Title: Insulin-like Growth Factor-2 increases de novo steroidogenesis in prostate cancer cells

Running title: IGF2 increases steroidogenesis in prostate cancer cells

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Abstract

Insulin-like growth factor-2 (IGF2) is a mitogenic foetal growth factor, commonly over-expressed in cancers, including prostate cancer (PC). We recently demonstrated that insulin can activate de novo steroidogenesis in PC cells, a major pathway for reactivation of androgen pathways and PC progression. IGF2 can activate the IGF-1 receptor (IGF-1R) or insulin receptor (INSR) or hybrids of these two receptors. We therefore hypothesised that IGF2 may contribute to PC progression via de novo steroidogenesis. IGF2 mRNA but not IGF2 receptor mRNA expression was increased in patient samples during progression to castrate resistant prostate cancer (CRPC) as was immunoreactivity to INSR and IGF1R antibodies. Treatment of androgen receptor (AR) positive PC cell lines LNCaP and 22RV1 with IGF2 for 48hr resulted in increased expression of steroidogenic enzyme mRNA and protein, including steroid acute regulatory protein (StAR), cytochrome p450 family member (CYP)17A1, aldo-keto reductase family member (AKR)1C3, and hydroxysteroid dehydrogenase (HSD)17B3. IGF2 treatment resulted in increased steady state steroid levels and increased de novo steroidogenesis resulting in AR activation as demonstrated by PSA mRNA induction. Inhibition of the IGF1R / INSR signalling axis attenuated the effects of IGF2 on steroid hormone synthesis. We present a potential mechanism for prostatic IGF2 contributing to PC progression by inducing steroidogenesis, and that IGF2 signalling and related pathways present attractive targets for PC therapy.

Introduction

Insulin-like growth factors (IGFs) play an integral role in the progression of many cancers (Pollak 2008a; Pollak 2008b), regulating proliferation and transformation, as well as inhibiting apoptosis (Pollak 2008a). Related growth factors insulin and IGF1 have both been implicated in PC progression (Lubik, et al. 2011b; Pollak 2008a); however, the role of IGF2 is less well explored. IGF2 expression is elevated in

Androgen deprivation therapy (ADT), which removes testicular androgens necessary for survival of prostate cells, is the standard treatment for advanced PC (So, et al. 2005). Local tumour production and/or retention of steroids have been shown to differ significantly to levels in the circulation and contribute to PC progression (So et al. 2005; Stanbrough, et al. 2006) so despite castrate levels of androgens in men undergoing ADT it has been shown by our group and others that the local prostatic production of steroids (and conversion of adrenal precursors) may be instrumental in driving PC progression towards the terminal castrate resistant PC (CRPC) (Locke, et al. 2008). The clinical success of the (CYP)17A1 inhibitor, Abiraterone, (Attard, et al. 2008; Attard, et al. 2011) highlights the importance of this pathway in PC progression. At this disease stage, circulating androgen levels are low, while the prostate tissue levels are high enough to reactivate the androgen receptor (AR), which may itself harbour mutations that give rise to constitutively active truncated receptors or increase AR sensitivity to low androgen and drive progression (Locke et al. 2008; Stanbrough et al. 2006). We recently reported that insulin, which rises in response to ADT, can drive expression of steroidogenesis
enzymes in PC cells, increase intracellular androgens and increase expression of PSA (Lubik, et al. 2011a). Given the similarities in signalling between insulin and IGF2 (Pandini et al. 2004), we hypothesised that the increased IGF2 in the tumour microenvironment may accelerate prostatic de novo steroidogenesis. IGF2 has been shown to initiate steroidogenesis in thecal (Spicer and Aad 2007) and adrenocortical cells (Fottner, et al. 1998). In this study we observed increased IGF2 transcript in clinical tumour samples which correlated with progression to castrate resistance in addition to increased expression of the receptors which facilitate IGF2 signalling: IGF1R and INSR. Our results demonstrate that IGF2 may accelerate prostate cancer progression through upregulation of steroidogenesis enzymes and enhanced steroid production in PC cell lines. Levels of steroidogenic enzyme mRNA and protein in LNCaP and 22RV1 cells are increased after IGF2 treatment and result in increased DHT secretion. Increased de novo steroidogenesis in LNCaP and VCaP cells was measured using radiolabelled substrate, which resulted in androgen-mediated reactivation of AR and upregulated expression of PSA.

Materials and Methods

Laser capture microdissection (LCM) and microarray analysis: Microarray analysis of mRNA was performed using samples of patient tissue after radical prostatectomy grouped into the following catagories: 14 primary prostate cancers from patients undergoing surgery with no therapy before surgery, 12 primary prostatectomy samples from patients receiving 1-3 months of neoadjuvant hormone therapy (NHT) before surgery, 5 primary prostate cancers after 5-6 months NHT before surgery, 4 primary prostate cancers after 8-9 months NHT before surgery, and 3 hormone refractory prostate cancers before surgery. Patients were further grouped according to the following risk factors: High (PSA >20, Gleason >7, Clinical stage T3-T4), Intermediate (PSA 10-20, Gleason 7, Clinical stage T2) and Low (PSA<10, Gleason <7, Clinical stage 1).
The array preparation was performed as previous described (Chi, et al. 2005). Tissues were flash frozen in OCT Compound (Tissue-Tek, VWR, Batavia, IL) and frozen sections (8µm) were cut and mounted on LCM slides (P.A.L.M. Microlaser Technologies, Germany), sections were briefly thawed and fixed with 95% ethanol at -25°C. Hematoxylin staining was followed by washes in DEPC water and then dehydration in 100% ethanol and LCM was performed on cancer cells using the P.A.L.M. Microlaser system. Total RNA was isolated (PicoPure RNA Isolation Kit, ARCTURUS, Carlesbad, CA) and amplified using the RiboAmp HS RNA Amplification kit (ARCTURUS), labelled with Cy5 using the AminoAllyl Message Amp IIa RNA Amplification Kit (Ambion, Streetsville, Ont), and fragmented with RNA Fragmentation Reagents (Ambion) prior to hybridization. Custom-designed microarrays of 34,580 (70-mer) human oligos representing 24,650 genes and 37,123 gene transcripts (Human Operon V3.0, Operon Technologies, Huntsville, Al) were supplied by the Microarray Facility of the Vancouver Prostate Centre. Scanned arrays were visualized using ImaGene 8.0 software (BioDiscovery, San Diego, CA). Feature data was subjected to background correction, print-tip-lowess within-array normalization and G quantile between-array normalization (Limma, R/Bioconductor software). Significant differences between treatment groups were assessed using linear regression and Benjamini-Hochberg multiple test correction to estimate the false discovery rate.

**Immunohistochemistry:** Immunohistochemical staining was conducted on sequential sections of the Gleason graded tissue microarray (TMA) on patients from a similar cohort, also receiving NHT therapy before radical prostatectomy. Samples were probed using INSR receptor, beta subunit, rabbit immunoaffinity purified IgG (Upstate Cell Signaling Solutions, Lake Placid), and IGF1R beta rabbit polyclonal antibody (Santa Cruz Biotechnology Inc, Santa Cruz, CA) with enzyme labeled biotin streptavidin system and solvent resistant DAB Map kit. Nonspecific reactivity was assessed by omission
of the primary antibody. The specificity of staining for INSR was confirmed by using placenta as a positive control. The slide was scanned with BLISS system (Bacus Lab, North Lombard, IL) and immunohistochemical (IHC) score staining intensity was evaluated by an independent pathologist. The biomarkers were scored using a 4 point scale scoring system by a pathologist. Descriptively, 0 represents no staining by any tumour cells, 1 represents a faint or focal, 2 represents a stain of moderate intensity in a convincing number of cells, and 3 represents intense staining by a sufficient number of cells expressing this antigen.

**Cells:** LNCaP and 22RV1 cells were purchased from ATCC and routine array analyses performed in our lab confirm expected gene profiles. Cells were maintained in phenol red-free RPMI 1640 (Invitrogen, Mulgrave, VIC), 5% (v/v) fetal bovine serum (FBS) (Invitrogen). VCaP cells were maintained in DMEM media (HyClone), 5% FBS. Cells were plated overnight in respective media containing 5% FBS before media was changed to media with 5% charcoal-stripped serum (CSS; Hyclone, Hudson, NH) for 24hrs prior to 24hr starvation in serum free media. Following optimisation of concentration and duration, cells were treated, with 85ng/ml IGF2 (Novozymes, Thebarton, SA) in 0.2% Bovine Serum Albumin (BSA) in SFM or 0.2% BSA (w/v) alone for 48-72 hr. IGF2 was refreshed at 24hr intervals as necessary. Serum levels of IGF2 range from 200-600 ng/ml in men approximately 65 years of age (with and without PC) (Harman, et al. 2000).

**Receptor inhibitor treatment:** LNCaP and 22RV1 cells were grown in 6 well plates for 24hr in FBS supplemented media before incubation in 5% CSS media for 24hr, and further 24hr incubation in serum free media. For AR inhibition, cells were incubated for 2hr with 25µM bicalutamide then 24hr with 85ng/ml IGF2 or 10nM DHT. For inhibition of receptor activation by IGF2, cells were incubated with
either 5µM BMS-754807 (Bristol-Myers Squibb) pan INSR / IGF1R inhibitor, or 12.5µg/ml of specific IGF1R inhibitor, CP-751,871 (Pfizer) for 2hrs before addition of IGF2. To determine the difference between IGF2 and 10nM insulin-induced steroid synthesis, 100nM INSR-specific inhibitor S661 (Novo Nordisk), was employed. Ketoconazole, 10µM (Sigma), demonstrated IGF2, or insulin induced steroidogenesis could be suppressed with this steroidogenic inhibitor. All treatments were normalized to vehicle control. LNCaP cells were then treated as above with 14C-acetate and IGF2 prior to *de novo* steroidogenesis analysis. 22RV1 cells, not treated with 14C-acetate, were analysed using DHT ELISA. All inhibitors were used with kind permission.

**Quantitative Real Time-PCR (QRT PCR):** QRT PCR was carried out using standard methods. Briefly, RNA was extracted from cell lines using TriReagent (Applied Biosystems, Melbourne, Australia), before reverse transcription with Superscript III RT (Invitrogen, Melbourne, Australia). QRT PCR was performed using SYBR Green detection on 7900HT Fast Real Time PCR System (Applied Biosystems). Primers used are listed in Table 1. Gene expression was normalized to the housekeeping gene, then expressed relative to vehicle control at the same time point. Data was analyzed with SDS 2.3 software by means of the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). Experiments were repeated a minimum of 5 times.

**Western blotting:** Protein extraction and western blotting was carried out as previously described (Lubik et al. 2011b). Briefly, cells were lysed in radioimmunoprecipitation assay (RIPA) buffer, proteins separated by SDS-PAGE and transferred to PVDF-FL membrane (Millipore, North Ryde, Australia). Antibodies were added in a 1:1 solution of Li-Cor blocking buffer (Li-Cor Biosciences, Lincoln, USA) and 0.1% Tween-20-PBS and incubated overnight at 4°C, before application of secondary antibody.
Blots were visualized using the Li-Cor Odyssey Imager. Antibodies used were as previously described (Lubik et al. 2011a). Experiments were repeated a minimum of three times.

**Steroid analysis:** LNCaP cells were grown in 15cm plates and treated with either 85ng/ml IGF2 in 0.5% BSA or 0.5% BSA in SFM for 48hrs. Two plates of treated cells were washed with PBS and combined to give a single sample. Steroids were extracted from the pellet with methyl-tert-butyl ether (MTBE)/Methanol/Water extraction, which was dried down and resuspended in acetonitrile, sonicated, dried down and resuspended in 50% methanol, and then sonicated and spun to remove any particulates. Samples were derivatized in 0.2M hydroxylamine HCl. Water equilibrated ethylacetate was used instead of MTBE/Methanol/Water for extraction of secreted steroids from media samples. All samples were run on the Waters Acquity Liquid Chromatography system and the Waters Quattro Premier LC/MS/MS, and analysed using BioLynx Software. Readings were normalized to cell pellet weight. Before extraction, d3T deuterated testosterone standard (0.015ng/ml final concentration) was added.

**Radio-labelled analysis of de novo steroidogenesis in LNCaP and VCaP cells:** Cells were grown in 6 well plates and treated as above. At the time of IGF2 treatment, 6µCi/ml ¹⁴C-acetate (PerkinElmer, Ontario) was added to each plate for 72hr after which time medium was analyzed for steroid content. Equal volumes of medium and hexane:ethylacetate (75:25) were incubated at room temperature for 1 hr and the organic phase was extracted twice. Samples were then dried and resuspended in 75ul 50% methanol. These samples were analysed on the Waters Alliance 2695 HPLC. Before extraction, d3T deuterated testosterone standard (0.015ng/ml final concentration) was added.
Steroid analysis using DHT ELISA: DHT secreted into the media by 22RV1 cells was evaluated using a DHT ELISA (BioCore Pty Limited) kit according to the manufacturer’s instructions. Limits of detection of this kit were 6pg/ml which is similar to that of our LC/MS/MS procedure.

Statistics: Statistical analyses were performed using ANOVA on Graphpad Prism software.

Results

IGF2 mRNA expression in prostate tissue from men undergoing neoadjuvant hormone therapy (NHT). We examined the levels of IGF2 mRNA expression in clinical tumour samples over the time of progression to castrate resistance (CRPC) in men undergoing NHT prior to radical prostatectomy. Prostastic tissue from patients undergoing radical prostatectomy after no NHT, 1-3 month NHT, 5-6 month NHT, 8-9 months NHT or having hormone refractory prostate cancers was examined by microarray analysis. Increased expression of IGF2 first occurred by 5-6 months. This reached statistical significance after 8-9 months and was maintained at CRPC (Fig 1a). IGF2 receptor (IGF2R) mRNA levels remained constant (Fig 1a), which suggests an altered ratio of free and bound IGF2 which may result in increased bioavailability of IGF2. Immunohistochemical staining of NHT samples showed an increase in IGF1R and INSR protein immunoreactivity, both of which may be subject to activation by IGF2 (Fig 1b,c). INSR demonstrated homogenous cytoplasmic staining within tumour cells and discontinuous staining in the basal cell layer of benign glands. IGF1R protein expression was apparent in basal and luminal cells of the benign glands as well as cancer cells. Immunoreactivity of IGF1R was localised predominantly to the cell membrane, with approximately double the staining in CRPC samples; no cytoplasmic staining was observed in early series samples (0-5 months) compared with occasional cytoplasmic staining in CRPC samples.
**IGF2 increases expression of steroidogenesis enzymes.** Because IGF2 has been shown, in other tissues, to induce expression of enzymes in the steroidogenesis pathways, we investigated its potential to induce steroidogenesis in AR positive prostate tumour cell lines, LNCaP and 22RV1. Working systematically through the steroidogenesis pathway (Fig 2), we observed a statistically significant ~2-fold increase in mRNA levels of steroid acute regulatory protein (*StAR*), *CYP17A1*, *SRD5A1*, and retinol 11-cis dehydrogenase *RDH5* (*p*<0.05) in LNCaP cells after 48 hours, as well as a 2 and 1.5-fold increase in aldo-keto reductase family member (*AKR*)1C3 (*p*=0.064) and *HSD17B3*, respectively (Fig 3a).

Parallel increases in protein expression of steroidogenesis enzymes were observed in LNCaP cells following IGF2 treatment (Fig 3b). Expression of the cholesterol chaperone protein StAR was significantly increased approximately 3-fold, along with a 2-fold increase in levels of the rate-limiting enzyme, CYP17A1 as well as HSD17B3 (Fig 3b). Levels of AKR1C3 and SRD5A proteins increased, although did not reach statistical significance. In contrast to increased mRNA levels, protein levels of RDH5 were unchanged at 48 hours.

In comparison to LNCaPs, 22RV1 cells showed a similar pattern of mRNA induction for StAR (1.8-fold, *p*>0.05), CYP17A1 (2.9-fold), AKR1C3 (2.6-fold, *p*>0.05), and RDH5 (Fig 3c). In contrast to LNCaPs, IGF2 induced an increase in CYP11A1 mRNA (1.5-fold), and HSD3B2 (2-fold, *p*<0.05), but not in HSD17B3 mRNA. Despite having similar magnitude changes in gene expression, much greater absolute levels of most steroidalogenic enzymes were detected in 22RV1 cells (Supp Fig 1).

Parallel changes in protein levels were observed in 22RV1 cells, as StAR increased 5-fold, CYP11A1 and CYP17A1 increased approximately 2-fold each, and HSD3B2 and AKR1C3 increased approximately 2-fold each (*p*<0.05) (Fig 3d). A small increase in HSD17B3 was also observed.
**IGF2 increases intracellular and secreted steroids.** High-performance liquid chromatography on LNCaP cell pellets extracted with MTBE was used to investigate differences in steroid content between IGF2 and vehicle treated cells. IGF2 treatment clearly increased intracellular steroid levels in LNCaP cells (Fig 4a, Suppl fig 3); intracellular DHEA and 17OH-progesterone increased 2-fold (p<0.05) and a 10-fold increase in androsterone (p<0.05), an intermediate in the backdoor steroid biogenesis pathway, was also observed. Pregnenolone and progesterone increased 5- and 3-fold, respectively (p<0.05) and intracellular testosterone increased 4-fold (p<0.05) from approximately 0.0131 to 0.053 ng/g cells with IGF2 treatment, which is consistent with our previous findings (Locke et al. 2008; Lubik et al. 2011b). These concentrations would be sufficient to activate the AR, as it has been shown that androgen concentrations of approximately $2.92 \times 10^{-6}$ ng/g may activate AR in prostate cancer cell lines (Gregory, et al. 2001). Interestingly, no change was observed in intracellular DHT.

In contrast, dramatic increases (~10-fold, p<0.05) in secreted steroids were observed for testosterone, DHT, and androsterone (Fig 4b, Supp fig 3). Small increases in DHEA and 17OH-progesterone were detected, as well as 5 and 9-fold increases in pregnenolone and progesterone, respectively. Notably, higher levels of steroids at the beginning of the steroidogenesis pathway are increased intracellularly, while the levels of more potent steroids and androgens increased further down in the pathway were measured in the media. Testosterone and DHT concentrations increased to 44.9pg/ml ($1.56 \times 10^{-10}$ mol/L) and 19.5pg/ml ($6.74 \times 10^{-11}$ mol/L) following IGF2 treatment, once again levels sufficient to activate the AR (Gregory et al. 2001; Locke et al. 2008; Titus, et al. 2005). Furthermore, IGF2 treatment was demonstrated to increase DHT secretion in 22RV1 cell medium (Fig 4c) from 100pg/ml to 135 pg/ml (p>0.05), well within the range necessary for AR activation. It is possible that IGF2 may activate the AR through mechanisms other than direct activation via steroidogenesis. To address this, our experiments
were performed in serum free medium, assuming that all androgens in the medium capable of inducing AR activation are newly formed. Therefore, it is unlikely that IGF2 activates the receptor in conjunction with the steroids present; our observations are that IGF2 does not enhance steroid activation of PSA (Supp fig 2).

**IGF2 increases induction of AR regulated genes.** We demonstrated that IGF2-mediated androgen biosynthesis could potentiate AR-mediated gene transcription, using PSA mRNA expression as a surrogate for AR activation in the presence and absence of the AR antagonist bicalutamide. We compared the potency of IGF2 to 10nM DHT (Fig 5a). PSA mRNA expression was doubled in LNCaP cells after IGF2 treatment (p<0.05) and we demonstrated that this PSA response was mediated through AR; the addition of bicalutamide abrogated the IGF2-induced increase in PSA expression. Following this we titrated PSA mRNA expression following IGF2 treatment with increasing concentrations of DHT (Fig 5b) and found that the concentration of IGF2 used in our studies is approximately as potent as 1.6x10^{-12}M DHT in PSA induction, which is consistent with our measurement of 6.74E^{-11}M in media.

**IGF2 increases de novo steroidogenesis in LNCaP and VCaP medium.** We measured *de novo* steroidogenesis by treating LNCaP or VCaP cells for 72hr with $^{14}$C-labeled acetate and subsequent HPLC and radiometric detection was used to measure *de novo* steroidogenesis. In VCaP cells, a 5-fold increase in androstenedione and a 3-fold increase in androsterone levels were detected after IGF2 treatment (Fig 6a, Supp fig 4). It is notable that a 3-fold increase in a steroid peak with a retention time close to that of progesterone was also detected (Supp fig 4). We have demonstrated that cold progesterone is present in LC/MS/MS of LNCaP cells in a similar concentration to that of testosterone and androsterone, and this peak proximal to progesterone is also similar in magnitude to those steroids.
It is possible that progesterone was compromised in our derivatization procedure. Furthermore, IGF2 treatment in VCaP cells increased pregnan3,20dione, 2.6-fold (p<0.05) (Fig 6a, Supp fig 4) and de novo cholesterol production, the building block of steroid synthesis (Leon, et al. 2010). As the method of extraction used in this experiment is more specific to steroids, the assessment of cholesterol levels should be considered more qualitative than quantitative.

IGF2-treated LNCaP cells significantly increased testosterone, 4.5-fold (p<0.05), as well as androstenedione, androsterone, and pregnan-3,17-diol-20-one (approximately 4-fold, p<0.05)(Fig 6b). In a similar manner to that observed in VCaP cells, there was an increase in the steroid peak resembling progesterone (approximately 3-fold, p<0.05) (Supp fig 4). IGF2 also induced a 2.4-fold increase in pregnan-3,20-dione (Fig 6b). De novo DHT synthesis was not detected in these experiments, as our current methods for steroid extraction of radio-labeled steroids are not effective for extracting DHT.

**Receptor blockade of IGF2.** Our IHC analysis of patient tissue microarrays demonstrated increased expression of INSR and IGF1R which correlated with PC progression; IGF2 can signal through both of these receptors or through hybrid INSR:IGF1R. In order to address the relative contribution of each receptor to IGF2-induced steroidogenesis, inhibitors of the insulin signalling axis, including the BMS-754807 tyrosine kinase inhibitor (Carboni, et al. 2009; Huang et al. 2010) and the highly specific IGF1R neutralizing antibody, CP-751,871 (Cohen, et al. 2005) were used and their effect on IGF2-induced steroidogenesis, assessed. BMS-754807 inhibits IGF1R and INSR activity with equal affinity (Carboni et al. 2009), whereas CP-751,871 is highly specific at inhibiting IGF1R activation, including hybrid receptors, with no effect on the INSR (Cohen et al. 2005). Optimal concentrations of inhibitors were determined empirically to assess effectiveness in each cell line, but were not optimised for comparison of efficacy.
At the concentrations used, IGF2-induced testosterone synthesis was completely abolished with CP-751,871, compared to 50% reduction with BMS-754807 (Fig 7a). An approximate 50% decrease in androstenedione, androsterone, and pregnan-3,17-diol-20-one was demonstrated with CP-751,871 treatment, with minimal change by BMS-754807 (Fig 7a). There was little impact by the inhibitors on pregnan-3,20-dione or the steroid peak close to progesterone. QRT PCR analysis of steroidogenesis enzyme mRNA in LNCaP cells showed that CP-751,871 treatment blocked CYP17A1 expression, which is upstream of androstenedione and may account for the decrease in that key steroid with the IGF1R inhibitor; whereas BMS did not appear to decrease CYP17A1 in LNCaP cells. Both inhibitors decreased levels of StAR, AKR1C3, and HSD17B3 (Fig 7b). This data suggests that inhibition of the IGF/insulin signalling axis, particularly IGF1R in LNCaPs reduces IGF2-mediated steroidogenesis.

The same concentrations of inhibitor used in 22RV1 cells, completely blocked IGF2-induced DHT secretion with both BMS-754807 and CP-751,871 (Fig 7c). IGF2-induced increases in StAR, CYP17A1, and AKR1C3 protein were equally blocked by both inhibitors (Fig 7d). Differences in endogenous, basal levels of enzymes, cholesterol and steroid metabolism between the cell lines may explain some of the differences in the relative sensitivity of LNCaPs, VCaPs and 22RV1s to inhibitor treatment (Locke, et al. 2009).

We have recently demonstrated that insulin induces steroidogenesis in PC cells (Lubik et al. 2011b). We compared the relative potency of 10nM insulin and 85ng/ml IGF2 on PSA mRNA induction in the presence and absence of multiple receptor inhibitors (Fig 8). Co-treatment with CP-751,871 reduced IGF2-induced PSA expression, as did BMS-754807 (Fig 8a). Specific inhibition of INSR (and hybrid receptors) with S-661 (Schäffer, et al. 2008) had very little effect of induction of PSA by IGF2, suggesting it signals predominantly through IGF1R over hybrid receptors in this cell line. Ketoconazole, a pan-CYP enzyme inhibitor (Locke et al. 2009) completely abolished PSA induction. In contrast,
induction of PSA by insulin was not inhibited by CP-751,871 (Fig 8b). It was however blocked by BMS-754804 treatment, as well as S-661, and ketoconazole. In summary, IGF2 and insulin, albeit with different preferential use of different receptors, both induced PSA expression which was inhibited with the steroidogenesis inhibitor, ketoconazole.

**Discussion**

Androgen deprivation therapy effectively reduces systemic androgens; however, elevated androgen levels persist within PC tumours (So et al. 2005). Past studies from our group and others indicate that one mechanism driving progression to castrate resistance, is the ability of PC cells to initiate a program of *de novo* steroidogenesis from cholesterol or other precursors, providing androgens to the tumour microenvironment at concentrations sufficient to activate the AR and promote PC growth, (Gregory et al. 2001; Leon et al. 2010; Locke et al. 2008; Locke et al. 2009), a process which has rationalised the recent addition of the CYP17A1 inhibitor, abiraterone, to advanced PC therapies. Understanding the mechanisms that regulate intratumoural steroidogenesis in prostate cancer is part of ongoing studies in our laboratory aimed at providing new therapies for this stage of disease.

Increased prostatic IGF2 mRNA and protein concentrations have previously been shown to increase during progression from normal to PIN to PC (Cardillo et al. 2003; Trojan, et al. 2006) and correlated to high Gleason scores (Cardillo et al. 2003; Pollak 2008a). In models of androgen deprivation, e.g. LNCaP xenografts in castrated mice, IGF2, IGF1R and INSR mRNA increases significantly from pre-castrate to PSA recurrence (Lubik et al. 2011a; Nickerson, et al. 2001). Our microarray analysis of clinical samples revealed a steady rise in IGF2 with duration of neoadjuvant hormone therapy which persisted in CRPC and contrasted with the static expression of IGF2R. Furthermore, studies have shown a correlation between elevated levels of prostatic IGF2 and DHT (Monti, et al. 1998). Given the similar signalling
pathways between insulin and IGF2, we hypothesised that increased local IGF2 coupled with increased receptors may accelerate local steroid synthesis.

Here, we demonstrate for the first time that IGF2 can promote steroidogenesis in prostate cancer cells, LNCaP and 22RV1, via upregulation of obligatory enzymes (mRNA and protein), coupled with increased total content and *de novo* synthesis of steroids to concentrations sufficient to activate the androgen receptor (Gregory et al. 2001; Locke et al. 2008). IGF2 increased mRNA expression of StAR, CYP17A1, AKR1C3, SRD5A1 and RDH5. The importance of increased StAR is a reflection of its key role ferrying cholesterol into the mitochondria for initiation of steroidogenesis. CYP17A1 catalyses several reactions in the pathway including conversion of pregnenolone, AKR1C3 and HSD17B3 can both convert androstenedione to testosterone and HSD17B3 also contributes to “backdoor” steroidogenesis through conversion of androsterone to androstanediol (Locke et al. 2008). The “backdoor” pathway differs from the classical pathway in that it bypasses testosterone in the formation of DHT. SRD5A1 and RDH5 catalyse the final reactions in the synthesis of DHT in the classical and “backdoor” pathways, respectively (Auchus 2004). Once made, DHT is the most potent activator of AR and its pathways, and vital to PC survival (So et al. 2005).

The observed differences between LNCaP and 22RV1 cells in IGF2-induced enzyme expression may be explained by their differing origins, LNCaP from lymph node metastasis and 22RV1s from a sub-clavicular metastasis (Horoszewicz, et al. 1980; Sramkoski, et al. 1999). The main difference between the two cells lines at 48hrs was the absence of CYP11A1 and HSD3B2 induction in LNCaPs (data not shown) in contrast to significant induction in 22RV1 cells. It’s possible that these early enzymes in the steroidogenesis pathway are upregulated rapidly in LNCaPs in response to IGF2, with levels restored by 48hrs. Alternatively, these enzymes may be more active / abundant in LNCaP cells. Studies have
demonstrated that steroidogenic PC cells will bypass inhibition to achieve steroidogenesis depending on cell needs and enzyme availability (Locke et al. 2009).

IGF1 can activate many pathways in PC cells, aside from steroidogenesis, which would contribute to AR-mediated progression. However, in contrast to IGF1, we have observed that IGF2 does not enhance steroid activation of PSA (Supp fig 2), as IGF1 has been reported to do (Kim and Coetzee 2004). Furthermore, we performed our experiments in serum free medium, assuming that all androgens in the medium capable of inducing AR activation, as measured by PSA expression, were newly formed by IGF2 stimulation and PSA induction was amplified by IGF2 in concert with pre-existing steroids. Furthermore, our preliminary studies with IGF1 suggested that IGF2 was a more potent enhancer of steroidogenesis than IGF1 (data not shown). This is similar to findings reported in human adrenocortical cells (l'Allemand, et al. 1996); however, because IGF2 clearly functions at least in part through IGF1R, it is plausible that IGF1 may also enhance steroidogenesis in PCa cells.

The modulation of both intracellular and extracellular steroid profiles in LNCaP cells by IGF2 compared to our earlier studies with insulin showed considerable differences between the growth factors. In our previous study, 10nM insulin increased intracellular testosterone levels 60-fold in LNCaPs, here, IGF2 increased testosterone levels 4-fold but IGF2 increased secreted DHT and testosterone approximately 10-fold, where insulin saw a smaller, 2-fold increase. In 22RV1 cells, DHT production induced by the two growth factors was similar; insulin increased DHT by 1.7 fold where IGF2 increased DHT 1.4-fold. BMS-754807, an equipotent inhibitor of INSR and IGF1R, prevented IGF2-induced steroidogenesis in 22RV1s but was less effective in LNCaP cells. One reason for this may be the relative abundance of IGF1R, INSR and hybrid receptors.

The varying amounts of steroidogenesis occurring in our cell lines, in absolute levels, as well as induction, may partially reflect their relative AR mutation status. LNCaP, VCaP and 22RV1 are
androgen responsive; however, each cell line expresses different forms of AR; LNCaP AR harbours the T877A mutation which renders it promiscuous (Veldscholte, et al. 1990), and 22RV1 cells express the less promiscuous AR mutation, H874Y, along with a truncated, constitutively active AR (Tepper, et al. 2002); whereas, VCaPs express wild type AR (Wu, et al. 2011). Interestingly, 22RV1s, which have the most cumulative AR mutations, appear to be most steroidogenic in regards to absolute levels of DHT and testosterone synthesis; furthermore, LNCaP cells produce more de novo T and DHT than VCaP cells, which are documented to have the least AR mutations. This supports the concept that steroidogenesis and AR mutations work synergistically to promote PCa (Knudsen and Penning 2010).

IGF2 signalling can be targeted by pharmacological agents currently in clinical trials (Huang et al. 2010). Inhibition of IGF1R leads to decreased concentrations of steroids downstream of CYP17A1 activity in LNCaP cells, which may indicate CYP17A1 upregulation by IGF2 is crucial to steroidogenesis. Further, a decrease in IGF2-induced SRD5A1 would account for the decrease in backdoor pathway steroids, as SRD5A1 may facilitate synthesis through the backdoor pathway, and decreased HSD17B3 and AKR1C3 would result in reduced testosterone (LNCaP) or DHT (22RV1) synthesis in the presence of treatments which target the insulin / IGF receptor signalling axis. Given the temporal changes in IGF2 expression with neoadjuvant hormone therapy, IGF2-induced steroidogenesis could also be targeted using CYP17A1 inhibitors, such as abiraterone, which have shown tremendous promise in the clinical setting (Attard et al. 2008; Attard et al. 2011). This is supported by our finding that the pan-CYP inhibitor, ketoconazole, blocked IGF2 activation of AR-mediated PSA expression.

In breast cancer cell lines, it has been shown that blocking either the INSR or the IGF1R can result in increased expression of the other and therefore may not hinder IGF2 related tumour progression (Ulanet, et al. 2010). Inhibition of both receptors, or of common downstream effectors, may have therapeutic potential for prostate cancer (Sayer et al. 2005). Both CP-751,871 and BMS-754807, which are in
clinical trials (Gualberto and Pollak 2009), demonstrate efficacy in reducing IGF2 induced steroidogenesis and AR activation. IGF2 activates numerous intracellular pathways leading to cancer cell survival, thus, combining receptor inhibitors and steroidogenesis inhibitors, such as abiraterone, may also help improve patient outcomes. In summary, IGF2 has been identified as a candidate prostate cancer gene target and we provide evidence that IGF2 activates de novo steroidogenesis in prostate cancer cell lines.

Conflicts of Interest: Michael Pollak consulted for Pfizer and for Bristol Myers and received research support from Pfizer. No other authors have conflicts of interest to declare.

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**Figure legends:**

**Figure 1: IGF2 and receptor expression in prostate cancer.** Prostatic tissues included primary tumours from patients undergoing radical prostatectomy with no therapy prior to surgery (NHT 0), following 1-3 months of Neoadjuvant Hormone Therapy (NHT) (NHT 1-3m), 5-6 months of NHT (NHT 5-6m), 8-9 months of NHT (NHT 8-9m), and 3 castrate resistant prostate cancers (CRPC). (a) IGF2 (red) and IGF2R (black) mRNA levels from patient samples were compared between groups, (mean + SE; *p<0.05). IHC staining of (b) IGF1R and (c) INSR from NHT naïve tumour (left) and CRPC tumour (right) show increased staining at CRPC. Staining intensity was scored from 0 to 3 and graphed (mean + SE; *p<0.05).

**Figure 2: Steroidogenesis pathways** indicating enzymes involved in both classical (left) and backdoor (right) pathways of DHT production.

**Figure 3: IGF2 upregulates expression of steroidogenic enzymes mRNA and protein.** (a) RNA from LNCaP cells treated with 85ng/ml IGF2 for 48hr, increased StAR, CYP17A1, SRD5A1 and RDH5 expression. Results analyzed by $\Delta\Delta$Ct method, normalized to RPL32 and then to vehicle-treated controls for the equivalent time point (n=5). (b) Quantitated densitometry of western blot from LNCaP lysates treated for 48hr, 85ng/ml IGF2 showed increased StAR, CYP17A1, HSD17B3, AKR1C3 and SRD5A1 (n=3). (c) Identical treatment in 22RV1 cells showed significant increase in StAR, CYP17A1, HSD3B2 and AKR1C3 mRNA (n=6). (d) and increased 22RV1 protein CYP11A1, CYP17A1, HSD3B2, AKR1C3 (n=3). Western blots were quantitated using GAPDH loading control (mean + SE; *p<0.05).
Figure 4: IGF2 treatment increased steroid production in LNCaP cells
LNCaP cells were treated with 85ng/ml IGF2 for 48hr (a) LC-MS used to identify and quantitate intracellular steroids showing a significant increase in 17-OH progesterone (17-OH Prog), testosterone (Test), androsterone (Andro), pregnanolone (Preg), and progesterone (Prog). (b) Extracellular steroids identified and quantitated by LC-MS in media detected significantly increased levels of testosterone (Test), androsterone (Andro), and pregnanolone (Preg) were detected. Steroid levels normalized to cell pellet weight and deuterated testosterone for extraction efficiency and compared to the vehicle time point control (n=6). (c) 22RV1 cells were treated with 85ng/ml IGF2 for 48hr, secreted significantly more DHT compared to vehicle-treated (n=6) (mean + SE; *p<0.05).

Figure 5: IGF2 increases expression of androgen receptor regulated genes
(a) Increased PSA mRNA following IGF2 treatment was compared to PSA induction by 10nM DHT. Both IGF2-induced and DHT-induced expression was blocked by AR antagonist bicalutamide. (b) PSA mRNA expression following IGF2 treatment was compared to with increasing concentrations of DHT (0.01pM-1nM). DHT induction of PSA mRNA was plotted as a function of concentration. The equation of the line was used to compare IGF2 induction. 85ng/ml IGF2 is equipotent to 1.6x10^{-12}M DHT for PSA induction. (n=3; mean + SE; *p<0.05).

Figure 6: IGF2 increases de novo steroidogenesis in prostate cancer cells.
Steroids were extracted from media from VCaP and LNCaP cells after 72hr incubation with 85ng/ml IGF2 and 6µCi/mL $^{14}$C acetate, quantitated by HPLC and radiometric detection. (a) VCaPs responded to IGF2 with increased steroid levels for pregnane3,20dione and a peak close to progesterone in retention
time, though trend of increase is evident for androstenedione, androsterone and cholesterol. (b) LNCaPs increased levels of every detected steroid in response to IGF2 treatment. Mean+SE of n=3; *p<0.05).

Figure 7: IGF2 increases de novo steroidogenesis in prostate cancer cells is blocked by inhibition of IGF1R and hybrid receptors.

(a) Medium was collected from LNCaP cells after 72hr incubation with 85ng/ml IGF2 and 6µCi/mL $^{14}$C acetate and in the presence or absence of BMS-754807 pan-INSR and IGF1R inhibitor (5µM) or anti-IGF1R antibody (12.5µg/ml) CP-751,871. Steroids were quantitated via HPLC / radiometric detection. Steroidogenesis was blocked by CP-751,871 and partially blocked by BMS-754807. (b) Both inhibitors potently inhibited mRNA expression of StAR, CYP17A1, AKR1C3, HSD17B3 in LNCaP cells. (c) Medium from 22RV1 cells following 48hr incubation with IGF2 in the presence or absence of CP-751,871 or BMS-754807 showed inhibition of IGF2 induced steroidogenesis with both inhibitors. (d) Western blot showed IGF2 increased steroidogenesis enzymes in 22RV1s following IGF2 treatment was blocked by CP-751,8871 or BMS-754807. Mean+SE of n=3; *p<0.05).

Figure 8: IGF2 and insulin induce AR activation was blocked by ketoconzole. PSA mRNA expression in LNCaP cells was measured in response to (a) 85ng/ml IGF2 treatment (b) 10nM insulin in the presence or absence of or 12.5µg/ml CP-751,871, 5µM BMS-754807 or 10µM ketoconazole. (a) IGF2 induction of PSA mRNA was blocked in the presence of inhibitors to IGF1R and hybrid receptors; whereas, (b) insulin-induced PSA was reduced in the presence of INSR inhibitors (100nM S661 and BMS-754807). Mean+SE of n=3; *p<0.05.
Figure A: mRNA expression of IGF2R and IGF2 across different treatment intervals.

Figure B: IGF1R expression in NHT 0 and CRPC samples. IHC Score comparison:
- NHT 0
- NHT <3m
- NHT 3-5m
- NHT >5m
- CRPC

Figure C: INSRb expression in NHT 0 and CRPC samples. IHC Score comparison:
- NHT 0
- NHT <3m
- NHT 3-5m
- NHT >5m
- CRPC
A. Intracellular

B. Secreted

C. 22RV1
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Supplementary Figure 1

Comparison of Ct values in LNCaP and 22RV1 cells (normalized to reference gene RLP32): 22RV1 cells displayed 2 to 12 times the normalized levels for most enzymes compared to LNCaPs, with much greater basal levels of AKR1C3. Exceptionally, CYP11A1 levels were higher in LNCaP cells than 22RV1.
Supplementary Figure 2

After 24 hours, 10nM progesterone strongly induces PSA expression in LNCaP cells, compared to ethanol vehicle, through the promiscuous AR of LNCaP cells (white bars). In order to test whether IGF2 mutually enhanced progesterone induction of PSA, LNCaP cells were co-incubated with 85ng/ml IGF2 (black bars). IGF2 did not enhance progesterone-mediated steroid induction of PSA.
Supplementary Figure 3

Supplemental Figure 3

IGF2 treatment increased steroid production in LNCaP cells. Representative absolute intracellular (A) and secreted (B) steroid concentrations LNCaP cells treated with 85ng/ml IGF2 for 48hr. As described in Figure 4, LC-MS used to identify and quantitate steroids dehydroepiandrosterone (DHEA), 17-OH progesterone (17-OH Prog), testosterone (Test), dihydrotestosterone (DHT), androsterone (Andro), pregnanolone (Preg), and progesterone (Prog).
Supplementary Figure 4

Representative spectra of steroids isolated from 2mL of media following 85ng/ml treatment of VCaP (a, b) or LNCaP cells (c, d). Steroids were extracted with equivolume hexane/ethanolacetate (75:25) and resuspended in 75µl of 50% methanol. Mix 10 standards and radiolabelled standards (e) (which included DHT, progesterone, and cholesterol) were used to identify steroid retention times (a) and peaks were quantified by measuring area under the curve. For VCaP cells, the control (a) was compared to 85ng/ml IGF2 treated (b) to calculate fold change. The same was done for LNCaPs, vehicle-treated control cells (c) being used to normalise IGF2 induced peaks (d). Steroid retention times were comparable in both LNCaP and VCaP cells and are identified from 1 to 8, with testosterone the first to elute from the column and cholesterol the last. De novo synthesised testosterone was detected in LNCaPs only. Cholesterol was detected to increase in VCaPs but not LNCaPs. One explanation may be that the $^{14}$C labelled acetate was rapidly converted to testosterone in LNCaPs, where time for steroidogenesis may be elongated in VCaPs.