metabolism, protein translation, cell growth, and division, and play a clear role in the resistance to PI3K/AKT-directed therapies in breast cancer (Braso-Maristany *et al.*, 2016; Le *et al.*, 2016) and prostate cancer (PCa) (Padi *et al.*, 2019; Song *et al.*, 2018). Although many of the specific targets and pathways that are regulated by the PIM kinases to drive cancer growth and progression have been examined (de Bock *et al.*, 2018; Malone *et al.*, 2019), investigating other potential mechanisms will provide new insights into novel therapeutic targets.

A stem cell gene expression signature has been shown to characterize poorly differentiated tumors from several types of human cancer (Ben-Porath *et al.*, 2008). Tumor-initiating cells expressing pluripotency factors, such as NANOG, octamer-binding transcription factor 4 (OCT-4), and SRY (sex-determining region Y)-box 2 (SOX2), play a significant role in the induction and maintenance of malignancy. Immortalized PCa epithelial cell cultures and DU145 human prostatosphere cells have been shown to express these stem cell genes (Gu *et al.*, 2007; Rybak *et al.*, 2011), which are enriched in high-Gleason grade PCa (Mathieu *et al.*, 2011), and predict the poorest overall survival (Markert *et al.*, 2011). PIM1 transcriptional levels in embryonic stem (ES) cells are controlled by STAT3 and leukemia-inhibitory factor, which are known to regulate stem cell genes (Aksoy *et al.*, 2007). When ES cells are fused to fibroblasts, the addition of IL-6 to stimulate these cultures upregulates PIM1 levels, which then cooperates with OCT-4, SOX2, and Krüppel-like factor 4 (KLF4) to

increase induced pluripotent stem cell frequency (Brady *et al.*, 2013). Consistent with a role in stem cells, transgenic mice overexpressing PIM1 upregulate hematopoietic stem/progenitor cell proliferation, while PIM1 knockout mice have impaired long-term hematopoietic repopulating capacity (An *et al.*, 2013). Together, these findings suggest that the PIM kinases could regulate stem cell genes to control cancer growth.

We demonstrate that the PIM1 protein kinase induces the long noncoding RNA (lncRNA) H19 expression and promotes induction of a stem cell signature in both T-cell acute lymphoblastic leukemia (T-ALL) and PCa cells. The lncRNA H19 is highly expressed in embryonic tissue and placenta and repressed after birth (Pachnis *et al.*, 1988; Poirier *et al.*, 1991), but is highly re-expressed in multiple cancers including both hematopoietic (Takeuchi *et al.*, 2007) and solid tumors including breast (Adriaenssens *et al.*, 1998), esophageal (Hibi *et al.*, 1996), bladder (Ariel *et al.*, 1995; Elkin *et al.*, 1995), lung (Kondo *et al.*, 1995), and endometrial and cervical (Lee *et al.*, 2003) cancers. H19 is encoded by the IGF2/H19-imprinted gene cluster located on human chromosome 11p15 (Zemel *et al.*, 1992), and its transcription is controlled by differentially methylated regions (DMRs) of the upstream DNA called 'imprinting control regions' (ICR). When hypomethylated, four specific DMRs upstream of H19 start site bind the transcription factor CCCTC-binding factor, which acts as insulator preventing IGF2 promoter activation and enhancing H19 transcription (Phillips and Corces, 2009). Here, we show that PIM1 induces epigenetic changes in DNA methylation in the control regions of H19, suggesting a mechanism by which it regulates the transcription of this lncRNA. The inhibition of pan-PIM kinase activity and the knockdown (KD) of H19 sensitize PCa cells to treatment with antiandrogen enzalutamide (Enza). In PCa and its highly aggressive variant neuroendocrine PCa (NEPC) (Davies *et al.*, 2018), which expresses elevated H19 levels as compared to adenocarcinoma (Ramnarine *et al.*, 2018), the combination of pan-PIM kinase inhibitors with concomitant H19 KD reduces tumor growth. These results delineate a unique signaling cascade driven by the PIM kinases that involves lncRNA and stem cell genes to regulate tumor growth.

2. Materials and methods

2.1. Cell culture

PC3, LNCaP, and DU145 were purchased from the American Type Culture Collection (ATCC, Baltimore, MD, USA); PC3-LN4 and human prostate fibroblast cell line BHP1S1 (Zemskova *et al.*, 2015) were cultured in RPMI supplemented with 2 mmol·L⁻¹ GlutaMAX (Life Technologies, Rockville, MD, USA) and 10% FBS (BioAbChem, Ladson, SC, USA) at 37 °C under 5% CO₂ as reported previously. The T-ALL cell lines HSB-2, DU.528, KOPT-K1, CUTLL1, HPB-ALL, and SUP-T1 (Padi *et al.*, 2017) were cultured in RPMI 1640 supplemented with 2 mM GlutaMAX (Life Technologies) and 10% FBS (BioAbChem) at 37 °C under 5% CO₂. LASCPC-01 (ATCC) was grown in HITES media containing RPMI, 5% FBS, 10 nM hydrocortisone, 10 nM beta-estradiol (Sigma-aldrich, St. Louis, MO, USA), insulin–transferrin–selenium (Life Technologies), and GlutaMAX (Life Technologies). hPrEC and murine prostate epithelial tumor cells (mPrEC) cells were cultured as described previously (Song *et al.*, 2018). Phoenix-Eco cells (a gift from J. Schatz, University of Miami Health System) and fibrosarcoma cell line FLYRD18/mCAT-IRES-Bleo (a gift from H.G. Wendel, Memorial Sloan Kettering Cancer Center) were cultured in DMEM + 2 mM glutamine + 10% FBS. All cell lines were maintained for no more than 6 months in culture and were routinely tested for Mycoplasma.

2.2. Organoid culture

The NEPC patient-derived organoids OWCM-155 were provided by H. Beltran and were cultured as previously described (Puca *et al.*, 2018). For murine organoids, cells were dissociated from the wild-type (WT) mouse prostate and cultured as organoids as described (Drost *et al.*, 2016). All studies involving the use of animals were approved by and conducted in accordance with the guidelines of the Institutional Animal Care and Use Committees at the University of Arizona Cancer Center. Both NEPC and murine organoids were replenished with fresh media every 3–4 days during organoid growth. Dense cultures with organoids ranging in size from 200 to 500 µm were passaged weekly. Organoid cultures were biobanked using Bambanker (Gibco, Life Technologies) at –80 °C.

2.3. RNA extraction, qPCR, and gene expression analysis

Total RNA was isolated from cells using TRIzol reagent (Invitrogen, Waltham, MA, USA; Cat #

15596–018). One microgram total RNA was reverse-transcribed by using i-Script cDNA Synthesis System Kit (Bio-Rad, Hercules, CA, USA; Cat # 1708891). To measure gene expression, real-time PCR was performed using SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad; Cat # 1725271), following the manufacturer's protocol. Expression level of each transcript was quantified by using Bio-Rad CFX96 Real-Time PCR Detection System. Quantitative real-time PCR (qPCR) assay was performed using the following primers (5'–3'):

h-H19-F: GCACCTTGGACATCTGGAGT,
h-H19-R: TTCTTTCCAGCCCTAGCTCA; primer
for H19 was designed based on Ref Seq ID
NR_002196.2; amplicon length: 171

h-KLF4-F: GGCCTACCGTAAACACACG,
h-KLF4-R: CTGGCAGTGTGGGTCATATC;
amplicon length: 140

h-NANOG-F: TTTGTGGGCCTGAAGAAACT,
h-NANOG-R: AGGGCTGTCTGAATAAGCAG;
amplicon length: 116

h-OCT-4-F: TCGAGAACCGAGTGAGAGG,
h-OCT-4-R: GAACCACACTCGGACCACA;
amplicon length: 125

h-SOX2-F: CCCTGTGGTTACCTCTTCCT,
h-SOX2-R: AGTGCTGGGACATGTGAAGT;
amplicon length: 136

h-PIM1-F: CGACATCAAGGACGAAAACATC,
h-PIM1-R: ACTCTGGAGGGCTATACACTC;
amplicon length: 137

h-PIM2-F: GAACATCCTGATAGACCTACGC,
h-PIM2-R: CATGGTACTGGTGTGAGAG;
amplicon length: 142

h-PIM3-F: GACATCCCCTTCGAGCAG,
h-PIM3-R: ATGGGCCGCAATCTGATC; ampli-
con length: 147

h-c-MYC-F: AAACACAACTTGAACAGCTAC,
h-c-MYC-R: ATTTGAGGCAGTTTACAT-
TATGG; amplicon length: 188

h-18S-F: GTAACCCGTTGAACCCATT,
h-18S-R: CCATCCAATCGGTAGTAGCG; ampli-
con length: 151

m-PIM1-F: GATCATCAAGGGCCAAGTGT,
m-PIM1-R: GATGGTTCCGATTCTTCA;
amplicon length: 122

m-OCT-4-F: TCAGGTTGGACTGGGCCTAGT,
m-OCT-4-R: GGAGGTTCCCTCTGAGTTGCTT;
amplicon length: 100

m-SOX2-F: GGTTACCTCTTCTCCCACTCCAG,
m-SOX2-R: TCACATGTGCGACAGGGGCAG;
amplicon length: 193

m-KLF4-F: CCAAAGAGGGGAAGAAGGTCTG,
m-KLF4-R: GTGCCTGGTCAGTTCATCGG;
amplicon length: 198

m-HPRT-F: AAGCTTGCTGGTGAAAAGGA,
m-HPRT-R: TTGCGCTCATCTTAGGCTTT;
amplicon length: 186

2.4. Immunoblotting

As previously described (Padi *et al.*, 2017), at the end of each experiment, cells were lysed in RIPA buffer (Cell Signaling Technology, Danvers, MA, USA, Cat # 9806S) with complete protease/phosphatase inhibitor cocktail (Cell Signaling Technology, Cat # 5872S). The protein concentration was determined by Bio-Rad DC Protein Assay (Bio-Rad). Western blots (WBs) were performed as described previously (Song *et al.*, 2018). The levels of PIM1 kinase (relative to ACTIN) and p-IRS1 (S1101) proteins (relative to IRS1) were quantified and normalized to their respective control samples using IMAGEJ (NIH, <https://imagej.nih.gov/ij/>) software.

2.5. Antibodies and reagents

Primary antibodies used for western blotting included anti-PIM1 (Cell Signaling Technology, Cat # 2907), anti-PIM2 (Cell Signaling Technology, Cat # 4730), anti-PIM3 (Cell Signaling Technology, Cat # 4165), anti-pIRS1-S1101 (Cell Signaling Technology, Cat # S1101 Cat # 2385), anti-insulin receptor substrate 1 (IRS1; Cell Signaling Technology, Cat # 06-248), anti-HA (Cell Signaling Technology, Cat # 14031), anti-SOX2 [(E-4) sc-365823; Santa Cruz Biotech, Santa Cruz, CA, USA], anti-OCT-4 [(C-10) sc-5279; Santa Cruz Biotech], and anti-NANOG [(5A10) sc-134218; Santa Cruz Biotech]. HRP-conjugated anti- β -actin (Cat # A3854) was purchased from Sigma-Aldrich. HRP-linked mouse IgG (Cat # NA931V) and rabbit IgG (Cat # NAV934V) were purchased from GE Healthcare Life Sciences (Princeton, NJ, USA).

Doxycycline (Dox) hydrochloride (Cat # D3447) was purchased from Sigma-Aldrich. AZD1208 (Cat # A13203) was purchased from Adooq Bioscience (Irvine, CA, USA). Enza (MDV3100; Cat # S1250) was purchased from Selleckchem (Houston, TX, USA). PIM447 was a gift from Novartis (Basel, Switzerland).

2.6. Plasmids

Knockdown of human H19 was performed using the lentiviral plasmids pLenti-siH19-GFP (Abm, Richmond, British Columbia, Canada, Cat # i009382) and pLenti-scrambled siRNA-GFP (Abm, Cat # LV015-G) as a control. These siH19 plasmids allow for direct nonviral plasmid transfection for immediate expression (siH19) and also package into lentiviral particles for high-efficiency transduction and stably integrated expression (shH19). Overexpression of human H19 was performed using pLenti-GIII-CMV-H19-GFP-2A-Puro (Abm, Cat # LV178008). The PIM1-expressing constructs and its K67M kinase-dead (KiD) mutants were described previously (Cen *et al.*, 2010). All the PIM1, PIM2, and PIM3 overexpression plasmids and siRNA to PIM1 were performed as previously described (Padi *et al.*, 2017; Song *et al.*, 2018). Transient transfection of siRNA and cDNA was performed using Lipofectamine 3000 (Invitrogen) and Xfect transfection reagent (Clontech, Takara Bio USA Inc., Mountain View, CA, USA). The SUP-T1 cells were engineered (SUP-T1E) using a fibrosarcoma cell line FLYRD18/mCAT-IRES-B (Ngo *et al.*, 2006) and infected with MigR1 and MigPIM1 following established procedures (Peters *et al.*, 2016).

2.7. Cell viability assay

LNCaP and PC3 cell lines were seeded into 96-well plates at a density of 5000 cells per well and allowed to grow under desired conditions. As described previously (Padi *et al.*, 2017), at the end of each experiment, cell viability was measured using XTT cell proliferation assay (Trevigen, Gaithersburg, MD, USA; Cat # 4891-025-K) following the manufacturer's protocol. Briefly, the XTT reagent was added to cell culture (1 : 2 dilution) and incubated for 4 h at 37 °C and 5% CO₂. The absorbance of the colored formazan product was measured at 450 nm.

2.8. Organoid growth assay

Organoids were dissociated with TrypLE (Invitrogen) into tiny cell clusters, plated (5000 cell clusters per well), and treated under desired conditions for 4–6 days. A real-time imaging system (IncuCyte™; Essen

Bioscience, Sartorius, Ann Arbor, MI, USA) was used to measure organoid growth. Images were captured every 12 h, and results were plotted as the percent average organoid growth vs time.

2.9. Luciferase assay

PC3 cells stably overexpressing Dox-inducible PIM1 (PC3 Tripz-PIM1) were plated in 96-well plates (2×10^4 cells per well) and transfected with a OCT-4 (proximal and distal enhancer) luciferase reporters (Addgene, Watertown, MA, USA) or Renilla (Promega, Madison, WI, USA) using Lipofectamine (Invitrogen). The plasmid sequences for OCT-4 distal and proximal enhancers were pGL3-human OCT-4 DE-SV40-Luc and pGL3-human OCT-4 PE-SV40-Luc and were a gift from J. Hanna (Addgene plasmid # 52414 and 52415, respectively) (Gafni *et al.*, 2013). The total plasmid DNA used was normalized to 0.5 µg per well by the addition of Renilla. At 24 h after transfection, luciferase activities were measured using a Dual-Luciferase Reporter Assay System (Promega) and a Glo-Max 96-well microplate luminometer (Promega). OCT-4 (proximal and distal promoter) luciferase activities were corrected by the corresponding Renilla luciferase activities. Results are expressed in arbitrary light units.

2.10. Lentiviral production and transduction

Lentiviral particle production and infection were performed as described previously (Tiscornia *et al.*, 2006). For infection of adherent PCa cells, 10^6 cells per well were seeded in six-well plates and infected with concentrated lentiviral particles 1 day after seeding. For lentiviral transduction, organoids were preincubated for at least 48 h with regular organoid media supplemented with Wnt-3a and Rho kinase inhibitor (Karthaus *et al.*, 2014). After 48 h, organoids were dissociated with TrypLE and spinoculated with lentiviral particles along with polybrene (8 µM final concentration) at 600 g for 1 h at 32 °C. After incubating organoid cells at 37 °C for 3 h, the cells were replated in Matrigel in ENR media without lentivirus and allowed to grow for several days.

2.11. Bisulfite sequencing

Genomic DNA from informative samples were treated with bisulfite (EpiTect Plus DNA Bisulfite Kit; Qiagen, Hilden, Germany; Cat No./ID: 59124) to convert unmethylated cytosines to uracils, whereas methylated cytosines are unaffected according to the manufacturer's protocol. Bisulfite-treated DNA was subsequently amplified using the forward 5'-

TGGGTATTTTGGAGGTTTTTTT-3' and reverse 5'-TCCCATAAATATCCTATTCCCA 3' primers.

2.12. Fluorescent-activated cell sorting

Cells were resuspended in 10% FBS/PBS to reach a concentration of 10^7 cells per milliliter. Twenty microliters of the cell suspension was stained with various antibodies diluted in 10% FBS/PBS for 1 h. Subsequently, cells were washed with 2% FBS/PBS and resuspended in 10% FBS/PBS for flow cytometry analysis (FACS). Antibodies used for our FACS analyses include APC anti-human CD24 antibody (Cat # 311117; Biolegend, San Diego, CA, USA), PE anti-human CD29 antibody (Cat # 303003; Biolegend), APC anti-human CD133 (Cat # 17-1338-41; eBioscience, San Diego, CA, USA), and APC anti-human CD49b (integrin alpha-2; Cat # 17-0500-41; eBioscience).

2.13. Affymetrix gene chip expression analysis

The microarray was performed as described previously (Padi *et al.*, 2017). Total RNA was extracted from six T-ALL cell lines using the RNeasy kit following manufacturer's instructions (Qiagen, Cat #74104). The Genomics Facility Core at University of Arizona Cancer Center performed quality control using the Agilent Bioanalyzer 2100 to confirm all RNA samples had RNA integrity numbers greater than seven and to quantitate the concentration. From the RNA, the Genomics Core produced labeled a DNA target using the WT PLUS Reagent Kit and hybridized it to the Affymetrix® HTA 2.0 Array (Santa Clara, CA, USA) overnight according to the manufacturer's instructions. Arrays were washed and scanned with the GeneChip Hybridization, Wash, and Stain Kit and an Affymetrix® Scanner 3000 (Santa Clara, CA, USA) following the manufacturer's instructions. The Affymetrix® TRANSCRIPTOME ANALYSIS CONSOLE v3.0 software (Affymetrix) was used to analyze resulting data file to identify differentially expressed genes between PIM inhibitor (PIM-i)-sensitive cells (HSB-2, DU.528, and KOPT-K1) and PIM-i-insensitive cells (CUTLL1, HPB-ALL, and SUP-T1) and generated a scatter plot of differentially expressed genes with the following criteria: fold change (linear) < -2 or fold change (linear) > +2, and ANOVA *P* value (condition pair) < 0.05.

2.14. Statistics

Values reported and shown in graphical displays are the mean ± standard deviation or standard error of the mean, as indicated. Comparisons of mean expression across groups were made using an unpaired two-

tailed Student's *t*-test. For all comparisons, *P* values < 0.05 were considered statistically significant.

3. Results

3.1. PIM protein kinase regulates the level of the lncRNA H19

We have chosen to examine two model systems to understand the mechanism by which the family of PIM kinases regulates tumor growth. T-ALL and PCa have been shown to be driven by increased PIM levels, and small molecule inhibitors of PIM decrease the growth of these tumor types. T-ALL cell lines can be divided into the early progenitor types (ETP-ALL), HSB-2, KOPT-K1, and DU.528, containing elevated levels of PIM kinase, and those that are more mature, SUP-T1, HPB-ALL and CUTLL1, and express lower levels of this protein kinase (Padi *et al.*, 2017). ETP-ALL cells are blocked in their growth by PIM-i, while the more mature T-ALLs, SUP-T1, and CUTLL1 are not (Padi *et al.*, 2017). To understand the genes or pathways that might be involved in the sensitivity of HSB-2, KOPT-K1, and DU.528 and the resistance of SUP-T1, HPB-ALL, and CUTLL1 to PIM-i, microarray profiling was carried out on these six T-ALL cell lines (Fig. 1A, Table S1). Among the genes most elevated in ETP-ALL cells whose growth is driven by PIM and sensitive to PIM-i therapy, the lncRNA H19 was identified (Fig. 1A). Using qPCR, we confirmed that H19 is highly expressed in the PIM-driven PIM-i-sensitive HSB-2 and DU.528 but at much lower levels in the PIM-i-insensitive SUP-T1 and CUTLL1 cells (Fig. 1B,C, Fig. S1). KD of PIM1 levels in HSB-2 with siRNA led to a significant decrease in cellular H19 level (Fig. 1D), demonstrating that PIM1 regulates H19 in these ETP-ALL cells. Conversely, PIM1 overexpression in the PIM-i-resistant cell line SUP-T1 induced increases in H19 levels (Fig. 1E). The PIM1 overexpression in SUP-T1E cells was confirmed by measuring p-IRS1 (S-1101; Fig. 1E). IRS1 is a known PIM kinase substrate, and its phosphorylation is regulated by PIM kinase activity (Song *et al.*, 2016).

A possible mechanism by which PIM regulates H19 levels is by modulation of the H19 DMR methylation. There is a striking difference in the methylation state of H19 between PCa and benign prostate hyperplasia (BPH). Eight percent of the CpGs in the DMRs and in the ICRs are methylated in BPH, while only 41% of CpGs are methylated in PCa (Paradowska *et al.*, 2009). Bisulfite sequencing demonstrates that while the

DMR is methylated in 100% of the SUP-T1 clones, PIM1-overexpressing SUP-T1 cells demonstrated a demethylation of the DMR in 30% of the clones analyzed, reflecting the loss of imprinting of H19 (Fig. S2A,B). This result suggests a potential mechanism for the suppression of H19 expression in SUP-T1 cells and its potential regulation by PIM1 kinase.

Since PIM1 kinases have been shown to drive the growth of PCa, we sought to address whether the PIM1 regulation of H19 levels was also present in this tumor type. Transient overexpression of PIM1 in human PCa cell lines PC3 and DU145 elevated H19 expression (Fig. 2A,B). To examine whether the kinase activity of this enzyme was needed, DU145 cells were transfected with PIM1 (WT) or a K67M KiD mutant (Cen *et al.*, 2010) (Fig. 2B). WT-PIM1, but not its KiD mutant, induced increases in H19 levels (Fig. 2B). Similarly, in human prostate stromal cell line BHPrS1 containing Dox-inducible PIM1 construct (Zemskova *et al.*, 2015), PIM1 induction resulted in increases in H19 levels, which were blocked by 24 h of PIM-i (AZD1208, 3 μ M) treatment (Fig. 2C). The effect of Dox induction and PIM-i treatment was confirmed by p-IRS1 phosphorylation (Fig. 2C). Similarly, PIM1-mediated H19 induction was also observed in normal human prostate-derived epithelial cells, hPrEC (Fig. 2D), or prostate tumor cell line PC3 (Fig. 2E). To check whether this H19 induction by PIM kinase is isoform-specific, we transduced PC3 cells with PIM1, PIM2, or PIM3 and measured the H19 levels. Each of the PIM kinase isoforms induced similar H19 level increases (Fig. S3A), indicating that each isoform was capable of regulating this lncRNA.

H19 and IGF2 are expressed from the same genetic locus (Gabory *et al.*, 2010). We observed no changes in IGF2 RNA expression when PIM1 kinase was transfected in PC3 cells (Fig. 2F). Since H19 has been shown to be directly activated by c-MYC (Barsyte-Lovejoy *et al.*, 2006), the increase in H19 upon PIM overexpression could be mediated by changes in c-MYC levels. It has been shown that the PIM1/PIM2 kinases synergize with c-MYC to induce tumorigenesis. It has been found that overexpressing PIM1 kinase decreased phosphorylation of Thr58 and enhanced phosphorylation at S62, whereas PIM2 caused S329 phosphorylation on c-MYC. These phosphorylation events caused by PIM1/PIM2 lead to increased protein stability and enhanced transcriptional activity of c-MYC (Kim *et al.*, 2010; Zhang *et al.*, 2008). However, in our model system, overexpression of c-MYC in DU145 cells did not induce H19, suggesting that PIM regulates H19 independently of c-MYC function (Fig. S4). Taken together, these data demonstrate that

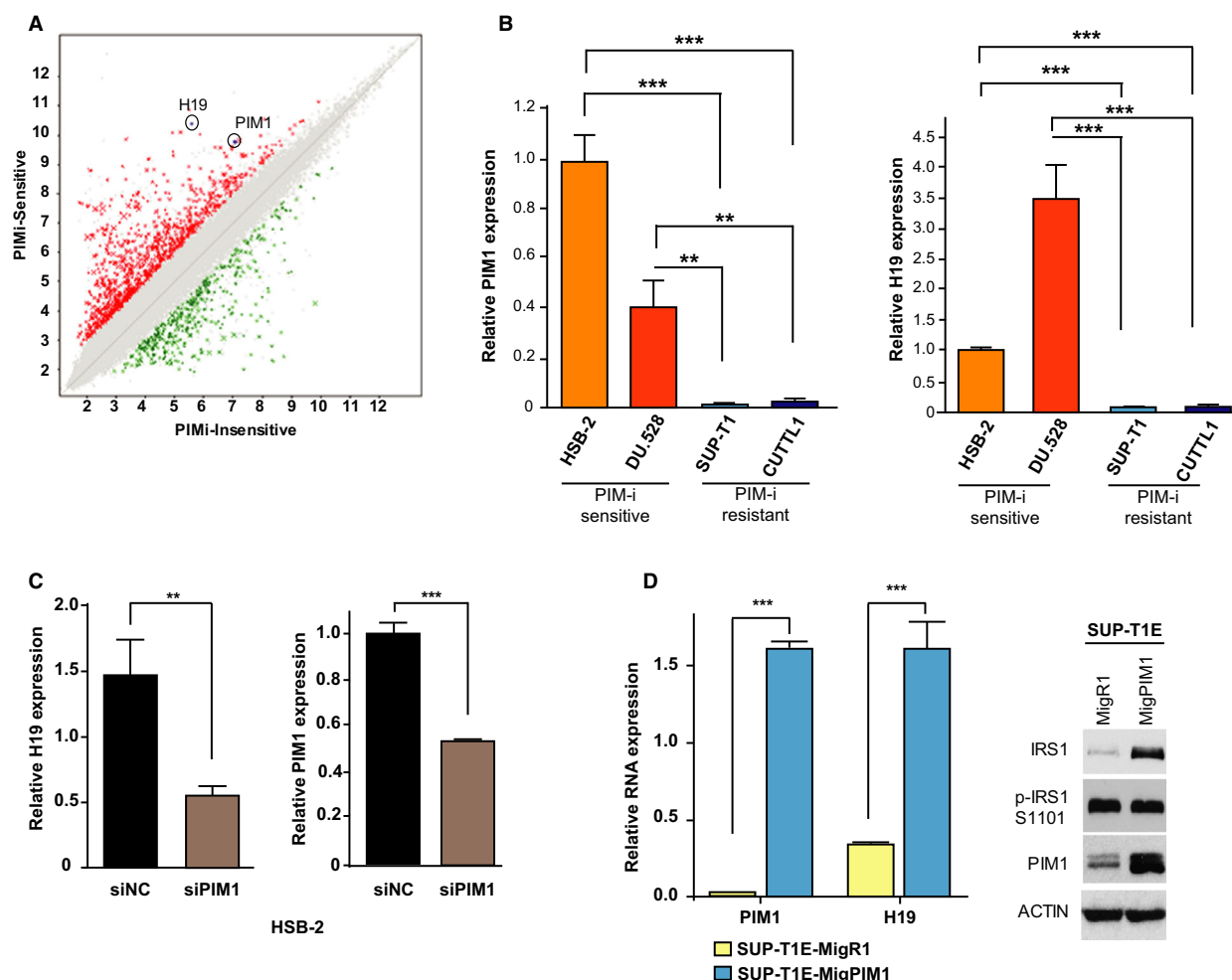


Fig. 1. PIM induces H19 expression in T-ALL cell lines. (A) Scatter plot of genes that are significantly different [fold change (linear) < -2 or $> +2$ and ANOVA P value (condition pair) < 0.05] comparing the RNA levels of PIM-i-sensitive cells with PIM-i-resistant T-ALL cell lines. H19 and PIM1 RNA are circled. Expression levels are visualized as color-coded with red indicating higher levels and green indicating lower levels of gene expression. (B) Relative PIM1 RNA expression in HSB-2, DU.528, SUP-T1, and CUTLL1. (C) Relative RNA expression of H19 in HSB-2, DU.528, SUP-T1, and CUTLL1. (D) Relative RNA expression of H19 and PIM1 in HSB-2 cells transfected with an siRNA to a negative control (siNC as control) or PIM1 (siPIM1). (E) Relative RNA expression of PIM1 and H19 in SUP-T1 cells that were engineered (SUP-T1E) for infection with control vector MigR1 (SUP-T1E MigR1) or PIM1-overexpressing vector MigPIM1 (SUP-T1E MigPIM1). The WB was performed with the indicated antibodies. ACTIN was used as loading control. (B–E) Relative RNA expression was normalized to 18S RNA levels. Values are mean $\pm$ SEM; $n = 3$; $**P < 0.01$, and $***P < 0.001$.

the lncRNA H19 expression is regulated by the PIM kinases in both hematopoietic and solid tumor types.

3.2. PIM overexpression is associated with a stem cell signature

H19 expression has been shown to positively correlate with the level of stem cell genes and pluripotency factors in various tumor types (Bauderlique-Le Roy *et al.*, 2015; Li *et al.*, 2016; Peng *et al.*, 2017; Zeira *et al.*, 2015). Comparison of the PIM-i-resistant (SUP-

T1 and CUTLL1) and PIM-i-sensitive (HSB-2 and DU.528) T-ALL cell lines revealed significantly elevated SOX2 expression in sensitive vs resistant T-ALL cells (Fig. S1). PIM1 overexpression in both T-ALL cell line—SUP-T1—and PCa cell lines—PC3 and DU145—was able to induce the expression of stem cell factors OCT-4, SOX2, NANOG, and KLF4 (Fig. 3A, B). At the protein level, PIM1 overexpression in murine PCa cells mPrEC (Song *et al.*, 2018) augmented the level of the stem cell genes Sox2, NANOG, and Oct-4 (Fig. 3C). The overexpression of PIM2 and

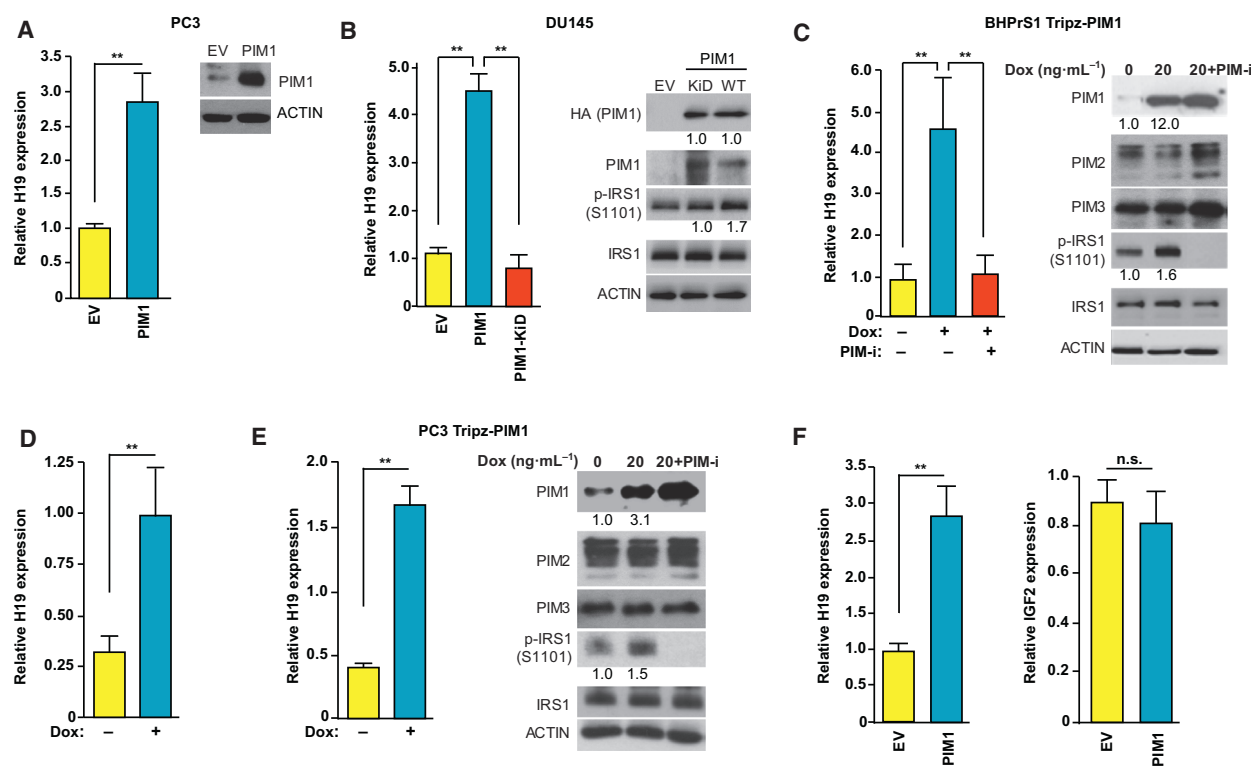


Fig. 2. PIM1 induces H19 expression in PCa cell lines. (A) Relative H19 RNA expression in PC3 cells transfected with EV as control or FUCRW-PIM1 (PIM1). PIM1 protein expression was validated by WB. (B) Relative H19 RNA expression in DU145 cells transfected with pcDNA3 vector encoding either PIM1 (PIM1) or a mutated PIM1-KiD. DU145 (EV) cells were used as control. Respective transfections and PIM1 overexpression were validated by WB. (C) Relative H19 RNA expression in BHP1S1 cells stably expressing Tripz vector encoding Dox-inducible PIM1 (BHP1S1 Tripz-PIM1). H19 expression level was measured by qPCR after Dox treatment (20 ng·mL⁻¹, 24 h) with or without overnight incubation with PIM-i (AZD1208, 3 μ M). The WB was performed with the indicated antibodies. (D) Relative H19 RNA expression in normal human prostate-derived epithelial cells, hPrEC cells stably expressing Tripz vector encoding Dox-inducible PIM1. H19 expression level was measured after Dox treatment (20 ng·mL⁻¹, 24 h) by qPCR. (E) Relative H19 RNA expression in PC3 cells stably expressing Tripz vector encoding Dox-inducible PIM1 (PC3 Tripz-PIM1). H19 expression level was measured after Dox treatment (20 ng·mL⁻¹, 24 h) by qPCR. WB was performed with the indicated antibodies. Inhibitor treatments are the same as in C. (F) Relative H19 and IGF2 RNA expression in PC3 cells transfected with EV as control or FUCRW-PIM1 (PIM1). (A–C, E) For WB: ACTIN was used as loading control. Numerical values shown under the blot are derived as described in Materials and Methods and calculated relative to the controls. (A–F) Relative RNA expression was normalized to 18S RNA levels. Values are mean $\pm$ SEM; $n = 3$; n.s., not significant, $^{**}P < 0.01$.

PIM3 in PC3 cells also led to increases in NANOG and OCT-4 gene expression (Fig. S3A). Using a luciferase reporter controlled by either the OCT-4 proximal or distal enhancers, increasing PIM1 levels in PC3 cells showed increased OCT-4 transcription and promoter activity when PIM1 was induced (Fig. 3D). To confirm that the PIM1-induced stem cell signature is associated with a stemness state, we analyzed PIM1-overexpressing cells for the enrichment of stem cell surface markers (CD24 (Weng *et al.*, 2019), CD29 (Lawson *et al.*, 2010; Vassilopoulos *et al.*, 2014), and CD49B (Erb *et al.*, 2018; Lawson *et al.*, 2010). We demonstrate that PIM1 overexpression in PC3 cells induces stem cell surface markers CD29, CD49b, and CD24, suggesting that increased PIM1 expression is capable of inducing a stem cell-like surface phenotype

through in part regulating these gene changes (Fig. S3B).

To evaluate the effect of PIM1 overexpression on growth of normal stem cells, we isolated WT mouse prostate organoids that have been shown to possess stem cell-like characteristics (Karthaus *et al.*, 2014). PIM1 overexpression in these organoids caused a significant increase in proliferation of these cells (Fig. 4A; PIM1-RFP vs EV-RFP, $P < 0.001$). As in PCa cell lines, overexpressing PIM1 in mouse organoids induced increases in H19 and the stem cell genes Klf4, Oct-4, and Sox2 (Fig. 4B). Importantly, in PC3-LN4 cells, a metastatic variant of PC3 cells that expresses elevated levels of the PIM kinases (Song *et al.*, 2018), H19 KD repressed the expression of NANOG, OCT-4, SOX2, and KLF4 (Fig. 4C).

To validate that the PIM1 increases in stem cell genes are mediated by H19, we knocked down H19 in PIM1-overexpressing SUP-T1E cells using either lentivirus encoding scrambled shRNA (shScr) as a control or shH19 (Fig. 4D). Our results indicated that the KD of H19 abrogated the PIM1-induced stem cell gene expression. Thus, these experiments help to establish a novel signaling cascade connecting PIM1, H19, and stem cell genes, suggesting that the PIM1 induction of stem cell genes may occur with the activity of H19.

3.3. PIM-i treatment reduces H19 expression and inhibits cell growth

To investigate the effect of pharmacological inhibition of PIM kinases on the levels of H19 in T-ALL cell lines, HSB-2, DU.528, and CUTLL1 were treated with the pan-PIM-i AZ1208 (3 μ M, 24 h). This treatment reduced H19 levels in the two ETP-ALL cell lines, HSB-2 and DU.528, by $\sim 60\%$, while it had no effect on H19 levels in the more mature and kinase inhibitor-resistant CUTLL1 cells (Fig. 4E). Similarly,

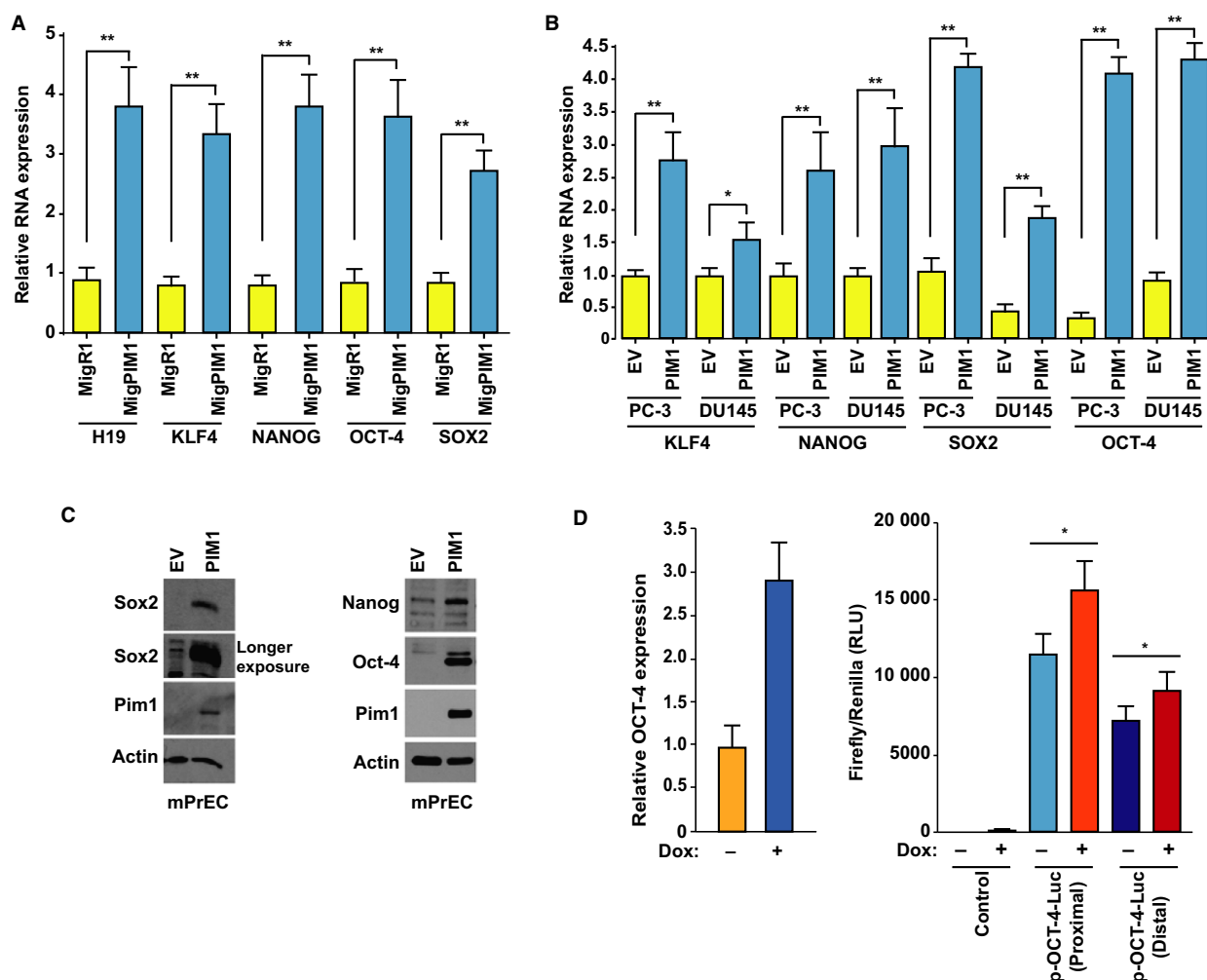


Fig. 3. PIM1 regulates stem cell genes in T-ALL and PCa cell lines. (A) Relative RNA expression of H19, KLF4, NANOG, OCT-4, and SOX2 in SUP-T1E cells transfected with control MigR1 vector (MigR1) or PIM1-overexpressing vector (MigPIM1). (B) Relative RNA expression of KLF4, NANOG, SOX2, and OCT-4 in PC3 and DU145 cells transduced with EV or PIM1 vector (PIM1). (C) WB using the identified antibodies on mPrEC cells in which Trp53 and Rb1 had been knocked out, and stably transduced with EV plasmid or FUCRW-PIM1 (PIM1) plasmid. (D) Relative OCT-4 RNA expression in Dox-inducible PC3-Tripz-PIM1 after Dox treatment (20 ng·mL⁻¹, 48 h). Relative Firefly/Renilla luciferase activity measurement after Dox treatment (20 ng·mL⁻¹, 48 h) in PC3-Tripz-PIM1 cells transfected with luciferase reporter plasmid (p-OCT-4-Luc) controlled by OCT-4 proximal (Proximal) and distal (Distal) enhancers. Untransfected PC3-Tripz-PIM1 cells were used as control. (A, B, D) Relative RNA expression was normalized to 18S RNA levels. Values are mean $\pm$ SEM; $n = 3$; * $P < 0.05$, ** $P < 0.01$.

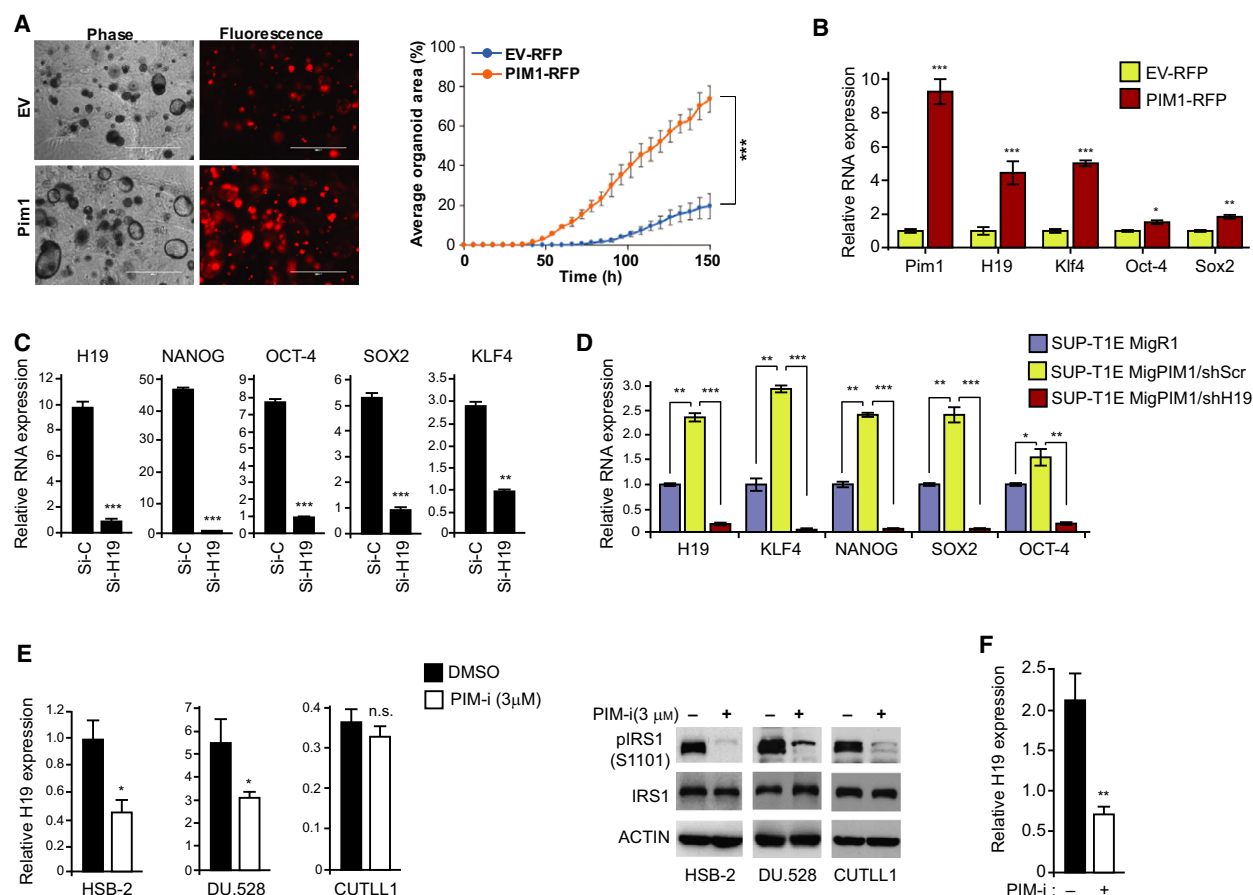


Fig. 4. PIM1 controls H19 expression and regulates tumor growth. (A) Mouse prostate organoid cells transduced with EV (EV-RFP) or FUCRW-PIM1 plasmid (PIM1-RFP; 5000/well) were plated for organoid growth analysis. Representative images of the growth analysis (left panel), scale bar: 1000 μm. Quantification of the organoid growth (right panel). (B) Relative RNA levels of Pim1, H19, Klf4, Oct-4, and Sox2 in mouse prostate organoids described in A. Relative RNA expression was normalized to Hprt RNA. (C) Relative RNA expression of NANOG, OCT-4, SOX2, and KLF4 in PC3-LN4 cells transfected with a control siRNA (si-C) and siRNA to KD H19 (si-H19). Relative RNA expression levels were normalized to ACTIN RNA. (D) Relative RNA expression of H19, KLF4, NANOG, OCT-4, and SOX2 in SUP-T1E cells transfected with control MigR1 vector (MigR1) or PIM1-overexpressing vector (MigPIM1). Mig PIM1 cells were further transfected with shScr and shH19. (E) Relative H19 RNA expression in HSB-2, DU.528, and CUTLL1 after PIM-i treatment (AZD1208, 3 μM, 24 h). DMSO was used for control treatment. Relative RNA expression levels were normalized to ACTIN RNA. WB of these cells with indicated antibodies for validation of PIM1 inhibition. (F) Relative H19 RNA expression in LNCaP cells treated with PIM-i (PIM447, 1 μM, 24 h). DMSO was used for control treatment. Relative RNA expression was normalized to 18S RNA unless specified otherwise. Values are mean ± SEM; $n = 3$; n.s., not significant, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

treatment of LNCaP cells (Fig. 4F) with PIM-i decreased H19 levels. Similar results were obtained in NEPC cell line LASCPC-01 where PIM447, another small molecule pan-PIM-i, significantly decreased H19, NANOG, OCT-4, and SOX2 expression in a dose-dependent manner (Fig. S5). Additionally, we observed that overexpression of PIM1 partially sensitized PIM-i-resistant SUP-T1E cells to PIM-i treatment (Fig. S6). These data demonstrate that PIM1 regulates H19 levels in PIM-dependent but not in PIM-i-insensitive cell lines.

To analyze whether the inhibition of PIM and the subsequent decrease in H19 affected cell growth in PCa, we performed XTT assay on PC3 cells with H19 KD and treated or not with a PIM-i. The combination of si-H19 and PIM-i significantly inhibited cell growth (Fig. 5A), while individual treatments had modest effects. NEPC is a variant of PCa that is clinically unresponsive to chemotherapy treatments. To test this combination treatment in this tumor type, we utilized NEPC patient-derived organoids OWCN-155 (Puca *et al.*, 2018), which have high levels of H19 (Singh

et al., manuscript in preparation). When compared to androgen-responsive PCa cell line LNCaP, these organoids have elevated PIM kinase expression (Fig. S7). Stable KD of H19 in these organoids (Fig. S8) results in a significant growth reduction (Fig. 5B). When H19 KD and PIM-i treatment are combined, there is an even further decrease in the NEPC organoid growth (Fig. 5B). These results demonstrate that kinase inhibition and decreases in this lncRNA can function together to inhibit PCa growth.

3.4. H19 blocks the ability of PIM-i to sensitize prostate cancer cells to hormone blockade

Patients diagnosed with advanced PCa initially respond to androgen deprivation therapy (ADT) but ultimately develop hormone-refractory disease that leads to death. Recent evidence suggests that increased expression of the reprogramming stem cell transcription factor SOX2 makes PCa cells resistant to hormone blockade by the antiandrogen Enza (Mu *et al.*, 2017). To investigate the role of PIM kinases in ADT, we analyzed the effect of Enza treatment on PIM1-overexpressing androgen-responsive PCa cell line LNCaP cells. Interestingly, we observed that while Enza treatment (48 h, 10 μ M) was cytotoxic to the empty vector (EV)-transduced control LNCaP cells (EV), overexpression of PIM1 caused resistance to Enza treatment (Fig. 5C). This result is consistent with our finding that PIM kinase activity stimulates increases in SOX2 (Fig. 3C). To explore this further, we examined the growth of LNCaP with control vector transduction (LNCaP-EV) and H19 overexpression (LNCaP-H19; Fig. S9), followed by treatment with increasing concentrations of PIM447 with or without Enza (2, 5 μ M) treatment for 72 h (Fig. 5D,E). The PIM-i treatment significantly sensitized the control LNCaP cells to growth inhibition by Enza (Fig. 5D). Notably, ComboSyn analysis (Chou, 2010) demonstrated that the PIM447 and Enza combination was highly synergistic in killing LNCaP cells. As shown in Fig. S9, the combination index values were < 1 for multiple PIM447 and Enza combination doses. Overexpression of H19 in LNCaP cells blocked the effect of PIM kinase inhibitor treatment (Fig. 5E), suggesting that the PIM-mediated control of H19 levels modulates Enza sensitivity in LNCaP cells.

4. Discussion

Our results identify a unique signaling cascade that regulates the transcription of stem cell genes in both T-ALL and PCa. PIM1 kinase induces the expression

of lncRNA H19, which in turn stimulates the transcription of the stem cell genes SOX2, NANOG, and OCT-4. We found that the expression of H19 is significantly higher in the T-ALL cell lines with high levels of PIM1 kinase and sensitivity to PIM-i (HSB-2, DU.528, and KOPT-K1) as compared to those with low PIM1 levels and insensitivity to these kinase inhibitors (CUTLL1, HPB-ALL, and SUP-T1). Since PIM kinases (Aksoy *et al.*, 2007; Jimenez-Garcia *et al.*, 2017; Xie and Bayakhmetov, 2016) and H19 (Peng *et al.*, 2017; Zimta *et al.*, 2019) have been both shown to be involved in the maintenance of stemness in solid tumors and hematopoietic malignancies, we decided to investigate whether there is a link between the two in the regulation of stem cell genes. We further demonstrated that PIM-i decreased H19 levels in PIM-i-sensitive cell lines (HSB-2 and DU.528) but has no effect on H19 levels in the PIM-i-insensitive cell line CUTLL1. Overexpression of PIM1 in SUP-T1 cells increased stem cell gene transcription through H19 and restored partial sensitivity to PIM-i. Further, we identified that the stem cell genes SOX2, KLF4, NANOG, and OCT-4 were induced as a result of H19 elevation. These findings were corroborated in PCa cells, indicating the PIM regulation of H19 and induction of stem cell genes in both solid and hematopoietic tumors.

Further analysis of the microarray data comparing the transcriptomes of PIM-i-sensitive vs PIM-i-insensitive cells revealed enrichment of several pathways important for stem cell progression and maintenance (Table S2) in the PIM-i-sensitive cell lines. Studies have shown the involvement of these pathways in maintenance of pluripotent stem cells both in solid tumors and in hematopoietic malignancies. Specific pathways of interest could be chemokine signaling pathway (P value = 0.00019) (Jiang *et al.*, 2017), VEGFA/ VEGFR2 pathway (P value = 0.0002) (O'Donnell *et al.*, 2016), and PI3K-Akt signaling pathway (P value = 0.045) (Madsen *et al.*, 2019) that are enriched in the PIM-i-sensitive cell lines and could be presumably more important in understanding the PIM-specific mechanism. Further experiments are needed to examine the role of PIM and H19 in regulating a broad segment of the genes regulating this stem cell phenotype.

The mechanism by which H19 mediates stemness in liquid and solid tumors (Jiang *et al.*, 2016; Peng *et al.*, 2017; Ren *et al.*, 2018; Sasaki *et al.*, 2018; Zhou *et al.*, 2019) is likely complex. Various studies have pointed out the 'sponge effect' of H19 in which this lncRNA decreases the bioavailability of miRNAs such as Let7, leading to an increase in their target gene expression

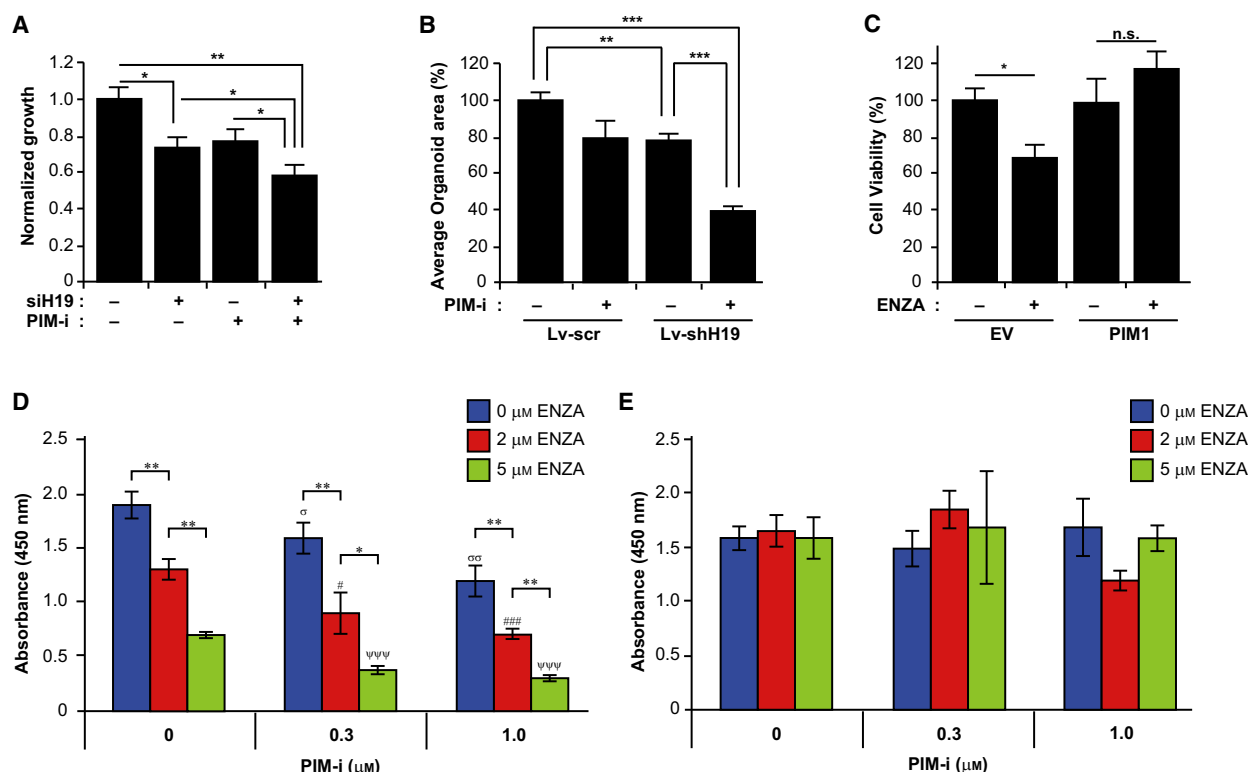


Fig. 5. PIM-i represses growth in combination with inhibition of H19 and sensitizes LNCaP cells to Enza. (A) Cell viability measured by XTT assay of PC3 cells treated with si-H19 and PIM-i (PIM447, 0.5 μM, 72 h). DMSO was used for the control treatment. Cell viability is represented as normalized growth (DMSO = 1). (B) Bar graphs showing growth response measured as percent average organoid area of OWCM-155 NEPC organoids with and without KD of H19 (Lv-shH19 vs Lv-scr) in combination with PIM1-i (PIM447, 3 μM, 4 days). DMSO was used for control treatment. (C) Percent cell viability of LNCaP cells stably expressing EV or FUCRW-PIM1 plasmid (PIM1), treated with Enza (MDV3100, 10 μM, 48 h). DMSO treatment was used as control. (D) Cell viability of LNCaP cells stably expressing EV (LNCaP-EV) treated with increasing concentrations of either Enza (MDV3100, 2 and 5 μM) or PIM-i (PIM447, 0.3 and 1 μM, 72 h) and combination, measured by XTT assay. DMSO was used to treat cells when no drug was added (0 μM). Values are mean ± SEM; $n = 3$; σ represents P values < 0.05 as compared to DMSO treatment. # represents P values < 0.05 as compared to 2 μM Enza. Ψ represents P values < 0.05 as compared to 5 μM Enza. * P < 0.05, ** P < 0.01, (E) Cell viability of LNCaP cells stably overexpressing H19 (LNCaP-H19) treated with Enza or PIM-i and combination, as stated in D. XTT assay was carried out 72 h after the start of drug treatment, and absorbance was measured at 450 nm. Results from cell viability in D and E were represented as absorbance at 450 nm. Values are mean ± SEM; $n = 3$; n.s., not significant, * P < 0.05, ** P < 0.01, *** P < 0.001.

(RAS, MYC, HMGA2, STAT3, and IL-10) (Peng *et al.*, 2017; Zhou *et al.*, 2017). H19 forms a double-negative circuit with let-7 and LIN28 in breast cancer cells, wherein H19 sponges the let7 miRNA that consequently releases and promotes the LIN28 expression. This increase in LIN28 expression further potentiates H19 expression, leading to the feedback circuit that acts to maintain the stem cell state in breast cancer (Peng *et al.*, 2017). The LIN28/let7 has also been associated with increasing the stem cell gene expression in oral squamous cell carcinoma (Chien *et al.*, 2015). The suppression of Let7 by LIN28 derepresses its target mRNAs—ARID3B and HMGA2, which then is thought to increase the transcription of stem cell genes OCT-4 and SOX2, through promoter binding.

However, further studies are needed to investigate the effect of H19 on this double-negative feedback loop in the context of PIM1-overexpressing PCa or T-ALL cells.

Alternatively, H19 may function to increase stem cell gene levels through epigenetic mechanisms. H19 has been documented to bind to *S*-adenosylhomocysteine hydrolase protein inducing genome-wide methylation changes by indirectly regulating *S*-adenosylmethionine-dependent methyltransferases (Zhou *et al.*, 2015). This H19-driven methylation could be sufficient to increase the transcription of stem cell genes. It has been demonstrated by our laboratory (Singh *et al.*, manuscript in preparation) and others that H19 physically interacts with the proteins in the

polycomb repressor complex 2 (PRC2) complex, for example, enhancer of zeste homolog 2, and increases histone H3K27me3 modifications (Fazi *et al.*, 2018). It is possible that similar to the lncRNA HOTAIR (Tsai *et al.*, 2010), H19 could bind both the PRC2 complex and lysine demethylases to mutually activate and repress gene transcription inducing a 'stem-like' state.

The PIM kinases may function to elevate H19 levels by regulating both transcription factor binding and DNA methylation. The promoter region of H19 contains transcription factor binding sites for HIF1 (Wu *et al.*, 2017), androgen receptor (AR; putative), E2F1 (Berteaux *et al.*, 2005), and OCT-4/SOX2 (Zimmerman *et al.*, 2013). In hypoxia, PIM kinases are elevated and increase HIF1 activity (Casillas *et al.*, 2018), thus having the potential to activate H19 transcription. H19 levels have been shown to be hormonally regulated and are inhibited by dihydrotestosterone in androgen-responsive cells (Berteaux *et al.*, 2004), thus pointing to an inverse relation between AR and H19. PIM1 can regulate AR levels by phosphorylation, leading to degradation of this protein (Linn *et al.*, 2012) potentially removing a negative regulator controlling the transcription of this lncRNA.

All the PIM isoforms phosphorylate p27 protein leading to its degradation, stimulating the cell cycle (Morishita *et al.*, 2008), which can indirectly inhibit RB activity. When RB1 activity is lost, E2F1 could bind to the H19 promoter and stimulate increases in its transcription (Berteaux *et al.*, 2005). In turn, H19 affects RB1 phosphorylation by regulating the expression of CDK4 and CCND1 genes (Ohtsuka *et al.*, 2016) that can act to further increase E2F1 activity. In murine ES cells, OCT-4 and SOX2 have been shown to cooperatively bind the ICR of the Igf2/H19 locus, resulting in a hypomethylated state, which stimulates increases in this lncRNA (Zimmerman *et al.*, 2013). Mass spectrometry on PIM1-treated ES cell samples revealed the relative abundance of phosphorylated OCT-4 peptides that contain putative PIM1 phosphorylation at S289 and S290, suggesting that PIM1 phosphorylates OCT-4 (Brumbaugh *et al.*, 2012). PIM kinase inhibitor treatment could block this phosphorylation inhibiting the ability of OCT-4 to work synergistically with SOX2. Consequently, the decreased binding of these two transcription factors could reduce H19 levels.

We showed that expression of PIM1 in T-ALL modulates the methylation status of H19 DMR, suggesting that the PIM kinase could act directly or indirectly as an epigenetic regulator. It has been suggested that PIM regulates DNA methylation. Phosphorylation of heterochromatin protein 1 γ (HP1) at Ser-93 by PIM1 promotes HP1 binding with histone H3K9me3, which

leads to heterochromatin formation and the suppression of gene transcription responsible for proliferation (Jin *et al.*, 2014). Further experiments are required to decipher the exact mechanism by which PIM controls H19 levels.

Prostate cancer cells can escape ADT through a change in lineage identity driven by elevated SOX2. Knocking down SOX2 can restore sensitivity to Enza *in vitro* and in mouse xenograft models (Mu *et al.*, 2017). Our findings are consistent with these observations in that small molecule PIM-i cells downregulate SOX2 and sensitize androgen-responsive LNCaP cells to Enza therapy. This result could be secondary to the ability of these inhibitors to decrease H19. A recent study (Lawrence *et al.*, 2018) showed that the combination of pan-PIM-i and RNA polymerase I inhibitor targeting ribosomal biosynthesis was effective against all four neuroendocrine-like AR-null patient-derived xenografts. These tumor cells exhibited heterogeneous mechanisms of resistance, including AR mutations and genomic structural rearrangements of the AR gene (Lawrence *et al.*, 2018).

5. Conclusions

Our data demonstrate that PIM kinase induction increases the lncRNA H19 and this in turn regulates stem cell genes, including the transcription factor SOX2, which plays a role in controlling the response to ADT. Additionally, we found that while elevated levels of PIM1 contribute resistance to ADT, the combination of a pan-PIM-i and H19 KD can reduce the tumor-forming capacity of highly aggressive NEPC. Together, these data suggest a novel pathway controlled by the PIM kinases whose inhibition could impact clinical outcomes.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

NS, VO and ASK wrote the manuscript. NS, SKRP, JJB, RP, JHS, and VO designed and carried out the experiments. KO and HB supplied essential reagents. NS, VO and ASK conceived the project.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Increased expression of PIM2 in PIM-i sensitive vs resistant T-ALL.

Fig. S2. PIM1 decreased the DNA methylation of H19 DMR in SUPT1 cells.

Fig. S3. PIM1 overexpression induces a stem cell like phenotype in PC3 cells.

Fig. S4. c-MYC expression has no effect on H19 levels.

Fig. S5. Pharmacological inhibition of PIM kinases reduces stem cell gene expression in NEPC cell line.

Fig. S6. PIM1 overexpression restores partial sensitivity in PIM-i resistant T-ALL cells.

Fig. S7. PIM kinase is highly expressed in NEPC.

Fig. S8. KD of H19 expression in NEPC organoid OWCN-155.

Fig. S9. Synergistic effect of pan-PIM-i with Enza in LNCaP/H19.

Table S1. Microarray expression analysis of six T-ALL cell lines.

Table S2. Pathway analysis of transcriptomes from Microarray data from six T-ALL cell lines.