# Supplemental Information

# Materials and Methods

*Tissue Specimens, Tissue Microarrays, cDNA Microarrays, and CGH Arrays.*

Tissue microarrays containing primary PCa (n=17) and metastatic PCa lesions (n=43) from 14 PCa patients, and paired BPH (n=48) *vs*. PCa tissues (n=48) from 48 patients were constructed at Department of Clinical Pathology and Cytology, Skåne University Hospital, Malmö. The mRNA expression data of cyclin A1 and aromatase (CYP19A1) were extracted from the dataset in the cBioPortal database ([51](#_ENREF_51),[52](#_ENREF_52)). The follow-up time from diagnosis to disease recurrence known as biochemical recurrence (BCR) ranged from 1 to 60 months was used for analysis of disease-free survival. The study was approved by the Ethics Committee, Lund University, and the Helsinki Declaration of Human Rights was strictly observed.

*Immunohistochemistry Analysis*

Immunohistochemistry on tumor tissue arrays was performed as previously described ([47](#_ENREF_47)). The staining procedure was performed using a semiautomatic staining machine (Ventana ES, Ventana Inc., Tucson, AZ). For immunohistochemical analysis of xenograft mouse organs, tissues or tumors were fixed in 4% paraformaldehyde for 24 hours and embedded in paraffin. For histology analysis, the sections were stained with hematoxylin-eosin (H&E) and were subjected to analysis using an Olympus BX51 microscopy. Immunostaining of tumor tissues using antibodies was performed as previously described ([48](#_ENREF_48)). The sections were viewed under an Olympus BX51 microscope at magnification of 20x or 40x. The slides were scanned and viewed; microphotographs were taken by using a high resolution scanner (ScanscopeCS, Aperio, Vista, CA). The staining intensity was scored as 0 (negative), 1 (weakly positive or positive), 2 (moderate positive), 3 (strongly or very strongly positive) using an arbitrary semi-quantitative scale.

*Cell culture*

An androgen-insensitive cell line, PC3M cells ([53](#_ENREF_53)), was kindly provided by Dr. J Fidler (Department of Urology, MD Andersson Cancer Center, Houston, Texas, USA). The cell lines were authenticated by the suppliers. The PC3M cells were received 2011, and fresh-frozen stocks were used for the experiments shortly after. The total span of years in using the cell line in our labs is approximately three years. PC3M cells were maintained in phenol red free RPMI 1640 medium (PAA Laboratories, Pasching, Austria) or in Ham’s F12 medium (PAA Laboratories, Pasching,Austria), supplemented with 10% fetal bovine serum (FBS) (PAA Laboratories, Pasching, Austria), 1% penicillin-streptomycin-neomycin (Life Technologies, Paisley, UK) and 2 mM L-Glutamine (PAA Laboratories, Pasching, Austria ).

*Generation of vectors and stable and transient transfection*

For transient transfection studies, pMSCV-cyclinA1-EGFP was generated by cloning the full-length (1.8 kB) human cyclin A1 cDNA into the *EcoRI* site of the pMSCV-EGFP construct (Clonetech Inc.). The empty pMSCV-EGFP control vector was designated as pMSCV-EGFP. Transient transfection was performed using a Microporator MP-100 (Digital-Bio Technology, Seoul, Korea) electroporation system according to the manufacturer’s instructions. For stable transfection, cells that stably overexpressed pMSCV-EGFP-A1 or pMSCV-EGFP empty vector were selected by culturing cells in medium containing G418 for two to four weeks.

*Prostate tumor spheroid formation assays*

PC3M cells were cultured in polyhema-coated flasks at 5000/ml in spheroid medium modified from protocols used for mammo-sphere formation ([54](#_ENREF_54)). The culture medium consisted of DMEM/F12 medium containing 1% B27 supplement, 20ng/mL epidermal growth factor, 20 ng/mL basic fibroblast growth factor, 5μg/mL insulin, and 4μg/mL heparin, 5μg/mL hydrocortisone and 1% penicillin/streptomycin. Cells in suspension were cultured for 7 days to attain first passage spheres. To dissociate the cells within the spheres, spheres were collected by gentle centrifugation at 100 x g for 4 min, and cells were suspended in 1x PBS containing 0.05% trypsin and incubated for 3 min. To generate second passage spheres, cells derived from the first passage spheres were plated again at a density of 5x103 cells/ml and grown in culture medium for 7 days as mentioned above.

*ALDEFLUOR assay*

The ALDEFLUOR kit (StemCell Technologies, Vancouver, British Columbia, Canada) was used to isolate the population with a high ALDH enzymatic activity (stem-like ALDHhigh cells). PC3M or PC3 cells were incubated with ALDEFLUOR reagent according to manufacturers’ protocol. Briefly, 1x106 cells were stained with 5µL of activated ALDEFLUOR reagent. As negative control, an aliquot of each sample was treated with 50 mmol/L diethylaminobenzaldehyde (DEAB), a specific ALDH inhibitor. ALDHhigh or ALDHlow populations were defined based on the presence or absence of DEAB. Cells were stained with 7AAD (BD Biosciences, San Jose, CA, USA) to distinguish viable cells from dead cells during the fluorescence-activated cell sorting and analysis on CyAn™ ADP flow cytometer or FACS Aria (Beckman Coulter, Miami, FL, USA). ALDHhigh vs. ALDHlow populations were sorted on FACS Aria (BD Biosciences). FACS data were analyzed using FCS Express software (DeNovo Software, Los Angeles, CA).

*Mouse model of PCa distant metastases*

The animal studies were approved by the Swedish Regional Ethical Animal Welfare Committee. Three sets of mouse experiments were performed. Athymic NMRI nude male mice (n=5 or n=7, per experiment group) aged 8–12 weeks and weight 25-27 gram each (Taconic Europe, Lille Skensved, Denmark) were used in each experimental setting. For each setting, mice were sub-lethally irradiated with two doses of 2 Gy administered 2 hours apart using a 137Cs source at a dose rate of 1Gy/min. In the first two repetitive experiments, equal amount of ALDHhigh cells were sorted from PC3M cells expressing pMSCV-EGFP or pMSCV-EGFP-A1 (1x105 /mouse) and were subsequently injected into mice via tail-veins. In the second setting, unsorted PC3M cells transfected with pMSCV- EGFP-cyclin A1 vector or pMSCV-EGFP-control vectors (2x106 cells/mouse) were suspended in 100µL PBS and were injected into tail vein of nude mice. For intracardiac injection to introduce tumor cells to bone marrow through systemic circulation without passing through lung and liver, 2x105 cells/mouse in 100µL PBS were injected into the left ventricle of anaesthetized mice (1% isoflurane through inhalation). The weights of mice were regularly measured and survival was followed. Mice were examined and photographs were taken using *in vivo* imaging device (IVIS imaging system, PerkinElmer, Massachusetts). Xenograft mice were injected IP with 30ug of HLA-ABC antibody conjugated with 680 DyLight NHS-ester (LifeTechnologies, Stockholm, Sweden) and the imaging was examined at the time course between 2 hours and 16 hours post-injection of the antibody as described (55). The end point of the animal experiments using intracardiac injection was 33 days. The animal welfare and guidelines of Swedish Regional Ethical Committee were strictly followed.

*Identification of metastatic PCa cells in the bone marrow of mice by FACS*

To identify metastatic PCa cells in the bone marrow of recipients after implantation of tumor cells, bone marrow cells from long bones of the recipient mice were isolated and were subjected to FACS analysis as described ([50](#_ENREF_50)). To further distinguish metastatic PCa cells from hematopoietic stem and progenitor cells (HSPC) in the bone marrow, the bone marrow cells were stained with HLA-ABC conjugated with fluorescin isothiocyanate (FITC), APC-conjugated Annexin V and 7AAD (BD Pharmingen of BD Biosciences, San Jose, CA, USA). The cells enriched with HLA-ABC were identified. Human xenograft prostate tumor cells, metastatic PCa from xenograft mice or normal bone marrow from nude mice were used as positive or negative controls for setting the gates. The proportion of the cells and cell viability were assessed by flow cytometry on CyAn™ ADP flow cytometer (Becton Coulter, Miami, FL, USA) or FACS Aria (BD biosciences). FACS data were analyzed with FCS Express software (DeNovo Software, Los Angeles, CA).

*Evaluation of ability of single cells from metastatic tumors of mice to form colony-forming unit using methylcellulose-based medium*

To assess the repopulating ability and differentiation potential of the BM progenitor cells or metastatic PCa cells in the bone marrow, we used a methylcellulose-based colony-forming assay according to the manufacturer’s description ([MethoCult™ GF M3434](http://www.stemcell.com/en/Products/All-Products/MethoCult-GF-M3434.aspx), Stem Cell Technologies, Vancouver, British Columbia, Canada). Single cell suspension were prepared as the following. Briefly, the femur bones in 1XPBS were crushed and subsequently pass through the filtered Cell Strainer with pore size of 80 µm (BD biosciences). Single cells were dispersed by running through the filtered Cell Strainer. Cells were re-suspended in 1 ml of Ham’s F-12 media with 50% of FBS (PAA Laboratories, Pasching, Austria) and 8% of DMSO (Sigma-Aldrich, Stockholm, Sweden). Cells from the total bone marrow (5x104) or xenograft tumor cells at various amount (from 1x104 to 1.25 x 106 cells) were added to 2 ml of semisolid methylcellulose-based medium containing: stem cell factor, IL-3, IL-6, and erythropoietin. Duplicate aliquots of the cell-containing media were dispensed into 35-mm dishes. Colonies containing 30 and more cells were scored after 14 days. Photographs of colonies were taken every 3 days.

*Preparation of single cell suspensions from xenograft tumors*

Tumors of 5 mm3 in size were cut into small pieces, and placed in solution containing 300 U/ml of collagenase, 100 U/ml of hyaluronidase, 5 mg/ml of dispase and 1mg/ml DNAseI (Stem Cell Technologies, Vancouver, British Columbia, Canada) followed by incubation on a shaker for 8 hours at 37 ºC. At the end of the incubation, cells were filtered through a 75 m filter and were washed in Ham’s F-12 media (PAA Laboratories, Pasching, Austria) containing 20% of FBS (PAA Laboratories, Pasching, Austria) to yield a single cell suspension.

*Immunoblot analysis and source of antibodies*

Protein lysates were prepared from PC3M or PC3 cells before or after transfection or treatment. Cells were lysed in ice-cold lysis buffer (150 mM NaCl, 50 mM Tris-HCL pH 7.5, 1% Triton X-100, 50 mM NaF, 0.1mM Na3VO4, 10 mM phenylmethylsulfonyl fluoride (PMSF)) and Complete Mini protease inhibitor cocktail (Roche, Basel, Switzerland). Protein samples (20-40 µg) were loaded and samples were separated on 12% SDS-PAGE gels and transferred onto nitrocellulose membranes (Hybond ECL, Amersham Pharmacia Biotech, Buckinghamshire, UK). The membranes were incubated with primary antibodies, followed by horseradish peroxidase (HRP)–conjugated secondary antibodies (Amersham Life Science, Alesbury, UK). Signals were visualized using the Enhanced ChemiLuminescence HRP substrate (Millipore Corp Sweden, Solna, Sweden). The following antibodies were used in this study: For cyclin A1, monoclonal anti-cyclin A1, (BD Pharmingen, and San Diego, CA), or polyclonal anti-cyclin A1 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-β-actin (Santa Cruz Biotechnology, CA).

*Immunofluorescence analysis*

For immunofluorescence analysis, cell suspensions were fixed on slides in 4% paraformaldehyde (Merck KGaA, Darmstadt, Germany) for 15 minutes at room temperature (RT). The slides were washed in 1x PBS twice and permeabilized in 0.5 % Triton X-100 (Sigma-Aldrich, Stockholm, Sweden) for 10 minutes at room temperature (RT). Non-specific staining was blocked by applying serum for 30 minutes at RT. The cells were then incubated with primary antibodies diluted in 1% BSA in PBST (0.05% Tween-20 in 1x PBS) at 4 ºC in a moist chamber overnight followed by the incubation with secondary antibodies. Secondary anti-rabbit conjugated to Alexa Fluor 488 (Invitrogen, Stockholm, Sweden) and anti-donkey conjugated to Rhodamine (Chemicon International Inc, Temecula, CA) or anti-goat conjugated to FITC antibodies (Invitrogen, Stockholm, Sweden) were used. Cells were counterstained with DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride) (SERVA Electrophoresis GmbH, Heidelberg, Germany). Primary antibodies including anti-HLA (Biosite) and anti-cyclin A1 (BD Pharmingen) were used. The cells were examined under an Olympus AX70 microscope (Lund, Sweden) equipped with a Nikon DS-U1 camera (Stockholm, Sweden). The microphotographs were taken and accessed using software ACT2U version 1.5.

*Treatment of ALDHhigh or ALDHlow cells with 17-β-estradiol or DTH*

For estrogen treatment, ALDHhigh or ALDHlow populations were sorted from PC3M cells and were maintained in 10% charcoal stripped medium (CSS) (Gibco, Life Technologies Corporation, Stockholm, Sweden) for 24 hour prior to treatment with 17-β-estradiol at 10 nM and DTH at 5 nM (Sigma-Aldrich Inc, Stockholm, Sweden).

*Co-culture of ALDHhigh or ALDHlow cells with the bone marrow and measurement of estradiol production*

For measurement of MTS, ALDHhigh or ALDHlow cells were cultured with serum-free medium containing 50% of the bone marrow extracts prepared from the bone marrow cells of the long bones of NMRI-nude mice. The cells were cultured for 48 hours and were subjected for MTS assay. For measurement of estradiol in ALDHhigh cells, bone marrow cells or coculture of ALDHhigh and bone marrow cells, cells were cultured in phenol red free RPMI 1640 supplemented with 10% serum for 24 hours, the ratio of ALDHhigh cells : bone marrow cells in the coculure was 6:1000 ratio. The cell culture supernates from each sample were measured using an immunoassay from Roche Diagnostics (Estradiol III) on a Cobas 6000 Analyzer (Roche Diagnostics, USA). To examine the effect of aromatase inhibitor in ALDHhigh cells and bone marrow cells, ALDHhigh cells were seeded in serum-free medium RPMI1640 and allow attaching to the plates. Cells were pretreated with 100 nM Type I aromatase inhibitor (Merck Millipore, Darmstadt, Germany) for 2 hours and the medium were replaced with bone marrow cells in the serum-free and phenol red free RPMI1640 and cultured for 24. The concentrations of estradiol in supernates were determined by Estradiol Assay kit (R&D Systems, Europe, Ltd, Abingdon, UK) according to manufacturer’s protocols. The absorbance values were determined on an Infinite M200 multimode microplate reader (Tecan Sunrise).

*MTS Proliferation Assay*

The effects of 17-β-estradiol or DTH or bone marrow on ALDHhigh or ALDHlow cells were determined using the nonradioactive tetrazolium dye-based proliferation assay (MTS) proliferation assay (Promega Biotech) according to the manufacturer’s protocol. Viability was determined by measuring the absorbance at 490-nm wavelength, on an Infinite M200 multimode microplate reader (Tecan Sunrise). 100 nM Type I aromatase inhibitor (Merck Millipore, Darmstadt, Germany) was used for inhibition of aromatase.

*Statistical analysis*

Statistical analysis was performed using the paired Student *t* test (Graphpad Prism Software, San Diego, CA); a *P* value of <0.05 was considered significant. Distribution of overall survival (OS) was estimated by the method of Kaplan-Meier, with 95% confidence intervals. Differences between survival curves were calculated using the log-rank test using statistical program (SPSS, 16.0).

1. **Supplemental Figure legends**

**Supplemental Figure 1. PC3M cells overexpressing cyclin A1 initiated macro-metastases in distant organs in xenograft mice.** (A). Kaplan-Meier survival analysis shows overall survival of two groups of mice which received PC3M cells expressing pMSCV-EGFP-A1 or pMSCV-EGFP. Differences in overall survivals between two groups were calculated using the log–rank test. (B). Metastatic tumors obtained from mice received PC3M cells expressing pMSCV-EGFP-A1 or pMSCV-EGFP. Histology pictures and microphotographs of tumor sections of metastatic tumors in prostate, liver and lung and the control organs are shown.

**Supplemental Figure 2. Evaluate bone marrow metastasis initiated by PC3M cells via intracardiac injection of tumor cells into mice.** (A). A schematic chart depicts the experimental procedure of cardiac injection of PC3M cells expressing control EGFP or EGFP-cyclin A1 vector into nude mice. The tumor cells were induced systemically into the circulation. (B). Representative images of the bioluminescent *in vivo* imaging to visualize the mice received PC3M cells expressing EGFP or PC3M cells expressing EGFP-A1 using IVIS imaging device. The tumors were visualized under CCD camera in imaging device. (C). Representative FACS plots show that the percentage of HLA-ABC-positive metastatic PCa cells in the bone marrow of the two groups of mice. The FACS settings using mouse bone marrow or PC3M tumors alone or together were performed. Representative FACS plots of the bone marrow from mice which did not receive PC3M tumor cells or mice which received PC3M cells expressing EGFP or EGFP-A1 are shown. (D). Percentages of HLA-ABC-positive PCa cells expressing EGFP or EGFP-A1 in the bone marrow of mice as determined by FACS are shown. SD± values indicate means of samples from 5 mice/group (*P*=0.359).

**Supplemental Figure 3. Evaluate bone marrow metastasis initiated by PC3M cells via intracardiac injection of tumor cells into mice.**

(A and B). Representative microphotographs of the bone marrow sections stained with antibodies against cytokeratin 5 (CK5). The representative metastatic lesions positive to the antibody against CK5 are indicated by the arrows. Percentage of CK5-positive cells in the bone marrow from xenograft mice received PC3M cells expressing EGFP or EGFP-A1 were evaluated. \* *P*= 0.016. (C and D). Representative microphotographs of the bone marrow sections stained with antibodies against cytokeratin 20 (CK20). The representative metastatic lesions positive to the antibody against CK20 are indicated by the arrows. Percentage of CK20-positive cells in the bone marrow from xenograft mice received PC3M cells expressing EGFP or EGFP-A1 were evaluated. \*\* *P*= 0.0014.