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## Androgen-stimulated UDP-glucose dehydrogenase expression limits prostate androgen availability without impacting hyaluronan levels

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### Abstract

UDP-glucose dehydrogenase (UGDH) oxidizes UDP-glucose to UDP-glucuronate, an essential precursor for production of hyaluronan (HA), proteoglycans, and xenobiotic glucuronides. High levels of HA turnover in prostate cancer are correlated with aggressive progression. UGDH expression is high in the normal prostate even though HA accumulation is virtually undetectable. Thus, its normal role in the prostate may be to provide precursors for glucuronosyltransferase enzymes, which inactivate and solubilize androgens by glucuronidation. In this report, we quantified androgen dependence of UGDH, glucuronosyltransferase, and HA synthase expression. Androgen dependent and independent human prostate cancer cell lines were used to test the effects of UGDH manipulation on tumor cell growth, HA production and androgen glucuronidation. Dihydrotestosterone (DHT) increased UGDH expression  $\approx 2.5$ -fold in androgen dependent cells. However, upregulation of UGDH did not affect HA synthase expression or enhance HA production. Mass spectrometric analysis showed that DHT was converted to a glucuronide, DHT-G, at a six-fold higher level in androgen dependent cells relative to androgen independent cells. The increased solubilization and elimination of DHT corresponded to slower cellular growth kinetics, which could be reversed in androgen dependent cells by treatment with a UDP-glucuronate scavenger. Collectively, these results suggest that dysregulated expression of UGDH could promote the development of androgen independent tumor cell growth by increasing available levels of intracellular androgen.

### Keywords

prostate cancer; hormone refractory; dihydrotestosterone; detoxification; hyaluronan

### Introduction

Prostate cancer is the third leading cause of cancer death in men (1). Although localized tumors can often be effectively treated with resection and/or androgen ablation, androgen insensitive recurrence is a major clinical problem (2). Neoplastic proliferation in the prostate epithelium is initially dependent on the presence of androgen stimuli, which activate androgen receptor (AR)-mediated transcription of genes that initiate and sustain mitotic cell division. Loss of

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response may occur for a variety of reasons, including AR loss of expression or constitutive activation, or oncogenic transformation through other growth control pathways (3).

Pathways involved in regulation of androgen availability have been investigated as an obvious link to hormone independent cancer progression. Typically, the focus of these studies has been the biosynthetic enzymes such as hydroxysteroid dehydrogenase and 5 $\alpha$ -reductase that complete activation of testosterone precursors to their potent growth stimulatory forms (4-7). Some therapeutic success has been achieved by targeting these enzymes, but excess hormones from other pathways can also be converted to potent androgens. UDP-glucuronosyltransferase (UGT) enzymes have received increasing attention because of their expression in hormone-sensitive target tissues, as well as their demonstrated capacity to effectively reduce lipophilic hormone and xenobiotic concentrations by glucuronidation, which inactivates the chemical substrate while also solubilizing it for urinary excretion (8-10). Specifically, studies have implicated polymorphisms in genes encoding both prostate UGT isoforms, UGT2B17 (11) and UGT2B15 (12), as genetic risk factors for prostate cancer. These findings are supported by cell culture experiments in which these 95% identical isoforms were shown to be androgen downregulated and to alter proliferation rate of cells by inactivation of androgens (4,13-15). Thus, the enzymes required for the glucuronidation pathway in prostate epithelium are a significant consideration in androgen availability.

UDP-glucose dehydrogenase (UGDH) catalyzes oxidation of UDP-glucose to UDP-glucuronate, the requisite precursor for all glucuronidation reactions. The importance of this enzyme in development is well defined by the lethality of targeted disruption in multiple organisms (16-21). Supplemental Figure 1 illustrates the central role of the enzyme in providing UDP-glucuronate for divergent incorporation into hyaluronan (22), modification of hormones or xenobiotics for solubilization and elimination (10), polymerization of heparan sulfate chains, as well as conversion to UDP-xylose to initiate proteoglycan production (23). In humans, many tissues express UGDH, but strong expression is specific to liver and prostate. Two independent studies have suggested that UGDH expression is stimulated in cultured cells by androgen treatment. One study noted estrogen-stimulated expression in breast tumor cells was AR dependent (24) and another group identified UGDH in a microarray analysis of genes expressed >2-fold in LNCaP prostate tumor cells treated with dihydrotestosterone (DHT), a potent natural androgen (25). A putative androgen responsive element has been identified at position -1569 in the upstream regulatory sequence of the human UGDH gene (25), but surprisingly, the strong potential for UGDH to influence intracellular androgen availability has been completely overlooked.

In this study we used androgen dependent and independent prostate cancer cell lines to examine the requirement for androgens in UGDH protein expression and subsequent HA production. Our findings suggest increases in UGDH protein levels, effected in androgen receptor dependent fashion, are not related to HA levels. Instead, UDP-glucuronate production by UGDH primarily contributed to neutralization of the elevated androgens in androgen dependent prostate tumor cells, and to increased proliferation rate in androgen independent cells. In addition, our results show that increased UDP-glucuronate levels can drive inactivation of androgens despite decreased glucuronosyltransferase expression, and that this capacity is lost in androgen independent tumor cells.

## Material and Methods

### Cell culture and reagents

HEK293 human embryonic kidney cells and LNCaP human prostate tumor cells were purchased from American Type Culture Collection (Manassas, VA). LNCaP C33 and C81 low and high passage LNCaP cells, respectively, were kindly provided by Dr. Ming-Fong Lin

(University of Nebraska Medical Center, Omaha, NE). PC3M-LN4 human prostate tumor cells, originally derived from PC3 cells by successive passaging through mouse orthotopic injection and culture of resultant lymph node tumor cell colonies to select for enhanced metastatic propensity, were obtained from Dr. Isaiah J. Fidler (M.D. Anderson Cancer Center, Houston, TX). 4-methylumbelliferone (4MU), 5- $\alpha$ -dihydrotestosterone (DHT) and methyltrienolone (R1881) were purchased from Sigma (St. Louis, MO). Charcoal stripped FBS was from Hyclone (Logan, UT). Antibodies were obtained as follows: polyclonal rabbit anti-human PSA (DakoCytomation, Glostrup, Denmark); mouse monoclonal anti-human  $\beta$ -tubulin (Sigma); IRDye 800 conjugated anti-rabbit IgG (Rockland, Gilbertsville, PA); IRDye 680 conjugated goat anti-mouse IgG (LI-COR Biosciences, Lincoln, NE).

### Development of anti-UGDH antibodies

Human UGDH expressed in *E. coli* as a 6-Histidine fusion protein and purified to homogeneity from soluble lysates by nickel-NTA chromatography (26) was used to raise polyclonal antibodies in New Zealand white rabbits (Covance Research Inc.). Specificity was verified by western analysis of serially diluted recombinant antigen, UGDH transfected cell lysates, and immunoprecipitated endogenous UGDH protein from multiple prostate tumor lines (Supp Fig 2).

### Subcloning of UGDH coding sequence and transient transfection of HEK cells

The hUGDH gene was amplified by PCR from a LNCaP cDNA library and ligated via Sac I and Sal I restriction sites to the pIRES2-EGFP eukaryotic expression vector (Clontech, Mountain View, CA). The UGDH D280N inactive point mutant was generated from this template using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Clones were sequenced at the University of Nebraska. HEK293 cells were transfected by the calcium phosphate method. The transfection efficiency was assessed by fluorescent EGFP expression at >80%.

### Androgen stimulation

LNCaP cells were subcultured to 50% confluence in phenol red-free RPMI 1640 supplemented with 1% charcoal stripped FBS (CS-FBS) for 48 hours (androgen free conditions). Similarly, for androgen stimulation experiments, PC3M-LN4 cells were subcultured in phenol red-free MEM supplemented with 10% CS-FBS for 48 hours. For dose response, DHT or R1881 was serially diluted from concentrated stocks into the respective media. Growth media were replaced with 3ml per well of media containing the indicated concentration of androgen. Cells were harvested after 48 hours for analyses.

### Western analysis

UGDH and  $\beta$ -tubulin were probed simultaneously in cell lysates with a mixture of anti-UGDH (1:5000) and anti  $\beta$ -tubulin (1:10,000). PSA and  $\beta$ -tubulin were detected simultaneously using anti-human PSA (1:1500) and anti  $\beta$ -tubulin. Following secondary incubation with IRDye 800 anti-rabbit IgG and IRDye 680 anti-mouse IgG (1:5000 dilutions), proteins were quantified by fluorescence emission using the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE). Signals for each wavelength were analyzed in red (700nm) and green (800nm). Tubulin-normalized UGDH signals from androgen-treated samples (DHT or R1881) were further normalized to the tubulin-weighted UGDH quantity in untreated samples.

### HA quantification

HA content of cell cultures was quantified by competitive binding assay as described previously (27). Prior to harvesting cells cultured in the presence of vehicle or DHT (10nM), conditioned media were collected and cells were counted. Media were serially diluted for

incubation with HA binding protein in HA-precoated plates. HA concentration was interpolated from a standard curve and normalized to cell number.

### Quantification of androgen glucuronide conjugates

Conditioned media from androgen treated cell cultures were ethanol extracted for glucuronide analysis by liquid chromatography and mass spectrometry (LC-MS) essentially as previously published ((28) and Supplementary Methods).

### Detection of UDP-glucuronosyltransferase expression

Total RNA was isolated from LNCaP C33 and C81 cells that had been cultured in 1% CS-FBS for 48 hrs and then treated with 10nM DHT for another 48 hrs. Equivalent amounts of total RNA were reverse transcribed using the Superscript III kit (Invitrogen). Primers were designed as previously published to amplify UGT2B15 (515 bp) and UGT2B17 (519 bp) transcripts (13,29), HAS2 (411 bp) and HAS3 (414 bp) transcripts (27,30), or UGDH (448 bp). Primer sequences are given in Supp Table 1.

### Cell proliferation assay

LNCaP C33 and C81 cells ( $4 \times 10^3$  cells/well) were cultured in 96-well plates in androgen free conditions. After 48 hrs, media were removed and replaced with media containing vehicle, 10 nM DHT, or 0.1 nM R1881, alone or in combination with  $1 \mu\text{M}$  4MU, and incubated another 48 hrs. Cell proliferation was assayed daily in replicate plates incubated for 4 hrs with WST-1 (10  $\mu\text{l}$  per well), after which absorbance at 440 nm was measured in a microplate spectrophotometer.

## Results

### UGDH expression is stimulated by androgens

To evaluate the effect of androgens on UGDH expression in prostate cancer, we initially compared the androgen-dependent LNCaP prostate tumor cell line and PC3M-LN4, an androgen-independent, AR negative line derived from PC3 (31). Cells were cultured in androgen free conditions for 48 hours prior to dosing with DHT, a potent naturally produced androgen, or R1881, a synthetic androgen. Levels of UGDH protein increased dose-dependently in LNCaP cells treated with either DHT or R1881 (Fig 1A and 1B, respectively). Ratiometric analysis of UGDH expression relative to  $\beta$ -tubulin levels showed UGDH protein was elevated by 2-3-fold in LNCaP cells cultured in 10 nM DHT when compared with vehicle treated cells (0 nM). Similarly, UGDH expression increased  $>3$ -fold in response to R1881 treatment. No further increases were obtained with concentrations of 50 and 100 nM (not shown), and we selected the 10 nM dose for subsequent experiments. Prostate specific antigen (PSA) was evaluated in the soluble lysates as a positive control for androgen stimulated gene expression and was found elevated 5-10-fold (Supp Fig 3). In contrast, neither DHT nor R1881 altered expression of UGDH (Fig 1C and 1D, respectively) or PSA (Supp Fig 4) in AR-negative PC3M-LN4 cells.

### UGDH overexpression stimulates HA production in HEK293 cells

UGDH expression is suppressed by hypoxia and/or chemical elevation of intracellular NADH (32), factors that additionally influence hyaluronan (HA) and general glycosaminoglycan metabolism (33), suggesting UGDH provision of precursor UDP-glucuronate may be rate limiting for these syntheses. We have shown that excess HA production and turnover by prostate tumor cells can promote tumor growth and angiogenesis (34-36), and that inhibition of HA biosynthetic enzymes reduces both tumorigenesis and metastatic dissemination (36, 37). Thus, we initially hypothesized that UGDH elevation in normoxic tumor cells would

dramatically increase HA production and tumorigenic potential. When we transfected LNCaP cells with human UGDH cDNA (not shown), we detected <2-fold increased UGDH protein in cell lysates and no effect on HA production. We screened numerous available cell lines and identified HEK293 cells as a line that expresses very little endogenous UGDH protein and produces virtually no HA. We transfected HEK293 cells with UGDH alone or in conjunction with a hyaluronan synthase 3 (HAS3) expression vector to determine whether the expression of UGDH could stimulate HA production above the level of the HAS3 enzyme alone. Transfection conditions were optimized to yield equivalent UGDH and/or HAS3 expression levels (Fig 2A), and conditioned media were evaluated for HA content (36). An inactive point mutant of UGDH (asp280asn, or D280N (26)) was used as a negative control for the specific activity of UGDH. Figure 2B illustrates that HEK293 cells transfected with vector (GFP) or UGDH constructs alone produced virtually no HA (<0.1  $\mu\text{g}$  per  $10^6$  cells). As we have seen in other cell types (35,38), HAS3 transfection alone stimulated HA synthesis  $\approx$ 50-fold. However, cotransfection with wild-type UGDH increased the yield of HA polymers by an additional 3-fold, which was not reproduced by the inactive UGDH mutant. Thus, although there is an existing pool of UDP-glucuronate precursors, elevation of UGDH expression directly influences the production of HA.

### **UGDH stimulation does not influence HA production by LNCaP cells**

To relate this finding to prostate cancer, we used a LNCaP lineage-derived cell culture model for loss of androgen dependence. This model was generated by culturing LNCaP cells at a low passage (designated C33) to ultimate androgen independence in high passage (designated C81) (39), importantly without altering AR expression (39,40). Whereas UGDH was elevated  $\approx$ 2.5-fold by DHT treatment in the low passage C33 cells (Fig 3A and Supp Fig 5), no significant change in UGDH expression was observed in C81 cells, consistent with the results in Figure 1. We additionally performed a time course to compare transcriptional activation of UGDH expression in C33 and C81 cells. RT-PCR analysis showed that DHT and R1881 stimulation of UGDH mRNA occurred within two hours, reaching maximal levels by  $\approx$ 12 hours, exclusively in C33 cells (Supp Fig 6). The culture media of these cells were then evaluated for HA content. Low passage LNCaP cells express HAS3 enzyme but synthesize little to no HA ((27), Fig 3B). In contrast to what we observed for HEK293 cells, no significant change in HA levels was measured in either C33 or C81 cells upon DHT treatment (Fig 3B), but the baseline level of HA secretion by the high passage C81 cells was approximately doubled. Thus, HA synthesis itself is not androgen sensitive, and a 2-3-fold increase in the level of UGDH protein is not sufficient to promote additional HA production in LNCaP cells.

### **Androgens are effectively solubilized for excretion by androgen dependent prostate tumor cells**

It has previously been suggested that the expression of UDP-glucuronosyltransferases in hormone dependent tissues such as the prostate is an autoregulatory mechanism to prevent sustained cellular response in the presence of large hormone excesses. Since UGDH is also highly expressed in those tissues, and is needed to provide precursors for UGT isozymes, we tested whether UGDH elevation by androgens would lead to increased production of androgen glucuronides. Several metabolites of DHT have been identified in LNCaP previously, including  $3\alpha$ -diol and its glucuronide (28), which are produced in significant quantities. We focused on DHT-glucuronides, the inactive products of UGT enzymes, as a readout for UGT activity. Elimination of DHT-glucuronide was increased in a dose dependent manner by DHT (Fig 4A). At the physiologically relevant concentration of 10 nM DHT, secretion of DHT-glucuronide occurred at a level six times higher for androgen sensitive C33 cells than for C81 cells. Significant androgen inactivation by C81 cells was detected only at elevated concentrations of DHT. Correlation of DHT-glucuronide production with androgen-stimulated UGDH expression was further examined by exposing cells to the glucuronidation scavenger 4-

methylumbelliferone (4MU (41)). Inclusion of 4MU had no effect on DHT-glucuronide levels of androgen-supplemented C81 culture media, but reduced the levels in C33 media by 35% (Fig 4B). UGDH expression was not affected by DHT treatment in C81 cells, so the content of DHT-glucuronide in C81 cultures can be considered basal DHT inactivation. Subtraction of this amount suggests 4MU competition blocked >50% of the androgen-stimulated DHT glucuronidation at the subsaturating concentration used in our study.

### **UDP-glucuronosyltransferases UGT2B15 and UGT2B17 are androgen suppressed**

Androgen glucuronidation is dependent on both precursor availability and the activity of UGTs, so we evaluated the effect of DHT on expression of the two isozymes expressed in prostate that use androgen substrates (13,42), UGT2B15 and UGT2B17. Conventional (Fig 5A) and quantitative real time (Fig 5B) RT-PCR analysis revealed comparable basal expression of both messages in LNCaP C33 and C81 cells. Both transcripts were reduced >60% in C33 cells by DHT treatment for 48 hours, but levels in C81 cells were not altered by DHT. This result provides strong evidence that UGDH activity is the rate limiting factor in solubilization of excess androgen from prostate tumor cells. Expression of hyaluronan synthase isozymes HAS2 and HAS3, previously shown elevated in highly metastatic PC3M-LN4 prostate tumor cells relative to poorly tumorigenic LNCaP cells, was unaltered by androgen starvation or DHT treatment (Fig 5A,5C) in C33 and C81 cells, consistent with unaffected HA production in these conditions (Fig 3C). However, as suggested by the doubled HA production in C81 cells relative to C33 cells, quantitative RT-PCR did reveal  $\approx 3.5$ -fold greater HAS3 expression in C81 cells, irrespective of culture conditions.

### **Excess availability of androgens increases proliferation rate of androgen dependent prostate tumor cells**

Altered growth kinetics of LNCaP C33 and C81 cells have been previously quantified (39, 40). To determine whether androgen elimination resulting from UGDH provision of glucuronidation precursors was a contributing factor in these growth differences, we compared DHT-stimulated growth in the absence and presence of the glucuronate scavenger, 4MU. Addition of DHT to C33 cells increased their growth rate to that of androgen free C81 cultures, and also modestly enhanced growth of C81 cells ( $\approx 10\%$ , Fig 6A). Treatment of cells in androgen free conditions with 4MU did not affect proliferation rate. Concurrent culture with DHT and 4MU also did not enhance growth of C81 cells beyond the level of DHT alone. However, inclusion of this general glucuronosyltransferase substrate in DHT-stimulated C33 cell cultures further increased growth by almost two-fold. Importantly, treatment of cells with the androgen analogue, R1881, which cannot be modified and inactivated by glucuronidation, stimulated growth of both C33 and C81 cells to a similar extent as DHT treatment but no further increase in growth rate occurred in the presence of 4MU (Fig 6B). Collectively, these results strongly support loss of androgen elimination (i.e.; increased intracellular androgen availability) as a component in development of androgen independence by prostate tumor cells.

## **Discussion**

Loss of epithelial androgen dependence in prostate cancer progression is a poorly understood phenomenon, and a source of aggressive recurrent cancer. Potential mechanisms underlying hormone independent growth of tumor cells may include altered expression of enzymes that regulate intracellular androgen availability. In this work, we tested the role of UGDH in provision of precursors for enzymes of the androgen catabolic pathway versus the hyaluronan biosynthetic pathway shown previously to correlate with metastatic prostate cancer progression. By comparing androgen dependent and independent cells derived from a common lineage to aggressive, metastatic cells from a different origin, we have demonstrated that UGDH is generally expressed in all lines, but the activity of UGDH can be further elevated in

AR dependent fashion. In androgen sensitive cells, this is insufficient to drive the accelerated synthesis of HA characteristic of metastatic cells. Instead, androgen dependent cells express little HA synthase, and UGDH activity contributes dramatically to inactivated androgen excretion. Furthermore, in the transition to androgen independent growth, the ability to regulate UGDH expression and androgen inactivation by this pathway is lost, implicating UGDH for the first time as a novel point of control in hormone-dependent cell growth.

An important aspect of our study is the validation of UGDH as an androgen-stimulated gene in androgen dependent prostate tumor cells. We found UGDH mRNA elevated by both DHT and R1881 within two hours, with significant increases in protein evident at 24 hours. These kinetics are consistent with previously published microarray data from LNCaP cells in the absence and presence of DHT (25). In addition, similar kinetics have been reported for PSA expression, in which case androgen-stimulated gene transcription and *de novo* protein synthesis were required (43). Involvement of the AR is further suggested by the failure of DHT or R1881 to impact UGDH levels in PC3M-LN4 cells, which lack AR and PSA expression. Thus, it is probable that androgens act primarily via AR-mediated transcriptional activation to increase UGDH levels in low passage LNCaP cells.

As the enzyme catalyzing formation of the rate-limiting precursor for HA biosynthesis in development, UGDH overexpression was postulated to factor significantly in the excessive HA accumulation that correlates with numerous human pathologies, including cancer. Accelerated HA production and turnover have been shown to predict invasive prostate cancer progression and PSA biochemical recurrence clinically (44), and also to promote prostate tumorigenesis and spontaneous metastasis in mice (45,46). Consistent with a direct role for UGDH in regulating HA levels, antagonism of UGDH function in cultured aortic smooth muscle cells (16) and in human keratinocytes (47) either by siRNA or 4MU, respectively, was shown to reduce HA production significantly in these HA rich cell types. In the current study, we observed that HA production is not stimulated by androgens in LNCaP cells at low or high passage, even though HAS3 transcription is elevated in the latter. Moreover, 2.5-fold stimulation of endogenous UGDH was not sufficient to drive additional HA production in prostatic cells, though coexpression of HAS3 in HEK293 cells yielded abundant HA production from the basal UDP-glucuronate pool that was increased upon coexpression of UGDH and HAS3. Altogether, it is apparent that complex mechanisms act to regulate HA production and more studies are needed to determine how and when UGDH and HAS function coordinately.

Glucuronosyltransferases UGT2B15/17 have been knocked down in mice and in LNCaP cell culture. In mice, which do not abundantly express UGT enzymes, targeted disruption leads to smaller prostate weight (14). In LNCaP cells, siRNA knockdown modestly reduces glucuronidation of DHT, leading to an accumulation of active androgens that increases expression of AR target genes such as PSA (13). On the other hand, a high level of UGT2B15 expression occurring in the human prostate has been reported to correlate with excess circulating androgen glucuronides (48). These results are consistent with our findings that the stimulated expression of UGDH protein can drive net increases in glucuronidation through elevation of available UDP-glucuronate. These modifications occur despite concurrent reduction of UGT2B15/17 mRNA levels, and only in androgen dependent fashion, since neither UGDH protein nor UGT2B15/17 mRNA levels are affected in androgen independent cells of the same lineage. With respect to UGT expression, our results are consistent with prior reports in which six and eight-day treatment with DHT reversibly suppressed UGT mRNA levels (4,15). In those studies, DHT-glucuronide levels were also reduced relative to those measured at our twoday endpoint. This is likely due to residual presence of UGT2B15/17 protein at day two, and is a function of both the length of androgen treatment and the duration of androgen depletion preceding DHT-glucuronide quantification. The difference in the dose



response we observed for UGDH stimulation by DHT and R1881 reflects reported differences in their intracellular stability and further underscores the relevance of the glucuronidation equilibrium: while R1881 is not modified by glucuronidation (49), DHT can be catabolized and its effective concentration diminished. Overall, despite suppression of UGT enzymes, the ability of androgen dependent cells to elevate UGDH and UDP-glucuronate still provides a mechanism for excess androgen inactivation that is lacking in androgen independent cells.

The general glucuronosyltransferase substrate, 4MU, has been widely used as an inhibitor of HA synthesis and tumor cell proliferation (41,47,50,51). Its mechanism of action is to serve as a low affinity competitor for UDP-glucuronate, diverting it from its normal biosynthetic pathways into UGT-mediated detoxification. As such, it is highly cytotoxic and dose responses are cell type specific. Whereas the administered concentrations for HA synthesis inhibition are on the order of 0.1-0.5 mM in fibroblasts and keratinocytes, we found these amounts were toxic to both androgen dependent and independent prostate tumor cells (not shown) and identified a range of 1-10  $\mu$ M as their threshold, which is probably dictated by their total UGT isoform expression. At this dose, treatment had no effect on DHT solubilization or growth rate of androgen independent cells, but significantly antagonized DHT elimination and thereby enhanced DHT-stimulated growth of low passage androgen dependent cells. These results raise the possibility that scavenger competitions occur during chemotherapeutic treatments. Xenobiotics may increase androgen potency in the prostate by placing demand on the UDP-glucuronate pool for xenobiotic inactivation, thereby effectively increasing bioactive androgen levels and further suppressing UGT. The long-term outcome could be enhanced androgen-mediated cell growth in the presence of less and less androgen, as well as more permanent suppression of genes involved in androgen clearance.

The phenomena described here may be a significant underlying factor in aggressive relapse of prostate cancer following androgen ablation therapy in human patients. AR mediated activation of cell proliferation has been shown to be dose responsive (52). Although AR expression is unchanged in the low and high passage LNCaP cells, altered proliferation response suggests differential ligand-dependent AR activity. This could be a manifestation of AR sensitivity to specific intracellular androgen concentrations, since cells treated with identical amounts of DHT responded with significant differences in rates of glucuronidation. Loss of ability to increase UGDH expression in response to androgens may be selected as a cellular adaptation for tumor cells to preserve individual androgen stores. Metastatic human prostate tumor cells in patients with castration resistant disease have been shown to contain more androgen than cells in primary tumor tissue and to express higher levels of enzymes involved in intracrine androgen synthesis. Thus, consistent with results of our study and others, tumor cells exhibit survival mechanisms that sustain androgen dependent signaling pathways in the absence of systemic androgen by optimizing local synthesis and retention (53). Additional studies are underway to determine the implication of UGDH control in prostate cancer.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## ABBREVIATIONS

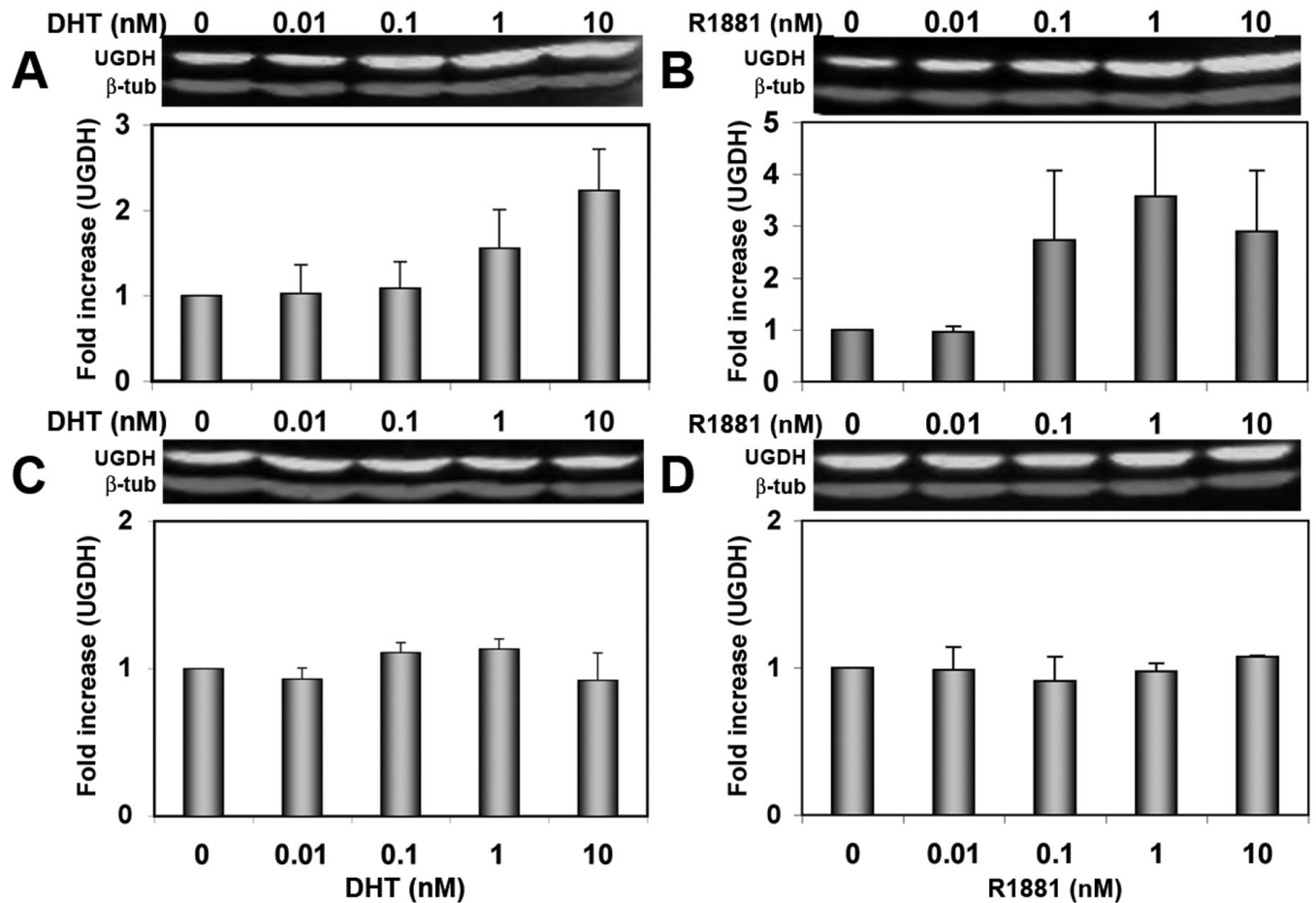
UGDH, UDP-glucose dehydrogenase; UGT, UDP-glucuronosyltransferase; HA, hyaluronan; HAS, hyaluronan synthase; DHT, 5- $\alpha$ -dihydrotestosterone; DHT-G, dihydrotestosterone glucuronide; AR, androgen receptor; PSA, prostate specific antigen; 4MU, 4-methylumbelliferone.

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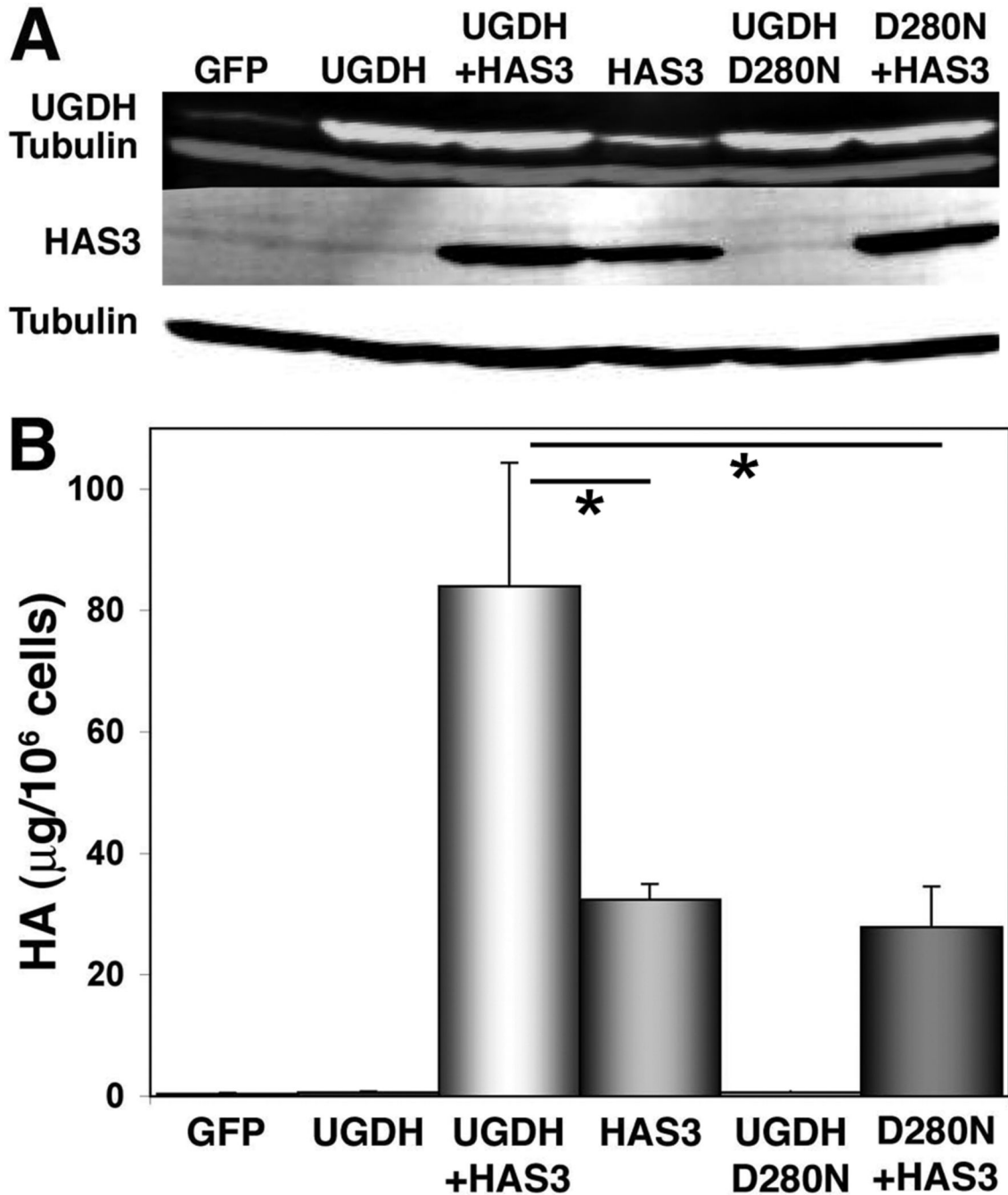
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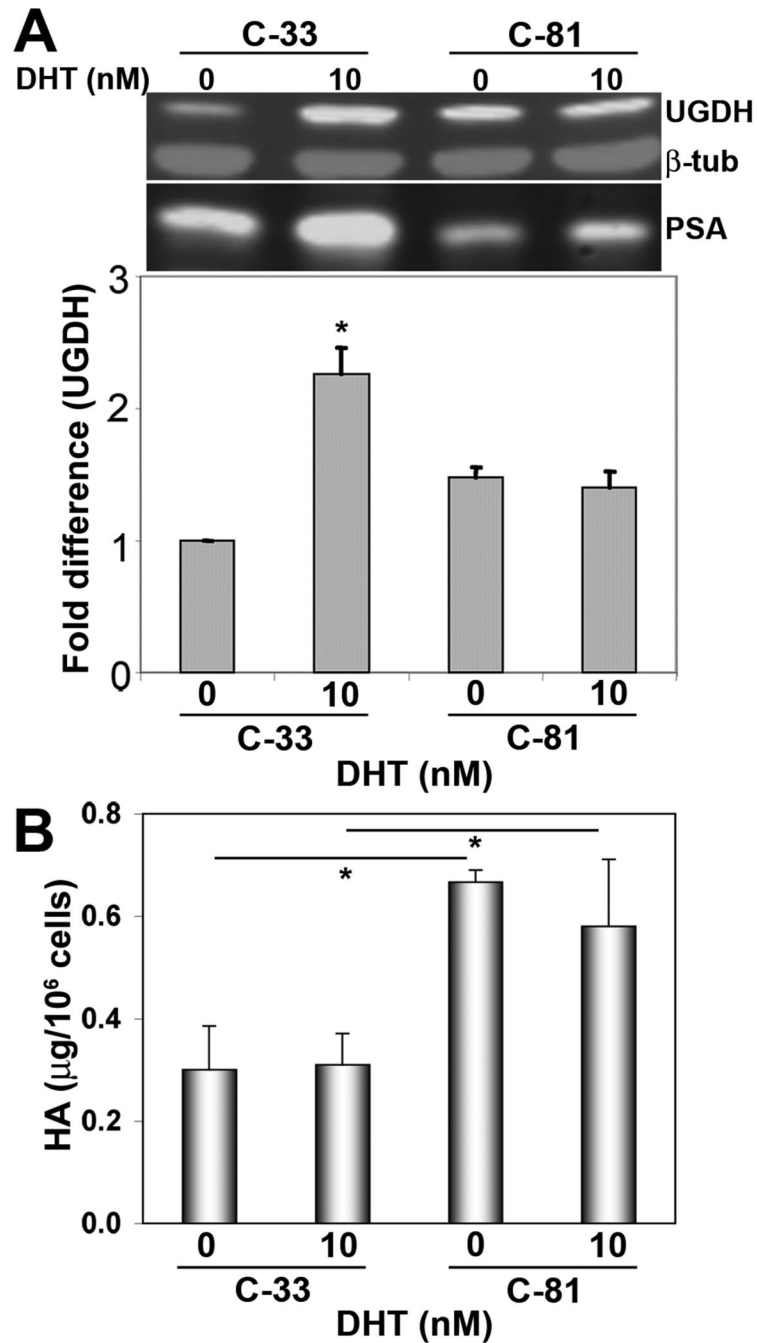
**Figure 1. UGDH expression is enhanced by androgens in LNCaP cells**

LNCaP (A,B) or PC3M-LN4 (C,D) cells were cultured in androgen-free media for 48 h prior to addition of DHT (A,C) or R1881 (B,D). After an additional 48 h, soluble lysates were analyzed by western blot. UGDH expression was quantified by fluorescence intensity normalized to  $\beta$ -tubulin signal using Odyssey software and plotted as fold difference (mean  $\pm$  SEM) with respect to untreated samples (0 nM). Blots represent one experiment analyzed in triplicate and repeated three times.



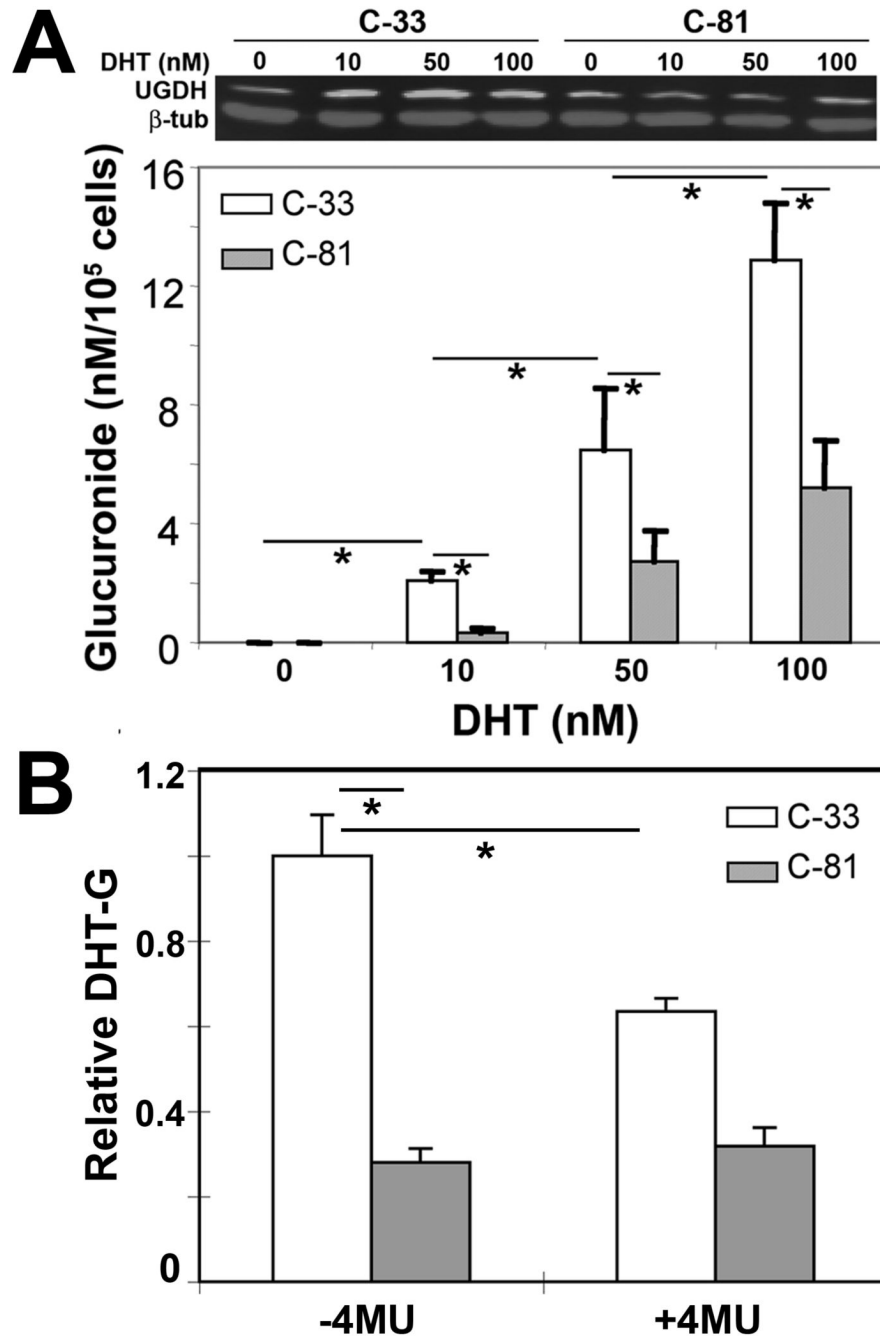
**Figure 2. Overexpression of UGDH promotes HA production in HEK293 cells**

HEK293 cells were transfected for 48 h with vectors expressing GFP (negative control), wild-type UGDH, an inactive point mutant of UGDH (D280N), the HA synthesizing enzyme HAS3, or cotransfected with UGDH+HAS3, and D280N+HAS3. (A) Western analysis of lysates (top) probed concurrently with anti-UGDH and anti-tubulin; membrane-enriched fractions probed for HAS3 (center) or tubulin (bottom). (B) Media of indicated transfectants were analyzed for HA content by competitive binding assay. One experiment done in triplicate is plotted (mean  $\pm$  SEM; \* $p$ <0.001), representative of three total repetitions.



**Figure 3. Androgen dependence is required for UGDH elevation in prostate tumor cells but does not affect HA production**

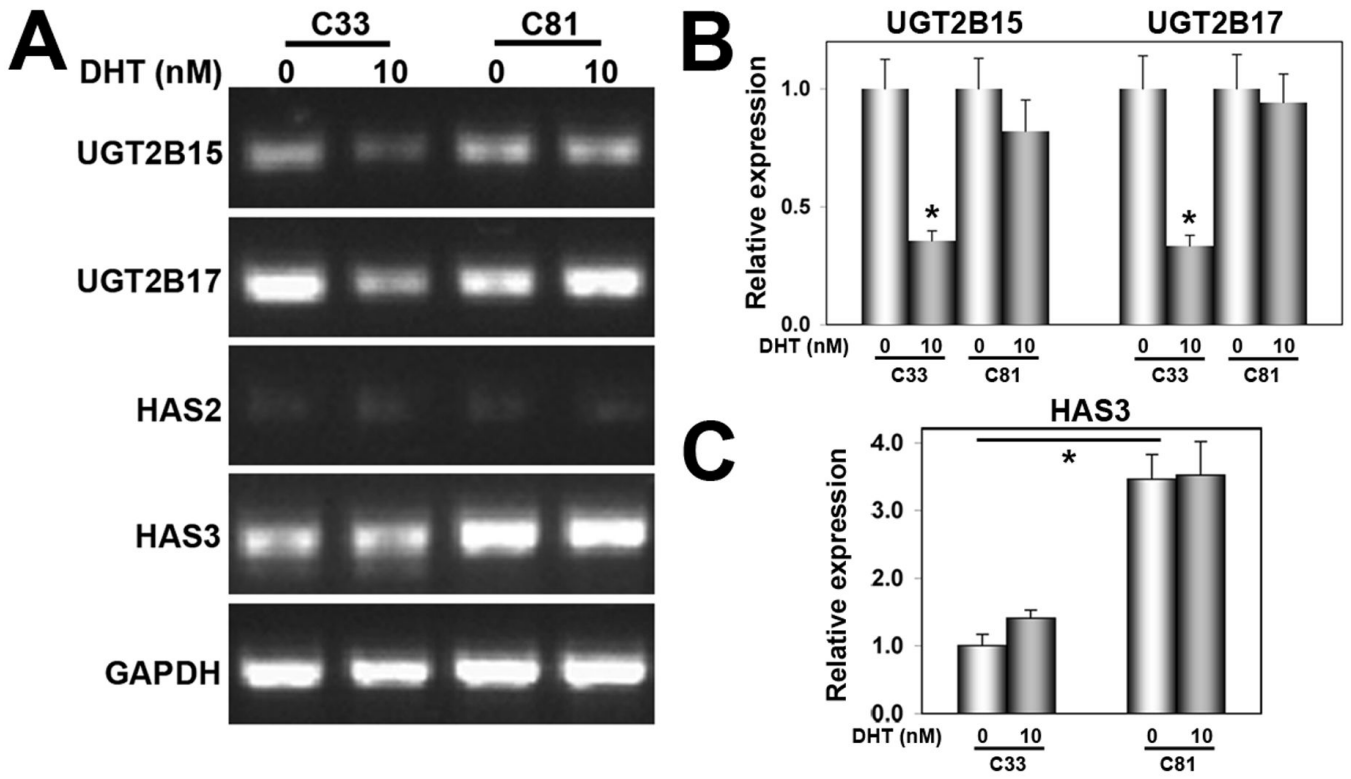
LNCaP C33 and C81 cells were cultured in the absence or presence of 10 nM DHT. (A) Soluble lysates were analyzed by western blot for expression of UGDH (upper panel) or PSA (lower panel). UGDH expression was normalized to  $\beta$ -tubulin and plotted as fold difference relative to untreated controls (mean  $\pm$  SEM; \* $p$ <0.01). (B) Conditioned media from triplicate wells of C33 and C81 cells, cultured with and without DHT were analyzed for HA content and normalized to manual cell counts (mean  $\pm$  SEM).



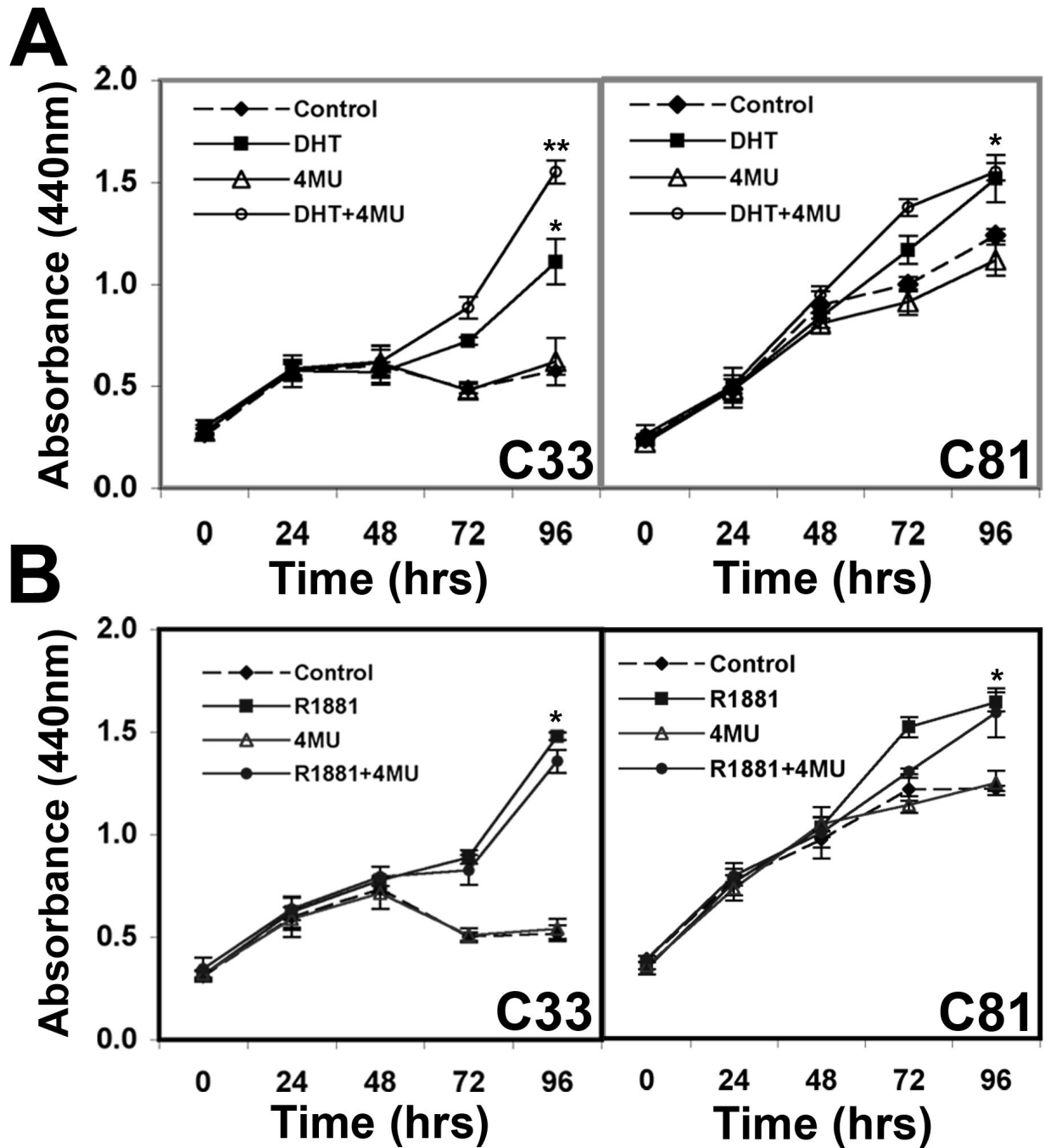
**Figure 4. DHT treated C33 prostate tumor cells secrete excess androgen in glucuronidated form** (A) LNCaP C33 and C81 cells were cultured in the absence and presence of DHT for 48 h. Soluble lysates were analyzed by western blot for UGDH and  $\beta$ -tubulin. Hormone content of conditioned media was quantified by LC-MS. DHT-glucuronide (DHT-G) levels were normalized to cell number in three separate experiments (mean  $\pm$  SD). Statistical significance was assessed by t-test for differences between untreated and DHT treated media, as well as those between C33 and C81 media at each DHT concentration, indicated by bars. \* $p$ <0.01. (B) DHT-G levels were quantified in 48h conditioned media of cells treated with DHT (10nM) in the absence or presence of 4MU (1 $\mu$ M). Results of triplicate experiments were normalized to



the mean DHT-G content of C33 androgen supplemented media. Mean  $\pm$  SD is plotted; \*  
p<0.01.



**Figure 5. Expression of UDP-glucuronosyltransferases (UGT) is significantly reduced while HA synthase (HAS) is unaffected by androgen treatment**  
 Total RNA from LNCaP C33 and C81 cells cultured in the absence and presence of 10nM DHT for 48 h was analyzed by conventional RT-PCR (A) and quantitative real-time PCR for UGT2B15 and UGT2B17 (B) or HAS3 (C). Mean ± SD is plotted; \*p<0.01.



**Figure 6. Androgen sensitivity of C33 and C81 proliferation**

LNCaP C33 and C81 cells were seeded in androgen depleted media. (A) After 48 hrs, media were removed and replaced with the same media containing vehicle only (control, filled diamonds), 1 $\mu$ M 4MU (open triangles), 10 nM DHT (filled squares), or both DHT and 4MU (open circles). In (B), R1881 (0.1nM) was used instead of DHT. Absorbance (440 nm) was measured daily in replicate plates following 4 h incubation with WST-1. Mean  $\pm$  SD is plotted. \*  $p < 0.01$  for control versus DHT or R1881 treated cells; \*\*  $p < 0.01$  for DHT+4MU treated cells relative to DHT treatment only.