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**14. ABSTRACT**

Prostate cancer (PCa) is the second highest cancer mortality among male cancer in USA. This disease is still incurable because PCa once becomes metastatic and develops drug resistance when cancer cells undergo epithelial-to-mesenchymal transition (EMT) and acquire cancer stem cell (CSC) phenotypes. Emerging evidence has shown that the presence of metastatic PCa is associated with CSC phenotype that is likely associated with its resistance to radiation or chemotherapy. The preliminary data from this study clearly demonstrate that several tumor suppressor microRNAs (miRNAs) involved in regulating these processes are often degraded by IFIT5. Also, elevated IFIT5 is associated with PCa malignancy. Thus, this study will delineate the mechanism of IFIT5 in tumor suppressor miRNA degradation and examine IFIT5 gene regulation. By determining its clinical correlation, this study will provide valuable biomarker(s) for lethality of PCa, which will an immediate impact on patient prognosis and selection for more suitable agent. The outcome of this study will provide a better understanding of miRNAs biogenesis associated with aggressive PCa exhibiting CSC phenotypes. Most importantly, the long-term the impact of this study will generate more effective therapeutic strategy of CRPC.

**15. SUBJECT TERMS**

NONE LISTED

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## 1. INTRODUCTION

MicroRNAs (miRNAs) are small non-coding RNA molecules that regulate gene expression by post-transcriptional degradation or translational repressions by recognizing the 3'-UTR sequence of target mRNA from the specific seed sequence (ca. 2-7 nucleotides) of miRNAs (1). miRNAs have been shown to regulate approximate 60% protein-coding genes via post-transcriptional suppression by facilitating mRNA degradation, or translational inhibition. In general, miRNAs are initially transcribed into a long primary transcript by RNA polymerase II similar to cellular mRNA, and sequentially processed by Drosha and Dicer-mediated endonuclease cleavage to become mature miRNA (2-4). Nevertheless, miRNA biogenesis becomes more complicated when miRNAs are derived from the same gene cluster controlled by the same promoter and yet some is processed with different efficiency at the precursor or mature level (5, 6), which adds more complexities into the scheme of gene regulation.

Epithelial-to-mesenchymal transition (EMT) is considered an initial step for cancer cells to acquire the metastatic potentials. In prostate cancer, many studies have demonstrated the relationship of the onset of EMT phenotypes with cancer metastasis. Knowing EMT as a normal physiologic process takes place during embryonic development, therefore, the cancer cells undergoing EMT appear to have higher potential to acquire cancer stem cell (CSC) phenotypes (7). However, the molecular mechanism(s) associated with EMT or CSC in prostate cancer is not fully understood.

Our preliminary data clearly indicated that elevated IFIT5 is associated with malignant prostate cancer and IFIT5 can target many miRNAs with tumor suppressive function in preventing EMT and CSC, in which IFIT5 can be as a potential therapeutic target. Therefore, it is critical to dissect the mechanism of IFIT5 in degrading miRNA or the regulation of IFIT5. Also, significant clinical correlation of elevated IFIT5 expression in prostate cancer specimens prompt us to explore IFIT5 as prognostic marker for prostate cancer.

REFERENCES


1. Aalto AP, Pasquinelli AE. (2012) Small non-coding RNAs mount a silent revolution in gene expression. Curr Opin Cell Biol., 24:333-340.
2. Bartel DP. (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. Cell, 116:281-297.
3. Feng Y, *et al*. (2011) Drosha processing controls the specificity and efficiency of global microRNA expression. Biochimica et Biophysica Acta, 1809:700-707.
4. Winter J, *et al*. (2009) Many roads to maturity: microRNA biogenesis pathways and their regulation. Nat. Cell Biol. 11:228-234.
5. Chaulk SG, *et al.* (2011) Role of pri-miRNA tertiary structure in miR-17~92 miRNA biogenesis. RNA Biol., 8:1105-1114.
6. Chakraborty S, *et al.* (2012) Pri-miR-17-92a transcript folds into a tertiary structure and autoregulates its processing. RNA, 18:1014-1028.
7. Garg M. (2015) Targeting microRNAs in epithelial-to-mesenchymal transition-induced cancer stem cells: therapeutic approaches in cancer. Expert Opin. Ther. Targets., 19:285-297.

## 2. KEYWORDS

Epithelial-to-mesenchymal transition, cancer stem cell, IFIT5, XRN1 microRNA, microRNA turn over.

## 3. ACCOMPLISHMENTS

**Major goals and accomplishments**

**Aim 1 Dissect the mechanism of IFIT5-mediated miRNA turnover.**

**Major Task:** Unveil new machinery of miRNA turnover**.**

**Milestone:** Manuscript on mechanism of miRNA turnover**.**

The manuscript has been published in Cancer Research this year.

**Aim 2 Determine the regulation of IFIT5 gene in prostate cancer progression.**

**Major Task:** Identify key regulator(s) and inducer(s) of IFIT5 gene expression**.**

**Subtask 1:** Determine the inhibitory mechanism of DAB2IP in regulating IFIT5 expression induced by IFN.

Completed with paper publication.

**Subtask 2:** Examine the mechanism of IFIT5 gene regulation in response to paracrine/juxtacrine stimulation.

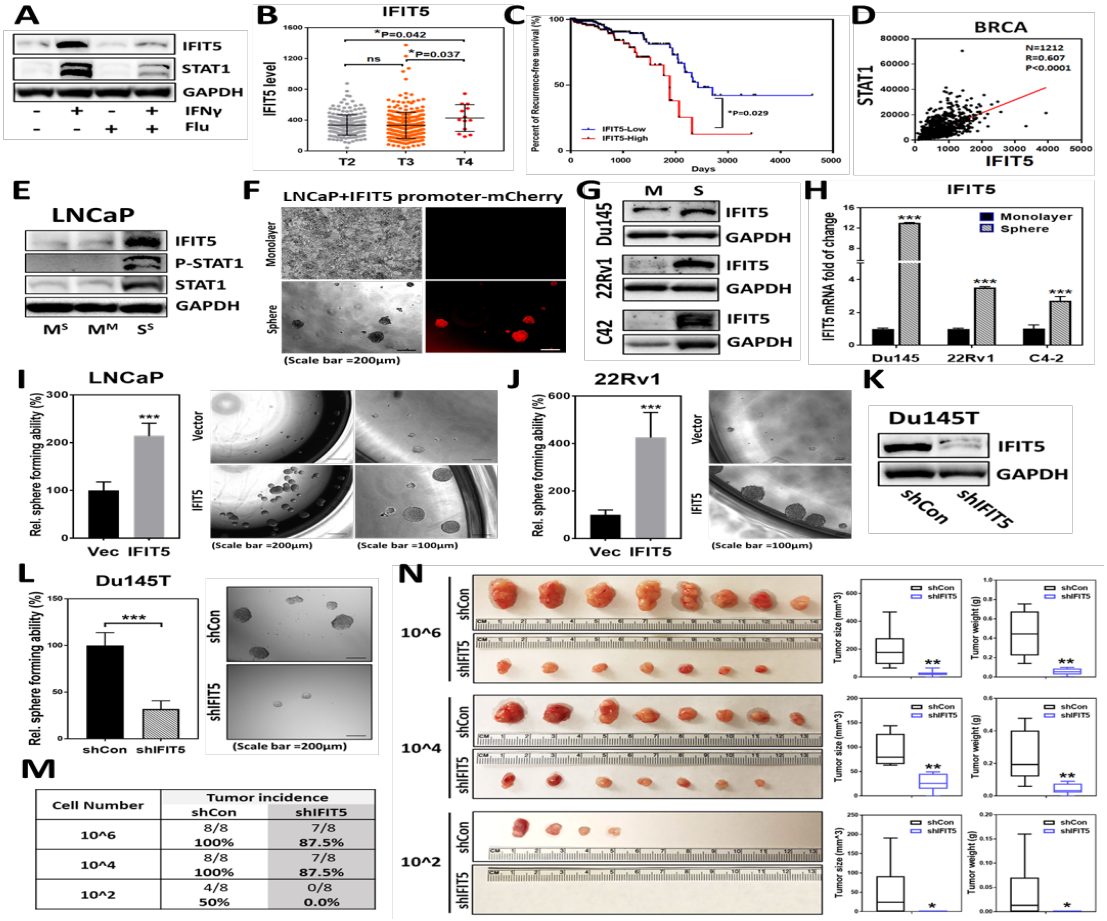
Completed with paper publication.

**Subtask 3:** Determine the role of IFIT5 in prostate cancer progression

The induction of IFIT5 protein and mRNA level is mediated through STAT1 signaling cascade (Fig. 1A). Also, clinical evidence demonstrated a significant elevation of IFIT5 in highly invasive prostatic tumors compared to lower stage specimens (Fig. 1B). Meanwhile, higher expression of IFIT5 also predict a poor survival rate in PCa patients (Fig. 1C). Moreover, a positive correlation between STAT1 and IFIT5 observed in PCa (Fig. 1D). Altogether, we determined to investigate whether IFIT5 is the key molecule relay to STAT1 signaling-mediated acquisition of stemness properties in PCa.
LNCaP or Du145 cells were initially seeded as adherent monolayer and incubated within sphere culture medium ($M^S$). Compared to monolayer culture medium ($M^M$), the sphere medium containing supplement B27, EGF and bFGF ($M^S$) does not induce or activate STAT1 in monolayer adherent culture (Fig. 1E). In contrast to $M^S$, the PCa cells cultured with sphere medium in ultra-low attachment plate ($S^S$) significantly enhanced the STAT1 activity, along with increased protein level of STAT1 and IFIT5 in both cell lines (Fig. 1E). Also, upregulation of mCherry fluorescent protein driven by IFIT5 promoter activity is also observed in the LNCaP tumor sphere, which is not seen while transfected LNCaP cells are cultured under adherent condition (Fig. 1F). In addition, a significant elevation of IFIT5 protein (Fig. 1G) and mRNA (Fig. 1H) is observed in Du145, 22Rv1 and C4-2 tumor spheres, compared to corresponding adherent monolayer culture. Elevated IFIT5 significantly facilitates sphere formation of both LNCaP and 22Rv1

lines in both size and number (Fig. 1I and 1J). In contrast, knockdown of IFIT5 in IFIT5-high Du145 cells (Fig. 1K) leads to significant attenuation of sphere forming ability (Fig. 1L). Consistent with *in vitro* observation, the incidence of Du145 tumor formatiom is significantly reduced in the absence of IFIT5 (Fig. 1M and 1N). In particular, at the low number of Du145 cells with IFIT5 knockdown failed to form any tumor, while the control cohort remains 50% of tumor incidence (Fig. 1M and 1N).

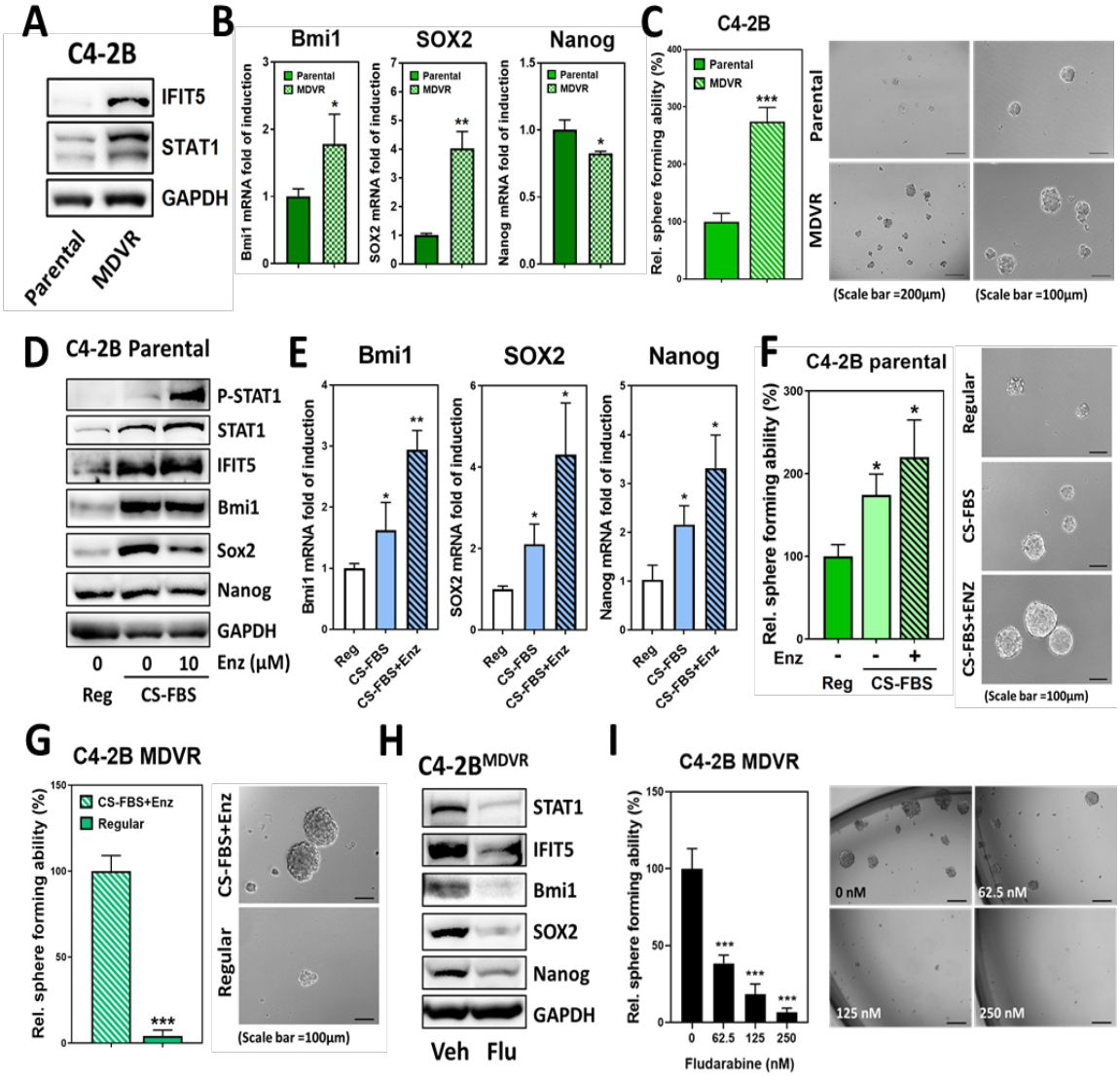
**Figure 1 IFIT5 facilitates the emergence of stemness properties in PCa** (A) Impact of Fludarabine-mediated STAT1 inhibition on IFNγ-induced IFIT5 protein upregulation in Du145 cell. (B) TCGA PCa dataset demonstrating IFIT5 expression level among T2 (n=216), T3 (n=314) and T4 (n=13) stage of PCa patients. (C) Kaplan-Meier survival curve of PCa patients grouped into IFIT5-high and IFIT5-low cohorts. (D) TCGA dataset demonstrating the clinical correlation of IFIT5 with STAT1 in Breast invasive carcinoma (BRCA, N=1212) (E) Induction of STAT1 activation and IFIT5 protein level in LNCaP tumor sphere cultured in sphere condition ($S^S$), compared to monolayer culture within regular ($M^M$) or sphere medium ($M^S$). (F) Expression of IFIT5 promoter-driven mCherry fluorescent protein in tumor sphere compared to monolayer culture. (G) IFIT5 protein upregulation in the sphere (S) derived from each PCa line compared to corresponding monolayer (M) culture. (H) Upregulation of IFIT5 mRNA level in the sphere (S) derived from each PCa line compared to corresponding monolayer (M) culture (***P<0.0001). (I-J) The impact of IFIT5-overexpression on tumor sphere formation compared to vector control (Vec). (***P<0.0001) (K) shRNA knockdown of IFIT5 in Du145 cells. (L) The impact of IFIT5-shRNA knockdown (shIFIT5) on sphere forming ability of Du145 cells, compared to control shRNA (shCon) (***P<0.0001). (M) The tumor incidence of shIFIT5 Du145 cells at $10^6$, $10^4$ and $10^2$ cells, compared to shCon cohort. (N) Quantified size and weight of subcutaneous shIFIT5 Du145 tumors, compared to shCon cohort (*P<0.05, **P<0.001).



Both STAT1 and IFIT5 proteins are significantly increased in C4-2B MDVR compared to the parental C4-2B line (Fig. 2A). Concurrently, significant elevation of both Bmi1 and Sox2 mRNA is seen in C4-2B MDVR line (Fig. 2B), leading to significantly enhanced sphere formation compared to the parental line (Fig. 2C). In contrast, no significant elevation of Nanog is observed in the resistant line. To investigate whether the acquired stemness properties in castration-resistant PCa lines is due to prolonged androgen-deprived condition, we cultured the parental C4-2B cells in 10% Charcoal Stripped-FBS-supplemented Phenol Red free RPMI medium (CS-FBS) without or with additional 10μM ENZ. In contrast to parental C4-2B cultured in regular condition (Reg, 10% regular FBS-supplemented RPMI medium), androgen deprivation significantly increased the protein and mRNA level of STAT1 and IFIT5, which is further elevated by an additional 10 μM ENZ treatment (Fig. 2D). Following an elevation of STAT1 and IFIT5, a significant upregulation of Bmi1, Sox2 and Nanog mRNA level is observed at 3$^{rd}$ week of culture under androgen-deprived condition, and further enhanced by an additional 10 μM ENZ (Fig. 2E), leading to significantly enhanced sphere forming ability in both size and number of C4-2B tumor sphere (Fig. 2F).

Castration resistant C4-2B MDVR cells cultured in regular FBS-supplemented medium for 2 weeks appears to have a significant downregulation of STAT1, IFIT5, Bmi1, Sox2 and Nanog mRNA level as well as attenuated sphere forming ability compared to androgen-deprived condition (Fig. 2G), suggesting AR signaling can abolish the stemness properties of C4-2B MDVR cells. Meanwhile, based on the elevation of STAT1 and IFIT5 under androgen-deprived condition (Fig. 2D), we also examine whether STAT1-driven signaling determines the lineage switch toward more progenitor-like state in castration-resistant PCa lines. Indeed, Fudarabine-mediated blockade of STAT1 signaling results in significant decrease of STAT1, IFIT5, Bmi1, Sox2 and Nanog in either C4-2B MDVR (Fig. 2H). Consequently, Fludarabine treatment leads to dose-dependent attenuation of self-renewal capacity in C4-2B MDVR line (Fig. 2I). Overall, this evidence indicates that emergence of STAT1 signaling under androgen-deprived condition may be a key driver of acquiring stemness properties in castration-resistant PCa lines.

**Figure 2 STAT1 signaling activation confers CSC properties in castration-resistance PCa** (A) Elevation of STAT1 and IFIT5 protein in C4-2B MDVR cells, compared to C4-2B parental line. (B) Expression level of Bmi1, Sox2 and Nanog mRNA in C4-2B MDVR cells, compared to C4-2B parental line (*P<0.05, **P<0.001). (C) The sphere forming ability of C4-2B MDVR cells, compared to C4-2B parental line (***P<0.0001). (D) Expression level of phosphorylated STAT1, STAT1, IFIT5, Bmi1, Sox2 and Nanog proteins in C4-2B cells cultured in CS-FBS-supplemented phenol Red free RPMI without (CS-FBS) or with 10μM Enzalutamide (CS-FBS+Enz), compared to regular culture condition (Reg). (E) Induction of Bmi1, Sox2 and Nanog gene upregulation in C4-2B cells cultured in CS-FBS-supplemented phenol Red free RPMI without (CS-FBS) or with 10μM Enzalutamide (CS-FBS+Enz), compared to regular culture condition (Reg). (*P<0.05, ***P<0.0001). (F) The sphere forming ability of C4-2B cells primarily cultured in CS-FBS-supplemented Pheno Red free RPMI without (CS-FBS) or with 10μM Enzalutamide (CS-FBS+Enz) for 2 weeks, compared to regular culture condition (Reg). (*P<0.05). (G) The sphere forming ability of C4-2B MDVR cells primarily cultured in regular condition for 2 weeks, compared with culture condition of CS-FBS-supplemented phenol Red free RPMI with 20μM Enzalutamide (CS-FBS+Enz) (***P<0.0001). (H) The expression level of STAT1, IFIT5, Bmi1, Sox2 and Nanog proteins in C4-2B MDVR cells treated with Fludarabine (500 nM, 48hrs). (I) The sphere forming ability of C4-2B MDVR cells treated with increased dose of Fludarabine during sphere culture.




In summary, we conclude that several IFN-inducible STAT1-driven genes are significantly upregulated in metastatic PCa and CRPC lines with acquired lineage plasticity. Overall, this study has identified the critical impact of STAT1-regulated IFIT5-mediated microRNA turnover on the acquisition of stemness properties of prostate cancer stem cells. We are preparing manuscript to report this outcome.

**Aim 3 Evaluate IFIT5 as a prognostic marker in prostate cancer patients.**

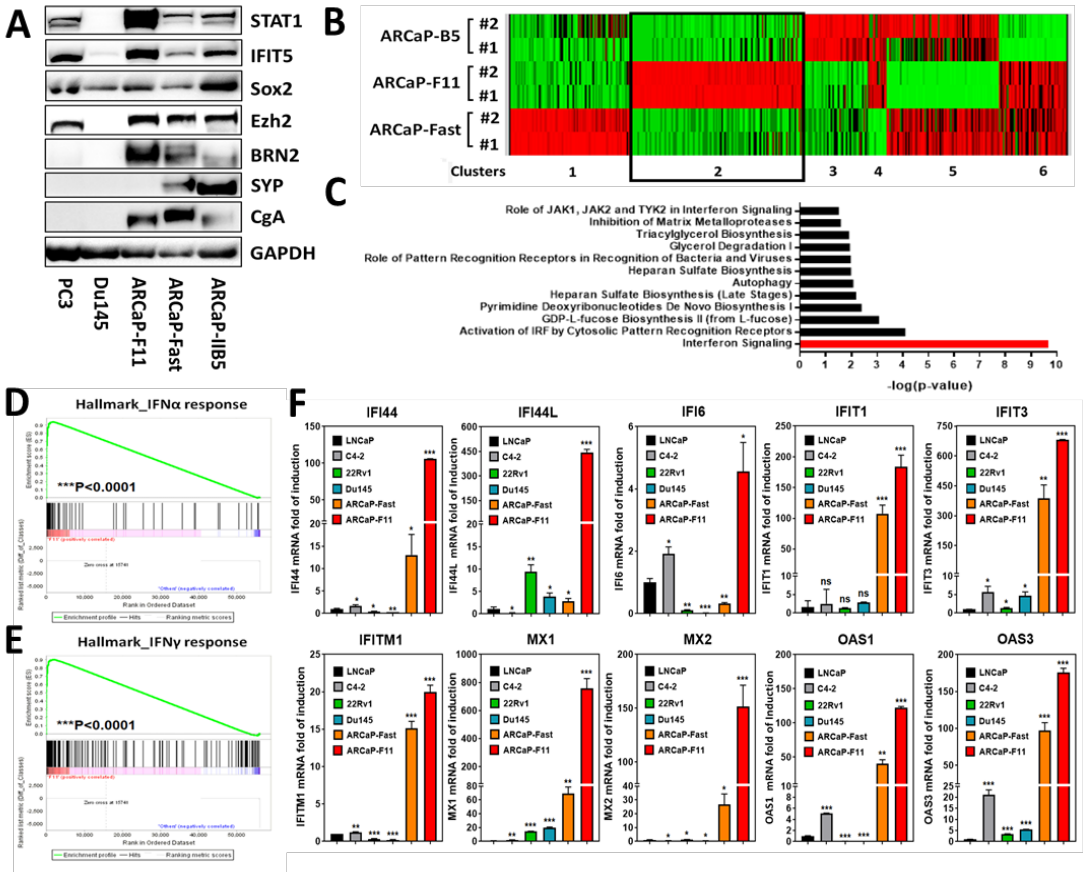
**Major Task:** Determine IFIT5 as a potential prognostic marker for prostate cancer.

Subtask 1: Collect clinical specimens and Study the clinical correlation of IFIT5 and miRNAs in prostate cancer progression.

By profiling several AR-negative PCa cell lines, expression of neural transcriptional factor (BRN2) and NE markers (SYP and CgA) are significantly higher in three ARCaP sublines (ARCaP-F11, ARCaP-Fast and ARCaP-IIB5) compared to castration resistant PC3 and Du145 (Fig. 3A). RNA-seq data indicate that

five different clusters were categorized by its expression level in different ARCaP sublines, and genes belong to the cluster 2 are grouped due to their highly expression in ARCaP-F11 (Fig. 3B). In addition, the Ingenuity pathway analysis demonstrates that most Cluster 2 genes are highly involved in the IFN signaling pathway (Fig. 3C). Suggesting IFN signaling-comprised STAT1-driven genes are highly elevated in ARCaP-F11 subline. Indeed, GSEA demonstrated that ARCaP-F11 has enriched level of genes involved in IFNα (Fig. 3D) and IFNγ response (Fig. 3E) compared to the other two ARCaP sublines. In addition, LNCaP, C4-2, 22Rv1, Du145, and ARCaP-Fast, ARCaP-F11 subline has the highest expression level of IFI44, IFI44L, IFI6, IFIT1, IFIT3, IFITM1, MX1, MX2, OAS1 and OAS3 genes (Fig. 3F).

**Figure 3 STAT1-IFIT5 signaling activation is emerged during the advanced progression of PCa toward CRPC and NePCa** (A) Screening of STAT1, IFIT5, SOX2, EZH2, BRN2, SYP and CgA protein levels among PCa lines. (B) Heat map illustrating the gene expression profile among three ARCaP sublines. (C) The ingenuity pathway analysis suggesting significantly enrichment of IFN signaling pathway genes in ARCaP-F11 subline, compared to ARCaP-Fast and ARCaP-B5 sublines. (D-E) GSEA identifying enrichment of upregulated genes associated with IFNα and IFNγ response in ARCaP-F11 subline, compared to ARCaP-Fast and ARCaP-B5 sublines.




## What opportunities for training and professional development have the project provided?

This project provides excellent training opportunities for molecular cell biology, tumor biology and pathohistologic techniques.

## How were the results disseminated to communities of interest?

Currently, we have published three manuscripts. Also, we are preparing two abstracts for submitting to SBUR meeting and Cold Spring Harbor symposium (JAK-STAT Pathways in Health & Disease) in 2020.

**What do you plan to do during the next reporting period to accomplish the goals?**
Currently, our progress is right on the target based on original SOW; we have completed Specific Aim 1 and 2. Currently, we are planning to conduct more experimental therapy experiments using animal model to strengthen the conclusion of this manuscript. In addition, we are working on public clinical database to determine clinical relevance of STAT1-JAK1 in PCa progression and evaluating the quality of immunohistochemical staining of IFIT5 in clinical specimens.

## 4. IMPACT

**What was the impact on the development of the principal discipline(s) of the project? What was the impact on other disciplines?**
Our study unveils several tumorigenic roles of IFN in prostate cancer including induction of epithelia-to-mesenchymal transition and cancer stem cell. This study also demonstrates that several tumor suppressor microRNAs (miRNAs) involved in regulating these processes are often degraded by IFIT5. Also, elevated IFIT5 is associated with prostate cancer malignancy. Thus, this study will delineate the mechanism of IFIT5 in tumor suppressor miRNA degradation, and examine IFIT5 gene regulation. By determining its clinical correlation, this study will provide valuable biomarker(s) for lethality of prostate cancer, which will an immediate impact on patient prognosis and selection for more suitable agent.
In addition, we are exploring the efficacy of several FDA-approved small molecule inhibitors with STAT1 and JAK1 specificity using in vivo model in hope to provide a new therapeutic strategy for therapy- and castration-resistant prostate cancer patients.

**What was the impact on technology transfer?**
Nothing to Report.

**What was the impact on society beyond science and technology?**
Nothing to report.

## 5. CHANGES/PROBLEMS

Nothing to report.

## 6. PRODUCTS

Lo, U., Lee, C.F., Lee, M.S., Hsieh, J.T. (2017) The role and mechanism of epithelial-to-mesenchymal transition in prostate cancer progression. Int. J. Mol. Sci., 18: pii: E2079. PMID28973968

Lin CJ, Lo UG, Hsieh JT. (2018) The regulatory pathways leading to stem-like cells underlie prostate cancer progression. Asian J Androl. doi: 10.4103/aja.aja_72_18. PMID: 30178777

Lo, U., Pong, R.C., Yang, D., Gandee, L., Hernandez, E., Dang, A., Lin, C-J., Santoyo, J., Ma, S-H., Sonavane, R., Huang, J., Tseng, S-F., Moro, L., Arbini A.A., Kaour, P., Raj, G., He, D., Lai, C.H., Lin, H., Hsieh, J.T. (2019) Interferon-γ induces epithelial-to-mesenchymal transition in prostate cancer via a new mechanism of microRNA processing. Cancer Research, 79:1098-1112.

## 7. PARTICIPANTS AND OTHER COLLABORATING ORGANIZATIONS

Name: U-Ging Lo
Project Role: Research Scientist
No Change

Name: Payal Kapur
Project Role: collaborator
No Change

**Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**
Nothing to Report.

**What other organizations were involved as partners?**
Nothing to Report.

## 8. SPECIAL REPORTING REQUIREMENTS

None.

## 9. APPENDIX

None