INTERLEUKIN-17 INDUCES MTA1 EXPRESSION IN DU145 CELLS

AN ABSTRACT

SUBMITTED ON THE FIFTH DAY OF APRIL 2018

TO GRADUATE PROGRAM IN BIOMEDICAL SCIENCES

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

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MASTER OF SCIENCE IN ANATOMY RESEARCH

BY

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ABSTRACT

Metastasis-associated protein 1 (MTA1) has been reported as a highly relevant protein with the process of cancer metastasis and progression. The MTA1 functioning as a co-regulator interacts with a spectrum of proteins and nucleosomes to modulate the expression of target genes in cancer-promoting processes. However, whether interleukin-17 (IL-17) can induce the expression of MTA1 in castration-resistant prostate cancer still needs to be elucidated. In this study, reverse transcription-polymerase chain reaction (RT-PCR) analysis and Western blot analysis showed that the expression of MTA1 in DU145 cells under IL-17 treatment was significantly higher than the control untreated group. The motility of DU145 cells was also increased with IL-17 treatment in the Wound Healing assays. These findings demonstrate that MTA1’s expression is induced by IL-17 in DU145 prostate cancer cell line, resulting in increased motility of DU145 cells. This study suggests that the expression of MTA1 may be up-regulated by IL-17 in castration-resistant prostate cancer cells.
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I. INTRODUCTION

Cancer cells always emerge from a local site of the human body. If detected early, the cancer is no longer an incurable disease and the primary tumor can be removed when metastasis has not happened. Metastasis is a pathologic process which involves a systematic series of steps in which neoplastic cells leave the original site and migrate to other sites of the body through circulatory system, implantation, and lymphatic transfer. By disseminating, implanting, invading and proliferating at the distant sites, cancer cells progressively become deteriorated and finally result in metastasis. Metastasis-associated gene (MTA) is a family of cancer progression-related genes, including MTA1, MTA1s, MTA-ZG29P, MTA2, MTA3, and MTA3L. Metastasis-associated protein 1 (MTA1), encoded by MTA1 gene, is the first product found in this family and has already been shown that MTA1 is over-expressed in a large range of human cancers, such as breast, gastric, and colorectal cancer. MTA1 physically interacts with histone deacetylase (HDAC) to form the nucleosome remodeling histone deacetylase (NuRD) complex, which has been shown to play an important role in regulating oncogenesis, angiogenesis, and tumor progression of a wide range of malignant tumors. In consequence, MTA1 is one of the most remarkable indicators associated with the cancer progression, aggressive phenotype, and poor prognosis. Moreover, increased MTA1 levels directly enhance tumor cell motility, invasion, and metastasis. Several lines of
evidence suggest that silencing or up-regulating MTA1 is highly correlated with the changing of phenotype in many different cancers. A previous study found that over-expressed MTA1 can down-regulate the expression of E-cadherin to promote tumor invasion in esophageal squamous cell carcinoma (ESCC)\textsuperscript{16}. Additionally, MTA1 has been reported to promote epithelial to mesenchymal transition (EMT) and metastasis via AKT/glycogen synthase kinase 3β (GSK3β)/β-catenin signaling in non-small-cell lung cancer cells\textsuperscript{13}. Through repression of the estrogen receptor α (ERα) and hypoxia-inducible factor-1α (HIF-1α), MTA1 accelerates breast cancer progression and changes the cancer cells to a more aggressive phenotype\textsuperscript{17}. Similarly, MTA1 expression highly correlates with prostate cancer progression\textsuperscript{18}, including angiogenesis\textsuperscript{19}, metastasis\textsuperscript{20}, and invasiveness\textsuperscript{21} in both \textit{in vitro} and \textit{in vivo} studies.

Interleukin-17 (IL-17, also named IL-17A), a pro-inflammatory cytokine produced by a kind of T-helper cells (T\textsubscript{H}17), belongs to the family of Interleukin-17 cytokines. IL-17 family includes IL-17A, IL-17B, IL-17C, IL-17D, IL-17E, and IL-17F\textsuperscript{22}. IL-17A plays an important role in pro-inflammatory responses, autoimmune diseases, and defense against bacterial, fungal and parasitic infections\textsuperscript{23}. In our laboratory’s previous studies, IL-17A has been shown to promote development of hormone-dependent and castration-resistant prostate cancer (CRPC) in mouse prostates\textsuperscript{24,25}. Furthermore, the previous findings demonstrated that matrix metalloproteinase 7 (MMP7) can mediate IL-17’s function in promoting prostate carcinogenesis via induction of EMT\textsuperscript{26}. Another study
in our laboratory showed that IL-17 and insulin/insulin-like growth factor 1 (IGF1) can promote adhesion of prostate cancer cells to vascular endothelial cells\textsuperscript{27}.

Whether IL-17 can induce the expression of MTA1 in prostate cancer remains largely unknown. Our laboratory has previously found that MTA1 protein levels were decreased in IL-17 receptor C (IL-17RC) knockout mouse prostates compared to IL-17RC wild-type mouse prostates\textsuperscript{25}, suggesting that MTA1 may be an IL-17 downstream gene. Therefore, in the present study, we examined whether the expression of MTA1 can be induced by IL-17 in CRPC cells \textit{in vitro}. Through quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis and Western blot analysis, we found that MTA1 was significantly up-regulated by IL-17 in DU145 cells. In addition, we showed that the increased level of MTA1 was correlated with increased motility of DU145 cells. These findings provide a basis for future studies on IL-17 and MTA1 in CRPC.
II. MATERIALS AND METHODS

Reagents and Materials. 10 mL serological pipets (Cat#: 12-106), 25 mL serological pipets (Cat#: 12-104), 60 x 15 mm tissue culture dishes (Cat#: 25-260), 96 x 22 mm tissue culture dishes (Cat#: 25-202), 1000 µL reach tips (Cat#: 24-165RL), 200 µL reach tips (Cat#: 24-150RL), 10 µL reach tips (Cat#: 24-121RL), 25 cm cell lifters (Cat#: 25-271), 1.7 mL microtubes (Cat#: 22-281), and trypsin 0.25% (Cat#: 08171014) were purchased from Genesee Scientific, El Cajon, CA, USA.

Cell Culture. Human prostate cancer cell lines (LNCaP, DU145, and PC-3) were obtained from the American Type Culture Collection (Manassas, VA, USA). Mouse castration-resistant prostate cancer cell line Myc-CaP/CR was a gift from Dr. Leigh Ellis and Dr. Roberto Pili (Roswell Park Cancer Institute, Buffalo, NY, USA). Mouse prostate cancer cell line MPC3 was a kind gift from Dr. Zhenbang Chen (Meharry Medical College, Nashville, TN, USA). All cell lines were stored in liquid nitrogen vapor phase within cryovials. To thaw the frozen cells, the cryovials containing the cells were removed from liquid nitrogen storage and immediately placed into a water-bath at 37°C. The cells were quickly thawed within less than 1 minute by gently swirling the vials at 37°C in the water-bath until there was just a small bit of ice left in the cryovials. The vials
were transferred into the laminar flow hood. Before opening the vials, the gloves and the outside of the cryovials were wiped with 70% ethanol. The thawed cells were transferred dropwise into a 15-ml centrifuge tube containing 5 ml of pre-warmed complete growth medium Dulbecco's Modified Eagle's Medium (DMEM; Caisson Laboratories, Inc., Smithfield, UT, Lot 09161016). The medium contained 10% fetal bovine serum (FBS; Gemini Bio-Products, West Sacramento, CA, lot A02F00G) without any antibiotics. The cell suspension was centrifuged at 200 x g for 5 minutes. After centrifuging, clarity of supernatant and visibility of a complete pellet were checked. The supernatant was removed by using a sterile disposable glass pipette through vacuum aspiration pump without disturbing the cell pellet at the bottom of the centrifuge tube. The cells were gently resuspended by adding 3 ml complete growth medium and transferred into 96-mm cell culture dishes. Four mL complete growth medium was added into each dish. The dishes were gently shaken to disperse the cells evenly. The cells were cultured in an incubator with humidified atmosphere of 5% CO2 at 37°C. For conducting the experiments, the cells were cultured in serum-free medium in 60-mm culture dishes and treated with or without recombinant human IL-17A (Cat# 7955-IL-025/CF, Fisher Scientific, Hampton, NH, USA) or mouse IL-17A (Cat# 421ML025CF, Fisher Scientific, Hampton, NH, USA) for the indicated time points.

**Western blot Analysis.** Proteins of the cells were extracted using RadiolImmu
Precipitation Assay buffer (RIPA lysis buffer) and were stored in -80°C. To denature the
protein, 3 x loading buffer containing 4% mercaptoethanol with the anionic denaturing detergent sodium dodecyl sulfate (SDS) was mixed with the protein samples and boiled at 95°C for 5 minutes. The samples were centrifuged at 1000 xg for 30 seconds at room temperature. 80 mg of protein of each group was loaded to 10% SDS-polyacrylamide gel electrophoresis. The Pre-Stained Rec protein ladder was loaded in the first well as a protein size marker. Electrophoresis was run in 1 x Tris-glycine running buffer for 3 hours at 200V. Transferring filter paper was soaked in 1 x transfer buffer. Polyvinylidene Fluoride (PVDF, Cat#: 10600023, GE Healthcare Life Sciences, Inc., Pittsburgh, PA, USA) membrane was soaked in 100% methanol for 2 minutes. The proteins were transferred from the gel to the membrane in a semi-dry transfer condition. A sandwich of paper/gel/membrane/paper was placed directly between positive and negative electrodes in TRANS-BLOT SD Semi-dry Transfer Cell (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The transfer was run for 30 minutes at 20V. The membrane was incubated in 5% non-fat dry milk diluted in 1 x Tris-buffered saline with 0.1% Tween 20 (TBS-T) buffer (25mM Tris-HCl, 125mM sodium chloride, and 0.1% Tween 20) for 1 hour at room temperature with agitation. Then, the membrane was incubated with 1:1000 dilution primary antibody (mouse anti-MTA1 monoclonal antibodies, Cat# sc-373765, Santa Cruz Biotechnology, Inc., Dallas, TX, USA) in 5% non-fat milk at 4°C with shaking overnight. After washing 3 times with 1 x TBS-T buffer for 10 minutes in each, the membrane was incubated with 1:5000 dilution IRDye 800CW or IRDye 680RD-conjugated secondary
antibodies (goat anti-mouse antibodies, Cat# c60107-06 or c40610-09, LI-COR Biosciences, Inc., Lincoln, NE, USA) at room temperature for 1 hour away from light. After washing 3 times with 1 x TBST buffer for 10 minutes each, the membrane was scanned using Odyssey Infrared Imager (Bio-Rad Laboratories, Inc.) for visualization. For loading control, the membrane was re-probed for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using anti-GAPDH monoclonal antibodies (Cat# 2955484, Millipore, Billerica, MA, USA).

Quantitative Real-time PCR. For quantitative real-time PCR, total RNA was isolated from cell lines using NucleoSpin RNA 250 preps Kit (MACHERY-NAGEL, Inc., Bethlehem, PA, USA). First-strand cDNA synthesis was performed using the PrimeScript™ RT reagent Kit (Cat# RR037A, Takara Bio USA, Inc., Mountain View, CA, USA). Quantitative real-time PCR was performed on the QuantStudio 3 (Applied Biosystems, Foster City, CA, USA) using the PowerUp™ SYBR Green Master Mix kit (Applied Biosystems, Cat#A25741) following the manufacturer's instructions. The following reaction mixtures were prepared in an optical plate (Cat# TCS0803, Bio-Rad Laboratories, Inc.): 10 µl PowerUp™ SYBR Green Master Mix (2X), 5 µl forward and reverse primers (500 nM for each primer), and 5 µl DNA template (approximately 10 ng) in nuclease-free dH2O. The plate was sealed with an optical adhesive cover (iCycler iQ® Optical tape, Cat#: 2239444, Bio-Rad Laboratories, Inc.) and then centrifuged briefly to spin down the contents and eliminate any air bubbles. The conditions for quantitative
PCR reactions were set up on QuantStudio 3 as following: one cycle of 50°C for 2 min, one cycle of 95°C for 2 min, 40 cycles of 95°C for 15 seconds, and 40 cycles of 60°C for 1 min. At the end of the PCR reactions, the samples were subjected to a melting analysis to confirm specificity of the amplicons. The MTA1 primer sequences were: Forward: 5’-GCAGCTGAAGCTGAGAGCAAGTTA-3’; Reverse: 5’-CCTTGACGTGTGAGCTGA-3’. For internal standard, the GAPDH primer sequences were: Forward: 5’-CCACTCCTCCACCTTTGAC-3’; Reverse: 5’-ACCCTGTTGCTGATGCAA-3’. The result of each group was normalized to its own GAPDH level by using the formula \( \Delta Ct (\text{Cycle threshold}) = Ct \text{ of target gene} - Ct \text{ of GAPDH} \). The fold change of mRNA level of each treatment group was calculated as: 
\[
\Delta \Delta Ct = \Delta Ct \text{ of target gene in the treatment group} - \Delta Ct \text{ of target gene in control group},
\]
and fold change \( = 2^{-\Delta \Delta Ct} \).

**Wound Healing Assay.** The DU145 cells were cultured in DMEM supplemented with 10% FBS in 60-mm tissue culture dishes until they reached at a density of approximately 100% confluence as a monolayer. The monolayer was scratched with a new sterile 1000-µl pipette tip across the center of each dish. While scratching across the surface of the well, the long-axial of the tip should always be perpendicular to the bottom of the well. The resulting gap distance (i.e., the wound) therefore equaled to the outer diameter of the end of the tip. After scratching, the dishes were gently washed twice with DMEM to remove the detached cells. The dishes were replenished with fresh medium.
Twenty ng/ml recombinant human IL-17 was added into the medium of the treatment group, while the control group was treated with 0.1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS). The control and IL-17 treatment groups had 3 dishes per group. Photos of the wounds were taken every 24 hours under EVOS FL Auto Microscopy (Life Technologies Inc., Carlsbad, CA, USA). Five points were selected and captured alone each wound. Horizontal gap distance of the migrating cells from the wounds was quantitatively evaluated using software ImageJ.

**Statistical analysis.** All data are presented as the mean ± standard deviation and compared using one-way analysis of variance (ANOVA) followed by Student-NeumanKeul's post-hoc test. Differences among group means were considered statistically significant at P < 0.05.
III. RESULTS

MTA1 expression in prostate cancer cell lines. To assess the expression of MTA1 in human and mouse prostate cancer cells, we firstly examined the protein levels using Western blot analysis. Initially, five different prostate cancer cell lines (LNCaP, DU145, PC-3, MPC3, and Myc-CaP/CR) were selected and cultured in complete cell culture medium. Analysis of the results showed high levels of MTA1 expression in 4 cell lines (LNCaP, PC-3, MPC3, and Myc-CaP/CR) (Figure 1). In contrast, DU145 cells expressed very low levels of MTA1 (Figure 1).

![Western blot analysis of MTA1 expression in PC-3, DU145, LNCaP, MPC3, and Myc-CaP/CR cell lines. GAPDH levels were not equal in this preliminary test.](image)

Figure 1.
IL-17 treatment increased the protein level of MTA1 in DU145 cells. LNCaP, PC-3, DU145, and MPC3 cells were seeded in 5 groups per cell line in 60-mm dishes. When the cell confluence reached approximately 80%, the cells were switched to serum-free DMEM for 24 hours (h) and then treated with 20 ng/ml recombinant human IL-17A (for human LNCaP, PC-3, and DU145 cell lines) or mouse IL-17A (for mouse MPC3 cell line), in order to determine which cell line was most responsive to IL-17 treatment. The five treatment groups were set up as: (1) control group (treated with same volume of PBS with 0.1% BSA); (2) treated with IL-17 for 8 h; (3) treated with IL-17 for 16 h; (4) treated with IL-17 for 24 h; and (5) treated with IL-17 for 36 h. The dosage of IL-17 treatment was based on the previous study. Following the treatment, all cells were harvested in RIPA lysis buffer to isolate the proteins. The protein expression levels of each prostate cancer cell line were determined by Western blot analysis. As shown in Figure 2, when treated with IL-17, the expression of MTA1 significantly increased in DU145 cells, whereas the protein levels of MTA1 in LNCaP, PC-3, and MPC3 cells were not changed significantly. Induction of MTA1 expression in DU145 cells by IL-17 treatment started at 8 h after IL-17 treatment and reached statistical significance at 36 h (Figure 2). In addition, we examined the expression levels of Snail, Slug, and E-cadherin, because the expression level of Snail, Slug, and E-cadherin may be changed by IL-17 treatment based on the previous study. However, we did not observe expression of Snail, Slug, and E-cadherin in DU145 cells (data not shown).
Figure 2. Western blot analysis of MTA1 expression after treatment with IL-17 for 8 h, 16 h, 24 h, and 36 h. (A) LNCaP cells; (B) MPC3 cells; (C) PC-3 cells; and (D) DU145 cells. Three representative blots were analyzed with Odyssey Infrared Imager software; the relative expression represents MTA1 band signals normalized by GAPDH band signals; data show the mean and standard deviations (error bars) of 3 blots. An asterisk (*) indicates a statistically significant difference (p<0.05, n=3).

**IL-17 enhanced MTA1 mRNA expression in DU145 cells.** To identify the role of IL-17 in the regulation of MTA1 mRNA expression in DU145 cells, we treated DU145 cells with 20 ng/ml recombinant human IL-17 for 8, 16, 24, and 36 h, and then isolated
72 h after making a wound in the monolayer culture, the gap distance of the wound was
assay to test the motility of DU145 cells with or without IL-17 treatment. We found that
IL-17 increased the migration ability of DU145 cells. We used Wound Healing

Figure 3. qRT-PCR analysis of mRNA expression of MTA1 in DU145 cells. An asterisk

* indicates a statistically significant difference (p<0.05). Data represent mean ±

standard deviation (error bar) of 3 independent experiments (n=3).

3 expression of MTA1 mRNA was up-regulated at all time points and was significantly
the total RNA and analyzed the mRNA expression using qRT-PCR as shown in Figure

13
dramatically decreased in IL-17 treatment group compared with the control group (Figure 4). Migration rates were assessed by measuring the horizontal distance between the wound edges at the same observed point every 24 h. The repair rates of wound closure were calculated by using the formula: repair rate $=1-n_t/n_0$ ($n_0$: original wound horizontal distance assessed at 0 h; $n_t$: horizontal distance between the wound edges assessed at the same point every 24 h) as previously described\textsuperscript{29}. The quantitative evaluation of wound closure showed statistically significant difference between IL-17 treatment group and the control group at 96 h ($P<0.05$, $n=3$) (Figure 5).

Figure 4. Wound Healing assays of DU145 motility. Wound closure of scrape-wounded cell monolayer was assessed every 24 h. Representative images (magnification, x 100) at 0, 24, 48, 72, and 96 h after wounding from one of three independent experiments are shown.
Figure 5. Repair rate of Wound Healing assay. Data represent mean ± standard deviation (error bar) of 3 independent experiments (n=3). An asterisk (*) indicates a statistically significant difference (p<0.05, n=3) between IL-17 treatment group and control group of DU145 cells.
IV. DISCUSSION

Since MTA1 gene was identified as a metastasis relevant gene in 1994\textsuperscript{30}, MTA1 has been found to be associated with a spectrum of tumor-promoting processes\textsuperscript{5}. Moreover, numerous studies have demonstrated that MTA1 is one of the most universally overexpressed protein associated with human cancers\textsuperscript{31}. However, the importance of MTA1's involvement in prostate cancer progression and metastasis has only been recognized recently. Dhar et al. have recently shown that MTA1 directly induces miR-22 (an epigenetic-microRNA), resulting in down-regulation of E-cadherin that plays an important role in invasiveness of prostate cancer\textsuperscript{3}. Butt et al. have demonstrated that pterostilbene sensitized tumor cells to suberoylanilide hydroxamic acid treatment through targeting MTA1/HIF-1α signaling\textsuperscript{18}. In another study, Kai et al. have shown that the tumors with MTA1 expression had higher proliferative indices and higher levels of VEGF, and became more vascularized\textsuperscript{19}. All of these studies strongly suggest that MTA1 plays a vital role in prostate tumor cell angiogenesis, invasion, and metastasis.

IL-17, known as a pro-inflammatory cytokine, is produced by the T-helper cell (T\textsubscript{H}17). Since Rouvier et al. first identified T\textsubscript{H}17 cell and isolated IL-17 from a rodent T-cell hybridoma in 1993\textsuperscript{33}, IL-17 family has been found with 6 family members, including IL-17A, IL-17B, IL-17C, IL-17D, IL-17E, and IL-17F\textsuperscript{22}. IL-17A plays important roles in a wide range of autoimmune diseases including psoriasis, rheumatoid arthritis, asthma,
lupus, and multiple sclerosis. Interestingly, IL-17 was reported to have both pro-tumorigenic role and anti-tumorigenic role. However, IL-17 has been demonstrated as a cancer promoter in many independent studies of animal models, such as colon cancer, breast cancer, skin cancer, lung cancer, and prostate cancer. The function of IL-17 has been shown to increase cellular proliferation, decrease apoptosis, and promote cancer angiogenesis, invasion, and metastasis. In the previous study, Zhang et al. have demonstrated that IL-17 enhances EMT and tumor cell invasion via IL-17-MMP7-EMT axis by disrupting E-cadherin/β-catenin complex and releasing β-catenin. E-cadherin regulates the properties of the cellular malignant phenotype, including invasion, adhesion, and filament systems. Another study has shown that MMP9 was induced by IL-17 in mouse model of lung cancer, resulting in increased cell motility. Moreover, Zhang et al. have shown that castrate IL-17RC mouse prostates had lower levels of expression of MTA1 compared with castrate IL-17RC mouse prostates.

Based on these previous studies, we aimed to investigate whether IL-17 induces MTA1 expression in CRPC cells and whether IL-17 acts through induction of MTA1 to increase the motility of CRPC cells. For this purpose, we first examined MTA1 expression in selected prostate cancer cell lines, including LNCaP, PC-3, DU145, Myc-CaP/CR, and MPC3. Four of the selected cell lines showed high levels of MTA1 expression except DU145 that expressed very low levels of MTA1. Secondly, we investigated if IL-17 enhances MTA1 expression in CRPC cell lines. We examined
protein and mRNA levels using Western blot analysis and qRT-PCR. MTA1 expression at protein and mRNA levels were significantly higher in IL-17 treatment group compared with the control group. However, the timing of MTA1 mRNA increase was not corresponding with MTA1 protein increase. Western blot showed that MTA1 expression was increased gradually over time of IL-17 treatment and reached the peak level at 36 h after IL-17 treatment. In contrast, the mRNA level of MTA1 reached the highest level at 16 h and then decreased, but remained higher than the control group until 36 h after IL-17 treatment. We speculated that the reasons may be that MTA1 undergoes ubiquitination and degradation by the RING finger E3 ubiquitin ligase constitutive photomorphogenesis protein 1 (COP1). A previous study has been shown that the half-life of MTA1 protein was about 1.5 h. Whether IL-17 can down-regulate the function of COP1 to decrease degradation of MTA1 still remains unknown. We believed that the protein level was gradually accumulated, whereas the transcriptional process has been reduced after 16 h.

Some previous studies have shown that MTA1 overexpression significantly enhanced the ability of migration, invasion, and metastasis of cancer cells. We examined whether the migration ability of DU145 cells was enhanced after IL-17 treatment using Wound Healing assay. We observed that the gap distance of the wound in the IL-17 treatment group was decreased over 24 h compared with the control group, which was statistically significant at 96 h (p<0.05, n=3). The results suggest that IL-17
enhanced DU145 cell migration ability. A previous study showed that the transcription of MTA1 was stimulated by transcriptional factors including NF-κB. IL-17 acts though the IL-17RA:IL-17RC receptor complex to activate NF-κB signaling. Thus, we speculated that MTA1 may be up-regulated to increase the motility of DU145 cells.

In summary, our study demonstrates that IL-17 induces MTA1 expression in DU145 cell line, thus enhancing DU145 cell migration ability. These findings imply that IL-17 may induce MTA1 expression to promote prostate cancer invasion and metastasis. Further studies are necessary to investigate the role of IL-17 and MTA1 in prostate cancer metastasis.
APPENDIX A: PROCEDURES

A. Procedure of cell subculture:

1. Remove the complete growth medium by using sterile disposable pipettes through vacuum aspiration pump in the cell culture hood when the cell confluency reaches to 80-90%.

2. Rinse the cell culture dish 2 times with 5 ml sterile 1x Dulbecco’s phosphate-buffered saline (DPBS) to remove all complete medium.

3. Add 2.0 to 3.0 mL of 0.25% (w/v) Trypsin-EDTA solution to cell culture dish and observe cells under an inverted microscope until more than 90% cell layer is dispersed (usually within 2 to 5 minutes in 37°C).

4. Add 6 ml pre-warmed complete growth medium to neutralize the cell suspension and disperse the medium by pipetting over the cell layer surface several times.

5. Transfer all solution to a sterile 15 ml centrifuge tube.

6. Centrifuge at 1000 x g for 5 minutes.

7. Remove the supernatant gently and resuspend the cell pellet in 3 ml pre-warmed complete growth medium and remove a sample for counting.

8. Determine the total number of cells and percent viability using a hemacytometer.
9. Dilute cell suspension to the seeding density recommended for the cell line, and pipet 7 ml pre-warmed complete growth medium into a new sterile cell culture dish. Recommended seeding density in 100-mm cell culture dish for 2 cell lines: DU145 (1.5x10^6) and PC3 (1.7x10^6).

10. Incubate the cells in the incubator with humidified atmosphere of 5% CO₂ at 37°C

B. Procedure for cell counting with hemocytometer:

1. Take 10 µl dilution prepared cell suspension in cell subculture procedure. The concentration range for cell counting with Neubauer chamber should between 250,000 to 2,500,000 cells/ml.

2. Wipe the glass cover and the Neubauer chamber using 70% ethanol.

3. Put the glass cover on the Neubauer chamber central area.

4. Put a sterile disposable tip at the end of the micropipette.

5. Adjust the micropipette to suck 10 µl cell suspension.

6. Push the pipette plunger slowly until it has arrived at the end of its travel.

7. Remove the pipette tip from the dilution and bring it to the Neubauer chamber.

8. Place pipette tip close to the glass cover edge, right at the center of the Neubauer chamber.
9. Release the plunger slowly watching how the liquid enters the chamber uniformly, being absorbed by capillarity.

10. Place the Neubauer chamber on the microscope stage.

11. Focus the microscope until a sharp image of cells has been shown.

12. Look for the first counting grid square. Totally 5 big squares need be counted.

13. Cells touching the upper and left limits should be counted, unlike cells touching the lower and right limits which should not be taken into account.

14. Concentration (cell/ml) = number of cells*10,000/number of squares*dilution.

C. Procedure for IL-17 treatment:

1. Cells were seeded for 5 groups in 60-mm cell culture dishes. The recommended range of seeding density of each cell line in 60-mm cell culture dishes is: DU145 (1.2-1.7 x 10^6) and PC3 (0.9-1.5 x 10^6).

2. When the cell confluence reaches to 80% observed with microscopy, the cells were switched with 3 ml serum-free DMEM for 24 hours then treated with recombinant IL-17A (20ng/ml).

3. The stock concentration of IL-17 is 20 mg/ml. Diluted to 20 µg/ml with 1 ml sterile PBS and 0.1% Bovine Serum Albumin (BSA) in the cell culture hood.
4. The five treatment groups were: (1) Control group (treated with same volume of PBS with 0.1% BSA); (2) treated with IL-17 for 8 hours (h); (3) treated with IL-17 for 16 h; (4) treated with IL-17 for 24 h; and (5) treated with IL-17 for 36 h.

5. Add 3 µl diluted IL-17 solution separately to each dish.

6. Put the dishes back to the cell culture incubator.

D. Procedure for Total Protein Extraction from the cells:

1. Take out the cells following the treatment process.

2. Tilt the cell culture dish on ice at 30° angle with the horizontal plane.

3. Add 5 ml iced PBS from top of the dish using disposable pipettes gently to wash the cells for 3 times.

4. Drain the PBS, then add 60 µl ice-cold RadioImmunoPrecipitation Assay Buffer (RIPA lysis buffer), which contains 50mM sodium fluoride, 0.5% Igepal CA-630 (NP-40), 10mM sodium phosphate, 150 mM sodium chloride, 25mM Tris (PH 8.0), 1 mM phenylmethysulfonyl fluoride, 2 mM ethylenediaminetetraacetic acid (EDTA), and 1.2 mM sodium vanadate.

5. Scrape adherent cells off the dish using a cold plastic cell scrape, then gently transfer the cell suspension into a pre-cooled micro centrifuge tube.

6. Maintain constant agitation for 30 minutes at 4°C.
7. Spin at 14000 x g for 15 minutes in a 4°C pre-cooled micro centrifuge.

8. Gently remove the tubes from the micro centrifuge and place on ice. Transfer the supernatant to a fresh tube kept on ice and discard the pellet.

9. The protein concentration is measured by Microspectrometer (ThermoFisher Scientific, Inc) following the manufacturer’s instruction.

10. Store at -20°C.

E. Procedure for protein assay:

1. Prepare dye reagent by diluting 1 part Dye Reagent Concentrate with 4 parts distilled, deionized (DDI) water.

2. Prepare three to five dilutions of a protein standard, which is representative of the protein solution to be tested. The linear range of the assay for BSA is 0.2 to 0.9 mg/ml.

3. Set up glass culture tubes for blank, 0, 20, 40, 60, 80 standards and samples. Protein solutions are normally assayed in duplicate.

4. Total volume of standard/sample + diluted dye is 2 mL; use the appropriate amount of BSA standard and 1 µl of sample to be measured.

5. Incubate for 10 minutes at room temperature.

6. Transfer the samples to a cuvette.
7. Measure the protein concentration by using the BioTek SYNERGY microplate reader.

8. Measure absorbance at 595 nm.

F. Procedure for total RNA extraction and purification from the cells:

1. After treatment, total RNAs were isolated by using NucleoSpin RNA purification Kit (MACHERERY-NAGEL).

2. Cells cultured in 60-mm dish were collected by centrifugation at 1000 x g in 4°C for 5 minutes.

3. Add 350 µl Buffer RA1 and 3.5 µl β-mercaptoethanol to the cell pellet or to ground tissue and vortex vigorously.

4. Place NucleoSpin Filter in a collection tube (2 ml), apply the mixture, and centrifuge for 1 minute at 11,000 x g.

5. Discard the NucleoSpin Filter and add 350 µl ethanol (70%) to the homogenized lysate and mix by pipetting up and down (5 times).

6. For each preparation take one NucleoSpin RNA Column (light blue ring) placed in a collection tube. Pipette lysate up and down 2-3 times and load the lysate to the column. Centrifuge for 30 seconds at 11,000 x g. Place the column in a new collection tube (2 ml).
7. Add 350 µl MDB (membrane desalting buffer) and centrifuge at 11,000 x g for 1 minute to dry the membrane.

8. Prepare DNase reaction mixture in a sterile 1.5 ml microcentrifuge tube: for each isolation, add 10 µl reconstituted DNase to 90 µl Reaction Buffer for DNase. Mix by flicking the tube.

9. Apply 95 µl DNase reaction mixture directly onto the center of the silica membrane of the column. Incubate at room temperature for 15 minutes.

10. Add 200 µl Buffer RAW2 to the NucleoSpin RNA Column. Centrifuge for 30 s at 11,000 x g. Place the column into a new Collection Tube.

11. Add 600 µl Buffer RA3 to the NucleoSpin RNA Column. Centrifuge for 30 s at 11,000 x g. Discard flow-through and place the column back into the Collection Tube.

12. Add 250 µl Buffer RA3 to the NucleoSpin RNA Column. Centrifuge for 2 minutes at 11,000 x g to dry the membrane completely. Place the column into a nuclease-free Collection Tube.

13. Elute the RNA in 60 µl RNase-free H₂O₂, (supplied) and centrifuge at 11,000 x g for 1 minutes.

G. Procedure for Reverse Transcription:
1. For cDNA synthesis, use PrimeScript™ RT reagent Kit (TaKaRa, Cat. # RR037A) according to the manufacturer’s instructions.

2. Prepare the following reaction mixture on ice in the microtube for each sample:

   2µl 5X PrimeScript Buffer, 0.5µl PrimeScript RT Enzyme Mix I, 0.5µl Oligo dT Primer (50 µM)*1, 0.5µl Random 6 mers (100 µM)*1, 500 ng total RNA, then adjust the total volume to 10µl by using RNase free dH2O.

3. Incubate the reaction mixture at 37°C for 15 minutes.

4. Heat the reaction mixture at 85°C for 5 seconds.

5. Incubate at 4°C
APPENDIX B: ABBREVIATIONS

CRPC: castration -esistant prostate cancer

MTA1: metastasis-associated protein 1

IL-17: Interleukin-17

HDAC: histone deacetylases

NuRD: nucleosome remodeling deacetylase

EMT: epithelial–to-mesenchymal transition

ESCC: esophageal squamous cell carcinomas

ER-α: estrogen receptor-α

HIF-1α: hypoxia-inducible factors-1α

MMP7: matrix metalloproteinase 7

MMP9: matrix metalloproteinase 9

IGF1: insulin-like growth factor 1

FBS: fetal bovine serum

DMEM: Dulbecco's modified Eagle's medium

RIPA: radioimmunoprecipitation assay

SDS: sodium dodecyl sulfate

TBS-T: tris-buffered saline and 0.1% Tween 20
GAPDH: glyceraldehyde 3-phosphate dehydrogenase

BSA: bovine serum albumin

DPBS: Dulbecco’s phosphate-buffered saline

AKT: protein kinase B

GSK3β: glycogen synthase kinase 3 beta

VEGF: vascular endothelial growth factor

YBX1: Y box binding protein 1

UBE2C: ubiquitin-conjugating enzyme E2 C

COP1: constitutive photomorphogenesis protein 1

NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells
LIST OF REFERENCES


BIOGRAPHY (VITA)

Mr. Ge Shen was born in Hubei province of China on July 8, 1991. He was raised by his father Changlin Shen and mother Huizhen Huang. After graduating from Yuxin High School in 2009, he entered the Southern Medical University, Changsha, China, and majored in Clinical Medicine. During his college time, he served as an intern in Guangdong General Hospital and Chinese People’s Liberation Army General Hospital for two years. He worked at Yanqing County Hospital for one year after graduation. In 2016, he entered the two-year program of Master’s Degree of Research Anatomy in the Department of Structural and Cellular Biology at Tulane University School of Medicine. He has learned experimental knowledge and practiced laboratory techniques from Dr. Zongbing You and the You Lab members.