

CHAPTER-3

3. MATERIALS AND METHODS

3.1 Benign prostate tumor sample collection

The protocol for collection and isolation of cells from human BPH patient tissue was approved by the Institutional ethical committee for human research (IECHR/2016-7) of the Department of Biochemistry, Faculty of Science and informed patient consent (See Annexure VI) was acquired to use the surgically excised tissue for research purposes only. The patients included in this study had benign prostate hyperplasia confirmed by a urethral obstruction (Urine flow intermittency), prostate volume/size and histological evaluation. All the patients included are in the age group of 50-70 years and did not receive any medical treatment for BPH before the surgery. Twenty surgically excised benign prostate tumors from transition zone were obtained during transurethral resection of the prostate (TURP), at GMCRI Medical Hospital, Vadodara, India. Briefly, tissues were washed several times with PBS containing Penicillin-Streptomycin mixture and minced finely using scissors and scalpels in sterile conditions. Tissue fractions were then collected and distributed for transcript, protein and cellular studies in respective medium (TRIzol™, Laemmli and DMEM/F12 (Gibco™)). For gene and protein expression, an equal amount of tissue samples was weight and distributed in respective vials for further processing. Prostate tissue chips were subjected to Smooth Muscle Cells (SMCs) isolation. BPH patient-derived SMCs were isolated using the previously described protocol.¹ Briefly, the tissue chips were washed with PBS containing 2x Penicillin-Streptomycin (PenStrep) solution several times followed by a fine chopping with sterile scalpel and surgical scissors. The chopped tissue was then resuspended in PBS solution and incubated for 10-15 mins at room temperature for removal of fat. The chips were then washed with fresh PBS twice and resuspended in DMEM/F12 media containing 1mg/ml Collagenase Type-I (Sigma) followed by the incubation of the tubes at 37°C with rotation for 2 hours. After incubation, cells and tissue chips were centrifuged, washed and seeded in a culture plate containing DMEM/F12 containing 10% FBS and 1x PenStrep.

Patient Id	Age	Other Diseases	Habit	Serum PSA	Prostate Volume	Histological Confirmation of TURP Tissue
BPH/01	65	-	Smoking	-	-	Fibrotic Stroma - BPH
BPH/02	65	Diabetes	Alcohol (Gluc 222mg/Ml)	-	-	Glandular Moderate BPH
BPH/03	65	-	-	1.30 ng/ml	-	Glandular severe BPH
BPH/04	54	-	-	-	-	Glandular Moderate BPH
BPH/05	58	-	-	-	-	Glandular severe BPH
BPH/06	70	-	-	-	-	Glandular severe BPH
BPH/07	67	-	-	-	-	Stromal BPH
BPH/08	65	-	-	-	-	Stromal BPH
BPH/09	54	-	-	-	-	Stromal BPH
BPH/10	60	-	-	-	-	Stromal BPH
BPH/11	70	High Blood Pressure	Smoking	-	-	Glandular severe
BPH/12	73	-	-	-	-	Glandular Moderate to severe BPH '
BPH/13	67	-	Smoking	-	-	Glandular BPH
BPH/14	60	-	Smoking	-	-	Glandular Mild BPH
BPH/15	60	-	-	-	-	Glandular BPH
BPH/16	65	-	-	-	123CC	Severe Glandular BPH
BPH/17	67	-	-	22.24ng/ml	54.2CC	Glandular severe BPH (No histological Prostate Cancer)
BPH/18	65	-	-	-	62GM	Glandular moderate to severe BPH
BPH/19	76	Diabetes	-	-	124GM	Glandular moderate to severe BPH
BPH/20	55	-	-	-	39.6gm	Glandular BPH

3.2 Prostate Adenocarcinoma dataset analysis on cBioPortal tool

Cancer genomics data analysis was performed on Prostate Adenocarcinoma patients using an open-access cancer database on cBioPortal (<http://www.cbioportal.org/>).^{2, 3} The tool enables access to molecular profiling of PCa patient-derived tumor samples. For present study, we used the Prostate Adenocarcinoma database of 500 patients submitted by TCGA, as the study covered gene and protein expression profiling of all the 500 patients.⁴ The search interface was provided with a user-defined customized set of specific genes to interactively explore its alterations and co-expression across TCGA samples. The search parameters included gene amplification and protein expression from TCGA data with the default setting. All the calculations and clustered heatmap generation were performed as per cBioPortal tool instructions.

3.3 Cell culture conditions

Isolated SMCs were maintained in DMEM-F12 media (Gibco#12500-062) supplemented with 1X penicillin-streptomycin (Gibco#15140-122), 1X Glutamax (Gibco#35050061) and 10% fetal bovine serum (FBS; Gibco#10270-106). Experiments performed with SMCs are on or before passage#6. Benign prostate tumor-derived epithelial (BPH) cell line and BPH tissue-derived primary stromal cells were maintained in DMEM-F12 (Gibco) growth medium; with 10% FBS in 5% CO₂ incubator (Thermo Scientific). Previously, we have isolated BPH epithelial cells in our lab, which is used in the present study on epithelial cells.¹ The epithelial cells was also maintained in DMEM/F12 media containing 10% FBS and 1XPenStrep and used for the experiments before passage#25. For experiments, the epithelial cells were supplemented with Charcoal Stripped Serum (CSS) prepared by adding 2% w/v Charcoal to FBS and incubated for 3 hours at 4°C followed by filter sterilized.⁵ Before the treatments, the cells were serum-starved with DMEM/F12 media for 12 hours and then the respective treatments of 10nM Testosterone Propionate (TP) (Sigma), 200µg/ml IGF1 (R&D Systems), and 50µM Nilutamide (Nil) (Sigma) were given under the appropriate culture conditions with as given in the experimental plan.

3.4 AR shRNA transduction

To inhibit the expression of AR, shRNA Lentiviral Transduction Particles (Sigma: AR-SHCLNV-NM_000044, TRCN0000314657, 10⁷ TU) was used. (Annexure V) Briefly, 20,000 cells were seeded in a 12-well culture plate containing DMEM/F12 media with 10% FBS+1x Pen-Strep (Gibco) solution and allowed them to adhere overnight. The

culture media was then replaced with 10% FBS and 8µg/ml Hexadimethrine bromide (Himedia) containing DMEM/F12 medium to enhance lentiviral mediated transduction. The viral particles (10^7 TU) were added to the concentration of 5µl / 200µl of particles in media and incubated for 24 hours at 37°C in a humidified CO₂ incubator. After incubation, the lentiviral particle-containing media was replenished with 400µl pre-warmed complete culture media (without PenStrep) containing 1µg/ml Puromycin (Sigma) and incubated for 5-10 days with replenishing media at every alternate day with increasing dose of Puromycin up to 8µg/ml. The puromycin resistant cells were confirmed for AR knockdown by immunoblotting followed by sub-culturing them at constant 2.5µg/ml for experiments.

3.5 Conditioned media preparation and stromal-epithelial indirect co-culture

BPH patient-derived cells were serum-starved in DMEM-F12 media for 48 hours (hrs). Post serum starvation, SMCs were replenished with serum-free media (SFM) (DMEM/F12 Gibco#12500-062) containing 10nM Testosterone Propionate (TP) and 50µM Nilutamide (Nil) for 48 hrs to activate and inhibit stromal-AR. Untreated CM (Control-CM), TP treated CM (TP-CM), and Nil treated CM (Nil-CM) were collected in a sterile 50ml tube and centrifuged at 4000rpm for 30min at 4°C to remove cell debris, followed by filtration through 0.22-micron filter. The CM was then stored at -80°C until the treatment of epithelial cells and other experimental use.

3.6 Enzyme-linked immunosorbent assay

Collected CM of each treatment group was thawed from -80°C and IGF1 was quantified by sandwich enzyme-linked immunosorbent assay kit (RayBiotech#ELH-IGF1-1), according to the manufacturer's instructions.

3.7 High-pressure liquid chromatography

High-performance liquid chromatography (HPLC; LC20AD, Shimadzu) with a C4 column (Genetix) was used to analyze stromal cells derived secretome. Solvent A was composed of 0.1% Trifluoroacetic Acid (TFA) in ultrapure water, and solvent B was composed of 0.1% TFA in Acetonitrile (ACN). A linear gradient with a flow rate of 0.3 mL/min was employed, using the following gradient: 1% B (at 7 min), 60% B (at 30 min), 100% B (at 42 min) for 10 min, followed by equilibration with 1% B. Fractions were concentrated with vacuum centrifuge (Speedvac, Savant, USA) and reconstituted with distilled water

(D.W.) and injected in LC system (Waters). The determination of the presence of peptides in the secretome was performed at 215 nm wavelength and data was captured by the HPLC system.

3.8 Cell proliferation assay by MTT

The proliferation of the epithelial cells was determined with the treatments of CM (Control-CM, TP-CM, and Nil-CM) and all three CM containing 10ng/ml Anti-IGF-1-monoclonal antibody (mAb). A separate treatment of 10nM TP, 20ng/ml IGF-1 and 50 μ M Nil were also given to respective epithelial cells. The temporal analysis was performed at 24, 48 and 72 hrs for each treatment group. In brief, approximately 2,500 cells were seeded in a 96 well plate and allowed them to adhere overnight. For assessing the cell proliferation by formazan conversion from MTT, the culture media was aspirated from the respective wells and time, followed by supplementation of 0.5mg/ml MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) (HiMedia#TC191) in SFM. The cells were then incubated for 3 hrs in a humid incubator containing 5% CO₂. MTT media was aspirated post-incubation and formazan crystal in the cells was dissolved in DMSO (Dimethyl sulfoxide) giving a purple colored product. The intensity of the colored product in all the groups was measured at 595 nm using MultiskanTM plate reader (Thermo Scientific). The percent cell proliferation was calculated by the comparison of absorbances across the groups and the analyzed values were plotted on graphs using GraphPad Prism v5.

3.9 Clonogenic assay

The clonogenicity of the epithelia cells treated with stromal cells derived CM along with the direct exposure of 10nM TP and 50 μ M Nil in 5% charcoal-stripped serum (CSS) was determined. Experimentally, around $\sim 20 \times 10^3$ BPH epithelial cells of (between passage#16-20) for secretome treatment and $\sim 2.5 \times 10^3$ BPH epithelial cells for direct TP/Nil treatment were seeded in a six-well plate with DMEM/F12 media with 10% FBS and incubated overnight at 37°C for adherence. The media was aspirated and supplemented with respective treatments for 6 days with regular change of respective treatment media every two days. On the 6th day, treatment media was aspirated and cells were washed with PBS. Cells were fixed with 4% formaldehyde for 10 minutes at RT followed by staining with 0.5% w/v crystal violet for 30 minutes. The solution was removed and washed with distilled water. The cells were then left to dry at RT. The images of the plate were captured

using external camera and magnified images were captured with 20X image resolution under a phase-contrast microscope.

3.10 Immunocytochemistry and Immunohistochemistry

BPH patient-derived SMCs and BPH epithelial cells were grown on a glass coverslip in a 3cm² culture plate containing growth medium. The culture media was aspirated, washed with Phosphate Buffer Saline (PBS) and fixed in 2% p-Formaldehyde at room temperature (RT) for 10 minutes followed by permeabilization with 0.1% Triton-X-100 for 5 minutes. Cells were blocked with the buffer containing 1% Bovine Serum Albumin (BSA) in PBS for 1 hour at RT. Fluorophore-labelled or unlabelled primary antibodies were prepared in 0.1% BSA containing PBS and treated to SMCs overnight at 4°C in dark. Primary Antibodies were removed by washing with 0.1% BSA containing PBS followed by incubation of Fluorophore-labelled secondary antibody, if unlabelled primary antibody was used. Cells were then mounted to a clean glass slide in mounting medium containing anti-fade (Sigma) and imaging was performed on the Nikon-T200 fluorescence microscope or Zeiss LSM710 Confocal microscope.

3.11 Matrigel Tumour-sphere assay

The tumor sphere assay was performed as per the protocol published by Bahmad *et al.*⁶ Briefly, Matrigel (Corning, USA) was thawed overnight at 4°C followed by the preparation of the Matrigel matrix with an 1:1 mixture of Matrigel and chilled DMEM/F12 incomplete media. Approximately 2500 BPH epithelial cells were added in the cold 120µl of Matrigel mixture. A layer of cells with Matrigel was prepared to surround the bottom edge of a 12-well tissue culture plate using cold tips and pipettes on a sterile coolant brick. The mixture was then allowed to solidify for 30 mins at 37°C in a CO₂ incubator. All the respective treatments were prepared in CSS medium and around 500µl of the treatment medium was applied at the hollow space in the center of plate. The treatment medium was replenished every alternative day until the end of the 8-Day treatment period. Bright-field images were captured on Nikon T200 inverted Phase-contrast microscope. The size of the tumor clusters was measured on Nikon NIS-Elements BR software.

3.12 Flow Cytometry/FACS

A single-cell suspension was prepared by scraping and pipetting. Cells were then fixed using 2% PFA for 10 minutes at RT followed by washing with phosphate buffer Saline.

Specific conjugated/unconjugated primary antibodies (Annexure-IV) were incubated for 1 hour at RT in 1% BSA containing PBS buffer. For unconjugated primary antibodies, fluorochrome-labelled Anti-mouse/rabbit secondary antibody was used (with isotype controls) after the incubation of primary antibody for 1 hour at RT. FACS was conducted using a FACS Aria-III (BD Biosciences, San Jose, CA) for cell culture experiments. Gating strategies were used to exclude cell doublets and debris by forward scatter (FSC) and side scatters (SSC). Unstained or secondary Ab controls were used for gating transmitted signals. All the data were analyzed by FlowJo™ Software (BD Biosciences, San Jose, CA).

3.13 Cell fractionation and euchromatin accessibility

Respective treatments were given to cells under appropriate culture conditions for specific incubation time is given in the work plan. Nuclear/cytoplasmic fractionation and chromatin accessibility experiments were performed as per the previously published protocol.^{7 8} Briefly, the treatment media was aspirated followed by chilled PBS (5 ml) wash. The cells were harvested by scraping in chilled PBS and collected in a pre-labeled cold tubes and kept it on ice till further steps. Cells were centrifuged at 600xg for 10 min to resuspend the cell pellet in 300µl Buffer-A (10mM HEPES, 10mM KCl, 1.5mM MgCl₂, 0.34M Sucrose, 10% Glycerol, 0.1% Triton-X100) containing protease inhibitors (1mM PMSF, 1X Protease Inhibitor Cocktail (Sigma), 10mM β-Glycerophosphate, 10mM NaF, and 1mM Sodium Orthovanadate). Post incubation of cells in Buffer-A on ice for 7 mins, samples were centrifuged at 2000xg for 5 min and collected the supernatant as cytoplasmic fraction in a fresh vial. The remaining pellet was washed and resuspended in 250µl Buffer-B (50mM Tris-Cl, 1mM EDTA, 420mM NaCl, 0.34M Sucrose, 10% Sucrose, and 0.5% Nonidet) containing protease inhibitors and incubated for 30min on ice followed by centrifugation at 16,000xg for 30min. The supernatant was collected as Nuclear fraction in a fresh vial. The remaining chromatin pellet was digested with Micrococcal nuclease (MNase) supplemented with 2 mM CaCl₂ using 0.06-unit MNase/1µg chromatin in Buffer A. The chromatin was pelleted at 1000 micrococcal nuclease per 1µg of chromatin. A small fraction of samples collected as High Chromatin Accessibility fraction. The chromatin was pelleted at 1000xg for 7 min and resuspended in 10 mM EDTA followed by addition of 0.5 M NaCl and incubation on vertical rotor for 45 min. The samples were then centrifuged at 16,000xg for 5 min and supernatant was collected as Intermediate

Chromatin Accessibility and Pellet was collected as Low Chromatin Accessibility Fraction. The samples were stored at -20°C until western blot with specific primary antibodies.

3.14 Gene expression

The cells/patient tissue were suspended in TRIZOL™ reagent (Invitrogen# 15596018) for total RNA isolation followed by cDNA synthesis using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems#4368814) as per the manufacturer's protocol. Gene expression was assessed using SyBr Green (Takara#RR820A) and target gene-specific primers (Annexure-I) using the ABI-7500™ qPCR system (Applied Biosystems). The isolation of miRNA was also achieved by TRIZOL reagent and cDNA was synthesized using NCODE VILO MIRNA cDNA synthesis kit (Invitrogen) as per the manufacturer's protocol. The miRNA expression was assessed using specific forward primers (Annexure-III) and Reverse primers of the Kit using qPCR.

3.15 Immunoblotting

BPH patient tissues were stored in laemmli buffer until sonication at 40% amplitude-2 Sec-ON 0.2Sec-OFF for 5 times in chilled condition. Sonicated tissue samples were then centrifuged at 10,000g to remove debris and stored at -20°C until western blotting. For the *in-vitro* study, cells were scraped in chilled PBS and centrifuged after completion of treatment time. Whole-cell lysates were extracted using laemmli buffer containing and sonicated for total protein extraction. Total protein concentration was determined by the Bradford method. 40µg of total protein was loaded in each SDS-PAGE gel. The proteins were transferred to a 0.22-micron nitrocellulose membrane and blocking was performed at RT for 1 hour and incubated with respective primary antibody overnight at 4°C. Horseradish peroxidase (HRP) labelled secondary antibody was incubated for 1 hour at RT and washed with PBS containing 0.001% Tween-20 followed by plain PBS washes. Blots were developed with Bio-Rad enhanced chemiluminescence reagent on Alliance™ (UVTECH Cambridge) Chemidoc instrument. Captured images were analyzed by measuring band intensities of control and treated groups normalized with loading control using ImageJ Software.

3.16 Endogenous immunoprecipitation (IP)

The assessment of protein-protein interaction was performed using the protocol of Dynabeads™ Protein-A (Invitrogen). Briefly, whole-cell lysates were extracted by the buffer containing 50mM of Tris pH8.0, 150mM of NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS and protease inhibitor cocktail (Sigma). The samples were pre-cleared by incubation of magnetic bead (mBead) with samples for 1 hour at 4°C to eliminate non-specific protein binding. The mBead-antibody (Ab) binding was achieved by the incubation of anti-AR/anti-β-CATENIN antibodies for 1 hr at RT. Pre-cleared lysates were then added to the mBead-ab complex and incubated for 12-15 hours at 4°C followed by elution and solubilization in laemmli buffer. The target proteins complexes were assessed by immunoblotting using specific antibodies.

3.17 Chromatin immunoprecipitation (ChIP)

10⁶ cells were seeded and treated with specific doses of TP, IGF1, and Nil (Section 3.3) for a specific time (Chapter-6) in a 15cm² tissue culture plate. Experimental preparations and execution were achieved as given in Pierce Magnetic ChIP Kit (ThermoFisher Scientific#26157) protocol. Briefly, the cells were cross-linked by adding 1% Paraformaldehyde to the culture media for 10 mins at RT followed by the addition of 125mM Glycine solution for 5 minutes at RT. The cells were scraped in chilled PBS buffer and collected in a conical tube followed by centrifugation and collection of the cells in 1.5 ml tubes and resuspension in ChIP specific buffers. Nuclei were isolated with MNase digestion and sonicated with 5 cycles of 11 amplitude for 10 seconds (2-sec ON/1-sec OFF) on ice. The isolated chromatin from the nuclei was incubated with Anti-AR and Anti-β-CATENIN Antibodies overnight with mixing at 4°C. Anti-Rabbit-IgG (CST) and Anti-RNA POLII (ThermoFisher) served as negative and positive control respectively during the ChIP experiment. The protein-DNA complex was isolated using ChIP Grade Protein A/G Magnetic Beads (ThermoFisher) followed by the digestion of proteins with proteinase K and the recovery of genomic DNA fragments. The binding of AR and β-CATENIN to gene-specific the promoters were analyzed using region-specific primers (Annexure-II) using qPCR (ABI-7500).

3.18 Statistical analysis

Correlation analysis was performed using Pearson correlation within patient protein expression study. The graphs represent Means±SEM values in the patient study and *in*

in vitro experiments. All groups are compared with control and analyzed with the Student t-test. The significance between time and treatment variables of the groups was performed with Two-Way ANOVA and Bonferroni post-test. The Statistical significance was considered with the p-values ≤ 0.05 .

3.19 References

- [1] Prajapati A, Gupta S, Bhonde R, Gupta S: Pluripotent stem cell within the prostate could be responsible for benign prostate hyperplasia in human. *J Stem Cell Res Ther* 2014, 4:2.
- [2] Cerami E, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA, Jacobsen A, Byrne CJ, Heuer ML, Larsson E, Antipin Y, Reva B, Goldberg AP, Sander C, Schultz N: The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. *Cancer discovery* 2012, 2:401-4.
- [3] Gao J, Aksoy BA, Dogrusoz U, Dresdner G, Gross B, Sumer SO, Sun Y, Jacobsen A, Sinha R, Larsson E, Cerami E, Sander C, Schultz N: Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. *Science signaling* 2013, 6:p11.
- [4] Cancer Genome Atlas Research N: The Molecular Taxonomy of Primary Prostate Cancer. *Cell* 2015, 163:1011-25.
- [5] Gulick T: Transfection using DEAE-dextran. *Current protocols in molecular biology* 1997, 40:9.2. 1-9.2. 10.
- [6] Bahmad HF, Cheaito K, Chalhoub RM, Hadadeh O, Monzer A, Ballout F, El-Hajj A, Mukherji D, Liu YN, Daoud G, Abou-Kheir W: Sphere-Formation Assay: Three-Dimensional in vitro Culturing of Prostate Cancer Stem/Progenitor Sphere-Forming Cells. *Frontiers in oncology* 2018, 8:347.
- [7] Baghirova S, Hughes BG, Hendzel MJ, Schulz R: Sequential fractionation and isolation of subcellular proteins from tissue or cultured cells. *MethodsX* 2015, 2:440-5.
- [8] Steinbach N, Hasson D, Mathur D, Stratikopoulos EE, Sachidanandam R, Bernstein E, Parsons RE: PTEN interacts with the transcription machinery on chromatin and regulates RNA polymerase II-mediated transcription. *Nucleic acids research* 2019, 47:5573-86.