

Rapid, Non-Destructive, Cell-Based Screening Assays for Agents That Modulate Growth, Death, and Androgen Receptor Activation in Prostate Cancer Cells

Peyman Tavassoli,^{1,3} Rob Snoek,³ Mira Ray,⁴ Leticia Gomez Rao,⁵
and Paul S. Rennie^{1,2,3*}

¹Department of Pathology & Laboratory Medicine, University of British Columbia, Vancouver, British Columbia, Canada

²Department of Urologic Sciences, University of British Columbia, Vancouver, British Columbia, Canada

³The Prostate Centre at Vancouver General Hospital, Vancouver, British Columbia, Canada

⁴Department of Oncology, London Health Science Centre, University of Western Ontario, Canada

⁵Department of Medicine, University of Toronto, Toronto, Ontario, Canada

BACKGROUND. We developed non-invasive, cell-based screening assays to rapidly and biologically assess factors that modulate prostate cancer growth and affect androgen receptor (AR) activity.

METHODS. LNCaP cells, which stably express enhanced green fluorescent protein (EGFP) either constitutively or upon AR activation, were treated with a variety of agents, and then monitored by fluorescence and MTS assays for dose-dependent changes in cell number and AR activity.

RESULTS. The assays were validated for rapid, fluorescence-based, quantitative measurement for the presence of growth and AR modulators. Using these assays, we found that osteoblast conditioned media (CM) enhanced prostate cancer cell growth, but not AR activity. After priming with androgen (<1 nM R1881), forskolin or the pesticide dichlorvos enhanced AR activation, whereas interleukin-6 (IL-6) inhibited it.

CONCLUSION. These non-destructive, cell-based assays enable rapid systematic monitoring of the effects of drugs or complex mixtures on prostate cancer cell growth and/or AR activity.

Prostate 67: 416–426, 2007. © 2007 Wiley-Liss, Inc.

KEY WORDS: lentivirus; apoptosis; osteoblast conditioned media; IL-6; dichlorvos; EGFP

INTRODUCTION

The development and proliferation of prostate cancer in its early stages depends on the presence of androgens and androgen receptor (AR) [1]. There is considerable evidence that even with androgen ablation, AR still plays an important role in progression of ablation-resistant prostate cancer [1–4]. The activation of AR at this stage may be due to the compensatory mechanisms of the multiplicity of endogenous factors such as co-regulators, cytokines, or growth factors [5–11], or due to the effect of exogenous factors. For example, it has been reported that many chemicals

present in the environment can mimic, antagonize or interfere with physiological actions of endogenous hormones such as androgens [12]. These exogenous

Grant sponsor: SPORE (Specialized Programs of Research EXCELLENCE); Grant number: 03-5199-504251; Grant sponsor: Prostate Cancer Research Foundation of Canada.

*Correspondence to: Dr. Paul S. Rennie, The Prostate Centre at Vancouver General Hospital, 2660 Oak Street Vancouver, BC, V6H 3Z6 Canada. E-mail: prennie@interchange.ubc.ca

Received 26 September 2006; Accepted 6 October 2006

DOI 10.1002/pros.20532

Published online 11 January 2007 in Wiley InterScience

(www.interscience.wiley.com).

substances have been called endocrine disruptors and include environmental contaminants [13–16], designer androgens [17,18], and natural products [19,20]. There is evidence that some of these endocrine disruptors can cause cancers in humans, including prostate cancer [21,22]. With a continually growing list of endogenous and exogenous substances that can alter AR transcriptional activity or prostate cancer cell growth, there is a need for a high throughput method to assess these activities.

Typically, AR transcription assays involve transient transfection of cell lines with one or more plasmids expressing AR and a reporter gene, followed by treatment with a variety of agents and subsequent measurement of reporter gene expression. However, transient transfection assays may not accurately reflect evenly distributed, steady-state levels of receptor, as transfection efficiencies can vary greatly between cells and between replicate assays. Also, these assays usually require a large number of cells, costly transfection materials and several experimental manipulations. In addition, transient transfections have time-limited responsiveness since the transgene is usually lost within 72 hr. For cell growth assays, procedures for determining cell number at given time points usually are lethal to the cells and hence, to obtain growth curves, one needs to perform several parallel yet independent experiments.

To circumvent these problems, we have created a novel approach to screen for agents that modulate prostate cancer cell growth or AR activity using stably transformed prostate cancer cells expressing an enhanced green fluorescence protein (EGFP) reporter under a constitutive or AR-regulated promoter. These assays are relatively high throughput, versatile, and far less variable than transient transfection techniques. While others have also developed in vitro bioassays using stable cell lines to quantify AR activity [21,23,24], the advantage of our system is that one can monitor and screen multifactorial components such as conditioned media (CM), growth factors, and potential therapeutic treatments over a time course in a rapid and non-destructive manner, and thereby find the optimal treatment conditions with respect to impact on prostate cell growth and AR activation.

MATERIALS AND METHODS

Cell lines, Media, and Additives

LNCAp human prostate adenocarcinoma cells and 293 T human embryo kidney cells were obtained from American Type culture collection (ATCC, Manasa, VA). PC3 human prostate cancer cells that stably express androgen receptor (PC3(AR)₂) were a kind gift from Dr. T.J. Brown, (University of Toronto, Ontario,

Canada). LNCAp cells were grown in RPMI 1640 medium supplemented by 5% heat inactivated fetal bovine serum (FBS), and Penicillin-Streptomycin (P-S) 1% (Life Technologies, Inc., Gaithersburg, MD). PC3(AR)₂ and 293 T cells were maintained in DMEM medium with 10% FBS and 1% P-S. Osteoblast-like SaOS-2 cells (ATCC) were cultured and induced to differentiate as previously described [25]. Briefly, cells were initially cultured in Ham's F-12 medium (Life Technologies) containing 28 mM HEPES pH 7.4, 1.4 mM CaCl₂, 2 mM glutamine, 10% FBS, 1% P-S. This is further supplemented with 10 nM dexamethasone and 50 µg/ml ascorbic acid. At day 8, 10 nM β-glycerophosphate was added and this addition was repeated at every medium change until mineralization, at approximately day 15. Subsequently, the media was switched to 5% charcoal stripped serum (CSS) supplemented Ham's F-12 media as above except without ascorbic acid and dexamethasone, for another 24 hr. CM from this source was stored at –80°C prior to experimentation. SaOS-2 differentiation was validated by alkaline phosphatase and Von Kossa staining, as before [26].

Generation of Stable Cell Lines Expressing EGFP

A lentiviral approach was used to generate either stable, constitutive EGFP-expressing LNCAp cells (LN-CMV-EGFP cells) or PC3(AR)₂ cells (PC3-CMV-EGFP) as described previously [27]. Briefly, 1.5 × 10⁶ 293 T cells were seeded onto 10 cm plates and co-transfected the following day by calcium phosphate precipitation method (Profection Mammalian Transfection Systems, Promega, Madison, WI) with 10 µg of lentiviral vector, Lv-CMV-EGFP; 10 µg of pCMVDR8.2, containing viral assembly sequences; and 5 µg of pMD.G, carrying the vesicular stomatitis virus envelope glycoprotein G (VSV-G). After 16 hr incubation, the medium was changed to 10% FBS-DMEM. The virus containing medium was collected after 48 hr, passed through a 0.45 mm filter to remove debris and then stored at –80°C. For lentiviral infection, LNCAp or PC3(AR)₂ cells were seeded onto 10 cm plates. When cell confluence was ~60%, 5 ml of virus containing medium was added. After 16 hr, the media was switched to 5% FBS RPMI and, 2 days after infection, cells expressing the EGFP transgene were checked under a fluorescent microscope.

Similar procedures were used for generating stable, AR-regulated EGFP-expressing LNCAp cells (LN-ARR₂PB-EGFP) using an androgen responsive probasin-derived promoter (ARR₂PB) instead of a CMV promoter for the transfer plasmid (Lv-ARR₂PB-EGFP). The ARR₂PB promoter, a gift from Dr. R. Matusik [28], contains two copies of probasin androgen

response region (PB ARR; –244 to –96) upstream of the minimal promoter and is the most potent form of PB promoter that has been shown to retain prostate-specific and hormone-regulated EGFP expression in LNCaP cells [27].

Selecting for Homogeneous EGFP Expressing Cells

To obtain populations of cells that show relatively homogeneous fluorescence, LN-CMV-EGFP, PC3-CMV-EGFP, and AR-activated LN-ARR₂PB-EGFP cells were sorted for high fluorescence levels using fluorescence activated cell sorting (FACS) at the Biomedical Research Centre of the University of British Columbia (Vancouver, BC, Canada). Briefly, about 2×10^7 trypsinized cells were re-suspended in cold phosphate buffered saline (PBS) supplemented with 0.02 mM EDTA and ranked based according to their EGFP expression. The highest fluorescence expressing cells were collected and cultured for subsequent assay development.

Cell Proliferation Assays

LN-CMV-EGFP cells were seeded into 96-well black plates (Nalge Nunc International, Rochester, NY) using 5% FBS, phenol red-free RPMI medium (Gibco, Life Technologies, Inc., Gaithersburg, MD) at different cell densities (5,000–20,000 cells per well) and assessed non-destructively over time by measuring fluorescence (EGFP) using a Fluoroskan Ascent FL (Thermo-Labsystem, Helsinki, Finland) at excitation and emission of 485 and 518 nm, respectively, every 24 hr and verified by fluorescent microscopy. Mean relative fluorescence unit (RFU) values were obtained from eight replicates of each assay condition. For treatment with CM from SaOS-2 cells, LN-CMV-EGFP cells were re-suspended with 50% or 100% of CM or control (5% CSS SaOS-2 media) and seeded at 20,000 cells/well. Fluorescent cells were assessed over time using the Fluoroskan. Cell numbers were determined at the termination of each experiment (day 4) using the MTS cell proliferation assay (CellTiter 96[®] Aqueous One Solution Reagent, Promega). In brief, 20 μ l of the reagent was added to attached cells in each well of the 96-well plate containing 100 μ l media and incubated for 2 hr at 37°C in 5% CO₂. Light absorbance of formazan was measured at 490 nm on a 96-well plate reader equipped with KC4 software (Biotek Instruments, Inc., Winooski, VT).

Measurement of Apoptotic Cells

Mitoxantrone was purchased from Sigma Chemicals (St. Louis, MO). Stock solutions of mitoxantrone (1 mg/ml) were prepared with PBS and diluted to the required

concentrations prior to each experiment. One hundred microliters of 5% FBS phenol red-free media RPMI containing 20,000 LN-CMV-EGFP cells were seeded into each well of a 96-well black plate. Twenty-four hours later, cells were treated with different mitoxantrone concentrations ranging from 0 to 10 μ M. Fluorescence was measured at time 0 and the cells were also monitored visually under a fluorescent microscope. After 48 hr, the media were gently transferred into a new multi well plate and 100 μ l of fresh media was added to the original plate, followed by fluorescence measurements of both plates. At the termination of each experiment, a MTS cell number assay was performed.

Androgen Receptor Transactivation Assays

LN-ARR₂PB-EGFP cells were grown in 5% CSS RPMI for 5 days to deplete cells of bio-available androgen and to reduce their EGFP expression to background levels. This was confirmed by fluorescent microscopy. Cells were then seeded at a density of 20,000 cells/well in 96-well black plates in 5% CSS phenol red-free RPMI, grown overnight, subsequently treated with different concentrations of the potent synthetic androgen, R1881 (methyltrienolone, Dupont, Boston, MA) and followed by fluorescent measurements every 24 hr. At day 3, experiments were terminated and MTS assays were performed. Using analogous protocols, 20 ng/ml interleukin-6 (IL-6) (Calbiochem-Novabiochem Corp., San Diego, CA), 25 μ M forskolin (FSK) (Sigma), and 0.1–1 μ M dichlorvos (AccuStandard, New Haven, CT), with or without R1881, were also tested in this system. For experiments with bicalutamide (casodex, a gift from AstraZeneca), LN-ARR₂PB-EGFP cells were grown for 5 days in CSS, seeded at 20,000 cells/well and incubated with bicalutamide (0–50 μ M) overnight. Following this, the cells were treated with 1 nM R1881 and then after 72 hr assessed for fluorescence and cell number as described above.

Statistical Analysis

Statistical analyses were performed using Student's *t*-tests with JMP IN software (version 4.0.2.-Academic).

RESULTS

EGFP Expression Directly Correlates With the Number of Cells

LNCaP cells were infected by lentiviral vector Lv-CMV-EGFP to create the LN-CMV-EGFP cell line. In these cells, the constitutively active CMV promoter drives EGFP expression, which is visible under

fluorescent microscopy and quantifiable by Fluoroskan fluorometry. Using this cell line, we sought to establish and to validate a fluorescence assay to estimate changes in prostate cancer cell numbers in response to various growth promoting or inhibiting agents. Due to differences in the relative multiplicity of infection of individual cells, variable expression of EGFP from low to high intensities was observed. To achieve a homogeneous population of LN-CMV-EGFP cells and therefore a robust growth assay system, these cells were sorted for high fluorescence expression by FACS. The sorted cells had a comparatively homogeneous pattern of EGFP expression as determined by a fluorescent microscope and remained stable expressers of EGFP even after more than 12 passages. Since the CMV promoter is constitutively active, higher EGFP expression levels is presumably due to a higher number of cells rather than any change in fluorescent intensity per cell. To test for a direct correlation between fluorescence and cell number, fluorescent LN-CMV-EGFP cells and non-fluorescent wild-type LNCaP cells were seeded at different densities in 96-well plates and the amount of fluorescence measured every 24 hr. Figure 1A shows that there was an increase in RFUs as the seeding density of the LN-CMV-EGFP cells was increased. After an initial delay of 24 hr, the RFU values

in each well containing LN-CMV-EGFP cells increased in a time-dependent manner, such that there was a linear increase ($R^2 > 0.90$) between 24 and 96 hr. To verify that the RFU increases indeed reflected increased cell numbers, MTS assays were performed at 96 hr. As expected, the number of both EGFP and non-EGFP expressing cells increased by day 4 in proportion to the number of cells initially seeded (Fig. 1B). To determine if the amount of EGFP fluorescence directly correlated with the number of cells, the corresponding cell numbers and RFU at day 1 and 4 were plotted. As shown in Figure 1C there is a linear correlation between cell number and RFU up to 30,000 cells/well ($R^2 = 0.99$).

PC3 human prostate cancer cells, which were infected by Lv-CMV-EGFP were also tested in a similar fashion. These cells were seeded at densities of 2,000 to 8,000 cells/well and fluorescence was measured every 24 hr as described above (Fig. 1D). Again, after an initial 24 hr delay, there was an apparent linear relationship between RFU levels and incubation time ($R^2 > 0.98$). After 4 days, cell numbers were determined by MTS assays and compared to RFU values, with a linear correlation between RFU and cell number estimates seen over the range of 2,000 to 17,000 cells/well ($R^2 = 0.99$) (data not shown). Overall, the linear

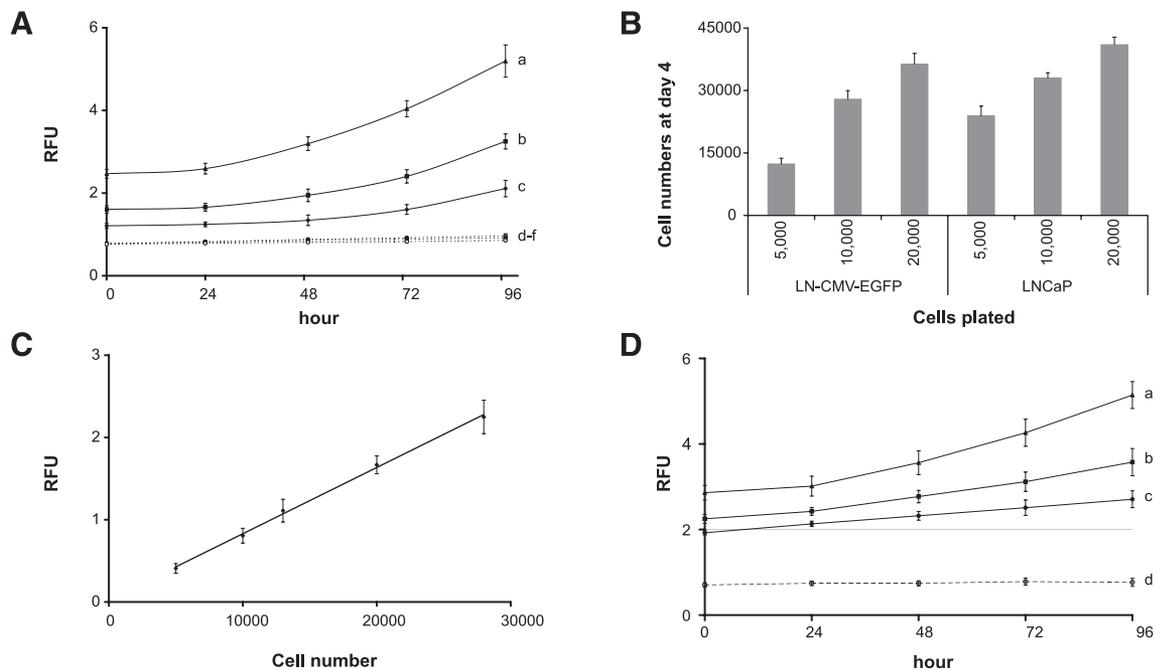


Fig. 1. Correlation of EGFP fluorescence with cell number. **A:** LN-CMV-EGFP cells were seeded in 96 black well plates at different densities (**a**) 20,000, (**b**) 10,000, (**c**) 5,000 cells/well among LNCaP cells at the corresponding numbers (**d–f**) using 5% FBS phenol red free RPMI. The EGFP was measured by fluorometry every 24 hr. **B:** Cell number assay using a MTS method at day 4 of both LN-CMV-EGFP and LNCaP cells based on seeded cell numbers. **C:** Plot of fluorescence (RFU) versus LNCaP-CMV-EGFP cell numbers **D:** PC3-CMV-EGFP cells were seeded at different densities (**a**) 8,000, (**b**) 4,000, (**c**) 2,000 cells/well versus (**d**) media into a 96-well plate and the fluorescence was measured every 24 hr.

correlation between EGFP expression and cell numbers in both LNCaP and PC3 EGFP-expressing cells indicated that these cells could provide the basis for a direct assay to non-destructively measure changes in cell number over time in living prostate cancer cells in response to various growth modulators or inhibitors.

Simultaneously Screening for Cell Death and Cell Viability

Having demonstrated that this fluorescence assay could be used for quantitation of prostate cancer cells under growth promoting circumstances, we next tested whether it could accurately estimate decreasing cell numbers in response to a cytotoxic agent. Mitoxantrone is an anthraquinone antineoplastic agent that has been extensively used to treat castrate-resistant prostate cancer [29] and has been shown to induce apoptosis in LNCaP cells [30]. To validate whether our cell-based assays could determine a dose response to mitoxantrone, LN-CMV-EGFP cells were plated at 20,000 cells/well and then treated with mitoxantrone concentrations ranging from 0 to 10,000 nM. After 48 hr, light microscopy revealed that wells with >1,000 nM mitoxantrone contained a mixture of alive and dead cells with some cells having apoptotic features such as cell shrinkage and nuclear fragmentation (data not shown). Under the fluorescent microscope, it was observed that viable, adherent LN-CMV-EGFP cells had EGFP fluorescence that was concentrated inside the cells, resulting in bright fluorescence wherever there was a live cell. By comparison, dead cells had lysed and their EGFP proteins had diffused into media. This diffused EGFP resulted in an elevated fluorescence background that could be easily seen with the fluorescent microscope. Since only upon cell death did EGFP appear in the media, the amount of fluorescence in the media should be an index of cell death.

Accordingly, after 48 hr treatment with mitoxantrone, the media from each well was transferred to a new plate and RFUs were measured. As is evident in Figure 2A, only background fluorescence was detected in the media after treatment with 0 to 100 nM mitoxantrone. However, significant media RFU was measured at >1,000 nM suggesting extensive cell death ($P < 0.05$). At 10,000 nM of mitoxantrone, all the cells were dead as determined by fluorescent microscopy and further validated by MTS assays.

To measure the proportion of living cells in the same samples, fresh media was added and RFUs measured in adherent cells. To confirm that the RFU values obtained were correlated with the number of surviving cells, MTS assays were performed and compared to the corresponding RFUs (Fig. 2B). At 0 nM mitoxantrone, the RFU levels of live cells had increased 30% in comparison to the average RFUs measured at the time of seeding (2.26 ± 0.14). Between 10 and 100 nM of mitoxantrone treatment, cells appeared to be in a quiescent state, since their mean RFU values were not significantly changed in comparison to that at seeding time and the MTS assay performed on these cells showed no significant difference in cell numbers between seeding time and after 48 hr treatment. However, at 1,000 nM mitoxantrone, there was 30% less RFU than at seeding time, consistent with a 30% decrease in cell numbers determined by the MTS assay. As the concentration of the drug increased, the RFU levels decreased in the same proportion as the cell number, such that at 10,000 nM mitoxantrone no live cells were detectable. It should be noted that there is an apparent reciprocal relationship between the RFU values measured in the surviving cells and that in the media, where the fluorescent contribution is from dead cells and that this assay enables one to estimate the relative number of both living and dead cells in the same sample.

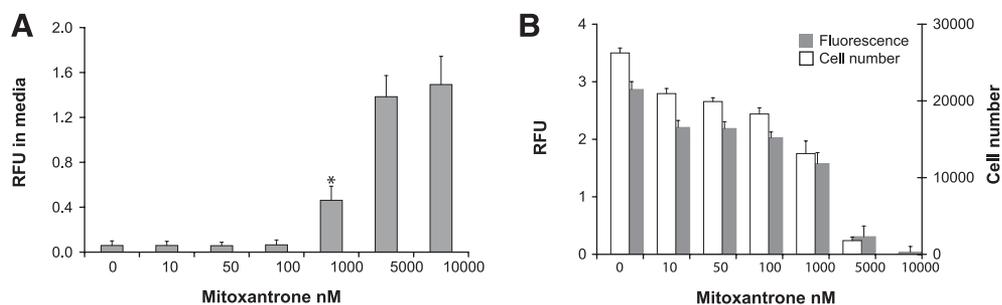


Fig. 2. Screening for cell viability and cell death using LN-CMV-EGFP cells. **A:** Twenty thousand LN-CMV-GFP cells were seeded in each well of a 96 black well plate overnight. Cells were then treated with mitoxantrone ranging from 10 to 10,000 nM for 48 hr. The media was then transferred to a new 96-well plate. The diffused EGFP (RFUs) in the media from lysed cells was measured. Asterisk indicates a significant difference ($P < 0.05$) at a 1,000 nM in comparison to lower levels. **B:** One hundred microliters of fresh media was added to each well and the EGFP from the attached viable cells measured. After the fluorescence was measured, a MTS assay was performed to determine the cell numbers/well. Both fluorescence and cell numbers at various mitoxantrone levels were plotted.

Osteosarcoma (SaOS-2) Conditioned Media Increases LN-CMV-EGFP Cell Proliferation

Having developed an assay that quantifies both cell proliferation and cell death, we next evaluated a biological sample with an unknown potential for growth modulation. In vitro and in vivo studies have shown that bone factors can accelerate human prostate cancer growth [6,31–36]. SaOS-2, a human osteoblast-like osteosarcoma cell line is capable of producing a variety of bone factors (osteoprotegerin, osteonectin, osteocalcin, bone sialoprotein, TGF- β , IGF-1, IL-6, IL-1) and mineralization in the presence of β -glycerophosphate [6,37–39]. Accordingly, we tested CM from differentiated SaOS-2 cells in our assay system. Figure 3A shows the RFUs measured over time for cells treated with different concentrations of steroid-depleted (charcoal-stripped) CM. While following the first 24 hr there were no significant differences among treatment conditions, after 48 hr, differences in fluorescence were detectable and became significant with 100% CM compared to non-CM at day 3 ($P < 0.05$). After 4 days, a significant RFU difference over control ($P < 0.05$) was also seen in experiments with 50% CM. The increased RFUs with 50% and 100% CM at day 4 were about 1.3- and 1.4-fold, respectively. This was confirmed by a MTS assay, which showed a similar fold increase in cell number (Fig. 3B). Taken together, these results indicate that, in a single time-course experiment, this assay can be used to non-destructively monitor the presence of secreted mitogens in complex mixtures (i.e., CM) and evaluate their relative potency in stimulating prostate cancer cell growth.

A Cell-Based Assay to Measure Changes in AR Activity

Since the AR plays a key role in prostate cancer growth and survival, we sought to establish a cell-based assay for evaluation of agents capable of

modulating endogenous AR activation in living prostate cancer cells. A lentivirus having an androgen responsive probasin-derived promoter upstream of an EGFP reporter gene (ARR₂PB-EGFP) was used to make the LNCaP cell line LN-ARR₂PB-EGFP. EGFP expression in this system is regulated by AR and thus, increased fluorescence should indicate increased AR transcriptional activity. To test this, LN-ARR₂PB-EGFP cells were first grown in charcoal steroid media to deplete cells of bio-available androgen. As before [27], EGFP fluorescence declined over time and by day 5 no fluorescent cells were observed (data not shown). This lag period is likely due to the time it takes for (i) remaining androgens to be metabolized, (ii) the AR to become transcriptionally inactive after translocation into cytoplasm, and (iii) all endogenous EGFP protein to be degraded. When EGFP fluorescence was undetectable, LN-ARR₂PB-EGFP cells were incubated with various concentrations of the potent synthetic androgen R1881 at concentrations ranging from 0.01 to 10 nM (Fig. 4A). After 24 hr of hormone treatment, cells exposed to 1 nM or higher concentrations of R1881 showed an increased amount of EGFP expression, which was visually confirmed by fluorescent microscopy. The EGFP expression levels increased over time in a dose-dependent manner and showed significant differences between 0.1 and 1 nM hormone at 48 hr ($P < 0.001$) and with 0.1 and 0.05 nM at 72 hr ($P < 0.001$). On a per cell basis, the mean RFU value at day 3 indicated that there was a higher fluorescence as the R1881 concentration increased (Fig. 4B). This indicates that the observed increase in EGFP expression was due to enhanced AR activation and not due to cell growth.

We next tested the effect of bicalutamide, an anti-androgen and antagonist of AR [40], to determine if this assay could also measure inhibition of androgen induced EGFP expression. Figure 4C shows changes over time in RFU values in LN-ARR₂PB-EGFP cells exposed to increasing concentrations of bicalutamide

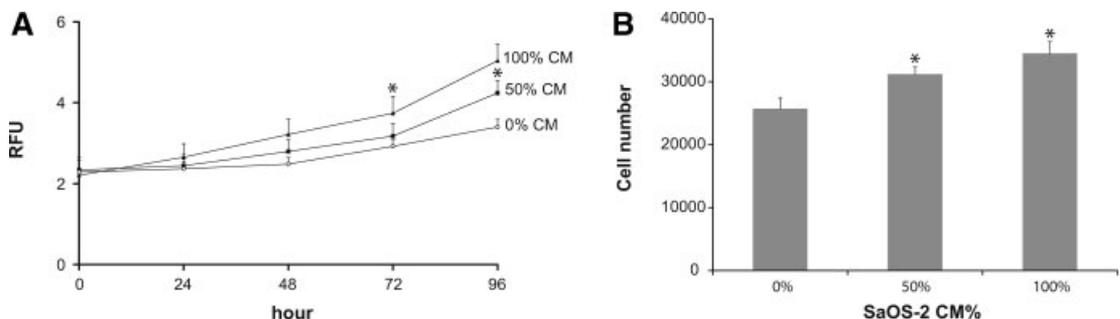


Fig. 3. Effects of osteoblastic conditioned media (CM) on EGFP expression in LN-CMV-EGFP cells. **A:** Twenty thousand LN-CMV-EGFP cells were seeded in a 96-well plate either suspended in 50% or 100% differentiated SaOS-2 CM media, or media alone (0% CM). The amount of fluorescence was measured every 24 hr. **B:** Cell number assay (MTS) was performed at day 4. Asterisks show significant differences in cell numbers of treatment conditions over controls ($P < 0.05$).

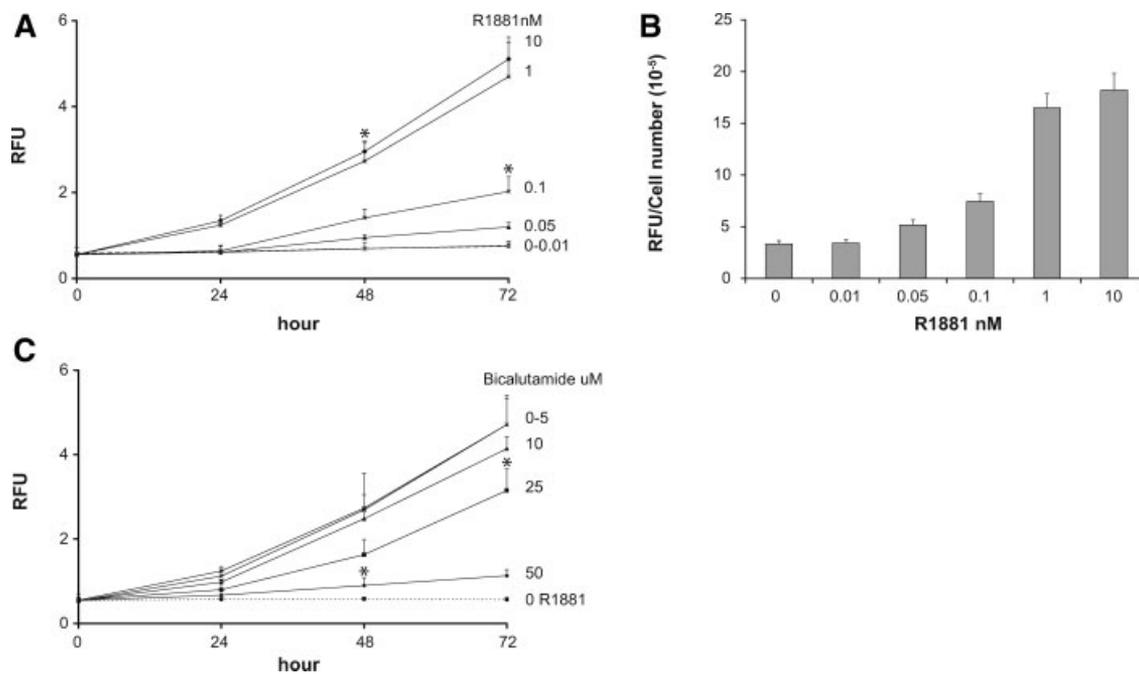


Fig. 4. Measurement of AR activity with LN-ARR2PB-EGFP cells. **A:** After 5 days growing in charcoal stripped serum (CSS) RPMI, 20,000 LN-ARR2PB-EGFP cells/well were seeded in a 96 black well plate and incubated with R1881 ranging from 0.05 to 10 nM of R1881. Fluorescence was measured every 24 hr. Asterisks show the significant difference between 0.1 and 1 nM R1881 at 48 hr ($P < 0.001$) and 0.1 and 0.05 at 72 hr ($P < 0.001$). **B:** After 72 hr cell numbers were determined using a MTS assay. The mean fluorescence/treatment at 72 hr was normalized to mean cell number/treatment and plotted against R1881 concentration. **C:** After 5 days growing in CSS, LN-ARR2PB-EGFP cells were seeded at 20,000 cells/well and incubated overnight with bicalutamide from 0 to 50 μ M before treatment with 1 nM R1881. Asterisks show significant difference with 50 and 25 μ M bicalutamide at 48 and 72 hr, respectively, compared to control.

in the presence of R1881. With 10 μ M bicalutamide there is a slight suppression of RFU levels after 3 days, whereas a significant decrease ($P < 0.05$) is seen at this time point when the drug concentration is raised to 25 μ M. By comparison, a significant inhibition of AR activity is observed after only 2 days of treatment with 50 μ M bicalutamide with a $>90\%$ decrease in androgen-induced RFU seen at 3 days. Thus, this assay can readily measure dose-dependent effects of agents that are able to decrease AR activity.

Assessment of Non-Steroidal Activation of AR

Having established an assay that is able to monitor endogenous AR activity in living cells, we next tested whether the effects of non-steroidal modulators of AR could also be assessed. Since we had found that CM from differentiated SaOS-2 cells could induce growth of LNCaP cells in a steroid depleted environment (Fig. 3A), we sought to determine whether this growth response was mediated via AR transactivation. Similar to that observed with the LN-CMV-EGFP cells, LN-ARR₂PB-EGFP cells exhibited significant enhancement of growth at day 3 when treated with 100% CM in the absence of hormone (as detected by a MTS assay,

$P < 0.05$). However, no significant changes of AR-induced fluorescent activity were seen ($P > 0.05$), even using CM concentrated to 400% by centrifugal filter devices (500MW cut-off). When combined with R1881, the amount of fluorescence seen could be attributed entirely to the presence of androgen (data not shown). Thus osteoblast CM induced growth of LNCaP cells through an AR-independent mechanism.

It has been reported that both IL-6 and FSK can modulate the AR transcriptional activity [5,7,8,11,41–44]. Both were added to LN-ARR₂PB-EGFP cells to determine if they could change the endogenous AR activity in this system. Neither reagent modulated AR activity in the absence of R1881, nor at R1881 concentrations lower than 0.1 nM (data not shown). However, as shown in Figure 5A, in the presence of ≥ 0.1 nM R1881, a 72 hr exposure to 25 μ M FSK increased RFUs 1.5-fold relative to R1881 alone ($P < 0.01$), consistent with reported observations of this androgenic priming requirement [41,43,44]. Conversely, IL-6 (20 ng/ml) in the presence of 0.1 nM R1881 resulted in a 40% decrease in fluorescence ($P < 0.01$) (Fig. 5A). This is in agreement with Jia et al. [42], who also observed an inhibition of AR transactivation with IL-6. Cell numbers at day 3 were determined by MTS

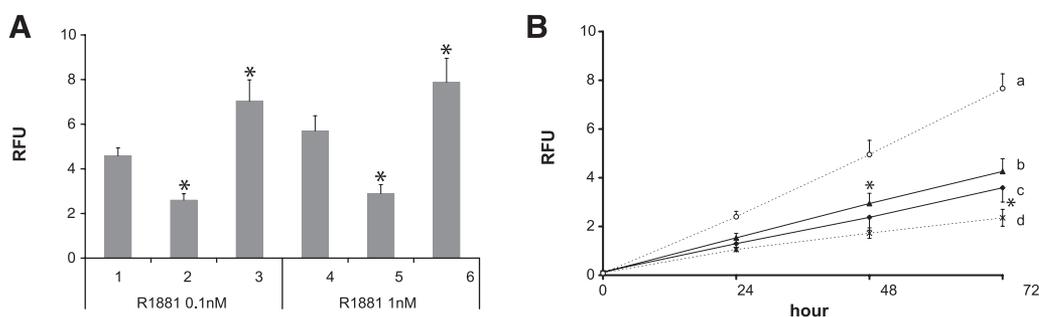


Fig. 5. Effects of FSK, the pesticide dichlorvos, and IL-6 on fluorescence levels in ARR2PB-EGFP cells. **A:** After 5 days growing in CSS, LN-ARR2PB-EGFP cells were seeded at 20,000 cells/well and treated with 0.1 or 1 nM R1881 alone (1 & 4), or in addition with 20 ng/ml IL-6 (2 & 5) or 25 μ M forskolin (3 & 6). The fluorescence, representing AR activity was measured every 24 hr. At $t = 72$ hr, in the presence of 0.1 or 1 nM R1881, fluorescence expressions of IL-6 and forskolin are significantly different over control ($P < 0.01$). **B:** LN-ARR2PB-EGFP cells were treated with (a) 1 nM R1881, (b & c) 1 and 0.1 μ M dichlorvos in the presence of 0.1 nM R1881, respectively, or (d) 0.1 nM R1881 alone.

assays and showed no significant differences among the above-treatment conditions (data not shown), indicating fluorescence measurements reflected AR activity and not cell growth. Thus, FSK enhanced and IL-6 inhibited endogenous AR transcriptional activation in the presence of 0.1 nM or 1 nM R1881.

We next tested the utility of this assay for ascertaining the effects of an environmental chemical contaminant on our androgen-regulated EGFP cells. Dichlorvos is a potential carcinogenic pesticide [45] and also a possible AR modulator through yet undetermined mechanism(s) [46]. This pesticide was used to treat LN-ARR₂PB-EGFP cells in the presence or absence of androgen. In the absence of R1881, different concentrations of dichlorvos did not affect AR activity (data not shown). After 48 hr in the presence of 0.1 nM R1881, 1 μ M dichlorvos significantly enhanced 0.1 nM R1881 induction of EGFP ($P < 0.05$), whereas fluorescence enhancement by 0.1 μ M of dichlorvos became significant by day 3 ($P < 0.01$) (Fig. 5B). Thus, dichlorvos is ineffective alone, but is able to enhance AR activity in the presence of low androgen levels. A future application of this system could be to screen various chemical compounds or natural products for modulation of AR activity.

DISCUSSION

Since there is an expanding number of chemicals, biological substances and cellular extracts that can alter prostate cancer cell growth and AR transcriptional activity, a high throughput, non-destructive method to identify these agents and to assess their activities in viable cells was developed using LNCaP and PC3 cell lines that had been lentiviral treated to stably express fluorescent EGFP reporters. To monitor cell growth or AR enhancement, EGFP was either driven by a constitutive CMV promoter (LN-CMV-EGFP or

PC3-CMV-EGFP cells) or by an ARR₂PB androgen responsive promoter (LN-ARR₂PB-EGFP cells), respectively.

After lentiviral infection of LNCaP or PC3 human prostate cancer cells with Lv-CMV-EGFP and subsequent enrichment of high fluorescence expression levels by FACS sorting, we found that, after 24–96 hr culturing in multi-well plates, there was a direct linear relationship ($R^2 = 0.99$) between levels of fluorescence and cell number (Fig. 1). To evaluate the accuracy and practical utility of this high throughput assay for assessment of agents that kill or inhibit the growth of prostate cancer cells, we tested the effects of a known cytotoxic drug, mitoxantrone [30,47], and found that there was direct concordance between the patterns and quantitation of cell death and apoptosis measured by both our non-destructive assay and MTS (non-viable) estimates (Fig. 2). Similarly, our fluorescent assay enabled accurate and reproducible measurement of prostate cell proliferating effects of complex mixtures such as osteoblast CM from differentiated SaOS-2 cells which induced a small (30%–40%), yet statistically significant ($P < 0.05$), increase in cell proliferation (Fig. 3), in agreement with reports that the bone environment is supportive both in survival and metastatic growth of prostate cancer cells [6,31–36]. In contrast to its growth promoting effects, CM did not alter endogenous AR activity, implying that the cell proliferation induced by osteoblast CM was through AR-independent pathways. Overall, our cell-based screening method can robustly measure factors that even subtly influence prostate cancer cell proliferation or cell death and therefore should be particularly useful for large scale, simultaneous assessment of multifactor treatments at different concentrations and time points, saving both time and costs. Furthermore, the non-destructive nature of this 96-well assay would enable multiple dynamic measurements of cellular and

molecular responses to the treatments, replacing several individual experiments.

The practical utility of applying a viable, cell-based assay to measure chemical and environmental agents that can modulate AR activity was tested by using lentiviral vectors containing a probasin-derived AR-responsive promoter to create the LNCaP cell line, LN-ARR₂PB-EGFP. Using these cells, we found that R1881, a potent synthetic androgen and AR agonist [48], could reproducibly induce EGFP expression in a concentration- and time-dependent manner (Fig. 4) over a large number of passages. As anticipated, bicalutamide, an effective anti-androgen known to block AR activation [40], was readily evaluable in this system (Fig. 4). Because these cells are both stable AR-inducible expressers and enriched by FACS selection, responses are far less variable than estimates using transient transfection systems.

While screening for established androgenic and anti-androgenic compounds validated this AR-bioassay, LN-ARR₂PB-EGFP cells also provided an opportunity to assess the effects of non-steroidal drugs or compounds that may modulate the AR-dependent gene transcription. There are conflicting reports as to whether the FSK can directly activate the AR in a ligand-independent fashion or whether it requires some form of androgen priming to see this agonistic effect [43]. Similarly, it remains unresolved whether the cytokine IL-6 is an androgen-independent agonist [5,8,11] or an antagonist [42] of AR. Since our AR functional screening assay is stable, reproducible, relies on a chromosomally integrated AR reporter system, and is not confounded by transient transfection manipulations, we felt that it could provide a more physiological judgment of these questions. While neither chemical was observed to modulate AR activity at in the absence of androgen in charcoal-stripped serum, in the presence of 0.1 nM or higher concentrations of R1881, IL-6 significantly inhibited AR activity whereas FSK stimulated AR transcriptional activity of EGFP (Fig. 5).

To broaden the screening application of our cell-based AR functional assay, we tested whether the pesticide dichlorvos could modulate AR activity in a prostate cancer cell. As with IL-6, there is some controversy as to whether this environmental contaminant is an AR agonist as demonstrated in transient transfections using LNCaP prostate cancer cells [49] or antagonist as shown in similar experiments with Chinese hamster ovary cells [46]. Our results clearly support an agonist role for dichlorvos, at least in the presence of priming amounts of androgen and in the context of human prostate cancer cells. In general, these experiments support the efficacy of using a stable, reproducible, and chromatin-integrated AR-inducible

reporter system for assessing the impact of drugs and chemicals on endogenous AR activity in prostate cancer cells. Furthermore, to assist in uncovering the mechanism of action of AR-interfering compounds, one can use this fluorescent assay to monitor AR activity over time in multiple sets of viable prostate cancer cells to determine the optimal time points and conditions for subsequent analyses such as gene expression microarrays or signal transduction assays.

CONCLUSIONS

The lentiviral-created, cell-based fluorescent assay systems developed in this study enable rapid, reproducible, time-dependent, and high-throughput screening and monitoring of chemicals or complex mixtures for their capacity to influence prostate cancer cell growth/death or AR-mediated signaling, under a variety of treatment conditions in a single experiment. After validation, we used these assays to illustrate the cytotoxicity of mitoxantrone, the AR-independent growth-promoting activity of CM from differentiated osteoblasts (SaOS-2 cells), the androgen priming requirement for FSK activation and IL-6 inhibition of AR activity, and finally, that the pesticide dichlorvos enhances AR transcription activity in a androgen-dependent manner.

ACKNOWLEDGMENTS

We thank the University of British Columbia graduate studies for the research-training award to P. Tavassoli.

REFERENCES

1. Heinlein CA, Chang C. Androgen receptor in prostate cancer. *Endocr Rev* 2004;2(25):276–300.
2. Culig Z, Klocker H, Bartsch G, Steiner H, Hobisch A. Androgen receptors in prostate cancer. *J Urol* 2003;170(4 Pt 1):1363–1369.
3. Setlur SR, Rubin MA. Current thoughts on the role of the androgen receptor and prostate cancer progression. *Adv Anat Pathol* 2005;12(5):265–270.
4. Gioeli D. Signal transduction in prostate cancer progression. *Clin Sci (Lond)* 2005;108(4):293–308.
5. Blaszczyk N, Masri BA, Mawji NR, Ueda T, McAlinden G, Duncan CP, Bruchovsky N, Schweikert HU, Schnabel D, Jones EC, Sadar MD. Osteoblast-derived factors induce androgen-independent proliferation and expression of prostate-specific antigen in human prostate cancer cells. *Clin Cancer Res* 2004;10(5):1860–1869.
6. Culig Z, Steiner H, Bartsch G, Hobisch A. Interleukin-6 regulation of prostate cancer cell growth. *J Cell Biochem* 2005;95(3):497–505.
7. Degeorges A, Tatoud R, Fauvel-Lafeve F, Podgorniak MP, Millot G, de Cremoux P, Calvo F. Stromal cells from human benign prostate hyperplasia produce a growth-inhibitory factor for LNCaP prostate cancer cells, identified as interleukin-6. *Int J Cancer* 1996;68(2):207–214.

8. Lu Y, Zhang J, Dai J, Dehne LA, Mizokami A, Yao Z, Keller ET. Osteoblasts induce prostate cancer proliferation and PSA expression through interleukin-6-mediated activation of the androgen receptor. *Clin Exp Metastasis* 2004;21(5):399–408.
9. Wang MH, Abreu-Delgado Y, Young CY. Effects of vitamin C on androgen receptor mediated actions in human prostate adenocarcinoma cell line LAPC-4. *Urology* 2003;62(1):167–171.
10. Nishimura K, Nonomura N, Satoh E, Harada Y, Nakayama M, Tokizane T, Fukui T, Ono Y, Inoue H, Shin M, Tsujimoto Y, Takayama H, Aozasa K, Okuyama A. Potential mechanism for the effects of dexamethasone on growth of androgen-independent prostate cancer. *J Natl Cancer Inst* 2001;93(22):1739–1746.
11. Ueda T, Mawji NR, Bruchofsky N, Sadar MD. Ligand-independent activation of the androgen receptor by interleukin-6 and the role of steroid receptor coactivator-1 in prostate cancer cells. *J Biol Chem* 2002;277(41):38087–38094.
12. Darbre P. Preface. *Best Pract Res Clin Endocrinol Metab* 2006;20(1):vii–x.
13. Kim HJ, Park YI, Dong MS. Effects of 2,4-D and DCP on the DHT-induced androgenic action in human prostate cancer cells. *Toxicol Sci* 2005;88(1):52–59.
14. Kavlock R, Cummings A. Mode of action: Inhibition of androgen receptor function—vinclozolin-induced malformations in reproductive development. *Crit Rev Toxicol* 2005;35(8–9):721–726.
15. Sonnenschein C, Soto AM. An updated review of environmental estrogen and androgen mimics and antagonists. *J Steroid Biochem Mol Biol* 1998;65(1–6):143–150.
16. Wetherill YB, Fisher NL, Staubach A, Danielsen M, de Vere White RW, Knudsen KE. Xenoestrogen action in prostate cancer: Pleiotropic effects dependent on androgen receptor status. *Cancer Res* 2005;65(1):54–65.
17. Handelsman DJ. Designer androgens in sport: When too much is never enough. *Sci STKE* 2004;2004(244):pe41.
18. Thevis M, Kamber M, Schanzer W. Screening for metabolically stable aryl-propionamide-derived selective androgen receptor modulators for doping control purposes. *Rapid Commun Mass Spectrom* 2006;20(5):870–876.
19. Casner PR. Saw palmetto for benign prostatic hyperplasia. *N Engl J Med* 2006;354(18):1950–1951; author reply 1950–1951.
20. Jiang C, Lee HJ, Li GX, Guo J, Malewicz B, Zhao Y, Lee EO, Lee JH, Kim MS, Kim SH, Lu J. Potent antiandrogen and androgen receptor activities of an *Angelica gigas*-containing herbal formulation: Identification of decursin as a novel and active compound with implications for prevention and treatment of prostate cancer. *Cancer Res* 2006;66(1):453–463.
21. Michelini E, Magliulo M, Leskinen P, Virta M, Karp M, Roda A. Recombinant cell-based bioluminescence assay for androgen bioactivity determination in clinical samples. *Clin Chem* 2005;51(10):1995–1998.
22. Ho SM, Tang WY, Belmonte de Frausto J, Prins GS. Developmental exposure to estradiol and bisphenol A increases susceptibility to prostate carcinogenesis and epigenetically regulates phosphodiesterase type 4 variant 4. *Cancer Res* 2006; 66(11):5624–5632.
23. Sonneveld E, Jansen HJ, Riteco JA, Brouwer A, van der Burg B. Development of androgen- and estrogen-responsive bioassays, members of a panel of human cell line-based highly selective steroid-responsive bioassays. *Toxicol Sci* 2005;83(1):136–148.
24. Araki N, Ohno K, Takeyoshi M, Iida M. Evaluation of a rapid in vitro androgen receptor transcriptional activation assay using AR-EcoScreen cells. *Toxicol In Vitro* 2005;19(3):335–352.
25. Rao LG, Kung Sutherland MS, Muzaffar SA, Wylie JN, McBroom RJ, Murray TM. Positive interaction between 17 beta-Estradiol and parathyroid hormone in normal human osteoblasts cultured long term in the presence of dexamethasone. *Osteoporos Int* 1996;6(2):111–119.
26. Rao LG, Liu LJ, Murray TM, McDermott E, Zhang X. Estrogen added intermittently, but not continuously, stimulates differentiation and bone formation in SaOS-2 cells. *Biol Pharm Bull* 2003;26(7):936–945.
27. Yu D, Jia WW, Gleave ME, Nelson CC, Rennie PS. Prostate-tumor targeting of gene expression by lentiviral vectors containing elements of the probasin promoter. *Prostate* 2004;59(4):370–382.
28. Zhang J, Thomas TZ, Kasper S, Matusik RJ. A small composite probasin promoter confers high levels of prostate-specific gene expression through regulation by androgens and glucocorticoids in vitro and in vivo. *Endocrinology* 2000;141(12):4698–4710.
29. Di Lorenzo G, De Placido S. Hormone refractory prostate cancer (HRPC): Present and future approaches of therapy. *Int J Immunopathol Pharmacol* 2006;19(1):11–34.
30. Miyake H, Hara S, Arakawa S, Kamidono S, Hara I. Optimal timing and dosage of chemotherapy as a combined treatment with androgen withdrawal in the human prostate LNCaP tumour model. *Br J Cancer* 2001;84(6):859–863.
31. Roodman GD. Mechanisms of bone metastasis. *N Engl J Med* 2004;350(16):1655–1664.
32. Koeneman KS, Yeung F, Chung LW. Osteomimetic properties of prostate cancer cells: A hypothesis supporting the predilection of prostate cancer metastasis and growth in the bone environment. *Prostate* 1999;39(4):246–261.
33. Zhou HE, Li CL, Chung LW. Establishment of human prostate carcinoma skeletal metastasis models. *Cancer* 2000;88(12 Suppl):2995–3001.
34. Gleave M, Hsieh JT, Gao CA, von Eschenbach AC, Chung LW. Acceleration of human prostate cancer growth in vivo by factors produced by prostate and bone fibroblasts. *Cancer Res* 1991;51(14):3753–3761.
35. Chiao JW, Moonga BS, Yang YM, Kancherla R, Mittelman A, Wu-Wong JR, Ahmed T. Endothelin-1 from prostate cancer cells is enhanced by bone contact which blocks osteoclastic bone resorption. *Br J Cancer* 2000;83(3):360–365.
36. Fu Z, Dozmorov IM, Keller ET. Osteoblasts produce soluble factors that induce a gene expression pattern in non-metastatic prostate cancer cells, similar to that found in bone metastatic prostate cancer cells. *Prostate* 2002;51(1):10–20.
37. Cillo JE Jr, Gassner R, Koepsel RR, Buckley MJ. Growth factor and cytokine gene expression in mechanically strained human osteoblast-like cells: Implications for distraction osteogenesis. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2000; 90(2):147–154.
38. Wang C, Duan Y, Markovic B, Barbara J, Howlett CR, Zhang X, Zreiqat H. Phenotypic expression of bone-related genes in osteoblasts grown on calcium phosphate ceramics with different phase compositions. *Biomaterials* 2004;25(13):2507–2514.
39. Spyrou P, Papaioannou S, Hampson G, Brady K, Palmer RM, McDonald F. Cytokine release by osteoblast-like cells cultured on implant discs of varying alloy compositions. *Clin Oral Implants Res* 2002;13(6):623–630.

40. Furr BJ, Valcaccia B, Curry B, Woodburn JR, Chesterson G, Tucker H. ICI 176,334: A novel non-steroidal, peripherally selective antiandrogen. *J Endocrinol* 1987;113(3):R7–R9.
41. Nazareth LV, Weigel NL. Activation of the human androgen receptor through a protein kinase A signaling pathway. *J Biol Chem* 1996;271(33):19900–19907.
42. Jia L, Choong CS, Ricciardelli C, Kim J, Tilley WD, Coetzee GA. Androgen receptor signaling: Mechanism of interleukin-6 inhibition. *Cancer Res* 2004;64(7):2619–2626.
43. Kim J, Jia L, Stallcup MR, Coetzee GA. The role of protein kinase A pathway and cAMP responsive element-binding protein in androgen receptor-mediated transcription at the prostate-specific antigen locus. *J Mol Endocrinol* 2005;34(1):107–118.
44. Sadar MD. Androgen-independent induction of prostate-specific antigen gene expression via cross-talk between the androgen receptor and protein kinase A signal transduction pathways. *J Biol Chem* 1999;274(12):7777–7783.
45. Van Maele-Fabry G, Laurent C, Willems JL. Dichlorvos and carcinogenicity: A systematic approach to a regulatory decision. *Regul Toxicol Pharmacol* 2000;31(1):13–21.
46. Andersen HR, Vinggaard AM, Rasmussen TH, Gjermandsen IM, Bonefeld-Jorgensen EC. Effects of currently used pesticides in assays for estrogenicity, androgenicity, and aromatase activity in vitro. *Toxicol Appl Pharmacol* 2002;179(1):1–12.
47. Liu QY, Rubin MA, Omene C, Lederman S, Stein CA. Fas ligand is constitutively secreted by prostate cancer cells in vitro. *Clin Cancer Res* 1998;4(7):1803–1811.
48. Ghanadian R, Auf G, Smith CB, Chisholm GD, Blacklock NJ. Androgen receptors in the prostate of the Rhesus monkey. *Urol Res* 1977;5(4):169–173.
49. Fedoruk MN. (Ph.D. thesis) The modulation of androgen action in prostate cancer by exogenous chemicals, Efflux Transporter P-glycoprotein and Y-Box Binding Protein-1, University of British Columbia, 2006, pp 89–126.