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TITLE:

Coculture System to Study Osteoblast and Osteoclast Interactions in Prostate Cancer Progression to Hormone Resistance and Metastasis to Bone

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KEYWORDS:

Prostate cancer progression, bone metastasis, androgens and estrogens, fibroblast growth factors, cell models in cancer, recurrence and refractory disease

SHORT ABSTRACT:

Prostate cancer bone metastasis is reactivated by fibroblast growth factors and nuclear receptor interactions, together with autocrine/paracrine factors in tumor microenvironment. A coculture cell model using the transwell system of cocultured cells was developed to determine the genes, FGFs and FGF receptors, potentially involved in the progression to bone metastasis.

LONG ABSTRACT:

Bone metastasis is a lethal phenotype of advanced stage prostate cancer that is demonstrated in 85% of all related deaths. Considered as an early event, steroid hormone ratio changes that occur during andropause may result in a disruption in the homeostatic osteoblast-osteoclast interplay that promotes bone colonization. Aberrant expression of fibroblast growth factors (FGFs) and their receptors (FGFRs) function initiate the activation of downstream pathways that play an intrinsic role in the induction, proliferation, de-differentiation, angiogenesis and survival of the tumorigenic prostate cells. To investigate the molecular mechanism of FGF action, pathways, receptors, and interactions with paracrine/autocrine factors, we established a

coculture model, using androgen-sensitive LNCaP and androgen-resistant C4-2 cells, to examine the role of osteoblast and osteoclast precursors within the bone microenvironment during the progression from androgen-dependence to androgen-independence and metastasis. As an interaction model of prostate cancer cells during osteogenesis, we performed the coculture assays of LNCaP and C4-2 cells with the osteoblast precursor line human Saos-2 cells and the osteoclast line human Thp-1 cells. We identified the FGFs, FGFRs, and downstream pathways that are induced by the presence of osteoblast and osteoclast precursors in androgen-sensitive and androgen-resistant prostate cancer tumor cell lines. Our findings are consistent with the changes in cell proliferation of cancer epithelial cells in the context of the osteoblast/osteoclast microenvironment. This innovative use of the coculture system is suitable for different research purposes, including pharmacological studies on hormone agonist/antagonist action for androgens, estrogens, glucocorticoids and Vitamin D nuclear receptor and coactivator/corepressor function in the context of bone stroma during tumor progression of prostate cancer to androgen independence and bone metastasis.

INTRODUCTION

Bone metastasis represents a lethal phenotype of advanced stage prostate cancer. Among the prostatic cancer-related deaths, 85% of the cases demonstrate the presence of bone metastasis^{1,2}. Considered as an early event in patients with colonized bone metastasis, this metastasis may be activated presumably by the shift in steroid hormone ratios during andropause^{3,4}. Active prostatic bone metastasis is characterized by osteoblastic activity occurring directly adjacent to the prostatic tumor tissue, and in proximity to the hip, spine, and pelvic bones³⁻⁵. Steroid hormone ratio changes furthermore result in a disruption in the homeostatic osteoblast-osteoclast interplay that promotes bone colonization⁶.

Prostate tumorigenic involvement of the bone may occur early in progression, and disseminated cells can be detected in the bone marrow niche in lower Gleason grades of the primary tumor^{7,8}. Within this changed prostate-bone microenvironment, prostate cancer cells secrete paracrine factors, including bone morphogenetic protein (BMP) and transforming growth factor-beta (TGF β), that modulate osteoblast differentiation^{9,10}. TGF beta and BMP interact synergistically with fibroblast growth hormones (FGFs) to promote osteolytic and osteoblastic lesions for the establishment of a metastatic bone niche^{6,11}. Fibroblast growth factors (FGFs) are a family of 23 polypeptide growth factor ligands that have been well recognized for their paracrine or autocrine actions in the development of prostatic cancer¹². Consisting of seven sub-families (1; 4; 7; 8; 9; 11 and 19), FGFs bind to cell-surface high-affinity tyrosine kinase receptors (FGFRs), which upon ligand binding, form dimers to transphosphorylate and activate the intracellular tyrosine kinase domains^{13,14}. Aberrant expression of FGFs and their receptors function initiates the activation of downstream pathways, such as mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K) and phospholipase C γ (PLC γ), that result in the induction, proliferation, de-differentiation, angiogenesis and survival of the tumorigenic prostate cells^{15,16}.

To further understand the molecular mechanism of FGF action, pathways, receptors, and interactions with paracrine/autocrine factors, we established a coculture model, using

androgen-sensitive Lymph Node Carcinoma of the Prostate (LNCaP) cells and androgen-resistant C4-2 cells, a subline of LNCaP cells that proliferate in a low androgen or castrated nude mice environment, to examine the role of osteoblast and osteoclast precursors within the bone microenvironment during the progression from androgen-dependence to androgen-independence and metastasis. As an interaction model of prostate cancer cells during osteogenesis, we performed coculture assays of LNCaP and C4-2 cells with the osteoblast precursor line human Sarcoma Osteogenic (Saos-2) cells and the osteoclast human monocytic leukemia cell line (Thp-1) cells. The Saos-2 cell line, originally derived from an 11-year old Caucasian osteosarcoma patient, has been well documented and widely accepted for the study of osteoblastic differentiation and molecular interactions¹⁷. Osteoblasts synthesize and remodel bone through the secretion of matrix proteins and mineralization that is associated with bone metastasis in prostate cancer, often referred to as “seed and soil”^{18,19}. The Saos-2 line is appropriate for the effect of osteoblastic activity studies as this cell type secretes bone-specific proteins as osteopontin and can produce mineralized bone extracellular matrix²⁰. The monocyte-like Thp-1 cell line, derived from a pediatric patient with acute monocytic leukemia, can be differentiated into osteoclast cells upon the stimulation with phorbol myristate acetate (PMA) and/or Vitamin D3 precursors²¹⁻²³. Although prostate cancer in bone is described as an osteoblastic disease due to the close interaction with osteoblasts in a paracrine manner, osteoclasts may be also very relevant for an early modulator of the establishment of the metastatic bone niche²⁴.

This coculture system is suitable for different research purposes, including pharmacological studies on hormone agonist/antagonist action for the nuclear receptor and coactivator/corepressor function of androgens, estrogens, glucocorticoids and Vitamin D in the context of bone stroma during tumor progression of prostate cancer to androgen independence and bone metastasis. This experimental design to control the absence and presence of different ligands in both cellular compartments enables a better understanding of the potential mechanism(s) by which hormones and bone-derived stromal cells regulate gene expression of specific gene networks for growth factors and nuclear receptor(s) activation and regulation of transcription in a short term paracrine fashion with bone-derived cells in metastasis. Therefore, this *in vitro* model identifies and characterizes potential molecular mechanisms that may be relevant to the progression of prostate cancer upon the arrival of circulating tumor cells to the bone metastasis niche.

PROTOCOL:

1. Cell Models

1.1. Prostate Cancer Cell Types: Use LNCaP cells for an androgen dependent or androgen sensitive cell line. Use C4-2 cells for androgen independent and recurrent tumor cells.

1.2. Bone Stroma Derived Osteoblasts Saos-2 and Osteoclasts Thp-1 Cells: Use Saos-2 cell line derived from human osteosarcoma in the presence of calcitriol to represent a model for osteoblast precursors. Use the Thp-1 cell line to represent macrophage-like cells that mimic

monocyte-derived macrophages and multinucleated osteoclast-like cells in the presence of phorbol esters (PMA) and vitamin D3.

Note: Besides the number of bone-derived cell lines available in banking facilities, there are no established cell lines that represent human osteoblast and osteoclast differentiation. Therefore, for these studies, it is important to consider the use of specific cell culture systems commercially available to isolate, propagate and differentiate human osteoclast and osteoblast precursors, as primary cell cultures previously described elsewhere.

2. Cell Cultures

Note: All of the following steps are performed under sterile conditions in a biosafety level 2 (BSL2) tissue culture hood.

2.1. Cell Cryopreservation and Thawing

Note: All cell lines used (LNCaP, C4-2, Saos-2 and THP-1) were cryopreserved under liquid nitrogen conditions at -180 °C. Cells can also be stored at -80 °C freezers for up to one year without any changes in cell viability as assessed by trypan blue exclusion assay. The thawing process to propagate cells must be performed fast by warming the cryogenic tubes at 37 °C for a maximum of 5 min.

2.1.1 Store LNCaP, C4-2 and Saos-2 cells in 2 mL cryogenic vials containing 1.5 mL of the cell suspension, with $3-4 \times 10^6$ cells per tube. Thaw the cryogenic vials at 37 °C for a maximum of 5 min.

2.1.2. Transfer the cells to a 15 mL conical centrifuge tube containing 10 mL of RPMI/DMEM culture medium mix (1:1 ratio).

2.1.3. Resuspend the Thp-1 cells at $2-4 \times 10^5$ cells/mL in RPMI media only. Unless otherwise indicated, supplement all tissue culture media with phenol red as pH indicator, 10% fetal bovine serum (FBS), 1% L-glutamine (0.5 mg/mL), 1% penicillin/streptomycin (100x stock solution) and 1% fungizone.

Note: The thawing procedure is important as dimethyl sulfoxide (DMSO) is used as cryopreservant.

2.1.4. Centrifuge the LNCaP, C4-2, Saos-2 and Thp-1 cells in their respective conical centrifuge tubes at 800 x g for 5 min at room temperature in a swing bucket rotor, and then remove the supernatant by aspiration.

2.1.5. Wash the centrifuged cells by resuspending the cell pellet in 10 mL of RPMI and centrifuge again at 800 x g for 5 min at room temperature in a swing bucket rotor.

Note: The resulting cell pellet is ready for resuspension in the specific media for each cell type.

2.2. Prostate Cancer Single Cell Culture

2.2.1. Resuspend the LNCaP and C4-2 cell pellets (step 2.1) in 1 mL of RPMI/DMEN mix media supplemented as above (step 2.1.3), using a filtered micropipette tip (p1000).

2.2.2. Transfer 500 μ L of each cell suspension to a 10 cm tissue culture plate that contains 10 mL of RPMI/DMEM supplemented as above (step 2.1.3).

2.2.3. Transfer the tissue culture plates to a 37 °C incubator under 5% CO₂ and 90% humidity.

2.2.3.1. During the transfer to the incubator, be careful not to splash the cells. Do not remove the plates from the incubator until the following day. Check the adherence and confluency of the cells after 24 h incubation.

2.2.4. Change the media at least twice a week and maintain the cells at 37 °C until reaching 80% confluence, usually in 10 days.

Note: To change the media for the adherent LNCaP and C4-2 cells, the culture medium is aspirated using a vacuum pump system in the tissue culture hood.

2.2.5. Wash the cells by adding 5 mL of phosphate buffered saline (PBS) at pH 7.4 and swirling the plate.

2.2.6. Remove the PBS by aspiration and add 10 mL of fresh culture medium corresponding to each cell line type using a 10 mL disposable pipette.

2.3. Bone-Derived Osteoblasts Saos-2 and Osteoclast Thp-1 Single Cell Culture:

Note: The Saos-2 cells are adherent cells and are cultured under the same conditions as LNCaP and C4-2 cells. Thp-1 cells proliferate in suspension and are cultured with RPMI media supplemented with phenol red as pH indicator, 10% fetal bovine serum (FBS), 1% L-glutamine (0.5 mg/mL), 1% penicillin/streptomycin (100x stock solution) and 1% fungizone.

2.3.1 Thaw the cells, transfer to a 100 mm tissue culture plate containing 10 mL of RPMI supplemented media and incubate at 37 °C. Check the cells every day until the cells reach confluency of 80%.

Note: Thp-1 cells proliferated in suspension are not firmly adherent to the tissue culture flask. Therefore, change the media by replacing half of the upper volume every 2 days. The cells reach 50% confluency after 10 days culture, as the replication rate is lower compared to LNCaP, C4-2 and Saos-2. It's better to change the culture medium by aspiration using a 10 mL pipette and suspending the cells in a 15 mL conical centrifuge tube.

2.3.2 Centrifuge the cells at 800 x g for 5 min at room temperature. Aspirate the supernatant. Add 1 mL of RPMI supplemented medium to resuspend the cells using filtered tips.

2.3.3 Transfer the cells to the original 75 cm² flask containing the remaining Thp-1 cells and supplement with 10 mL of fresh RPMI media.

Note: Steps 2 and 3 decrease the loss of cells when changing the RPMI supplemented media every 2 to 3 days.

2.4 Cell Harvesting

2.4.1 Aspirate the media from the plates containing LNCaP, C4-2 and Saos-2 cells using the vacuum pump and wash with 5 mL of PBS to remove FBS.

2.4.2 Aspirate the PBS and treat the cells with 1 mL of trypsin (1x: 0.25% trypsin and 1 mM EDTA in PBS) for 5 min at 37 °C.

2.4.3 Tap the plates, after the incubation, to ensure the cells are detached, and add 3 mL of FBS to inactivate the trypsin. Transfer the resuspended cells immediately using a p1000 micropipette to a 15 mL conical centrifuge tube.

2.4.4 Centrifuge the cells at 800 x g for 5 min at room temperature and resuspend with 2 mL of RPMI to determine the number and viability of the cells using trypan blue exclusion assay in a Neubauer chamber.

Note: Live cells that exclude the trypan blue in each quadrant of the Neubauer chamber are counted in each of the 4 quadrants. The average number of cells is multiplied by the dilution factor of 20,000. The result is the number of viable cells per each milliliter of media. The number of experiments will depend on the number of cells obtained. LNCaP and Thp-1 can be expanded using 1:2 dilutions. The C4-2 and Saos-2 cells can be expanded using 1:3 dilutions during each passage. The passage number is critical for LNCaP, Saos-2 and Thp-1 cells.

2.4.5 Resuspend the Thp-1 cells by aspirating the media up and down several times. Perform the trypsin digestion for 1-2 min, as described in steps 2.4.1 to 2.4.4, as Thp-1 cells grow in suspension and are lightly attached to the tissue culture plate surface.

3. Co-Culture Assays and Hormone Treatments

3.1 Seed the LNCaP and C4-2 cells into a 12-well plate format (diameter of 22.1 mm for each well), with 2×10^5 cells in each well. First, add 1 mL of DMEM/RPMI supplemented media to each well and add the resuspended cells on top. Swirl the plate to spread the cells.

3.2 Seed the Saos-2 and Thp-1 cells at 1×10^5 cells per well, using the insert for the 12-well transwell coculture system (diameter of 12 mm for each insert) with the supplemented media for each cell type.

3.3 Incubate for 24 h at 37 °C in a 5% CO₂ incubator.

Note: The cells reach confluence after 24-36 h incubation.

3.4 Synchronize the cells for an additional 24 h at 37 °C in the 5% CO₂ incubator by changing the media to RPMI, which is free of phenol red and without FBS.

Note: Cells synchronize in G⁰ due to reduced levels of FBS and confluency. At this point, cells are ready for several downstream experiments, including hormone treatment, DNA/RNA isolation, protein purification and transcription assays.

3.5 Change the media to phenol red-free RPMI supplemented with 10% dextran-coated charcoal treated FBS (cdFBS) containing ethanol as control, 10 nM dihydrotestosterone (DHT), or DHT in the absence or presence of 100 nM androgen antagonists flutamide or bicalutamide.

Note: Working hormone solutions are prepared just before use as the hydrophobic ligands will bind to the plastic walls of the conical centrifuge tubes. In general, each experimental condition requires 10 mL of RPMI supplemented with 10% cdFBS (RPMI/cdFBS).

3.6 Add 10 mL of RPMI/cdFBS to a 15 mL conical centrifuge tube and add 11 µL of filter sterilized ethanol as control for the wells without ligands. Add 10 µL of ethanol and 1 µL of 10⁻⁴ M DHT working stock solution to another 10 mL of media for the 10 nM DHT treatment of the cells.

Note: For antagonist treatment, the ethanol is replaced by 10 µL of 10 mM flutamide or bicalutamide working solution to obtain the 10 nM DHT treatment in the presence of 100 nM antagonist flutamide or bicalutamide (**Figure 1**). In the lower chamber of the coculture, use 1 mL of RPMI/cdFBS media, and 0.5 mL in the upper chamber or insert (**Figure 1**).

3.7 Incubate the cells for 36 h in the 5% CO₂ incubator at 37 °C before harvesting for downstream assays, as described in step 5.

4. Cell Harvesting in the Co-Culture System

4.1. Wash the cells with 300 µL of 1x PBS in each well prior to the treatment with 100 µL of 1x trypsin, for 5 min in the 5% CO₂ incubator at 37 °C.

4.2. Add 300 µL of FBS to inactivate trypsin and transfer the entire volume of each well into 1.5 mL properly labeled centrifuge tubes for each condition.

4.3. Centrifuge at 800 x g for 5 min at 4 °C. Aspirate the supernatant, carefully leaving the cellular pellet intact for an additional wash with 300 µL of 1x PBS.

4.4. Fix the cells, resuspending the pellet in 500 µL of 4% paraformaldehyde (PFA) for 30 min at 4 °C.

4.5. Wash the fixed cells by resuspending the cell pellet in 10 mL of PBS.

5. Downstream Assays Following Hormone Treatments

5.1. Cell Proliferation

5.1.1. Perform mitotic index coupled to flow cytometry assay using mitosis-specific phosphorylation of histone H3-phosphorylated (p) Serine 10 labeling and propidium iodide incorporation to quantitate mitotic index and DNA content.

Note: The anti-HistoneH3 pSer10 antibody is a rabbit monoclonal directly conjugated to the green fluorescent dye that specifically labels mitotic cells. Propidium iodide staining of DNA is the classic means of cell cycle analysis by DNA content.

5.1.2. Resuspend the pellet above in 200 µL of 1x permeabilization solution (20 µL of triton X-100 to 1.98 mL of 5% BSA blocking solution) for 5 min at room temperature, previous to the antibody treatment and IP staining assays.

Note: This is to permeabilize the cells for the incorporation of the antibody within the fixed cells.

5.2. Flow Cytometry

5.2.1. Calibrate the flow cytometer (using beads containing 6 and 8 different peak) for green fluorescent dye in the FL-1 channel (Ex/Em: 495/519nm) and propidium iodide fluorescence in FL-2 channel (Ex/Em: 493/636nm) to determine proliferation rate and DNA incorporation (cell cycle), respectively.

5.2.2 Analyze each sample using the histogram of four quadrants after 10,000 events.

5.3. Global Gene Expression Profiling

5.3.1. Isolate total RNA using an RNA purification kit (for isolating high-quality total RNA) from each lower and an upper transwell by adding 500 µL of tryzol reagent directly to lyse the cells.

5.3.2. Reverse transcribe mRNA to cRNA using biotin-UTP labelled nucleotide and a kit that is a complete system for generating biotinylated, amplified RNA for hybridization.

5.3.3. Hybridize the biotinylated cRNAs to a high-density silica bead-based microarray for differential gene expression, which has a capacity for the analysis of above 34,000 genes.

5.3.4. Scan the array in the bead array reader. Calculate the relative changes in mRNA gene expression using fold change (FC).

5.3.5. Use the free distribution software MultiExperiment Viewer v4.9 (tm4MeV) to analyze changes in gene expression using heat maps²⁵.

Note: Genes with $FC \geq |1.9|$ are considered to be significant and subject to heat map and Venn diagrams analysis.

REPRESENTATIVE RESULTS:

The coculture method was optimized to determine the effect of hormone on prostate cancer progression to recurrence, in the context of bone stroma derived osteoblasts and osteoclasts cells. Although the scheme and timing of the cell coculture model is relatively complex due to the different rates of proliferation for each cell type, it is possible to achieve a sufficient number of cells to perform the specific experiments in the range of 2-3 months. Therefore, the propagating and maintenance of cell stock in culture is very important. As depicted in **Figure 1**, the prostate cancer LNCaP/C4-2 pair and the osteoblast (Saos-2)/osteoclast (Thp-1) precursors pair are propagated under specific cell culture conditions (**Figure 1A**). When the estimated number of cells is reached, and the confluency is close to 80-90%, the cells are subjected to trypsin digestion, and seeded overnight in the 12-well format for LNCaP/C4-2 cells, and in the transwell format for Saos2/Thp-1 cells (**Figure 1A**). Synchronization of the seeded cells is achieved using RPMI media without serum for 24-36 h (**Figure 1A**). The use of media without phenol red is very important to avoid estrogenic and cortisol agonistic activity of the pH indicator. To determine the effect of Saos-2 or Thp-1 in the absence or presence of different hormone agonists and antagonists on nuclear receptor mediated transcriptional activity or other downstream experiments, the cells are incubated with charcoal dextran coated fetal bovine serum to reduce and maintain low levels of circulating sex steroid hormones normally present in the regular fetal bovine serum (**Figure 1B**). The diagram (**Figure 1B**) describes an experiment whereby the effect of Saos-2 and Thp-1 on AR mediated transcriptional activity was determined in the absence and presence of the androgen antagonists flutamide and bicalutamide.

The model coculture shows that the proliferation rate in LNCaP and C4-2 is increased when in coculture with Saos-2 cells, as assessed by propidium iodine incorporation and flow cytometry (**Figure 2A**). The effect of short-term coculture in the propidium iodine incorporation in LNCaP and C4-2 due to the presence of Thp-1 is a minor decrease (**Figure 2A**). Confocal microscopy indicates that Saos-2 cells modify AR and SRC-1 coactivator immune localization (**Figure 2B**). However, the molecular mechanisms and the biological consequences of altering or modifying the coregulator interactions with the receptor is still under investigation. The effect of Saos-2 and Thp-1 cells on the global gene expression in both prostate cancer cell lines was studied in

the absence and presence of androgens using a high-density silica bead-based microarray for differential gene expression, which has a capacity for the analysis of over 34,000 genes.

The **Figure 3** represents changes in gene expression for fibroblasts growth factor (FGFs), their receptors (FGFRs), and the downstream pathways that results from FGFR activation. The Saos-2 cells increase FGF20 and FGF1 in LNCaP (**Figure 3A**). However, Thp-1 increases FGF2 expression, in a ligand dependent manner. The ligand-dependent activation of PLCG2 is repressed by both Saos-2 and Thp-1 (**Figure 3B**). The expression of the modulator of proliferation SPRY3 is increased in the presence of Saos-2 only (**Figure 2B**). The expression changes in C4-2 differ from LNCaP. Specifically, Saos-2 increases the expression of FGF2 and FGFR2 (**Figure 3C**). Thp-1 increases the expression of several FGFs, including FGF14; 8; 11; and 12 (**Figure 3C**). These changes in gene expression appear relevant for changes in proliferation as Saos-2 decreases expression of SPRY3 and increase expression of Sos2 (**Figure 3D**).

FIGURE LEGENDS:

Figure 1: Representative diagram of the osteoblast and osteoclast coculture model for prostate cancer bone metastasis in vitro. (A) The step-1 outline the model of monocultured cell in 10 cm plates for prostate cancer (LNCaP and C4-2) and bone stroma derived osteoblastic precursor Saos-2 cells. The osteoclastic precursors Thp-1 cells is propagated and maintained in a 75 cm² tissue culture flask, as these cells are non-adherent. After trypsin treatment, cells can be seeded overnight in a 12-well format for prostate cancer cells and in the transwell system for Saos-2 and Thp-1 cells. Then, cells are synchronized for 24 h using RPMI media that lack phenol red as pH indicator and without fetal bovine serum. (B) In step 2, the transwell containing the Saos-2 and the Thp-1 cells are transferred to the 12-well format that contains the prostate cancer cells to render the coculture system using the inserts. It is very important to plan ahead regarding whether the hormone treatment will be performed upon transferring the insert to the 12-well format containing the tumor cells, or whether the treatment will be performed after 24 h of cocultures. This pre-planning is important as the media containing hormone must be prepared just prior to use. The hormone treatment shown is in absence (control) and presence of DHT (10 nM final concentration from 0.1 mM stock), and in combination with the anti-androgens flutamide (F) and bicalutamide (B) at 100 nM final concentrations.

Figure 2: Representative flow cytometry and confocal microscopy analysis of cancer cells in coculture. (A) Flow cytometry assays were used to determine the changes in cell proliferation using the incorporation of propidium iodide (PI) and phosphorylation of H3-Ser (not shown). The relative changes in PI incorporation indicate that Saos-2 cells change the proliferation rate of LNCaP and C4-2 cells, in the absence of androgens. In the presence of Thp-1, the changes in PI incorporation in the tumor cells are minimal. (B) The expression of ligand-bound androgen receptor and SRC-1 coactivator in C4-2 cells, either alone or in the presence of Saos-2 cells, was detected using Cy3-labelled secondary antibodies for SRC-1 and FITC-labeled secondary antibody for androgen receptor (green). The Dapi staining was used as signal for nuclear localization. The yellow signal in the nuclei of C4-2 indicated co-immune localization of AR and SRC-1.

Figure 3. Representative effect of Saos-2 and Thp-1 on the androgen-induced gene expression for growth factors and their downstream pathways. The changes in the global mRNA gene expression signatures induced by 10 nM DHT final concentration was determined using total RNA isolated from LNCaP (A-B) and C4-2 (C-D) cells cultured alone (+/- DHT), or in combination with Saos-2 (+/- Saos-2) or Thp-1 (+/- Thp-1) cells. The relative changes in gene expression were determined using fold change |FC|, by taking the measurement of the signal obtained in the presence of ligand, and dividing it by the measurement of signal obtained in the absence of ligand. The ratio between the signals was ranked and ordered to obtain a heat map of the changes in gene expression using the T4MeV software that provide a hierarchical clustering with unsupervised analysis and Euclidean distance with average link. The heat maps highlight the low (green values) and increased (red values) expression of specific genes. The hierarchization separated the expressions of LNCaP and C4-2 cells, depending upon the change in expression (FC) when treated with hormone (DHT) and also in coculture with Saos-2 and Thp-1. The changes of growth factors (A and C) and growth factor pathways (B and D) in LNCaP and C4-2, respectively, represent the effect of Saos-2 and Thp-1 in tumor cells.

DISCUSSION:

Current research has shown that while prostate cancer has often been described as being osteoblastic, there is also an osteoclastic interaction due to the changes in the bone remodeling activity upon the arrival of circulating tumor cells. To further understand the mechanisms of osteogenesis in the prostate microenvironment, we developed a coculture model (Figure 1) using the transwell system to examine the paracrine effect of bone stroma-derived cells on androgen receptor mediated transcriptional activity, growth factors and their receptors expression, as well as the effect on additional nuclear receptors and coregulators of RNA pol II mediated transcription (coactivators/corepresors). This model is innovative for the initial testing of different products in all phases of development, from the initial prototype and patenting to potential clinical trials.

Prostate cancer cell type models representing the progression of prostate cancer to androgen independence, recurrence and bone metastasis were used, and include the lymph node derived carcinoma of the prostate (LNCaP) that represents an androgen dependent or androgen sensitive adenocarcinoma cell line. LNCaP is derived from a lymph node metastasis of a 50-year old patient. The LNCaP-C4-2B tumor cell line (termed C4-2) represents the androgen independent, androgen-resistant or recurrent prostate tumor cells that can proliferate in the presence of the low circulating levels of androgens. The C4/2 cell line was derived from LNCaP cells through tumor xenografts that acquired the property to proliferate in castrated immune compromised *nude* male mice. A second system that represents prostate cancer progression to androgen independence is the Case Western Reserve CWR22 cell and the CWR22R tumor pair. These cells can propagate as tumor xenografts using SCID mice. *In vitro* studies are limited to primary cell cultures²⁶. Two cell types that represent bone-derived osteoblasts and osteoclasts were used to determine the effect on prostate cancer cells in a coculture transwell system. The Saos-2 tumor cell line is derived from human osteosarcoma and can differentiate to osteoblasts in the presence of calcitriol to form mineralized matrixes in culture, representing a model for

osteoblast precursors. The Thp-1 tumor cell line is a human monocytic cell line derived from an acute monocytic leukemia patient and is used as a monocyte model. Thp-1 cells can differentiate into macrophage-like cells, which mimic native monocyte-derived macrophages. In addition, TRAP positive multinucleated osteoclast-like cells can be generated using high concentrations of PMA and Vitamin D3. Thus, the Saos-2 and Thp-1 cellular models representing the osteoblast and monocyte-macrophage system can be used to determine the potential roles of bone marrow activity and bone metastasis of prostate cancer cells.

Through the use of LNCaP and C4-2 cell lines, in conjunction with Saos-2 and Thp-1 cell lines that simulate osteoblasts and osteoclasts, respectively, we were able to identify new regulatory sites in coregulators (small peptides) that interact with nuclear receptors in androgen-sensitive and androgen-resistant prostate cancer tumor cells, in the absence and presence of increasing number of osteoblasts and osteoclasts precursors (**Figure 2**). These precursors change the expression of genes involved in tumor progression through a mechanism that involves the direct interaction of the activators with regulatory sites of the androgen receptor transcription complex (**Figure 2 and 3**), when in the context of increased number of osteoblasts and osteoclasts precursors (**Figures 2 and 3**).

One critical step of the protocol is when Thp-1 cells are seeded and manipulated during the experimental processes. The Thp-1 cells grow in suspension and do not attach as firmly to the tissue cultured plate as other cell types. The media must be changed twice a week, and the change of media must be performed carefully to prevent cell loss. To address this challenge, we recommend aspirating the medium into a new 50 mL conical centrifuge tube and spinning down at low speed to collect the floating cells. The new media added to this tube, which will contain the cells in suspension, can be added back to the plate. Another critical experimental step is the hormone treatment of the plated cells. Specifically, cells need to be treated with media lacking the serum, or media containing charcoal dextran coated treated fetal bovine serum (cdFBS), to lower circulating androgens and other sex steroid hormones, as well as additional small peptides and hormones present in the untreated or regular fetal bovine serum. This 24 h treatment is important to lower the level of hormones in order to reach basal conditions for gene expression, transcription activation, and growth factors expression that are secreted by the tumor cells in the absence and presence of bone stroma derived cells, in an androgen-dependent manner. Time courses and increasing hormone concentrations, either androgens or other hormones, are important variables to be considered to achieve results between 24, 36 and 48 or more hours, depending on the assessment and the specific details of the experiment to be performed. In our case, short-term interactions for 36 h were sufficient to observe the changes in gene expression due to the presence of androgens agonists and antagonists, in the absence or presence of Saos-2 and Thp-1 cells in cocultures. Long-term exposure in the coculture condition are limited due to poor cell survival after two weeks. Nevertheless, long-term experiments may be achieved using a different transwell format, such as the 10 cm plate. The use of direct cocultured cells further redefine the direct cell-cell contact and interaction between tumor cells and bone stroma derived cells. However, dissecting specific pathways using direct coculture for each cell represents a barrier in defining what is occurring in each specific cell type. When the cells are together in coculture, they cannot be taken apart

efficiently, even with laser capture microscopy. This is the reason that the transwell method is highly relevant to define the specific change(s) in each cell type in the context of paracrine signal in bone metastasis. The transwell system can also be used to determine cell migration of the tumor cells plated on the transwell, and tagged with green or red fluorescent protein encoded in mammalian expression vectors.

We intend to use this method to further analyze the effect of drugs, such as small peptides and other molecules, in addition to bicalutamide, flutamide, and mifepristone, on the androgen receptor complex with coregulators and downstream pathways that enable cancer cells to colonize and proliferate in the context of bone stroma osteoblasts and osteoclasts. We will use short-term cultures to mimic the interactions of circulating tumor cells upon arrival to the bone stroma, as well as long-term cultures to determine changes in gene expression under different experimental conditions. An important future step will be the use of differentiated osteoclasts and osteoblasts cells active in the remodelling of bone to mimic the effect of bone matrix. Thus, we will be able to evaluate the effect of changing estrogen to androgen ratios on osteoclast/osteoblast activity in the context of bone resorption and cancer cell proliferation during the progression to androgen independence, recurrence, and castration-resistant state of the disease. Molecular medicine is the new frontier in the development of therapies for a curative and definitive cancer treatment. The significant impact for the patients is to develop personalized treatments using primary or isolated circulating tumor cells, in order to eliminate unnecessary, costly and ineffective treatments, as well as increase quality of life. Thus, the use of the *in vitro* coculture system has the potential to change the paradigm of therapies focused on the androgen axis for androgen-resistant prostate cancer and other malignancies such as breast cancer. The concept that altered pathways in an androgen regulated tumor microenvironment influence the proliferation of epithelial cells through coregulators is innovative, and will generate an insightful perspective regarding the role of androgens and nuclear receptor coregulators in carcinogenesis and bone metastasis.

DISCLOSURES:

The authors have no conflicts of interest to disclose.

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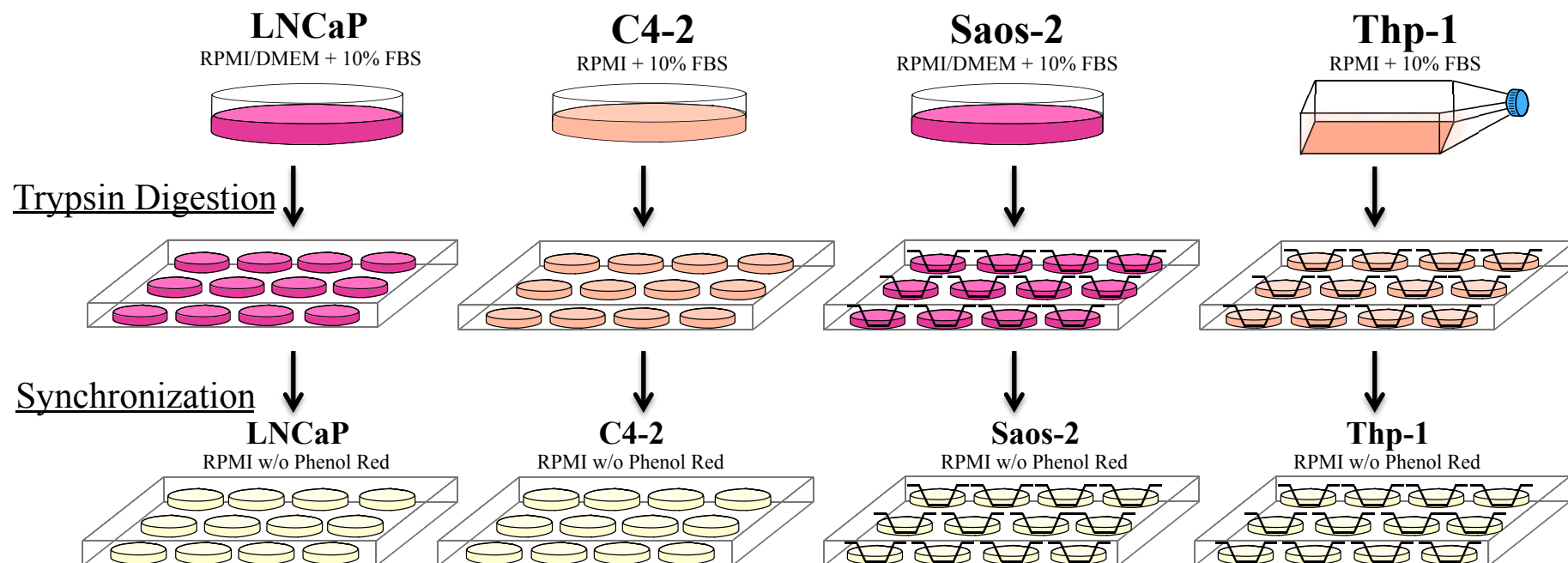
REFERENCES:

1. Hess K.R. *et al.* Metastatic patterns in adenocarcinoma. *Cancer*. **106** (7), 1624-33 (2006).
2. Coleman, S.J., Bruce, C., Chioni, A.M., Kocher, H.M., Grose, R.P. The ins and outs of fibroblast growth factor receptor signalling. *Clin Sci (Lond)*. **124** (4), 217-31 (2014).
3. Hu, Y., Yu, X., Xu, G., Liu, S. Metastasis: an early event in cancer progression. *J Cancer Res Clin Oncol*. **143** (5), 745-757 (2017).

4. Tang L. *et al.* Associations between polymorphisms in genes related to estrogen metabolism and function and prostate cancer risk: results from the Prostate Cancer Prevention Trial. *Carcinogenesis*. **39** (2), 125-133 (2018).
5. Maciel, M.J. *et al.* Computed tomography-guided percutaneous biopsy of bone lesions: rate of diagnostic success and complications. *Radio Bras*. **47** (5), 269-74 (2014).
6. McNerney E.M., Onate S.A. New insights in the role of androgen-to-estrogen ratios, specific growth factors and bone cell microenvironment to potentiate prostate cancer bone metastasis. *Nuclear Receptor Research*. **2**, 101186 (2015).
7. Murray, N.P., Reyes, E., Tapia, P., Badinez, L., Orellana, N. Differential expression of matrix metalloproteinase-2 expression in disseminated tumor cells and micrometastasis in bone marrow of patients with nonmetastatic and metastatic prostate cancer: theoretical considerations and clinical implications -an immunocytochemical study. *Bone Marrow Research*. **2012**, 259351 (2012).
8. Villagran M.A. *et al.* Bone stroma-derived cells change coregulators recruitment to androgen receptor and decrease cell proliferation in androgen-sensitive and castration-resistant prostate cancer cells. *Biochem Biophys Res Commun*. **467** (4), 1038-45 (2015).
9. Chen G., Deng, C., Li Y.P. TGF- β and BMP signaling in osteoblast differentiation and bone formation. *Int J Biol Sci*. **8** (2), 272-88 (2012).
10. Wu, M., Chen, G., Li Y.P. TGF- β and BMP signaling in osteoblast, skeletal development, and bone formation, homeostasis and disease. *Bone Res*. **4**, 18009 (2016).
11. Jeong H.M., Cho, S.W., Park, S.I. Osteoblasts are the centerpiece of the metastatic bone microenvironment. *Endocrinol Metab (Seoul)*. **31** (4), 485-492 (2016).
12. Oulion, S., Bertrand, S., Escriva H. Evolution of the FGF gene family. *Int J Evol Biol*. **2012**, 298147 (2012).
13. Xu, R., Rudd, T.R., Hughes, A.J., Siligardi, G., Fernig, D.G., Yates, E.A. Analysis of the fibroblast growth factor receptor (FGFR) signalling network with heparin as coreceptor: evidence for the expansion of the core FGFR signalling network. *FEBS J*. **280** (10), 2260-70 (2013).
14. Wesche, J., Haglund, K., Haugsten, E.M. Fibroblast growth factors and their receptors in cancer. *Biochem J*. **437**, 199-213 (2011).
15. Corn, P.G., Wang, F., McKeenan, W.L., Navone, N. Targeting fibroblast growth factor pathways in prostate cancer. *Clin Cancer Res*. **12**, 5856-5866 (2013).
16. Huang Z. *et al.* Two FGFR kinase molecules act in concert to recruit and transphosphorylate PLC γ *Mol Cell*. **61** (1) 98-110 (2016).
17. Czekanska, E.M., Stoddart, M.J., Richards, R.G., Hayes, J.S. In search of an osteoblast cell model for in vitro research. *Eur Cells Mater*. **24**, 1-17 (2012).
18. Rucci, N., Angelucci, A. Prostate cancer and bone: the elective affinities. *Biomed Res Int*. **2014**, 167035 (2014).
19. Langeley, R.R., Fidler, I.J. The seed and soil hypothesis revisited — the role of tumor-stroma interactions in metastasis to different organs. *Int J Cancer*. **128** (11), 2527-2535 (2011).
20. Nannuru, K.C., Singh, R.K. Tumor-stromal interactions in bone metastasis. *Curr Osteoporos Rep*. **8** (2), 105-13 (2010).
21. Bosshart, H. Heinzelmann, M. THP-1 cells as a model for human monocytes. *Ann Transl Med*. **4** (21), 438 (2016).

- 613 22. Park, K.Y. *et al.* Patient specific proteolytic activity of monocyte-derived macrophages and
614 osteoclasts predicted with temporal kinase activation states during differentiation. *Integr Biol*
615 *(Camb)*. **4** (12), 1459-69 (2012).
- 616 23. Daigneault, M. Preston, J.A., Marriott, H.M., Whyte, M.K., Dockrell, D.H. The identification
617 of markers of macrophage differentiation in PMA-stimulated THP-1 cells and monocyte-derived
618 macrophages. *PLoS One*. **5** (1), e8668 (2010).
- 619 24. Sottnik, J.L., Keller, E.T. Understanding and targeting osteoclastic activity in prostate cancer
620 bone metastases. *Curr Mol Med*. **13** (4), 626-639 (2013).
- 621 25. <http://www.tm4.org>.
- 622 26. Godoy A.S. *et al.* Altered Corepressor SMRT Expression and Recruitment to Target Genes as
623 a Mechanism that Change the Response to Androgens in Prostate Cancer Progression. *Biochem*
624 *Biophys Res Commun*. **423**, 564-570 (2012).

A) Step-1: Monocultured Cells



B) Step-2: Cocultured Cells

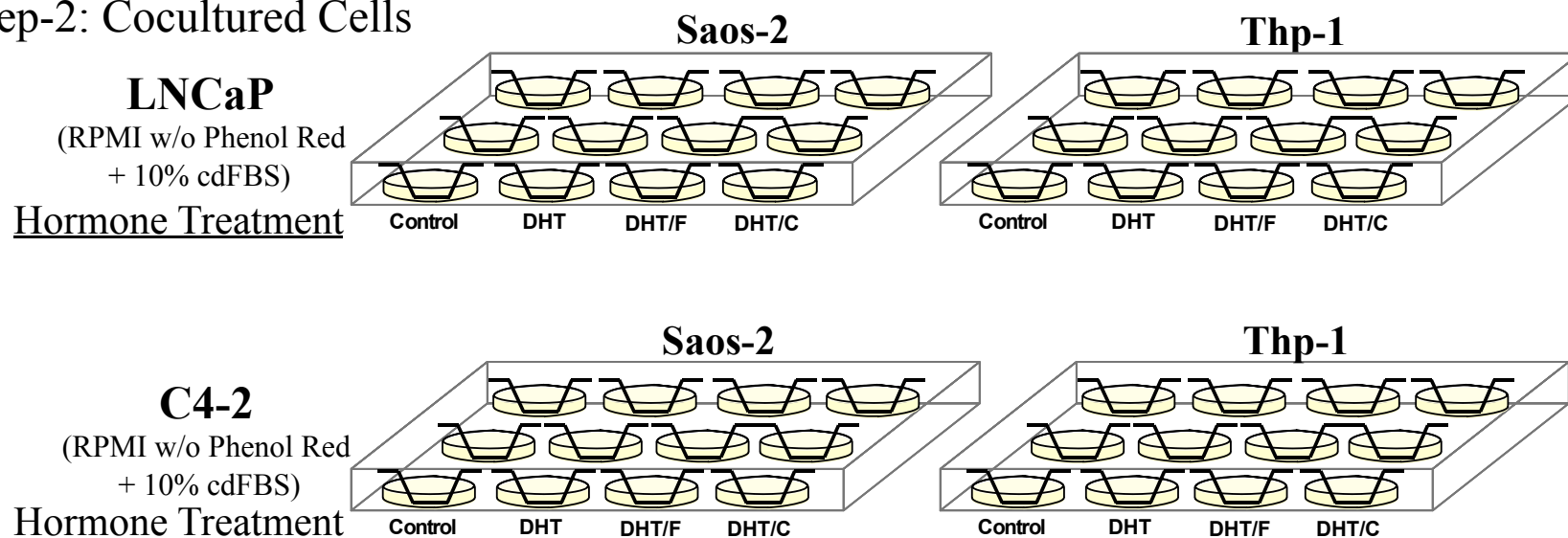


Figure 3

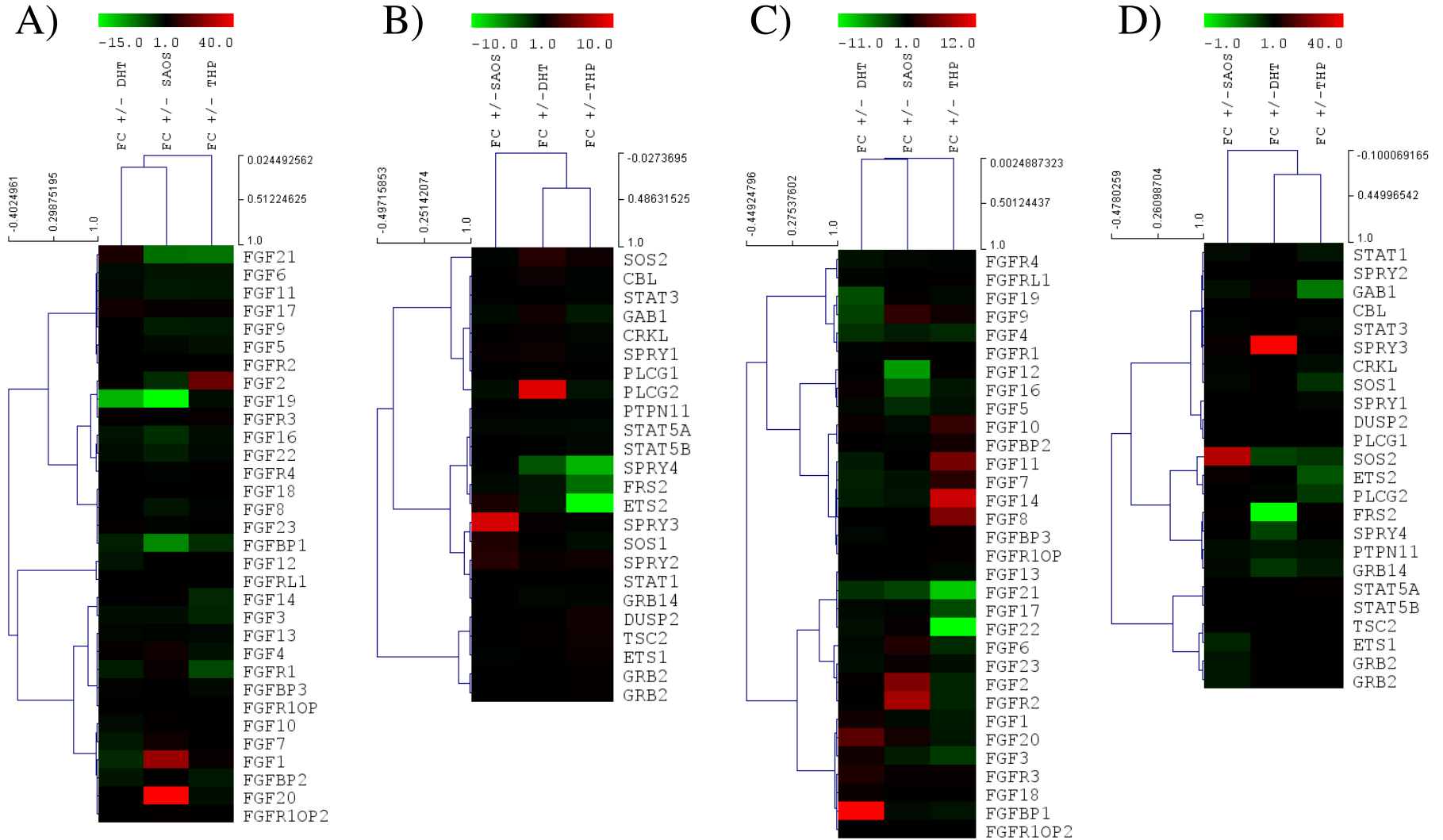
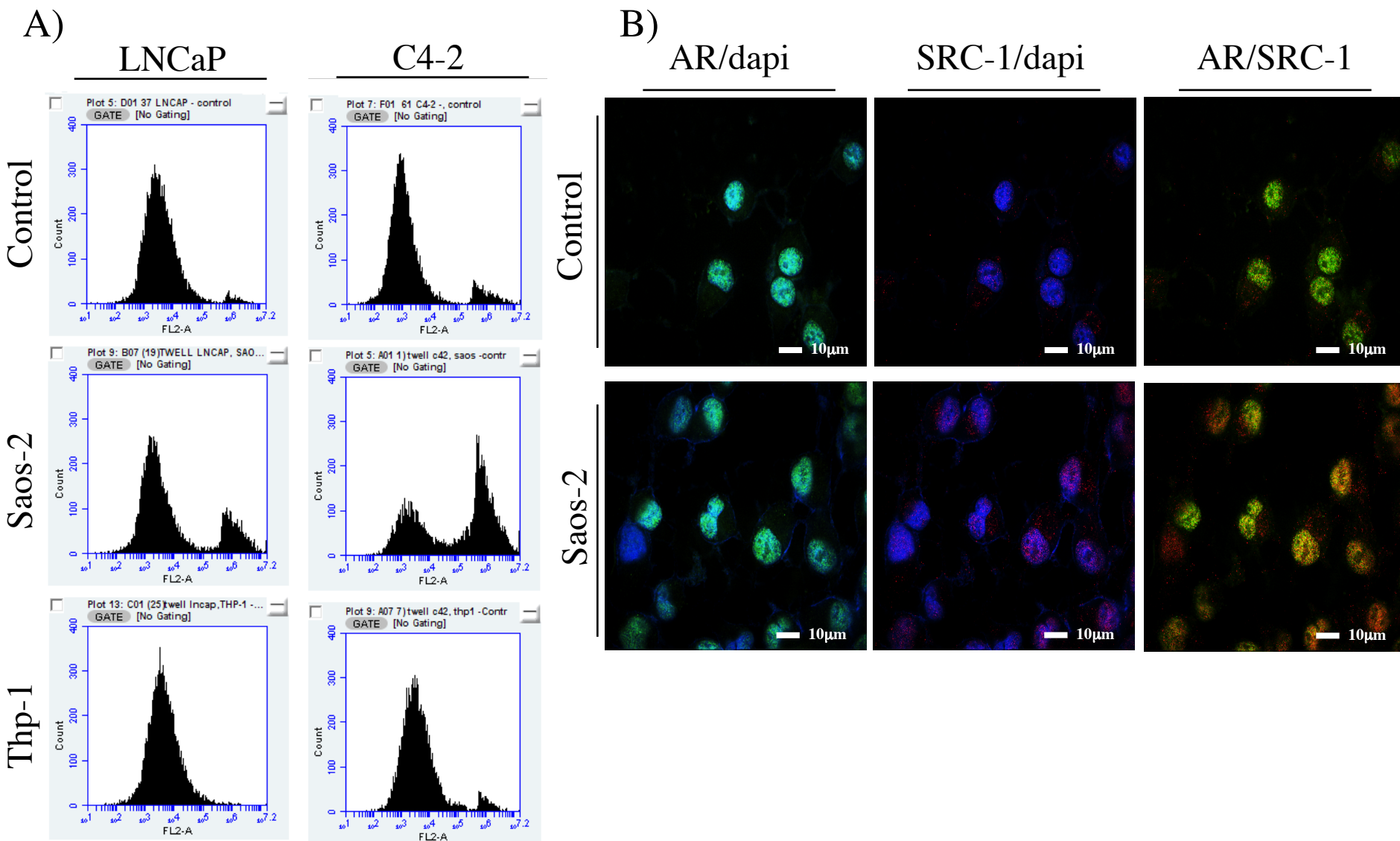
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Figure 2



Name of Reagent/ Equipment	Company	Catalog Number
Water-jacketed CO ₂ incubator	Autoflow	NU-8700E
Flow Cytometer	BD Biosciencies	23-13347-02 Accuri C6
Centrifuge legend micro 21R	Thermo Scientific	TSO-M21
Centrifuge eppendorf	Eppendorf	58040000323
Confocal Microscopes	Olympus	IX81/IX81-ZDC
Neubauerchamber	Hirschmann	8020385
		F144801; F123600;
Micropipette	Pipetman Gilson	F1123602
Cryogenic vial	Corning	430488
Tips	Biopointe	361-4050
Tips	Biopointe	351-4050
Tips	Biopointe	332-4050
Tube	Falcon	352097
Tube	Falcon	352098
Serological Pipettes	Falcon	53300-523
Tissue Culture Dish	Corning	25382-428
Tissue Culture flask	Falcon	3013
12 Well Format Tissue Culture Plates	Falcon	353043
12 Transwell Format Tissue Culture Plates	Corning	3460-clear
Eppendorf Tubes	Axygen	MCT-150-C
RPMI-1640 w/ 25mM Hepes, w/L-Glutamine	Corning	45000-414-1
DMEM High, w/o L-Glutamine & Sod.Pyr.	Corning	45000-326
	HyClone, Thermo	SH30605.01
RPMI 1640, without L-Glutamine and without Phenol Red	Scientific	
Fetal bovine serum, USDA Certified	Corning	35-010-CV
FBS (Charcoal Stripped Serum)	Corning	35-072-CV
L-glutamine	Corning	45000-676
Amphotericin B	Corning	45000-808
Penicillin Streptomycin	Corning	45000-652
Gentamicine	Corning	45000-636
Dimethyl Sulfoxide	Sigma life Science	D4540-500ML

PBS 1X	Corning	21-040-CVR
0.25% trypsin	Corning	45000-664
Ethanol absolute	Merck	200-578-6
Flutamide	Sigma Life Science	F 9397
Bicalutamide	Sigma Life Science	B9061
Dihydrotestosterone	Sigma Life Science	D-077
Paraformaldehyde	Sigma Life Science	p6148-500G
Histone H3 pser10 + Propidium iodide Kit	Abcam	ab151282
Bead 6 Peak flow citometer standarization	BD Biosciencies	653145
Bead 8 Peak flow citometer standarization	BD Biosciencies	559123
VECTASHIELD HardSet Antifade Mounting Medium with D Vector Laboratories		H-1500
Bovine Serum Albumin - Fraction V	Rockland	BSA-50
Triton x-100	Sigma Life Science	9002-93-1
Antibody Androgen Receptor	Abcam	Ab 9474
Antibody KAT13A/SRC-1	Abcam	Ab 2859
Fluorescein (FITC) AffiniPure Goat Anti-Rabbit IgG (H+L)	Jackson ImmunoResearch	111-095-003
Cy3 AffiniPure Donkey Anti-Rabbit IgG (H+L)	Jackson ImmunoResearch	711-165-152
HumanHT-12 v4 Expression BeadChip Kit	Illumina	BD-103-0204
MultiExperiment Viewer v4.9 (tm4MeV)	WebMeV	www.mev.tm4.org

Comments/Description

37 °C

Flow Cytometer Accuri C6

21R Model

Refrigerated Centrifuge 5803R

IX81 model

0.0025 mm²

P2; P20; P1000

2 mL

1000 µL

200 µL

10 µL

15 mL

50 mL

10 mL

100mm x 20 mm

25-cm²

22.1 mm Diameter Inserts

12 mm Diameter Inserts

1.5 mL

1000 mL

1000 mL

500 mL

500 mL

50 mL

200mM Solution; 100 mL

0.25mg/mL Solution; 100 mL

10 mg/mL Solution; 100 mL

50 mg/mL; 100 mL

500 mL

500 mL

2.21 mM EDTA, 1x; 100 mL

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March 12, 2018

Nandita Singh
 Editorial Manager
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 Journal of Video Experimental (JoVe)

Dear Ms. Singh:

Please find the attached article submission entitled **“Coculture system to study osteoblast and osteoclast interactions in prostate cancer progression to hormone resistance and metastasis to bone.”** This manuscript, as well as supporting figures and materials table, have been revised according to the recommendation of the editor and reviewers. In addition, the relevant protocols to be featured in the video have been highlighted in yellow.

Rebuttal and Responses to Reviewer Comments

Reviewer #1

1. We incorporated the wording recommended into the title. The new title is: **“Coculture system to study osteoblast and osteoclast interactions in prostate cancer progression to hormone resistance and metastasis to bone.”**
2. “Rephrase the subtitles 1.2 (line 151) and 2.3 (line 216) specifying the cell types they have used.” We agree that the cell lines do not represent osteoblast and osteoclast differentiation. As suggested, we incorporated the use of human derived primary cell culture as an alternative means to represent osteoblast and osteoclast cells. We rephrased subtitles 1.2 and 2.3 to specify cell types used.
3. “I suggest the authors to rephrase this subtitle as “Gene expression profiling.” We incorporated the wording recommended for subtitle 5.3.
4. “Please clarify Fig.1 labeling as “Saos-2” and “Thp-1” as they have noted in Fig.2.” We incorporated “Saos-2” and “Thp-1” into Fig 1, as recommended.



University of Concepcion

Sergio A. Onate, Ph.D.

Director

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Reviewer #2

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1. We agree that bone metastasis is only present in metastatic prostate cancer patients. Therefore, we acknowledged in the long abstract and introduction that bone metastasis **may** be considered to be an early event in patients with colonized bone metastasis. We also agree that, although the clinical data points to the change in androgen-estrogen ratios affect estrogen receptor function, the specific experiment(s) in vivo remain to be determine, i.e. how the modification of E to T ratio change tumor cell proliferation, migration and bone metastasis using tumor cell heart injection. With regards to the

Gleason score, we had used the Gleason score described in the reference #7, but in acknowledgement of the reviewer's observation, we have changed the text to read, "disseminated cells can be detected in the bone marrow niche in lower Gleason grades of the primary tumor^{7,8}" With regards to the reviewer's concern of the introduction being "over-generalized" and containing "a number of incorrect statements," and in addition to changes specified above, the supporting references #3 and 4 have been updated to support the concept that metastasis is an early event, which may be influenced by changing steroid hormone ratios (. Hu, Y., Yu, X., Xu, G., Liu, S. Metastasis: an early event in cancer progression. J Cancer Res Clin Oncol. 143 (5), 745-757 (2017); Tang L. et al. Associations between polymorphisms in genes related to estrogen metabolism and function and prostate cancer risk: results from the Prostate Cancer Prevention Trial. Carcinogenesis. 39 (2), 125-133 (2018)).

2. "The methods outlined are extremely detailed and are written in the text of a protocol rather than an outline of what has been performed:" The format used for the protocol described in the manuscript was in compliance with the requirements of JoVE.

3. "There are a number of spelling errors in the text including "cytometry", misspelled as "cytometry" and on line 191, it should read, "RPMI/DMEM". We corrected "cytometry," and added "RPMI/DMEM" as recommended.

Please contact me if any additional changes are required.

Sincerely,

A handwritten signature in blue ink, appearing to read 'Sergio A. Onate'.

Sergio A. Onate, Ph.D.