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ARTICLE *in* PROTEOMICS - CLINICAL APPLICATIONS · JUNE 2014

Impact Factor: 2.96 · DOI: 10.1002/prca.201300098 · Source: PubMed

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REVIEW

Proteomic and other analyses to determine the functional consequences of deregulated kallikrein-related peptidase (KLK) expression in prostate and ovarian cancer

Ruth Anna Fuhrman-Luck[1,2], Munasinghage Lakmali Silva[1*], Ying Dong[1], Helen Irving-Rodgers[3], Thomas Stoll[4], Marcus Lachlan Hastie[4], Daniela Loessner[1], Jeffrey John Gorman[4] and Judith Ann Clements[1,2]*

[1] Institute of Health and Biomedical Innovation, Translational Research Institute, Queensland University of Technology, Brisbane, Australia
[2] Australian Prostate Cancer Research Centre – Queensland, Translational Research Institute, Queensland University of Technology, Brisbane, Australia
[3] School of Medical Science, Griffith University, Gold Coast, Australia
[4] Protein Discovery Centre, QIMR Berghofer Medical Research Institute, Brisbane, Australia

Rapidly developing proteomic tools are improving detection of deregulated kallikrein-related peptidase (KLK) expression, at the protein level, in prostate and ovarian cancer, as well as facilitating the determination of functional consequences downstream. MS-driven proteomics uniquely allows for the detection, identification, and quantification of thousands of proteins in a complex protein pool, and this has served to identify certain KLKs as biomarkers for these diseases. In this review, we describe applications of this technology in KLK biomarker discovery and elucidate MS-based techniques that have been used for unbiased, global screening of KLK substrates within complex protein pools. Although MS-based KLK degradomic studies are limited to date, they helped to discover an array of novel KLK substrates. Substrates identified by MS-based degradomics are reported with improved confidence over those determined by incubating a purified or recombinant substrate and protease of interest, in vitro. We propose that these novel proteomic approaches represent the way forward for KLK research, in order to correlate proteolysis of biological substrates with tissue-related consequences, toward clinical targeting of KLK expression and function for cancer diagnosis, prognosis, and therapies.

Received: September 30, 2013
Revised: November 23, 2013
Accepted: November 30, 2013

Keywords:
Cancer / Degradomics / Kallikrein / Protease

Correspondence: Dr. Judith Ann Clements, Institute of Health and Biomedical Innovation, Queensland University of Technology at the Translational Research Institute, 37 Kent Street Woolloongabba, QLD 4102, Australia
E-mail: j.clements@qut.edu.au
Fax: 07-3443-7770

Abbreviations: CA125, cancer antigen 125; **KLK**, kallikrein-related peptidase; **MMP**, matrix metalloproteinase; **PICS**, Proteomic Identification of protease Cleavage Sites; **PROTOMAP**, PROtein TOpography and Migration Analysis Platform; **PSA**, prostate-specific antigen; **SHBG**, serum hormone binding globulin; **TAILS**, Terminal Amine Isotopic Labeling of Substrates; **TGF**, transforming growth factor; **uPA**, urokinase-type plasminogen activator

1 Kallikrein-related peptidases (KLKs)

KLKs are secreted serine peptidases with multifaceted expression patterns and physiological functions [1–3]. In humans, the 15 *KLK* genes (*KLK1–15*) are tightly arranged in a tandem array on chromosome 19q13.3–19q13.4, representing the largest contiguous cluster of protease genes within the human genome [4–7]. *KLK* genes are transcribed and translated in many tissues, including the prostate (KLK1–5, KLK9, KLK11 and KLK13–15) and ovary (KLK1, KLK6–8, KLK10–11 and KLK14), the subject of this review, as well as in fluids,

*These authors have contributed equally to this study.
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such as seminal plasma (KLK1–5, KLK7, KLK9–15) and ovarian ascites (KLK5–7, KLK9–12) [3].

The function of active KLKs is irreversible and, therefore, regulated by many post-translational mechanisms [8]. The full-length forms of these peptidases are secreted as proteolytically inactive precursors that require cleavage of an N-terminal peptide for activation. KLKs can be activated by other KLKs, or by other proteases, including matrix metalloproteinases (MMPs), plasmin and urokinase-type plasminogen activator (uPA) [9–13]. Active KLKs display either trypsin- (KLK1, KLK2, KLK4–6, KLK8, and KLK10–15) [9, 14, 15] or chymotrypsin-like (KLK3, KLK7, and KLK9) [8, 14] enzymatic activity. Their activity is tightly regulated by endogenous inhibitors, such as serpins, kazal-type inhibitors, zinc ions, and α2-macroglobulin [16–19].

Complementing the broad expression profile of KLKs, their function has been implicated in many physiological processes. KLK-mediated proteolysis is required for activation, inactivation, and/or modification of the activity of various substrates, including other enzymes, growth factors, and their binding proteins, cytokines, ECM proteins, and cell surface receptors, discussed below. By playing such a pivotal role in regulatory response cascades, KLKs initiate and mediate key cellular functions, such as tissue remodeling, cell proliferation, migration, and angiogenesis [8, 20].

The proteolytic mechanism of KLK action in most cellular functions has not been validated, hence, the link between most KLK substrates and their induced cellular processes can only been inferred. Until recently, most established KLK substrates have been identified in biochemical assays, that is, by incubating a purified or recombinant protease with its putative substrate. Although this represents significant progress, such studies do not necessarily reflect the events occurring in, or the true biological substrates involved in, the complex extracellular microenvironment. The application of proteomics-based technologies is a significant advancement in this field in so far as these tools allow for assessing KLK action in a complex protein milieu, identifying biologically relevant KLK substrates in these processes, and delineating novel KLK-mediated cellular functions with which these substrates are associated. In this review, we will focus on the KLKs involved in prostate and ovarian cancer and their proteolytic function therein. We will also draw upon additional information, where appropriate, that relates to other members of this large peptidase family.

2 Deregulated KLK expression in prostate and ovarian cancer

Considering the diverse cellular processes regulated by KLKs, it is not surprising that deregulated expression of these peptidases is associated with pathological conditions, including cancer [1]. Notably, KLK3, or prostate-specific antigen (PSA), has been employed as a clinical biomarker of prostate cancer for decades [21, 22]. The "PSA test" has reduced mortality of men [23], however, has come under criticism due to the lack of knowledge regarding the functional contribution of this protease in cancer progression [24]. Many prostate tumors remain undetected and/or progress to end-stage disease, typically bone metastasis, which is incurable [25]. Other prostate-expressed KLKs (KLK1–2, KLK4–5, KLK10–11, and KLK15) show promise as adjunct or independent biomarkers for this disease and, along with KLK3, functional assessment has highlighted their potential as targets for novel prostate cancer therapy [26]. In detecting ovarian cancer, a disease characterized by late stage diagnosis of bulky intraperitoneal tumor burden and high mortality rates [27–29], cancer antigen 125 (CA125), or mucin 16, has been long utilized as a serum biomarker, although its specificity remains to be improved [30, 31]. Strikingly, recent studies have shown a closer relationship of KLK5, KLK6, and KLK7 expression in ovarian cancer tissues with the remaining tumor size following surgery, tumor grade and stage, compared to CA125 [32], emphasizing the potential utility of KLKs as tumor biomarkers in combination with CA125 [33, 34]. Consistently, KLK4–8, KLK10–11, and KLK13–14 show potential as biomarkers and therapeutic targets in this cancer [27–29, 35, 36]. Thus, KLKs hold promise as diagnostic, prognostic, or therapeutic targets in prostate and ovarian cancer.

3 Proteomics as a tool for global analysis of KLK production and function in prostate and ovarian cancer

3.1 MS-based KLK biomarker discovery

Rapidly evolving technologies for proteomic analysis represent a novel advancement with the promise of accelerating the field of biomarker discovery and its clinical applications. MS-based techniques have already been employed to identify novel biomarkers of prostate and ovarian cancer, which may serve to overcome the lack of specificity and sensitivity of the PSA and CA125 test, respectively [37–41]. The human proteome is a complex and diverse system, due in part to the large number of PTMs. Therefore, proteins provide a far richer and more specific source of potential biomarkers than the transcripts from which they are derived. This rich and complex pool of potential protein biomarkers can be analyzed by MS-based techniques, which uniquely allow for detection, identification, and quantification of thousands of proteins (and a range of PTMs), simultaneously, within a complex protein background.

Readily accessible biological fluids are the preferred sources of cancer biomarkers and MS-based analysis of biological fluids has identified KLKs in seminal plasma (KLK2–3, KLK11) [42], expressed prostatic secretions (KLK1–3, KLK11, KLK13) [43, 44], urine (KLK1, KLK3, KLK11, and KLK13) [45–47], serum (KLK1–3, KLK5–8, KLK11–15) [48], and ascites (KLK5–8, KLK10–11) [49, 50]. Proteomic methods can be substituted for currently employed methods of detection

of known markers and will be applied to identify and detect novel markers in the future. MRM is a technique, whereby selected protein-specific precursor peptides (MS^1) are fragmented (MS^2) and certain fragmentation product ions are isolated for quantification. As precursor peptides are protein-specific, and because reporter ions are isolated from the majority of interfering ions prior to quantification, the technique is sensitive and specific, with the relative abundance of selected fragmentation ions representative of the abundance of protein in the sample. MRM consistently identified the association of KLK3 with prostate cancer, comparable to ELISA-based detection methods used in the clinic [51,52]. Another study employed 2D-LC-MS/MS, with iTRAQ labeling, to show that KLK11 is decreased ~2.5-fold in prostate tissue from men with prostate cancer, versus those with benign prostatic hyperplasia. However, KLK11 was only identified by one unique peptide in this study, albeit reproducibly between replicates [53].

Other KLKs are yet to be confirmed as prostate cancer biomarkers by proteomic approaches; however, the sensitivity and specificity of MRM approaches may highlight extended KLK biomarker utility in the future. To this end, MRM protocols have recently been developed for identifying KLK2 in seminal fluid [54]. Proteomic approaches may also serve to refine diagnosis using current biomarkers. For example, MRM analysis showed sialylated PSA and KLK6 to be enriched in prostate [55] and ovarian cancer [56], respectively. Additionally, MS-identified KLK3 variants in seminal fluid that were not detected by existing antibodies, a finding that may be of clinical significance as these otherwise undetected KLK3 variants may correlate with prostate cancer [57].

Indeed, the application of unbiased 2D-LC-MS/MS analysis and MRM techniques, confirmed the expression of well-documented KLKs in ovarian cancer including KLK6, KLK7, and KLK9 [49, 50, 58]. In addition, MS-based identification of the above mentioned KLKs in cell lines derived from serous adenocarcinoma, but not in clear cell carcinoma, affirmed a correlation of KLK expression with the most aggressive and lethal ovarian cancer histotype, serous epithelial carcinoma [50]. Of clinical relevance, KLK6 and KLK8 have been identified in ascites from women with ovarian cancer, by shotgun MS-driven proteomics, that is, digestion of a complex protein pool with trypsin, prior to sample fractionation using LC and identification of eluted peptides by MS/MS. Presence of these KLKs in ascites has been subsequently confirmed using MRM [59]. Of note, ascites occurs at a later stage of the disease and may have a different proteomic profile to that of the early stage tumor microenvironment. Although several KLKs have been shown as potential biomarkers in the early detection of ovarian cancer, further validation is required to implement these KLKs as ovarian cancer biomarkers in the clinic. In the future, these MS-based techniques may be applied in serum, for detection of this tumor, in particular for those at an earlier stage.

The activity, and not mere expression, of proteases, such as KLKs, may also be exploited to detect cancer. Recently, in conjunction with its proposed role in inhibiting angiogenesis, levels of proteolytically active KLK3 in prostatic fluid were inversely correlated with aggressiveness of prostate cancer [60]. The reverse may apply for KLKs that function to promote cancer progression. Endogenous protease-generated protein fragments may also serve as tumor biomarkers [61]. Understanding KLK proteolysis is vital in further underpinning the full potential of KLKs as cancer biomarkers, as well as their utility as therapeutic targets. Evaluation and validation of KLKs, and KLK-generated peptides, as biomarkers, will advance our ability to utilize these KLKs in clinical applications.

3.2 MS-driven approaches for defining the KLK degradome

Proteomic analysis of biological fluids, cells, and tissues constitutes a valuable tool in studying the mechanisms of disease progression. A limitation of KLK research to date is that almost all identified KLK substrates that are implicated in cancer progression have been determined by biochemical assays. Hence, whether this reflects the KLK biological substrate repertoire in the complex extracellular microenvironment remains to be explored. Indeed, the number of biochemically identified KLK substrates to date is likely much fewer than those in vivo. This has led to the application of globally unbiased proteomic analyses of KLK substrates in complex cell, tissue, and/or biological fluid derived proteomes. A summary of these approaches, along with those used for biomarker discovery, is detailed in Figs. 1–3. The corresponding KLKs analyzed by degradomics is listed in Table 1. These approaches have enormously enhanced our understanding of the function of KLKs and will be integral to future discoveries.

3.2.1 Subsite-centric degradomics and detecting protease-substrate binding

Global profiling of KLK substrates has most simply involved assessing the preferred KLK cleavage motifs, using synthetic or cell-derived peptide libraries, or phage/bacterial display, and performing in silico analysis to map these to human proteins, thereby identifying potential substrates [62–64]. Almost all KLKs have been assessed for their proteolytic subsite preferences using the above methods, from which some studies derived novel protein substrates, which were validated in biochemical assays. For example, KLK4 was predicted and confirmed to activate pro-KLK3 and degrade insulin-like growth factor binding proteins [63]. KLK14 was similarly predicted and validated to hydrolyse laminin α5 and collagen-IV [65].

A novel proteomic approach to predict protease substrates based on their cleavage site is called Proteomic Identification of Protease Cleavage Sites (PICS) [66]. In PICS analysis, proteolysis of a cell-derived peptide library is quantified by selectively isolating (affinity chromatography) and identifying (MS/MS) tagged neo N-termini, generated by the protease

Table 1. Proteomic technologies which have been used to identify kallikrein-related peptidase (KLK) substrates

Method	Reference(s)
Subsite-centric degradomics and detecting protease-substrate binding to determine KLK substrates	
Phage display	
KLK1	[103]
KLK2	[64]
KLK3	[104]
KLK6	[103, 105]
KLK14	[65]
Bacterial display	
KLK7	[106]
Peptide library	
KLK1	[107, 108]
KLK2	[109]
KLK3	[110]
KLK4	[16, 62, 63, 110]
KLK5	[62, 110]
KLK6	[110]
KLK7	[110]
KLK8	[111]
KLK10	[110]
KLK11	[110]
KLK12	[112]
KLK13	[62]
KLK14	[62, 113]
Yeast-two-hybrid[a)]	
KLK4	[82]
PICS	
KLK4,7	Our unpublished data
2D-PAGE and shotgun MS-driven approaches to determine KLK substrates	
Affinity capture-MS/MS[a)]	
KLK3	[88]
2D-PAGE	
KLK4	Our unpublished data
LC-MS/MS	
KLK12	[86]
PROTOMAP	
KLK4	Our unpublished data
N-terminomics to determine KLK substrates	
TAILS	
KLK4–7	[81]

a) Indicates that the technique was used for unbiased assessment of substrate interacting partners.
PICS, Proteomic Identification of protease Cleavage Sites; PROTOMAP, PROtein Topography and Migration Analysis Platform; TAILS, Terminal Amine Isotopic Labeling of Substrates.

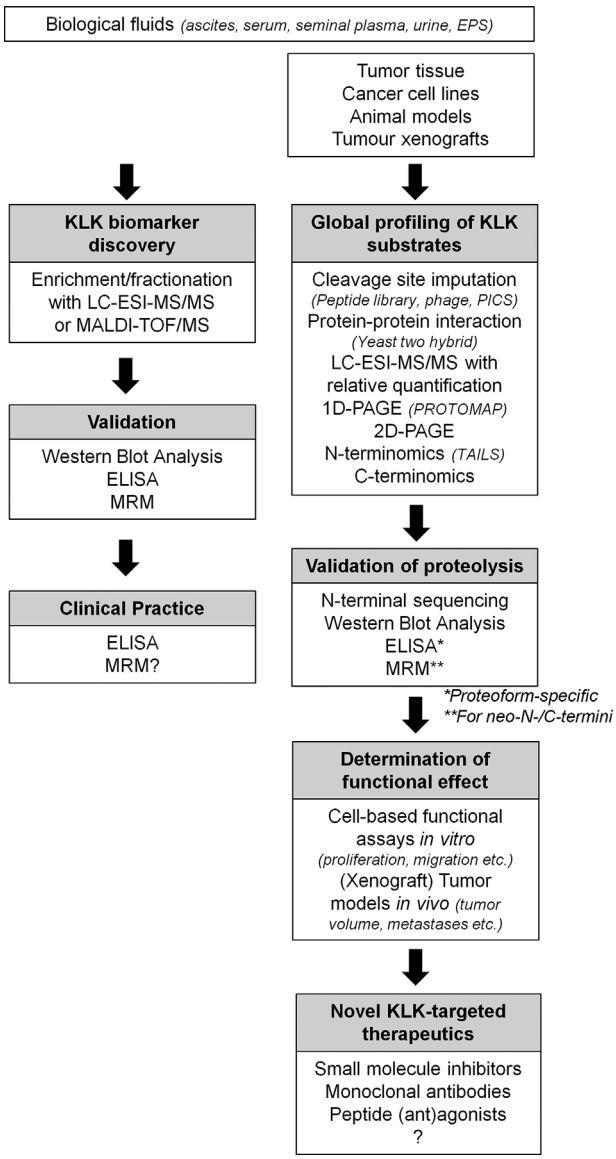

Figure 1. Schematic detailing the workflow of MS-driven kallikrein-related peptidase (KLK) biomarker or substrate identification and validation. Brackets indicate examples corresponding to each category. EPS, expressed prostatic secretions; PICS, Proteomic Identification of protease Cleavage Sites; PROTOMAP, PROtein TOpography and Migration Analysis Platform; TAILS, Terminal Amine Isotopic Labeling of Substrates.

of interest, to determine the preferred protease cleavage site specificity. Recently our group used the PICS platform, along with a custom bioinformatics-based extension that accounts for structural accessibility of the cleavage site, to decipher the substrates of KLK7 in vitro (our unpublished data). Interestingly, we observed most known KLK7 substrates, such as fibronectin, fibrinogen, and E-cadherin, along with novel targets. Among the identified novel substrates were cell adhesion molecules, basement membrane proteins, cell-ECM interacting molecules and factors that reduce apoptosis in cancerous cells, suggesting that KLK7-mediated proteolytic processing facilitates cancer advancement.

To date, biochemical assays have shown that KLKs process several ECM proteins, growth factors, cytokines, and other proteases and are associated with cancer progression. For instance, KLK4–5 and KLK14 cleave the basement membrane-specific collagen (type-IV) and, with KLK13, process the

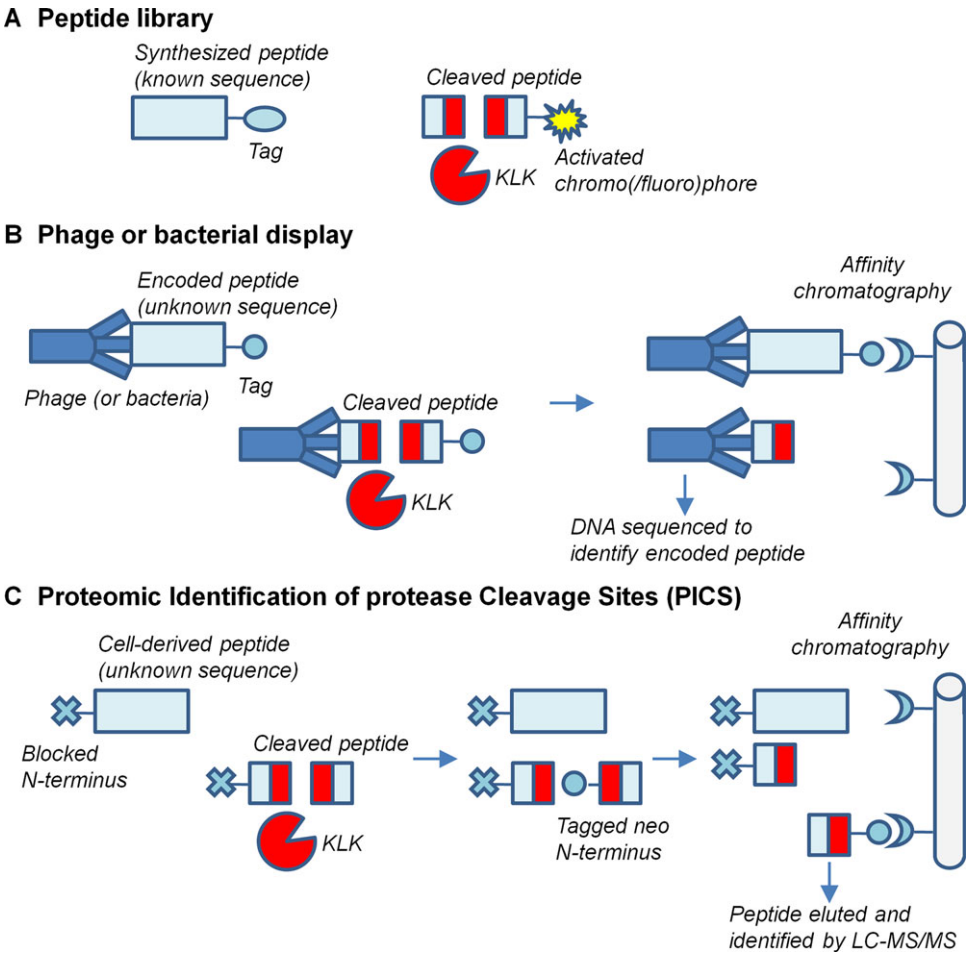

Figure 2. Peptide-centric approaches to kallikrein-related peptidase (KLK) substrate profiling (degradomics). (A) Peptide chemistry is used to generate a peptide library, whereby individual wells of an assay plate contain unique peptides. KLK hydrolysis activates choromo(/fluoro)genic peptide tags, thus the rate of choromo(/fluoro)genic peptide release is proportional to the rate of KLK-induced peptide hydrolysis. (B) A library of phage or bacteria expressing proteins, whose genes have been modified to include sequences coding for different peptides, is generated. These proteins are localized to (displayed on) the phage or cell surface and loss of the protein N-terminal tag, following KLK-mediated hydrolysis within the peptide sequence, allows affinity capture to eliminate tagged proteins, enriching for phage or bacteria encoding peptide substrates. The DNA from these phage or bacteria is subsequently sequenced, to determine the identity of the peptide substrates which they encode. (C) A cell-derived protein pool is treated with trypsin to generate a peptide library. Amine groups are blocked, prior to KLK proteolysis, allowing KLK-generated N-termini to be specifically tagged, following KLK digestion. Tagged peptides are captured by affinity chromatography and identified by LC-MS/MS. For each approach (A–C), bioinformatics may be used to determine the preferred KLK cleavage site and infer putative protein substrates based on this sequence.

fibrillar type I collagen, in vitro [67–70]. KLK2–5 and KLK13–14 cleave fibronectin, in vitro, and KLK3, KLK5, and KLK13–14 process laminin [67–72]. In addition to ECM proteins, KLK7 cleaves and activates the cytokine, pro-interleukin (IL)-1β [73], which alters the morphology of mesothelial cells to aid invasion of epithelial ovarian cancer cells into the peritoneal membrane [74]. In addition, KLKs, such as KLK6 [75] and KLK7 [76], can shed the ectodomain of E-cadherin generating fragments that have been observed abundantly in metastatic epithelial ovarian cancer and ascites, suggesting their role in ovarian cancer dissemination [77]. Interestingly, the above-mentioned PICS proteomic substrate screening technique identified a considerable number of these proteins as putative KLK7 substrates, highlighting the utility of the PICS approach.

PICS profiling additionally revealed that KLK7 hydrolyses members of the family of integrins that modulate activation of transforming growth factor (TGF)-β1 signaling in the ovarian cancer microenvironment. Importantly, the integrin αvβ6 heterodimer activates latent TGF-β1 and is expressed only in cancerous ovarian tissues [78]. TGF-β1 is involved in keratinocyte growth through modulating the expression levels of integrin α5β1, αvβ5, α2β1, α3β1, and αvβ6 heterodimers [79]. Our group showed that KLK7 promotes multicellular aggregate formation and paclitaxel chemoresistance via increasing the levels of the integrin α5β1 heterodimer in

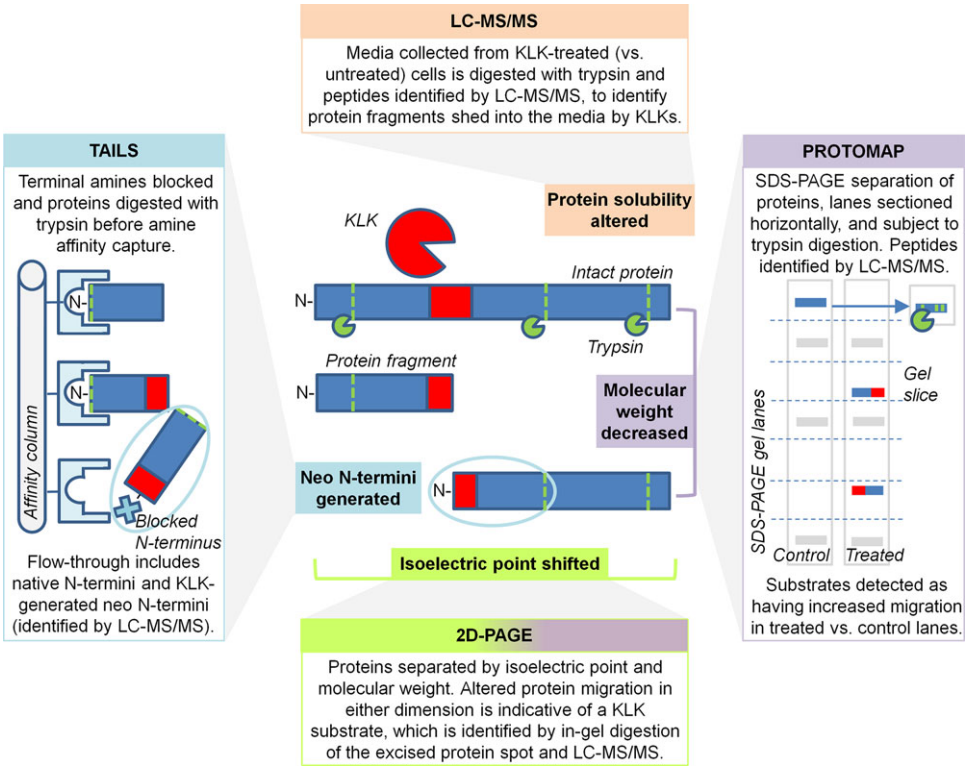

Figure 3. Protein-centric approaches to kallikrein-related peptidase (KLK) substrate profiling (degradomics). PROTOMAP, PROtein Topography and Migration Analysis Platform; TAILS, Terminal Amine Isotopic Labeling of Substrates.

ovarian cancer cells [36], suggesting a potential relationship between KLK7-mediated chemoresistance, TGF-β1, and integrin signaling in ovarian cancer. Furthermore, the Terminal Amine Isotopic Labeling of Substrates (TAILS) proteomic approach [80] that detects substrates by enriching for and identifying neo-N-termini generated by substrate proteolysis, showed a relationship between co-expression of KLK4–7, and TGF-β1 activation and expression in an ovarian cancer cell line [81]. Of note, KLK5 has been shown, biochemically, to directly contribute to TGF-β1 activation. Thus, TGF-β1 activation may have occurred indirectly, through KLK7-mediated integrin processing, and directly, through KLK5 proteolysis, in these cells. Collectively, these findings serve to support our results obtained by the PICS approach, above.

A related approach to phage/bacterial display in determining protein–protein interactions is yeast-two-hybrid screening. Routinely, for yeast-two-hybrid assays, yeast are engineered to co-express the protein of interest (bait) with one of a library of cDNA sequences (prey), for example, those corresponding to the human genome. The genes for the "bait" and "prey" are fused with the binding and activating domain of a transcription factor, respectively, so that interaction between the translated "bait" and "prey" proteins is required for the transcription factor to initiate transcription of its corresponding reporter gene. The genes encoding "bait"-interacting "prey" may be identified by DNA sequencing. This technique was not employed to determine KLK substrates, but instead, interacting partners of serum hormone binding globulin (SHBG), with which KLK4 was found to interact [82]. Analysis by our group showed that the interaction between KLK4 and SHBG was that of KLK4 binding to its substrate prior to subsequent proteolysis. Interestingly, KLK4 proteolysis of SHBG did not affect the ability of the latter to sequester androgen, although it cleaved SHBG into two distinct components, the biological relevance of which is still not clear [82]. This is a rare example whereby unbiased assessment of substrate-interacting proteins detected an interaction with a KLK, hence this could be classified as a unique form of unbiased determination of KLK proteolysis.

3.2.2 2D-PAGE and shotgun MS-based degradomics

Although subsite-centric imputations are insightful first-pass analyses into the KLK degradome, substrates identified by these techniques still require validation in a complex proteome background mimetic of that observed in vivo. Additionally, techniques used to detect protein–protein binding may not detect the transient interaction between protease and substrate. 2D-PAGE provides the ability for global profiling of KLK substrates, within such a biologically mimetic proteome background. 2D-PAGE entails separation of a complex protein mixture by p*I* (IEF) in the first dimension and molecular weight (SDS-PAGE) in the second dimension. Differential migration of proteins in either dimension upon protease treatment is essentially restricted to protease substrates, which may be identified by in-gel digestion and identification by MS/MS. To explore the role of KLK4 proteolysis at the site of bone metastasis, we have employed 2D-PAGE of a KLK4-treated osteoblast cell-derived mineralized matrix [83], and

identified both known and novel KLK4 substrates in the bone matrix (our unpublished data). KLK4 is known to degrade proteins of the mineralized tooth enamel matrix, in vitro, thus the ability of KLK4 to degrade the bone matrix was not surprising [84, 85]. Additional proteomic studies may similarly implicate other KLKs to play a role in bone metastasis.

Recently, conditioned media harvested from breast cancer cells treated with KLK12 was analyzed using shotgun proteomics, to identify proteins shed into the media, representing putative KLK12 substrates [86]. This study identified novel membrane-associated KLK12 substrates, including CCN proteins. Cell-based functional assays confirmed that KLK12 cleaved CCN1 (or 5) in CCN-1(-5)-TGF-β1 complexes, releasing TGF-β1 in a concentration-dependent manner [86]. A similar technique has been used to assess the consequences of PSA proteolysis on prostate cancer cells. However, the whole cell lysates, and not secreted proteins, from these PSA-treated prostate cancer cells were quantified for relative changes in protein abundance, thus identifying events downstream of extracellular KLK3 proteolysis [87].

Finally, affinity capture chromatography, coupled with LC-MS/MS, identified KLK3 to interact with galectin-3 on the surface of prostate-expressed vesicles, or "prostasomes," in seminal fluid. Galectin-3 was confirmed as a novel KLK3 proteolytic substrate and proteolysis of this glycoprotein in seminal fluid was reduced following proteolytic inhibition of KLK3, hence galectin-3 appears to be a definitive biological KLK3 substrate [88]. This study constitutes another example where identification of a KLK substrate was determined in a reverse manner by a global screening approach used to identify binding proteins of what was eventually found to be a KLK substrate. Prostasomes are functionally implicated in cancer-progression and these vesicles may provide a unique source of KLK substrates which function to drive carcinogenesis [89, 90]. Thus, traditional shotgun proteomic approaches have been highly useful to establish novel KLK substrates, from an array of different biological sources.

More recently, novel MS-driven degradomic techniques have been developed to more effectively assess proteolysis in a complex proteome background. These approaches supercede 2D-PAGE and traditional shotgun MS, as they enrich for protease-generated products, thus improving the sensitivity of detection, and/or provide detail regarding the protein cleavage site and/or the sequence and stability of proteolytically released protein products. These emerging technologies have been recently reviewed [91]. One such approach is an application of shotgun proteomics, known as the PROtein TOpography and Migration Analysis Platform (PROTOMAP) [92], a robust method utilizing the resolution of 1D SDS-PAGE with the high sensitivity of LC-MS/MS, whereby the distance of migration within a given time is coupled with the peptide sequence and spectral data by LC-MS/MS, offering an unbiased view of proteolytic cleavage events [92]. All cleavage fragments are theoretically captured with this approach, however, are more easily isolated and identified, as compared to 2D-PAGE. Time-course experiments can also be used to determine which protein fragments are stable and hence likely to be bioactive in their own right. This technology has been successfully applied to immortalized human T-lymphocyte cell apoptosis, whereby many established and novel caspase substrates (>150) were detected [92, 93].

We have used the PROTOMAP approach to identify novel KLK4 substrates in secretions from prostate cancer cell lines that represent early- and late-stage disease, as well as secretions from prostatic fibroblasts (our unpublished data). Previously, the identification of prostatic KLK4 substrates has been highly biased toward those derived from the prostate epithelium, where prostate cancer is believed to originate. However, prostate fibroblasts secrete the majority of prostatic ECM proteins, which likely constitute a rich source of substrates for prostate cancer cell-expressed KLKs, such as KLK4, as the tumor invades the surrounding stroma and ECM. In assessing KLK4 substrates in prostate fibroblasts secretions, using PROTOMAP, we have distinguished known KLK substrates previously identified biochemically, such as insulin-like growth factor binding proteins and some collagens. We have also identified several novel substrates, including growth factors and their regulators, ECM proteins, cytokines, and other proteases (our unpublished data). In addition, we have mapped the downstream transcriptional changes occurring in KLK4-treated prostate fibroblasts. Collectively, combining transcriptomic and degradomic analyses has confirmed a role for KLK4 in established pathways, such as the uPA/uPA receptor signaling axis, as well as novel pathways that have not previously been associated with KLK function. Of note, as reported above for other KLKs, we also identified the TGF-β1 signaling axis as a key KLK4 target (our unpublished data). TGF-β1 is one of the key growth factors implicated in transforming normal fibroblasts into cancer-associated myofibroblasts, a process integral to cancer progression as the latter phenotype is both permissive and supportive of cancerous outgrowth [94]. Thus, KLK4 and indeed other KLKs that are yet to be screened for fibroblast interactions using similar approaches, may contribute toward cancer progression via modulation of the stromal microenvironment.

A range of other cell types exist in the prostate stroma, including endothelial cells, with which KLK3 has been found to interact, repressing angiogenesis [95]. To this end, KLK3-activating peptides have been suggested for prostate cancer therapy [96]. Identification of KLK substrates produced on and by the range of cells within the cancer stroma will help to further our understanding of the diversity of KLK function in prostate cancer progression, including that in bone metastases. The future of cancer therapy has been suggested to involve agents that target both cancer cells and stromal tissue [97], hence the effect of KLKs need to be understood in both components for maximum therapeutic benefit. To this end, novel MS-based substrate profiling approaches can facilitate analysis of proteolytic events within the complex extracellular microenvironment and will be a highly valuable tool for future KLK research.

3.2.3 Degradomics using neo-N-(or C-) terminal enrichment strategies

Several other MS-driven proteomic techniques use chemical labeling of emergent N-(or C-)termini from protease cleavage events and chromatographic enrichment to identify these neo-N-(or C-)termini and impute the corresponding protein substrate [91]. These techniques are highly sensitive, as the complexity of the peptide pool is significantly reduced prior to identification by MS. Additionally, the precise protease cleavage site may be determined from such approaches, which can be used to rule out substrates likely cleaved by proteolytically activated proteases in the sample. One such approach, TAILS [80], has been successfully employed to identify MMP substrates in cell-derived proteomes and biological fluids [98,99].

The TAILS approach was first applied to KLKs in identifying pro-KLK7 as a proteolytic target of astacin metalloproteases [100]. Recently, Schilling et al. has used this approach to define the degradome of KLK4–7 in ovarian cancer cells overexpressing all four KLKs, simultaneously [81]. This is the most comprehensive study of KLK proteolysis to date as it uses an unbiased approach to map the KLK degradome of four KLKs in a biologically mimetic protein pool. Therein, TGF-β1 was identified as cleaved and its signaling axis altered in KLK4–7 overexpressing, but not vector control-transfected, ovarian cancer cells. Other novel substrates included TGF-β1 binding proteins and cathepsins, while several previously reported classes of substrates were also identified, such as ECM proteins (laminin and some collagens) and proteases (such as MMPs). As exemplified by this study, similar applications will likely propel the KLK field toward divulging the KLK degradome, in a biological context, as well as highlighting novel pathways of KLK function based on the substrates identified.

3.3 Challenges and opportunities in applying proteomics to define KLK function, aimed at targeting KLKs as a strategy for cancer therapy

MS-driven degradomics, although applied in only few studies in the KLK field, has identified a large body of novel KLK substrates in prostate and ovarian cancer. Even including those known KLK substrates determined biochemically, we do not believe this constitutes an exhaustive list of KLK substrates in human physiology and disease, and anticipate that further application of these techniques will identify additional biologically relevant KLK substrates. However, despite the advances conferred by MS-driven degradomics, most KLK substrates identified by novel and traditional approaches remain to be validated as KLK-regulated effectors of cancer-associated cellular pathways. Indeed, putative KLK substrates identified by proteome-wide substrate profiling must first be validated by alternative approaches, such as biochemical incubation of recombinant or purified protease and substrate, or Western blot analyses of KLK-treated conditioned media. Following its validation, the effect of this proteolytic interaction on cancerous pathology should be examined. To this end, and before we can realize the potential of KLKs as therapeutic targets, we must address the challenges in delineating the definitive biological KLK degradome and, particularly, the functional consequences of KLK-mediated proteolysis in cancer.

Importantly, given the homology between KLKs, it is likely that many share similar substrates, although the nature of this processing may differ. Clinical trials using MMP inhibitors exemplified the importance of understanding redundancy in protease cascades when targeting proteases for cancer therapy [101], hence the overlap between KLK proteolytic networks and other interacting protease classes will need to be mapped to identify nodes most amenable to therapeutic intervention. This will potentially require the application of combinations of specific KLK inhibitors, such as those described for KLK4 and KLK7 [16,102]. In characterizing substrate properties following proteolysis by various KLKs, a range of methods can be employed, including cell treatments with intact substrate versus KLK-cleaved substrate fragments, or with blocking peptides to mask the KLK cleavage site in selected substrates, followed by functional cell-based assays. Determining the resulting functional consequences of KLK proteolysis has been poorly achieved in the KLK field. Despite many studies showing cellular consequences of KLK overexpression or knockdown, neither the proteolytic activity, nor the underlying proteolytic mechanism, is reported, and so any observed effects cannot be separated from a potential nonproteolytic KLK function. It is, therefore, important that the proteolytic activity of KLKs is monitored during the time-course of these cell-based functional assays and the use of experimental controls that discriminate proteolytic from non-proteolytic actions, such as the application of proteolytically inactive KLK variants or small molecule protease inhibitors, is employed.

A final challenge of defining the KLK degradome exists in confirming substrate proteolysis and its downstream consequences, in vivo. As a step toward relevant biological significance, KLK substrate profiling may be performed using KLK-overexpressing or -knockdown tumor xenograft models. KLK knockdown models are ideal as they draw a comparison between an absence of proteolytic activity and endogenous levels of active protease, as compared to degradomic studies, to date, which have profiled the KLK substrate repertoire following treatment with sometimes super-physiological amounts of active exogenously-added KLKs, or KLKs constitutively over-expressed by transformed cell lines. Alternatively, patient tissues that have high endogenous KLK expression can be compared with those with low KLK expression. However, although limiting the amount of treatment protease is beneficial to reflect biology, it is important that the amount of protease used is not too low so as to exceed the detection limit of substrate reporter fragments for individual methods. Additionally, the complexity of the in vivo microenvironment may make substrate profiling in such tissues

difficult, and in vivo validation may instead need to take the form of targeted MRM- or antibody-based detection of the KLK-produced byproducts inferred from less complex in vitro systems. The application of such techniques, for in vivo validation of protease-substrate interactions determined in vitro, is crucial for confident identification of the biological KLK degradome.

In conclusion, using the sensitivity and high-throughput capabilities of MS-driven approaches, the interaction between KLKs and their substrates, derived from the complex tumor microenvironment, can begin to be defined. However, even those substrates that have been identified in a biologically mimetic context, using novel MS-driven degradomic approaches, must further be scrutinized for the functional consequence of their proteolytic activity on cancer progression. Thus, the fields of biochemistry, cell biology, and proteomics must be integrated for successful analysis of KLK action in prostate and ovarian cancer, with the goal of targeting these proteases as a therapeutic strategy.

We gratefully acknowledge Dr. Oded Kleifeld for his helpful review of this manuscript. Unpublished data cited herein was acquired using proteomic infrastructure in the QIMR Berghofer Protein Discovery Centre provided through the Australian Government National Collaborative Infrastructure Scheme (NCRIS) via Bioplatforms Australia and the Queensland State Government. We thank Dr. Scott Stansfield and Mr. Carson Stephens for contributions toward the cited unpublished work. Distinguished Professor Judith Clements is in receipt of a National Health and Medical Research Council (NHMRC) Principal Research Fellowship. Miss Fuhrman-Luck and Miss Silva are receiving Australian Post-graduate Award funding and Miss Fuhrman-Luck receives financial support from the Queensland Government Smart Futures Fund and Queensland University of Technology. This work was funded by grants-in-aid from the NHMRC, Prostate Cancer Foundation of Australia, Australia-India Strategic Research Fund, the Cancer Council Queensland and Wesley Research Institute Foundation.

The authors have declared no conflict of interest.

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