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Anterior gradient 2 and *3* – two prototype androgen-responsive genes transcriptionally upregulated by androgens and by oestrogens in prostate cancer cells

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Androgens and oestrogens have been implicated in prostatic carcinogenesis and tumour progression. Although the actions of androgens have been studied extensively, the mechanisms underlying oestrogen signalling in prostate cancer are not fully understood. In the present study, we analyzed the effect of androgens and oestrogens on the expression of *anterior gradient 2* (*AGR2*) and *anterior gradient 3* (*AGR3*), comprising two highly-related genes encoding secretory proteins that are expressed in prostate cancer and one of which (*AGR2*) has been associated with tumour metastasis. Quantitative reverse-transcriptase PCR and western blot analysis showed androgen induction of AGR2 and AGR3 in three androgen receptor positive cell lines, starting at concentrations of 0.1 nM. Both AGR genes were also transcriptionally activated by $\geq$ 5 nM oestradiol but not by isotype selective or nonselective oestrogen receptor agonists in DUCaP cells that harbour a high-level of wild-type androgen receptor. A functional androgen receptor but not oestrogen receptor turned out to be required for both androgen and oestrogen regulation. This pattern of androgen and oestrogen regulation was confirmed in VCaP cells and was also observed for *FKBP5*, a well-characterized androgen-regulated gene. Genome-wide chromatin-immunoprecipitation studies coupled with deep sequencing identified androgen receptor binding sites localized in the distal promoter and intron regions of the *AGR2* and *AGR3* genes, respectively. The androgen responsiveness of these enhancers was verified by luciferase reporter gene assays and site-directed mutagenesis analysis. Androgen treatment also induced p300 and RNA Pol II recruitment to androgen receptor enhancers of *AGR2* and initiated local chromatin remodelling and the formation of RNA Pol II-containing androgen receptor transcription complexes.

Abbreviations
AGR2, anterior gradient 2; AGR3, anterior gradient 3; AR, androgen receptor; ARE, androgen-responsive element; ChIP, chromatin-immunoprecipitation; ChIP-seq, ChIP-coupled sequencing; ChIP-qPCR, ChIP-quantitative real-time PCR; E_2, 17ß-oestradiol; ER, oestrogen receptor; ICI, ICI 182 780; PCa, prostate cancer; PSA, prostate-specific antigen; qRT-PCR, quantitative reverse transcriptase-PCR; siRNA, small interfering RNA; TSS, transcriptional start site.

Introduction

Androgen receptor (AR) signalling plays an important role in the development and overall function of normal prostate tissue, and promotes the growth and survival of prostate cancer (PCa) cells [1]. Therefore, treatment of locally advanced, relapsed or metastasized PCa is based on inhibiting AR transcriptional activity, primarily through deprivation of androgen by chemical castration or through antiandrogens. Despite initial good responses being achieved in most patients, they eventually develop castration-resistant prostate tumours, in most cases retaining active but dysregulated AR signalling [2]. Understanding the downstream effector molecules that are regulated directly or indirectly by AR is thus important for unravelling the molecular mechanisms of AR signalling in PCa development and progression, as well as for providing therapy targets or disease biomarkers.

The targets of AR include those essential for the secretion function of the prostate, such as prostate-specific antigen (PSA) and KLK2, which are also the best-studied androgen-regulated proteins [3,4]. Androgens also regulate those molecules involved in proliferation and survival pathways that are crucial for tumour development, such as cyclin-dependant kinases 2 and 4, P16 and caspase 2 [5,6]. One of the most significant recent advances in the understanding of the function of androgen in promoting PCa development is the identification of the recurrent chromosomal translocations that result in the androgen-driven expression of fusion ETS (E-twenty six transformation specific) transcription factors such as ERG [7].

Intriguingly, several known androgen-responsive genes, such as the fusion gene for *TMPRSS2-ERG*, as well as the genes for *PSA, KLK2* and *NKX3.1*, were also reported to be oestrogen-regulated either through the oestrogen receptor (ER) [8] or through promiscuous activation of mutated AR [9]. Anterior gradient 2 (AGR2) is one such protein whose expression has been reported to be regulated by both androgens and oestrogens [10–12]. AGR2 is a protein disulfide isomerase that is essential for the isomerization and maturation of the intestinal mucin MUC2 [13]. It is overexpressed in a variety of tumours, including prostate [10,12, 14,15] and breast tumours [11], and is associated with prognosis [15]. AGR2 was found to be induced in oestrogen-treated normal breast tissue [16] and upregulated by oestradiol in MCF-7 and ZR-75-1 breast cancer cells [11,15]. In the PCa cell line LNCaP, it is upregulated by androgens and oestrogens, although less efficiently by the latter steroid hormone [12]. The AGR2 transcript was found to be elevated in urine sediment samples of PCa patients [10]. Its overexpression in PCa cells leads not only to cell cycle arrest and inhibition of growth but also to an enhancement of migration [10]. The tumourigenic function of AGR2 is associated with cell growth, survival and metastases [11,15,17], as reviewed recently [18].

Compared to AGR2, much less is known about anterior gradient 3 (AGR3), a protein with 71% sequence similarity to AGR2, for which the gene is located close to the *AGR2* gene on chromosome 7p21 [17]. Similar to AGR2, it also has a predicted protein disulfide isomerase function [19]. AGR3 was initially identified in breast cancer cell membranes [20] and found to be associated with ER positive breast tumours [17]. It is also upregulated in some ovarian cancers and was suggested as a tumour marker for that cancer type [21, 22]. Little is known about the hormone regulation of this protein in human tumours.

In the present study, we analyzed the steroid hormone regulation of AGR2 and AGR3 in PCa cells, especially their regulation via androgens and oestrogens. The main finding of the present study was the characterization of both AGR genes as androgen-responsive genes. AR binding sites within both genes were identified using chromatin-immunoprecipitation-coupled sequencing (ChIP-seq). The androgen responsiveness of these sequences was verified by luciferase reporter gene assays and site-directed mutagenesis analysis. Androgen induced p300 and RNA Pol II recruitment to the enhancers of AGR2, indicating local chromatin remodelling, namely the formation of RNA Pol II-containing AR transcriptional complexes bridging distal enhancers to the proximal promoter, which is a model of androgen regulation that is also seen in the gene for PSA [3]. In addition to androgen regulation, 17ß-oestradiol (E_2) turned out to be the only oestrogen among several isotype selective or nonselective agonists that mediates the induction of the AGR genes and the results of the present study show that this occurs via promiscuous activation of the wild-type AR.

Results

Androgen regulation of AGR2 and AGR3 expression in PCa cells

Androgen treatment of PCa cell lines 22Rv1, LNCaP and DUCaP confirmed the androgen regulation of AGR2 expression (Fig. 1A) [10,12]. To investigate whether its close homologue AGR3 is also androgen-responsive, these AR^+ PCa cell lines were incubated

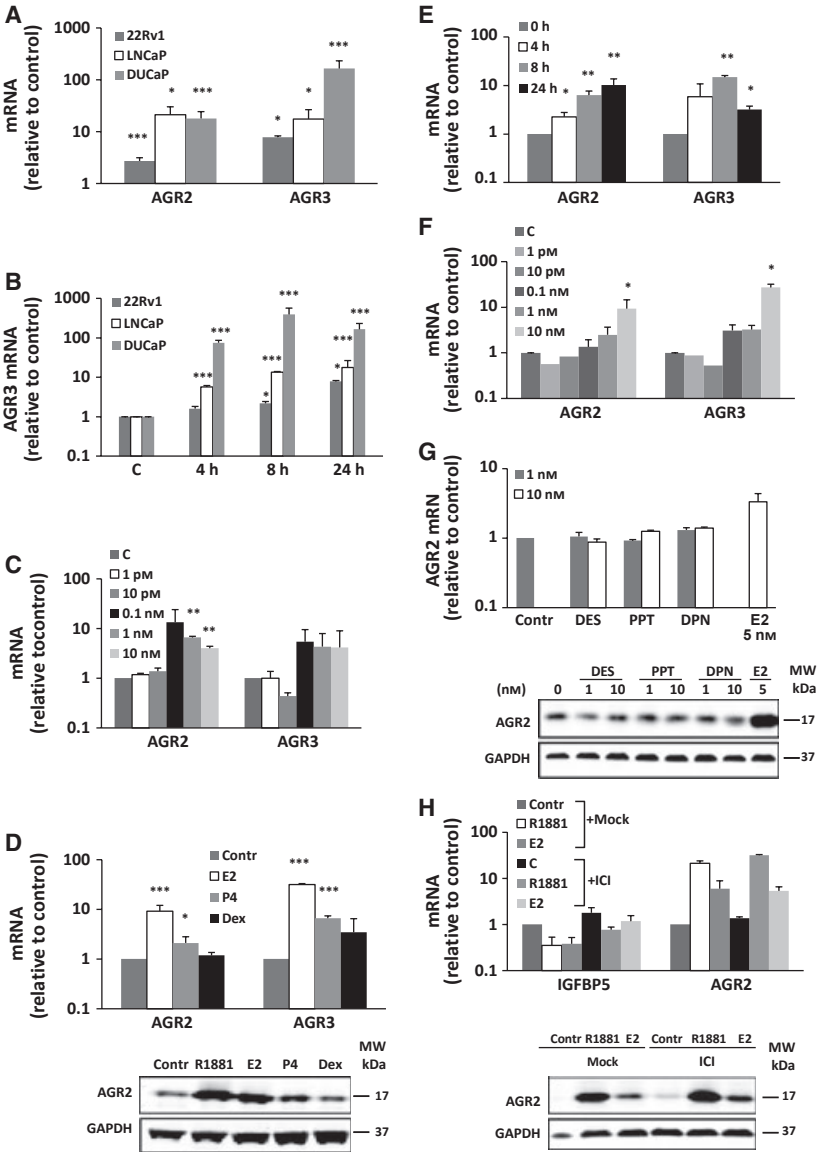

Fig. 1. Androgen and oestrogen regulation of AGR2 and AGR3 expression in PCa cells. Upregulation of AGR2 and AGR3 expression by androgens and other steroid hormones was measured in AR positive PCa cell lines by real-time PCR and western blotting. (A) Prostate cancer cells 22Rv1, LNCaP and DUCaP were treated with the synthetic androgen R1881 (1 nM) for 24 h or (B) for the times indicated and the induction of AGR2 and AGR3 mRNAs was determined by real-time PCR. (C) The androgen dose–response on AGR2 and AGR3 transcript levels was measured in DUCaP cells after 24 h of incubation with the indicated doses of R1881. (D) Effect of 10 nM concentrations of the non-androgen steroid hormones 17β-oestradiol (E_2), progesterone (P4) and dexamethasone (Dex) on the expression of AGR2 and AGR3 mRNA and AGR2 protein levels in DUCaP cells. (E) Time course and (F) concentration dependence regulation of AGR genes by E_2 in DUCaP cells. (G) AGR2 transcript and protein levels were measured in DUCaP cells after 24 h of treatment with the natural ER agonist E_2, the nonselective synthetic ER agonist diethylstilbestrol (DES), the ERα subtype-specific agonist 1,3,5-tris(4-hydrox-phenyl)-4-propyl-1H-pyrazole (PPT) and the ERβ subtype-specific agonist 2,3-bis(4-hydroxyphenyl)propionitrile (DPN). (H) AGR2 transcript and protein levels were determined after 24 h of incubation with the ER antagonist ICI alone (control, C) or together with E_2 (10 nM) or R1881 (1 nM) or with the hormones alone. The mRNA of the oestrogen repressed gene *IGFBP5* served as a control for the inhibitory effect of the ER antagonist ICI. AGR2 and AGR3 mRNAs were determined by qRT-PCR using the primers listed in Table 2. The house-keeping gene *HPRT1* transcript served as an internal control for mRNA quantification. GAPDH was used as a loading control for western blotting protein analysis. Histograms represent the mean ± SD transcript levels of two or three independent experiments. Western blots are representative of two or three independent experiments. Statistical differences were calculated using Student's *t*-test. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

with synthetic androgen R1881 for 24 h (Fig. 1A). AGR3 was significantly upregulated after treatment in all three cell lines, showing 8-, 18- and 165-fold induction in 22Rv1, LNCaP and DUCaP, respectively (Fig. 1A). Despite strong androgen-mediated induction, the basal expression of AGR3 is low (approximately 1000-fold lower than AGR2). In comparison, the relative basal AGR2 and AGR3 mRNA expression levels are listed in Table 1 for a panel of five AR positive PCa cell lines.

The temporal dynamics of AGR3 induction was examined in time course assays with 1 nM R1881 treatment. Induced AGR3 expression was observed in all three cell lines upon 4 h of androgen treatment (Fig. 1B). In 22Rv1 and LNCaP cells, AGR3 induction reached peak levels after 24 h (8- and 18-fold induction, respectively; Fig. 1B). By contrast, AGR3 mRNA levels peaked after 8 h of hormone treatment in DUCaP cells (393-fold induction) with a sustained elevated expression thereafter (165-fold at 24 h; Fig. 1B).

By contrast to LNCaP cells that express a promiscuous, mutated AR, DUCaP cells express wild-type AR at a high level [23], and both AGR2 and AGR3 were rapidly and robustly upregulated by androgen in these cells. To focus on regulation by wild-type AR, DUCaP cells were predominantly employed in subsequent experiments. Of note, this cell line was isolated from a dura metastasis of a PCa patient and represents tumours harbouring the fusion gene for *TMPRSS2-ERG*, which is a gene rearrangement found in approximately 50% of all prostate tumours. In dose–response assays, the highest induction of both AGR2 and AGR3 expression was observed at concentrations as low as 0.1 nM R1881 (13- and 5-fold, respectively; Fig. 1C); higher concentrations of 1 and 10 nM showed no further increase. This confirmed the androgen responsiveness of both *AGR* genes, although the fold induction was lower compared to the dynamics experiment. This probably reflects some variance of cell culture conditions or the low basal level of AGR3 expression. Collectively, these data indicate that AGR2 and AGR3 are rapidly upregulated in response to androgen, suggesting that both are primary AR targets.

Table 1. Basal level of AGR2 and 3 mRNA expressions in different PCa cell lines normalized to the housekeeping gene *TBP*.

Cell line	AGR2 (mean ± SD)	AGR3 (mean ± SD)
22Rv1	0.41 ± 0.07	0.0011 ± 0.0002
LNCaP	1.48 ± 0.36	0.0011 ± 0.0005
DUCaP	16.56 ± 3.83	0.0083 ± 0.0033
VCaP	55.79 ± 19.50	0.1435 ± 0.0114
LAPC-4	0.49 ± 0.10	0.0016 ± 0.0007

Table 2. Real-time PCR primer sequences.

Gene	Sequence (5′- to 3′)	Location	Product size (bp)
AGR2	F: CGACTCACACA AGGCAGGT R: GCAAGAATGCTG ACACTGGA	Spans exons 1 and 2	73
AGR3	F: GCTTTGGGTCTC TGCCTCTTAC R: TTGACAATCCTC CAGGTGATGA	Spans exons 2 and 4	201
HPRT1	F: TGACACTGGCA AAACAATGCA R: GGTCCTTTTCAC CAGCAAGCT	Spans exons 6 and 7	94
TBP	F: CACGAACCACG GCACTGATT R: TTTTCTTGCTGC CAGTCTGGAC Probe: Fam-TCT TCA CTC TTG GCT CCT GTG CAC A-Tamra	Spans exons 4 and 5	89

Oestrogen regulation of AGR2 and AGR3 in PCa cells

AGR2 and AGR3 expression has been associated with ER positive breast cancer [17]. We therefore investigated whether AGR2 and AGR3 are regulated by oestrogen and other steroid hormones in DUCaP cells, which also express very low α and higher β isoforms of the ER (data not shown). Both AGR2 and AGR3 were significantly upregulated at the mRNA level by 10 nM E_2 (9- and 31.5-fold, respectively) and, to a lower extent, also by progesterone (two- and sevenfold) but not by the synthetic glucocorticoid dexamethasone (10 nM; Fig. 1D). A similar finding was observed for AGR2 at the protein level (Fig. 1D).

Similar to the rapid induction of AGR2 and AGR3 expression in response to androgen (Fig. 1B), AGR2 and AGR3 mRNA levels were induced after 4 h of E_2 treatment with an induction of two- and six-fold, respectively (Fig. 1E). However, although 0.1 nM R1881 was sufficient to dramatically upregulate AGR2 and AGR3 expression (Fig. 1C), a 100-fold higher concentration of E_2 (10 nM) was required to induce the expression of AGR2 and AGR3 (11- and 27-fold respectively; Fig. 1F).

To determine which ER isotype(s) were responsible for E_2-mediated induction of AGR2 and AGR3, DUCaP cells were incubated with isotype-specific or nonselective synthetic ER agonists (Fig. 1G). Although the natural ER agonist E_2 strongly induced AGR2 expression at both the mRNA and protein levels,

neither ER isotype selective (1,3,5-tris(4-hydrox-phenyl)-4-propyl-1H-pyrazole as ERα agonist and 2,3-bis (4-hydroxyphenyl)propionitrile as ERβ agonist), nor a nonselective synthetic agonist (diethylstilbestrol), modulated the expression of AGR2 (Fig. 1G). Moreover, the nonselective ER antagonist ICI 182,780 (ICI) could not attenuate E_2-mediated expression of AGR2 at both mRNA and protein levels (Fig. 1H), although this drug effectively blocked the effect of E_2 on the known oestrogen-repressed gene for insulin-like growth factor-binding protein 5 (*IGFBP5*) [24]. Taken together, these data suggest that E_2-mediated upregulation of AGR2 and AGR3 may be ER-independent.

AR-dependent androgen and oestrogen regulation of AGR2 and AGR3

We hypothesized that E_2-mediated expression of AGR2 and AGR3 may occur via promiscuous activation of wild-type AR rather than ER activation. To investigate this possibility, small interfering RNA (siRNA)-mediated knockdown of AR and AR inhibition by the antiandrogens flutamide and bicalutamide was employed. AR siRNA dramatically reduced AR protein levels as expected (Fig. 2A). Following AR knockdown, androgen induction of AGR2 and AGR3 mRNAs was attenuated and androgen induction of AGR2 protein was attenuated in DUCaP cells (Fig. 2A). Similar findings were observed in LNCaP cells (data not shown). Furthermore, the antiandrogen flutamide attenuated the androgen induction of AGR2 protein, as well as AGR2 and AGR3 mRNAs, in a dose-dependent manner in DUCaP cells (Fig. 2B). Interestingly, androgen stimulation also significantly decreased AR expression (Fig. 2A), which is consistent with the findings of another study performed in VCaP cells that showed downregulation of AR expression by androgens through histone modification [25]. AR knockdown or inhibition of AR transcriptional activity by an antiandrogen also attenuated the E_2-mediated expression of AGR2 (Fig. 2C,D), supporting the hypothesis that E_2-induced expression is also mediated via the AR. In the LNCaP cell line harbouring a mutant, promiscuous AR, oestrogen activation of AR has been described [26]. To exclude this possibility in DUCaP cells, their AR was re-sequenced and a wild-type sequence was confirmed.

To exclude the possibility that oestrogen induction via the AR is a cell type-specific phenomenon, we also investigated androgen and E_2 induction of AGR2 and AGR3 in wild-type AR expressing VCaP cells. This also allowed an analysis of AGR3 protein regulation because VCaP was the only cell line expressing significant amounts of AGR3 protein among a panel of six PCa cell lines (Fig. 2E). Both AGR2 and AGR3 mRNAs were induced by androgen and E_2 and induction was blocked by the simultaneous addition of an AR-blocking antiandrogen in VCaP cells (Fig. 2F). The antiandrogen decreased androgen stimulation of AGR3 mRNA from 6.8- to 1.7-fold and abolished oestrogen-induced expression from 2.6- to 1.0-fold. The AGR3 protein levels showed the same pattern (Fig. 2F).

The observed steroid hormone regulation pattern is not restricted to AGR2 and AGR3. The well characterized androgen-regulated gene *FKBP5* [27] displayed the same induction by androgen and oestrogen in both cell lines DUCaP and VCaP, and an antiandrogen significantly blocked induction by both steroid hormones (Fig. 3A).

DUCaP cells express a very high level of endogenous AR. To investigate whether this high AR level is required for E_2 regulation, the AR negative PC-3 PCa cell line was transfected with an androgen-driven luciferase reporter vector and increasing amounts of a wild-type AR expression vector. A significant induction of reporter gene activity was observed in cells transfected with a low amount (0.01 μg) of AR and stimulated with 1 nM R1881 (1.8-fold, $P = 0.02$; Fig. 3B). E_2 also significantly induced reporter gene activity but only in cells treated with $\geq$ 5 nM E_2 and with high exogenous AR expression (0.25 μg of AR expression vector; induction of 1.6- and 2.4-fold for 5 and 10 nM E_2 treatment, respectively). The AR level required for E_2 induction is similar to the level found in DUCaP cells and much higher that in LNCaP cells (Fig. 3B). Collectively, these results suggest that a high expression level of endogenous wild-type AR in DUCaP cells is responsible for E_2-mediated stimulation of the expression of AGRs and other AR responsive genes.

Identification of AR regulatory sequences within AGR2 and AGR3 genomic regions

AGR2 and AGR3 are rapidly induced (within 4 h) by R1881 in DUCaP cells, characterizing their respective genes as immediate–early androgen-responsive. To identify putative AR-binding sites and thus potential androgen-responsive elements (AREs) within the promoter regions of *AGR2* and *AGR3*, DUCaP cells were treated for 1 h with 1 nM R1881 and subjected to ChIP-seq as an unbiased approach aiming to locate all AR binding sites within the DUCaP genome. This approach identified > 40 000 putative AR-binding sites which were then annotated to the nearest genes. Prominent binding sites within several classic primary

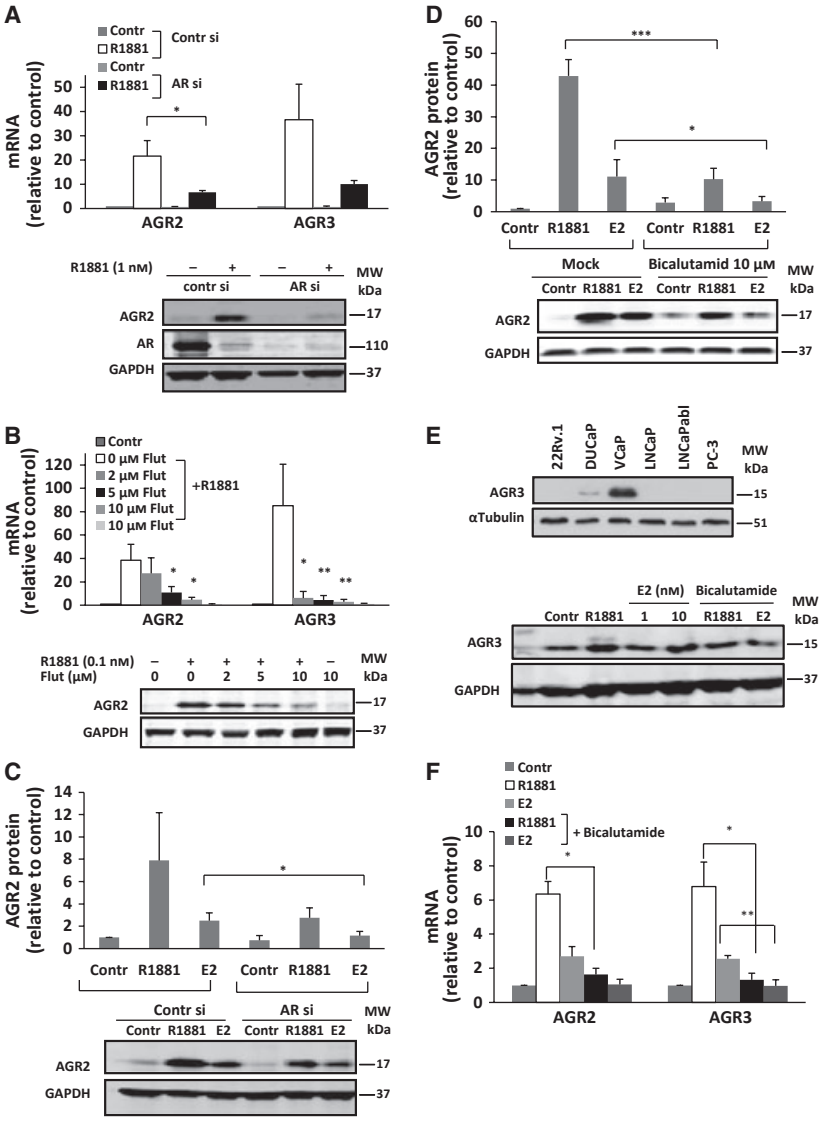

Fig. 2. Androgen and oestrogen regulation of AGR2 and AGR3 is mediated through the AR. AR function for hormone regulation of AGR2 and AGR3 was investigated in DUCaP and VCaP cells using AR knockdown by siRNA (AR si) treatment or inhibition of AR transcriptional activity with the antiandrogens flutamide (Flut) or bicalutamide. Antiandrogens were added 1 h before hormone stimulation. SiRNA knockdown of AR was initiated 2 days before the start of hormone treatment and luciferase siRNA (Contr si) served as a control. (A–D) Androgen receptor function was blocked by siRNA knockdown (A, C) or antiandrogen treatment (B, D) followed by treatment with androgen (R1881, 1 or 0.1 nM; A, B) or with oestrogen or androgen (E_2, 10 nM; R1881, 1 nM; C, D) for 24 h in DUCaP cells. Subsequently, cells were harvested and AGR2 and AGR3 transcripts and AGR2 protein levels were analyzed using qRT-PCR and western blotting, respectively. The house-keeping gene *HPRT1* transcript served as an internal control for mRNA quantification. GAPDH was used as a loading control for western blot protein analysis. (E) AGR3 expression in different PCa cell lines was analysed by western blotting and α-tubulin was used as a loading control. (F) Androgen and oestrogen-regulated AGR3 protein and mRNA expression were analysed in VCaP cells. The cells were stimulated by either 0.1 nM R1881 or 10 nM E_2 together with (or without) antiandrogen (bicalutamide) pre-treatment. *TBP* transcript served as an internal control for mRNA quantification. GAPDH was used as a loading control for western blotting analysis. Values in diagrams represent the mean ± SD of at least two independent experiments. Western blots are representative images. Statistical differences were calculated using Student's *t*-test. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

androgen-responsive genes (e.g. the genes for *PSA, KLK2, FKBP5* and *NKX3.1*) confirmed that this approach was successful.

As hypothesized, putative AR-binding sites were identified in the *AGR2* and *AGR3* gene regions. Four putative AR-binding sites were found in the distal

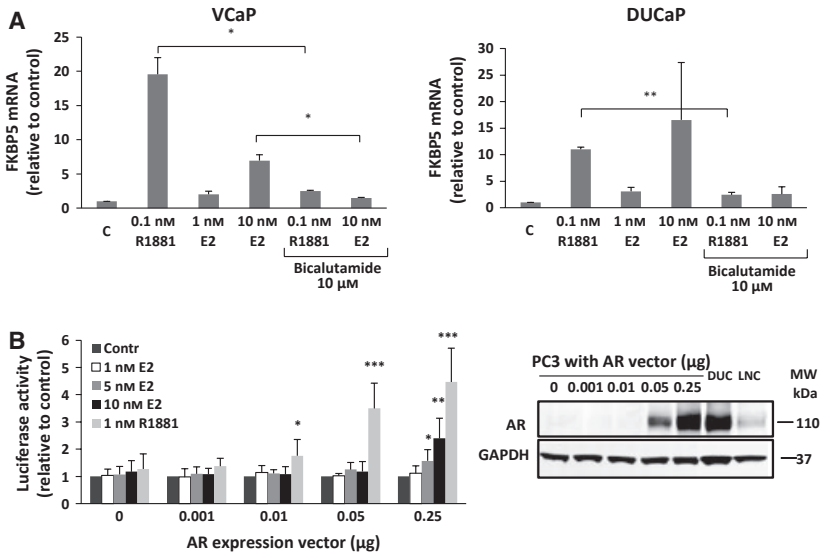

Fig. 3. AR-mediated oestrogen regulation of *FKBP5* and androgen-responsive reporter gene expression in PCa cells with a high level of AR protein. (A) Androgen and oestrogen regulation of FKBP5 was analysed in VCaP and DUCaP cells. Cells were pre-treated with antiandrogen (bicalutamide) or mock control followed by stimulation with steroid hormones for 24 h for VCaP cells or 8 h for DUCaP cells. Subsequently, cells were harvested for mRNA quantification using real-time PCR. *TBP* transcript served as an an internal control for mRNA quantification. (B) AR-negative PC-3 PCa cells were grown in 24-well plates and transfected with the indicated amounts of the AR expression vector PSG5-AR together with 0.25 μg of the androgen-responsive luciferase reporter vector ARE2-PGL3B. SV40 enhancer/promoter driven renilla luciferase reporter vector PGL4.73 was used as a transfection control. PSG5 vector without an insert served as a filling vector to adjust for equal amounts of plasmid DNA. One day after transfection, the cells were treated with the indicated concentrations of E_2 or R1881 for 24 h and subsequently processed for dual luciferase assays. The firefly luciferase activity was normalized to renilla luciferase activity and the regulation of hormone-treated samples was calculated in relation to the solvent control (Contr)-treated samples. Values represent the mean ± SD of 2–4 independent experiments. Western blots are representative images of two independent experiments. Statistical differences were calculated using Student's *t*-test. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

promoter region of *AGR2* located 11, 5.5, 8 and 17 kb upstream of the transcriptional start site (TSS) and designated *AGR2* S1–S4, respectively, according to the signal intensity of the ChIP-seq (Fig. 4A). Unlike the multiple AR binding sites of *AGR2*, only one strong AR binding site was identified in *AGR3* and designated *AGR3* S1. It localized to the first intron 1 kb downstream of the TSS (Fig. 4B). ChIP-PCR with independent samples confirmed that *AGR2* S1–S4 and *AGR3* S1 represented bona fide AR-binding loci (Fig. 4C). Consistent with hormone stimulation experiments, E_2 treatment also induced AR loading onto all *AGR2* and *AGR3* enhancers, as well as onto the well-defined enhancer for the gene for *PSA*, which was included as a control (Fig. 4C).

To investigate the dynamics over time of AR recruitment to *AGR2* and *AGR3* enhancers, DUCaP cells were treated for different durations with 1 nM R1881 and subsequently subjected to ChIP-quantitative real-time PCR (ChIP-qPCR). AR recruitment to all five loci was rapid (5–10 min), reaching peak levels after approximately 1 h of androgen stimulation and declined after 16 h of treatment (Fig. 4D). Of the four AR-binding sites in *AGR2*, *AGR2* S1 and S2 demonstrated the highest enrichment by ChIP-qPCR (97- and 98-fold, respectively), which is consistent with their high signal intensities in ChIP-seq analysis (Fig. 4A). Enrichment of *AGR3* S1 (28-fold) was lower than that of *AGR2* S1 and S2 and only comparable to enrichment of *AGR2* S3 (20-fold). *AGR2* S4, which exhibited the lowest signal intensities by ChIP-seq, also showed the lowest enrichment of AR (six-fold).

AR-binding sites in *AGR2* and *AGR3* contain functional androgen-responsive elements

We next investigated whether the AR-binding sites within *AGR2* and *AGR3* contain functional AREs. Accordingly, *AGR2* S1–S4 and *AGR3* S1 sequences were inserted into a luciferase reporter vector upstream of an SV40 minimal promoter. Relative to an empty vector control, *AGR2* S1–S4 or *AGR3* S1 had no significant effect on luciferase activity in the absence of androgens but, in the presence of 1 nM R1881, a 1.6–31-fold increase in luciferase activity was observed (Fig. 5A).

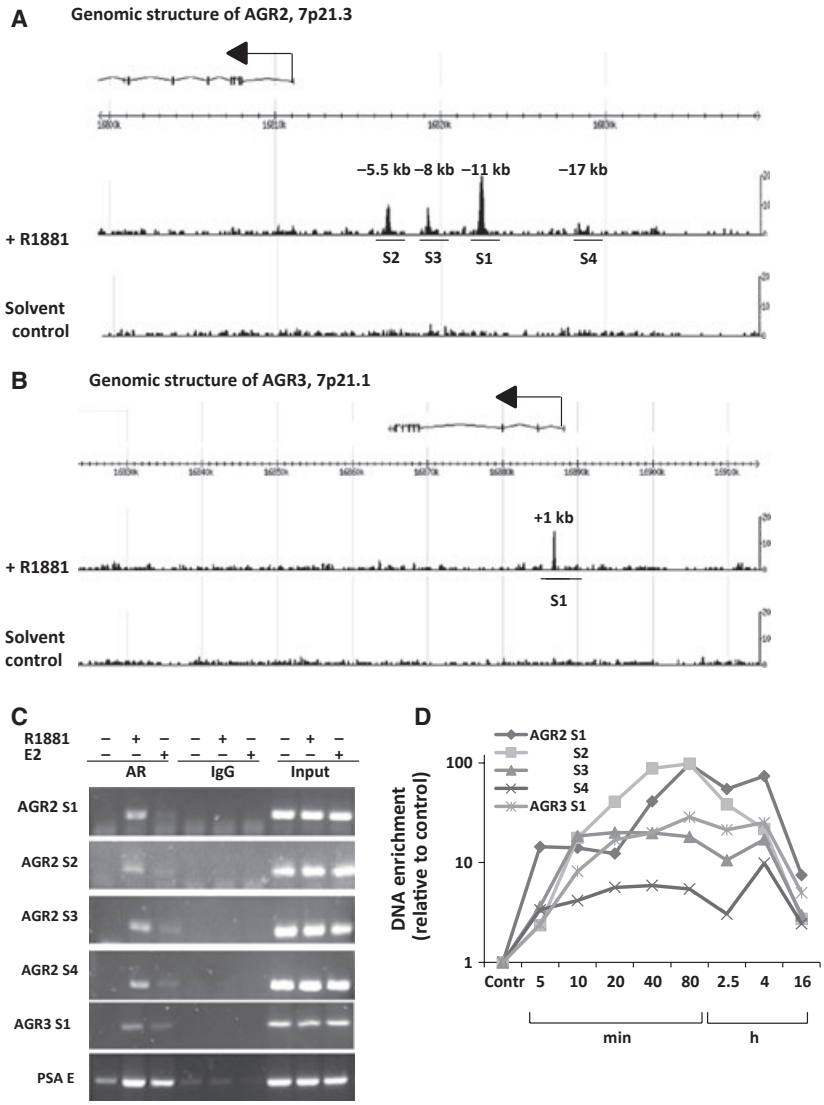

Fig. 4. Identification and verification of AR regulatory elements in *AGR2* and *AGR3* genes. AR binding sites within the *AGR2* (A) and *AGR3* (B) genes were identified by ChIP and subsequent sequencing of isolated DNA fragments. The panels from top to bottom represent (1) a scheme of the *AGR2* (A) or *AGR3* (B), gene regions on chromosome 7 (7p21.3 and 7p21.1, respectively); (2) genomic sequence frequencies of androgen stimulated anti-AR immunoprecipitated; and (3) control solvent-treated anti-AR immunoprecipitated DNAs. Genomic locations are indicated on the *x*-axis and genomic sequence frequencies are shown on the *y*-axis. Four AR binding sites upstream of the AGR2 transcription start site were designated as *AGR2* S1–S4 and one AR binding site of *AGR3* was designated as *AGR3* S1. (C, D) PCR and qPCR with AR-precipitated samples were used to confirm the AR recruitment to ChIP-seq identified *AGR2* and *AGR3* binding sites. DUCaP cells were stimulated with 1 nM R1881, 10 nM E_2 or control solvent (C) for 1 h or treated with 1 nM R1881 for the duration axis in (D). Subsequently, cross-linked chromatin was immunoprecipitated with an anti-AR antibody or an isotype IgG control antibody. The precipitated DNAs were then subjected to either normal PCR amplification followed by electrophoresis in 2% agarose gel and visualization of the PCR product by SYBR Green staining (C) or to qPCR for quantification of androgen stimulated AR enrichment to the binding sites relative to control solvent-treated samples (D). The input was the total genomic DNA used for AR-ChIP and served as normalization control for the AR precipitated DNA. The *PSA* enhancer fragment located 4 kB upstream of the *PSA* transcription start site served as a positive control (C). Values in (D) represent the mean values of three or four independent experiments. The SDs are not shown for clarity, although they ranged from 1 to 51 for *AGR2* S1, 1 to 67 for *AGR2* S2, 0 to 13 for *AGR2* S3, 0 to 2 for *AGR2* S4 and 1 to 17 for *AGR3* S1.

Of the four AR binding sites within *AGR2*, *AGR2* S1 induced the highest fold change in luciferase activity (5.6-fold, $P < 0.001$) with *AGR2* S2–S4 mediating weaker responses, with an induction of 1.8-, 1.7- and 3.0-fold, respectively (Fig. 5A). *AGR3* S1 resulted in a 30-fold induction of luciferase activity ($P = 0.002$) (Fig. 5A).

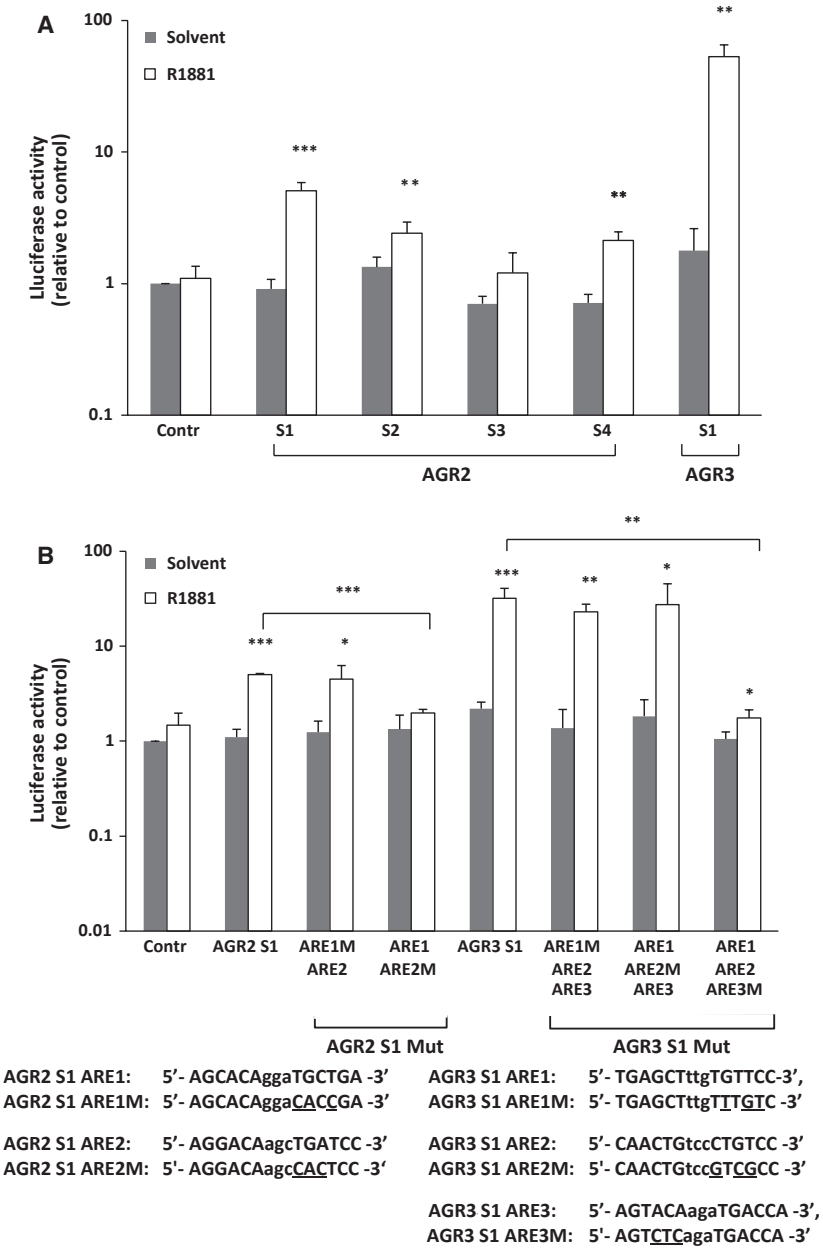

Fig. 5. Functional verification of AR regulatory sequences identified in the *AGR* genes. Identified AR-binding DNA fragments were amplified by PCR and cloned upstream of the SV40 promoter into the enhancer site of the PGL3P firefly luciferase reporter vector as described in the Experimental procedures. Putative AREs in the binding sites *AGR2* S1–S4 and *AGR3* S1 were identified by *in silico* analysis. (A) Vectors harbouring the AR binding sites *AGR2* S1–S4 and *AGR3* S1 were transfected into DUCaP cells. The renilla luciferase vector PGL4.73 served as a transfection control. PGL3P vector without an enhancer insert served as the baseline control. One day after transfection, cells were treated with either 1 nM R1881 or solvent for 24 h and were thereafter processed for dual luciferase assays. The firefly luciferase activity was normalized to the renilla luciferase transfection control activity and related to the activity of the baseline control (Contr). (B) To test the functionality of the AREs in AR enhancer sequences AGR2S1 (two putative AREs) and AGR3S1 (three putative AREs), these elements were mutated by site-directed mutagenesis and the effect on androgen regulation was assessed in the reporter gene assay. Analysis and calculations were performed identically to those in (A). Reporter gene assay values represent the mean ± SD of three or four independent experiments. Statistical differences were calculated using Student's *t*-test. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

To localize the androgen-responsive elements within these regulatory sites, the bioinformatics tools MATINSPECTOR [28] and PROMO [29] were applied. Putative AREs were found in all five AR-binding enhancers: three in *AGR3* S1, two in each of *AGR2* S1 and *AGR2* S3, and one in each of the other sequences. AREs in *AGR2* S1 and *AGR3* S1, which mediated highest androgen regulation in reporter gene analysis, were chosen for site-directed mutagenesis to verify their functional activity. In the *AGR2* S1 sequence, inactivation of ARE2 but not of ARE1 abrogated androgen-induced luciferase activity (Fig. 5B), indicating that ARE2 was responsible for androgen stimulation. Of the three AREs in the *AGR3* S1 enhancer, ARE3 is the most essential one for androgen regulation because inactivation of this ARE abolished androgen induction most significantly with an drop in induction from 14.6- to 1.7-fold.

To rule out a cell type-specific activity of the AR enhancers driving AGR2 and AGR3 expression in DUCaP cells, we confirmed their function in AR negative PC3 cells by exogenous expression of AR. In addition, we also confirmed E_2 induction at high AR protein levels under the same experimental setting.

PC3 cells were transfected with luciferase reporter vectors containing the *AGR2* S1 or *AGR3* S1 enhancers or their ARE mutated counterparts together with a low (50 ng) or a high (250 ng) amount of an AR expression vector or a control expression vector. The results obtained confirmed the function of the enhancers and the putative AREs (Fig. 6). Furthermore, although the synthetic androgen stimulated reporter gene activity at lower and higher AR protein levels, E_2 stimulated at the higher level only, thus confirming the dependence on high AR protein levels for E_2 induction. As expected, mutation of the most functional AREs of both enhancers decreased hormone induced reporter gene activities.

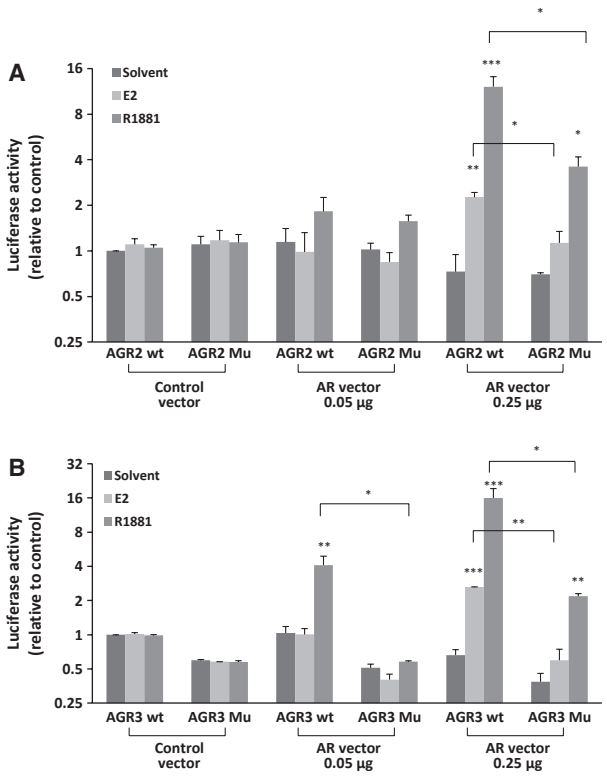

Fig. 6. AR-mediated oestrogen regulation of *AGR2* and *AGR3* enhancers. Firefly reporter vectors containing *AGR2* S1 and *AGR3* S1 sequences were transfected into PC-3 cells together with 0.05 or 0.25 µg AR expression vector to test the responsiveness of reporter vectors to androgen and oestrogen. Renilla luciferase reporter vector PGL4.73 was used as a transfection control. The PSG5 vector without an insert served as a filling DNA. (A) Wild-type *AGR2* S1 (*AGR2* wt) vector or the vector with mutation inactivated ARE2 of *AGR2* S1 fragment (*AGR2* Mu; described in the legend of Fig. 5) were used. One day after transfection, the cells were treated either with 10 nM E_2 or 1 nM R1881 for 24 h and subsequently processed for dual luciferase assays. (B) Wild-type *AGR3* S1 (*AGR3* wt) vector or the vector with mutation inactivated ARE3 of the *AGR3* S1 fragment (*AGR3* Mu; described in legend to Fig. 5) were transfected and hormone-treated as described in (A). Firefly luciferase activity was normalized to renilla luciferase activity and the regulation of hormone-treated samples was calculated in relation to the solvent control (Contr)-treated samples. Values represent mean ± SD of two or three independent experiments. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Histone modification and androgen-induced chromatin remodelling of the regulatory elements

Verification of AR-driven enhancer activity of *AGR2* S1–4 and *AGR3* S1 sequences lead us to investigate other chromatin events present at these enhancers that might cooperate with androgen regulation. Because histone H3K4Me1 has been reported as one of the few chromatin markers to be heavily enriched at cell type-specific active enhancers [30], we used the ChIP-qPCR assay to assess the presence of this marker on AGR enhancers. The *PSA* enhancer (*PSA* E) was used as a positive control (Fig. 7A). Strong histone H3K4Me1 enrichment (100–200-fold relative to an IgG precipitated sample) was seen in all four *AGR2* enhancers without androgen stimulation. This represented an enrichment that was two- to four-fold higher than that seen for the *PSA* enhancer. The *AGR3* enhancer showed only minimal enrichment (four-fold), indicating a much less active chromatin status compared to *AGR2* enhancers. Androgen treatment generally had little effect on the H3K4Me1 status of these enhancers.

Similar to the presence of H3K4Me1 at the enhancers, several genome-wide studies also showed the enrichment of transcriptional coactivator histone acetylase p300 at cell type-specific enhancers [30,31]. Because the recruitment of p300 to promoters and to enhancers was reported to coordinate AR transactivation of the genes for *PSA* and *KLK2* [32,33], we aimed to determine whether it was also the case for the regulation of AGR genes. Again, we used the ChIP-qPCR assay to assess p300 enrichment to *AGR* enhancers (Fig. 7B). P300 enrichment to the *PSA* enhancer was confirmed. After 40 min of androgen treatment, five- to six-fold higher p300 recruitment was seen at both the *AGR2* S1 and *AGR2* S2 sites. The enrichment was further increased for *AGR2* S1 and peaked at 10-fold after 4 h of treatment. The *AGR2* S3 and *AGR2* S4 sites also showed a minimum of two-fold p300 loading after 40 min or 4 h of androgen treatment. Interestingly, p300 enrichment to these *AGR2* enhancers also diminished to basal levels after 16 h of androgen treatment in parallel to AR binding (Fig. 4D). At the *AGR3* S1 enhancer, p300 showed only a minimal enrichment of 1.4-fold after 40 min and 4 h of androgen administration (Fig. 7B).

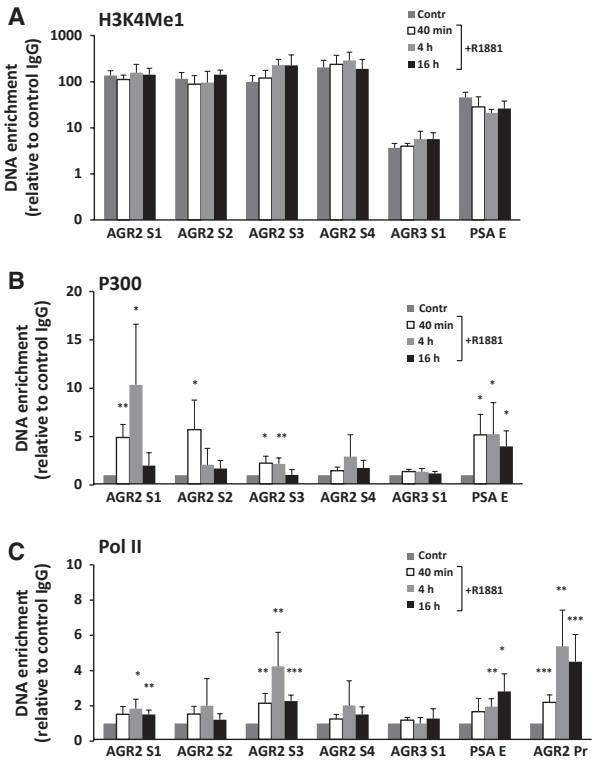

Fig. 7. AR binding sites in the *AGR* genes are located within active chromatin regions and androgen induces the recruitment of cofactors and RNA poymerase II. ChIP-qPCR was used to analyze the enrichment of the active chromatin histone marker H3K4Me1 (A), the transcriptional coactivator histone acetyl-transferase p300 (B) and RNA Pol II (C) on the AR binding sites of the *AGR2* and *AGR3* genes in cells treated with 1 nM R1881 for the time points indicated. The *PSA* 4-kb enhancer served as a positive control. The ChIP-qPCR analysis was performed as described in the legend to Fig. 3D. The enrichment of H3K4Me1 was normalized to control IgG precipitated DNA (A). Androgen stimulated p300 and RNA Pol II enrichment to the enhancers was normalized to the control solvent-treated samples (B, C). The *AGR2* proximal promoter region (*AGR2* Pr) was used as a positive control for RNA Pol II binding. The histograms represent the mean ± SD of three or four independent experiments. Statistical differences were calculated using Student's *t*-test. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

Previous studies on the *PSA* and *KLK2* promoters showed a direct looping interaction between the proximal and distal binding transcription factors in a single complex. RNA Pol II has been observed to interact with both the proximal promoter and the far upstream enhancers of genes [3,33]. To determine whether such an interaction is also important for androgen regulation of AGR2 and AGR3, RNA Pol II binding to their AR enhancers was analyzed by Pol II-ChIP and a region close to the *AGR2* proximal promoter (*AGR2* Pr, +50/+137) was used as a positive control (Fig. 7C). As expected, after 40 min of androgen treatment, there was a two-fold higher RNA Pol II recruitment to the *AGR2* proximal promoter to initiate transactivation. With time, Pol II was enriched further and remained at the proximal promoter until 16 h, when the experiment was terminated. The *AGR2* S3 enhancer was maximally loaded four-fold by RNA Pol II after 4 h of treatment. A lower level (up to two-fold) of recruitment of RNA Pol II was seen for the *AGR2* S1, S2 and S4 enhancers. Unlike the sustaining strong recruitment of RNA Pol II to the *AGR2* proximal promoter after 16 h, enrichment to the AR enhancers of *AGR2* was already diminished at this time. These results indicate that, although far away from the transcription start site, the AR enhancers in *AGR2* were brought into proximity with the proximal promoter through the formation of the transcription complex and the bridging of RNA Pol II. Interestingly, significant androgen induced Pol II recruitment could not be seen at the *AGR3* S1 enhancer site, which is just 1 kb downstream of the TSS, indicating a mechanism that differs from the formation of a looped structure.

Discussion

Previous studies have reported androgen and oestrogen regulation of AGR2 expression in prostate and breast cancer cells [10–12,15]; however, the detailed mechanism, as well as the regulation of its close homologue AGR3, remains unknown. The present study performed in AR positive cell lines consistently showed androgen regulation of AGR2 and AGR3 expression. Besides regulation by androgens, we also observed an E_2 response and established the underlying mechanism. The effect of E_2 was found not to be mediated via the ER but through AR activation at concentrations $\geq$ 5 nM in conjunction with a high AR level. This pattern of hormone activation is not unique for AGR2 and AGR3 but was also confirmed for the well-characterized androgen-regulated gene *FKBP5* in DUCaP and VCaP PCa cells. Oestrogen was previously reported to induce the expression of androgen-responsive genes, such as the genes for *PSA, KLK2* or *NKX3.1* in LNCaP cells [9,34], via the promiscuous, mutated AR-V877A that these cells harbour. Therefore, we focused on steroid hormone regulation in DUCaP and VCaP cells that both express wild-type AR [23]. Overexpression of AR coactivator ARA70 or co-stimulation with sex hormone-binding globulin has also been reported to mediate E_2-induced AR transcriptional activity [35,36]. In the present study, E_2 alone (and none of the ER agonists that we tested) could induce androgen-regulated genes in DUCaP cells. This finding is in agreement with other studies

reporting that E_2 (but not any other synthetic oestrogen agonist) can bind AR at super-physiological concentrations and is able to induce AR transcriptional activity [35].

Oestrogen induction of AGR2 was previously studied in breast cancer cells. The induction by E_2 reported for that cell type is weak compared to the robust effects observed in the PCa cell lines in the present study. In MCF-7, LCC9 and ZR-75-1 cells, E_2 induced AGR2 by two-fold or less at the mRNA or protein level [11,15] and a luciferase reporter gene construct employing the proximal −1594 to −94 bp promoter also showed only a two-fold induction of reporter gene [15]. An enhanced AGR2 induction was obtained with a combination of E_2 and the anti-oestrogen tamoxifen, raising the question of whether this is a classical ER regulation.

The findings of the present study also raise the question of whether oestrogen regulation of androgen-responsive genes via AR is a general pattern for tumour cells that express a high level of wild-type AR. The overexpression of AR alone in PC-3 cells to a level similar to that endogenously expressed in DUCaP cells was sufficient to trigger oestrogen regulation of androgen-responsive reporter gene expression, confirming the hypothesis that a high level of AR in DUCaP cells mediates oestrogen regulation. In agreement with these results, the same pattern of androgen and oestrogen regulation was confirmed in VCaP cells that also express a high level of wild-type AR. Prostate cancer cell lines with lower AR expression levels often harbour mutant promiscuous ARs that respond to oestrogens and other steroid hormones at lower concentrations, such as LNCaP or 22RV1 cells [37,38]. The results of the present study are in line with other studies reporting that increased AR expression could sensitize cells to low levels of androgen, as well as change the agonist spectrum [39,40]. There is accumulating evidence that AR is overexpressed in a vast majority of prostate tumours that relapse after hormone ablation treatment [41] and alteration of the AR pathway has been found in 56% of primary tumours and 100% of tumour metastases [42]. Because there is residual androgen in prostate tissue of patients under androgen ablation therapy [43], activation of overexpressed AR by residual androgens and promiscuous activation by other steroid ligands is considered to be one of the major therapy escape mechanisms [44]. In this context, the results of the present study suggest that oestrogens might support AR activation in this situation.

Both *AGR2* and *AGR3* were characterized as primary responsive genes of AR in PCa cells [45], providing additional models for studying the mechanism of AR regulation. In concordance with other reports showing that a majority of the AR regulatory sequences are located outside the proximity promoters [46], our ChIP-seq analysis identified several AR binding and functionally active sites of the two genes, in the distal promoter and the first intron, respectively. A rapid AR loading of all these enhancers after 5–10 min of androgen treatment was observed and, as in the case of the *PSA* and *KLK2* enhancers [33,34], the AR enrichment peaked at approximately 1 h and diminished thereafter, indicating a transient transcription complex recruitment.

In an attempt to characterize chromatin remodelling at the enhancers that coordinated androgen regulation of AGRs, active enhancer markers H3K4Me1 and p300 were chosen [30,31,47]. We found that all four *AGR2* enhancers are highly occupied with the H3K4Me1 marker, whereas the *AGR3* enhancer yielded 50-fold less enrichment. This indicates a less active chromatin status of *AGR3*, which corresponds with a much lower basal expression level of the AGR3 compared to AGR2. Androgen also induced the recruitment of p300 to the AR binding sites of *AGR2* but not to *AGR3* enhancers. p300 enhances transcription by recruitment of additional activating factors and through its acetyltransferase activity. For the gene *AGR3*, this function appears to be exerted by other histone-modifying enzymes and/or cofactors.

Despite the close proximity of the *AGR2* and *AGR3* genes on chromosome 7p21 and androgen-responsive enhancers in both genes, their expression, especially at the protein level, is distinct and variable in different PCa cell lines. AGR3 protein, for example, is expressed at a significant level only in VCaP cells and at a very low level in DUCaP cells, whereas, in all other PCa cell lines investigated, AGR3 protein was not visible in western blot analysis. By contrast, AGR2 protein was detected, although at various levels, in several PCa cell lines [10]. The relative mRNA levels parallel the protein abundance in the different cell lines. Uncoupled expression of AGR2 and AGR3 proteins was also found in different ovarian cancer cells [21]. In prostate tissue, AGR2 protein is elevated in PCa cells, especially in low grade tumours and displays a rather uniform expression pattern [10]. By contrast, the expression of AGR3 in primary prostate tumours is highly heterogeneous, with high protein levels in a small fraction of tumour cells among a majority of AGR3 negative cells (G Schäfer, ACB Cato & H Klocker unpublished results). This somehow mirrors the black and white picture of AGR3 expression seen in the cell lines.

The discordant expression pattern suggests that additional factors and cell type-specific effects modulate

the expression of AGR2 and AGR3. At the protein level, the two AGR subtypes can be aligned for approximately 94% of their sequence with a maximal identity of 65%. A bioinformatics comparison of the whole gene sequences, including 20 kb of the upstream sequence, revealed that 100% of the sequence length can be aligned with 47% sequence identity and 29% of the alignment consisting of gaps. Given these data, the relationship between AGR2 and AGR3 appears rather distant on the protein, as well as the DNA level, which suggests a potential gene duplication event far back in time. The regulatory differences between AGR2 and AGR3 as described in the present study and by Gray *et al.* [21] are not only attributable to the different numbers and locations of the active AREs, but also have to be interpreted in the light of the proximal transcription factor binding site profiles, which can work as 'docking stations' to recruit the distal co-activators. We calculated the differential profile for 5 kb upstream of both promoters using STRAP (http://www.ncbi.nlm.nih.gov/pubmed/20127973) and found more than 50 transcription factor matrices with a *P*-value difference >1 order of magnitude.

The finding that AR regulatory elements function as distal enhancers rather than proximal promoters is not unique to the *AGR2* gene, and is also seen in the androgen regulation of PSA and TMPRSS2 [3,46]. In the well studied model of the gene for *PSA*, AR binds to AREs both in the proximal promoter and in the 4-kb distal enhancer region, and both sites interact with each other through a common RNA Pol II-containing AR transcription complex [3]. This model of communication between distant AR enhancers and the proximal promoter is also seen in the present study of the *AGR2* gene, with the difference that there are four AR regulatory enhancers instead of one, and also that there is no binding site in the proximal promoter. This implies that transcription factors binding within the proximal promoter work as docking molecules to recruit the distal AR-containing coactivator complexes and RNA Pol II to assemble the transcriptional machinery on the proximal promoter. Candidate factors are the forkhead transcription factors, FoxA1-3, and Ets transcription factor, SPDEF, all of which were reported to regulate AGR2 expression [48,49]. Putative transcription factor binding sites were identified *in silico* in the proximal promoter at −46/−30 for Ets and at +8/+28 for forkhead proteins.

The direct interaction between the distal AREs and the proximal promoter involving RNA Pol II does not appear to be the only model for androgen regulation because RNA Pol II occupancy does not mirror the loading of AR in the *FKBP5* gene, where the AR enhancers are located in the fifth and sixth introns more than 60 kb downstream of the TSS [27,50]. A similar situation can be seen in AGR3 transactivation, with the functional ARE located 1 kb downstream of the TSS in the first intron. It is not known yet whether a loop structure could be applied only to genes with regulatory elements upstream of the TSS. Further efforts are required aiming to determine the mechanism of androgen regulation with enhancers downstream of the TSS.

In conclusion, our characterization of the androgen regulation of AGR2 and AGR3 provides additional models for studying androgen and AR with respect to gene regulation, as well as in prostate carcinogenesis and tumour progression, and provides evidence for promiscuous activation of a high level of wild-type AR by oestradiol.

Experimental procedures

Antibodies and reagents

Reagents and steroid hormones were obtained from Sigma Aldrich (St Louis, MO, USA) unless otherwise indicated. For western blotting, the antibodies used were: AGR2 (ab43043; Abcam, Cambridge, UK), AGR3 (ab82400; Abcam), αTubulin (sc-8035; Santa Cruz Biotechnologies, Santa Cruz, CA, USA), AR (MU256-UC; Biogenex, San Ramon, CA, USA), GAPDH (CH-MAB374; Chemicon, Temecula, CA, USA), IRDye800-conjugated goat anti-mouse (611-132-122; Rockland, Gilbertsville, PA, USA) and Alexa Fluor 680-conjugated goat anti-mouse (a21058; Invitrogen, Carlsbad, CA, USA). For ChIP, the antibodies employed were: AR (3202; Cell Signaling, Danvers, MA, USA; UB 06-680; Millipore, Billerica, MA, USA), H3K4Me1 (ab8895; Abcam), p300 (sc-585 X; Santa Cruz Biotechnology), RNA Pol II (sc-899 X; Santa Cruz Biotechnology) and normal rabbit IgG (sc-2027; Santa Cruz Biotechnology). The synthetic androgen R1881 was purchased from New England Biolabs (Ipswich, MA, USA), oestradiol and ICI 182780 from Tocris (Bristol, UK), and ER agonists and AR antagonists from Sigma-Aldrich. Nanofectin siRNA was purchased from PAA (Paching, Austria), Lipofectamine 2000 was obtained from Invitrogen, proteinase K from Roche (Basel, Switzerland), protein A sepharose beads from GE Healthcare (Upsala, Sweden), and primers and siRNAs from GenXpress (Vienna, Austria) or Applied Biosystems (Foster City, CA, USA).

Cell culture and transfection

Human PCa cell lines LNCaP, 22Rv1, VCaP and PC-3 were obtained from ATCC (Manassas, VA, USA). DUCaP was a generous gift from Dr Jack Schalken (Center for Molecular Life Science, Nijmegen, The Netherlands). DUCaP and

VCaP cells were originally derived from different metastasis tissues of the same patient with hormone refractory PCa and shared similar molecular characterization, such as a high level of wild-type AR, low PSA expression and p53 mutation [23,51,52]. For routine culture, the cells except VCaP were maintained in RPMI 1640 (PAA) supplemented with 10% fetal bovine serum (PAA) and 2 mM L-glutamine (Invitrogen). VCaP cells were cultured in DMEM low glucose medium (PAA), supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 1.4 $mg \cdot mL^{-1}$ D-glucose. For steroid hormone treatment, cells were seeded in phenol-red free RPMI 1640 (Fischer, Logan, UT, USA) supplemented with 5% (LNCaP and 22Rv1) or 10% (DUCaP, VCaP) charcoal/dextran-treated fetal calf serum (Fischer) for up to 3 days before incubation with the indicated reagent at the stated concentration and duration.

Semi-confluent DUCaP and LNCaP cells were transfected with 20 nM siRNA for 48 h or plasmid DNA for 24 h using the Nanofectin siRNA kit (PAA) or Lipofectamine 2000 (Invitrogen) in accordance with the manufacturer's instructions. Next, the cells were treated with either R1881 or E_2 at the indicated concentrations and durations. The target sequences of the siRNAs were 5′-GACCUACCGAG GAGCUUUC-3′ for the AR transcriptional start region and 5′-GCACUGCUACUCUUCAGCA-3′ for the AR LBD region. Luciferase siRNA was used as a control.

RNA isolation, cDNA synthesis and real-time qPCR

RNA isolation, cDNA synthesis and qPCR were performed as described previously [10]. qPCR using cDNA or ChIP-isolated DNA as a template was carried out using SYBR Green chemistry or a Fam-labelled Taqman probe detection on an ABI 7500 Fast machine (Applied Biosystems). Forty amplification cycles were performed with denaturation at 95 °C for 15 s, followed by annealing and extension at 60 °C for 1 min. The FKBP5 primers and Taqman probe (Hs01561006_m1) were obtained from Applied Biosystems; other primers were self-designed and the sequences are given in Tables 2 and 3.

Western blotting

Western blotting of total cell extracts normalized for protein content via the Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA) was performed as described previously [10]. Primary antibodies were incubated overnight at 4 °C in 0.2% Tween-20 in $NaCl/P_i$ at the concentrations: AGR2 (dilution 1 : 1000), AGR3 (dilution 1 : 1000), αTubulin (dilution 1 : 10 000), AR (dilution 1 : 100) and GAPDH (dilution 1 : 100 000). After washing, membranes were incubated with fluorophore-conjugated secondary antibodies (dilution 1 : 5000) before signal detection using the Odyssey system (LiCor Biosciences, Lincoln, NE, USA).

ChIP-sequencing and PCR

Chromatin immunoprecipitation was performed as described previously [32]. In brief, DUCaP cells were treated with 1 nM R1881, 10 nM E_2 or vehicle equivalent for 1 h before formaldehyde cross-linking, DNA sonication and immunoprecipitation with two combined AR antibodies, or antibodies against H3K4Me1, p300 or RNA Pol II. Normal rabbit IgG was used as a control. After pulling down the antibody–protein–DNA complex using protein A sepharose beads, the complex was digested and reverse cross-linked with 500 $\mu g \cdot mL^{-1}$ proteinase K by shaking at 56 °C overnight. DNA was purified with ChIP DNA kit (Zymo Research, Irvine, CA, USA) and eluted in 25 μL of elution buffer.

ChIP AR-precipitated DNA samples were used either for deep sequencing or PCR amplification. Preparation of the libraries and sequencing were performed using the Solexa

Table 3. Primer sequences for amplification of AR binding sites in the *AGR* genes.

	Sequence (5′- to 3′)	Location	Annealing temperature (°C)	Product size (bp)
PSA enhancer	F: GGGGTTTGTGCCACTGGTGAG	4 kb promoter	65	366
Positive control	R: GGGAGGCAATTCTCCATGGTTC			
AGR2 enhancer	S1 F: TGAGGCCCGTTTTACCTACTT	11 kb promoter	60	171
	S1 R: AAGAGGCAAAGTGAGTGACGA			
	S2 F: AGGGTCAGACTCGAACTGCT	5.5 kb promoter	60	154
	S2 R: TTCCATCGAAATGATATGCTG			
	S3 F: CAAAGTGAAAATGGCCACAA	8 kb promoter	60	148
	S3 R: GTGGCATCTTGTCACCTGAA			
	S4 F: GGGTTGGCAGAGAAAATGAA	17 kb promoter	60	242
	S4 R: CCCATTGTCTAATCGCCAGT			
AGR2 proximal promoter	AGR2 Pr F: GCAGCACTAGTGGGTGGGATTG	Transcription start site	60	87
	AGR2 Pr R: GATGCGGTCCAAGCTTCTG			
AGR3 enhancer	F: CCTGGGTTGAGCTTTGTGTT	Intron 1	60	239
	R: CCCTCCTTTCCTGTCCTTTC			

sequencing platform (GAII analyzer; Illumina, San Diego, CA, USA) in accordance with the manufacturer's instructions. Briefly, ChIP-DNA was end-repaired, adaptor ligated and PCR amplified, followed by cluster generation and sequencing-by-synthesis using the Genome Analyzer (Illumina). ChIP-DNAs were also subjected to amplification by either 40 cycles of normal PCR or real-time PCR using primers targeting ChIP-seq identified AR binding fragments of the *AGR2* and *AGR3* genes (Table 3). The *PSA* enhancer region 4 kb upstream of the transcriptional start site was used as a positive control. PCR products were separated on a 2% agarose gel and visualized by staining with SYBR Green (dilution 1 : 5000; Fisher Scientific, Vienna, Austria).

Bioinformatics analysis

Raw sequencing data were analyzed using PIPELINE analysis software (Illumina), bases were called by Bustard and aligned to the unmasked human reference genome (NCBI v36, hg18) using BOWTIE [53]. All reads that could be mapped to multiple locations in the human genome, such as repetitive elements, were discarded. The MACS tool was used to identify AR-enriched regions in a genome-wide manner [54]. Read-data were visualized using a local installation of the GENERIC GENOME BROWSER (http://promotion.molgen.mpg.de/gb2/gbrowse/human/).

Reporter vector construction and site-directed mutagenesis

AR bound enhancer fragments of *AGR2* and *AGR3* were PCR amplified with the primers listed in Table 2 using Phusion high fidelity DNA polymerase (Finnzyme, Espoo, Finland) and inserted into the pSC-B entry vector (Stratagene, La Jolla, CA, US). Inserts were size verified by *Kpn*I and *Sac*I restriction and sequencing, followed by ligation into a PGL3 promoter plasmid (Promega, Madison, WI, USA) using the Rapid DNA Ligation Kit (Roche) to generate firefly luciferase reporter vectors. Putative AREs were deleted using the QuikChange II Site-Directed Mutagenesis Kit (Stratagene). The mutagenic primers used were 5′-gctgAGCACAggaCACCGAgggaatggtgc-3′ and 5′-ggagtcAGGACAagcCACTCCtgctgaagtag-3′ for *AGR2* S1 ARE1 (AGCACAggaTGCTGA) and ARE2 (AGGACAagcTGATCC), respectively, and 5′-ctgggtTGAGCTttgTTTGTCagagtcaactgtccc-3′, 5′-ccagagtCAACTGtccGTCGCCtgtactaaaatccacc-3′ and 5′-cagtctttaaAGTCTCagaTGACCAaggccttaaggtac-3′ for *AGR3* S1 ARE1–3 (TGAGCTttgTGTTCC, CAACTGtccCTGTCC and AGTACAagaTGACCA).

Luciferase reporter gene assay

Cells were seeded in 10% charcoal/dextran-treated fetal calf serum at a density of approximately 50% confluence 24 h before transfection. For DUCaP transfection, cells were transfected with firefly luciferase reporter or site-directed mutagenic vectors. PGL3 promoter plasmid without insert was used as a control. For transfection of PC-3 cells, increasing amounts of AR expression vector PSG5-AR (0, 0.001, 0.01, 0.05 and 0.25 μg) were transfected together with androgen-responsive firefly reporter vector ARE2-PGL3B or *AGR2* and *AGR3* reporter vectors. PSG5 vector without insert was used as a filling vector. PGL4.73, an SV40 enhancer/promoter-driven renilla luciferase reporter vector (Promega), was used as a transfection control. Twenty-four hours after transfection, cells were treated with R1881 or E_2 at the concentrations indicated for 24 h, followed by cell lysis and processing for quantification of luciferase activity using the Dual Luciferase Reporter Assay kit (Promega). Firefly luciferase activity was normalized to renilla luciferase activity.

Statistical analysis

Numerical data are presented as the mean ± SEM of at least two or three independent experiments. Statistical differences were calculated using Student's *t*-test. $P < 0.05$ was considered statistically significant.

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