Forkhead box protein A1 regulates UDP glucuronosyltransferase 2B15 gene transcription in LNCaP prostate cancer cells

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Running Title: FOXA1 regulates $UGT2B15$ basal gene expression

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**ABBREVIATIONS:** 3α-diol, 5α-androstane-3α,17β-diol; 3β-diol, 5α-androstane-3β,17β-diol; ADT, androsterone; ChIP, chromatin immunoprecipitation; EMSA, electrophoretic mobility shift assays; DHT, dihydrotestosterone; FBS, fetal bovine serum; FOX, forkhead box; GAPDH, glyceraldehyde phosphate dehydrogenase; HNF, hepatocyte nuclear factor; HSD, hydroxysteroid dehydrogenase; PBS, phosphate buffered saline; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; SNP, single nucleotide polymorphism; UGT, UDP-glucuronosyltransferase.
Abstract

The UDP glucuronosyltransferases (UGT) 2B15 and 2B17 are the major UGTs involved in the inactivation and elimination of the active androgens, dihydrotestosterone and testosterone. Although regulation of these UGT genes by various endogenous and exogenous ligands, including steroid hormones and bile acids, is well documented, the mechanisms controlling their basal gene expression are poorly understood. We recently reported that Forkhead box protein A1 (FOXA1) regulates the basal expression of the UGT2B17 gene in prostate cancer cells. Herein we show that FOXA1 also regulates basal expression of the UGT2B15 gene in the prostate cell line, LNCaP. FOXA1 binds to a site -208 to -217 bp relative to the UGT2B15 translation start site, as shown by electromobility shift and chromatin immunoprecipitation assays. Mutation of this site prevents binding and substantially decreases basal UGT2B15 promoter activity. Silencing of FOXA1 expression by siRNA significantly reduced UGT2B15 transcript levels, further confirming a crucial role of FOXA1 in controlling UGT2B15 gene expression. As local inactivation of active androgens by UGT2B15 and UGT2B17 has been shown to be a major determinant of androgen response and signalling activity, regulation of the UGT2B15 and 2B17 genes by FOXA1 may have an important role in the maintenance of androgen homeostasis within prostate cancer cells.
Introduction

Androgen signalling through the androgen receptor is crucial for human prostate growth and function; however, excessive androgen signalling within the prostate is implicated in prostate cancer development and progression (Kaarbo et al., 2007). The prostate has all the essential steroidogenic enzymes necessary to produce active androgens locally from circulating adrenal precursor sex steroids. For example, testosterone can be synthesized in the prostate from either androstenedione by 17β-hydroxysteroid dehydrogenase (HSD) or androst-5-ene-3β,17β-diol by 3β-HSD/(Δ5/Δ4 isomerase), and it can be further transformed into the most potent androgen 5α-dihydrotosterone (DHT) by 5α-reductase (Labrie et al., 2005). In addition, the prostate also expresses steroid metabolizing enzymes, including DHT-inactivating enzymes (Barbier and Belanger, 2008). The expression of 3α-HSD and 17β-HSD within the prostate permits the local interconversion between DHT and its metabolites androsterone (ADT), 5α-androstane-3α,17β-diol (3α-diol), and 5α-androstane-3β,17β-diol (3β-diol) (Labrie et al., 2005). This pathway is considered to be reversible and is at least partially depending on the redox status within the cellular context. The influence of this pathway on the intracellular levels of active androgens within the prostate has not yet been fully investigated. However, the expression of UGT2B15 and UGT2B17, two major androgen-conjugating enzymes from the UGT superfamily, leads to the irreversible inactivation of DHT and its metabolites within the prostate through glucuronidation (Chouinard et al., 2004).

UGT2B15 and UGT2B17 are 95% identical in amino acid sequence but possess unique substrate specificities towards 5α-reduced androgens (Turgeon et al., 2000). UGT2B17 conjugates both the 3- and -17-hydroxy positions of androgens, such as the 3-OH of ADT and the 17-OH of 3α-diol or DHT, whereas UGT2B15 specifically glucuronidates the 17-hydroxy
position of androgens, including the 17-OH of 3α-diol and DHT (Dubois et al., 1999; Turgeon et al., 2001). In the prostate, UGT2B17 is expressed in the basal cells of the alveoli and is thought to be involved in glucuronidation of DHT, ADT and 3α-diol in these cells, whereas UGT2B15 is exclusively expressed in the luminal cells, where it only inactivates DHT, as the enzymes for the formation of ADT and 3α-diol are not present in these cells (Barbier and Belanger, 2008). As glucuronidation of androgens and their metabolites facilitates their excretion, UGT2B15 and 2B17 help reduce intracellular DHT concentrations and consequently prevent excessive androgen signalling within the prostate (Chouinard et al., 2008). Indeed, it has been recently shown that the glucuronidation of androgens by UGT2B15 and 2B17 is a major determinant of the androgen response in prostate cancer LNCaP cells as evidenced by the reported enhanced expression of androgen target genes in UGT2B15/17-deficient cells compared to control cells in the presence of DHT (Chouinard et al., 2007).

Given the crucial role of UGT2B15 and 2B17 enzymes in the local inactivation of active androgens, it is important to decipher the molecular mechanism(s) that regulate their expression within the prostate. Accumulating evidence strongly suggests that the expression of the human UGT2B15 and 2B17 genes are negatively regulated at the transcriptional level by a number of endogenous and exogenous ligands in Androgen Receptor (AR)-positive human prostate cancer cell lines. These include AR-mediated suppression of UGT2B15 and 2B17 expression by natural (DHT) and synthetic (R1881) androgens (Chouinard et al., 2006; Bao et al., 2008), the farnesoid X receptor-mediated repression by farnesoid X receptor activators such as chenodeoxycholic acid, GW40, and androsterone (Kaeding et al., 2008b), and the vitamin D receptor-mediated down-regulation by Calcitriol (1α, 25-dihydroxyvitamin D3), the active metabolite of vitamin D (Kaeding et al., 2008a). However, in contrast to these effects of ligands, the mechanisms regulating basal UGT2B15 and 2B17 gene expression are poorly understood.
Forkhead box (FOX) proteins are highly conserved transcriptional regulators implicated in cancer development and progression. FOXA proteins consist of three isoforms, namely FOXA1 (HNF3α), FOXA2 (HNF3β), and FOXA3 (HNF3γ) (Mincheva et al., 1997; Myatt and Lam, 2007). FOXA1 appears to act as a transcriptional facilitator by binding to its cognate response elements as a monomer in the regulatory regions of target genes and enhancing the access of other transcription factors (Lupien et al., 2008).

We recently reported that FOXA1 is a major regulator of the basal expression of $UGT2B17$ gene in prostate cancer cell lines (Hu et al.). In this study we report that FOXA1 is also essential for the basal transcriptional activity of the $UGT2B15$ gene in LNCaP cells.
Materials and Methods

Generation of *UGT2B15* Promoter Luciferase Reporter Deletion Constructs and Mutants:

Sixteen pGL3-derived luciferase reporter constructs carrying varying lengths of the *UGT2B15* proximal promoter were used in transient transfection in the present study (Fig. 1). Fourteen of these constructs were previously reported, namely 2B15-2716/-3Luc, 2B15-747/-3Luc, 2B15-705/-3Luc, 2B15-595/-3Luc, 2B15-556/-3Luc, 2B15-458/-3Luc, 2B15-412/-3Luc, 2B15-361/-3Luc, 2B15-310/-3Luc, 2B15-253/-3Luc, 2B15-202/-3Luc, 2B15-154/-3Luc, 2B15-101/-3Luc, and 2B15-54/-3Luc (Hu and Mackenzie, 2009). The other two constructs were generated with the construct 2B15-747/-3Luc as template. The *UGT2B15* promoter regions from nucleotides -3 to -511 and -3 to -648 were amplified by PCR using the forward primers 2B15-511For 5'-'AGCCATGGTACCAGTGAAGTAAAATTTCGT-3' and 2B15-648For 5'-'AGCCATGGTACCAAGGGTCCAGAAAATGC-3', respectively, and the common reverse primer as previously reported (Hu and Mackenzie, 2009). Underlined are the KpnI restriction site added to the 5'-end of these primers to facilitate subsequent cloning. The resultant amplicons were ligated into the KpnI and MluI sites of the pGL3-basic vector to create the promoter constructs 2B15-511/-3Luc and 2B15-648/-3Luc. Mutagenesis was performed using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) and a complementary pair of primers. Using the forward primer 2B15-FOXA1-MT1 with sequences of 5'-GACTAGAGTAATTGCCCCCCTAAAAGAACACC-3', the FOXA1 site was mutated from 5'-TGTAAACATAAA-3' to 5'-TGCCCCCTAAA-3' (mutated nucleotides are underlined) on two constructs 2B15-253/-3Luc and 2B15-458/-3Luc to generate their respective mutants 2B15-253/FOXA1-MT/-3Luc and 2B15-458/FOXA1-MT/-3Luc. The promoter sequences of all reporter constructs were confirmed by DNA sequencing.
Transient Transfection and luciferase Reporter Assay: The LNCaP human prostatic cancer cell line, one of the most frequently used in vitro model of human prostate cancer, was obtained from The American Type Culture Collection (Manassas, VA), and routinely maintained in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 5% (v/v) fetal bovine serum (FBS) at 37 °C in a 5% CO2/95% air environment. All transfections were performed using Lipofectamine 2000 (Invitrogen). Exponentially growing LNCaP cells were trypinized and subsequently plated in 24-well plates at a density of 2 x 10^5 cells per well in 800 μl of RPMI medium containing 5% FBS. Transfections were conducted when culture reached approximately 50-60% confluence. Briefly, 500 μl of medium was aspirated from each well and replaced with 300 μl of serum-free RPMI transfection mixture containing 0.5 μg of each reporter construct and 25 ng of pRL-null plasmid, which served as an internal control to normalize transfection efficiency. Sixteen hours post-transfection, 300 μl of medium was aspirated from each well and replaced with 500 μl of fresh RPMI medium supplemented with 5% FBS. Forty-eight hours post-transfection, cells were lysed in passive lysis buffer and analyzed for firefly and Renilla luciferase activities using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI) according to the manufacturer’s instructions and a Packard TopCount luminescence and scintillation counter (PerkinElmer Life and Analytical Sciences, Waltham, MA). Transfections were performed in triplicate and all experiments were repeated at least twice.

Electrophoretic Mobility Shift and Supershift Assays (EMSAs): The FOXA1 expression vector was prepared by cloning full-length FOXA1 cDNA into the EcoRI site of the mammalian expression vector pCMX-PL2. With this FOXA1 expression plasmid as template, recombinant FOXA1 proteins were synthesized using the T<T> Quick Coupled Transcription/Translation kit.
according to the manufacturer’s instructions (Promega). EMSAs were performed with 2B15 promoter-specific probes, which contained either a wild-type FOXA1 site (designated 2B15-FOXA1) with the sense sequence of 5’-TAATTGTAAACATAAAAGAACACCAAACACACTAA-3’ (the FOXA1 site is underlined), or a mutated FOXA1 site (2B15-FOXA1-MT2) with the sense sequence being 5’-TAATTGCGCACATAAAAGAACACCAAACACACTAA-3’ (mutated nucleotides are underlined). The anti-FOXA1 antibody (H-120) for supershift assays was purchased from Santa Cruz Biotechnology (Santa, Cruz, CA). EMSA experiments were carried out essentially as reported elsewhere (Hu and Mackenzie, 2009).

**Chromatin Immunoprecipitation Assay and Quantitative Real-Time PCR (ChIP-qPCR):**

ChIP-qPCR was performed as reported elsewhere (Hu and Mackenzie, 2009). In brief, LNCaP cells were grown in RPMI medium supplemented with 5% FBS. When culture reached 90% confluence, cells were crosslinked by 1% formaldehyde and subsequently quenched by 125 mM glycine solution. Cells were lysed, sonicated, and then subjected to immunoprecipitation with 10 μg of each specific antibody or equivalent amounts of the rabbit preimmune IgG control. The resulting chromatin precipitates were captured by protein A Sepharose CL-4B beads (GE Healthcare), and subsequently were eluted from these beads following several washes in different buffers as reported (Hu and Mackenzie, 2009). Cross-linking was reversed by heating the eluates at 65°C overnight. The resulting DNA/protein precipitates were digested with proteinase K, followed by phenol-chloroform extraction and ethanol precipitation. The DNA pellets were dissolved in 50 μl of Tris-EDTA buffer. Quantitative real-time PCR (qPCR) was performed with 2 μl of each of the resultant DNA samples as templates and locus-specific primers. qPCR primers for both the *UGT2B15* promoter (Hu and Mackenzie, 2009) and the control locus (chr7:...
72,190,955-72,192,054) (Carroll et al., 2006) were the same as previously reported. Antibodies against ERα (HC-20) or FOXA1 (H-120, the same as used in EMSAs), and the rabbit preimmune IgG control (sc-2027) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

**siRNA Knockdown Experiments:** On-TARGETplus SMARTpool siRNA against FOXA1 (NM_004496, designated anti-FOXA1 siRNA) and On-TARGETplus Non-targeting pool siRNA (non-target siRNA) were purchased from Dharmacon RNAi Technologies (Lafayette, CO). LNCaP cells were maintained in RPMI 1640 medium supplemented with 5% FBS. Cells grown at logarithmic phase were harvested and seeded into six-well culture plates at a density of 1x10⁶ cells per well in 2 ml of RPMI medium, consisting of 1.5 ml of cell suspension in serum-containing RPMI medium and 0.5 ml of the serum-free transfection mixture, containing 8 μl of Lipofectamine (Invitrogen) and 400 nM of either anti-FOXA1 siRNA or non-target siRNA. Transfection was conducted for 24-48 hours, followed by the addition of 2 ml of fresh RPMI medium with 5% FBS to each well. Cells were harvested 68-72 hours post-transfection, and mRNA levels of target genes were measured by quantitative real-time RT-PCR as described elsewhere (Hu and Mackenzie, 2009). RT-PCR primers were 5’-GAAGATGGAAGGGCATGAAACCA-3’ (forward) and 5’-TGGCATAGGACATGTGAAGGACG-3’ (reverse) for FOXA1 and 5’-GAAGGTGAAGGTCGGAGTC-3’ (forward) and 5’-GAAGATGGTGATGGGATTTC-3’ (reverse) for GAPDH. Those for UGT2B15 and 18S rRNA were the same as previously reported (Congiu et al., 2002). Data from 18S rRNA transcripts were used as a reference to normalize the amount of total RNA amplified in each reaction.
Results and Discussion

As the *UGT2B15* and *UGT2B17* genes share considerable sequence homology between their proximal promoters (Fig. 1), it is likely that they are regulated by some transcription factors in common. We have recently shown that FOXA1 regulates the basal activity of the *UGT2B17* promoter in LNCaP cells (Hu et al.). We have also shown that a single base change (the A/G SNP at -155 bp) within the *UGT2B17* FOXA1 site can alter promoter activity up to 13-fold. Although the equivalent putative FOXA1 site of the *UGT2B15* promoter does not contain this particular SNP, it does differ by a single base to that of *UGT2B17* (Fig. 1). As single base changes can diminish FOXA1 binding and substantially reduce promoter activity, it was necessary to confirm that the *UGT2B15* promoter was active and was regulated by FOXA1. It was also important to determine whether other sites in the UGT2B25 proximal promoter were involved in control of basal *UGT2B15* promoter activity.

For this purpose, a series of sixteen luciferase reporter constructs carrying varying lengths of the *UGT2B15* proximal promoter were generated. As shown in Fig. 2, reporter constructs with *UGT2B15* promoter lengths ≤ 202 bp possessed very low activities compared to the background activity of the promoter-less pGL3-basic vector, when transfected into LNCaP cells. In contrast, constructs with *UGT2B15* promoter lengths >253 bp possessed activities that were approximately 15-fold higher over that of the construct 2B15-202-3Luc (*p > 0.001*). These results suggested that the *UGT2B15* promoter between -202 to 253 bp harbors a positive regulatory *cis*-acting element(s), which is pivotal to its basal transcription activity in LNCaP cells and that similar elements are not present further upstream (up to -2716 bp). Based on a comparison to the *UGT2B17* promoter, this 52-bp region contains the putative *UGT2B15* FOXA1 site (5’-TGTAACATATAAA-3’), located between nucleotides -218 to -207 relative to the translation start site of the *UGT2B15*
gene. To determine whether this FOXA1 site contributes to the high activity of *UGT2B15* promoters >253 bp in length, we generated 2B15-253/FOXA1-MT/-3Luc bearing a mutated FOXA1 site (5’-TGCCCCCTAAA-3’, mutated sequences are underlined). When transfected into LNCaP cells, this construct had greatly diminished promoter activity in comparison to its unmutated counterpart (*p* < 0.001) (Fig. 2). To further verify its functionality, we mutated this FOXA1 site in another construct 2B15-458/-3Luc to create the mutant 2B15-458/FOXA1-MT/-3Luc. When transfected into LNCaP cells, this mutation also resulted in a large reduction in promoter activity compared to its wild-type counterpart (*p* < 0.01) (Fig. 2). Altogether, these results clearly demonstrate that basal *UGT2B15* promoter activity is dependent on an intact FOXA1 site, and that transcription factors which bind outside this region, do not have a major role in basal *UGT2B15* expression in LNCaP cells, under our experimental conditions.

There are three FOXA proteins, A1, A2 and A3, which bind to the same consensus sequence (Lai et al., 1990; Lai et al., 1991). However, as only FOXA1 protein expression has been demonstrated in LNCaP cells (Mirosevich et al., 2006), we undertook EMSAs to see whether FOXA1 bound to the *UGT2B15* FOXA1 site using in vitro transcribed/translated recombinant FOXA1 protein.

Incubation of a *UGT2B15* promoter probe (nucleotides -222 to -188) containing the putative FOXA1 site with recombinant FOXA1 protein led to the formation of a FOXA1-specific protein/DNA complex (labelled A) (Fig. 3A, lane 2), as evidenced by the formation of the supershifted complex (labelled SS) upon the addition of the anti-FOXA1 antibody (Fig. 3A, lane 4). Furthermore, the absence of complex A in the negative control (Fig. 3A, lane 1), as well as disruption of this complex in competition assays in the presence of a 100-fold molar excess of unlabelled probe (Fig. 3A, lane 3), provide further evidence that FOXA1 binds to its cognate site in the *UGT2B15* promoter. To prove the direct involvement of the putative FOXA1 site in the
formation of complex A, further EMSAs were performed using probes with a mutated FOXA1 site. As shown in Fig. 3A, lane 5, mutating the putative FOXA1 site in the UGT2B15 promoter almost completely disrupted complex A formation, thereby confirming the necessity of this site in forming the FOXA1-specific protein/DNA complex A.

Having established that recombinant FOXA1 binds to the UGT2B15 promoter in vitro, we next performed chromatin immunoprecipitation assays followed by quantitative real-time PCR (ChIP/qPCR) to see whether endogenous FOXA1 proteins in LNCaP cells could be constitutively present at the FOXA1 site within the UGT2B15 promoter. ChIP assays were performed with specific antibodies against FOXA1 or Estrogen Receptor (ER) α, and the rabbit preimmune IgG control for normalizing against possible non-selective background immunoprecipitation. FOXA1 occupancy on the UGT2B15 promoter was quantified with the FOXA1-containing region of the UGT2B15 promoter spanning nucleotides -381 to -135 relative to the translation start site. As shown in Fig. 3B, we observed a ~5.5-fold enrichment of the UGT2B15 promoter in the samples precipitated from the anti-FOXA1 antibody over the IgG-precipitated controls ($p < 0.001$). By contrast, there was no enrichment of this promoter region in the samples precipitated from the anti-ER$\alpha$ antibody, used as a negative control in these experiments. There was also no binding to the negative control locus (Carroll et al., 2006). Collectively, these ChIP assays provided evidence for the in vivo binding of FOXA1 to the UGT2B15 promoter region harbouring the FOXA1 site in LNCaP cells.

The involvement of FOXA1 in UGT2B15 gene transcription was further investigated by siRNA silencing technologies. We transfected siRNAs targeting FOXA1 mRNA into LNCaP cells and subsequently measured the mRNA levels of target genes using quantitative real-time RT-PCR. As shown in Fig. 3C, transfection of LNCaP cells with anti-FOXA1 siRNA significantly decreased the levels of endogenous FOXA1 transcripts to 48% of that in control
cells treated with non-targeting siRNAs ($p < 0.01$). This reduction of FOXA1 mRNA resulted in an approximately 3-fold decrease in $UGT2B15$ mRNA levels ($p < 0.001$), whereas there was no significant effect on the mRNA levels of the negative control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Taken together, these siRNA silencing experiments strongly suggest that FOXA1 has a positive regulatory role in $UGT2B15$ gene transcription in LNCaP cells.

In summary, the present study identifies a functional FOXA1 site within the proximal $UGT2B15$ promoter that is important for basal $UGT2B15$ gene transcriptional activity in LNCaP cells. Although differing by one base to the equivalent site in the $UGT2B17$ gene, the $UGT2B15$ FOXA1 site is closer in sequence to the high activity $UGT2B17$ promoter allele (-155A) and thus sustains significant promoter activity. Hence, our results show for the first time that FOXA1, a pioneering transcription factor (Lupien et al., 2008), plays a crucial role in controlling basal expression of $UGT2B15$ in prostate cancer cells. Together with our previous demonstration of the importance of FOXA1 in regulating basal $UGT2B17$ gene expression (Hu et al.), these findings provide new insights into our understanding of the molecular mechanisms that determine androgen glucuronidation capacity and hence androgen homeostasis and signalling in the prostate. As UGT2B15 and UGT2B17 are the major enzymes inactivating and eliminating active androgens by glucuronidation, the control of $UGT2B15$ gene expression by FOXA1 would be of particular significance for individuals homozygous for the $UGT2B17$ deletion, where UGT2B15 is the only enzyme in this organ with the capacity to effectively glucuronidate active androgens (McCarroll et al., 2006).
References


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Legends for Figures

FIG. 1. The putative FOXA1 site is highly conserved between the *UGT2B15* and *UGT2B17* promoters. Shown are the sequences of the *UGT2B15* and *UGT2B17* promoter regions containing the putative FOXA1 site. Of note, there is a single C/T (bold) base difference within the FOXA1 site between these two promoters and a -155A/G SNP (underlined) within this site in the *UGT2B17* promoter. The sequences of the promoter regions are numbered on the left relative to the translation start sites of the respective genes.

FIG. 2. Luciferase analyses of successively deleted or mutated *UGT2B15* promoter reporter constructs identify a functional FOXA site that is required for basal promoter activity in LNCaP cells. A series of luciferase reporter constructs (Luc) carrying varying lengths of the *UGT2B15* proximal promoter is shown on the left. For better comparisons, the data from the two mutated constructs 2B15-253/FOXA1-MT/-3Luc and 2B15-458/FOXA1-MT/-3Luc, both of which contained a mutated FOXA site, were plotted next to their respective unmutated constructs. LNCaP cells were plated into 24-well plates in RPMI medium supplemented with 5% FBS. When culture reached 50-60% confluence, cells were transfected with 500 ng of each of the indicated reporter constructs and 25 ng of pRL-null plasmid as an internal control for normalizing transfection efficiency. Forty-eight hours post-transfection, cells were lysed and assayed for firefly and Renilla luciferase reporter gene activities as described under Materials and Methods. The luciferase activities of the *UGT2B15* promoter constructs are expressed as the mean firefly/Renilla luciferase ratio relative to that of the control pGL3-basic vector (Luc), which was
arbitrarily set at a value of 1. Data shown are representative experiments performed in triplicate with error bars representing 1 S.D. **p < 0.01 and ***p < 0.001.

FIG. 3A. FOXA1 binds to the UGT2B15 FOXA1 site both in vitro and in vivo and positively regulates UGT2B15 gene transcription in LNCaP cells. 3A: EMSA assays for in vitro binding, experiments were performed using 1 μl of in vitro transcribed/translated recombinant FOXA1 proteins and 50,000 cpm (~1 ng) of the indicated 32P-labeled probes as described under Materials and Methods. In supershift assays, 2 μg of anti-FOXA1 antibody (lane 4) were added. For competition assays, unlabelled probes (cold) were added at a 100-fold (lane 3) molar excess of labeled probe before the addition of labeled probe. Shown are the specific FOXA1-DNA retarded complexes (A), the FOXA1 antibody supershifted complexes (SS), the non-specifically retarded complexes (B). The complexes present in the negative control (lane 1) were non-specific and resulted from unknown proteins present in the rabbit reticulocyte lysate from the TNT Quick Coupled Transcription/Translation kit. 3B. ChIP assays for in vivo binding, cells cultured in RPMI medium supplemented with 5% FBS were cross-linked by 1% formaldeyde and subsequently subjected to chromatin immunoprecipitation assays by antibodies against FOXA1 or ERα, or by the rabbit preimmune IgG control. Quantitative real-time PCR was then used to measure the precipitated DNA of target regions as described under Materials and Methods. Data were expressed as the-fold enrichment in DNA samples precipitated with 10 μg of each of the indicated antibodies compared to that (set as a value of 1) in the control samples, which were precipitated from equivalent amounts of the rabbit preimmune IgG control. Data plotted are from a representative experiment performed in triplicate, the error bars representing 1 S.D. *** p < 0.001. 3C. Knockdown of FOXA1 mRNA levels by siRNA is associated with reduced UGT2B15 gene expression in LNCaP cells. LNCaP cells were transfected with either FOXA1 siRNAs or
non-target siRNAs in RPMI-medium supplemented with 5% FBS. Sixty-eight hours after transfection, cells were harvested, and target mRNAs were measured by quantitative real-time RT-PCR as described under Materials and Methods. After normalizing to 18S rRNA, the relative mRNA levels of target genes in cells transfected with FOXA1 siRNAs were expressed as a percentage of that (set as a value of 100%) in control cells treated with non-target siRNA. Experiments were performed in triplicate and repeated at least twice in independent experiments. Data plotted are representative experiments in triplicate with error bars representing 1 S.D. **p < 0.01.
Figure 1

| UGT2B15   | -236 TTGC -TTGACTAGAGTAATTGTAAACATAAAAGGAAACCCAAACA |
| UGT2B17-A | -177 TTGCCCTTGACTAGAGTAATTGTAAATATAAAGAAACCCAAACA    |
| UGT2B17-G | -177 TTGCCCTTGACTAGAGTAATTGTAAATATAAAGAAACCCAAACA    |

FOXA1
Figure 2

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Figure 3

A

32p-probe  2B15-FOXA1  2B15-FOXA1-MT2

100x probe (cold)
Anti-FOXA1 antibody
FOXA1 proteins

Free probe

B

C

Effects on mRNA levels by siRNA