Supplementary information

Material and methods

Reagents

Casodex® was purchased from Toronto Research Chemicals and Naringenin from Sigma, MDV3100 for *in vitro* study, and SB431542 were purchased from Selleck Chemicals. LY294002 was purchased from Cayman Chemicals, and MMP9 inhibitor from EMD Millipore. Human recombinant TGF-β was purchased from Cell Signaling. ASC-J9®, CTS, and the MDV3100 used for *in vivo* studies were synthesized by Dr. Defeng Xu in Shanhai JaioTong University. All other chemicals and solvents used in this study were of reagent grade or high performance liquid chromatography (HPLC) grade.

3D Invasion Assay.

The 3D invasion assay was modified from the previous publication (1). Briefly, 1 x 10^4 cells in 200 µl media containing 1% matrigel were plated into the collagen/matrigel mixture coated 48-well plate. The DHT, anti-androgens, and ~~the~~ inhibitors were added as described, and the media were replaced every 4 days. The spheres with protrusion were considered as positive for cell invasion. After 2 wks, the spheres with/without protrusion were recorded in each well under the Olympus IX70 inverted microscope. Each experiment was repeated twice and each experiment was done in triplicates.

Detection of Luciferase Signaling by IVIS System

The xenografted mice were injected with D-luciferin (Gold Biotechnology) at 150 mg/kg in DPBS by i.p. at 10 min before acquiring the images. The animals were anesthetized and maintained using continuous flow of 2% isoflurane (Abbott Laboratories, North Chicago, IL), and placed onto the stage inside the camera box. Detection of the primary and metastasis tumors with luciferase signals were performed by the IVIS ImagingSystem (Xenogen, Alameda, CA). Images and measurements of bioluminescent signals were acquired and analyzed using Living Image software (Xenogen). The images were over-exposed for 30 sec to detect the potential metastatic signals. The luciferase signals were displayed as a pseudocolor overlay onto a gray scale animal image with the unit photons/second (ph/sec). The bioluminescence under 5x10^4 ph/sec was considered as the background. After euthanizing the mice, the primary tumors were removed and the luciferase signals on the mice were acquired again for 30 sec to

confirm the visible lymph node metastases and to evaluate the potential invisible metastasis, such as in the bone. The metastatic lesions were further confirmed by various methods as tumor lesions.

Western Blot

The cells were washed with 1x PBS and scraped into a lysis buffer containing the proteinase inhibitor cocktail (Roche). Protein concentrations were measured with the BCA protein reagent (Pierce Chemical, Rockford, IL). Approximately 50 μg of protein/lane were loaded and run on the polyacrylamide gel with a Tris/glycine running buffer system and then transferred onto a polyvinylidene difluoride membrane. The antibodies (Akt, phosphorylated Akt, and phosphorylated Smad3, from Cell signaling; MMP9, from Abcam; Smad3, from BD Bioscience; TGF-β, and GAPDH, from Santa Cruz Biotechnology) with dilutions of 1:500 to 1:1,000 were incubated overnight in the cold room. The horseradish peroxidase-conjugated secondary antibody (Pierce Chemical, Rockford, IL) was used and the signals were detected by adding the enhanced chemiluminescence Western blotting detection reagents (Amersham Biosciences). The quantification of Western blotting results was done by Image Lab statistic software (Bio-Rad).

Quantitative PCR

Total RNAs were extracted from PCa cells using Trizol (Invitrogen). Reverse transcription was performed using the iScript reverse transcription kit (Bio-Rad). Quantitative real-time polymerase chain reaction (PCR) analyses using the comparative CT method were performed on an ABI PRISM 7700 Sequence Detector System using the SYBR Green PCR Master Mix kit (Perkin Elmer, Applied Biosystems, Wellesley, MA) according to the manufacturer's instructions. After an initial incubation at 50ºC for 2 min and 10 min at 95ºC, amplification was performed for 40 cycles at 95ºC for 20 sec, 65ºC for 20 sec, and 72ºC for 30 sec. Specific primer pairs were determined with the Primer-Express program (Applied Biosystems). The PSA primer pairs were 5'-AGG CCT TCC CTG TAC ACC AA-3' and 5'-GTC TTG GCC TGG TCA TTT CC-3'. The TGF-β primer pairs were 5'-TGC TAA TGG TGG AAA CCC AC-3' and 5'-ATC GCC AGG AAT TGT TGC TG-3'. The Smad3 primer pairs were 5'-ATC CTG CCT TTC ACT CCC C-3' and 5'-GCC TTC TCG CAC CAT TTC TC-3'. The MMP9 primer pairs were 5'-GAC GCC GCT CAC CTT CAC TC-3' and 5'-CTT GCC CAG GGA CCA CAA CTC-3'. The normalization control used was β-actin, and the primers used were 5'-TCA CCC ACA CTG TGC CCC ATC TAC GA-3' and 5'-CAG CGG AAC CGC TCA TTG CCA ATG G-3'.

Immunohistochemistry

The tumor tissue samples were fixed in 4% neutral buffered paraformaldehyde and embedded in paraffin. The primary antibodies of the rabbit anti-TGF-β (Santa Cruz), the rabbit anti-p-Smad3 (Cell Signaling), the rabbit anti-MMP9 (Abcam), and anti-PSA (Santa Cruz) were used for staining. The primary antibody was recognized by the biotinylated secondary antibody (Vector), and visualized by VECTASTAIN ABC peroxidase system and peroxidase substrate DAB kit (Vector). The positive staining signals were semiquantitated by Image J software.

X-Ray and CT-Scan

The mice bones with luciferase signals were evaluated radiographically using a Faxitron X-ray system (Faxitron X-ray Corporation, Wheeling, IL). The regions with increased bone density were further evaluated by microCT using a Scanco vivaCT40 scanner with a 55 kVp source as we have previously described (Naik et al., 2009; Wu et al., 2008). Tibiae were scanned at a resolution of 12 μm with a slice increment of 10 μm. Images were reconstructed at identical thresholds to allow 3-dimensional structural rendering of the lesions.

Reference

1. Xiang B, Muthuswamy SK. Using three-dimensional acinar structures for molecular and cell biological assays. Methods Enzymol 2006;406:692-701.