

CHAPTER II

MATERIALS AND METHODS

Animals and tissues

CD-1 and LPB-Tag 12T-7f mice on a CD-1 background were housed in the animal care facility at Vanderbilt University Medical Center in accordance with the National Institutes of Health (NIH) and institutional guidelines for laboratory animals. CD-1 males and females were purchased from Harlan (Indianapolis, IN). The 12T-7f mouse model for prostate cancer has been described in detail elsewhere [9, 10]. This model was generated by utilizing the long probasin promoter (-10806 to +28 bp, LPB) to target the Large T antigen (*Tag*) gene to the mouse prostate. Small t antigen is not expressed in this model. Tumor incidence is 100% in mice heterozygote for the LPB-Tag transgene and Tag expression occurs specifically in prostate epithelial cells. Tumor growth is rapid, progressing to lesions similar to human low-grade prostatic intraepithelial neoplasia (LGPIN) and high-grade prostatic intraepithelial neoplasia (HGPIN). Metastases are rarely seen. CD-1 as well as 12T-7f mice were bred to provide normal CD-1 and 12T-7f prostates at 1, 2, 3, 4, 5, 6, 10 and 15 and 40 weeks (wk) of age. The number of CD-1 prostates dissected for the early times points were approximately 900 for 2 wks, 450 for 3 wks and 300 for 4 wks of age, resulting in a total of 1,650 mouse dorsal lobes dissected. The CD-1 dorsal prostates were pooled into three groups of 300 for 2 wk, 150 for 3 wks of age and 100 for 4 wks of age. The same number of prostates was dissected for the 12T-7f mice, kept separate until genotyping was completed and

then separated into three pools for each time point as described above. As age increased, the prostatic lobes were larger and the number of prostates required per group decreased. All data presented is generated from the dorsal prostate (DP). For immunohistochemical analysis, individual prostatic lobes were fixed in 10% buffered formalin and subjected to standard processing and paraffin embedding.

Antibodies and Reagents

Mouse anti-human CK8 and mouse anti-human Vimentin were obtained from Sigma (St. Louis, MO). Mouse anti-human E-cadherin, mouse anti-human p120 were from BD Transduction Laboratories (San Diego, CA); Rabbit anti-human AR, goat anti-human PSA, rabbit anti-human Vimentin and rabbit anti-human E-cadherin were from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-human adipophilin, mouse anti-human CD59 were obtained from RDI (Flanders, NJ). Rabbit anti-stathmin, rabbit anti-phospho-p38MAPK and psmad2/3 were obtained from Cell Signalling Technology (Danvers, MA). Rabbit polyclonal anti-p-ser16-stathmin/rabbit polyclonal anti-p-ser63-stathmin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and rabbit polyclonal anti-p-ser25-stathmin/ rabbit polyclonal anti-p-ser38-stathmin were gifts from Dr. Andre Sobel, Paris). Goat anti-human Smad2 was obtained from Santa Cruz Biotechnology(Santa Cruz, CA). Elite Vectastain ABC kit was from Vector Laboratories Inc. (Burlingame, CA). Alexa Fluor 594 or Alexa Fluor 488-conjugated donkey anti-rabbit, mouse or goat secondary antibody and CellTracker Green CMFDA were obtained from Molecular Probes (Eugene, OR). Recombinant human TGF- β 1 was from R&D

systems, Inc. (Minneapolis, MN). Epithelial growth factor (EGF) was from Invitrogen Life Technologies (Carlsbad, CA).

Two-Dimensional Gel Electrophoresis and Mass Spectrometry

Tissue proteins were extracted in lysis buffer (7M urea, 2 M thiourea, 4% CHAPS, 30mM Tris, 5mM magnesium acetate). One hundred micrograms of protein from 3 wk developing CD-1 prostates, 15 wk adult CD-1 prostates and 15 wk 12T-7f prostate tumors were labeled with 200 picomoles of Cy2, CY3, or Cy5 (Amersham Biosciences, Piscataway, NJ) respectively for 30 minutes on ice in the dark. Samples were treated with 2 μ l of 10mM lysine for 10 minutes on ice in the dark to quench the reactions. The three samples were then combined and added to an equal volume of 2X rehydration buffer (7 M Urea, 2 M thiourea, 4% CHAPS, 4mg/ml DTT) supplemented with 0.5% immobilized pH gradient (IPG) buffer 4-7. Standard 2D gel electrophoresis was performed using an IPGphor first-dimension isoelectric focusing unit and 24cm 4-7 IPG strips (Amersham Biosciences, Piscataway, NJ) to co-resolve combined samples labeled with Cy2/3/5. The samples were reduced and alkylated with 1% DTT and 2.5% iodoacetamide in equilibration buffer (6M urea, 30% glycerol, 2% SDS, 50mM Tris pH 8.8) before second dimension separation on a 12% SDS-PAGE using an Ettan DALT 12 unit (Amersham Biosciences, Piscataway, NJ). Hand-cast SDS-PAGE gels were used for second dimension separation using low fluorescence glass plates, with one glass plate presilanized (bind-silane, Amersham Biosciences, Piscataway, NJ) to affix the polymerized gel to only one of the glass plates.

CyDye-specific images were acquired in the 2D 2920 Master Imager (Amersham Biosciences, Piscataway, NJ) using mutually exclusive excitation/emission wavelengths. DeCyder Differential In-gel Analysis software (Amersham Biosciences, Piscataway, NJ) was used to calculate individual protein spot volume ratios. Mean values were calculated from a modeled normal distribution of all spot volume relations. Two standard deviations calculated from the mean were used to identify protein spot features with significant abundance changes within the 95% confidence interval.

2D-gels were stained with Sypro Ruby Red (Molecular Probes) according to the manufacturer's protocol. Proteins of interest were robotically excised and digested in-gel with trypsin protease (Promega) using Ettan Spot Picker and Digester Workstations (Amersham Biosciences, Piscataway, NJ). Peptides were reconstituted in 10 μ l of 0.1% trifluoroacetic acid. C18 ziptip pipette tips (Millipore, Billerica, MA) were used to desalt/concentrate into 2 μ l of 60% acetonitrile and 0.1% trifluoroacetic acid. A Voyager 4700 (Applied Biosystems, Foster City, CA) was used to perform Matrix-assisted laser desorption/ionization, time-of-flight (MALDI-TOF) mass spectrometry. Peptide mass maps, acquired in reflectron mode averaging 2,000 laser shots per spectrum, were internally calibrated to within 20 ppm mass accuracy using trypsin autolytic peptides (m/z = 842.51, 1045.56 and 2211.10). MASCOT (www.matrixscience.com) and ProFound (prowl.rockefeller.edu) database search algorithms were used to interrogate human sequences in the SWISS-PROT and NCBItr databases respectively. Masses of tryptic peptides were used to identify proteins from MALDI-TOF. The search algorithm allowed

for carbamidomethylation of cysteine, partial oxidation of methionic residues and one missed trypsin cleavage.

Cell Culture

DU145 cells were cultured in MEM media (Gibco, Grand Island NY) supplemented with 10% FBS (Hyclone, Logan UT), 2mM glutamine (Gibco), 0.1 mM non-essential amino acid (Gibco) and 1.0 mM sodium pyruvate (Gibco). PC-3 cells were cultured in F-12K Nutrient mixture supplemented with 10% FBS. LNCaP cells were cultured in RPMI 1640 media supplemented with 10% FBS, 1.25g/500 ml of glucose and 1.0 mM sodium pyruvate. BPH1 cells were cultured in RPMI 1640 media supplemented with 5% FBS. NeoTag cells were cultured as described previously [36]. HeLa and HeLa-AR cells were cultured in low glucose DMEM (Gibco) supplemented with 10% FBS. NMuMG cells were maintained in DMEM supplemented with 10% FBS, 4.5g/L glucose and 10 μ g/ml insulin. For the androgen and anti-androgen treatments, LNCaP cells were cultured in 24-well plates at a density of 10⁵ cells/well and upon reaching 80% confluency were treated with androgens and anti-androgens. Cells were harvested 24 hours later in RIPA buffer and processed for western blot analysis as described below.

Primary Prostate Epithelial Cell Culture

Human prostate tissue samples were cut into small pieces in 1 ml medium (RPMI 1640 supplemented with 5% FBS) using scissors and forceps. Samples were then gently rocked for 30 minutes at room temperature (RT) and spun down at 500g for 5 minutes in a tabletop centrifuge maintained at RT. The medium was aspirated and the tissue re-

suspended in 1ml medium (RPMI 1640 supplemented with 20% FBS) and shaken overnight at RT. Next day the tissue was spun at 500g for 5 min at RT, re-suspended in 1 ml culture medium. The culture medium was prepared by supplementing RPMI 1640 with 2.5% charcoal stripped, heat-inactivated FBS, 20mM HEPES buffer, 100 units/ml penicillin, 100 µg streptomycin, 0.25 µg/ml amphotericin B, 50 µg/ml gentamycin, 56 µg/ml bovine pituitary extract, 1x insulin-transferrin-selenium, 10ng/ml epidermal growth factor, and 50 ng/ml cholera toxin. The tissue pieces were plated on 60 mm plates. Any excess medium was removed from the plate. The tissue pieces were spread evenly on the plate with forceps, cultured at 37°C, 5% CO₂ overnight. Medium was changed to larger volume (2-3 ml/60 mm plate) to avoid drying of tissue. Cell growth and color of medium was followed on a daily basis. Medium was changed the medium every 2-4 days. Cells were passaged after 7-10 days in culture.

Western Blot Analysis

Cell proteins were isolated using RIPA buffer and concentration determined using the BCATM Protein Assay Kit (Pierce, Rockford IL). Twenty microgram of total protein were separated by hand cast 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto HybondTM ECLTM Nitrocellulose membranes (Amersham Pharmacia Biotech, Uppsala, Sweden). The membranes were blocked with 5% skim milk (BD, Sparks, MD) and incubated with a 1:1000 dilution of primary antibody in 5% skim milk. Membranes were washed and incubated with 1:10,000 dilution of horseradish peroxidase-linked anti-rabbit IgG (Amersham Pharmacia Biotech, Uppsala, Sweden). Proteins were visualized in ECL-plus solution (Amersham

Pharmacia Biotech, Uppsala, Sweden) and exposed on HyperfilmTM ECLTM (Amersham Pharmacia Biotech, Uppsala, Sweden) for 3-5 minutes. To ensure equal amount of protein was loaded in each lane, the membrane was stripped using 100mM 2-mercaptoethanol, 2% SDS, 62.5mM Tris-HCL for 30 minutes at 70⁰C, washed, blocked and subjected to immunodetection as outlined above using mouse polyclonal anti-GAPDH (1:5000) as primary antibody (IMGENEX, San Diego, CA).

Real-Time RT-PCR Analysis

Stathmin mRNA levels in developing and in adult CD-1 prostates were compared to those in 12T-7f mouse tumors. cDNA was generated from 2 µg total RNA from 1, 2, 3, 4, 5, 6, and 10 wk CD-1 and 12T-7f prostates and used in PCR reactions to determine stathmin expression levels. The following primers with an annealing temperature of 58⁰C were used in the PCR reaction: forward primer, 5'-CACCATGGCTTCTTCTGATATCCAGG-3'; reverse primer, 5'-AAATTAGTCAGCTTCAGTCTCGTCAGC-3'. The standard curve was generated using serial dilutions of pGEM-TEasy-Stathmin plasmid containing full length stathmin cDNA. The human 18S gene was subcloned into pGEM-TEasy (Promega) and served as internal standard. PCR amplification was performed using SYBR Green PCR Core Reagent (Applied Biosystems, Foster City, CA), followed by analysis of melting curves to validate the real-time RT-PCR data and agarose gel electrophoresis of an aliquot from each RT-PCR product to monitor purity of the specific RT-PCR product. Stathmin concentrations (in µM) were determined and standardized to the 18S product from the same sample.

Tissue Recombination Assay

Tissue recombination experiments using NeoTag cells were performed as previously described [36]. Briefly, 100,000 epithelial (NeoTag) and 300,000 mesenchymal (rat embryonic urogenital mesenchyme (UGM)) cells were combined in suspension. The cell mixtures were pelleted and reconstituted in 50 μ l of neutralized type I rat tail collagen prepared as described previously. Recombinants were incubated at 37°C for 15 minutes to allow solidification of the collagen plug and subsequently incubated in RPMI1640 medium supplemented with 5% FBS at 37°C overnight and grafted under the renal capsule of male athymic nude mice. NeoTag cells without UGM were used as control recombinants. After 4 weeks, mice were euthanized and tissue recombinants were dissected and processed for immunohistochemistry by standard methods.

Gleason Pattern Tissue Microarray (TMA)

Slides (H&E) from radical prostatectomy specimen (from 1989 to 2003) were obtained from the Vancouver General Hospital. The patients had no prior treatment. Benign and cancer lesions were identified and marked in donor paraffin blocks using matching H&E reference slides. The TMA was constructed using a manual tissue micro arrayer (Beecher Instruments, Silver Spring, MD). Each marked block for benign and cancer was sampled 4 times with a core diameter of 0.6 mm arrayed in rectangular pattern with 1 mm between the centers of each core, creating a quadruplet TMA layout which was arrayed by increasing Gleason pattern from 3 to 5 (Table 1). To score stathmin staining, the number of stathmin positive cells per core was counted and the cores from

each patient averaged and graphed. A total of 4 cores/patient or 200 cores from 50 patients were included in the TMA.

CMFDA Staining

HPE cells were washed with PBS on reaching the desired confluence and treated with 5 ml of 10 μ M dye (CMFDA from Molecular Probe) in CO₂ -independent medium (GIBCO) for 45 minutes at 37⁰C. Cells were washed twice with PBS and incubated overnight with fresh growth media. To determine whether the dye has leaked out of cells, the media containing the dye was transferred to an unstained set of cells and incubated for 45 minutes at 37⁰C. The plates were then checked for staining using a fluorescence microscope.

Alamar Blue assay

Rate of proliferation of HPE and EMT cells were compared by the Alamar Blue (Biosource, Camarillo, CA) assay. Alamar Blue is a ready-to-use dye solution that shifts color from blue to pink, depending on the number of oxido-reduction reactions in the cell system. The degree of dye reduction is proportional to the number of viable cells, and therefore cell number¹⁹. HPE and EMT cells were cultured in triplicate overnight in 96-well plates at an initial density of 1 \times 10³ cells/well in 100 μ l of medium. Alamar Blue dye was added (10 μ L/well) and absorption at 570/630 nm was determined using an ELISA plate reader (Dynex Technologies, Chantilly VA). Baseline readings were taken from wells containing medium and Alamar Blue only.

Invasion Assay

1.0×10^5 LNCap, HPE, EMT, or PC-3 cells were suspended in 200 μ l of RPMI medium and seeded in the upper chamber (Becton Dickinson) of a transwell. Medium in the lower chamber was replaced with RPMI-1640 medium containing 5% FBS and incubated for 72 hours. Following the removal of the non-invading cells from the upper surface of the Matrigel with a cotton swab, the invasive cells were fixed, stained with 0.5% crystal violet, and counted under a microscope.

Transfection

Transfection with SmartPool siRNA was performed according to manufacturer's protocol. Briefly, DU145 cells were plated at a density of 2.6×10^6 cells/78 cm² of surface area. siRNA transfections were performed in 100mm plates. Twelve hundred picomole of stathmin or control siRNA was added per plate in a total transfection volume of 12.0 ml. Stathmin knockdown was confirmed by western blot analysis 24 hours after transfection.

To over-express stathmin in NMuMG cells, a full length mouse stathmin cDNA in pDream2.1 vector (Genscript, Piscataway, NJ) was transfected using Lipofectamine 2000 (Invitrogen, Carisbad CA). transfection was performed according to manufacturer's protocol. Briefly, NMuMG cells were plated in 24-well plates and cultured till 90-95% confluent. Stathmin cDNA vector and Lipofectamine were diluted separately in Opti-MEM I using a DNA (μ g) to Lipofectamine 2000 (μ l) ratio of 1:3. After 5 minutes of incubation at room temperature, diluted DNA was combined with diluted Opti-MEM I and incubated for an additional 20 minutes at room temperature. One hundred microlitre

of the complex thus obtained were added to each well and mixed gently by rocking back and forth. Cells were incubated at 37°C in a CO₂ incubator for 48 hours prior to testing for transgene expression by western blot analysis.

Immunohistochemistry (IHC) and Immunofluorescence (IF) and Confocal Microscopy

Archival human prostate specimens as well as CD-1 and 12T-7f mouse prostate samples were formalin-fixed and paraffin-embedded. Five micron tissue sections were cut, deparaffinized and subjected to antigen retrieval by immersing the slides in Antigen Unmasking Solution (Vector Laboratories, Burlingame, CA), microwaving for 10 minutes and allowing the slides to cool to room temperature in the buffer. The slides were then washed twice in distilled water prior to IHC analysis. Slides were incubated with a 1:50 dilution of rabbit polyclonal anti-stathmin antibody (Cell Signaling Technology, Boston, MA) for standard IHC analysis and a combination of anti-stathmin and monoclonal mouse anti-p63 antibody (NeoMarkers, Fremont, CA) for double IF microscopy overnight at 4°C. For standard IHC, sections were processed and developed using DakoCytomation LSAB[®]+ System-HRP (Dako North America Inc., Carpinteria, CA). The sections were counterstained with hematoxylin, dehydrated and mounted with Permount[®] (Fisher Scientific, Hampton, NH). For double IF analysis, sections were blocked using 3% donkey serum and 3% BSA in phosphate buffered saline (PBS). After incubating with primary antibodies, sections were washed and incubated with donkey anti-rabbit Alexa Fluor 488 (green) and donkey anti-mouse Alexa Fluor 594 (red) (Molecular Probes) secondary antibodies to detect stathmin and p63 respectively. Sections were washed and mounted in Vectashield[®] mounting medium containing DAPI

(Vector Laboratories, Burlingame, CA). All digital images were captured using the Zeiss Axiovision Camera and software attached to a Zeiss Imager M1 microscope. The Tissue Microarray was stained for stathmin utilizing the Discovery XT Autostainer (Ventana Medical System Inc, Tuscan Arizona) and DABMap.

Cultured cells were harvested by trypsinization and attached onto glass slides (VWR) by incubation at 37°C overnight. Slides were fixed in 4% paraformaldehyde diluted in PBS for 15 min, washed three times in PBS, and permeabilized for 5 min on ice in PBS + 0.1% Triton X-100. Slides were blocked in PBS + 3% BSA +3% donkey serum for 30 minutes at room temperature. The slides were incubated with primary antibodies for 1 hour at room temperature, and subsequently incubated with Alexa Fluor 594 and/or Alexa Fluor 488-conjugated donkey anti-rabbit, mouse or goat secondary antibody (Molecular Probes) for 1 hour at room temperature. Then, cells were washed in PBS and mounted using Vectashield mounting medium containing 4,6-diamidino-2-phenylindole (DAPI) to counter-stain nuclei (Vector Laboratories). Images were captured with a Leica fluorescence microscope equipped with a digital camera. To demonstrate specificity of staining, control slides were stained as described above with the omission of either the primary antisera or the secondary antibodies. For confocal microscopy, the slides were washed, mounted in 50% glycerol, and imaged with a Zeiss LSM510 Meta Laser Scanning microscope. Stacks were acquired with LSM510 software; merged images were made with NIH ImageJ. All confocal images were acquired with a Plan-Apochromat 63x/1.4 oil differential interference contrast microscope objective at the following settings: wavelength 488 nm 5%, 543 nm 37%, and 633 nm 10%; filters: Ch2-

1: BP 505-550, Ch3-2: BP 560-615, and ChS1-3: 649-756 with a pinhole of 281 micrometers. Images have identical microscope settings between treatment groups. Image brightness/contrast was not altered between treatment groups; thus, intensity comparisons could be made.