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PRECLINICAL STUDY

# Coordinated steroid hormone-dependent and independent expression of multiple kallikreins in breast cancer cell lines

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**Abstract** The regulation of gene expression by steroid hormones plays an important role in the normal development and function of many organs, as well in the pathogenesis of endocrine-related cancers. Previous experiments have shown that many kallikrein genes are under steroid hormone regulation in breast cancer cell lines. We here examine the coordinated expression of multiple kallikrein genes in several breast cancer cell lines after steroid hormone stimulation. Breast cancer cell lines were treated with various steroid hormones and kallikrein (*KLK*/hK) expression of hK3 (prostate-specific antigen, PSA), hK5, hK6, hK7, hK8, hK10, hK11, hK13, and hK14 was analyzed at the RNA level via RT-PCR and at the protein level by immunofluorometric ELISA assays. We identified several distinct hK hormone-dependent and hormone-independent expression patterns. Hormone-specific modulation of expression was seen for several kallikreins in BT-474, MCF-7, and T-47D cell lines. hK6 was specifically up-regulated upon estradiol treatment in all three cell lines whereas PSA expression was induced by dihydrotestosterone (DHT) and norgestrel stimulation in BT-474 and T-47D. hK10, hK11, hK13, and hK14 were specifically up-regulated by DHT in T-47D and by estradiol in BT-474 cells. Bioinformatic analysis of upstream proximal promoter sequences for these hKs did not identify any recognizable hormone-response elements (HREs), suggesting that the coordinated activation of these four hKs represents a unique expression ''cassette'', utilizing a common hormone-dependent mechanism. We conclude that groups of human hKs are coordinately expressed in a steroid hormone-dependent manner. Our data supports clinical observations linking expression of multiple hKs with breast cancer prognosis.

**Keywords** Kallikreins · Breast cancer · Gene expression · Steroid hormones · Hormone-dependent expression

**Abbreviations**

| | |
|---|---|
| KLK | Kallikrein gene |
| HK | Kallikrein protein |
| HRE | Hormone-response element |
| ELISA | Enzyme-linked immunosorbent assay |
| PSA | Prostate-specific antigen |
| AR | Androgen receptor |
| PR | Progesterone receptor |
| ER | Estrogen receptor; bp, base pair |
| DHT | Dihydrotestosterone |
| DFP | Difunisal phosphate |
| ECM | Extracellular matrix |
| IGF | Insulin-like growth factor |
| IGFBP | Insulin-like growth factor binding protein |
| FBS | Fetal bovine serum |

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## Introduction[1]

Steroid hormones, in particular estrogens, play an essential role in breast cancer development and their involvement in breast cancer tumorigenesis is associated with an increase in breast epithelial cell proliferation, thus facilitating malignant transformation [2]. All 15 kallikrein genes show differential expression patterns in many cancers at the mRNA and protein levels and many kallikreins have been examined as prognostic indicators in breast cancer. Previous studies have found that there is a close association between steroid hormone stimulation of cancer cell lines and kallikrein gene expression [3, 4].

Steroid hormones exert their effect by binding to their cognate hormone receptor. Upon binding to the receptor, the hormone-receptor complex translocates into the nucleus and activates gene transcription via binding to specific DNA sequences known as hormone-response elements (HREs) [5]. HREs are usually found in upstream promoter regions and recruit coactivators/corepressors to the general transcriptional machinery to modulate transcriptional activation. Hormone receptors, in particular the androgen and progesterone receptors (AR and PR, respectively) recognize very similar DNA *cis*-elements, however, the estrogen receptor (ER) binds to a quite unique sequence [6, 7] . Therefore, the sensitivity/expression of a particular kallikrein in a cell line to any given steroid hormone is dependent upon both the presence of the hormone receptor and consensus HRE binding sites.

By far, the kallikrein whose regulation by steroid hormones has been most thoroughly studied is *KLK3 (PSA)*. Initially, two androgen response elements (ARE-I and ARE-II) were identified in the upstream promoter region (–170 and –400 bp), functionally tested and found to be active in LNCaP, a prostate cancer cell line [8, 9]. An additional ARE was found at –4316 bp, which induced a dramatic increase in *KLK3* transcription, in comparison to ARE-I and ARE-II [10] . AREs have also been identified in *KLK2*, including one at –170 bp and another in an enhancer region approximately 3000 bp upstream from the transcriptional start site, a similar organization of regulatory elements to *KLK3* [11, 12]. The androgen-dependent expression of PSA and hK2 represents the ''classical'' regulatory mechanism of members of the kallikrein gene family. Along with androgen sensitivity in prostate cancer cell lines, *KLK2* and *KLK3* expression is also up-regulated by androgens and progestins in the breast cancer cell lines BT-474, T-47D, and MFM 223 [13]. *KLK4* was also found to be up-regulated by androgens in the prostate cancer cell line LNCaP [14]. Putative AREs have been identified in the immediate upstream promoter region of *KLK4*, however, they have not been functionally tested. Such similarities could account for the shared expression patterns seen between these three genes, especially in the androgen sensitive organ, the prostate [15, 16].

Until now, hormone-dependent kallikrein gene expression studies have either been limited to individual kallikrein genes or to specific cancer cell lines. There is now evidence indicating presence of multiple kallikreins in breast-associated biological fluids and tissue extracts (normal and cancerous) and expression levels that correlate with steroid hormone receptors [17–20]. Therefore, we selected several breast cancer cell lines representing benign (BT-20), solid tumor (BT-474, T-47D, and MCF-7) and metastatic variants (MDA-MB-468 and MDA-MB-231) to investigate the hormone-dependent regulation of multiple kallikrein family members. Our results indicate that several kallikreins (*PSA*, *KLK5*, *KLK6*, and *KLK8)* are regulated in a classical HRE-dependent manner. However, kallikreins 10, 11, 13, and 14, are up-regulated by different hormones in BT-474, T-47D, and MCF-7 cell lines, but always in a parallel fashion. This unique coordinated expression of four kallikrein family members by steroid hormones has not been reported before and defines a new hormone-dependent regulatory mechanism for the kallikrein locus.

## Materials and methods

### Cell lines

The following breast cancer cell lines were obtained from the American type culture collection (ATCC), Rockville MD: BT-20, BT-474, T-47D, MCF-7, MDA-MB-468, and MDA-MB-231.

### Steroids and inhibitor compounds

All steroid hormones and the steroid antagonist cyproterone acetate were obtained from Sigma Chemical Co., St. Louis, MO. The steroid hormone antagonist ICI 182,780 was purchased from Tocris Cookson, Inc., Ballwin MO, while Mifepristone (RU 486) and Nilutamide were gifts from Roussell UCLAF, Paris, France. Steroid and inhibitor stock solutions and dilutions were prepared in 100% ethanol.

[1] In this article kallikrein genes are denoted as *KLK1...KLK15* and kallikrein proteins as hK1...hK15, in accordance with the current nomenclature [1]

Cell culture: hormone stimulations and blocking studies

All cell lines were cultured in phenol-red-free RPMI 1640 media supplemented with fetal calf serum (11%), at 37°C, 5% $CO_2$ in plastic culture flasks. Once confluent, $1 \times 10^6$ cells were seeded into 6-well plates with 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**

The following steroid hormones were used for all stimulations: dihydrotestosterone (DHT), Norgestrel, 17$\beta$-estradiol, aldosterone, and dexamethazone. Cells were incubated with each hormone added once ($10^{-8}$ M final concentration) for 24 h for RNA analysis and for 7 days for measuring secreted kallikrein protein production in cell supernatants. All stimulations were performed in triplicate.

**Blocking studies**

The cell lines BT-474 and T-47D were cultured as described in the stimulation experiments. To block steroid hormone receptors, blockers for different hormones ($10^{-6}$ M final concentration) were added for 1 h into the culture media, to which the cells were then stimulated with either estradiol (BT-474) or DHT (T-47D). After 24 h, the cells were harvested for total mRNA extraction. Blocking experiments were repeated at least twice.

RNA extraction and RT-PCR

Total RNA was extracted from breast cancer cells using TRIZOL reagent (Invitrogen, Carlsbad, CA) following the manufactures 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, CA) on first strand cDNA for multiple kallikreins. Table 1 lists the primers and expected product size for each kallikrein PCR. An equal amount of each PCR product was run out on 0.9% agarose gels and visualized by ethidium bromide staining.

Quantification of hKs in cell culture supernatants

The concentration of each hK was measured with specific and quantitative immunofluorometric ELISA assays 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

**Table 1** Kallikrein primer sequences used for RT-PCR

| Kallikrein | | Sequence | Predicted size (bp) |
|---|---|---|---|
| *PSA (hK3)* | Forward | 5′ CCCACTGCATCAGGAACAAAAGCG 3′ | 600 |
| | Reverse | 5′ GGTGCTCAGGGGTGGCCAC 3′ | |
| *KLK5* | Forward | 5′ GTCACCAGTTTATGAATCTGGGC 3′ | 330 |
| | Reverse | 5′ GGCGCAGAACATGGTGTCATC 3′ | |
| *KLK6* | Forward | 5′ GAAGCTGATGGTGGTGCTGAGTCTG 3′ | 450 |
| | Reverse | 5′ GTCAGGGAAATCACCATCTGCTGTC 3′ | |
| *KLK7* | Forward | 5′ CCGCCCCACTGCAAGATGAATGAG 3′ | 450 |
| | Reverse | 5′ AGCGCACAGCATGGAATTTTCC 3′ | |
| *KLK8* | Forward | 5′ GCCTTGTTCCAGGGCCAGC 3′ | 420 |
| | Reverse | 5′ GCATCCTCACACTTCTTCTGGG 3′ | |
| *KLK10* | Forward | 5′ GGAAACAAGCCACTGTGGGC 3′ | 470 |
| | Reverse | 5′ GAGGATGCCTTGGAGGGTCTC 3′ | |
| *KLK11* | Forward | 5′ CTCGGCAACAGGGCTTGTAGGG 3′ | 460 |
| | Reverse | 5′ GCATCGCAAGGTGTGAGGCAGG 3′ | |
| *KLK13* | Forward | 5′ GGAGAAGCCCCACCCACCTG 3′ | 440 |
| | Reverse | 5′ CACGGATCCACAGGACGTATCTTG 3′ | |
| *KLK14* | Forward | 5′ CACTGCGGCCGCCCGATC 3′ | 485 |
| | Reverse | 5′ GGCAGGGCGCAGCGCTCC 3′ | |
| $\beta$-actin | Forward | 5′ ATCTGGCACCACACCTTCTA 3′ | 850 |
| | Reverse | 5′ CGTCATACTCCTGCTTGCTG 3′ | |

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 with biotinylated antibodies, alkaline phosphatase-conjugated streptavidin was added. Finally, diflunisal phosphate (DFP) and terbium-based detection solutions were added and fluorescence was measured with the Cyberfluor 615 Immunoanalyzer (MDS Nordion, Kanata, ON, Canada). The calibration and data reduction were performed automatically. More details for the ELISA assays used have been described elsewhere as follows: PSA [21], hK5 [22], hK6 [23], hK7 [24], hK8 [25], hK10 [26], hK11 [27], hK13 [28], and hK14 [29].

## Results

### Kallikrein gene regulation by steroid hormones in human breast cancer cell lines

Previous studies have focused on individual kallikreins and hormone responsiveness within one or two cell lines [13, 19, 27, 30, 31]. However, such an approach does not address the multi-parametric measurements that are currently being undertaken to study the prognostic and diagnostic value of multiple kallikreins in various cancer types. We therefore extended the previous studies on the hormonal regulation of human kallikreins by including several breast cancer cell lines and most members of this multigene family. Breast cancer cell lines were chosen to be representative of several origins (benign, solid tumor and metastatic). From previous studies, it became clear that breast cancer cell lines showed the greatest steroid hormone sensitivity. Another aspect of kallikrein expression in breast tumors was the correlation to hormone-receptor expression. Shown in Table 2 are the relative levels of three prominent receptors associated with endocrine-related cancer progression and prognosis, the androgen receptor (AR), estrogen receptor (ER) and progesterone receptor (PR).

**Table 2** Relative sex hormone receptor levels in breast cancer cell lines used in this study

| Cell line | ER | AR | PR | References |
|---|---|---|---|---|
| BT-20 | – | – | – | [32] |
| T-47D | + | + | ++ | [33] |
| BT-474 | + | + | + | [33] |
| MCF-7 | ++ | + | + | [33] |
| MDA-MB-468 | – | ND | – | [34] |
| MDA-MB-231 | – | – | – | [35, 36] |

All human tissue kallikreins were analyzed by RT-PCR and by immunofluorometric ELISAs. We first analyzed the expression of PSA in our breast cancer cell lines upon steroid hormone stimulation. As shown in Fig. 1, PSA shows specific DHT and Norgestrel up-regulated expression patterns. This expression pattern in T-47D and BT-474 cells has been previously reported by our laboratory and highlights the stimulatory effect of DHT and the synthetic androgenic progestin Norgestrel on PSA production [13]. Other cell lines, most notably MCF-7, which possess a functional androgen receptor, did not show PSA expression upon stimulation with either DHT or Norgestrel. The failure for MCF-7 to express PSA after hormone treatment may be due to other factors, as discussed later.

*KLK5*, *KLK6*, and *KLK8* were primarily up-regulated by 17$\beta$-estradiol and, to a lesser degree, by other steroid hormones in several cell lines (Fig. 2). *KLK6* was up-regulated by 17$\beta$-estradiol in T-47D, BT-474, and MCF-7. *KLK5* also shows up-regulation after estradiol stimulation but limited to BT-474 and MCF-7 cell lines. *KLK8* is also up-regulated after estradiol stimulation, but this is limited to the cell lines MCF-7 and T-47D. MCF-7 produces much higher concentrations of hK5 and hK6 (Fig. 2B), which is most likely a result of the higher levels of the estrogen receptor in this cell line versus T-47D and BT-474 (Table 2). However, the relative changes in the kallikrein levels upon estradiol stimulation are similar. hK6 protein shows an approximate 13-fold increase in BT-474, an 11-fold increase in MCF-7 and an 8-fold increase in T-47D. hK5 protein levels in MCF-7 and BT-474 were increased by 12 and 13-fold, respectively. Finally, hK8 levels show about a 5-fold increase in T-47D and a 4-fold increase in MCF-7. This kallikrein is not produced in BT-474 cells and hK5 is not produced in T-47D cells, in accordance with the RT-PCR data (Fig. 2A, B). The estrogen receptor-negative cell lines BT-20 and MDA-MB-231 failed to show any expression of these three kallikreins and PSA upon hormone stimulation (data not shown). The expression of various kallikreins in the cell line MDA-MB-468 is discussed separately later.

### Coordinated multiple kallikrein expression

Hormonal stimulation of different breast cancer cell lines revealed a unique expression pattern for kallikreins 10, 11, 13, and 14. The cell lines BT-474, T-47D, and MCF-7 are all hormone-sensitive regarding

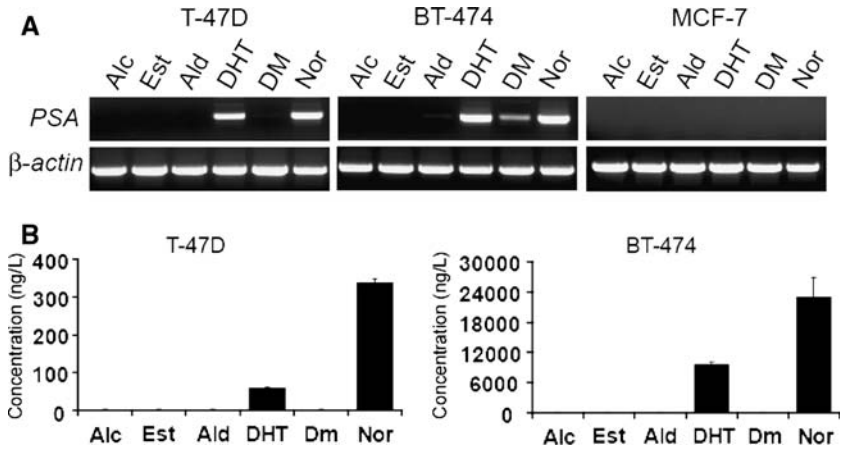


**Fig. 1** PSA expression profile in breast cancer cell lines. (**A**) RT-PCR analysis of *PSA* expression in the hormone-responsive cell lines T-47D, BT-474, and MCF-7. *PSA* shows specific DHT and norgestrel sensitive up-regulation in T-47D and BT-474, but not in MCF-7 cells. Actin expression was used as a control of RT-PCR analysis. (**B**) PSA protein production in T-47D and BT-474 cells was quantified by ELISA in tissue culture supernatants. Alc, ethanol; Est, 17$\beta$-estradiol; Ald, aldosterone; DHT, 5$\alpha$-dihydrotestosterone; Dm, Dexamethazone; Nor, Norgestrel

expression of these kallikreins, however, by different types of hormones (Fig. 3). Whereas, *KLK10*, *KLK11*, *KLK13*, and *KLK14* show mainly up-regulation by estradiol in BT-474 cells, in T-47D, the same kallikreins are mainly up-regulated by DHT. Moreover, whereas a single hormone up-regulated these four kallikreins in BT-474 and T-47D, MCF-7 cells express *KLK10*, *KLK11*, *KLK13*, and *KLK14* almost equally upon estradiol, DHT and Norgestrel stimulation. With the exception of hK13, the kallikreins hK10, hK11, and hK14 show roughly parallel protein expression patterns to their RNA profiles (Fig. 3A, B). In BT-474 cells, hK10 and hK11 show an approximate 18–20-fold increase and hK14 an 8-fold increase upon estradiol stimulation. Similarly, in T-47D cells, a 4–6-fold increase of these three proteins was seen upon DHT stimulation. hK13 protein levels were below the detection limit of our ELISA method and are thus not shown in Fig. 3B.

The specificity of estradiol to stimulate *KLK10* and *KLK11* in BT-474 cells and of DHT in T-47D cells via their respective hormone receptors was tested by performing blocking experiments using antagonists of estrogens (ICI 182,780), androgens (cyproterone acetate) and progestins (mifepristone). The antagonists were added alone or in concert with the stimulating hormone, followed by RT-PCR for *KLK10* and *KLK11*, 24 h later. As illustrated in Fig. 4, the expression of these kallikreins is dependent upon both the hormone and their cognate receptor, as ICI 182,780 can specifically block *KLK10* and *KLK11* expression in BT-474 cells in the presence of estradiol, but the other antagonists cannot. Similar results are seen with DHT stimulation of *KLK10* and *KLK11*, with the androgen antagonist cyproterone acetate in T-47D cells. These results are consistent with the notion that these four kallikreins are co-regulated in a hormone-dependent manner, through their respective activating receptors, in these cell lines.

## Hormone-independent kallikrein expression

While some cell lines show hormone-dependent kallikrein gene expression (BT-474, MCF-7, and T-47D) other cell lines do not produce any kallikreins regardless of hormone stimulation (BT-20 and MDA-MB-231). However, the association of kallikreins and clinical cancer manifestation is often linked to dysregulated kallikrein expression [3, 37, 38]. Of particular significance is the cell line MDA-MB-468 which expresses several kallilkreins in a hormone-independent manner.

Different from the other cell lines tested for kallikrein expression, the MDA-MB-468 cell line, although lacking any hormone receptors, expresses an abundance of these kallikreins regardless of the presence of any steroid hormone (Fig. 5). *KLK5* is most strongly expressed in MDA-MB-468 followed by *KLK6*, *KLK10*, *KLK7*, *KLK8*, and to a lesser degree *KLK11*. Concordance between mRNA and protein expression is seen in Fig. 5A, B. Of note is that MDA-MB-468 is a metastatic cell line, suggesting that the dysregulated expression of these kallikreins may be correlated with tumor aggressiveness. The switch form hormone-dependent kallikrein gene expression to hormone-independent expression has been observed

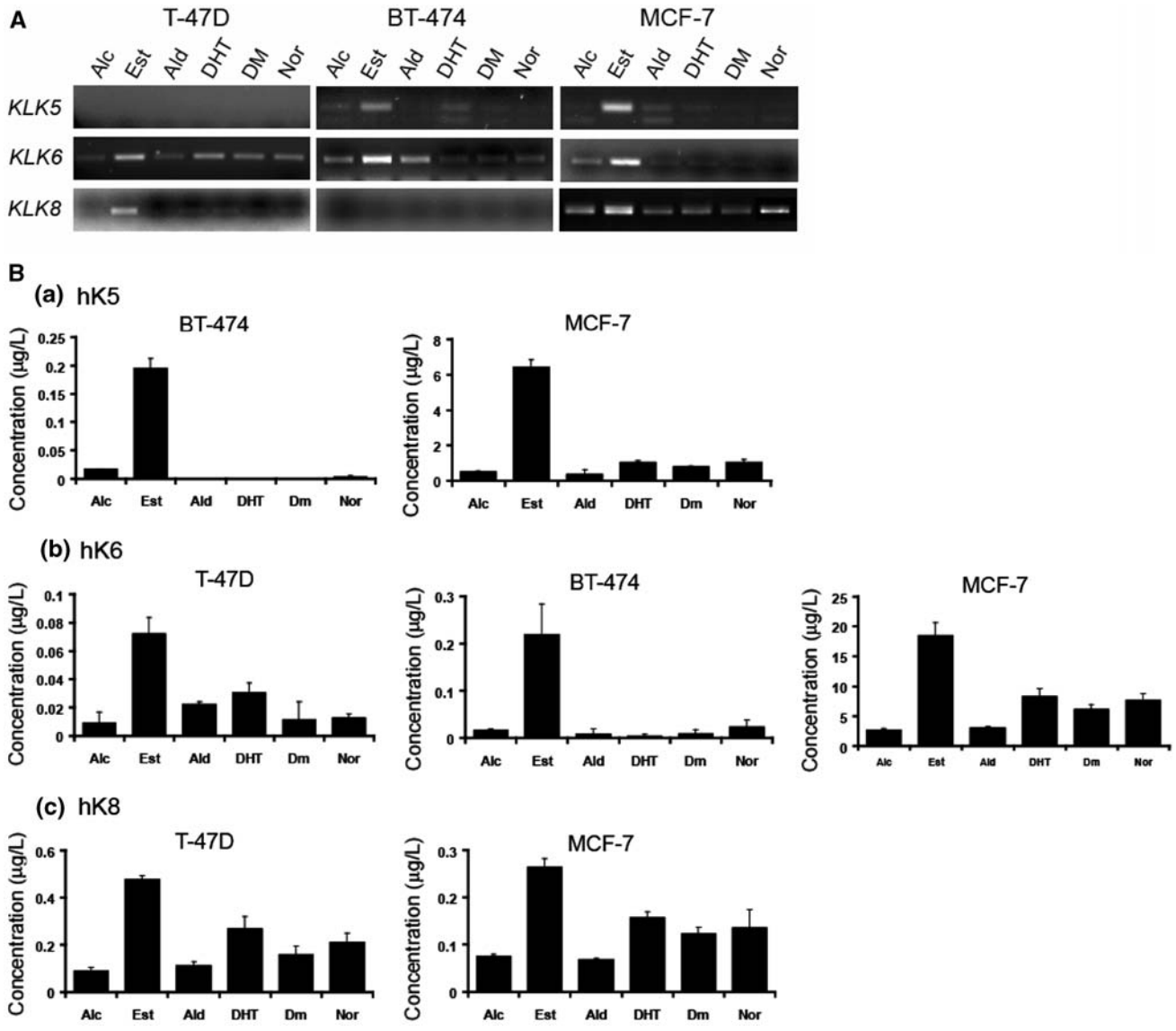


**Fig. 2** Estradiol-stimulated kallikrein expression. (**A**) RT-PCR analysis of *KLK5*, *KLK6*, and *KLK8* genes shows selective up-regulation by estradiol in all three hormone sensitive breast cancer cell lines. *KLK6* is up-regulated in all three lines, but *KLK5* and *KLK8* expression is increased in two cell lines, and shown. (Actin control, see Fig. 1) (**B**) Protein production of hK5, hK6, and hK8 as measured by ELISA assays in tissue culture supernatants

clinically for PSA (hK3) in prostate cancer. The conversion from hormone-dependent to hormone-independent expression has been correlated to several mutations including constitutive MEK-ERK activity and loss of the tumor suppressor protein *phosphatase and tensin homologue deleted from chromosome 10* (*PTEN*) [39, 40].

## Discussion

We describe a detailed analysis of kallikrein expression profiles in breast cancer cell lines. The characterization of the hormone-dependent and hormone-independent expression profiles of the tissue kallikreins in the breast cancer cell lines studied are clinically relevant to the progression, hormonal dependence and prognosis of breast cancer. We have extensively published the clinical significance of individual kallikreins as potential and viable biomarkers in a number of cancers [3, 4, 41], however multi-parametric kallikrein expression for assessing prognosis is currently underway for several endocrine-related malignancies, including breast, ovarian and prostate cancers [42]. Clearly, the expression of several kallikreins is steroid hormone-dependent, with sex hormones (estrogens, androgens and

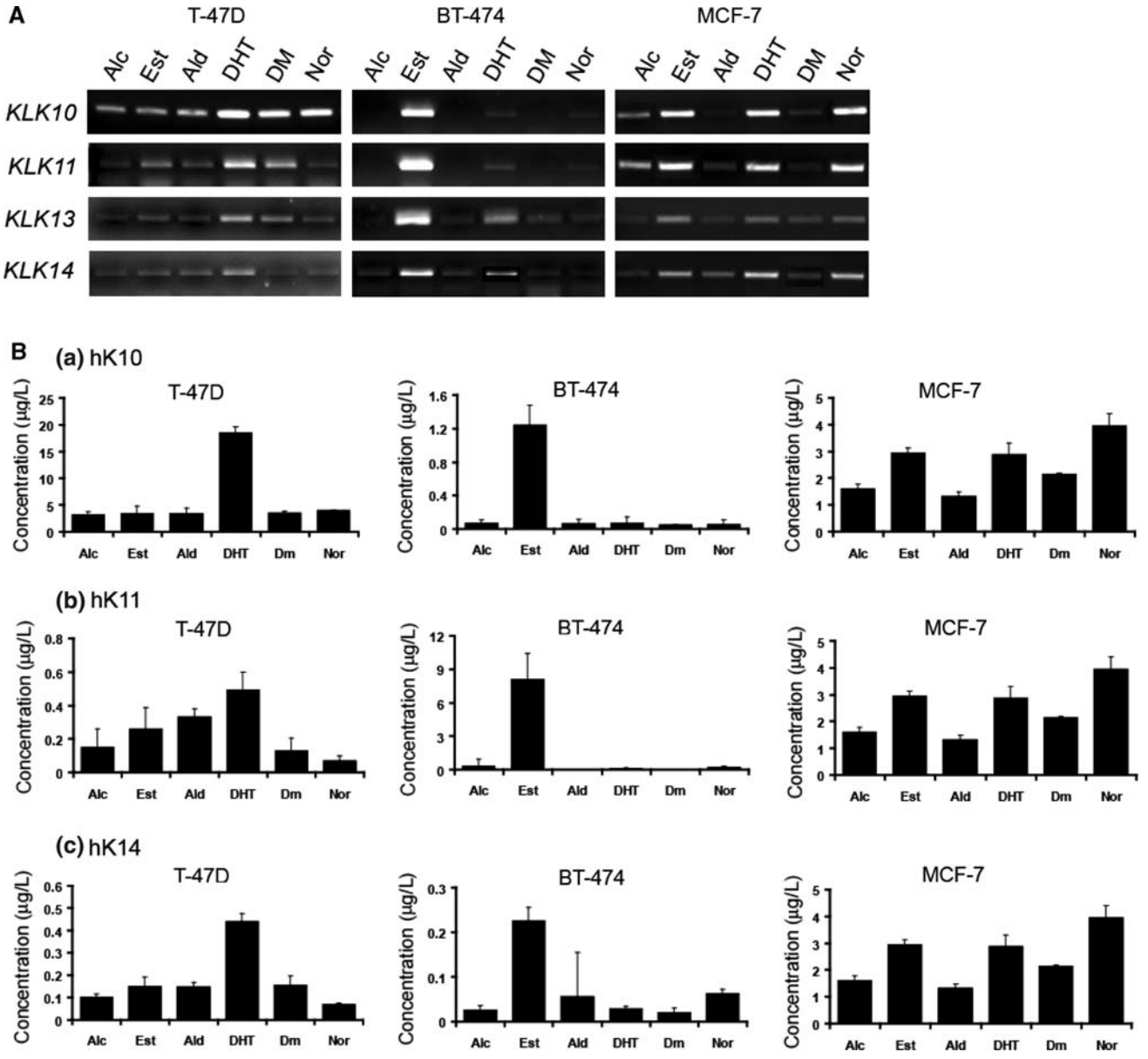


**Fig. 3** ''Cassette-type'' kallikrein expression. A. *KLK10*, *KLK11*, *KLK13* and *KLK14* mRNAs are up-regulated by different hormones in T-47D, BT-474 and MCF-7 cells. These kallikreins are all up-regulated by DHT in T-47D, estradiol in BT-474 and DHT, estradiol and Norgestrel in MCF-7 cells. (Actin control, see Fig. 1) (**B**) Protein expression profiles, as assessed by ELISAs on tissue culture supernatants

progestins) playing a major role, in comparison to glucocorticoids (dexamethazone) and mineralocorticoids (aldosterone).

MCF-7, T-47D, and BT-474 show selected, estradiol-specific up-regulation of *KLK5*, *KLK6*, and *KLK8.* This observation would suggest presence of functional estrogen receptors in these cell lines. Despite possessing functional hormone receptors, PSA is not expressed or modulated by steroids in MCF-7 cells, *KLK5* in T-47D cells and *KLK8* in BT-474 cells. This would suggest that either genetic or epigenetic aberrations in the kallikrein locus are affecting the expression of these kallikreins, or that these kallikreins are expressed at such low abundance that our assays cannot detect them. Overall, although hormone-receptor levels frequently correlate with kallikrein expression levels, as seen in MCF-7, which has high levels of estrogen receptors and secretes much more hK5 and hK6 than the other hormone responsive cell lines, this observation does not hold true for the other kallikreins. Altogether, this study underscores the need to further characterize the underlying mechanisms by

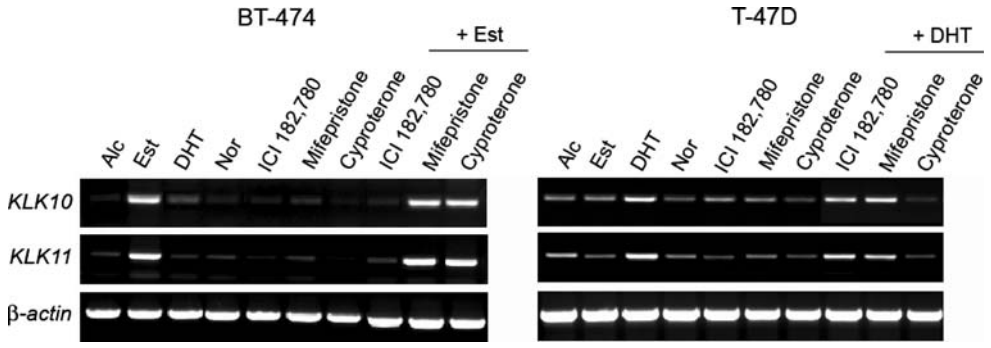


**Fig. 4** *KLK10* and *KLK11* expression decrease by respective hormone antagonists in T-47D and BT-474 cells upon hormone stimulation. RT-PCR performed on RNA extracted from hormone plus antagonist-treated cells, reveals that the hormone specific up-regulation of *KLK10* and *KLK11*, can be effectively suppressed. Left panel: Suppression of *KLK10* and *KLK11* expression by ICI 182,780 in estradiol-stimulated BT-474 cells. Right panel: Suppression of *KLK10* and *KLK11* expression by cyproterone acetate in DHT-stimulated T-47D cells

which these kallikreins are transcriptionally regulated, in order to understand their physiological significance in cancer progression and their connection to hormone-refractory cancer.

Scanning of up to 6 kbupstream proximal promoter regions by bioinformatics (Signal Scan, http://www.bimas.dcrt.nih.gov/molbio/signal/) failed to identify estrogen response elements (EREs) in the estradiol-up-regulated *KLK5*, *KLK6*, and *KLK8* genes. For *KLK10*, previous studies failed to identify either androgen or estrogen-specific HREs that are necessary for the differential expression patterns observed in T-47D and BT-474 [43]. The difficulties arising from traditional analysis of promoter deletion constructs include the possibility that transcriptional gene activation may require the coordinated binding of a number of co-transcription factors along with the hormone receptor or be mediated indirectly via other hormone-dependent *trans*-activating factors. Our data suggests the latter, having identified a number of transcription factors that are also required for the hormone-dependent activation of a number of kallikreins (our upublished data).

Kallikrein regulation is further complicated by the observed coordinated ''cassette-type'' expression of multiple kallikreins by a number of different hormones. These kallikreins include hK10, hK11, hK13, and hK14. As observed in BT-474 cells, these kallikreins are under the regulation of estradiol, whereas in T-47D they are up-regulated upon DHT stimulation, and they are nearly equally up-regulated by estradiol, DHT, and Norgestrel in MCF-7. The failure of BT-474 to show specific DHT-dependent expression of these four kallikreins, as seen in T-47D (the same being said for T-47D and estradiol stimulation), is not a result non-functional androgen receptor, as BT-474 cells clearly show androgen-dependent up-regulation of PSA (Fig. 1). Moreover, although apparently lacking proximal upstream HREs, these kallikreins are sensitive to hormone-receptor antagonists such as cyproterone acetate in T-47D cells stimulated by DHT, and ICI 182,780 in estradiol-treated BT-474 cells (Fig. 5).

The traditional understanding of kallikrein (e.g. *PSA)* up-regulation involves the interaction of a hormone receptor with an upstream HRE. It is not clear whether the coordinated ''cassette-type'' expression of *KLK10*, *KLK11*, *KLK13*, and *KLK14* is regulated by similar or different molecular mechanisms in each cell line, and whether this phenomenon is linked to single hormone-dependent *trans*-activating factors. Recently, several studies suggest that signal transduction pathways may influence the hormonal regulation of kallikrein gene expression. The AR has been shown to be activated by several pathways including MEK through the RAS pathway, AKT kinases and PKC, which sensitize the receptor to low circulating levels of androgen [44–46]. Using RAS effector-loop gain-of-function RAS mutant stable cell lines, it has been shown that constitutive MEK activation can hyper-induce PSA protein expression in LNCaP cells under normal levels of androgen [47, 48]. Constitutive MEK activity was also correlated with the switch of prostate cancer cells lines from an androgen-dependent to an androgen-independent state [39, 48].

It has been found that approximately 30% of all breast cancers either have a deletion or mutation in the gene encoding the tumor suppressor protein PTEN. PTEN is a negative regulator of AKT function, inducing in increases in cell growth and proliferation [49]. The role of PI3K/AKT pathway in regulating kallikrein expression has been studied in LNCaP prostate cancer cell lines. LNCaP is a PTEN-deficient

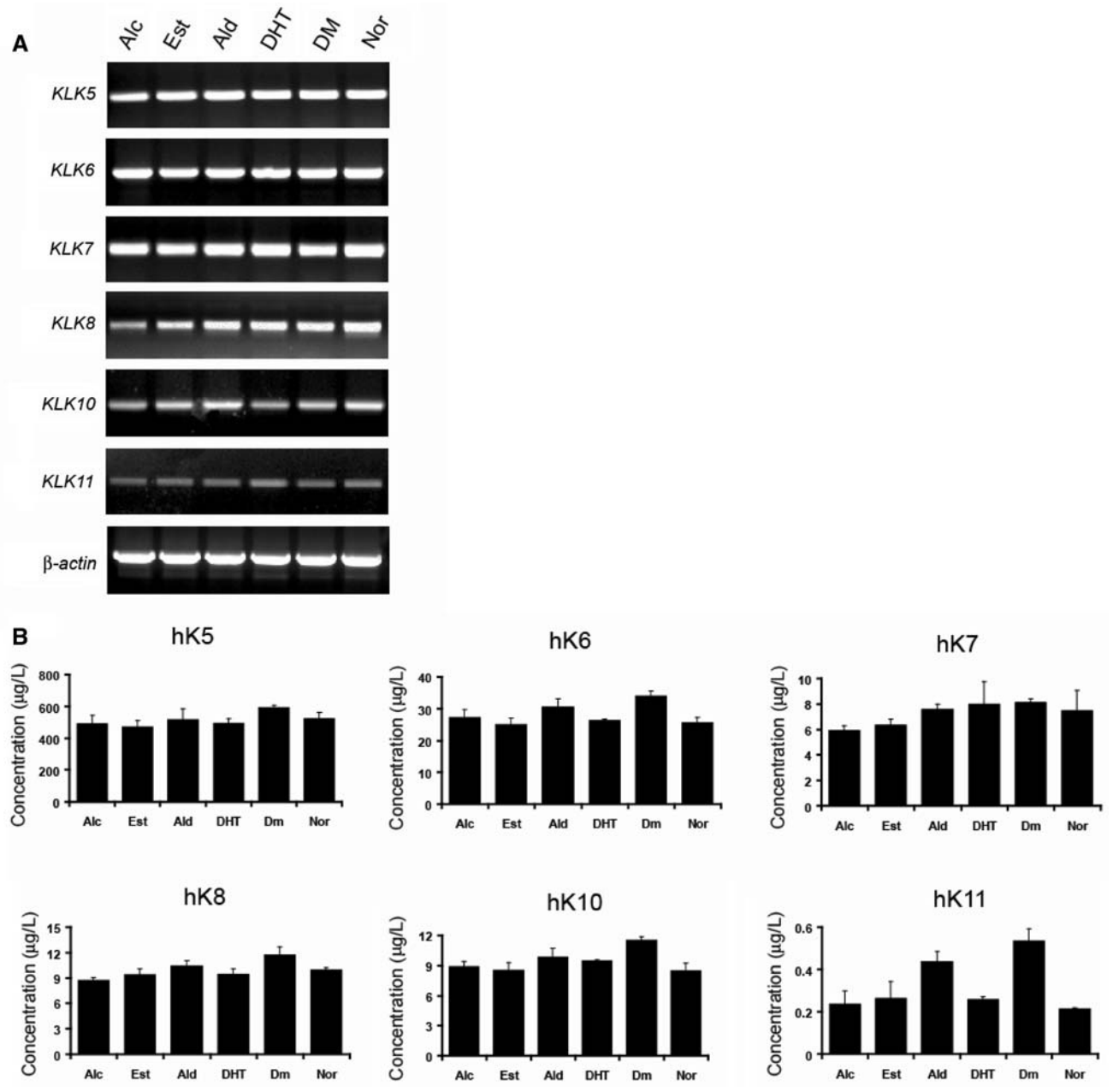


**Fig. 5** Hormone-independent kallikrein expression in MDA-MB-468 cancer cells. (**A**) RT-PCR of kallikreins 5, 6, 7, 8, 10 and 11 indicates that kallikreins are not regulated by steroid hormones. $\beta$-actin is used as a control. (**B**) ELISA assays of these kallikreins in tissue culture supernatants correlate the with RT-PCR data

cell line and it has been shown that either transient PTEN expression activity or PI3K inhibitors can repress PSA expression in the presence of DHT stimulation, by regulating the transcriptional activity of the androgen receptor [50]. Thus, the actions of single or integrated signal transduction pathways may explain how several hormones may activate the same kallikrein genes. It is currently being investigated whether other kallikreins are also influenced by the RAS-MEK-ERK and PI3K/AKT signal transduction pathways, with preliminary results suggesting that these pathways can regulate the expression of kallikreins in breast cancer cell lines that show both hormone sensitivity and dysregulated expression.

Many kallikreins have been assessed as prognostic indicators in breast cancer [3]. As observed in the metastatic cell line MDA-MB-468, several kallikreins are expressed regardless of hormone stimulation. Such

kallikrein dysregulation may be an indication of tumor progression, transforming from a hormone-dependent to hormone-independent state and to a more aggressive phenotype. Therefore, the transition to hormone-independent kallikrein expression could be explained by a mechanism that incorporates components that are required for hormone-dependent expression. As noted in Table 2, MDA-MB-468, MDA-MB-231, and BT-20 are sex hormone receptor-negative, whereas MDA-MB-468 highly overexpresses several kallikreins, but BT-20 and MDA-MB-231 fail to express any. The reason may be linked to specific mutations of MDA-MB-468. This cell line is PTEN-negative and overexpresses EGFR, common alterations found in more aggressive breast cancer types [51, 52]. The metastatic properties of MDA-MB-231, which does not express *KLKs*, must therefore lie in other alterations that are not related to kallikrein expression.

The application of phage-display technology has identified several kallikrein substrates that may have physiological relevance in tumorigenesis [53–55]. Many of the identified substrates suggested that kallikreins are able to cleave extracellular matrix (ECM) proteins including, laminin α-5 chain precursor, matrilin-4, and collagen IV [53]. In-vitro analysis has shown that hK5, hK6, and hK13 are also able to hydrolyze a variety of ECM proteins including, laminin, fibronectin and collagen I, II, and III [56–57]. Prostate cancer cells overexpressing PSA and hK4 showed both increases in cell migration, linked to loss of E-cadherin [58]. There are also several non-ECM-related proteins which are hydrolyzed by kallikreins. hK2, PSA, and hK5 may be regulators of the insulin-like growth factors (IGFs) in prostate carcinogenesis. It has been shown that hK2, PSA, and hK5 are IGFBP proteases that can collectively degrade IGFBP2, IGFBP3, IGFBP4, and IGFBP5, resulting in release of IGF1, which, in turn, can interact with the IGF1 receptor, stimulating growth of normal, stromal, and malignant prostate cells [32, 34]. Finally, another kallikrein substrate subgroup that has been studied is the kallikreins themselves. hK5 can activate pro-PSA and pro-hK2 [55]. Therefore, the discovery that these kallikreins are able to hydrolyze a number of different substrates, taken together with the dysregulated expression of these proteins in breast, prostate and ovarian cancer, raises the possibility that kallikreins could contribute to the invasiveness and/or progression of these cancers.

Characterization of the mechanisms mediating the coordinated expression of kallikreins in specific tissues and their dysregulation in several cancers will certainly help to answer the above questions. Our observation of coordinated kallikrein expression is an indication of the existence of a complex regulatory mechanism that not only controls the expression of these genes, but also their downstream physiological function.

## References


1. Diamandis EP, Yousef GM, Clements J et al (2000) New nomenclature for the human tissue kallikrein gene family. Clin Chem 46:1855–1858
2. Butt AJ, McNeil CM, Musgrove EA et al (2005) Downstream targets of growth factor and oestrogen signalling and endocrine resistance: the potential roles of c-Myc, cyclin D1 and cyclin E. Endocr Relat Cancer 12(Suppl 1):S47–S59
3. Borgono CA, Diamandis EP (2004) The emerging roles of human tissue kallikreins in cancer. Nat Rev Cancer 4:876–890
4. Borgono CA, Michael IP, Diamandis EP (2004) Human tissue kallikreins: physiologic roles and applications in cancer. Mol Cancer Res 2:257–280
5. Aranda A, Pascual A (2001) Nuclear hormone receptors and gene expression. Physiol Rev 81:1269–1304
6. Baniahmad A (2005) Nuclear hormone receptor co-repressors. J Steroid Biochem Mol Biol 93:89–97
7. Moehren U, Eckey M, Baniahmad A (2004) Gene repression by nuclear hormone receptors. Essays Biochem 40:89–104
8. Riegman PH, Vlietstra RJ, van der Korput JA et al (1991) The promoter of the prostate-specific antigen gene contains a functional androgen responsive element. Mol Endocrinol 5:1921–1930
9. Cleutjens KB, van Eekelen CC, van der Korput HA et al (1996) Two androgen response regions cooperate in steroid hormone regulated activity of the prostate-specific antigen promoter. J Biol Chem 271:6379–6388
10. Schuur ER, Henderson GA, Kmetec LA et al (1996) Prostate-specific antigen expression is regulated by an upstream enhancer. J Biol Chem 271:7043–7051
11. Murtha P, Tindall DJ, Young CY (1993) Androgen induction of a human prostate-specific kallikrein, hKLK2: characterization of an androgen response element in the 5′ promoter region of the gene. Biochemistry 32:6459–6464
12. Yu DC, Sakamoto GT, Henderson DR (1999) Identification of the transcriptional regulatory sequences of human kallikrein 2 and their use in the construction of calydon virus 764, an attenuated replication competent adenovirus for prostate cancer therapy. Cancer Res 59:1498–1504
13. Magklara A, Grass L, Diamandis EP (2000) Differential steroid hormone regulation of human glandular kallikrein (hK2) and prostate-specific antigen (PSA) in breast cancer cell lines. Breast Cancer Res Treat 59:263–270
14. Nelson PS, Gan L, Ferguson C et al (1999) Molecular cloning and characterization of prostase, an androgen-regulated serine protease with prostate-restricted expression. Proc Natl Acad Sci USA 96:3114–3119
15. Obiezu CV, Shan SJ, Soosaipillai A et al (2005) Human kallikrein 4: quantitative study in tissues and evidence for its secretion into biological fluids. Clin Chem 51:1432–1442
16. Obiezu CV, Soosaipillai A, Jung K et al (2002) Detection of human kallikrein 4 in healthy and cancerous prostatic tissues by immunofluorometry and immunohistochemistry. Clin Chem 48:1232–1240
17. Yousef GM, Scorilas A, Nakamura T et al (2003) The prognostic value of the human kallikrein gene 9 (KLK9) in breast cancer. Breast Cancer Res Treat 78:149–158

18. Yousef GM, Scorilas A, Kyriakopoulou LG et al (2002) Human kallikrein gene 5 (KLK5) expression by quantitative PCR: an independent indicator of poor prognosis in breast cancer. Clin Chem 48:1241–1250
19. Yousef GM, Borgono CA, Scorilas A et al (2002) Quantitative analysis of human kallikrein gene 14 expression in breast tumours indicates association with poor prognosis. Br J Cancer 87:1287–1293
20. Chang A, Yousef GM, Scorilas A et al (2002) Human kallikrein gene 13 (KLK13) expression by quantitative RT-PCR: an independent indicator of favourable prognosis in breast cancer. Br J Cancer 86:1457–1464
21. Ferguson RA, Yu H, Kalyvas M et al (1996) Ultrasensitive detection of prostate-specific antigen by a time-resolved immunofluorometric assay and the Immulite immunochemiluminescent third-generation assay: potential applications in prostate and breast cancers. Clin Chem 42:675–684
22. Yousef GM, Polymeris ME, Grass L et al (2003) Human kallikrein 5: a potential novel serum biomarker for breast and ovarian cancer. Cancer Res 63:3958–3965
23. Diamandis EP, Scorilas A, Fracchioli S et al (2003) Human kallikrein 6 (hK6): a new potential serum biomarker for diagnosis and prognosis of ovarian carcinoma. J Clin Oncol 21:1035–1043
24. Kishi T, Soosaipillai A, Grass L et al (2004) Development of an immunofluorometric assay and quantification of human kallikrein 7 in tissue extracts and biological fluids. Clin Chem 50:709–716
25. Kishi T, Grass L, Soosaipillai A et al (2003) Human kallikrein 8, a novel biomarker for ovarian carcinoma. Cancer Res 63:2771–2774
26. Luo LY, Grass L, Howarth DJ et al (2001) Immunofluorometric assay of human kallikrein 10 and its identification in biological fluids and tissues. Clin Chem 47:237–246
27. Diamandis EP, Okui A, Mitsui S et al (2002) Human kallikrein 11: a new biomarker of prostate and ovarian carcinoma. Cancer Res 62:295–300
28. Kapadia C, Chang A, Sotiropoulou G et al (2003) Human kallikrein 13: production and purification of recombinant protein and monoclonal and polyclonal antibodies, and development of a sensitive and specific immunofluorometric assay. Clin Chem 49:77–86
29. Borgono CA, Grass L, Soosaipillai A et al (2003) Human kallikrein 14: a new potential biomarker for ovarian and breast cancer. Cancer Res 63:9032–9041
30. Luo LY, Grass L, Diamandis EP (2000) The normal epithelial cell-specific 1 (NES1) gene is up-regulated by steroid hormones in the breast carcinoma cell line BT-474. Anticancer Res 20:981–986
31. Yousef GM, Diamandis EP (1999) The new kallikrein-like gene, KLK-L2. Molecular characterization, mapping, tissue expression, and hormonal regulation. J Biol Chem 274:37,511–37,516
32. Rehault S, Monget P, Mazerbourg S et al (2001) Insulin-like growth factor binding proteins (IGFBPs) as potential physiological substrates for human kallikreins hK2 and hK3. Eur J Biochem 268:2960–2968
33. Cohen P, Graves HC, Peehl DM, Kamarei M, Giudice LC, Rosenfeld RG (1992) Prostate-specific antigen (PSA) is an insulin-like growth factor binding protein-3 protease found in seminal plasma. J Clin Endocrinol Metab 75:1046–1053
34. Peterson G, Barnes S (1991) Genistein inhibition of the growth of human breast cancer cells: independence from estrogen receptors and the multi-drug resistance gene. Biochem Biophys Res Commun 179:661–667
35. Yasui LS, Hughes A, Desombre ER (2001) Cytotoxicity of 125I-oestrogen decay in non-oestrogen receptor-expressing human breast cancer cells, MOA-231 and oestrogen receptor-expressing MCF-7 cells. Int J Radiat Biol 77:955–962
36. Edwards DP, Kuhnel B, Estes PA et al (1989) Human Progesterone receptor binding to mouse tumor virus deoxyribonucleic acid:dependence on hormone and nonreceptor nuclear factor(s). Mol Endocrinol 3:381–391
37. Craft N, Shostak Y, Carey M et al (1999) A mechanism for hormone-independent prostate cancer through modulation of androgen receptor signaling by the HER-2/neu tyrosine kinase. Nat Med 5:280–285
38. Corey E, Quinn JE, Buhler KR et al (2003) LuCaP 35: a new model of prostate cancer progression to androgen independence. Prostate 55:239–246
39. Oka H, Chatani Y, Kohno M et al (2005) Constitutive activation of the 41- and 43-kDa mitogen-activated protein (MAP) kinases in the progression of prostate cancer to an androgen-independent state. Int J Urol 12:899–905
40. Koksal IT, Dirice E, Yasar D et al (2004) The assessment of PTEN tumor suppressor gene in combination with Gleason scoring and serum PSA to evaluate progression of prostate carcinoma. Urol Oncol 22:307–312
41. Obiezu CV, Diamandis EP (2005) Human tissue kallikrein gene family: applications in cancer. Cancer Lett 224:1–22
42. Oikonomopoulou K, Scorilas A, Michael IP et al (2006) Kallikreins as markers of disseminated tumour cells in ovarian cancer – a pilot study. Tumour Biol 27:104–114
43. Luo LY, Grass L, Diamandis EP (2003) Steroid hormone regulation of the human kallikrein 10 (KLK10) gene in cancer cell lines and functional characterization of the KLK10 gene promoter. Clin Chim Acta 337:115–126
44. Lin HK, Yeh S, Kang HY, Chang C (2001) Akt suppresses androgen-induced apoptosis by phosphorylating and inhibiting androgen receptor. Proc Natl Acad Sci USA 98:7200–7205
45. Rochette-Egly C (2003) Nuclear receptors: integration of multiple signalling pathways through phosphorylation. Cell Signal 15:355–366
46. Blok LJ, de Ruiter PE, Brinkmann AO (1998) Forskolin-induced dephosphorylation of the androgen receptor impairs ligand binding. Biochemistry 37:3850–3857
47. Bakin RE, Gioeli D, Sikes RA et al (2003) Constitutive activation of the Ras/mitogen-activated protein kinase signaling pathway promotes androgen hypersensitivity in LNCaP prostate cancer cells. Cancer Res 63:1981–1989
48. Bakin RE, Gioeli D, Bissonette EA et al (2003) Attenuation of Ras signaling restores androgen sensitivity to hormone-refractory C4-2 prostate cancer cells. Cancer Res 63: 1975–1980
49. DeGraffenried LA, Fulcher L, Friedrichs WE et al (2004) Reduced PTEN expression in breast cancer cells confers susceptibility to inhibitors of the PI3 kinase/Akt pathway. Ann Oncol 15:1510–1516
50. Nan B, Snabboon T, Unni E et al (2003) The PTEN tumor suppressor is a negative modulator of androgen receptor transcriptional activity. J Mol Endocrinol 31:169–183
51. She QB, Solit D, Basso A et al (2003) Resistance to gefitinib in PTEN-null HER-overexpressing tumor cells can be overcome through restoration of PTEN function or pharmacologic modulation of constitutive phosphatidylinositol

3′-kinase/Akt pathway signaling. Clin Cancer Res 9:4340–4346
52. Panigrahi AR, Pinder SE, Chan SY et al (2004) The role of PTEN and its signalling pathways, including AKT, in breast cancer; an assessment of relationships with other prognostic factors and with outcome. J Pathol 204:93–100
53. Felber LM, Borgono CA, Cloutier SM et al (2005) Enzymatic profiling of human kallikrein 14 using phage-display substrate technology. Biol Chem 386:291–298
54. Cloutier SM, Chagas JR, Mach JP et al (2002) Substrate specificity of human kallikrein 2 (hK2) as determined by phage display technology. Eur J Biochem 269:2747–2754
55. Michael IP, Sotiropoulou G, Pampalakis G et al (2005) Biochemical and enzymatic characterization of human kallikrein 5 (hK5), a novel serine protease potentially involved in cancer progression. J Biol Chem 280:14,628–14,635
56. Kapadia C, Ghosh MC, Grass L et al (2004) Human kallikrein 13 involvement in extracellular matrix degradation. Biochem Biophys Res Commun 323:1084–1090
57. Ghosh MC, Grass L, Soosaipillai A et al (2004) Human kallikrein 6 degrades extracellular matrix proteins and may enhance the metastatic potential of tumour cells. Tumour Biol 25:193–199
58. Matsumura M, Bhatt AS, Andress D et al (2005) Substrates of the prostate-specific serine protease prostase/KLK4 defined by positional-scanning peptide libraries. Prostate 62:1–13