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Intracellular Signaling Pathways Regulate Hormone-Dependent Kallikrein Gene Expression

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Key Words
Kallikreins · Breast cancer · Gene expression · Intracellular signaling pathways · Steroid hormones · Chemical inhibitors, hormone-dependent expression · Transcription factors · Androgen receptor

Abstract
Objectives: Our aim was to examine how certain signal transduction pathways influence the regulation of hormone-dependent kallikrein *(KLK)* gene expression in androgen-sensitive breast cancer cell lines. ***Methods:*** We used the breast cancer cell lines T47D and BT474, treated with steroid hormones or various pathway inhibitors. KLKs were quantified by ELISA. RT-PCR, Western blots and immunoprecipitations were used to assess transcript and protein levels. ***Results:*** *PSA, KLK10, KLK11, KLK13* and *KLK14* are upregulated upon androgen stimulation in the T47D cell line. The expression of *PSA, KLK10* and *KLK11* was repressed by the MEK1/2 inhibitor U0126 and the PI3K inhibitor Wortmannin in the presence of the hormone, thus implicating the RAS/MEK/ERK and PI3K/AKT signaling pathways in regulating hormone-dependent *KLK* gene activation. Analysis of inhibitor-treated cells revealed changes in c-MYC expression with a pattern parallel to *KLK* gene expression. Chromatin immunoprecipitations identified androgen-dependent recruitment of specific transcription factors to the KLK proximal promoters, including c-MYC binding to *PSA* and *KLK11*. ***Conclusion:*** The hormone-specific upregulation of *PSA, KLK10* and *KLK11* in the breast cancer cell line T47D is dependent on major intracellular signaling pathways. This work provides a new dimension to the regulation of these cancer-related genes and the potential for new therapeutic targeting strategies.

Introduction

The hormone-dependent regulation of human tissue kallikreins (KLKs) has been extensively studied, with almost all KLKs showing either estrogen or androgen sensitivity in several breast cancer cell lines [1–4]. The usefulness of *KLKs* as biomarkers for breast and prostate cancer has also been extensively reviewed [5, 6]. However, the mechanisms of hormone-dependent regulation of these genes/proteins remain to be elucidated. KLK levels in biological fluids as well as breast and prostate cancer tissues show similar expression profiles which are related to stage and type [5]. Therefore, similar regulatory pathways of *KLK* expression may be involved in both prostate and breast cancer. In general, genes can be regulated by a variety of mechanisms, including transcriptional RNA processing, and translational events. The factors regulating gene expression are interdependent upon various cellular processes, especially signal transduction pathways. In turn, these pathways, and the resulting gene expres-

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sion, contribute to cellular differentiation, cell proliferation and development, as well as progression of disease.

The role of intracellular signaling pathways in hormone-dependent gene expression has been most extensively studied for prostate-specific antigen *(PSA)* and for regulating androgen receptor (AR) activity in prostate cancer cell lines. The homodimerization and translocation of AR to the nucleus, followed by recruitment to the *PSA* promoter, is dependent upon the binding of the androgen ligand and on several key phosphorylation events of the receptor and its release from cytoplasmic heat shock proteins (HSP70 and HSP90) [7]. AR is phosphorylated at a number of sites, including Ser 81, Ser 93, Ser 650, Ser 662 and Ser 803 by PKC, Ser 515 by ERK and Ser 210 by AKT [8]. AR has also recently been shown to be phosphorylated at tyrosine residues via c-SRC [9]. The phosphorylation of the receptor at these residues stabilizes dimerization of the ligand/receptor complex, marking it for translocation to the nucleus and binding to androgen response elements (AREs) and initiation of androgen-dependent gene expression [10, 11].

Increasing in vitro and clinical evidence has correlated the activity of AR, and its phosphorylation via intracellular signaling pathways, with prostate disease progression [12–14]. Bakin et al. [15] showed, by using RAS effector loop gain-of-function RAS mutant stable cell lines, that constitutive MEK activity can hyperinduce PSA protein expression in LNCaP cells in the presence of low levels of the synthetic androgen R1881. The phosphorylation of AR by ERK positively modulates the expression of AR-dependent genes, the recruitment of cofactors and causes increase in prostate cancer cell growth [13]. Clinically, activated MEK and ERK have also been correlated to advanced-stage and -grade prostate cancer [16].

AKT activity has been shown to have both positive and negative effects on the phosphorylation and activity of AR [17, 18]. The activity of AKT promotes both survival and cell proliferation by phosphorylating and inactivating proapoptotic pathways [17]. Along with AR activity, AKT also regulates several downstream pathways via phosphorylation, which, in turn, also promote androgen-dependent gene expression, including GSK3-β, IKKs/NF-κB and mTOR/4EBP/p70S6K [18–25]. The progression of prostate cancer from hormone-dependent to hormone-refractory state has also been correlated with an increase in AKT activity [14, 26]. High-passage LNCaP cells or cells that have undergone hormone ablation or antiandrogen treatment become androgen independent and have higher levels of basal AKT activity [27]. In vivo loss or inactivation of phosphatase and tensin homologue deleted from chromosome 10 *(PTEN)*, a negative regulator of the PI3K/AKT pathway, is common in prostate cancer and is associated with greater risk of recurrence. Other common prostate cancer genetic changes that have been correlated with either hyperactivity of PI3K/AKT and MEK/ERK pathways include overexpression of EGFR and RAS mutations [19, 22, 28].

The above-mentioned AR-dependent gene regulation pathways are also commonly found in breast cancers. *PTEN*-deficient cells are no longer sensitive to current therapeutic agents such as CCI-779 and tamoxifen [29, 30]. *Her2/neu* overexpression is a common marker associated with breast cancer progression. Although extensive, the role of intracellular signaling pathways, as they relate to androgen-dependent gene expression, has been limited to *PSA* and prostate cancer. Therefore, using our accumulated knowledge of KLK expression profiles and their coordinated hormone-dependent gene expression in breast cancer, we utilized selective inhibitor treatment approaches to specific cell signaling pathways that were implicated in PSA regulation, to analyze the expression profiles of other androgen-regulated KLKs in breast cancer cells. Our results indicate that although several KLKs may be under the androgenic influence, they show differential expression patterns upon inhibitor treatments, as well as in recruiting different transcription factors to their proximal promoter in a manner that correlates to the observed selective inhibitor effects.

Materials and Methods

Cell Lines

T47D and BT474 breast cancer cell lines were obtained from American Type Culture Collection (Rockville, Md., USA).

Steroids and Inhibitor Compounds

Dihydrotestosterone (DHT) was obtained from Sigma Chemical Co. (St. Louis, Mo., USA). The selective inhibitors and concentrations used are listed in online supplement table 1 (www.karger.com/doi/10.1159/000135686) and were purchased from EMD Biosciences (San Diego, Calif., USA).

Cell Culture: Hormone Stimulations and Blocking Studies

T47D and BT474 cell lines were cultured in phenol red-free RPMI 1640 media supplemented with FBS (10%) at 37°C, 5% CO_2 in plastic culture flasks. Once confluent, 1×10^6 cells were seeded into 6-well plates in the same medium to allow the cells to adhere. Twenty-four hours after plating, the medium was changed to RMPI supplemented with 10% charcoal-dextran-stripped FBS and incubated for an additional 24 h. The following day, the medium was changed to fresh RMPI/charcoal-dextran-stripped FBS for stimulation and inhibitor studies.

Stimulation Experiments

Cells were incubated with DHT (10^{-8} M final concentration) for 24 h for RNA analysis and for 4 days for measuring secreted KLK protein production in cell supernatants. All stimulations were performed in triplicate. These conditions were optimized for our experimental analysis.

Inhibitor Studies

T47D and BT474 cells were cultured as described in the stimulation experiments. Selective inhibitors were added either as a single dose for RNA and lysate analysis or daily, for 3 days; then, condition media were collected on the fourth day for KLK protein analysis. Concentrations used in inhibitor studies are listed in online supplement table 1 (www.karger.com/doi/10.1159/000135686), unless noted otherwise in the text or the figures.

RNA Extraction and RT-PCR

Total RNA was extracted from breast cancer cells using TRIZOL reagent (Invitrogen, Carlsbad, Calif., USA) following the manufacturer's instructions. RNA concentration was determined spectrophotometrically and 5 μg of total RNA was reverse transcribed into first-strand cDNA using the Superscript™ First-Strand Synthesis kit (Invitrogen) using an oligo(dT) primer. PCRs were carried out using Qiagen HotStar Taq Polymerase (Qiagen, Valencia, Calif., USA) on first-strand cDNA, for multiple KLKs. Online supplement table 2 (www.karger.com/doi/10.1159/000135686) lists the primers and expected product size for each KLK PCR. An equal amount of each PCR product was run on 0.9% agarose gels and visualized by ethidium bromide staining.

Quantification of KLKs in Cell Culture Supernatants

The concentration of each KLK was measured with specific and quantitative immunofluorometric ELISA developed in our laboratory. In brief, 96-well polystyrene plates were first coated with 500 ng/well of an hK-specific capture antibody. After overnight incubation, the plates were washed, 50 μl of culture supernatant or standards and equal volume of assay buffer were added and incubated at room temperature for 2 h. Plates were washed and biotinylated antibodies were subsequently added. Following incubation and wash, alkaline phosphatase-conjugated streptavidin was added. Finally, diflunisal phosphate and terbium-based detection solutions were added and fluorescence was measured with the Envision time-resolved fluorometer (Perkin-Elmer, Boston, Mass., USA). The calibration and data reduction were performed automatically. More details for the ELISA used have been described elsewhere [31].

Western Blotting

T47D cells were treated with selective inhibitors overnight, washed twice with 1× PBS and lysed using 1× cell lysis buffer supplemented with 1× complete protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Protein concentrations were determined using the BCA protein assay kit (Pierce Biotechnology, Rockford, Ill., USA). For Western blot analysis, 20 μg of cleared cell lysate was used. Antibodies used for Western blot analysis, using manufacturer's protocols, included β-actin (C4), AR (N-20), c-JUN (D), c-FOS (6-2H-2F), NF-κB [p65 (A), p50 (E10)] and ATF2 (N-96; all purchased from Santa Cruz Biotechnology, Santa Cruz, Calif., USA) as well as c-MYC (Upstate, Charlottesville, Va., USA).

Co-Immunoprecipitation of AR-Interacting Proteins

T47D cells were stimulated for 24 h and lysed in immunoprecipitation lysis buffer (0.1 M Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 1× complete protease inhibitors). One hundred micrograms of cleared cell lysate was used per AR co-immunoprecipitation (co-IP) assay. AR co-IPs were carried out overnight at 4°C. An 8% protein A sepharose slurry was added to each sample and incubated at room temperature for 90 min. Beads were then washed 3 times with wash buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% Tween 20) and resuspended in 100 μl of 1× SDS gel loading buffer. Samples were denatured by boiling, resolved on a 4–12% SDS-acrylamide gel and transferred to nitrocellulose membrane for Western blot analysis.

Chromatin Immunoprecipitations

Thirty million T47D cells were seeded on 15-cm plates and cultured as mentioned above. Cells were then stimulated for 24 h and chromatin was fixed and collected using ChIP-IT Enzymatic Kit (Active Motifs, Carlsbad, Calif., USA) according to the manufacturer's recommendations. Primers used in the chromatin immunoprecipitations (ChIP) are listed in online supplement table 3 (www.karger.com/doi/10.1159/000135686).

Results

MEK/ERK and PI3K/AKT Pathways Positively Regulate KLK Expression

The breast cancer cell lines T47D and BT474 were chosen as they both show enhanced *PSA* expression upon DHT stimulation. We were interested in analyzing the role of intracellular signaling pathways on the regulation of KLKs, previously reported for prostate cancer cell lines. Our previous work has also shown that along with *PSA*, other *KLKs*, such as *KLK10, KLK11, KLK13* and *KLK14,* are also sensitive to androgen stimulation in T47D cells; BT474 was used as a control of *PSA* expression [2, 3, 32]. T47D and BT474 cells were treated with the MEK1/2 and PI3K inhibitors U0126 and Wortmannin, respectively, in the presence and absence of DHT. Both inhibitors significantly reduced the secretion of PSA protein into the conditioned media in the presence of DHT (fig. 1a). KLK10 protein levels of T47D-DHT plus inhibitor-treated cells were also reduced, with a pattern similar to that observed for PSA (fig. 1b). These observations suggest that these 2 signaling pathways exert a positive influence on the regulation of PSA and KLK10 secretion by androgens. RT-PCR analysis was carried out on T47D-DHT-stimulated cells with or without U0126 and analyzed transcript expression of *PSA, KLK10, KLK11, KLK13* and *KLK14* (fig. 1c). The results indicated that these KLKs are influenced by the MEK/ERK pathway at the level of transcription. Our findings that both MEK/ERK and

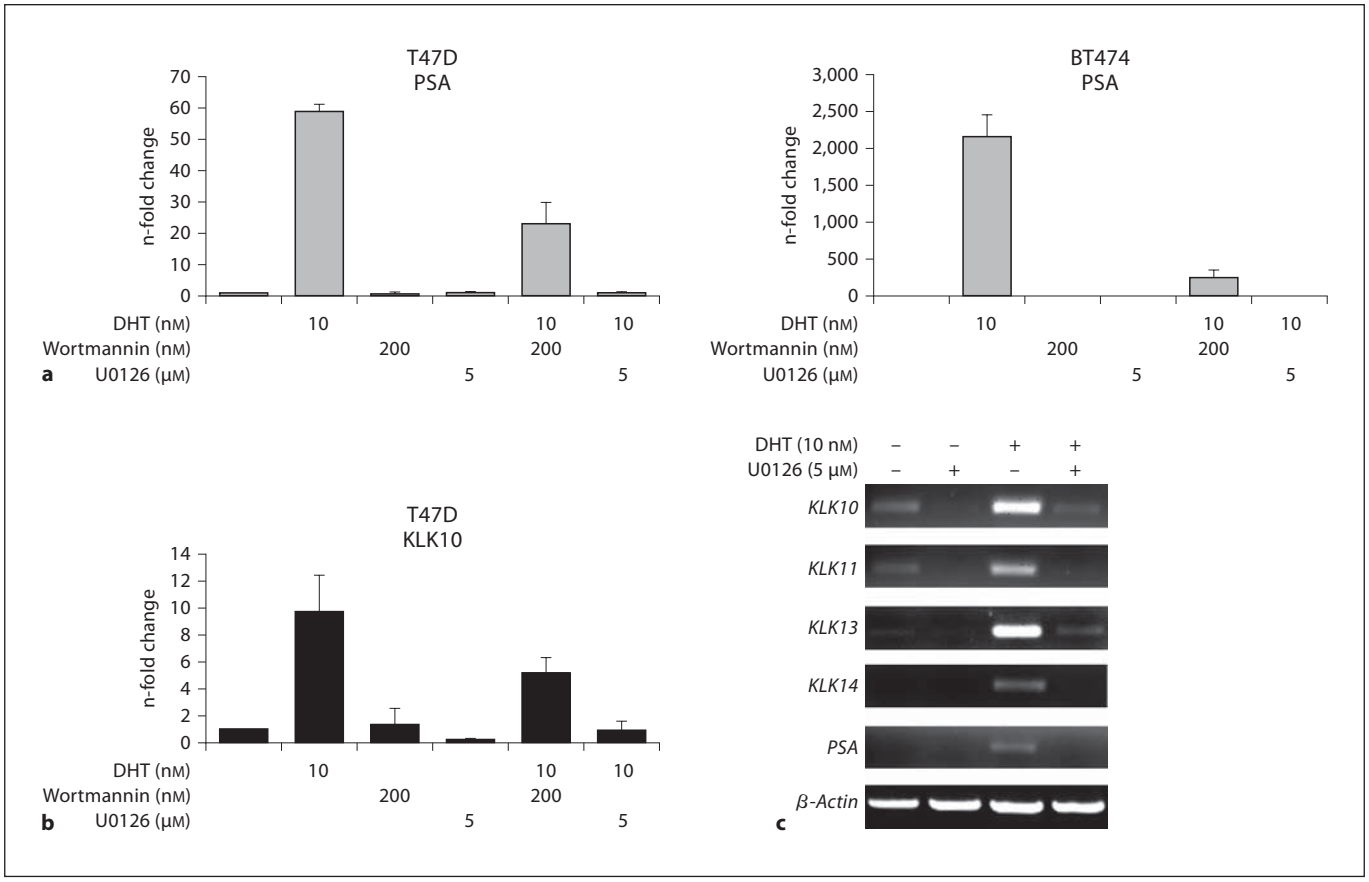
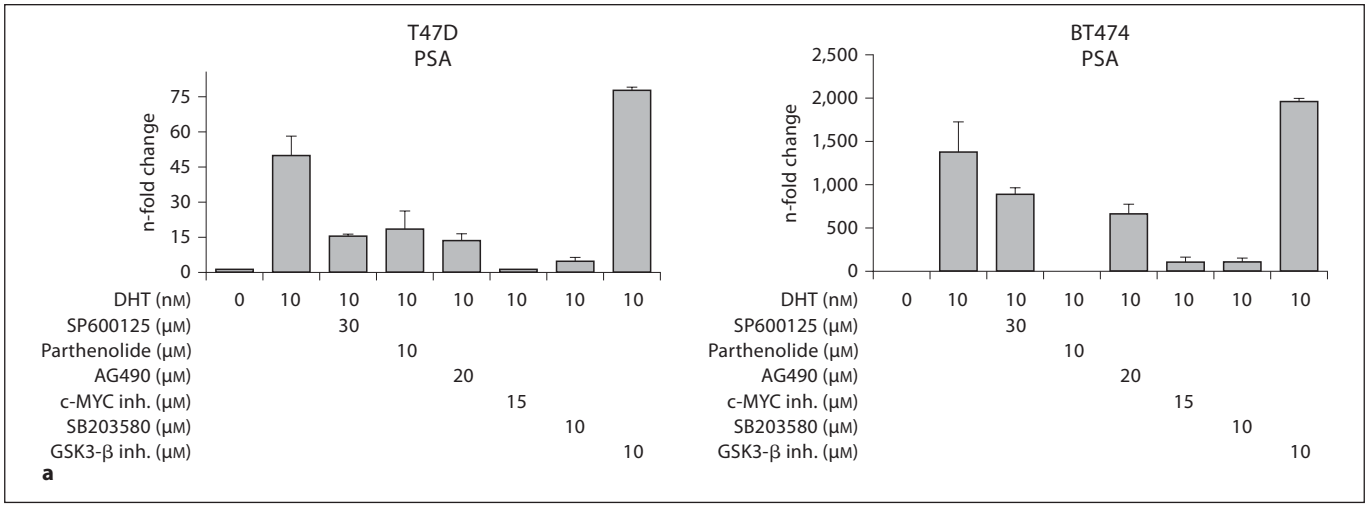


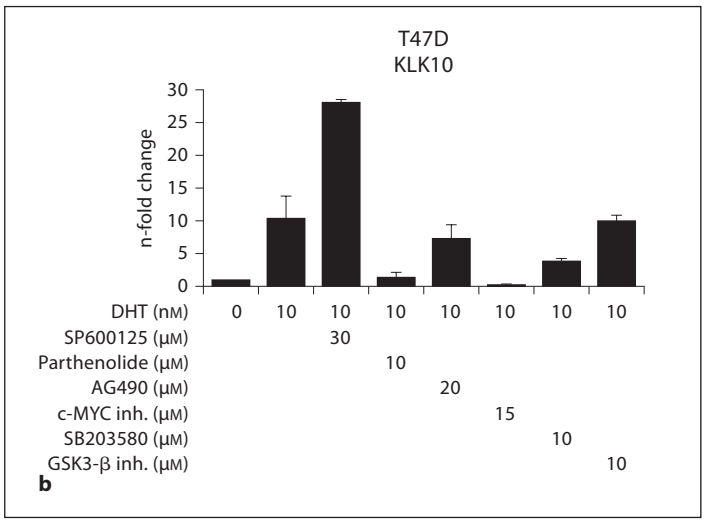
Fig. 1. Changes in KLK expression upon MEK1/2 and PI3K inhibition in 2 hormone-sensitive breast cancer cell lines. **a** T47D and BT474 cell lines were stimulated with DHT in the presence of either the MEK1/2 inhibitor U0126 or the PI3K inhibitor Wortmannin, and changes in PSA in supernatants were analyzed by ELISA. **b** KLK10 protein expression profiles of T47D cells treated with the 2 inhibitors. **c** RT-PCR analysis of *PSA, KLK10, KLK11, KLK13* and *KLK14* of T47D-treated cells. β-Actin is used as loading control; n-fold changes were calculated based on values in nonstimulated cells.

PI3K/AKT pathways are regulating a number of different KLKs support our previously suggested model [4] that groups of hormone-sensitive KLKs are regulated via similar pathways that may influence the activity of the receptors and/or other downstream transcription factors that are also necessary for *KLK* gene expression. In further experiments we chose to focus on *PSA, KLK10* and *KLK11* expression.

Selective Inhibitor Treatments of Downstream Transcription Factor Activity

We next expanded our studies by selecting chemical inhibitors that would block the activity of certain transcription factors that were reported to regulate PSA expression in prostate cancer cell lines. These factors could potentially interact with proximal promoter elements identified bioinformatically by screening upstream DNA sequences using Signal Scan (http://www-bimas.cit.nih.gov/molbio/signal/). Such inhibitors included parthenolide (an NF-κB inhibitor) [33], SP600125 (an inhibitor of the JNK pathway and AP-1 transcription factor activity) [34], SB203580 (a p38MAPK inhibitor) [35], AG490 (an inhibitor of JAK/STAT activity) [36], α-4-dibromoacetophenone (a GSK3-β inhibitor) [37] and (Z,E)-5-(4-ethylbenzylidine)-2-thioxothiazolidin-4-one (an inhibitor of c-MYC activity that blocks its interaction with MAX) [38]. T47D and BT474 cells were treated with inhibitors in the presence of DHT, and PSA protein secretion was analyzed (fig. 2a). All inhibitors repressed PSA expression, further suggesting that the pathways and downstream factors which these inhibitors block, positively influence DHT-stimulated PSA expression in breast cancer cells.





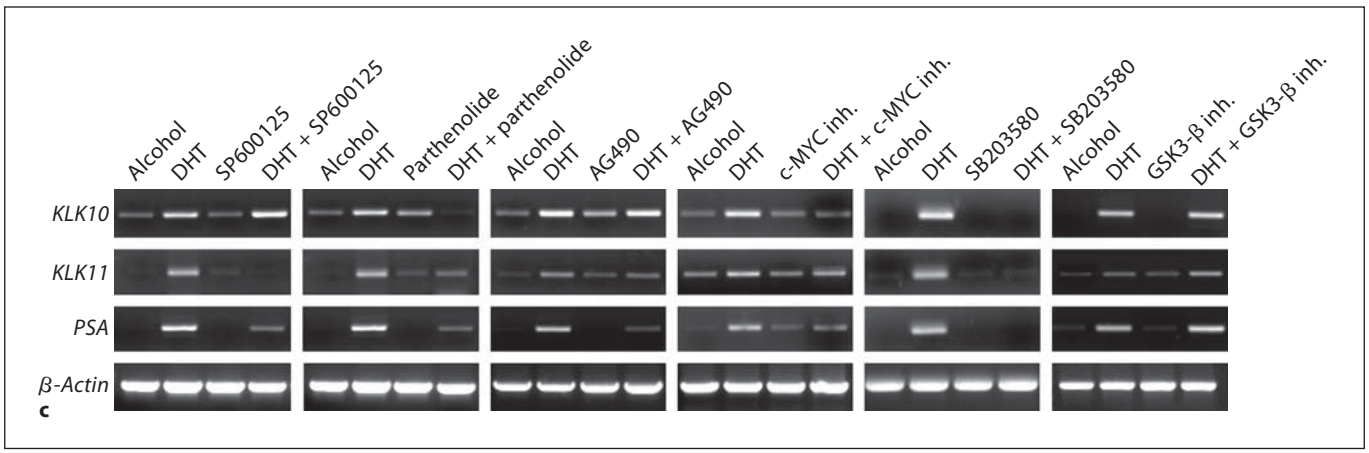


Fig. 2. Expanded selective small-molecule compound inhibitor treatments. **a** T47D and BT474 cell lines were stimulated with DHT in the presence of each inhibitor and PSA expression was analyzed by ELISA in condition media. **b** T47D cells were treated with inhibitors followed by analysis of KLK10 protein by ELISA in the presence of DHT. **c** RT-PCR analysis of *PSA, KLK10* and *KLK11* of T47D cells treated with various inhibitors. Concentrations are shown in online supplement table 1 (www.karger.com/doi/10.1159/000135686) or in brackets. Inh. = Inhibitor.

Although the GSK3-β inhibitor resulted in an increase in PSA production, further investigation reveals that the pathway positively influences PSA expression (see below). These findings are consistent with the current literature on PSA regulation in prostate cancer cells. We further identified that c-MYC activity may be required for PSA expression. We then analyzed KLK10 protein expression of DHT plus inhibitor-treated T47D cells (fig. 2b). The inhibitor SP600125 in the presence of DHT elevated KLK10 levels, suggesting that the JNK pathway is a negative regulator of androgen-stimulated KLK10 expression, whereas AG490 and the GSK3-β inhibitors did not alter KLK10 expression significantly and therefore do not seem to be required for the regulation of this KLK. RT-PCR results confirmed that the protein expression patterns for *PSA* and *KLK10* are transcriptional events and that *KLK11* expression patterns are roughly parallel to those observed for *PSA* (fig. 2c). The observation that PSA and KLK11 may share similar pathways for their hormone-dependent regulation is not surprising as it has been reported that these 2 KLKs are both highly expressed in prostate cancer cells [31].

Changes in KLK Expression Are Not a Result of Apoptosis or Cell Death

Concentrations selected for inhibitor effects on *KLK* gene expression were based on literature reports. Therefore, to preclude that any changes in KLK expression levels may be a result of apoptotic or necrotic cell death, we tested for changes in DNA fragmentation or changes in the intracellular protein lactate dehydrogenase in cell supernatants upon inhibitor treatments (fig. 3a and b). We did not observe DNA fragmentation with any of the inhibitors used for either T47D (fig. 3a) or BT474 (data not shown), nor were there any significant changes in lactate dehydrogenase levels versus the alcohol vehicle control upon inhibitor treatments (fig. 3b). Morphological changes were observed in the cell line T47D upon SP600125 and SB203580 treatments (fig. 3c). However, the changes in KLK expression are not due to deleterious effects on the viability of the cells upon inhibitor treatments.

Changes in c-MYC Protein Levels Parallel Those Observed for PSA and KLK11

Since the selected inhibitors have been well characterized for their ability to specifically block the activity of potential downstream transcription factors, we examined whether changes in the expression of these factors correlated with changes in KLK levels upon DHT stimulation plus inhibitor treatments. We analyzed T47D lysates by Western blot after DHT stimulation and inhibitor treatment for the following transcription factors: AR, ATF2, NF-κB p65/p50/p105, c-MYC, c-JUN and c-FOS. Aside from c-MYC, we could not discern any changes in transcription factor expression levels upon DHT stimulation plus inhibitor treatments (fig. 4; data not shown for c-FOS). c-MYC expression was elevated upon DHT stimulation and GSK3-β inhibitor treatments. Altogether, the changes seen for c-MYC are consistent with those reported in the literature. c-MYC possesses AREs and is sensitive to androgen stimulation; further, it is regulated by transcription factor components of the GSK3-β pathway that includes β-catenin [23, 39, 40]. GSK3-β negatively regulates the activity of β-catenin via phosphorylation and it has been shown that β-catenin regulates the expression of c-MYC. Therefore, the observation of GSK3-β inhibition positively correlates with the pattern of c-MYC expression and with changes in PSA and KLK11 expression levels. This raises the possibility that c-MYC may be a key regulator of the transcriptional expression of these 2 KLKs.

Low Levels of the NF-κB Inhibitor Parthenolide Increase both PSA and c-MYC Expression

Dose-response curves were constructed with the T47D cell line for all tested inhibitors to minimize cell death. At 10 μM parthenolide (the concentration used in the above experiments) PSA expression was dramatically reduced in the presence of DHT. However, at lower concentrations (1–2 μM parthenolide) we observed a 2-fold increase in PSA secretion in the presence of DHT in comparison to DHT alone (fig. 5a). Moreover, the enhancement of PSA expression at low levels of parthenolide was repressed by the addition of SP600125, SB203580 and c-MYC inhibitors (fig. 5b), suggesting that the increased expression is dependent on the activity of these downstream transcription factors. The resulting increase in PSA with low-level parthenolide treatment also seems to be a transcriptional event (fig. 5c). To further investigate this phenomenon, we performed Western blots for the transcription factors in cells treated with DHT and parthenolide (fig. 5d). Only c-MYC expression correlated with PSA changes. We thus hypothesized that c-MYC may directly participate in pathways regulating PSA expression.

Characterizing the AR Transcriptional Complex

Our results suggest that certain transcription factors may be involved in androgen-dependent expression of *PSA, KLK10* and *KLK11*. We then examined whether

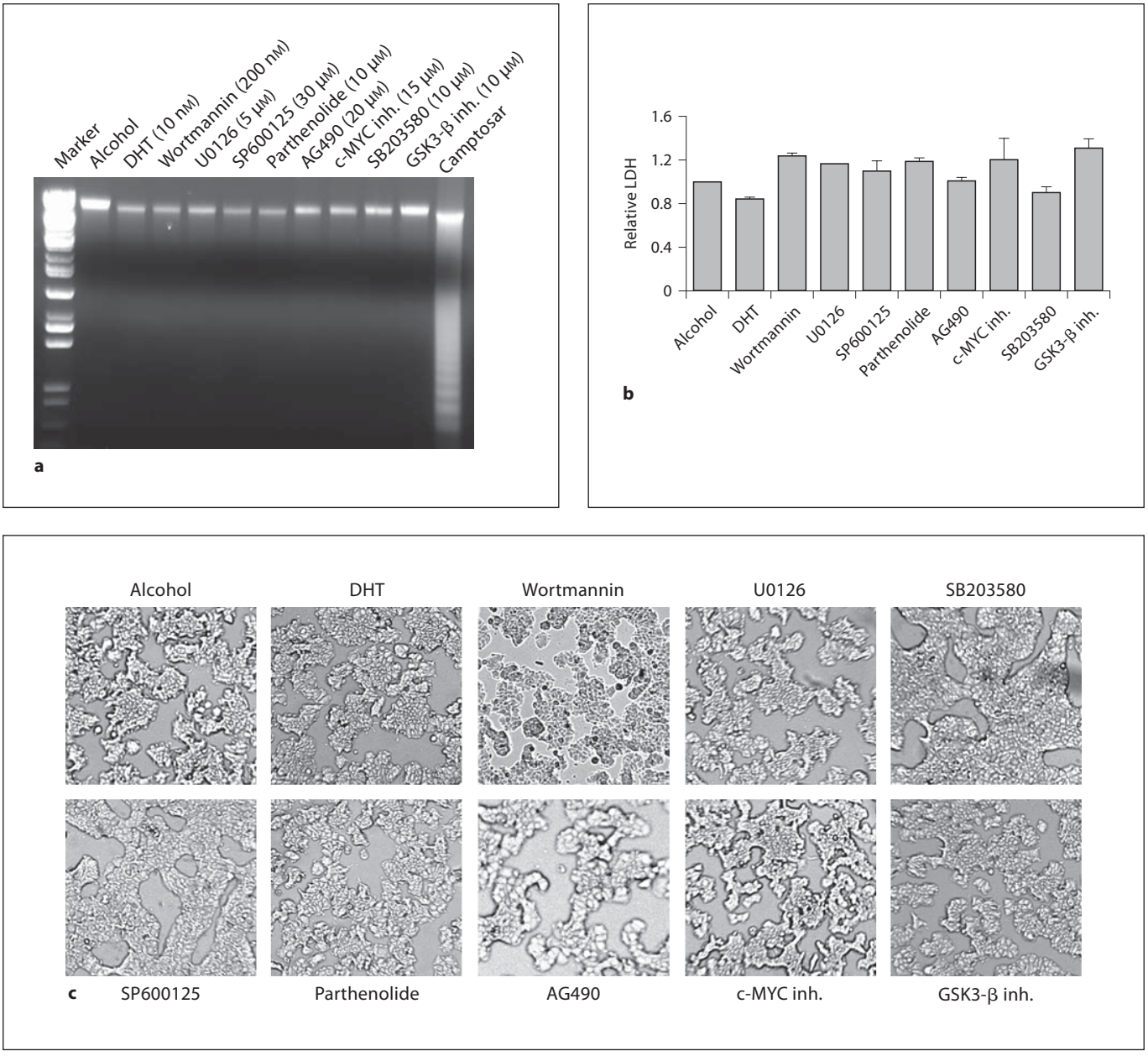


Fig. 3. Determination of cytotoxicity of chemical inhibitors of T47D-treated cells. **a** DNA fragmentation analysis for determination of apoptotic events of inhibitor-related cells. Campostar, a marketed derivative of camptothecin, was used as positive control for apoptosis of T47D cells. **b** Analysis of relative lactate dehydrogenase levels of T47D inhibitor-related cells for cell necrosis events. **c** Analysis of morphological changes of T47D cells upon inhibitor treatments. Inh. = Inhibitor; LDH = lactate dehydrogenase.

these factors were present within an active AR transcriptional complex. T47D cells, with or without DHT stimulation, were cultured and co-IP of lysates was carried out for c-MYC, c-FOS, ATF2 and NF-κB p65 subunit to examine their interaction with AR. Results are shown in figure 6a. AR was co-immunoprecipitated with c-MYC, c-FOS and ATF2 upon DHT stimulation, suggesting that these transcription factors may be part of the active AR transcriptional complex. We show for the first time that c-MYC is present in an active AR transcriptional com-

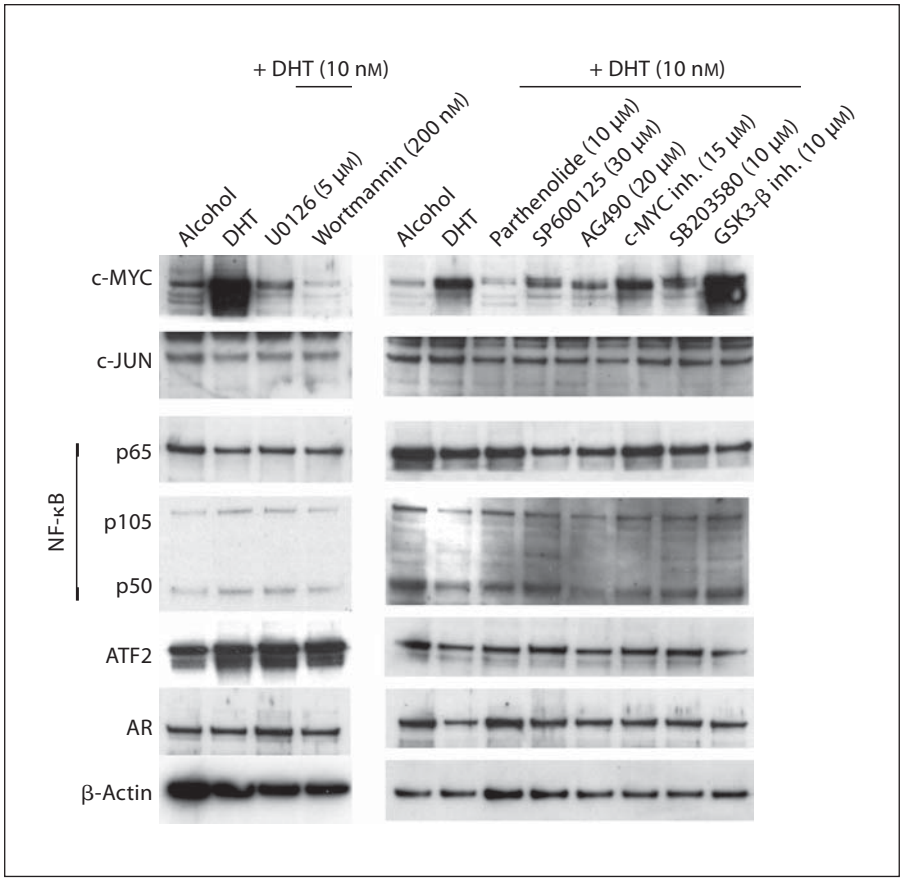


Fig. 4. Analysis of transcription factor expression of T47D inhibitor-treated cells. Western blot analysis of transcription factors whose activity is affected by the chemical inhibitor used in the study (c-FOS is not shown; however, there are no changes in the expression levels upon inhibitor treatments). For discussion see text.

plex, required for the expression of androgen-dependent *KLK* genes. Although it has been shown that NF-κB p65 subunit is required for the transcriptional regulation of *PSA* via direct binding to upstream promoter elements [25], we did not see co-IP with the active AR complex.

c-MYC, c-FOS and ATF2 Are Differentially Bound to KLK Promoter Elements

Our co-IP experiments could not show if c-MYC, c-FOS and ATF2 within an active AR transcriptional complex are directly bound to KLK promoter elements or activate other factors required for *KLK* gene expression. Therefore, ChIPs were carried out to identify direct binding of these transcription factors to proximal KLK promoter elements of androgen-stimulated T47D cells. Aside from AR binding to 2 AREs within the immediate *PSA* promoter, bioinformatic analysis of up- and downstream sequences of *PSA*, *KLK10* and *KLK11* identified several potential *cis*-binding elements for c-MYC, c-FOS and ATF2. Therefore, we carried out 500-bp PCR scanning of the transcription factor-immunoprecipitated chromatin. Figure 6b shows the results of the ChIP experiments. Along with observing AR binding to the first 500 bp of the *PSA* proximal promoter, as predicted, we also identified that c-MYC was bound to the same segment of DHT-stimulated chromatin. We also identified a second c-MYC binding site located approximately –1,000 to –1,500 bp upstream from the *PSA* transcriptional start site. The second c-MYC binding site was also predicted by bioinformatics. Finally, for the *PSA* promoter, we also identified an ATF2 binding site between –500 and –1,000 bp. These results suggest that c-MYC is recruited to the *PSA* promoter in an androgen-dependent process via association with the active AR complex to activate *PSA* expression. ChIP analysis for *KLK10* promoter elements identified 2 previously unrecognized potential AREs with 2 segments showing positive PCRs for AR binding, 1 upstream (approx. –2,000 to –2,500 bp) and 1 downstream (approx. +1,000 to +1,500 bp). Also of interest was the observation of c-FOS binding to a *KLK10*-unstimulated chromatin segment, with the binding lost after DHT treatment. The result of c-FOS binding to the *KLK10* pro-

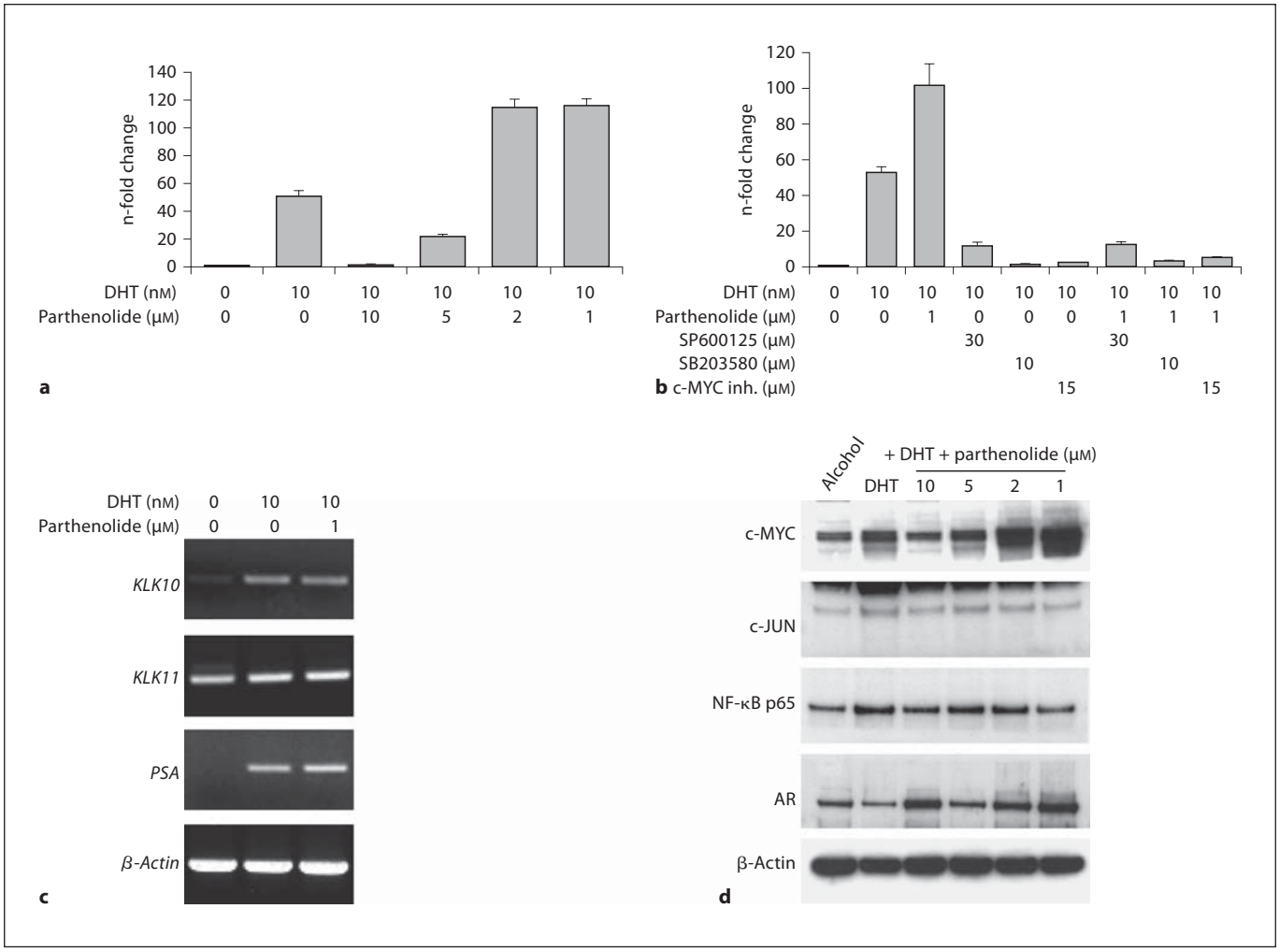
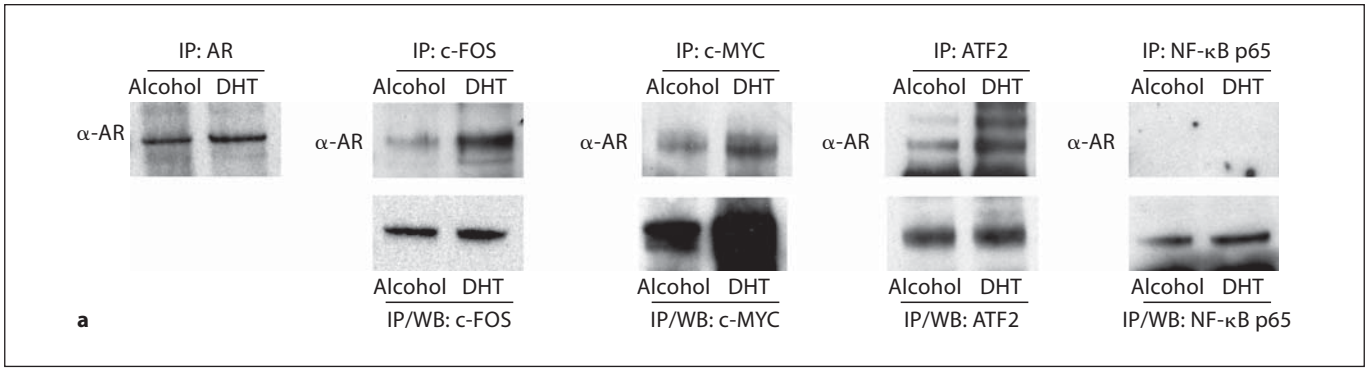


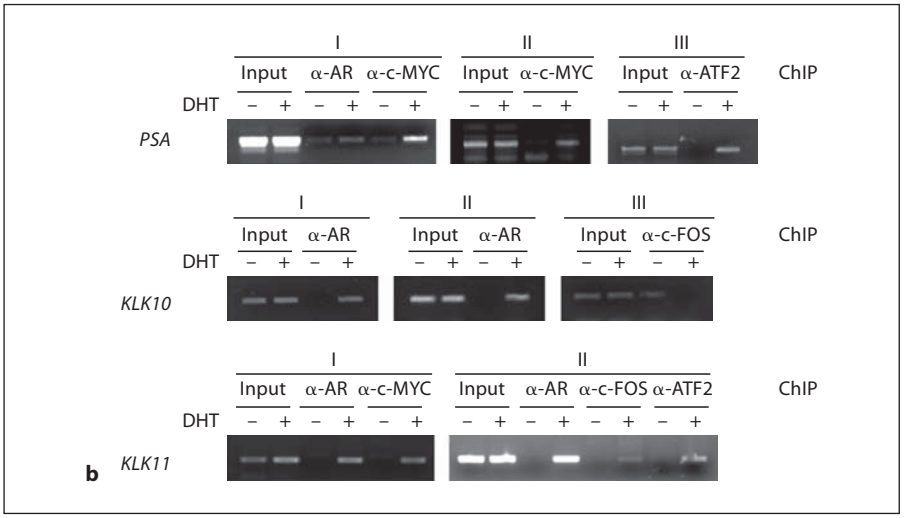
Fig. 5. Enhanced PSA expression with DHT and low levels of parthenolide. **a** PSA protein levels increase with low levels of the NF-κB inhibitor parthenolide in T47D cells. **b** Enhanced PSA expression with 1 μM parthenolide is repressed by the addition of chemical inhibitors to JNK, p38 MAPK and c-MYC, suggesting that PSA expression is still dependent upon other transcription factor activity. **c** RT-PCR analysis of DHT plus 1 μM parthenolide-treated T47D cells show enhanced *PSA* and *KLK11* transcription, but not *KLK10*. **d** Western blot analysis of selected transcription factors shows that c-MYC is overexpressed when cells are treated with DHT and low concentrations of parthenolide.

moter would correlate with our findings that treatment with SP600125, in the presence of DHT, showed greater activation of *KLK10* versus DHT alone, strengthening the suggestion that AP-1/c-FOS transcription factor activity negatively regulates *KLK10* expression. Analysis of *KLK11* also identified 2 previously unrecognized potential AREs, 1 immediately upstream (approx. +1 to –500 bp) and 1 located approximately –1,000 to –1,500 bp from the *KLK11* transcriptional start site. Furthermore, we also identified c-MYC, c-FOS and ATF2 binding to chromatin segments of the *KLK11* proximal promoter.

Discussion

Our data from analyzing androgen-dependent *KLK* gene regulation in breast cancer cells have identified many cell signaling pathways that influence gene expression. The mechanisms underlying the hormone-dependent regulation of the human tissue KLKs are of importance, since these enzymes are used as biomarkers for diagnosis, progression and therapeutic efficacy in breast and prostate cancers [5]. We have previously shown that several KLKs are coordinately expressed upon hormone





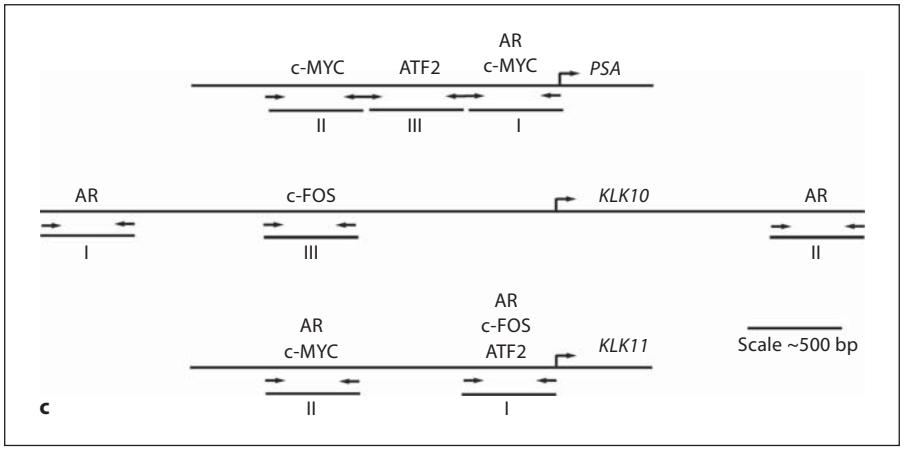


Fig. 6. Characterization of AR complexes and ChIPs. **a** Upon DHT stimulation of T47D cells, AR selectively co-immunoprecipitates with c-MYC, c-FOS, ATF2 and c-JUN (data not shown). Although it appears that NF-κB activity is required for *PSA*, *KLK10* and *KLK11* gene expression, NF-κB p65 subunit did not co-immunoprecipitate with AR. **b** Several transcription factors are bound to promoter regions of *PSA*, *KLK10* and *KLK11* proximal promoter. Specifically, 2 c-MYC binding sites in *PSA* proximal promoter as well as a c-MYC binding site in *KLK11* promoter regions were identified, but not in *KLK10* promoter regions. Also, positive PCRs identified potential AREs for both *KLK10* and *KLK11* promoter regions. **c** Schematic diagram of the location of the ChIP primers for the 3 genes analyzed. ChIP PCR primers (online suppl. table 2, www.karger.com/doi/10.1159/000135686) were designed to amplify approximately a 500-bp fragment. WB = Western blot.

stimulation in breast cancer cells [3]. Although *PSA* regulation has been extensively studied in prostate cancer cell lines and prostate cancer tissues, its expression is not exclusive to this tissue [3, 41–44]. There are several shortcomings of using prostate cancer cell lines to study *PSA* and KLK regulation. First, *KLK2* and *PSA* are the only KLKs that have been shown in prostate cancer cell lines to be influenced by androgen stimulation. Therefore, the selection of a cell line to study other *KLKs* together with *PSA* would be more advantageous. Second, the most com-

monly used prostate cancer cell lines, LNCaP and 22Rv1, have mutations in AR and accumulate PSA protein in the condition media over long periods of time without DHT stimulation. Thus, these cell lines could be considered to some extent to be androgen independent. Finally, *PTEN* is frequently mutated in almost all available prostate cancer cell lines, including LNCaP and PC3 [21, 45]. As others have shown, the unregulated PI3K/AKT signaling pathway can influence PSA expression [17, 18, 24]. Therefore, to circumvent the above deficiencies, we selected breast cancer cell lines to study androgen-dependent *KLK* gene regulation, keeping in mind that breast and prostate cancers share similar KLK expression profiles.

We used selective inhibitors to repress pathways and transcription factor activities, to analyze androgen-dependent *KLK* gene expression. Each inhibitor has been well characterized for its ability to block specific components of each respective downstream target. We initiated our study by blocking MEK/ERK and PI3K/AKT pathways with U0126 and Wortmannin, respectively, of androgen-stimulated breast cancer cells and noticed a marked reduction in all KLKs analyzed (fig. 1). As already mentioned, these 2 pathways have been shown to be positive regulators of androgen-dependent *PSA* expression in prostate in cancer cell lines [12, 21, 23]. Here, we also show that they positively influence the androgen-dependent activation of coordinately expressed *KLK10* and *KLK11* in breast cancer cells. Inhibition of these 2 pathways also corresponded with a repression of the androgen stimulation of c-MYC expression (fig. 4). Another critical factor that altered *KLK* gene expression and c-MYC expression was the inhibition of NF-κB and GSK3-β pathways. The inhibitor parthenolide blocks the activation and translocation of NF-κB components into the nucleus, subsequently repressing gene expression [33]. Using high concentrations of the inhibitor, *KLK* gene expression was blocked, along with c-MYC expression. However, at lower concentrations of the inhibitor, and in conjunction with DHT stimulation, we observed an increase in *PSA* and *KLK11* levels, much higher than DHT stimulation alone. Low concentrations of parthenolide also induced an increase in c-MYC expression (fig. 5). It is unknown why low concentrations of parthenolide are associated with increased expression of both KLK and c-MYC in an androgen-dependent manner. The current literature on this inhibitor is devoid of any work using such low concentrations. The inhibition of the GSK3-β pathway also resulted in marked changes in KLK and c-MYC expression levels. Another link between c-MYC and *KLK* gene expression is the inhibition of the interaction between c-MYC and MAX and the formation of an active complex that also repressed *KLK* gene expression. Taken together, our data point towards a correlation between KLK expression and c-MYC expression and activity, culminating in our observation that c-MYC is recruited to the *PSA* proximal promoter in an androgen-dependent manner via association with the active AR transcriptional complex. c-MYC is also recruited to the *KLK11* proximal promoter. This is significant, as our results show parallel expression patterns for *PSA* and *KLK11*. Further dissection of putative transcription factor-binding sites, by site-directed mutagenesis of KLK promoter reporter constructs, will expand our understanding of the dynamic relationship between these transcription factors and the active AR transcriptional complex.

The association between c-MYC and cancer progression has been reviewed extensively. As a transcription factor, c-MYC has been shown to target (directly and indirectly) the expression of several genes required in cell cycle regulation, apoptosis, metabolism, differentiation and cell adhesion [46–48]. Cell cycle regulatory targets for c-MYC include *cyclin A*, *cyclin D1*, *cyclin E* and *cdc25a*. Also, c-MYC promotes entry into S phase. Along with cell cycle regulatory proteins, several translation initiation factors are upregulated, including eIF4E and eIF2α, 2 proteins required for the initiation and formation of the translation complex. Overexpression of c-MYC, using a retroviral vector, was sufficient for transformation of normal prostatic epithelium to a metastatic tumor, underlying another characteristic associated with cancer progression, cell adhesion and differentiation.

KLK10 is downregulated in more advanced stages of breast cancers. *KLK10* has been shown to be most highly expressed in normal breast and prostate epithelial cells and is often referred to as a tumor suppressor marker [6]. This gene is also regulated by steroid hormones and, like *PSA* and *KLK11*, is upregulated by androgens. However, we have shown that the regulation of *KLK10* differs from *PSA* and *KLK11*. The most significant difference is that *KLK10* is negatively regulated by the JNK/AP-1 pathway. The JNK inhibitor SP600125 represses *PSA* and *KLK11* in the presence of DHT, but increases *KLK10* expression. AP-1 transcription factor activity and the differential regulation between *KLK10* and the other KLKs is also illustrated in our ChIP experiments, with c-FOS bound to the KLK promoter of unstimulated cells. First, such hormone-dependent regulation of coordinately expressed KLKs has not been observed before. Second, JNK inhibition offers a unique pathway for therapeutic targeting by decreasing poor prognostic KLK expression levels *(PSA*

and *KLK11)* and increase in the expression of the tumor suppressor KLK, *KLK10*.

In conclusion, we used breast cancer cell lines to increase our understanding on the androgen-dependent regulation of *KLK* gene family members. The mechanisms underlying regulation of *KLK* gene expression can be potentially exploited for therapeutic targeting of both breast and prostate cancer.

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