DISTRIBUTION OF HUMAN TISSUE KALLIKREIN-RELATED PEPTIDASES IN TISSUES AND BIOLOGICAL FLUIDS: LOCALIZATION, HORMONAL REGULATION AND PHYSIOLOGICAL FUNCTIONS IN THE FEMALE REPRODUCTIVE SYSTEM

By

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A thesis submitted in conformity with the requirements for the Degree of Doctor of Philosophy
Graduate Department of Laboratory Medicine and Pathobiology
University of Toronto

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Distribution of Human Tissue Kallikrein-Related Peptidases in Tissues and Biological Fluids: Localization, Hormonal Regulation and Physiological Functions in the Female Reproductive System

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Doctor of Philosophy 2008

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ABSTRACT

Human tissue kallikrein-related peptidases (KLK) are fifteen genes located on chromosome 19q13.4, encoding hormonally regulated, secreted serine proteases with trypsin/chymotrypsin-like activity. I identified expression of many KLKs in tissues throughout the female reproductive system and in cervico-vaginal fluid (CVF).

The female reproductive system is hormonally regulated during the menstrual cycle, suggesting KLKs may also be regulated by these hormones. Measurement of KLKs levels in CVF and saliva samples throughout the menstrual cycle revealed a peak in expression following ovulation in both fluids. Progesterone levels rise during this period suggesting KLK regulation by progesterone during the menstrual cycle.

Using proteomic techniques, I resolved the CVF proteome to identify potential KLK substrates. Among 685 proteins identified, several cell-cell adhesion molecules, cervical mucins and defense-related proteins were found.

KLKs play a role in the desquamation of skin corneocytes through cleavage of cell-cell adhesion proteins. The vaginal epithelium undergoes cyclical changes during
the menstrual cycle involving desquamation of cells upon rising progesterone levels. The post-ovulatory peak in KLK expression suggests that KLKs may contribute to cell desquamation during the menstrual cycle.

Cervical mucus acts to block the uterus from vaginal microorganisms. Around ovulation, cervical mucus loses viscosity to facilitate sperm passage through the cervix. Proteolytic enzymes are thought to aid in this mucus remodelling. Our immunohistochemical studies localized KLK expression to the mucus secreting cervical epithelium and I investigated KLK processing of cervical mucin proteins in vitro. KLKs 5 and 12 were found to cleave mucins, suggesting their potential involvement in cervical mucus remodelling.

CVF plays a role in host defense. KLKs are known to process the antimicrobial cathelicidin protein in skin and I investigated whether KLKs may also process antimicrobial proteins found in CVF. KLK5 was found to cleave defensin-1 alpha, in vitro, suggesting KLKs may aid in defense of the female reproductive system.

Here I provide evidence of potential physiological roles for KLKs in the female reproductive system: in desquamation of vaginal epithelial cells, remodelling of cervical mucus and processing of antimicrobial proteins. These findings suggest KLKs may function in female fertility, in pathological conditions such as vaginitis and in host defense.
Acknowledgements

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Thank you to my MSc. supervisor, Dr. Lois Mulligan, for taking a chance on me as a young graduate student, with no experience, in the first place. You have been a wonderful mentor.

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<tr>
<td>aa</td>
<td>amino acid</td>
</tr>
<tr>
<td>ACN</td>
<td>acetonitrile</td>
</tr>
<tr>
<td>ACPT</td>
<td>testicular acid phosphatase gene</td>
</tr>
<tr>
<td>ACT</td>
<td>$\alpha_1$-antichymotrypsin</td>
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<tr>
<td>ALP</td>
<td>alkaline phosphatase</td>
</tr>
<tr>
<td>AMC</td>
<td>7-amino-4-methylcoumarin</td>
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<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
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<tr>
<td>ARE</td>
<td>androgen response element</td>
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<tr>
<td>AT</td>
<td>$\alpha_1$-antitrypsin</td>
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<tr>
<td>BCA</td>
<td>bicinchoninic acid</td>
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<tr>
<td>Bis-Tris</td>
<td>2-[bis(2-hydroxethyl)amini]-2-(hydroxymethyl)propane-1,3-diol</td>
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<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BPE</td>
<td>bovine pituitary extract</td>
</tr>
<tr>
<td>BPH</td>
<td>benign prostatic hyperplasia</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
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<tr>
<td>CID</td>
<td>collision-induced dissociation</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
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<tr>
<td>DFP</td>
<td>diflunisal phosphate</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagles medium</td>
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<td>DMSO</td>
<td>dimethylsulfoxide</td>
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<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<td>DSC</td>
<td>desmocollin</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<td>DSG</td>
<td>desmoglein</td>
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<td>DTT</td>
<td>dithiothreitol</td>
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<td>ECM</td>
<td>extracellular matrix</td>
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<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<td>EGF</td>
<td>epidermal growth factor</td>
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<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<tr>
<td>EMT</td>
<td>epithelial-mesenchymal transition</td>
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<tr>
<td>ER</td>
<td>estrogen receptor</td>
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<tr>
<td>ES</td>
<td>electrospray</td>
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<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
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<td>FSH</td>
<td>follicle stimulating hormone</td>
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<td>GO</td>
<td>genome ontology</td>
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<td>GnRH</td>
<td>gonadotrophin releasing hormone</td>
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<tr>
<td>GST</td>
<td>glutathione-s-transferase</td>
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<tr>
<td>h-CAP-18</td>
<td>human cathelicidin protein-18</td>
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<td>HEK</td>
<td>human embryonic kidney</td>
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<tr>
<td>HGF</td>
<td>hepatocyte growth factor</td>
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<tr>
<td>hGH</td>
<td>human growth hormone</td>
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<td>HPLC</td>
<td>high-performance liquid chromatography</td>
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<td>HREs</td>
<td>hormone response elements</td>
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<td>IE</td>
<td>immunoexpression</td>
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<td>IGF</td>
<td>insulin growth factor</td>
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<td>IGFBP</td>
<td>insulin-like growth factor binding protein</td>
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<td>IPI</td>
<td>international protein index</td>
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<td>IR</td>
<td>insulin receptor</td>
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kb     kilobase pairs
kDa     kiloDalton
ψKLK    KLK pseudogene
KLK     human tissue kallikrein-related peptidase gene
KLK     human tissue kallikrein-related peptidase protein
LC      liquid chromatography
LCR     locus control region
LEKTI   lymphoepithelial Kazal-type related inhibitor
LH      leutinizing hormone
MALDI   matrix-assisted laser desorption ionization
MBP     myelin basic protein
MMP     matrix metalloprotease
MS      mass spectrometry
MS/MS   tandem mass spectrometry
mRNA    messenger ribonucleic acid
MUC     mucin
NES1    normal epithelial cell-specific 1 gene
NSCLC   non-small-cell lung carcinoma
PAI     plasminogen activator inhibitor
PAR     protease-activated receptor
PBS     phosphate-buffered saline
PCR     polymerase chain reaction
PNGaseF Peptide: N-Glycosidase F
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<tr>
<td>PSA</td>
<td>prostate-specific antigen</td>
</tr>
<tr>
<td>PR</td>
<td>progesterone receptor</td>
</tr>
<tr>
<td>p value</td>
<td>probability value</td>
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<tr>
<td>PVDF</td>
<td>polyvinylidene diflouride</td>
</tr>
<tr>
<td>RPMI</td>
<td>Rossman-Park-Memorial-Institute</td>
</tr>
<tr>
<td>RSL</td>
<td>reactive site loop</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcriptase-polymerase chain reaction</td>
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<tr>
<td>SCX</td>
<td>strong cation-exchange</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecylsulfate-polyacrylamide gel Electrophoresis</td>
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<td>Serpin</td>
<td>serine protease inhibitor</td>
</tr>
<tr>
<td>SLPI</td>
<td>secretory leukocyte protease inhibitor</td>
</tr>
<tr>
<td>SNP</td>
<td>single nucleotide polymorphism</td>
</tr>
<tr>
<td>SPINK5</td>
<td>serine protease inhibitor kazal-type 5</td>
</tr>
<tr>
<td>TBS-T</td>
<td>tris-buffered saline-Tween</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>uPA</td>
<td>urokinase plasminogen activator</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
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Chapter 1: Introduction
1.1 **Proteases**

Proteolytic enzymes (proteases) are defined as enzymes which catalyze peptide bonds (1) and are generally divided into two categories: exopeptidases and endopeptidases. Exopeptidases cleave amino acids from the N-terminus or C-terminus of proteins whereas endopeptidases cleave proteins internally (1). Proteases can be divided into families in which all members share amino acid sequence similarity in the region of the molecule required for enzymatic activity. Proteases are also classified into clans which contain groups of families, all of which descended from a common ancestral protein (1). Within a clan members share similar three dimensional structures and arrangement of the catalytic residues. The clans are named with two letters: the first letter representing the type of catalytic activity exhibited by the proteases, either: serine, cysteine, aspartic, or metallo, based on the active amino acid involved in hydrolysis (1). The second letter is arbitrary.

1.2 **Serine proteases**

Serine proteases, which contain an active serine residue, play roles in coagulation, immunity and digestion and are grouped into eleven clans based on their evolutionary relationships (1). Members of clan SA contain catalytic triad residues ordered histidine, aspartic acid and serine. Within clan SA, family S1 contains all endopeptidases with structural similarity to trypsin (1). This is the largest family and contains trypsin, chymotrypsin, complement components, coagulation factors, granzymes, plasmin, urokinase plasminogen activator and tissue kallikreins (1;2).
1.3 Hydrolysis of peptide bonds by serine proteases

The mechanism of hydrolysis of a peptide bond by a serine protease occurs in two steps: acylation and deacylation. Acylation involves nucleophilic attack by an oxygen molecule within a hydroxyl group of the catalytic serine (serine 195) on the carbonyl carbon of the peptide bond to be hydrolyzed. Serine 195 donates a proton (from the hydroxyl group of the attacking oxygen molecule) to histidine 57 and aspartic acid 102 helps to orient histidine 57 correctly and neutralize the histidine’s developing positive charge. As a result, the substrate becomes covalently attached to the enzyme in an intermediate tetrahedral complex in which the carbonyl carbon of the substrate (the acid portion) is esterfied to the catalytic serine and the amine portion of the substrate is hydrogen bonded to histidine 57. This complex is stabilized by hydrogen bonds formed between two NH groups on the enzyme and the negatively charged carbonyl oxygen (the oxyanion) of the substrate. This site is referred to as the oxyanion hole.

The second stage of the reaction is called deacylation and involves hydrolysis of the enzyme substrate complex by water. Histidine 57 accepts a proton from a water molecule leaving a hydroxyl group free to attack the carbonyl carbon of the substrate-enzyme complex. Once again, a tetrahedral intermediate is formed following which histidine 57 donates a proton to the oxygen atom of serine 195 and the acidic component of the substrate is released. At this point, the enzyme is free to begin catalysis again.
1.4 Human tissue kallikrein-related peptidases (KLK)

As mentioned, human tissue kallikrein-related peptidases are members of family S1 of clan SA of serine proteases. These enzymes are the focus of this work and are the only serine protease which will be discussed further.

1.4.1 KLK history

The original kallikrein protein was discovered in the 1930’s by Werle and colleagues who identified high levels of this protein in pancreatic tissue (4). In the 1980’s, two additional kallikrein genes were identified, human glandular kallikrein 2 (5) and prostate-specific antigen (PSA) (6). The kallikrein family was thought to consist of only these three genes which mapped to chromosome 19q13.4 (7;8). In the late 1990’s, the kallikrein gene family was found to contain twelve additional family members, KLK4-15 and KLK1-3 became known as the “classical” kallikreins (9;10).

1.4.2 KLK locus

Kallikrein-related peptidase genes are found in a contiguous cluster spanning approximately 265 kb on chromosome 19q13.4 (10;11) and are known to be the largest contiguous cluster of protease genes in the human genome (12). The fifteen KLK genes are organized in tandem with no interfering non-kallikrein genes and the locus is bound centromerically by testicular acid phosphatase gene (ACPT) (13) and telomerically by Siglec-9 – a member of the sialic-acid binding Ig-like lectin (Siglec) family (14) (Figure 1.1). With the exception of KLK2 and KLK3, all kallikrein genes are transcribed from telomere to centromere (15).
1.4.3 Phylogeny of the kallikreins

Kallikrein gene families have been characterized in seven families, including: human, mouse, rat, chimpanzee, dog, pig and opossum (16). All families have at least one copy of KLK5-15 genes, whereas variability and duplication exists mainly among the classical kallikreins, KLK1-3. The klk family is larger in the mouse and rat genomes with an excess of genes, resulting from duplication, located between klks 1-15 (11). KLK1 is conserved among the mouse, rat, dog and pig genomes and there are no mouse, rat, dog, pig or opossum orthologues to KLKs 2 or 3 (11). Interestingly, the opossum genome is also missing and orthologue to KLK4 (16). The KLK genome is fully conserved in the chimpanzee (16).

Five main subfamilies are found to exist within the kallikrein locus and are suggested to share recent ancestry: KLK4, 5, 14; KLK9, 11, 15; KLK10, 12, KLK6, 13, and KLK8, 1, 2, 3 (16).
**Figure 1.1: The KLK Locus**

The 15 KLK genes and 1 pseudogene are organized in tandem on chromosome 19q13.4. The “classical” KLKs (KLKs 1-3) are shown in orange. The direction of transcription is represented by the arrow representing each gene.
1.4.4 KLK gene structure

Within the KLK family, members share 30-50% similarity at the DNA and amino acid level, with the exception of KLKs 2 and 3 which share 80% similarity (10). KLKs are structurally similar in that they are all composed of five coding exons of similar coding length (13) (Figure 1.2). The intron lengths are variable, however the intron phases, I-II-I-O, are conserved among all members. Structural differences between KLKs are found mostly in non-coding regions; KLK4-15 contain variable length untranslated (UTR) regions at the 5′ prime end, which are not found in the three classical KLKs (13;17) and UTR beyond the stop codons vary in length (12). The classical KLKs (1-3) also contain an additional 9-11 amino acid “kallikrein loop” not found in any of the other KLKs (13).

With the exception of KLK14, alternative transcripts have been identified for each of the KLKs. Most splicing events occur within coding regions and most often involve exon skipping (18). In addition, consensus GT-AG splice sites are found to be conserved in most KLKs (2).
All KLKs contain 5 coding exons of similar length and intervening introns of variable length. The intron phase is conserved among all KLKs. KLKs 4-15 contain variable length 5' UTR regions and, in all KLKs, 3' UTR lengths vary. The location of catalytic triad residues, H, D and S in exons 2, 3 and 5 respectively is conserved among all members of the KLK family.
1.4.5 Single nucleotide polymorphisms (SNPs) within the KLK locus

SNPs, the most common type of variation within the human genome (19). SNPs account for 86% of the polymorphisms in the KLK locus, the majority of which (42%) are located in non-coding regions or UTR. 3.8% of the SNPs in the KLK locus are found in coding regions (20). Non-synonomous SNPs have been validated in KLKs 1, 2, 3, 4, 5, 8, 10, 11, 14 and 15, of which the largest number are located in KLK1 (20).

1.4.6 KLK protein structure

KLK genes encode secreted, serine proteases with either trypsin-like or chymotrypsin-like specificity (13). Characteristic of serine proteases, KLK proteins contain the typical “catalytic triad” residues, histidine, aspartic acid, and serine, encoded in exons 2, 3 and 5 respectively (Figure 1.3) (13). Maximum similarity between KLKs exists around these catalytic triad residues as well as in the position and number of cysteine residues which are integral in the formation of disulfide bonds within the KLK proteins (12).

KLKs are translated as pre-pro enzymes containing a signal peptide of 16-34 amino acids in length at their N-termini, which targets them to the endoplasmic reticulum for secretion (11) (Figure 1.4). The signal peptide is followed by a pro-peptide of 4-37 amino acids in length, which is followed by the mature, catalytically active form of the protein (13). The signal peptide is removed when the proteins are released from the secretory pathway leaving the inactive zymogen. Removal of the pro-peptide by proteolytic cleavage causes a conformational change and opening of the active site, resulting in the enzymatically active form of the enzyme (11). With the exception of KLK4, all
KLKs contain an arginine or lysine residue preceding the pro-peptide cleavage site, indicating that trypsin-like cleavage is required for removal of the pro-peptide and subsequent KLK activation (13). This cleavage can be mediated by the KLK protein itself (auto-activation), by other KLKs or by other trypsin-like enzymes.

Based on the amino acid sequences, the molecular weights of the KLKs are expected to be between 23,000 and 26,000 Da, however molecular weights tend to vary because of N-glycosylation of many KLKs (2). The majority of KLKs have been confirmed as being N-glycosylated in either their native form or recombinant form such as: KLK1 (21), KLK3 (22), KLK4 (23), KLK5 (24), KLK6 (our unpublished observations), KLK7 (25), KLK8 (26), KLK11 (observations by Carolyn Goard, undergraduate thesis), KLK12 (my unpublished observations), KLK13 (27), and KLK15 (28).

Crystal structures have been analyzed for KLK1 (29), KLK3 (30), KLK4 (31), KLK5 (32), KLK6 (33) and KLK7 (34;35). KLK structures appear quite similar based on these structural analyses all consisting of two adjoining six-stranded β-barrels, two alpha-helices and variable surface loops. The catalytic triad is consistently located along the junction of the two β-barrels with the active site running perpendicular to this junction. Variability exists among the surface loops and is thought to contribute to individual KLK substrate specificities.
Figure 1.3: KLK Protein Structure

KLKs are translated as pre-proenzymes containing a signal peptide which is cleaved upon their exit from the secretory machinery, resulting in secretion of the inactive proenzyme. Activation of the enzyme occurs upon removal of the pro-peptide through enzymatic cleavage.
1.4.7 KLK substrate specificity

The substrate specificity of serine proteases is determined by the amino acid found in the S1 binding pocket (36), which is found six amino acids N-terminal of the catalytic serine residue, amino acid 189 (by chymotrypsin numbering (37). The majority of KLKs (KLKs 1, 2, 4, 5, 6, 8, 10, 11, 12, and 13) contain an aspartate or glutamate at this site, able to bind positively charged amino acids, conferring trypsin-like specificity and a preference to cleave following arginine or lysine residues (Table 1). KLKs 3, 7 and 9 contain large bulky amino acids in the S1 position (serine, asparagine and glycine, respectively) conferring chymotrypsin-like specificity (13). Experimental verification of tryptic-like and chymotryptic-like specificities has been performed for all KLKs except KLKs 9 and 10 (2). Specific substrate specificities have been studied for some KLKs using techniques such as: phage display (38-41), combinatorial libraries, fluorescence resonance energy transfer (FRET) peptide libraries (42;43) and kinetic assays (44-47).
Table 1.1: Amino acid present in S1 binding pocket and substrate specificity of each KLK.

<table>
<thead>
<tr>
<th>KLK</th>
<th>Amino acid of Substrate Binding Pocket&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Confirmed Substrate Specificity&lt;sup&gt;1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>D</td>
<td>trypsin-like</td>
</tr>
<tr>
<td>2</td>
<td>D</td>
<td>trypsin-like</td>
</tr>
<tr>
<td>3</td>
<td>S</td>
<td>chymotrypsin-like</td>
</tr>
<tr>
<td>4</td>
<td>D</td>
<td>trypsin-like</td>
</tr>
<tr>
<td>5</td>
<td>D</td>
<td>trypsin-like</td>
</tr>
<tr>
<td>6</td>
<td>D</td>
<td>trypsin-like</td>
</tr>
<tr>
<td>7</td>
<td>N</td>
<td>chymotrypsin-like</td>
</tr>
<tr>
<td>8</td>
<td>D</td>
<td>trypsin-like</td>
</tr>
<tr>
<td>9</td>
<td>G</td>
<td>unconfirmed chymotrypsin-like</td>
</tr>
<tr>
<td>10</td>
<td>D</td>
<td>unconfirmed trypsin-like</td>
</tr>
<tr>
<td>11</td>
<td>D</td>
<td>trypsin-like</td>
</tr>
<tr>
<td>12</td>
<td>D</td>
<td>trypsin-like</td>
</tr>
<tr>
<td>13</td>
<td>D</td>
<td>trypsin-like</td>
</tr>
<tr>
<td>14</td>
<td>D</td>
<td>trypsin and chymotrypsin-like</td>
</tr>
<tr>
<td>15</td>
<td>E</td>
<td>trypsin-like</td>
</tr>
</tbody>
</table>

<sup>1</sup> Taken from (2;13)
1.4.8 KLK regulation by steroid hormones

Many KLKs have been shown to be regulated by steroid hormones in various cancer cell lines (2,48). Steroid hormones bind to nuclear receptors, which dimerize and act as ligand-induced transcription factors (49). Transcriptional control of target genes by steroid hormone-receptor complexes can be either direct, or indirect. Steroid hormone receptors contain DNA binding domains and direct control of transcription is achieved by steroid hormone-receptor complex recognition and binding to specific cis-acting DNA sequences, referred to as hormone response elements (HRE), located in the 5’ regulatory regions on target genes (49). Steroid hormone-receptor complexes can also control transcription of target genes indirectly through their interactions with trans-acting transcription factors.

Regulation of KLKs 2 and 3 by androgens has been studied extensively in both breast and prostate cancer cells and in vivo. Androgen response elements (ARE) have been identified in the promoter regions of KLK2 (50) and KLK3 (51). KLKs 1, 6, and 10 have been shown to be regulated by estrogens (48). An estrogen response element has been identified in the KLK1 promoter (52), however no HRE have been identified in the promoter regions of KLKs 6 and 10. KLKs 10, 11, 13, 14 have been shown to be coordinately regulated by dihydrotestosterone (DHT, an androgen) in several breast cancer cell lines (48), however no HRE have been identified in the promoter regions of any of these genes, suggesting the action of trans-acting factors. It has also been suggested that a cis-acting locus control region (LCR) may exist which results in coregulation of many KLKs in the rat salivary gland (53).
Recently, KLKs have been shown to be regulated by glucocorticoids, particularly in breast and cervical cancer cell lines (54). In general, KLKs 5, 6, 8, 10 and 11 have been found to be co-upregulated in several breast cancer cell lines and co-downregulated in several cervical cancer cell lines by the synthetic glucocorticoid, dexamethasone (my unpublished observations).

1.4.9 Epigenetic control of KLKs by methylation

Hyper-methylation of CpG islands within the promoter of a gene or within the coding region of a gene results in transcriptional silencing. Methylation of CpG islands within the *KLK10* promoter have been shown to be responsible for downregulation of KLK10 mRNA and protein expression in breast, prostate and ovarian cancer cells (55). Similarly, *KLK10* expression was found to be significantly downregulated in gastric cancer tissues compared to normal gastric tissues using *in situ* hybridization (56). Reduced *KLK10* expression in gastric cancer tissues was suggested to be caused by methylation of CpG islands in exon 3. In addition, KLKs 5, 6, 11 and 12 are proposed as being regulated by DNA methylation in certain cancer cell lines (57;58).

1.4.10 Post-translational regulation of KLKs

Proteases catalyze reactions irreversibly which results in a need for strict control of their function. KLK functions are controlled through several mechanisms such as: zymogen activation, internal cleavage and endogenous inhibitors.

All proteases are synthesized as inactive zymogens containing a pro-peptide which sterically blocks the active site, rendering substrates unable to bind. Cleavage of the pro-peptide changes the conformation of the enzyme’s
substrate binding pocket and active site therefore allowing the substrate to bind. Cleavage of the pro-peptide can be achieved by the enzyme itself by autolysis, or by other enzymes. Autoactivation has been shown for several KLKs including, KLK2 (59), KLK4 (60), KLK5 (45), KLK6 (61), KLK12 (47) and KLK13 (27). Initiation of autolysis or cleavage of the pro-peptide by an independent enzyme can be controlled by several mechanisms including the presence or absence of specific enzyme cofactors and changes in pH.

Several KLKs (2, 3, 6, 7, 13, 7, 14) have also been shown to undergo internal cleavage, resulting in degradation and inactivation of the enzyme (2). This internal cleavage can occur by the enzyme itself (autolysis) or by another enzyme.

Many KLKs are inhibited by endogenous inhibitors such as serpins (alpha-2 antitrypsin, alpha-1 antichymotrypsin, plasminogen activator inhibitor, antithrombin III and alpha 2 antiplasmin) (2). KLKs 1, 2, 3, 5, 6 and 13 have been shown to form complexes with these inhibitors in biological fluids such as serum, ascites, cerebrospinal fluid and breast milk (2). Interaction of KLKs and serpins occurs by one of three pathways: 1) the inhibitory pathway, whereby a complex forms between the enzyme and the inhibitor which deforms and irreversibly inactivates the protease. 2) the substrate pathway whereby the serpin is cleaved by the protease and does not result in inhibition of the protease and 3) the trap pathway in which a large inhibitor binds to the enzyme, not rendering it inactive, but sterically blocking binding of other substrates.

In addition to serpins, KLK activity is also known to be controlled by the lympho-epithelial kazal-type-related inhibitor (LEKTI) in skin (KLKs 5, 6, 7, 13 and
14 (62-64). KLKs 2, 3, 5, 12 and 14 have been shown to be reversibly inhibited by the divalent cation, zinc, which binds to these KLKs at two allosteric sites (45-47).

1.4.11 KLK tissue expression patterns

KLK expression levels have been examined for all KLKs at the mRNA and protein level in multiple panels of human tissues and biological fluids (65) and will be discussed in detail in chapter 3. However, in general, KLKs can be divided into three groups based on their expression patterns (Table 1.2): very restricted KLKs (KLKs 2 and 3) (expressed at high levels in one tissue); restricted KLKs (KLKs 5, 6, 7, and 8) (expressed relatively highly in two or three tissues and at lower levels in various other tissues); widely expressed KLKs (KLKs 1, 4, 9, 10, 11, 12, 13, 14 and 15) (expressed at varying levels in a wide range of tissues) (65). KLKs are often found co-expressed in tissues, for example: KLKs 2, 5, 6, 8, 9, 11, 14 and 15 in the breast, KLKs 6 and 11 in the central nervous system (CNS), KLKs 1, 4, 5, 6, 7, 9, 10, 11, 12 and 13 in the cervix, KLKs 1, 3, 6, 7, 9, 11 and 15 in the kidney, KLKs 1, 6, 7, 10, and 11 in the ovary, KLKs 1, 2, 3, 4, 5 and 11 in the prostate, KLKs 1, 5, 6, 7, 10, 11, 13 and 15 in the salivary gland, KLKs 1, 5, 6, 7, 8, 9, 10, 11, 14 and 15 in the skin and KLKs 1, 5, 6, 7, 9, 10, 11, 12, 13 and 14 in the vagina (65). KLKs are also co-expressed in many biological fluids including seminal plasma, breast milk, and cervico-vaginal fluid (65).

Immunohistochemical analysis has shown that KLKs are mostly localized in the cytoplasm of granular epithelium and are most likely secreted from these glands (66).
Table 1.2: Abundance patterns of kallikreins, categorized according to levels in adult tissues.

<table>
<thead>
<tr>
<th>Tissue Abundance$^{1,2}$</th>
<th>Very Restricted (tissue)</th>
<th>Restricted (tissue)</th>
<th>Wide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>KLK2 (prostate)</td>
<td>KLK5 (skin, salivary, breast, esophagus)</td>
<td>KLK1</td>
</tr>
<tr>
<td></td>
<td>KLK3 (prostate)</td>
<td>KLK6 (brain/CNS)</td>
<td>KLK4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>KLK7 (esophagus, heart, liver, skin)</td>
<td>KLK9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>KLK8 (breast, esophagus, skin, tonsil)</td>
<td>KLK10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>KLK13 (esophagus, tonsil)</td>
<td>KLK11</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>KLK12</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>KLK14</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>KLK15</td>
</tr>
</tbody>
</table>

$^1$ data taken from (65)

$^2$ see chapter 3 for further discussion.
1.4.12  KLK participation in proteolytic cascades

KLK coexpression and coregulation in tissues and biological fluids suggests that they may participate in proteolytic cascades, similarly to the complement and coagulation cascades. Proteolytic cascades are carried out in three phases: initiation, propagation and execution (67). Initiation occurs by autolysis of an initiator zymogen enzyme which then activates intermediary propagating enzymes. These propagating enzymes process and activate executor zymogens and it is these enzymes which ultimately cleave the end product in a controlled manner (67). Partial proteolytic cascades involving KLKs have been delineated in the skin and seminal plasma. KLKs 2, 3, 5 and 14 are implicated in the seminal plasma cascade in which KLKs 5 and 14 are suggested as the initiating enzymes (68;69). KLKs 5, 7 and 14 have been shown to participate in a cascade in the skin and similarly, KLKs 5 and 14 are thought to be the initiating enzymes in this cascade (62;70).

1.4.13  Physiologic Roles for KLKs

1.4.13.1  The classical KLKs

Physiologic functions are relatively well established for the classical KLKs. KLK1 cleaves low molecular weight kininogen resulting in the release of bradykinin which binds to bradykinin B1 and B2 receptors. This binding mediates processes such as blood pressure, smooth muscle contraction, vascular permeability, electrolyte balance and inflammation (71).

As mentioned above, KLKs 2 and 3 participate in a proteolytic cascade in seminal plasma (68). KLKs 2 and 3 are the executor KLKs in this cascade and
are specifically responsible for the cleavage of semenogellin proteins resulting in liquefaction of the seminal clot following ejaculation (72;73).

1.4.13.2 KLK roles in skin physiology: desquamation

KLKs 5, 7 and 14 participate in a proteolytic cascade in skin, as previously mentioned (70). These KLKs play a role in the desquamation of skin corneocytes through their cleavage of desmosomes containing cell-cell adhesion molecules such as desmogleins and desmocollins (62;74;75).

Desmosomes are disc-shaped, intercellular junctions between epithelial cells (76) which are connected to the cytoskeleton through intermediate filaments (77). Desmosomes are composed of two types of cadherins, desmocollins (DSC) and desmogleins (DSG). There are three DSC isoforms (DSC1-3) and three DSG isoforms (DSG1-3) encoded by genes on chromosome 18q12.1 (77). DSC2 and DSG2 are ubiquitously expressed in all tissues where desmosomes are formed. DSCs and DSGs 1 and 3 are more restricted in their expression and found in stratified squamous epithelia of the epidermis, esophagus, cervix and vagina.

Structurally, DSCs and DSGs are similar in that they are synthesized as precursor proteins containing a signal peptide and propeptide and they all contain four extracellular cadherin repeats in their extracellular domains, a transmembrane domain and an intracellular domain. DSCs and DSGs primarily differ in their cytoplasmic domains (Figure 1.4).

Desmosomes are composed of two main portions, the extracellular core domain (ECD) and the inner plaque region. The ECD is largely composed of the extracellular domains of DSGs and DSCs as shown in Figure 1.5 (77).
Figure 1.4: Schematic diagram of DSG and DSC proteins

DSGs and DSCs are synthesized as precursor proteins containing a signal peptide (SP) and a propeptide (P). DSGs and DSCs contain an extracellular domain with four cadherin (EC) repeats. They contain a justamembrane extracellular anchor domain (EA) and a transmembrane domain (TM). The intracellular domain is made up of an intracellular cadherin-specific domain (ICS), a proline-rich linker (IPL) and a repeating unit domain (RUD). DSGs contain an additional DSG terminal domain (DTD).
**Figure 1.5: Schematic diagram of a desmosome**

The adhesive interface formed by the interaction of the extracellular domains of desmosomal cadherins is represented between the two plasma membranes (PM) of opposing cells. The extracellular domains of DSGs and DSCs extend into the extracellular space between the two cells.

1.4.13.3  **KLKs in skin pathologies**

KLK levels are known to be upregulated in many skin disorders, such as psoriasis, atopic dermatitis and peeling skin syndrome (78-80). In the autosomal recessive skin disorder, Netherton syndrome, mutations in the *SPINK5* gene, encoding the LEKTI protein, result in truncation of this protein and loss of inhibitory domains responsible for KLK inhibition. This results in uninhibited serine protease activity and overdesquamation of skin corneocytes in these patients (62,81-83).

1.4.13.4  **Antimicrobial roles for KLKs in skin**

KLKs have recently been shown to play a role in host defense in skin and sweat through cleavage of the antimicrobial human cathelicidin protein, hCAP-18 (84). hCAP18 is the only human member of the cathelicidin family (85) and is expressed in the epithelium of many tissues, including those in the cervix and vagina (86). hCAP18 is composed of an N-terminal cathelin domain and C-terminal LL-37 antimicrobial domain; proteolytic cleavage from the cathelin domain liberates the active LL-37 antimicrobial peptide.

KLK5 has been shown to cleave hCAP18 and liberate active LL-37 (84) and both KLK5 and 7 are capable of further digesting LL-37 into smaller antimicrobial peptides in sweat (84).

1.4.13.5  **KLK4 in tooth development**

Dental enamel formation consists of three phases: secretory, transition and maturation. KLK4 is found to be expressed from the beginning of the transition stage and throughout the maturation stage (87). KLK4 is responsible for degradation and removal of enamel proteins, such as amelogenin, during the
maturation stage. The removal of enamel proteins is necessary for sufficient mineral deposition and subsequent hardening of enamel.

KLK4 has also been implicated in amelogenesis imperfectas (AI), an autosomally recessive inherited disorder characterized by faulty tooth enamel development (88). A mutant form of KLK4 has been found in patients with AI containing a point mutation at nucleotide 2142. This mutation results in production of a truncated protein, 153 amino acids in length, which lacks the catalytic serine residue required for enzymatic activity (88). Phenotypically this mutation results in a discoloration of enamel thought to be the result of increased matrix protein retention due to inappropriate processing by KLK4 (89).

1.4.13.6 KLK6 in the central nervous system

KLK6 is known to be expressed in the central nervous system (CNS), where it was found abundantly expressed in neurons and oligodendrocytes (90). Physiological substrates for KLK6, such as myelin basic protein (91) and the amyloid protein (44) have been identified in the CNS. Increased levels of KLK6 have been found in inflammatory CNS lesions, suggesting that KLK6 may promote the demyelination of neurons and therefore may play a role in the development of multiple sclerosis (90). In contrast, decreased expression of KLK6 has been found in brain tissue from Alzheimer’s patients compared to tissue from normal patients (92) and KLK6 has been proposed as a potential biomarker for Alzheimer’s disease (93).

1.4.13.7 KLK processing of human growth hormone

KLKs 5-8 and 10-14 have been shown to colocalize with human growth hormone (hGH) in the pituitary gland and have been demonstrated to cleave
hGH *in vitro* (94). KLK activity was shown to be controlled by LEKTI, in the pituitary gland, similarly as in skin. Growth retardation is one hallmark characteristic of Netherton syndrome which, as mentioned above, is caused by loss of the *SPINK5* gene and resulting LEKTI protein (64). It has been suggested that the loss LEKTI may result in over-processing of hGH by KLKs and contribute to the growth retardation phenotype (94).

### 1.4.14 KLKs as signaling molecules

KLK signaling through kinins has been quite well characterized. KLKs 1, 2 and 12 have been shown to release lysyl-bradykinin from kininogens. Vasoactive kinin is then free to bind and signal through two G-protein coupled receptors (GPCR), B1 and B2 (95). Binding of kinins to their receptors can control a variety of functions, depending on the target organ, such as: vasodilation, smooth muscle relaxation and inflammation (71).

Signaling through the urokinase plasminogen activator (uPA)-urokinase plasminogen activated receptor (uPAR) system has been demonstrated for KLKs 2 (96) and 4 (60). The uPA family consists of serine proteases involved in the degradation of the basement membrane and extracellular matrix (ECM), implicating these enzymes in tumour cell invasion and metastasis (97). uPA is translated as a proenzyme requiring cleavage by a trypsin-like enzyme for its activation. As mentioned, KLKs 2 and 4 have been shown to cleave and activate uPA which catalyzes the conversion of plasminogen into plasmin and subsequent activation of several matrix metalloproteases (MMPs). These active MMPs are responsible for degradation of the ECM leading to cell detachment and migration. KLKs 2 and 4 are expressed in the prostate and are suggested to play a role in
malignant processes in the prostate through the pathway described above (60;96;97).

KLKs have also been shown to signal through protease-activated receptors (PARs), a family of four cell surface, G-protein coupled receptors (PAR 1-4) (98). Cleavage within the N-terminal domain of PARs creates a tethered ligand able to activate the receptor and initiate downstream signaling (98).

In some cases, proteolytic cleavage occurs downstream of the N-terminal tethered ligand site resulting in disarming and inactivation of the receptor. PARs are known to be involved in regulation of certain functions such as: homeostasis, inflammation and pain.

KLKs 4, 5, 6 and 14 have been shown to cleave and activate PAR2 leading to calcium release (99;100). PAR2 plays a role in inflammation and nociception, suggesting a potential role for KLKs in these processes. KLK4 was specifically shown to induce phosphorylation and activation of extracellular regulated kinase (ERK)1/2 downstream of PAR2 (100). KLK14 was also shown to activate PAR4 and cause platelet aggregation, and was found to cleave and disarm PAR1 (99).

Recently, KLKs 4 and 5 were shown to cleave and activate hepatocyte growth factor activator (HGFA) (101). Hepatocyte growth factor (HGF) known to play a role in tumour progression through binding to its target receptor, the MET receptor tyrosine kinase (102). HGFA is a potent activator of HGF and is secreted as an inactive zymogen. Proteolytic cleavage results in the generation of two chains, a light chain and a heavy chain, of which the light chain exhibits enzymatic activity (103). KLKs 4 and 5 were found to cleave and activate HGFA
leading to activation of HGF and subsequent phosphorylation and activation of the MET receptor \(^{(101)}\). KLKs 4 and 5 were also shown to be inhibited by a member of the kunitz-type-serine protease family, the HGFA inhibitor type 1 (HAI-1) \(^{(101)}\). These results suggest that KLKs may contribute to malignancy through activation of the MET signaling pathway, which is known to be important in invasive tumour growth \(^{(101\text{-}102)}\).

**1.4.15 KLKs and cancer pathophysiology**

As previously mentioned, KLKs are known to be dysregulated in many hormone-related malignancies, such as breast, prostate and ovarian cancer \(^{(104)}\). As an example, KLKs 4, 5, 6, 7, 8, 10, 11, 13, 14 and 15 have all been shown to be overexpressed in either ovarian cancer tissues, serum from ovarian cancer patients or in ovarian cancer cell lines, at the mRNA or protein level \(^{(104)}\). As such, several KLKs have been implicated in malignant progression through several possible mechanisms.

Epithelial-mesenchymal transition (EMT) is an important step in cancer progression to a malignant phenotype \(^{(105)}\) and a key marker for cells undergoing EMT is the loss of the E-cadherin adhesion molecule \(^{(105)}\). KLKs 3 and 4 have been shown to be involved in the EMT of prostate cancer cells. Stable expression of these KLKs in PC3 prostate cancer cells resulting in these cells acquiring a more invasive phenotype as well as mesenchymal characteristics, such as the loss of E-cadherin expression \(^{(106)}\).

Insulin growth factors (IGF) are mitogenic peptides which have been shown to signal through the insulin growth factor receptor (IGFR) and control processes such as: normal and malignant cell proliferation, as well as
differentiation, apoptosis and transformation (107). Before IGF can signal via the IGFR, they must be released from insulin growth factor binding proteins (IGFBP). Several KLKs, including KLKs 2, 3, 4, 5, and 14 have been shown to cleave insulin growth factor binding proteins (IGFBP) which may result in tumour growth (45;46;108;109).

Degradation of the extracellular matrix (ECM) is a crucial step in the invasion and malignant spread of cancer cells. KLKs are thought to play a similar role to MMPs in their ability to participate in processing of ECM proteins. KLKs 4, 5, 6, 7, 8, 13 and 14 have all been shown to degrade ECM components directly (45;46;110-114). Some KLKs are thought to influence ECM degradation indirectly. KLK1 is able to activate MMP-2 and MMP-9 which in turn degrade collagen (104). As mentioned above, KLKs 2 and 4 activate the uPA-uPAR system which can also result in degradation of the ECM.

In contrast to the cancer promoting roles for KLKs outlined above, KLKs 3 and 10 have been implicated in tumour suppression. Specifically, KLK3 has been shown to activate TGFβ, which is a suppressor of growth and inducer of apoptosis (115). KLK3 has also been shown to stimulate conversion of estradiol into its less potent form, estrone, in breast cancer cells and to inhibit growth of MCF-7 breast cancer cells (116). KLK10 is found to be expressed in normal breast epithelial cells and downregulated in breast cancer cells (117). When KLK10 was expressed in MDA-MB-231 (KLK10 negative) breast cancer cells, KLK10 was found to suppress anchorage-independent growth and found to inhibit tumour formation when these cells were injected into nude mice (117).
1.4.16  **KLKs as cancer biomarkers**

Biomarkers are endogenous molecules that, when measured in tissues or biological fluids, can indicate risk for presence of a specific malignancy. KLKs have been studied extensively for their potential roles as cancer biomarkers. KLK3 (prostate-specific antigen or PSA) has had the greatest impact clinically and is used widely for the diagnosis and monitoring of prostate cancer (118). Increased levels of PSA are found in the serum of prostate cancers as a result of leakage into serum upon destruction of the basement membrane of the prostate by cancer cells (119). Unfortunately, PSA levels can also be increased in the serum of patients with benign prostate hyperplasia (BPH), reducing the specificity of PSA as a marker (119). The ratio of KLK2 to free PSA (PSA unbound to inhibitors) in serum has been shown to increase discrimination of prostate cancer to BPH and has demonstrated the ability to discriminate between low and high grade tumours (119).

KLKs 6 and 10 are proposed markers for ovarian cancer and are associated with poor prognosis (120;121). In addition, presurgical levels of these KLKs have been shown to increase the diagnostic sensitivity of cancer-antigen 125 (CA-125) in patients with early stage ovarian cancer (120;121).

A multiparametric analysis of KLK expression in the serum of non-small-cell lung carcinoma (NSCLC) patients found that in this lung cancer subtype, KLKs 5, 7, 8, 10 and 12 levels were lower than in normal serum (122). In contrast, levels of KLKs 11, 13 and 14 were highly upregulated. This study also found that expression levels of KLKs 11 and 12 correlated with cancer stage and
that higher levels of KLKs 11, 12, 13 and 14 correlated with a higher risk of NSCLC.

1.4.17 KLKs as therapeutic targets

Given that KLKs are known be dysregulated in certain malignancies and are thought to contribute to neoplastic progression it is possible that they may have utility as therapeutic targets. Firstly, inhibition of KLK action may be useful in preventing their pro-neoplastic action, however this inhibition must be specific. Inhibitors specific to KLKs 2 \((123)\) and 14 \((123)\) have been developed. In both instances, serpins previously shown to inhibit these KLKs were modified at their reactive-site loop, the portion which interacts with the enzyme. Amino acids within this reactive-site of antichymotrypsin were replaced with sequences selected as ideal substrates for either KLK2 or KLK14 by phage-display technology \((39;40)\). This resulted in a serpin inhibitor very specific to the target KLK.

The highly restricted tissue specificity of KLK3 to the prostate has been harnessed as another therapeutic potential. KLK3 has been used to selectively cleave and activate prodrugs, such as doxorubicin \((124)\), vinblastine \((125)\) and thapsigargin \((126)\) used in chemotherapy for prostate cancer.

1.5 Anatomy of the female reproductive system

The principle organs of the female reproductive system are the ovaries, fallopian tubes, uterus and vagina (Figure 1.6).
1.5.1 Ovaries

The ovaries consist of two small organs located in the pelvic cavity. Their primary functions are the monthly production of ova and to produce and secrete hormones (127).

1.5.2 Fallopian tubes

The fallopian tubes are hollow muscular tubes and consist of three regions, the infundibulum, the ampulla and the isthmus. The infundibulum is the portion closest to the ovary and contains fimbriae projections which extend into the pelvic cavity. This portion of the fallopian tube is also lined with cilia which beat towards the entrance of the tube, the ampulla. The isthmus extends from the ampulla and passes through the wall of the uterus, opening into the uterine cavity. The muscular nature of the fallopian tube allows for peristaltic-like contractions which, along with the cilia, allow for transport of the ova from the ovary to the uterus at ovulation. The fallopian tube is also the location where sperm and ova meet and fertilization most often takes place (127).
Figure 1.6: Anatomy of the Female Reproductive System
1.5.3 Uterus

The uterus is responsible for the nourishment, support and protection of the developing embryo and fetus during pregnancy. The uterus consists of two main parts, the body and cervix. The body is the largest and main portion of the uterus and the cervix forms the inferior portion of the uterus, separating the uterus from the vagina. The cervix is a tube-like structure which extends into the vagina. The uterine wall is divided into an outer muscular portion called the myometrium and an inner glandular layer called the endometrium. The endometrium responds to monthly hormonal changes during the uterine or menstrual cycle (127;128).

1.5.4 Vagina

The vagina is a tube which connects the vestibule with the cervix, the entrance to the uterus. The vaginal wall consists of 3 layers: a fibrous outer layer, a muscular middle layer and an inner mucosal layer. The inner mucosa is composed of stratified squamous epithelial cells with multiple layers of basal and parabasal cells. The vagina serves three main purposes: 1) as a passageway for the elimination of menstrual fluids, 2) to receive sperm during sexual intercourse and 3) forms the lower portion of the birth canal.

1.5.5 Cervico-vaginal fluid

The vagina contains cervico-vaginal fluid (CVF), which is composed of fluids secreted and cells shed from the vaginal epithelium, cervical epithelium, fallopian tube and endometrium. CVF plays an important role in host defense (129;130) and under normal physiological conditions CVF contains many different types of bacteria, mostly dominated by lactobacillus (128;131).
Lactobacillus is capable of growing in normal acidic CVF (between pH 3.8 and 4.5) and produces substances including lactic acid and hydrogen peroxide which help to maintain a healthy state in the vagina by preventing growth of pathogenic bacteria. Changes in the vaginal ecosystem can lead to a decrease in the number of lactobacilli present, allowing other bacteria to dominate and infections to develop (128).

CVF is known to play an important role in host defense (129) and has been shown to contain antimicrobial substances such as cationic peptides (132), lysozyme (133), lactoferrin (134;135), secretory leukocyte protease inhibitor (SLPI) (136-138), human neutrophil peptides (136), human β-defensins (139), hornerin (136) and other members of the S-100 family of proteins (136).

1.5.6 The Ovarian and Menstrual Cycles

The ovarian and menstrual cycles are intricately connected and controlled by steroid hormones (Figure 1.7). At puberty, the pituitary gonadotrophin
Figure 1.7: Diagram of the Hormonal Changes occurring during the Menstrual Cycle
A) Changes in FSH and LH pituitary hormones, B) Development of the ovarian follicle and corpus luteum, C) Changes in estrogen and progesterone hormone levels, D) Changes in the endometrial lining.
releasing hormone (GnRH) stimulates the production of follicle stimulating hormone (FSH) which triggers to beginning of the ovarian cycle then and on a monthly basis from this point until menopause (140). Rising FSH levels trigger primitive follicles within the ovary to develop into primary follicles. Follicular cells form several layers around the ovum and produce and secrete estrogen as they increase in number (127). At the same time in the uterus, rising estrogen levels stimulate proliferation of the endometrial glands and increased vascularization. At ovulation, peaking estrogen levels cause a surge in leutinizing hormone (LH) levels which triggers the rupture of the follicle and release of the ovum into the pelvic cavity (127). The ovum is then swept into the fallopian tube by beating cilia. Ovulation occurs midcycle at approximately day 14 of a standard 28 day cycle. Following ovulation, under LH stimulation, the empty follicle collapses creating a structure called the corpus luteum. The corpus luteum produces progesterone, which stimulates the uterus to prepare for pregnancy (127). This is referred to as the secretory phase of the menstrual cycle marked by increased secretions by the endometrial glands (127). If no pregnancy occurs, levels of estrogen and progesterone fall dramatically as the corpus luteum degenerates and the menstrual phase is triggered, causing destruction of the functional endometrium. Falling estrogen and progesterone levels also trigger GnRH to begin the cycle once again.

1.5.7 The menstrual cycle and the vaginal epithelium

The vagina is a tube which connects the vestibule with the cervix, the entrance to the uterus. The vaginal wall consists of 3 layers: a fibrous outer layer, a muscular middle layer and an inner mucosal layer. The inner mucosa is
composed of stratified squamous epithelial cells with multiple layers of basal and parabasal cells. The vaginal mucosa thickens upon estrogen stimulation at puberty and from then until menopause responds cyclically to hormonal changes during the menstrual cycle (128;131). Estrogen production peaks prior to ovulation and causes the vaginal epithelial cells to mature and become cornified, thinner and flatter. Progesterone stimulation following ovulation inhibits this maturation. Following menopause the decrease in estrogen results in less mature cells and subsequent shedding of vaginal epithelial cells (131).

1.5.8 The cervical epithelium and cervical mucus

Unlike the vagina, the cervix is lined with mucus-secreting columnar epithelial cells which secrete cervical mucus (141). The cervical mucosa does not undergo desquamation during the menstrual cycle (128). However, the amount and type of mucus secreted by the cervical mucosa does change throughout the menstrual cycle (128). During ovulation, mucus secretions are less viscous to facilitate sperm penetration of the uterus and subsequent fertilization (128). During the luteal phase or during pregnancy the cervical mucosa contains mucus glands which proliferate and produce more viscous secretions to prevent microorganisms from entering the uterus.

1.5.9 Mucins

Mucin (MUC) proteins are the major components of mucus. MUCs are highly glycosylated proteins, containing tandemly repeated amino acid domains which are rich in serine and threonine residues, resulting in MUCs being primarily O-glycosylated (142). Thirteen MUC genes have been identified until now and
can be characterized into three groups based in their structural properties: membrane spanning, gel-forming and small soluble.

The primary MUCs found in cervical mucus are MUCs 4 and 5B (143). MUC4 is a membrane-spanning mucin which is cleaved extracellularly resulting in the extracellular portion present in secretions (144). MUC5B is secreted and a member of the gel-forming group (145).

1.5.10 Cervical mucus and changes over the menstrual cycle

The opening to the cervix is filled with a substance referred to as cervical mucus, which is important for host defense and for reproductive function (146). This mucus works to prevent the ascension of microorganisms from the vagina into the uterus (147). Cervical mucus is primarily secreted from the columnar cells of the endocervical epithelium and is composed mostly of water, electrolytes (primarily sodium chloride) and proteins such as mucins, enzymes and antibacterial proteins (148). As mentioned, the major mucin proteins found in cervical mucus are mucin 4 and mucin 5B (143) which form the main structural components of cervical mucus.

The composition and pH of cervical mucus change throughout the menstrual cycle in response to changes in hormone levels. In the period before ovulation the pH of cervical mucus is around 6.2 and has a viscous consistency composed of compact fibre structures (149). In response to estrogen during ovulation, the volume of mucus increases and becomes watery and less viscous (150), the pH of mucus increases to around 7.6-7.8 (151) and levels of mucins 4 and 5B increase (143). This highly hydrated mucus allows for migration of sperm through the cervix into the uterus (150). Mucin 4 and 5B levels are found to
Inversely correlate with progesterone levels and to fall in the post-ovulatory period when progesterone levels are increased (143).

Cervical mucus is found in all women and in non-pregnant women it is a viscous fluid (147). Following conception, increasing progesterone levels cause the mucus to become thick and form a semi-solid plug which blocks the entrance into the uterus from the vagina and prevents microorganism from entering the amniotic fluid from the vagina (147;152). The cervical mucus plug is also found to have an antimicrobial effect and contains antimicrobial proteins such as secretory leukocyte protease inhibitor (SLPI), lysozyme, calprotectin, lactoferrin and defensins (147).

1.6 **Enzyme linked immunosorbant assay (ELISA) immunoassay**

Immunoassays are useful for the quantification of substances from complex mixtures using specific antibodies for the detection of their conjugate antigens. In general there are two ELISA configurations: competitive and non-competitive (153). All ELISAs used in this study were non-competitive, “sandwich” type assays, employing one antibody for capture of the specific KLK of interest and a second unique antibody for detection of that KLK. The specific configurations used in this study are detailed in section 3.2.3.

In general, non-competitive assays utilize an excess of antibody for quantification of a specific antigen. Excess antibody allows for most (all) of the antigen to be in complex with the antibody and results in quantification of the antigen-antibody complex (154).

The solid phase used in our assays was white polystyrene in a 96-well plate format, which bound capture antibody hydrophobically. After 16 hours of
incubation, any unbound or loosely bound capture antibody was washed from the plates. Samples or calibrators were diluted in bovine serum albumin (BSA) as a blocking agent. The purpose of the blocking agent was to prevent non-specific binding between the antigen (KLK) and the polystyrene plate (153). Once added to the coated plate, the specific KLK of interest was immunoextracted from either the calibrators or samples by the capture antibody and all other constituents were washed away. The amount of KLK present was quantified in the detection step of the assay using an excess of labeled detection antibody. Most often the detection antibody was labeled with biotin through the process of biotinylation, resulting in covalent attachment of biotin to the amino groups present in the antibody (153). Detection of the biotin-labeled antibody was achieved through a specific interaction with streptavidin linked- alkaline phosphatase. Diflusinal phosphate (DFP) was then added as a substrate for alkaline-phosphatase, resulting in cleavage of phosphate from DFP, leaving DF. Fluorescence was achieved through the addition of a solution containing terbium and EDTA and the formation of a complex between DF, EDTA and terbium (155). Excitation of this complex at a wavelength of 337 nm, and measurement of fluorescence at 615 nm using a time-resolved fluorometer resulted in fluorescence which could be quantified. Time –resolved fluorescence can be achieved through the use of fluorescent labels with both short and long-lasting signals, such as terbium (155). Upon initial excitation, short signals are ignored which allows scattered excitation to be eliminated. The long-lasting signals remain and are able to be measured with high sensitivity. A standard curve can be drawn based on fluorescent signals from KLK calibrators.
1.7 **Proteomic analysis of human biological fluids**

A proteome describes the set of proteins encoded by a particular genome (156). Analysis of human biological fluid proteomes is now possible through proteomic techniques, specifically protein/peptide fractionation and mass spectrometry (157).

The complex nature of biological fluids requires fractionation strategies aimed at reducing this complexity. The most common methods of fractionation are SDS-PAGE and chromatographic methods (158). Trypsin is the most common enzyme used for protein digestion prior to mass spectrometry because trypsin is very specific (cleaving after arginine and lysine residues), efficient and creates peptide sequences of approximately twenty amino acids in length, which is ideal for mass spectrometry analysis (159).

Mass spectrometers measure the mass-to-charge ratios (m/z) of gas-phase ions and consist of: an ion source, a mass analyzer and a detector (160). The ion source is responsible for vapourization and ionization of the sample which generally employs one of two techniques: electrospray ionization (ES) or matrix assisted laser desorption ionization (MALDI). ES was used in this study and will be described further. ES produces gas phase ions from solution based samples and are often coupled to liquid chromatographs (LC). Peptides are separated by the LC (often reversed phase chromatography) before they reach the ionizing source (160). Ionization is achieved as the peptide-containing solution moves through an electric field.

Following ionization, the ions move to the mass analyzer, which separates and detects ions based on their m/z ratio (159). There are four basic types of
mass analyzers: ion-trap, time-of-flight, quadrupole and fourier transform ion cyclotron resonance (160). An ion-trap mass analyzer was used in this study and will be discussed further. Ions of the same m/z enter the trap at the same time, are held within the ion-trap and subjected to oscillating electric fields (159). Ions become excited and are ejected from the trap based on their m/z ratios and are then detected by the detector (159). The most abundant ions are selected for tandem MS (MS/MS analysis) and peptide sequencing. These ions are isolated in the trap and fragmented by collision-induced dissociation (CID) within the trap (160). Fragmentation results in breaking of peptide bonds within the peptide and in the formation of daughter ions. So-called “b” and “y” ions are created depending on whether the peptide is fragmented from the N-terminal end or the C-terminal end and this series of ions creates a spectrum (159). Each peptide fragment in a series differs from another fragment by one amino acid. The peptide sequence can be determined by analyzing the mass difference between peaks in a spectrum (159). This analysis is usually performed by database search programs, such as Mascot, which compare the experimental spectrum with a database of theoretical spectrums and assign probabilities to matches (160).

1.8 Rational and hypotheses

1.8.1 Development of an ELISA immunoassay for KLK15

Thus far, KLK15 protein levels in tissues and biological fluids have not been examined. This is due to the lack of reagents for monitoring KLK15 protein levels. The physiological function of KLK15 is currently unknown, and PSA is the only proposed KLK15 substrate (161). To further elucidate this protein's tissue
expression pattern, well-characterized, recombinant protein is required. Recombinant KLK15 can be used for production of specific monoclonal and polyclonal antibodies and for development of a KLK15 immunoassay.

1.8.2 Global KLK expression in human tissues and biological fluids

Physiologic functions have been relatively well-established for the three classical KLKs (1, 2, and 3); however the physiologic roles of the other KLKs remain largely unknown. In order to better characterize the physiologic functions of KLKs, knowledge of their expression patterns is essential. Until now, a global study examining the expression of all KLKs at the protein level was not possible, due to the lack of KLK-specific reagents. Here, I utilized specific and sensitive ELISA immunoassays to examine global KLK expression patterns in multiple panels of human tissues and biological fluids.

1.8.3 Immunohistochemical localization of KLKs in the female reproductive system

Co-expression of KLKs was found in tissues of the female reproductive system including the fallopian tubes, cervix, vagina and endometrium. In addition, relatively large levels of several KLKs were found in CVF. Given that CVF is composed of fluids originating from the endometrium, fallopian tube, cervix and vagina I wanted to examine the immunohistochemical localization of KLKs within the female reproductive system. This information will help elucidate potential KLK functions in cervico-vaginal physiology.

1.8.4 Hormonal regulation of KLKs in cervico-vaginal physiology

Cervico-vaginal physiology is largely regulated by hormonal changes during the menstrual cycle. I hypothesized that KLKs may also be regulated by
these hormonal changes and may play a physiological role during the menstrual cycle.

1.8.5  **Proteomic analysis of human cervico-vaginal fluid (CVF)**

Until recently, limited information was available on the proteomic profile of CVF. I set out to resolve the proteome of CVF for the purpose of identifying potential KLK substrates and to provide clues to potential KLK functions in CVF.

1.8.6  **Potential roles for KLKs in cervico-vaginal physiology**

1.8.6.1  **A role for KLKs in desquamation**

Through proteomic analysis of CVF I identified several cell-cell adhesion molecules such as, desmoglein-1 (DSG1), desmocollin-2 (DSC2) and desmocollin-3 (DSC3). These proteins are most likely found in CVF as a result of cyclical changes in the endometrial and vaginal epithelium during the menstrual cycle. Vaginal epithelial cell desquamation takes place following ovulation when progesterone levels rise and estrogen levels fall (131). Given that KLK levels increase during this period, I hypothesized that KLKs may play a role in vaginal epithelial cell desquamation through cleavage of DSGs and DSCs, similarly to their role in skin physiology.

1.8.6.2  **A role for KLKs in cervical mucus remodelling**

The opening to the cervix is filled with a substance referred to as cervical mucus. This mucus works to prevent the ascension of microorganisms from the vagina into the uterus (147). Cervical mucus is primarily composed of water and also contains proteins, primarily mucins, but also enzymes and antibacterial proteins (148). The primary cervical mucin proteins are mucins 4 and 5B (162).
The composition and pH of cervical mucus changes throughout the menstrual cycle, in response to changing hormone levels. It has been suggested that proteolytic enzymes may affect the physical properties of mucin proteins causing the changes in mucus observed over the menstrual cycle (163). I hypothesize that KLKs are capable of processing mucin proteins and may be involved in remodelling of cervical mucus.

1.8.6.3 A role for KLKs in vaginal host defense

KLKs have recently been shown to play a role in host defense in skin and sweat through cleavage of the antimicrobial human cathelicidin protein, hCAP-18 (84). I hypothesize that KLKs may also contribute to antimicrobial activity within CVF through processing of additional antimicrobial proteins found in CVF, such as defensins.
Chapter 2: Development of an ELISA Immunoassay for KLK15

This work has been published in the following article,


G. Sitoropoulou provided us with a clone stably expressing KLK15 in yeast cells.

L. Grass was responsible for the production of the KLK15 mouse polyclonal antibody.

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2.1 Introduction and rationale

Kallikrein 15 (KLK15, prostinogen, ACO protease) is the most recently cloned member of the kallikrein gene family, and maps between the two classical kallikreins, KLK1 and KLK3 on the kallikrein locus (164). mRNA studies indicate that KLK15 is highly expressed in the thyroid, salivary and adrenal glands, prostate, and colon (164). KLK15 has also been found to be up-regulated by steroid hormones in the prostate cancer cell line, LNCaP (164).

The function of the protein encoded by KLK15, (KLK15), is currently unknown. However, KLK15 is predicted to be a secreted protein (13). Preliminary functional studies indicate that KLK15 is a trypsin-like serine protease, preferring to cleave after arginine and/or lysine (161). KLK15 has also been shown to cleave and activate pro-PSA (KLK3) into active PSA (161;164), indicating that perhaps KLK15 may be involved in an enzymatic cascade within the prostate (165).

Many kallikreins have been found to be useful cancer biomarkers (104) as discussed in the introduction (chapter 1). mRNA studies indicate that KLK15 may also have some utility as a cancer biomarker. KLK15 has been shown to be up-regulated in cancerous versus non-cancerous prostate tissues, as well as in more aggressive prostate tumours, at the mRNA level (164), indicating it may be useful for distinguishing between more, or less aggressive forms of prostate cancer. KLK15 may also serve as an unfavourable marker for ovarian cancer, as it was found to be up-regulated in cancerous versus benign ovarian tumours (166). KLK15 was found to be a predictor of reduced progression-free, and overall survival for ovarian cancer (166). For breast cancer however, mRNA
studies suggest that \textit{KLK15} may serve as a predictor of longer progression-free and overall survival (167).

Thus far, the clinical utility of \textit{KLK15} as a cancer biomarker has been studied only at the mRNA level, and KLK15 protein levels in tissues and biological fluids have not been examined. This is due to the lack of reagents for monitoring KLK15 protein levels. The physiological function of KLK15 is currently unknown, and PSA is the only proposed KLK15 substrate (161).

To further elucidate this protein’s tissue expression pattern, well-characterized, recombinant protein is required. Recombinant KLK15 can be used for production of specific monoclonal and polyclonal antibodies and for development of a KLK15 immunoassay.

2.2 Materials and methods

2.2.1 Cloning of KLK15 into a mammalian expression vector

\textit{KLK15} mRNA was obtained from LNCaP prostate cancer cells (purchased from ATCC, Manassas VA), by Trizol (Invitrogen Canada Inc., Burlington, ON) extraction, as per the manufacturer’s instructions. \textit{KLK15} mRNA was reversed transcribed into first strand cDNA using superscript first strand synthesis (Invitrogen Canada Inc., Burlington, ON). \textit{KLK15} cDNA (NM_017509) was amplified by PCR using the forward primer 5’-caccaggatggtgacaagttg 3’ and reverse primer 5’-gtcacttcctcttcatggtttccc-3’. PCR was performed in a 25 µL reaction mixture containing 15 ng cDNA, 10mM Tris-\(\text{HCl}\) (pH 8.3), 50 mM KCl, 1.5 mM MgCl\(_2\), 200uM deoxynucleoside triphosphates, 100 ng of primers and 2.5 U of \textit{pfu} turbo DNA polymerase (Stratagene, La Jolla, CA). The PCR conditions were 94°C for 2 min, followed by 94°C for 1 min, 66°C for 1 min, 72°C for 1 min,
and a final extension at 72°C for 10 min. The PCR product spanned the entire coding sequence of KLK15 and was subsequently cloned into the pcDNA 3.1-v5-HIS-Topo vector (Invitrogen Canada Inc, Burlington, ON), using the manufacturer’s recommended method and in frame with the start and stop codons of the KLK15 sequence which were used as translation signals. The correct sequence of the above construct was confirmed by sequencing.

### 2.2.2 Production of KLK15 in human embryonic kidney (HEK 293) cells

HEK293 cells were grown to confluency in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen Canada Inc, Burlington, ON) containing 10% fetal bovine serum (FBS). The KLK15-pcDNA3.1 construct was introduced into the HEK 293 cells using Fugene 6 transfection agent (Roche Canada, Mississauga, ON), as per the manufacturer’s recommendations. pcDNA3.1 is neomycin (G418) resistant, and 48 hours following transfection, 30µg/mL G418 was added to the medium, as a positive selection agent. Massive cell death occurred within 2 weeks of G418 addition. Viable clones were visualized under the light microscope, marked, and picked via pipetting. Stable clones were picked and grown to confluency in DMEM containing 10% FBS and 15 µg/mL G418. KLK15 expression was monitored by western blotting, using a rabbit polyclonal antibody developed in our laboratory against recombinant KLK15 protein produced in *E. coli*. The clone which produced the highest levels of KLK15 (clone C4) was characterized further. Once confluent in serum containing medium, C4 cells were grown in serum free CD CHO (BD Biosciences, Mississauga, ON) medium containing 15 µg/mL G418, for 10 days, after which the cells were pelleted by centrifugation and the supernatant was retained for purification.
2.2.3 KLK15 purification using cation-exchange and reversed-phase chromatography

Recombinant KLK15 was purified from HEK 293 cell culture supernatant using two stages of chromatography. Firstly, cation exchange chromatography was performed using an SP sepharose fast flow column (5 mL; GE Healthcare, Mississauga, ON) and secondly, reversed phase chromatography was performed using a C₄ column (Grace Vydac, Hesperia, CA). Briefly, SP sepharose beads previously activated with 1M NaCl were equilibrated with 50 mM sodium acetate (pH 5.2). C4 cell supernatant was pumped through the SP sepharose column at a flow rate of 1.0 mL/min to allow for protein binding to the beads. The beads were then washed with 50 mM sodium acetate (pH 5.2). KLK15 was eluted using a step gradient starting with a linear gradient from 0-200 mM NaCl elution over 20 minutes, followed by constant 200 mM NaCl over 20 minutes. This step was followed by a second linear gradient from 200mM-1M NaCl over 40 minutes. KLK15 was eluted around 400 mM NaCl. Trifluoroacetic acid, as an ion-pairing agent, was added to this eluate (final concentration 10 mL/L), which was then loaded on a C₄ column equilibrated with 1mL/L trifluoroacetic acid in water. A step gradient increasing from 28-40% in 1% increments over 80 min of acetonitrile in 1mL/L trifluoroacetic acid was then performed. The fraction containing KLK15 was concentrated by evaporation of the acetonitrile. The purified material was separated by SDS-PAGE using NuPAGE Bis-Tris 4-12% gradient gels (Invitrogen Canada Inc., Burlington, ON) and stained with Simply blue safestain ® (Invitrogen Canada Inc. Burlington, ON) to assess its purity and molecular mass. Bands stained from this purified sample were subjected to
electrospray ionization tandem mass spectrometry and N-terminal sequencing analysis to confirm their identity as KLK15 and their N-terminal sequence as the proform of KLK15.

2.2.4 Confirmation of KLK15 by mass spectrometry

Coomassie stained bands were excised and destained with 300 mL/L acetonitrile in 100 mmol/L ammonium bicarbonate. Each band was then reduced (10 mmol/L dithiothreitol in 50 mmol/L ammonium bicarbonate, pH 8.3) and alkylated (55 mmol/L iodoacetamide in 50 mmol/L ammonium bicarbonate, pH 8.3) before overnight trypsin digestion (Promega, Madison, WI). Peptide fragments were then extracted with 50 mL/L acetic acid, evaporated to dryness on a Savant concentrator, and reconstituted in 10 µL of a solution of methanol-water-acetic acid (500:495:5 by volume).

All nanoelectrospray mass spectrometry experiments were conducted on a Q-Star (PE/Sciex, Sunnyvale, CA) hybrid quadrupole/time-of-flight instrument, for high resolution and online tandem mass spectrometry (MS-MS) experiments. MS-MS experiments on tryptic peptides identified in survey scans were conducted using a nanoelectrospray source. Precursor ions were selected by the first quadrupole while a pusher electrode was pulsed (frequency ~7 kHz) to transfer fragment ions formed in the radiofrequency-only quadrupole cell to the time-of-flight analyzer. Mass spectral resolution was typically 9000–10 000. A scan duration of 1 and 2 s was set for conventional and MS-MS mass spectral acquisition, respectively. Collisional activation was performed using nitrogen collision gas with typically a 30-V offset between the DC voltage of the entrance.
quadrupole and the radiofrequency-only quadrupole cell. Data were acquired and processed using LC Tune and Biomultiview programs from PE/Sciex.

2.2.5 N-terminal sequencing

Purified KLK15 was resolved by SDS-PAGE using NuPAGE Bis-Tris 4-12% gradient gels (Invitrogen Canada Inc., Burlington, ON) and transferred to polyvinylidene difluoride (PVDF) membrane (GE Healthcare, Mississauga, ON). After the transfer, the membrane was stained with Coomassie blue R-250 (0.1% solution in 40% methanol) for 10 min and de-stained in 50% methanol. KLK15 bands were excised and subjected to automated N-terminal Edman degradation consisting of 5 cycles of Edman chemistry on an ABI 492 Procise cLC sequencer (Applied Biosystems, Foster City, CA), followed by analysis of resultant phenylthiohydantoin (PTH)-amino acid residues on an HPLC column.

2.2.6 Production of KLK15 specific polyclonal antibodies

Purified, recombinant KLK15 produced in HEK 293 cells was used as an immunogen to immunize BALB/c mice, and New Zealand white rabbits. KLK15 (100 µg) was injected subcutaneously into mice and rabbits. The protein was diluted 1:1 in complete Freund’s adjuvant (Sigma Aldrich, St. Louis MO) for the first injection and in incomplete Freund's adjuvant for the subsequent injections. Injections were repeated six times at 3-week intervals. Blood was drawn from the animals and tested for antibody generation. To test for production of anti-KLK15 polyclonal antibodies, I used the following assay. 50 ng of purified KLK15 protein diluted in 50 mmol/L Tris buffer, pH 7.80, was immobilized on 96-well white ELISA plates, and incubated overnight. The rabbit serum (immune and non-immune) was then applied to the plates in different dilutions ranging from
Chapter 2

Development of KLK15 immunoassay

1:10,000 to 1:1,000,000. After 1 hour incubation, the plates were washed 6 times in washing buffer (9 g/L NaCl, 0.5 g/L Tween 20 in 10 mmol/L Tris buffer, pH 7.40). 100 µL/well of alkaline phosphatase-conjugated goat anti-rabbit IgG, Fc fragment-specific (Jackson ImmunoResearch, West Grove, PA), diluted 1:5000 in 6% BSA was added to each well and incubated for 45 min. The plates were then washed as above. Diflunisal phosphate [100 µL of a 1 mmol/L solution in substrate buffer (0.1 mol/L Tris pH 9.1), 0.1 mol/L NaCl and 1 mmol/L MgCl$_2$] was added to each well and incubated for 10 min. Developing solution (100 µL, containing 1 mol/L Tris base, 0.4 mol/L NaOH, 2 mmol/L TbCl$_3$, and 3 mmol/L EDTA) was pipetted into each well and mixed for 1 min. The fluorescence was measured with a time-resolved fluorometer, the Cyberfluor 615 Immunoanalyzer (MDS Nordion, Ottawa, ON). The calibration and data reduction were performed automatically, as described in detail in the introduction (see section 1.6).

Blood was also drawn from the animals and tested for antibody generation by Western blotting. Blood was diluted 3000 fold in 1% milk in TBST (25 mM Tris-HCl, pH 8.0, 125 mM NaCl, 0.1% Tween 20) for western blotting.

2.2.7 Treatment of recombinant KLK15 with N-glycosidase F (PNGase F)

KLK15 was treated with PNGase F, according to the manufacturer’s recommended method (New England Biolabs, Ipswich, MA). Briefly, 10µg of purified KLK15 was incubated 1:1 with denaturation buffer for 10 minutes at 100°C. 10X G7 buffer and NP-40 (1/10) were then added to the reaction mixture, along with 1uL of PNGase F, and the reaction was incubated at 37°C for 1 hour. Samples were stored at -20°C until needed.
2.2.8 Development of a KLK15 immunoassay

A monoclonal antibody against full length, human recombinant KLK15 (produced in the murine myeloma cell line NSO) clone 820, was obtained from R&D Systems (Minneapolis, MN) (as a pre-release reagent). White polystyrene microtiter plates were coated with the monoclonal antibody (100 µL of coating antibody solution containing 250 ng of antibody diluted in 50 mmol/L Tris buffer, pH 7.8 in each well) by incubation overnight at room temperature. The plates were then washed two times with washing buffer (9 g/L NaCl and 0.5 g/L Tween 20 in 10 mmol/L Tris buffer pH 7.40). KLK15 calibrators or samples were then pipetted into each well (50 µL/well along with 50 µL of assay buffer (60 g/L BSA, 50 mmol/L Tris (pH 7.80), 0.5 g/L sodium azide, 25 mL/L normal mouse serum, 100 mL/L normal goat serum, and 10 g/L bovine IgG), and incubated for 1h at room temperature with shaking. The plates were then washed with wash buffer six times. KLK15 rabbit polyclonal antibody was then diluted 3000 fold in assay buffer and 100 µL were added to each well. After 1 hour incubation at room temperature with shaking, the plates were washed six times in wash buffer. 100 µL/well of alkaline phosphatase-conjugated goat anti-rabbit IgG (Fc fragment specific) (Jackson ImmunoResearch, West Grove, PA), diluted 5000 fold in assay buffer were then added to each well, and incubated for 45 min, and plates were washed as above. Diflunisal phosphate was then added and the assay was completed as described above.

2.2.8 Fractionation of seminal plasma with size exclusion HPLC

HPLC fractionation was performed using an Agilent 1100 (Agilent, Mississauga, ON) system and a gel filtration column (TSK gel G3000SW;
TOSOH Bioscience, Bellfonte PA). The mobile phase was 0.1M Na$_2$SO$_4$, 0.1M NaH$_2$PO$_4$ pH 6.8. A flow rate of 0.5 mL/min was used and the column was calibrated with molecular mass standards (Bio-rad Laboratories, Richmond, CA) containing thyroglobulin (670 kDa), IgG (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa) and cyanocobalamin (1.4 KDa). 200 µL of recombinant KLK15 (500 µg/L diluted in 6% BSA) and 300 µL of seminal plasma (diluted 1:1 in 0.1 M NaH$_2$PO$_4$, 0.1 M Na$_2$SO$_4$ pH 6.5) were fractionated and HPLC fractions of 0.5 mL were collected and analyzed for KLK15 and KLK3 using the KLK15 immunoassay described above and the KLK3 immunoassay described below (see section 3.2.3).

2.3 Results

2.3.1 Recombinant KLK15 produced in human embryonic kidney (HEK 293) cells

cDNA encoding the proform of KLK15 was cloned into the mammalian expression vector, pcDNA3.1-v5-His-topo; the construct was designed so that pro-KLK15 would be secreted into the cell culture supernatant. A stable cell line expressing pro-KLK15 was created and pro-KLK15 was purified from the cell culture supernatant by successive chromatographic steps. Purified pro-KLK15 was resolved by SDS-PAGE and stained with Coomassie blue, as shown in Figure 2.1A. A western blot of purified pro-KLK15 was performed using a KLK15 rabbit polyclonal antibody, produced previously in our laboratory (raised against E. coli-produced KLK15) (Figure 2.1B). pro-KLK15 produced by HEK 293 cells appears as a diffuse band of approximately 38 kDa molecular mass. The 38 kDa band was confirmed as KLK15 by electrospray ionization tandem mass
spectrometry. A total of ten peptides were identified, providing coverage of the entire KLK15 protein (Table 2.1). Edman degradation performed on the 38 kDa band revealed the N-terminal sequence of recombinant KLK15, D G D K L L, matching the first five amino acids of the proform of KLK15, as previously reported (164).

Table 2.1: Peptides identified by mass spectrometry analysis of recombinant KLK15 produced by HEK 293 cells.

<table>
<thead>
<tr>
<th>KLK15 form</th>
<th>Peptides identified</th>
<th>Location¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mammalian</td>
<td>LLGDECAPHSQPWQVALYER</td>
<td>22-42</td>
</tr>
<tr>
<td></td>
<td>FNCGASLISPHWVLSSAHCQSR</td>
<td>45-66</td>
</tr>
<tr>
<td></td>
<td>LGEHNLKR</td>
<td>72-79</td>
</tr>
<tr>
<td></td>
<td>RDGPEQLR</td>
<td>80-87</td>
</tr>
<tr>
<td></td>
<td>DGPEQLR</td>
<td>81-87</td>
</tr>
<tr>
<td></td>
<td>LNPQVRPAVLPTPR</td>
<td>118-130</td>
</tr>
<tr>
<td></td>
<td>CPHPGEACVVGGLVSHNEPHTGASPR</td>
<td>131-158</td>
</tr>
<tr>
<td></td>
<td>LTNTMVCAGAEGR</td>
<td>188-200</td>
</tr>
<tr>
<td></td>
<td>VCHYLEWIR</td>
<td>242-250</td>
</tr>
</tbody>
</table>

¹Amino acid numbering based on Genbank accession # NM_017509
Figure 2.1 Coomassie stained SDS-PAGE and a Western Blot showing Recombinant KLK15 protein produced by HEK 293 cells

Purified, recombinant KLK15 produced in HEK 293 cells was resolved by SDS-PAGE and the protein was either A) stained with Coomassie blue, or B) transferred to nitrocellulose for western blotting with a KLK15 polyclonal antibody (raised against *E. coli* KLK15, produced in our lab). KLK15 appears as a diffuse band of approximately 38 kDa. On the left panel are molecular mass standards in kDa.
2.3.2 Glycosylation status of recombinant KLK15

The diffuse appearance of KLK15, visualized by Coomassie staining, is characteristic of a glycosylated protein. The larger than expected size of KLK15, 38 kDa (predicted to be 30 kDa) also suggests glycosylation of KLK15 produced by HEK 293 cells. KLK15 has two predicted glycosylation sites at amino acids 171, and 232 (164).

To assess the glycosylation status of KLK15 produced in HEK 293 cells, I treated KLK15 with the deglycosylation enzyme PNGaseF. Figure 2.2 shows the results of PNGaseF treatment. Before PNGaseF treatment, the multi-molecular mass bands, of approximately 38 kDa, are present when stained with Coomassie blue (left panel), and they are immunoreactive when immunoblotted with a KLK15 polyclonal antibody (right panel). Following treatment with PNGaseF, the larger multi-molecular mass bands are no longer present, and instead, a single lower molecular mass band of 30 kDa is present. This single band is visible when stained with Coomassie blue, and immunoreactive when immunoblotted with the KLK15 polyclonal antibody. The shift in molecular mass indicates that KLK15 is glycosylated.

2.3.3 Production of KLK15 antibodies

Recombinant KLK15 produced by HEK 293 cells was used as an immunogen in rabbits and mice, for production of polyclonal antibodies. Figure 2.3 shows the results of western blotting using our polyclonal rabbit (Figure 2.3A) and mouse (Figure 2.3B) antibodies. Recombinant KLK15 proteins produced by us in *E. coli*, *P. pastoris*, and HEK 293 cells, as well as recombinant KLK15
Figure 2.2 Coomassie stained SDS-PAGE and a Western blot showing recombinant KLK15 before and after treatment with PNGaseF to assess glycosylation status of the protein.

10 μg of purified KLK15 produced in HEK 293 cells were treated with PNGaseF. The treated protein was separated by SDS-PAGE alongside untreated protein. One gel was stained with Coomassie blue (Simply blue safestain, Invitrogen) (left panel), and the other was transferred to nitrocellulose for western blotting (right panel). The 38 kDa band is seen on the Coomassie stained gel and by western blotting with our rabbit polyclonal antibody (raised against KLK15 produced in E. coli), in the untreated protein. A single, 30 kDa band can be seen in the lanes containing the PNGaseF treated protein, by both Coomassie staining and western blotting.
Figure 2.3: Western blots show that KLK15 mouse and rabbit polyclonal antibodies recognize yeast, HEK 293, R&D Systems Inc., and E. coli - produced recombinant KLK15 protein.

Recombinant KLK15 produced in yeast cells, HEK 293 cells, by R&D Systems Inc. and in E. coli cells was resolved by SDS-PAGE and transferred to nitrocellulose for western blotting. A) Western blotting with our rabbit polyclonal antibody raised against HEK 293 produced KLK15 protein, B) western blotting with a mouse polyclonal antibody raised against HEK 293 produced KLK15 protein. Note that both polyclonal antibodies recognize KLK15 produced in yeast (33 kDa), mammalian cells (38 kDa (HEK 293), 40 kDa (R&D Systems), and E. coli (30 kDa). Both antibodies were used at a dilution of 1:3000.
purchased from R&D Systems (produced in murine NSO cells) were resolved by SDS-PAGE and blotted with each of the above antibodies. The results shown in Figure 2.3 indicate that both polyclonal antibodies recognize all four forms of KLK15, but with less efficiency in the case of yeast protein.

2.3.4 KLK15 specific immunoassay

The KLK15 immunoassay was developed and optimized as described in the materials and methods section. Pure, recombinant KLK15 was diluted in 6% bovine serum albumin (BSA) to produce six assay calibrators of the following concentrations: 0, 0.05, 0.2, 1.0, 5.0, 20.0 µg/L. The lower detection limit of this assay was 0.05 µg/L, and a typical calibration curve is shown in Figure 2.4. Cross-reactivity was assessed against the other fourteen human kallikreins, each at a concentration of 1000 µg/L (all produced in our laboratory). Our immunoassay shows 0.04% and 0.2% cross-reactivity with recombinant KLK5 and KLK9 respectively and no detectable cross-reactivity with the other human kallikreins.

2.3.5 Size fractionation of KLK15 by size-exclusion HPLC

To determine the molecular mass of endogenous KLK15, as determined by the KLK15 immunoassay, samples were fractionated, according to size, using a gel filtration column. Fractions were then analyzed by our ELISA assay.

Gel filtration chromatography was performed for recombinant KLK15 and seminal plasma. In both cases, one immunoreactive peak eluted around fraction 39, corresponding to a molecular mass of approximately 38 kDa (Figure 2.5A, B). Eluted seminal plasma fractions were also subjected to analysis by a KLK3
Figure 2.4: A Typical calibration curve for the KLK15 immunoassay, showing the lower detection limit of the immunoassay as 0.05 ug/L.
Figure 2.5: Graphs showing elution time for recombinant KLK15 and endogenous KLK15 from seminal plasma samples by gel filtration chromatography

Samples of recombinant KLK15 or seminal plasma were separated by size exclusion chromatography using a gel filtration column. Fractions were collected and analyzed by ELISA for KLK15. Panel A shows that recombinant KLK15 elutes around fraction 39, corresponding to a 38 kDa protein. Panel B shows that endogenous KLK15 in seminal plasma also elutes around fraction 39, corresponding to a 38 kDa protein. Panel C shows that endogenous KLK3 (PSA) elutes around fraction 42, corresponding to a 32 kDa protein. Note that fractions were diluted 106-fold for KLK3 measurement, and KLK15 values were multiplied by 10 (panel C only), for clearer representation.
(PSA) immunoassay to rule out cross-reactivity due to the very high levels of KLK3 in seminal plasma. KLK3 eluted as a single, distinct peak around fraction 42, corresponding to a molecular mass of approximately 32 kDa (Figure 2.5C).

2.4 Discussion

Human kallikrein 15 although cloned in 2001 (164), has received little attention thus far, particularly at the protein level. This can be attributed to the lack of suitable reagents. I developed tools useful for characterizing the tissue expression of this protein and for determining whether KLK15 has clinical utility as a biomarker for prostate and/or ovarian cancer, as suggested by mRNA studies (164;166-168). Here I describe, for the first time, production and characterization of recombinant KLK15 protein, KLK15 specific antibodies, and a sensitive and specific KLK15 immunoassay.

Previously, our group developed recombinant KLK15 protein using an E. coli expression system and used this protein to produce a polyclonal antibody. This antibody was used to screen mammalian cells in this study for KLK15 expression and was capable of recognizing the glycosylated KLK15 protein produced by HEK 293 cells (data not shown). In contrast to a prokaryotic expression system such as E. coli, the mammalian expression system (HEK 293 cells), used here, ensured correct protein folding and post-translational modifications. HEK 293 cells were specifically used to create a stable cell line, primarily because of their high transfection efficiency, allowing for production of large amounts of secreted, recombinant KLK15. Recombinant KLK15 produced by HEK 293 cells is glycosylated (Figure 2.2). KLK15 has two potential
glycosylation sites, and glycosylation was confirmed by treatment of KLK15 with PNGase F.

Using recombinant KLK15, we developed mouse and rabbit polyclonal antibodies, able to recognize, on western blots, KLK15 proteins produced from a variety of sources such as yeast, mammalian cells, and *E. coli*. We have employed our rabbit polyclonal antibody in conjunction with a mouse monoclonal antibody (obtained as a pre-release reagent from R&D Systems Inc.) to develop a sandwich-type KLK15 immunoassay. This assay is highly specific and sensitive, showing minimal or no cross-reactivity with the other fourteen human kallikreins.

Given that KLK15 has been shown to be expressed in the prostate (164) and is predicted to be a secreted protein, it is expected to be expressed in seminal plasma. I found KLK15 to be present in seminal plasma, albeit at low levels. Size exclusion HPLC indicates that seminal plasma contains an immunoreactive peak of 38 kDa. This molecular mass suggests that endogenous KLK15 is glycosylated, similarly to recombinant KLK15 produced in HEK 293 cells, and that our immunoassay detects free, endogenous KLK15, not KLK15 in complex with protease inhibitors. However, it is possible that KLK15 is partially complexed with inhibitors; similarly to other kallikreins, such as KLK3, KLK6, and KLK5 (24;169;170), and that these complexes are not recognized by our immunoassay. Size exclusion HPLC revealed an immunoreactive peak of 32 kDa molecular mass, when analyzed for KLK3 (PSA) (Figure 2.5C), confirming that PSA and KLK15 are distinct proteins in seminal plasma.
This study confirms, for the first time, that KLK15 is a glycosylated protein of approximately 38 kDa in mass, produced in the prostate and secreted into seminal plasma. Our technology will aid in the further characterization of KLK15 protein expression, delineation of KLK15’s physiological function and its role as a potential cancer biomarker.
Chapter 3: Global KLK Expression in Human Tissues and Biological Fluids

This work has been published in the following article,


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3.1 Introduction and rationale

Individual KLKs or groups of KLKs are thought to have either quite restricted or broad expression patterns. These proteins appear to be highly expressed in one or a few tissues but are also found at lower levels in a wide variety of other tissues (2;13). It has been shown that groups of kallikreins are often co-expressed in tissues (2). For example, KLK2, 3, 4, 11, and 15 are highly expressed in prostate tissue, at the mRNA level (13). Kallikrein co-expression has also been confirmed in some biological fluids, suggesting their potential involvement in proteolytic cascades (165). In some cases, kallikrein cascades have been partially delineated, such as in seminal plasma (KLK2, 3, 5 and 14) (68;69), and skin (KLK5, 7, 14) (62;70).

Physiologic functions have been relatively well-established for the three classical kallikreins (KLK1, 2, 3). Briefly, KLK1 is known to cleave kininogen to kinin (71), which binds to B1 and B2 receptors, resulting in vasodilation and other responses (95). KLK2 and 3 are known to be involved in liquefaction of the seminal clot following ejaculation, by cleaving semenogelins (72). The physiologic roles of the other kallikreins remain largely unknown.

In order to better characterize the physiologic functions of kallikreins, knowledge of their expression patterns is essential. Until now, a global study examining the expression of all KLKs at the protein level was not possible, due to the lack of kallikrein-specific reagents. Here, I utilized specific and sensitive ELISA immunoassays to examine global kallikrein expression patterns in human tissues and biological fluids.
3.2 **Materials and methods**

3.2.1 **Tissue extracts**

Seven adult and three fetal tissue sets were examined. All tissues were collected at autopsy, performed a maximum of 24 h following death, and were stored at -80°C until use. Adults were 50-70 year old deceased individuals due to heart failure or myocardial infarction and fetuses were spontaneously aborted at gestational ages of 13-18 weeks. Tissue extracts were prepared by pulverizing approximately 0.2 g of each tissue in liquid nitrogen into fine powders. Extraction buffer (2 mL; 50 mM Tris-HCl buffer pH 8.0, containing 150 mM NaCl, 5 mM EDTA, and 10 mL/L NP-40 surfactant) was added to the tissue powders and the mixture was incubated on ice for 30 minutes, with vortexing every 10 minutes. Mixtures were centrifuged at 14,000 g at 4°C for 30 minutes. The supernatants (cytosolic extracts) were collected and stored at -20°C until use. Our procedures have been approved by the Institutional Review Boards (IRB) of Mount Sinai Hospital and the University Health Network, Toronto, Canada.

3.2.2 **Biological fluids**

The biological fluids (seminal plasma, breast milk, follicular fluid, amniotic fluid, breast cancer cytosol, breast cyst fluid, cerebrospinal fluid (CSF), cervico-vaginal fluid, urine, ovarian cancer ascites) were anonymous leftovers of samples submitted for routine biochemical testing, or collected with informed consent and IRB approval, and stored at -80°C until use. Amniotic fluids were collected between 15 and 23 weeks gestation. CVF samples were from healthy women between 20 and 30 years of age. Urines were collected from 6 men and 6 women and verified as negative for signs of infection.
3.2.3  KLK-specific ELISA immunoassays

All ELISA immunoassays used in this study were “sandwich” type, with one antibody used for capture, and another one used for detection. Three classes of immunoassays were used in this study, employing either monoclonal-monoclonal, monoclonal-polyclonal, or polyclonal-polyclonal configurations. In Table 3.1, I present the type and sources of all antibodies used in the ELISA assays. All assays were highly specific, with < 1% cross-reactivity with non-cognate KLKs.
### Table 3.1: Sources of antibodies used in ELISA assays.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Capture (code)</th>
<th>Detection (code)</th>
<th>Lowest Standard (µg/L)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kallikrein 1</td>
<td>AP-polyclonal(^1)</td>
<td>AP-polyclonal</td>
<td>0.2</td>
<td>Julie Chao, MUSC</td>
</tr>
<tr>
<td>Kallikrein 2</td>
<td>monoclonal (HK1G 586.1)</td>
<td>monoclonal (8311)</td>
<td>0.005</td>
<td>Beckman Coulter Inc., (Mississauga, ON)</td>
</tr>
<tr>
<td>Kallikrein 3</td>
<td>monoclonal (8301)</td>
<td>monoclonal (8311)</td>
<td>0.005</td>
<td>Medix Biochemica, (Kauniainen, Finland)</td>
</tr>
<tr>
<td>Kallikrein 4</td>
<td>monoclonal (10F4-1G6)</td>
<td>polyclonal</td>
<td>0.2</td>
<td>in-house</td>
</tr>
<tr>
<td>Kallikrein 5</td>
<td>monoclonal (2A4)</td>
<td>monoclonal</td>
<td>0.05</td>
<td>in house; R&amp;D Systems, (Minneapolis, MN)</td>
</tr>
<tr>
<td>Kallikrein 6</td>
<td>monoclonal (27-4)</td>
<td>monoclonal E24</td>
<td>0.2</td>
<td>in house</td>
</tr>
<tr>
<td>Kallikrein 7</td>
<td>monoclonal (73-1)</td>
<td>monoclonal (8301)</td>
<td>0.2</td>
<td>in house</td>
</tr>
<tr>
<td>Kallikrein 8</td>
<td>monoclonal (19-10)</td>
<td>monoclonal (20-64)</td>
<td>0.2</td>
<td>in house</td>
</tr>
<tr>
<td>Kallikrein 9</td>
<td>monoclonal (M1G1-E11)</td>
<td>polyclonal</td>
<td>0.5</td>
<td>in house</td>
</tr>
<tr>
<td>Kallikrein 10</td>
<td>monoclonal (B14)</td>
<td>monoclonal (5D3)</td>
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<td>in house</td>
</tr>
<tr>
<td>Kallikrein 11</td>
<td>monoclonal (18-1)</td>
<td>polyclonal</td>
<td>0.2</td>
<td>in house</td>
</tr>
<tr>
<td>Kallikrein 12</td>
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<td>polyclonal</td>
<td>5</td>
<td>in house</td>
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<td>Kallikrein 13</td>
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<td>monoclonal (27-1)</td>
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<td>in house</td>
</tr>
<tr>
<td>Kallikrein 14</td>
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<td>polyclonal</td>
<td>0.2</td>
<td>in house</td>
</tr>
<tr>
<td>Kallikrein 15</td>
<td>monoclonal (820)</td>
<td>polyclonal</td>
<td>0.05</td>
<td>R&amp;D Systems (Minneapolis, MN); in house</td>
</tr>
</tbody>
</table>

\(^1\)AP, affinity-purified
3.2.3.1 Monoclonal-monoclonal ELISA configuration

The immunoassays for KLKs 2, 3, 5, 6, 7, 8, 10, and 13 were based on this configuration. Generally, white polystyrene microtiter plates (Greiner bio-one, Monroe, NC) were coated with 500 ng/well of monoclonal coating antibody in 100 µL of coating buffer (50 mM Tris-HCl, pH 7.8). The plates were incubated overnight, and then washed twice in wash buffer (10 mM Tris-HCl, pH 7.4, containing 150 mM NaCl and 0.5 mL/L Tween 20). Fifty µL of recombinant protein standards or samples, diluted in a 6% bovine serum albumin (BSA) solution were then added to the wells along with 50 µL of assay buffer (made with 6% BSA, containing 25 mL/L normal mouse serum, 100 mL/L normal goat serum, and 10 g/L bovine IgG). Plates were incubated for 2 hours with continuous shaking, and then washed six times in wash buffer as described above. One hundred µL/well of biotinylated monoclonal detection antibody (50 ng), diluted in assay buffer were added to each well and incubated for 1 h with continuous shaking. Plates were then washed six times as described above. One hundred µL (5 ng) of alkaline phosphatase-conjugated streptavidin, diluted in 6% BSA were added to each well, and incubated for 15 min with continuous shaking. Plates were washed six times, and 100 µL of diflunisal phosphate solution (0.1 M Tris-HCl pH 9.1, containing 1 mM diflunisal phosphate, 0.1 M NaCl, and 1 mM MgCl$_2$) were added to each well, and incubated for 10 min with continuous shaking. One hundred µL of developing solution (1mM Tris, 0.4 M NaOH, 2 mM TbCl$_3$, 3 mM EDTA) were then added to each well and mixed for 1 minute. The fluorescence was measured with a time-resolved fluorometer, the Cyberfluor 615 Immunoanalyzer (MDS-Nordion, Ottawa, ON). Calibration and
data reduction were performed automatically, as described above (see section 1.6).

3.2.3.2 Monoclonal-polyclonal ELISA configuration – version 1 (for KLK4, 12 and 14)

These assays follow the same procedure described above, up until addition of the detection antibody. Then, 100 µL of polyclonal antiserum, diluted 1,000 fold in assay buffer was added and plates were incubated for 1 h as above. Plates were then washed six times and 100 µL of alkaline phosphatase-conjugated goat-anti-rabbit antibody (Jaskson Immunoresearch, Westgrove, PA), diluted 3,000-fold in assay buffer, were added to each well, and incubated for 45 min with continuous shaking. Substrate and developing solution were then added, and plates were read as described above.

3.2.3.3 Monoclonal-polyclonal ELISA configuration – version 2 (For KLKs 9, 11, and 15)

These assays follow the same procedure as described above, up until addition of the detection antibody. Then, 100 µL of biotinylated polyclonal antibody, diluted in assay buffer were added, and plates were incubated for 1 h as above. Plates were then washed six times and 100 µL of alkaline phosphatase-conjugated streptavidin in 6% BSA were added to each well, and incubated for 15 min with continuous shaking. The assay was then completed as described above.
3.2.3.4 Polyclonal-polyclonal ELISA configuration (for KLK1)

This assay follows the same procedure as the monoclonal-monoclonal assay configuration, but with use of an affinity-purified polyclonal antibody, kindly provided by Dr. Julie Chao, Medical University of South Carolina.

3.2.4 Total RNA extraction and RT-PCR for KLKs

Total RNA isolated from various tissues was obtained from Clonetech (Mountain View, CA) with the exception of adipose, cervical and vaginal RNA, which was obtained from Ambion (Austin, TX). cDNA was produced from the total RNA using the Superscript III First-strand synthesis kit (Invitrogen, Canada Inc., Burlington, ON) employing an oligo-dT primer. PCR reactions were performed in a mixture containing 1 µL cDNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 200 µM deoxynucleoside triphosphates, 100 ng of primers (listed in table 3.2) and 2.5 U of Hot Start taq polymerase (Qiagen Inc., Mississauga, ON) on an Eppendorf thermocycler (Eppendorf, Westbury, NY). The cycling conditions were 95 °C for 15 min to activate the Taq polymerase followed by 35 cycles of 94 °C for 30 s, the annealing temperature for 30 s, 72 °C for 30 s and a final extension at 72 °C for 10 min. Equal amounts of PCR products were electrophoresed on 1.5% agarose gels and visualized with ethidium bromide staining. The primers used and expected lengths of product are listed in Table 3.2.
### Table 3.2: Primers used for RT-PCR of kallikreins from tissues.

<table>
<thead>
<tr>
<th>KLK gene</th>
<th>Sequence</th>
<th>Annealing Temp. (°C)</th>
<th>Length of Product, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>KLK1</td>
<td>F: CTCCTGGAGAACCACACCCGCC&lt;br&gt;R: GCGACAGAAGGCTATTGAGGG</td>
<td>65</td>
<td>419</td>
</tr>
<tr>
<td>KLK2</td>
<td>F: GGCAGGGCTGCTGTCACGTAC&lt;br&gt;R: CAACATGAAACTCTGTCACCTTCTC</td>
<td>60</td>
<td>479</td>
</tr>
<tr>
<td>KLK3</td>
<td>F: CCCCAGCTCAGAAACAAAGGC&lt;br&gt;R: GGTGCTCAGGGTGTGGGCCAC</td>
<td>65</td>
<td>601</td>
</tr>
<tr>
<td>KLK4</td>
<td>F: GCAGCAGTCATTGCAATAGAAAC&lt;br&gt;R: AACATGCTGGGTGTACAGCG</td>
<td>60</td>
<td>437</td>
</tr>
<tr>
<td>KLK5</td>
<td>F: GTCACCAGTTTATGAATCTG&lt;br&gt;R: GCCGCAGAACATGTTGTCATC</td>
<td>60</td>
<td>328</td>
</tr>
<tr>
<td>KLK6</td>
<td>F: GAAGCTGATGGTGGTGGTGGAGCTC&lt;br&gt;R: GTCAAGGGAATACCATCTCTATGTC</td>
<td>60</td>
<td>454</td>
</tr>
<tr>
<td>KLK7</td>
<td>F: CCGGCTCTGCAAGATGAATGAG&lt;br&gt;R: AGCGCAGCATGGAATTTTCC</td>
<td>65</td>
<td>454</td>
</tr>
<tr>
<td>KLK8</td>
<td>F: CCTTTGTTCCAGGGCCAC&lt;br&gt;R: GCATCTCTCAGCAGTTCTTGGG</td>
<td>65</td>
<td>416</td>
</tr>
<tr>
<td>KLK9</td>
<td>F: TCTTCCCCACCTGGCTTCAAC&lt;br&gt;R: CGGGTGCTGAGAGGGGCAG</td>
<td>65</td>
<td>409</td>
</tr>
<tr>
<td>KLK10</td>
<td>F: GGAACAAGCCACTGTTGGG&lt;br&gt;R: GAGGATGCTTTAGGGGAGG</td>
<td>60</td>
<td>468</td>
</tr>
<tr>
<td>KLK11</td>
<td>F: CTCTGGCAACAGGGGCTTGAGG&lt;br&gt;R: GCATCGCAAGGTTGTGGAGG</td>
<td>60</td>
<td>461</td>
</tr>
<tr>
<td>KLK12</td>
<td>F: TCAGCCAGGACACACCCCG&lt;br&gt;R: GCCCTTCTTTTATA</td>
<td>65</td>
<td>905</td>
</tr>
<tr>
<td>KLK13</td>
<td>F: GAGAAGCGCCACCCACAC&lt;br&gt;R: CAGGATCCACAGACCATCTTG</td>
<td>65</td>
<td>441</td>
</tr>
<tr>
<td>KLK14</td>
<td>F: CACTGCGGCCGCCCAC&lt;br&gt;R: GCGAGGCACGCGCTTC</td>
<td>65</td>
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</tr>
<tr>
<td>KLK15</td>
<td>F: CTACGGACAGTCAGCTG&lt;br&gt;R: GACACCGGCTTTGTTG</td>
<td>65</td>
<td>459</td>
</tr>
</tbody>
</table>

1 F: forward primer; R: reverse primer
3.3  Results

3.3.1  Tissue extracts

Seven sets of adult human tissue extracts and three sets of fetal tissue extracts were assayed for KLK protein levels using specific ELISAs for each KLK. Colour-coded summaries of global KLK protein levels in adult tissues, fetal tissues, and fluids are shown in Figures 3.1, 3.2 and 3.3. KLK mRNA levels in adult tissues are shown in Figure 3.4.

KLK1 was found to be most highly expressed in the pancreas and salivary gland, followed by the colon and small intestine. Lower levels were found in the esophagus, kidney, lymph node, prostate, stomach, thyroid, ureter and vagina. In fetal tissues, highest levels were seen in the pancreas and colon, and lower levels in the skin, small intestine, spleen and stomach.

KLK2 was found primarily in the prostate, with relatively high expression levels. KLK3 was also found primarily in the prostate, with much lower levels in the adrenal gland, breast, colon, heart, muscle, spleen, stomach, testis, thyroid and ureter. KLK3 was only detected in the fetal adrenal gland. KLK4 showed relatively low expression in a wide variety of tissues, adult and fetal.
Chapter 3  Global expression of KLKs in tissues and fluids

![Diagram of Kallikrein expression in tissues](image)

**Figure 3.1  Global expression of KLKs in Adult Tissues**

KLK protein levels were quantified in tissue extracts using specific ELISAs for each KLK. KLK levels are indicated by the colour codes at the bottom of the figure.
Figure 3.2: Global KLK Expression in Fetal Tissues

KLK protein levels were measured in fetal tissue extracts using ELISA assays for each KLK. KLK levels are represented by the colour codes shown at the bottom of the figure.
Figure 3.3  Global KLK Expression in Biological Fluids

KLK protein levels were quantified in biological fluids using ELISA assays for each KLK. KLK levels are represented by the colour codes shown at the bottom of the figure.
Figure 3.4  KLK mRNA levels in Adult Tissues

KLK levels were quantified by RT-PCR. Relative mRNA levels are represented by the colour codes at the bottom of the figure.
KLK5 was found most highly expressed in the skin (adult and fetal), with lower levels of expression in the breast, esophagus, and salivary gland. KLK6 is most highly expressed in the brain and spinal cord. KLK6 also showed relatively low expression in a wide array of other adult and fetal tissues.

KLK7 is most highly expressed in skin tissue, both adult and fetal. Relatively high levels of KLK7 were also found in the esophagus and heart, with lower levels in the adrenal gland (adult and fetal), kidney (adult and fetal), and liver. KLK8 is most highly expressed in the esophagus, skin (adult and fetal) and tonsil, with lower levels in the adrenal gland (adult and fetal), breast, kidney (adult and fetal), fetal liver, and salivary gland. Highest levels of KLK9 were seen in heart (adult and fetal) and fetal cartilage. KLK9 was also found to be expressed in a wide variety of other tissues, both adult and fetal.

KLK10 was found highly expressed in the tonsil and skin (adult and fetal). Lower levels were seen in the brain, cervix, esophagus, fallopian tube, lung (adult and fetal), salivary gland, fetal thymus and trachea (adult and fetal). KLK11 was found to have a broad tissue expression pattern, with most abundant expression in the prostate and testis. Relatively high levels of KLK11 were also found in the esophagus, ovary, stomach (adult and fetal), tonsil, fetal thymus, and trachea (adult and fetal). KLK11 was also found at lower levels in a number of other tissues, adult and fetal. The highest levels of KLK12 were found in bone marrow and bone, followed by adult and fetal colon and stomach. KLK12 was also widely expressed at relatively moderate levels in a variety of other adult tissues. KLK13 was found to be highly expressed in the esophagus, and tonsil. Lower levels
were seen in the cervix, salivary gland, and vagina. KLK13 was also broadly expressed at relatively low levels in other adult and fetal tissues.

KLK14 showed a broad expression pattern, with the highest levels in fetal skin, and cartilage. KLK14 was also found in an array of other tissues, the most prominent being breast, skin and vagina. KLK15 is most highly expressed in the breast, adult and fetal skin, and fetal stomach. Lower levels were found in the adrenal gland (adult and fetal), brain, heart (adult and fetal), kidney (adult and fetal), liver (adult and fetal), prostate, salivary gland and spinal cord, as well as in several other fetal tissues.

3.3.2 Biological fluids

Kallikreins were quantified in biological fluids using ELISA assays. A summary for all KLKs is found in Figure 3.3.

KLK1 was found at high levels in urine, with lower levels in seminal plasma and saliva. Relatively high levels of KLK2 were found in seminal plasma, with trace amounts detected in breast milk, breast cancer cytosol, breast cyst fluid, saliva and urine. Very high levels of KLK3 were found in seminal plasma with relatively low levels in urine. KLK4 was primarily found in seminal plasma with lower levels found in breast milk and urine. Trace amounts of KLK4 were found in several other fluids.

Breast milk and ascites fluid from ovarian cancer patients contained, on average, the highest levels of KLK5, with lower levels found in seminal plasma, breast cyst fluid, follicular fluid, breast cancer cytosol, amniotic fluid, saliva, CVF, CSF and urine. KLK6 was detected at high levels in breast milk and CSF, with lower levels in breast cyst fluid, ascites, saliva, and CVF extract. Trace amounts
of KLK6 were found in seminal plasma, follicular fluid, breast cancer cytosol, amniotic fluid, and urine. KLK7 was found at highest levels in breast milk, with lower levels in seminal plasma, breast cancer cytosol, ascites, saliva and CVF extract. The highest levels of KLK8 were found in breast milk, with lower levels in follicular fluid, ovarian cancer ascites, saliva, and CVF extract. KLK9 was found primarily in breast milk, with low levels in seminal plasma, amniotic fluid and CSF. Trace amounts of KLK9 were found in follicular fluid, breast cancer cytosol, breast cyst fluid, ascites, saliva, and urine.

KLK10 was found at high levels in saliva, with lower levels in breast cyst fluid, ovarian cancer ascites and CVF extract. The highest levels of KLK11 were found in seminal plasma, with relatively low levels in all other fluids tested. Moderate levels of KLK12 were found in breast milk, breast cancer cytosol, CVF extract and saliva. KLK13 was found at highest levels in CVF extract, seminal plasma and saliva. Relatively low levels of KLK13 were found in all other fluids tested. KLK14 was found most abundantly in seminal plasma, amniotic fluid, and saliva, with lower levels detected in all other fluids tested. KLK15 was found primarily in breast milk, with lower levels in seminal plasma, breast cancer cytosol, and saliva.

3.3.3 RT-PCR

For comparative purposes, I have also assessed global KLK expression by RT-PCR in one adult tissue set. The data are summarized in Figure 3.4. I classified the expression by semiquantitative scoring, based on visual comparison of band intensities of ethidium bromide-stained agarose gels.
3.3.4 Tissue specificity of KLK expression

Based on the quantitative data of KLK protein expression in diverse adult tissues, I separated KLK expression into 3 categories, as follows. Very restricted expression (expression at comparatively high levels in one tissue, with lower levels (<1%) in other tissues), restricted expression (expression at comparatively high levels in 2-4 tissues, with lower levels (<20%) in other tissues), and wide expression (comparatively high levels in 5 or more tissues). The data are shown in Table 3.3.
Table 3.3: Abundance patterns of kallikreins, categorized according to levels in adult tissues.

<table>
<thead>
<tr>
<th>Tissue Abundance</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Very Restricted (tissue)</strong></td>
</tr>
<tr>
<td>KLK2 (prostate)</td>
</tr>
<tr>
<td>KLK3 (prostate)</td>
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<tr>
<td></td>
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</table>
3.4 Discussion

The protein expression patterns of the fifteen kallikreins can be divided into three classes; highly restricted, restricted, and wide expression (Table 3.3). Our most pertinent findings are presented in Figures 3.1-3.3. In general good concordance between KLK transcript and protein levels was observed, however in some cases, discrepancies between mRNA transcript and protein abundance existed. These discrepancies are most likely explained by degradation of KLK proteins, or by the fact that KLKs are secreted and therefore may not be present in high abundance in cytosols where their levels were measured.

3.4.1 KLK1

The primary role of KLK1 is the conversion of kininogen to Lys-bradykinin (71), resulting in several physiological responses, such as vasodilation and smooth muscle contraction (95). KLK1 has previously been shown to be expressed at both the mRNA and protein level in the aorta (171), colon (172), kidney (173), lung (174), pancreas (175), salivary gland (175) and trachea (174), and at the mRNA level in the adrenal gland (176) and skin (177). I confirmed these findings.

Consistent with KLK1 expression in the salivary gland and prostate, and with previous studies (175) I found relatively high levels of KLK1 in saliva and seminal plasma. I also found high levels of KLK1 in urine, consistent with previous findings (95).

3.4.2 KLK2 and KLK3

KLK2 and KLK3 are highly expressed in the prostate and found in seminal plasma, as expected. KLK2 and KLK3 expression in other tissues is much lower.
Given that KLK2 is a known activator of KLK3, KLK2 and KLK3 co-expression suggests participation in a common cascade in the prostate and other tissues (72). This also fits with the finding of KLK2 and KLK3 co-regulation by androgens (48;178). KLK2 and KLK3 were found in breast milk, breast cytosol, breast cyst fluid, saliva, and urine but at much lower levels than in seminal plasma. The twelve urine samples represented 6 female and 6 male samples. KLK3 levels were found higher in male urine samples as expected.

3.4.3 KLK4

KLK4 does not appear to be highly expressed by any of the tissues tested, when compared with other kallikreins. I confirmed previous findings of low KLK4 expression in the brain, breast, cervix, liver, prostate, salivary gland, and thyroid (179). With the exception of the liver, KLK4 mRNA data corresponded well with KLK4 protein expression in the tissues listed above, as well as with previous findings (180), and our own data. I confirmed previous findings (179) of KLK4 secretion into seminal plasma and urine.

3.4.5 KLK5

KLK5 was originally cloned as human stratum corneum tryptic enzyme (HSCTE) (181), and has a role in skin desquamation. I found highest levels of KLK5 expression in adult and fetal skin. KLK5 has been proposed as a potential biomarker for breast and ovarian cancer (182), and has been shown to be differentially regulated in testicular and lung cancer, at the mRNA level (183;184). I found moderate KLK5 expression in breast and testis and low KLK5 expression in lung. KLK5 protein expression data shows good concordance with mRNA findings.
KLK5 was detected in breast milk, breast cyst fluid, seminal plasma, saliva, cervico-vaginal fluid extract and ovarian cancer ascites (24). Traces were found in follicular fluid, breast cancer cytosol and amniotic fluid.

### 3.4.6 KLK6

We confirmed previous findings of KLK6 expression in a large number of tissues. Our protein expression data shows good concordance with both our own, and previously published mRNA data (185). I found KLK6 expression to be highest in the brain and spinal cord, with moderate to high levels in the breast, fallopian tube, kidney, lung, and salivary gland, as previously reported (186). KLK6 was previously found in seminal plasma, breast milk, breast cancer cytosol, breast cyst fluid, amniotic fluid, ovarian cancer ascites, and CSF (169;186). I confirmed the above findings and further report relatively high levels of KLK6 in cervico-vaginal fluid extract and traces in follicular fluid and saliva.

### 3.4.7 KLK7

KLK7 was originally purified as stratum corneum chymotryptic enzyme (HSCCE) and is involved in the skin desquamation process (187). I found high KLK7 expression in adult and fetal skin. I also confirmed previous findings of KLK7 expression in the heart, esophagus, kidney, lung, and salivary gland (184;188;189). KLK7 is significantly expressed in the adult heart at both the mRNA and protein level. This finding was not previously reported.

We have also confirmed previous findings of KLK7 secretion into seminal plasma, breast milk, amniotic fluid, ovarian cancer ascites, saliva, CSF, and urine (188), and in addition, cervico-vaginal fluid extract.
3.4.8 KLK8

KLK8 was originally cloned from a human skin cDNA library (190), and the protein was found in skin as a desquamation-related protease (191). I found KLK8 protein in the esophagus, kidney, ovary, salivary gland, skin, tonsil, breast, and cervix, as previously reported (192). Fetal tissue expression of KLK8 in the kidney, and skin was also confirmed (192). I identified KLK8 expression in the adrenal gland, and liver at both the mRNA and protein level, in both adult and fetal tissues. KLK8 has been previously detected in breast milk and amniotic fluid (192). I have confirmed KLK8 secretion in these two fluids and have also detected KLK8 in ovarian ascites, saliva, cervico-vaginal fluid extract and CSF.

3.4.9 KLK9

Little is known about the expression pattern of KLK9, particularly at the protein level. KLK9 has previously been shown to be expressed in the brain, liver, lung, small intestine, spinal cord, thymus, trachea, prostate, testis and breast at the mRNA level (193), and primarily expressed in the testis and seminal vesicle at the protein level (194). I confirmed KLK9 protein expression in the brain, liver, small intestine, spinal cord, trachea, prostate, testis, and breast. In addition, KLK9 was also found in a wide range of other tissues, both adult and fetal in concordance with our mRNA findings.

The secretion of KLK9 into biological fluids has not been studied to date. Here, I report KLK9 presence in breast milk and at lower levels in amniotic fluid, CSF, ovarian cancer ascites, saliva, breast cancer cytosols, seminal plasma and urine.
3.4.10 KLK10

*KLK10* was originally cloned from breast epithelium and was named normal epithelial cell-specific gene (NES1) (195). KLK10 has been shown to have clinical utility as a breast and ovarian cancer biomarker (104). I confirmed previous findings of KLK10 expression in the cervix, fallopian tube, liver, lung, salivary gland, and skin (196), in concordance with our own mRNA findings, and previous findings (195). I was unable to confirm KLK10 expression in breast tissue by our criteria (at least 50% of all tissues tested should be positive) although KLK10 was found in two out of five breast tissues examined, with a mean value of 600 ng/g (data not shown). Most of the tissue samples collected were from post-menopausal women. This could explain why KLK10 was not found in all breast tissues examined, given the lower steroid hormone levels in these women. I found KLK10 to be relatively highly expressed in the adult brain and tonsil, which has not been previously shown.

We have also confirmed previously published results (196) on presence of KLK10 in fluids, such as breast milk, seminal plasma, amniotic fluid, CSF, and ovarian cancer ascites. In this study, highest levels of KLK10 were found in saliva, followed by ovarian cancer ascites and cervico-vaginal fluid extract, findings not previously reported.

3.4.11 KLK11

KLK11 shows a broad tissue expression pattern. As previously shown (197), KLK11 protein is expressed most abundantly in the prostate, and is secreted at high amounts into seminal plasma. High levels of KLK11 were found in diverse tissues and fluids, including the lung, which has previously been
reported at the mRNA level (198). The protein expression pattern of KLK11 was in concordance with mRNA findings. I found relatively high levels of KLK11 in cervico-vaginal fluid extract, breast milk and ascites from ovarian cancer patients which have not been previously shown, although KLK11 has been proposed as an ovarian cancer biomarker (197).

3.4.12 KLK12

The protein expression pattern of this kallikrein has not been characterized, however one study reported KLK12 expression in microvascular endothelial cells (MVECS) (199), with reduced MVEC KLK12 expression in the presence of systemic sclerosis. I show here that KLK12 has a wide expression pattern in adult tissues but restricted expression in fetal tissues, such as the colon, albeit at relatively high levels. KLK levels in biological fluids are moderate.

3.4.13 KLK13

KLK13 has not been widely studied, however, KLK13 expression was previously found in the breast, esophagus, kidney, prostate, salivary gland, skin, testis, thyroid, tonsil, trachea, ureter and lung, with highest levels seen in esophagus and tonsil (200). A recent study by our group (62), has also suggested a role for KLK13 in skin desquamation. I confirmed these previous findings. I also confirmed a previous report on KLK13 secretion in seminal plasma (200) and extend these findings to saliva and cervico-vaginal fluid extract.

3.4.14 KLK14

Previous studies have been primarily focused on KLK14 expression in skin and its role in skin desquamation through proteolytic cascades with kallikreins 5, and 7 (62;70). I confirmed KLK14 expression in both adult and fetal skin; KLK14
is expressed more highly in the latter. KLK14 has also been previously shown to be expressed in breast, prostate, brain, lymph node, lung, testis, and stomach (201). I confirmed KLK14 expression in the breast, prostate, brain, lung, and stomach; however I did not detect KLK14 in the lymph node or testis. Previous studies looked at KLK14 expression in only one tissue set. I believe the multiple panels examined here provide a more accurate representation of the KLK14 expression profile. KLK14 was found in a wide variety of other tissues, particularly of fetal origin, suggesting that KLK14 may play a developmental role. KLK14 was found primarily in seminal plasma, amniotic fluid (201) and cervico-vaginal fluid extract.

3.4.15 KLK15

Little is known about KLK15. A previous study by our group showed very low levels of KLK15 protein in the thyroid gland, colon, and prostate, and secretion of KLK15 primarily into seminal plasma (28). Analysis of multiple tissues sets in this study has revealed that KLK15 expression is highest in the breast, and fetal skin. Low levels of KLK15 were detected in the prostate, as previously published (28). I confirmed KLK15 secretion into seminal plasma, and even higher levels in breast milk. KLK15 tissue protein expression is not always in concordance with KLK15 mRNA presence. For example KLK15 mRNA is found most highly expressed in colon tissue, whereas we did not detect KLK15 protein in the colon. This may be due to degradation of the protein by autolysis or by other proteases within the colon.

KLK15 was found to be expressed at low levels in several tissues but more widely in fetal tissues than adult tissues, similarly to KLK14.
3.4.16 Kallikrein co-expression patterns

Our studies have revealed many tissues of kallikrein co-expression. Previous studies reported kallikrein co-expression in the prostate and seminal plasma, as well as in skin (104). Proteolytic cascades involving the co-expressed kallikreins have previously been described in skin and seminal plasma (62;68-70). Our results pinpoint to many other potential areas of KLK co-expression and possibly, cooperation. These data are shown in Figures 3.1-3.3. Many KLKs are co-expressed in the skin, salivary gland, prostate, CNS and breast, as previously reported. Here, I report additional tissues, as shown in Figure 3.1. Co-existence in biological fluids is shown in Figure 3.3. These data should be valuable in future efforts to build, or complement, proteolytic cascade pathways involving several KLKs and cross-talks with other proteolytic systems. These data also complement previous findings of KLK co-regulation by steroid hormones.
Chapter 4: Immunohistochemical Localization of KLKs in the Female Reproductive System
4.1 Introduction and rationale

KLKs are secreted serine proteases which are produced primarily by the glandular epithelium of KLK-expressing tissues (66). I found relatively large levels of several KLKs in CVF extract (65) which is a pool of fluids originating from the fallopian tube, endometrium, cervix and vagina (130).

The lining of the fallopian tube contains a single layer of ciliated and non-ciliated columnar epithelial cells. The non-ciliated cells produce secretions which are propelled towards the uterus by the ciliated cells. This action aids in carrying the ovum down the fallopian tube towards the uterus. These secretions also provide nutrition to the ovum for its time in the fallopian tube (128;202).

Histologically, the endometrium can be divided into a functional zone, closest to the uterine cavity, and a basilar zone, adjacent to the muscular underlying myometrium. The functional zone is rich in glands and makes up most of the endometrial thickness. The appearance of the endometrium changes depending on the phase of the menstrual cycle: menses, proliferative or secretory. The functional zone of the endometrium is destroyed during menses. The proliferative phase following menses involves multiplication of uterine glands, regeneration and revascularization of the functional zone. During the secretory phase the uterine glands enlarge and increase their rate of secretion. The secretory activity peaks approximately 12 days after ovulation at which time the glandular activity declines and if pregnancy does not occur, menses ensues and the cycle begins once more (127;128).

The canal portion of the cervix is lined by a single layer of columnar mucus-secreting cells, whereas the portion of the cervix exposed to the hostile
environment of the vagina is composed of a thick stratified squamous epithelium as is the vagina (202). The epithelium of the vagina itself is stratified squamous and undergoes cyclical changes under hormonal stimulation during the menstrual cycle (128). The female external genitalia contains two glands called the glands of Bartholin which open into the vestibule and secrete mucus (128).

Given that I found many KLKs expressed in CVF extract and that CVF is made up of materials from several origins, I wanted to investigate the immunohistochemical localization of KLKs in the female reproductive system, specifically the fallopian tube, endometrium, cervix and vagina. Knowledge of KLK localization will aid in determining potential KLK functions in CVF and in cervico-vaginal physiology.

4.2 Materials and methods

4.2.1 Immunohistochemistry

Staining was performed on 4 µm thick paraffin sections of tissues fixed in buffered formalin. A streptavidin-biotin protocol was employed using the DAKO Envision automated LSAB kit (Dako, Cambridgeshire, United Kingdom). The following rabbit polyclonal antibodies and dilutions were used: KLK5, 1:300; KLK6, 1:600; KLK11, 1:600; KLK12, 1:1000; KLK13, 1:400. The Trilogy antigen retrieval system (Cell Marque, Rocklin CA) was used for antigen exposure.

Staining included deparaffinization in warm xylene for 5 min with two changes of xylene at room temperature. This was followed by rehydration by transfer through graded alcohols. Endogenous peroxidase activity was blocked with 0.5% H₂O₂ in methanol for 10 min. The sections were pretreated with Trilogy © solution (Cell Marque, Rocklin CA) for antigen retrieval and then
incubated overnight at 4 °C with the KLK specific antibodies in 3% BSA. The sections were then washed twice in 50 mM Tris (pH 7.6) and the biotinylated Link (Dako, Cambridgeshire, United Kingdom was applied for 15 min. A streptavidin-peroxidase conjugate (Dako) was then added for 15 min, following which the enzymatic reactions was developed in a freshly prepared solution of 3,3-diaminobenzidine tetrahydrochloride using DAKO Liquid DAB Substrate-Chromogen Solution for 10 min.

Negative controls were performed for all studied tissues by omitting the primary antibody or by replacing it by non-immune serum (dilution 1:500). The stained sections were reviewed by a trained pathologist.

4.3 Results

4.3.1 Fallopian tubes

A diffuse, cytoplasmic staining of all KLKs was found in the secretory and ciliated cells of the epithelium (Figures 4.1, A-F). KLK12 immunoexpression (IE) was stronger than the IE of the other KLKs (Figure 4.1E).

4.3.2 Endometrium

All KLKs were IE in the epithelium of the endometrium in both the proliferative and secretory phases. The staining was cytoplasmic with a characteristic infranuclear distribution (Figures 4.2, A-F). KLK11, KLK12 and KLK13 showed a stronger IE (Figures 4.2, E-F).
Figure 4.1: Immunohistochemical expression of KLKs in the epithelium of the fallopian tube

The immunolocalization of the KLKs is indicated by arrows. A: Non-immune serum x400 (no staining); B: KLK5 x400; C: KLK6 x400; D: KLK11 x400; E: KLK12 x400; F: KLK13 x400.
Figure 4.2: Immunohistochemical expression of KLKs in the epithelium of the endometrium

Localization of the KLKs is indicated by arrows. A: Non-immune serum x400 (no staining); B: Proliferative endometrium, KLK5 x200; C: Proliferative endometrium, KLK6 x400; D: Secretory endometrium, KLK11 x400; E: Proliferative endometrium, KLK12 x400; F: Proliferative endometrium, KLK13 x200.
4.3.3 Cervix

Cytoplasmic immunoexpression of the five KLKs was observed in the mucin-secreting epithelium of the endocervix and the tubular cervical glands. KLKs 11 and 12 were strongly expressed, while KLKs 5, 6 and 13 were moderately immunoexpressed (Figures 4.3, A-F).

4.3.4 Vagina

The stratified squamous epithelium of the vagina showed a full-thickness IE, with varying intensities for the different KLKs analyzed. KLK12 IE was the strongest, followed by KLK13 IE (Figures 4.4, E & F). KLKs 5, 6 and 11 were weakly immunoexpressed. The epithelium of the Batholin’s glands, both the ductal and the mucus-secreting columnar of the acini, showed a moderate to strong IE for all KLKs (Figures 4.4, B-D). KLK5 IE was stronger in the ductal epithelium than in the mucous-secreting columnar cells of the acini (Figure 4.4 B).
Figure 4.3: Immunohistochemical expression of KLKs in the epithelium of the endocervix

Localization of the KLKs is indicated by arrows. A: Non-immune serum x200 (no staining); B: KLK5 x400; C: KLK6 x400; D: KLK11 x400; E: KLK12 x400; F: KLK13 x200.
Figure 4.4: Immunohistochemical Localization of KLKs in the Vagina

A: Vaginal squamous epithelium, non-immune serum x 400 (no staining); B: Strong KLK5 immunohistochemical expression by the ductal epithelium of Bartholin’s glands (arrow) and weaker expression by the mucous-secreting acinar cells (arrowhead) x400; C: KLK6 immunohistochemical expression by the mucous-secreting acinar columnar cells of Bartholin’s glands (arrow); D: KLK11 immunohistochemical expression by the ductal epithelium (arrow) and the mucous-secreting acinar cells (arrowhead) x400; E: KLK12 immunohistochemical expression in the squamous vaginal epithelium (arrow) x400; F: KLK13 immunohistochemical expression in the squamous vaginal epithelium (arrow) x400.
4.4 Discussion

The presence of large levels of many KLKs in CVF extract is explained by the fact that I found KLKs to be immunoexpressed by the epithelium of all studied tissues (endometrium, endocervix, vagina, Bartholin’s glands and fallopian tubes). Each of these tissues contributes to the CVF milieu through secretions or exfoliation of cells. Furthermore, the higher levels of some KLKs (mainly KLK12 followed by KLK11) in the CVF extract, as well as in tissue extracts matches with our findings of stronger immunoexpression of these KLKs in the corresponding tissues.

Specifically, KLK localization in the endometrium suggests that KLKs may play a role in the remodelling of the functional zone during the menstrual cycle. Increased secretion by the endometrial glands during the secretory phase of the menstrual cycle suggests that increased levels of KLKs may be secreted during this phase of the menstrual cycle. This will be further discussed in chapter 5.
Chapter 5: Hormonal Regulation of KLKs in Cervico-vaginal Physiology
5.1 Introduction and rationale

All KLKs have been shown to be under some form of steroid hormone regulation, at the mRNA and protein level, in some cancer cell lines (2;48). Many KLKs are found to be dysregulated in hormone-dependent malignancies such as breast, ovarian and prostate cancer (104).

We have shown that KLKs are expressed by the epithelium of the fallopian tube, endometrium, cervix and vagina and have identified relatively large levels of several KLKs in human Cervico-vaginal fluid (CVF) extract (65). Given that the menstrual cycle is a hormonally regulated process, I believe that KLKs may be regulated by hormonal changes during the menstrual cycle. Salivary levels of KLKs 1 and 3 as well as serum levels of KLK3 have previously been shown to be altered by hormonal changes during the menstrual cycle in women (203-205). Here I have analyzed KLK levels throughout the menstrual cycle in CVF extract and saliva from a normal cycling premenopausal women. KLK levels in CVF extract from pregnant women were also measured. During pregnancy, steroid hormone levels are dramatically increased (127), suggesting that KLK levels may be altered by these rising hormone levels. Lastly, I also analyzed expression and hormonal regulation of KLKs in cultured human vaginal epithelial cells.

5.2 Materials and methods

5.2.1 CVF and saliva sample collection

Tampons were provided to a healthy, 30 year old, female volunteer, who was not pregnant. The subject was asked to insert the tampon into her vagina for 1 hour, every other day for an entire menstrual cycle. The tampon was then
removed and stored in 50 mL plastic conical tubes (BD Biosciences, Mississauga, ON), at -20°C until use.

CVF was collected from pregnant women using a polyester vaginal swab. The swab was rolled across the posterior vaginal fornix to absorb fluid. The swab was then inserted into 1 mL of sterile phosphate-buffered saline (PBS) and stored at -80 °C until use.

Saliva samples were collected from one female over one menstrual cycle. Male saliva samples were collected on the same day as female samples as control. Saliva was mixed 1:1 with PBS (pH 7.2) and stored at -20 °C until use.

Our protocols have been approved by the Institutional Review Boards of Mount Sinai Hospital and the University of Toronto.

### 5.2.2 CVF extraction

Tampons were used to collect CVF were thawed, 20 mL of sterile PBS was added to the tube with the tampon, and was mixed by rotation for 14 hours. The extract was removed from the tampon using a 20 mL syringe, which was used to squeeze the fluid out of the tampon. The CVF samples were stored at -20°C until use.

### 5.2.3 KLK ELISA immunoassays

The ELISA immunoassays used to measure KLK levels in hormonally stimulated vaginal epithelial cells, CVF extract and saliva were described above (see sections 2.2.3).

### 5.2.4 Analysis of trypsin-like activity in CVF and saliva

Total trypsin-like activity in CVF extract and saliva was measured using the fluorogenic substrate, Valine-Proline-Arginine-amino-4-methylcoumarin
CVF extract or saliva was diluted 20 fold in 100 mM Tris, 100 mM NaCl (pH 8.0) and 0.2 mM VPR-AMC in a total volume of 100 µL. Fluorescence was measured at an excitation wavelength of 355 nM and emission wavelength of 460 nM. Enzymatic activity is expressed as the fluorescence units per minute time per microgram of total protein in each CVF extract or saliva sample.

5.2.5 Steroid hormones

All hormones were purchased from Sigma-Aldrich (St. Louis, MO). All steroid hormone stock solutions (10⁻⁵ M) and dilutions were prepared in 100% ethanol.

5.2.6 Cell line

VK2 vaginal epithelial cells were purchased from the American Type Culture Collection (ATCC, Manassas VA). This epithelial cell line was established from the normal vaginal mucosa of a premenopausal woman. The cells were immortalized with the retroviral vector LXSN-16E6E7 and are characteristic of stratified squamous, non-keratinizing epithelia.

5.2.7 Cell culture

VK2 (vaginal epithelial) cells were maintained in keratinocyte serum free medium supplemented with EGF and BPE (Invitrogen Canada Inc., Burlington, ON). All cells were grown to between 60 and 90% confluence at which point cells were seeded at a density of 500,000 cells/well in a 6-well plate. Cells were left for 24 hours after which medium was removed and replaced with RPMI containing 10% charcoal-dextran stripped FBS. At this point, cells were hormonally stimulated once with either alcohol (< 1% ethanol final concentration
as a control), dexamethasone, norgestrel or estradiol (all at $10^{-8}$ M final concentration). Cells were incubated for 7 days, following which the supernatant was collected and frozen at -20°C until use. All hormonal stimulations were performed in triplicate.

5.2.8 Statistical analysis

The detection limit of each immunoassay was < 0.2 ug/L. This detection limit was used to calculate fold changes in KLK expression upon hormonal stimulation in VK2 cell supernatant in which KLK levels were undetectable, in the absence of hormonal stimulation. KLK levels undetectable by immunoassay upon alcohol stimulation were considered to be expressed at the lowest level of detection by the immunoassay.

Statistical analysis was performed using Prism software (version 4.02). The differences in mean expression levels between alcohol and each of the hormonal stimulations were calculated using one-way analysis of variance (ANOVA) followed by Dunnett’s post-hoc analysis. Differences in means with p values less than 0.05 were considered to be statistically significant.

Differences in mean KLK levels in CVF extract from pregnant women versus non-pregnant women were compared using the Mann-Whitney test. Differences in means with p values less than 0.05 were considered statistically significant.

5.2.9 Immunoprecipitation and western blotting for analysis of steroid hormone receptor status in VK2 cells

VK2 vaginal epithelial cells and T-47D breast cancer cells (for control expression of receptors) were grown to confluence in 75 cm$^2$ flasks. Cells were
then washed twice with cold PBS (pH 7.2) following which 1 mL of cell lysis buffer (20 mM Tris-HCl (pH 7.8), 150 mM NaCl, 1% NP-40, 2 mM Na₂EDTA, 10 mM PMSF, 10 ug/mL aprotonin, 10 ug/mL leupeptin) was added to the cells. Cells were then scraped off the bottom of the plate and transferred to a 1.5 mL tube. Lysates were incubated on ice for 15 minutes following which they were centrifuged at 13,000 rpm for 15 minutes at 4 °C to pellet membranous portions. Total protein levels of each lysate were determined using the Pierce BCA protein assay kit (Pierce, Rockford IL) according to the manufacturer’s instructions.

250 µg of each lysate was incubated with 2 µg of progesterone receptor antibody (AB-52 mouse monoclonal from Santa Cruz Biotechnology, Santa Cruz, CA) and 2ug of estrogen receptor antibody (62-A3 mouse monoclonal from Cell Signaling, Danvers MA) for 1 hour at 4°C on a nutator. 25 µL of protein A/G linked agarose (Santa Cruz Biotechnology, Santa Cruz, CA) was then added and samples were incubated for an additional 2 hours at 4°C on a nutator. Protein A/G beads were then washed 3 times with cold PBS (pH 7.2) and centrifuged between each wash for 30 seconds at 13,000 rpm. Protein A/G beads were then taken up in 30 µL of 4X SDS-PAGE sample buffer (Invitrogen Canada Inc., Burlington, ON) containing 100 mM DTT. Samples were then boiled for 10 minutes at 100 °C to release proteins from the protein A/G beads. Proteins were resolved by SDS-PAGE using NuPAGE Bis-Tris 4-12% gradient gels (Invitrogen Canada Inc., Burlington, ON) after which proteins were transferred to Hybond-C blotting membrane (GE Healthcare, Mississauga, ON). Western blotting for the progesterone receptor and estrogen receptor α was performed using specific antibodies for these proteins. The progesterone receptor antibody used was PR
(AB-52) (Santa Cruz Biotechnology, Santa Cruz, CA) and the estrogen receptor antibody was ERα (62A3; Cell Signaling, Danvers, MA).

For western blotting membranes were incubated with either the PR antibody (diluted 500-fold in 1% milk in TBST, incubated for 1 hour at room temperature) or the ER α antibody (diluted 1000-fold in 5% milk in TBST, incubated overnight at 4 °C), after which membranes were washed 3 times for 15 minutes each in TBST. Membranes were then incubated in alkaline-phosphatase conjugated goat-anti-mouse (Jackson Immunoresearch, West Grove PA) diluted 5000-fold in 1% milk in TBST for 1 hour at room temperature. Membranes were washed again, as above, and fluorescence was detected using a chemiluminescent substrate (Diagnostics Products Corp. Los Angeles, CA).

5.3 Results

5.3.1 Hormonal regulation of KLKs in CVF and saliva over the menstrual cycle

CVF extract and saliva samples were collected from a female and male subject over the course of the menstrual cycle (male subject, saliva only). KLK levels were measured in the CVF extract and saliva samples using ELISA immunoassays for each KLK. KLK levels in CVF extract and female saliva were normalized for total protein levels in each sample and are expressed as µg/g of total protein, shown graphically in Figures 5.1 A, B and 5.2. In CVF extract, levels of KLKs 5, 6, 7, 11 and 12 were found to peak around day 20 (Figure 5.1), following ovulation, which was estimated to have occurred on day 16 in these cycles which were 30 days in length. KLK levels were found to peak at day 25 in female saliva (Figure 5.2). There were no consistent changes in KLK levels over
30 days in male saliva (Table 5.1), however KLK7 and KLK10 levels were higher on days 27 and days 12 respectively, which could be due to changes in androgen levels.

Table 5.1: KLK levels in male saliva over 30 days

<table>
<thead>
<tr>
<th>KLK</th>
<th>mean (µg/g T.P.)</th>
<th>S.D. (µg/g T.P.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>5.2</td>
<td>2.3</td>
</tr>
<tr>
<td>6</td>
<td>10.3</td>
<td>2.0</td>
</tr>
<tr>
<td>7</td>
<td>41</td>
<td>29</td>
</tr>
<tr>
<td>8</td>
<td>9.3</td>
<td>4.8</td>
</tr>
<tr>
<td>10</td>
<td>62</td>
<td>37</td>
</tr>
<tr>
<td>11</td>
<td>103</td>
<td>41</td>
</tr>
<tr>
<td>12</td>
<td>2.0</td>
<td>0.9</td>
</tr>
<tr>
<td>13</td>
<td>4.2</td>
<td>1.2</td>
</tr>
</tbody>
</table>
Figure 5.1: KLK Levels in CVF over the menstrual cycle

KLK levels were measured using ELISA immunoassays specific for each KLK of interest. Measured KLK levels were normalized for total protein levels and KLK levels are expressed as microgram of KLK per gram of total protein. Expression of KLKs 5, 6, 7, 11 and 12 was found to peak around day 20 of the menstrual cycle during both cycles. Ovulation was estimated to have occurred on day 16 during both cycles which were 30 days in length. Ovulation typically occurs 14 days before the onset of the next menstrual period.
Figure 5.2: KLK levels in female saliva over the menstrual cycle

KLK levels were measured using ELISA immunoassays specific for each KLK of interest. Measured KLK levels were normalized for total protein levels and KLK levels are expressed as microgram of KLK per gram of total protein. Levels of KLKs 6, 7, 8, 10, 12 and 13 were found to peak at day 25 of the menstrual cycle.
5.3.2 Changes in trypsin-like activity in CVF extract and saliva over the menstrual cycle

General trypsin-like activity present in CVF extract and saliva (male and female) samples collected over the menstrual cycle was measured using the trypsin-like substrate, VPR-AMC. This is the preferred substrate for the majority of KLKs found in CVF extract and saliva. Activity levels are expressed as fluorescence units (FU) released per minute per microgram of total protein in the sample. Trypsin-like activity was found to peak around the time of ovulation (midcycle) in CVF extract (Figure 5.3 A, B) and on day 25 in female saliva (Figure 5.4). Trypsin-like activity remained fairly constant over 30 days in male saliva (Table 5.2), however there was a decrease in activity on day 25.

<table>
<thead>
<tr>
<th>Day</th>
<th>Activity (FU/min/µg T.P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>31</td>
</tr>
<tr>
<td>10</td>
<td>22</td>
</tr>
<tr>
<td>12</td>
<td>24</td>
</tr>
<tr>
<td>20</td>
<td>22</td>
</tr>
<tr>
<td>22</td>
<td>26</td>
</tr>
<tr>
<td>25</td>
<td>13</td>
</tr>
<tr>
<td>27</td>
<td>34</td>
</tr>
</tbody>
</table>
Figure 5.3: Trypsin-like activity in CVF over the menstrual cycle.

Total trypsin-like activity in CVF was measured using the fluorogenic substrate, valine-proline-arginine (VPR)-AMC. CVF was diluted 20 fold in 100 mM Tris, 100 mM NaCl (pH 8.0) and 0.2 mM VPR-AMC in a total volume of 100 μL. Fluorescence was measured at an excitation wavelength of 355 nm and emission wavelength of 460 nm. Enzymatic activity is expressed as the fluorescence units per minute time per microgram of total protein in each CVF sample. A) Trypsin-like activity over menstrual cycle #1, B) Trypsin-like activity over menstrual cycle #2.
Figure 5.4: Trypsin-like activity in female saliva over the menstrual cycle.

Total trypsin-like activity in saliva was measured using the fluorogenic substrate, valine-proline-arginine (VPR)-AMC. Saliva was diluted 20-fold in 100 mM Tris, 100 mM NaCl (pH 8.0) and 0.2 mM VPR-AMC in a total volume of 100 uL. Fluorescence was measured at an excitation wavelength of 355 nM and emission wavelength of 460 nM. Enzymatic activity is expressed as the fluorescence units per minute time per microgram of total protein in each saliva sample.
5.3.3 KLK levels in CVF from pregnant women versus non-pregnant women

CVF extract samples were collected from 7 non-pregnant women and 47 pregnant women and were measured for KLK levels using ELISA immunoassays specific for each KLK. Mean KLK levels between the two groups were compared using the Mann-Whitney test. KLKs 5, 6, 7, 8, 10, 11, 12 and 13 were all found to be higher in pregnant CVF extract versus non-pregnant CVF extract (Table 5.3), however only levels of KLKs 10, 11 and 12 reach statistical significance and are shown in bold and graphically in Figure 5.5.

<table>
<thead>
<tr>
<th>KLK</th>
<th>pregnant (ug/g TP)</th>
<th>non-pregnant (ug/g TP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>127</td>
<td>74</td>
</tr>
<tr>
<td>6</td>
<td>573</td>
<td>286</td>
</tr>
<tr>
<td>7</td>
<td>129</td>
<td>88</td>
</tr>
<tr>
<td>8</td>
<td>42</td>
<td>20</td>
</tr>
<tr>
<td><strong>10</strong></td>
<td><strong>263</strong></td>
<td><strong>123</strong></td>
</tr>
<tr>
<td><strong>11</strong></td>
<td><strong>1516</strong></td>
<td><strong>521</strong></td>
</tr>
<tr>
<td><strong>12</strong></td>
<td><strong>3859</strong></td>
<td><strong>282</strong></td>
</tr>
<tr>
<td>13</td>
<td>543</td>
<td>310</td>
</tr>
</tbody>
</table>
Figure 5.5: Comparison of KLK levels in CVF from pregnant versus non-pregnant women

KLK levels were measured in CVF, collected from pregnant and non-pregnant women, using specific ELISA immunoassays for each KLK. Mean KLK levels were compared between CVF from pregnant women and CVF from non-pregnant women using the Mann-Whitney test. Significantly higher levels of KLKs 10 (p=0.0018), 11 (p=0.0022) and 12 (p=0.0004) were found in CVF from pregnant women compared to CVF from non-pregnant women.
5.3.4 Consitutive expression and hormonal regulation of KLKs in vaginal epithelial cells

KLKs were found to be constitutively expressed in the culture supernatant of human vaginal epithelial cells (VK2) as outlined in Table 5.4. KLKs were found to be hormonally downregulated by dexamethasone and/or estradiol in VK2 cells. KLKs 5, 6 and 7 were found to be downregulated by dexamethasone (Figure 5.6) and KLKs 6 and 11 were downregulated by estradiol.

Table 5.4: Constitutive Expression and Hormonal regulation of KLKs in vaginal epithelial cells

<table>
<thead>
<tr>
<th>Hormone</th>
<th>KLK</th>
<th>Constitutive Level (mean ± SD)</th>
<th>Regulated Level (mean ± SD)</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>dexamethasone</td>
<td>5</td>
<td>4324 ± 444</td>
<td>↓ (2959 ± 327) ‡</td>
<td>- 1.5</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>30 ± 0.8</td>
<td>↓ (19 ± 1.3) ‡</td>
<td>- 1.6</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>417 ± 19</td>
<td>↓ (277 ± 14) ‡</td>
<td>- 1.5</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>308 ± 61</td>
<td>↓ (245 ± 32) ‡</td>
<td>- 1.3</td>
</tr>
<tr>
<td>Estradiol</td>
<td>6</td>
<td>30 ± 0.8</td>
<td>↓ (24 ± 2.0) ‡</td>
<td>- 1.3</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>308 ± 61</td>
<td>↓ (197 ± 61) ‡</td>
<td>- 1.6</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>51 ± 0.4</td>
<td>↓ (48 ± 1.8) †</td>
<td>- 1.1</td>
</tr>
</tbody>
</table>

† p < 0.05
‡ p < 0.01
Chapter 5

Hormonal regulation of KLKs

Figure 5.6: Co-downregulation of KLKs by dexamethasone and estradiol in VK2 vaginal epithelial cells

VK2 cells were stimulated with either alcohol (as a control), dexamethasone, norgestrel or estradiol. KLK levels were measured using specific ELISA immunoassays for each KLK. KLKs 5, 6 and 7 levels were all significantly downregulated by dexamethasone treatment. KLKs 6 and 11 levels were significantly downregulated by estradiol treatment.

** signifies p < 0.01, * signifies p < 0.05.
5.3.5 Steroid hormone receptor status of VK2, vaginal epithelial cells

To analyze the sex steroid hormone receptor status of VK2 cells, cell lysates were prepared and immunoprecipitated with either a progesterone receptor (PR) or estrogen receptor (ERα) antibody. PR/ER positive T47-D breast cancer cells were used as a positive control. The western blots in Figure 5.7 show that VK2 cells do not express either of the PR isoforms. VK2 cells show the same immunoreactive doublet bands at 66 kDa (the appropriate size for ERα) as found in T47-D cells indicating that they express the ERα.
Figure 5.7: Western blots showing immunoprecipitated PR and ER from VK2 and T47-D cells.

VK2 and T47-D lysates were immunoprecipitated with either a PR or ER α antibody. T47-D cells are known to express both receptors and were used as a positive control. Samples were resolved by SDS-PAGE using NuPAGE Bis-Tris 4-12% gradient gels and transferred to Hybond-C blotting membrane. Membranes were immunoblotted with either the PR (A) or ER α (B) antibody. T47-D cells express both isoforms of the PR and VK2 cells express neither (A). T47-D and VK2 cells showed immunoreactive bands at approximately 66 kDa, the appropriate size for the ERα indicating that VK2 cells express ER α.
5.4 Discussion

KLK genes are known to be transcriptionally regulated by steroid hormones (2). Kallikreins are also known to be differentially expressed in hormone-related malignancies, such as breast, ovarian and prostate cancer (48;206).

Given that many KLKs are expressed in CVF extract, I chose to examine the hormonal regulation patterns of KLKs in the context of cervico-vaginal physiology. Cervico-vaginal physiology is largely regulated by hormonal changes during the menstrual cycle. I hypothesized that KLKs may also be regulated by these hormonal changes and may play a physiological role during the menstrual cycle.

CVF extract and saliva samples were collected from a premenopausal woman over the course of the menstrual cycle. KLK levels were measured in these samples using ELISA immunoassays for the KLKs of interest. I chose to measure KLK levels in saliva, in addition to CVF extract, because hormonal levels in saliva are often measured when monitoring fertility status in women (207;208). During the menstrual cycle the levels of KLKs 5, 6, 7, 11 and 12 were found to increase immediately following the time of ovulation (midcycle) in CVF extract (Figure 5.3) and were found to peak at day 25 in saliva (Figure 5.4). These results were similar to those found by analysis of KLK1 and KLK3 levels in saliva and serum, respectively, over the menstrual cycle (203;205). Following ovulation, estrogen levels begin to fall and progesterone levels peak (131) suggesting that KLKs 5, 6, 7, 11 and 12 may be regulated by progesterone during the menstrual cycle. In saliva, progesterone levels are found to peak
around day 25 (209), further suggesting that KLKs are regulated by progesterone during the menstrual cycle.

During pregnancy, hormone levels increase dramatically, particularly progesterone which can be increased up to 100 times the normal level in pregnant women (210). I found higher KLK levels in CVF extract from pregnant women compared to CVF extract from non-pregnant women (Table 5.3; Figure 5.5). These results also suggest that KLKs may be regulated by progesterone in the female reproductive system.

The majority of KLKs found in CVF extract and saliva, with the exception of KLK7, have trypsin-like specificity. I therefore hypothesized that trypsin-like activity may also be altered throughout the menstrual cycle. I measured general trypsin-like activity in CVF extract and saliva throughout the menstrual cycle using the fluorogenic trypsin-like substrate, VPR-AMC. Tyspin-like activity peaked in CVF extract following ovulation (Figure 5.5), as expected, given that KLK levels also peak during this period. Tryptsin-like activity was also measured in saliva over the menstrual cycle and was found to peak at day 25 in saliva as expected given the sharp increase in the levels of many KLKs on day 25 (Figure 5.6). Chymotrypsin-like activity was also measured in the CVF extract and saliva samples using a chymotrypsin-like specific substrate, however no chymotrypsin-like activity could be measured in these samples (data not shown).

As mentioned, KLK genes are known to be transcriptionally regulated by steroid hormones (2). Given that many KLKs appear to be regulated by hormonal changes during the menstrual cycle, I chose to examine KLK regulation by steroid hormones in cultured human vaginal epithelial cells.
KLKs were found to be primarily regulated by the synthetic glucocorticoid, dexamethasone and by estrogen in this cell line (Table 5.4; Figure 5.6). I did not find KLKs to be regulated by progesterone, as expected, given that KLKs appear to be regulated by progesterone during the menstrual cycle based on our above data. The glucocorticoid receptor is ubiquitously expressed whereas the expression of sex hormone receptors is cell-type specific. The steroid hormone receptor status of VK2 cells is currently unknown. I investigated whether VK2 cells express the estrogen and/or progesterone receptor through immunoprecipitation and western blotting, using T-47D breast cancer cell lysates as a positive control for ER and PR expression. VK2 cells were found not to express the PR (Figure 5.7) which explains why they were unresponsive to progesterone treatment. Estrogen responsiveness of these cells can be explained by their expression of ERα (Figure 5.7).

KLK regulation by dexamethasone in vaginal epithelial cells is interesting given that GC have been shown to play a role in controlling vaginal epithelial cell differentiation (211). This finding is also intriguing given that glucocorticoids are often used to treat conditions affecting the vagina, such as vaginitis (131). This will be discussed further in chapter 8.
Chapter 6: Proteomic Analysis of Human Cervico-vaginal Fluid (CVF)

This work has been published in the following article,


C.R. Smith performed the mass spectrometry.

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6.1 Introduction and rationale

CVF is comprised of fluid originating from the vagina, as well as other fluids flowing into the vagina, such as cervical mucus, endometrial and oviductal fluids (130). CVF plays an important role in innate defense (129). CVF has been shown to contain antimicrobial substances, such as cationic peptides (132), lysozyme (133), lactoferrin (134;135), secretory leukocyte protease inhibitor (137;138), human neutrophil peptides, and human β-defensins (139). These substances play a role in host protection against invading microbes and viruses, including the HIV virus (129). I recently identified many members of the KLK family present in CVF from non-pregnant women (65).

The CVF proteome has primarily been studied in search for potential markers for pregnancy-associated conditions such as pre-term labour and intra-amniotic infections. For example, increased levels of fetal fibronectin in the CVF of pregnant women has been suggested to be predictive of pre-term delivery (212). Decreased levels of inflammatory cytokines measured in CVF have also been shown to be associated with pre-term delivery (213).

Until now, the human non-pregnant CVF proteome has not been systematically delineated and the physiologic function of KLKs in CVF is currently unknown. I performed proteomic analysis by different pre-fractionation methods and compiled a list of 685 unique proteins present in human CVF for the purpose of identifying potential KLK functions and substrates.
6.2 Materials and methods

6.2.1 Collection and extraction of CVF from healthy volunteers

The protocol for collection and extraction of CVF was described above (see sections 5.2.1 and 5.2.2). 5 mL of each of 4 CVFs were pooled together for the pooled experiments. 2.5 mL of an individual CVF sample was used for the gel experiment (see below).

6.2.2 Preparation of samples for SDS-PAGE fractionation

CVF samples were desalted using a disposable PD-10 desalting column (GE Healthcare, Mississauga, ON), according to the manufacturer’s recommendations. Briefly, the column was equilibrated with 200 mM ammonium bicarbonate. 2.5mL of vaginal fluid was then applied to the column, and allowed to flow through. 3.5 mL of 200 mM ammonium bicarbonate were then added to the column, and the eluate collected.

The PD-10 column eluate was lyophilized to dryness using a Modulyod freeze drying system (Thermo Fisher Scientific, Ottawa, ON) and then resuspended in 350 µL of water. A 30 µL aliquot was then resolved by SDS-PAGE, and stained using Simply Blue Safe Stain ® (Invitrogen Canada Inc., Burlington, ON), according to the manufacturer’s protocol. Thirty-four-36 evenly sized bands were then cut from the gel, comprising the entire lane of the stained protein sample.

6.2.3 In-gel preparation of proteins for mass spectrometry

Individual gel bands were placed in 1.5 mL microfuge tubes. To shrink the gel band, enough acetonitrile (ACN) was added to cover the band, and incubated at room temperature for 10 min. The ACN was then removed, and 10 mM DTT
(Sigma-Aldrich, St. Louis, MO) was added to cover the gel band and reduce the proteins present. Gel bands were incubated with DTT for 10 minutes at 50°C and then at room temperature for an additional 20 minutes.

Following reduction, DTT was discarded and alkylation was performed by adding 100 mM iodoacetamide (Sigma-Aldrich, St. Louis, MO) to cover the gel band. Gel bands were incubated with iodoacetamide for 15 min at room temperature, in the dark. Iodoacetamide was then discarded and 0.5 µg of trypsin (Promega, Madison, WI) were added, along with sufficient 50 mM ammonium bicarbonate to cover the gel, and incubated overnight at 37°C.

6.2.4 Preparation of samples for SCX fractionation

CVF was dialyzed using a 3,500 molecular weight cut-off tubing (Spectrum Laboratories Inc., Rancho Dominguez, CA) for 24 hours in 1 mM ammonium bicarbonate, in order to remove salts. Dialysis buffer was changed twice during this 24 hour period. The sample was then lyophilized to dryness using a Lyophilizer (Modulyod Freeze Drying System (Thermo-Fisher Scientific, Ottawa, ON). Samples were then denatured with 8M urea (Fisher Scientific, Ottawa, ON), and reduced with 13 mM DTT at 50°C for 30 minutes. Alkylation was then performed with iodoacetamide (125 mM) followed by incubation at room temperature for 1 hour, in the dark, with constant shaking. The sample was then desalted using a NAP5 column (GE Healthcare, Mississauga, ON). Five-hundred µL of eluate from the NAP5 column was lyophilized to dryness, followed by trypsin digestion (1 µg/100 µg of total protein) overnight at 37°C. The sample was then lyophilized to dryness.
6.2.5 SCX Liquid chromatography

The lyophilized sample was reconstituted in 120 µL of mobile phase A (0.26 M formic acid in 10% acetonitrile), and loaded directly onto a polySULFOETHYL A™ column with a 2.0 µm pore size and 5 µm diameter (The Nest Group Inc., Southborough, MA). A one hour fractionation was performed using high performance liquid chromatography (HPLC), with an Agilent 1100 system (Agilent, Mississauga, ON). A linear gradient was used with 0.26 M formic acid in 10% acetonitrile as running buffer and 1 M ammonium formate as elution buffer. Forty, 200 µL fractions were collected, and pooled into 8 fractions. Each of the 8 fractions was concentrated to 100 µL using a SpeedVac system.

6.2.6 Mass spectrometry

For samples fractionated by SCX, the peptides in each fraction were purified with a ZipTipC18 pipette tip (Millipore, Ottawa, ON), and eluted in 4 µL buffer B (90% ACN, 0.1% formic acid, 10% H2O, 0.02% TFA). Eighty µL buffer A (95% H2O, 0.1% formic acid, 5% ACN, 0.02% TFA) were added to each sample and 40 µL of each sample were injected onto a 2 cm C18 trap column (inner diameter 200 µm). For samples fractionated by SDS-PAGE, 40 µL of sample (not zip-tipped) were injected as above. Peptides were eluted from the trap column onto a resolving analytical C18 column (inner diameter 75 µm) with an 8 µm tip (New Objective, Woburn, MA). This liquid chromatography was set-up online to a 2-D linear ion trap (LTQ, Thermo Inc., Ottawa, ON) mass spectrometer, using a nanoelectrospray ionization source (ESI). Samples prepared by SCX and SDS-PAGE were run for 120 min and 30 min gradients, respectively. The eluted peptides were subjected to tandem mass spectrometry.
Data files were collected using Mascot Daemon (v2.1.03, Matrix Science, Boston MA) and extract_msn, using the following parameters: min. mass 300, max. mass 4,000, automatic precursor charge selection, min. peaks 10 per MS/MS scan for acquisition and min. scans per group of 1.

6.2.7 Data analysis

The resulting raw mass spectra were analyzed using Mascot (V 2.1.03, Matrix Science, Boston, MA) and X!Tandem (V 2006.04.01.2, Proteome Software, Portland, OR) search engines, and searched against the non-redundant IPI Human database (Version 3.24). Up to one missed cleavage was allowed and searches were performed with fixed carbamidomethylation of cysteines and variable oxidation of methionine residues. A fragment tolerance of 0.4 Da and parent tolerance of 3.0 Da were used in searches with each search engine, and trypsin was chosen as the digesting enzyme. The resulting files were loaded into Scaffold (Proteome Software, Portland, OR), a bioinformatics program which validates each MS/MS by assigning protein and peptide probabilities based on chosen parameters. I chose 80% probability of protein identification and 95% probability of peptide identification, based on the peptide prophet algorithm (214). Each protein was assigned a cellular localization based on information from Swiss Prot, and genome ontology (GO) databases.

6.2.8 Genome ontology (GO) databases.

To calculate the false-positive rate, identical searches to the ones performed above were performed using the “sequence-reversed” IPI human database (Version 3.24). The false positive rate was calculated as: Number of
peptides identified by the reverse database/(Number of peptides identified by the forward database + reverse database)*100.

6.2.9 Functional and pathway analysis of identified proteins

Functional and pathway analysis was performed using Ingenuity Pathways Analysis (Ingenuity® Systems, www.ingenuity.com) and identified functions and/or diseases that were most significant to the set of proteins identified.

394/685 genes were eligible for analysis in which the Fischer's exact test was used to calculate a p-value determining the probability that each function or interaction assigned to the set of proteins is due to chance alone.

6.3 Results

6.3.1 Identification of proteins by mass spectrometry: SDS-PAGE gel fractionation

A CVF sample from one individual (2.5 mL), and a pooled sample from four individuals (performed in duplicate, 5 mL per duplicate), were analyzed. Two-hundred and eighty-two proteins were identified from the single CVF sample and 181 proteins from the pooled sample, after combining the two duplicates.

6.3.2 Identification of proteins by mass spectrometry: SCX fractionation

A pooled sample was used and this experiment was performed in duplicate. Four hundred and forty-two proteins were identified using this method (from both duplicates).

6.3.3 Identification KLKs

Analysis of the same five CVF samples used in this study by ELISA identified the following members of the kallikrein family: KLKs 1, 5, 6, 7, 8, 10, 11, 12, 13, and 14. KLKs 6, 11, 12 and 13 were the most abundant. I was able to
identify KLKs 6, 7, 10, 11, 12, and 13 by mass spectrometry. The number of peptides identified and sequence coverage are shown in Table 6.1.

Table 6.1: Kallikrein proteins identified in CVF by mass spectrometry and ELISA.

<table>
<thead>
<tr>
<th>Kallikrein</th>
<th>Concentration (^1) (µg/L)</th>
<th># of peptides</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>599</td>
<td>7</td>
</tr>
<tr>
<td>7</td>
<td>247</td>
<td>2</td>
</tr>
<tr>
<td>10</td>
<td>155</td>
<td>2</td>
</tr>
<tr>
<td>11</td>
<td>1,690</td>
<td>5</td>
</tr>
<tr>
<td>12</td>
<td>3,188</td>
<td>1</td>
</tr>
<tr>
<td>13</td>
<td>1,750</td>
<td>7</td>
</tr>
</tbody>
</table>

\(^1\) Median values by ELISA based on 5 samples
6.3.4 Reproducibility between duplicates

Analyses of the pooled CVF samples prepared by SDS-PAGE and SCX were performed in duplicate. Venn diagrams illustrating the number of proteins found in each replicate and the overlap between duplicates are found in Figures 6.1A and B. For the samples prepared by SCX, there was 54% reproducibility between the proteins identified; 36% reproducibility was found with the samples prepared by SDS-PAGE.

6.3.5 Overlap of proteins between experiments

A total of 685 unique proteins were identified when combining the results of all experiments. Three hundred and forty-one (49.8%) of the proteins were identified by 2 or more peptides (Table 6.2). Figure 6.2 shows the overlap between experiments. Sixty-six proteins were identified in CVF by all three experiments.

<table>
<thead>
<tr>
<th># of peptides identified</th>
<th>SDS-PAGE single sample</th>
<th>SDS-PAGE pooled sample</th>
<th>SCX pooled sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>102</td>
<td>51</td>
<td>249</td>
</tr>
<tr>
<td>2</td>
<td>56</td>
<td>29</td>
<td>93</td>
</tr>
<tr>
<td>3</td>
<td>35</td>
<td>18</td>
<td>39</td>
</tr>
<tr>
<td>4</td>
<td>13</td>
<td>13</td>
<td>23</td>
</tr>
<tr>
<td>5</td>
<td>11</td>
<td>14</td>
<td>11</td>
</tr>
<tr>
<td>&gt;5</td>
<td>65</td>
<td>56</td>
<td>27</td>
</tr>
<tr>
<td>Total</td>
<td>282</td>
<td>181</td>
<td>442</td>
</tr>
</tbody>
</table>

1 The overlap of proteins in each sample is presented in Figure 6.1.
Figure 6.1: Venn Diagrams of the Reproducibility between duplicates for each fractionation method

The numbers refer to the number of proteins identified.
A) Number of proteins identified in each replicate and overlap for SCX-fractionated samples,
B) Number of proteins identified in each replicate and overlap for SDS-PAGE-fractionated samples
Figure 6.2: Venn diagram outlining the overlap of proteins identified in CVF between 3 experimental approaches.

The numbers refer to the number of proteins identified. The diagram illustrates the overlap of identified proteins between the different fractionation methods used: SDS-PAGE gel and SCX.
6.3.6 Cellular localization of identified proteins

The identified proteins were classified according to cellular localization using Genome Ontology (GO) classifications. These results are shown graphically in Figure 6.3. 23% of the proteins identified are not classified according to GO. 21% of identified proteins are classified as extracellular, 12% as nuclear, and 9.8% as membrane-associated proteins.

6.3.7 Calculation of false-positive error rate

When raw mass spectra were searched against the “sequence reversed” IPI human database, 5 peptides were identified. I calculated the false-positive rate to be approximately 0.2%.

6.3.8 Analysis of biological function

The potential molecular functions of identified genes/proteins were analyzed using Ingenuity Pathways Analysis (Ingenuity ® Systems, www.ingenuity.com). 394/685 genes were eligible for molecular function and pathway analysis. The top 15 molecular functions are shown in Figure 6.4. Major categories include cellular movement, immune response, cell death, cell-to-cell signaling and cellular assembly and organization.
Figure 6.3: Graphical Representation of Genome Oncology (GO) Classifications of Identified Proteins.

Proteins identified in this study with respect to genome ontology (GO) classifications. In brackets are the numbers of proteins per location.
Figure 6.4: The top 15 functions of gene products identified in CVF

Using Ingenuity Pathways Analysis software, the potential functions of proteins identified in CVF were analyzed. The top 15 functions are shown here and include: cellular movement, immune response, cell death, cell-to-cell signaling, and cellular assembly.
6.4 Discussion

We identified 685 unique proteins in human CVF extract from healthy individuals using two different sample preparation methods, followed by mass spectrometry and bioinformatic analysis. Two recent studies by Dasari et al. (215) and Pereira et al. (216) identified 205 proteins in CVF from pregnant women, 132 of which were also identified by us in this study. Another study, utilizing 2D-SDS-PAGE and mass spectrometry, identified approximately 400 protein spots. This study analyzed the CVF proteome of pregnant women at term (217), and only positively identified 15 proteins which were common to all 5 women studied. Ten of the 15 proteins identified by this group were also identified with our study. Differences between the proteins identified in these studies and our study are likely due to changes in the CVF proteome as a result of pregnancy.

We feel confident that none of the proteins identified in CVF extract in this study were the result of seminal contamination. Subjects confirmed abstinence from unprotected sexual intercourse for at least two weeks prior to sample collection. As well, I analyzed each CVF sample in this study for KLK3 (prostate-specific antigen; PSA), an excellent marker for seminal contamination with levels ranging from $10^7$-$10^8$ µg/L in seminal plasma. I identified trace amounts of KLK3 in these CVF samples (< 5 µg/L). Much larger levels would have been expected if the samples were contaminated with seminal fluid.

We identified members of the kallikrein family of serine proteases in human CVF extract by ELISA. KLK1, 5, 6, 7, 8, 10, 11, 12, 13, and 14 have all been found in human CVF extract by ELISA, with KLK6 (median 600 µg/L), 11
Proteomic analysis of human CVF

(median 1,690 µg/L), 12 (median 3,188 µg/L) and 13 (median 1,750 µg/L) found at highest levels (65). In this study, I positively identified KLK 6, 7, 10, 11, 12, and 13 in CVF extract (Table 6.1). Five of the 6 KLKs were identified by more than 2 peptides.

Two different sample fractionation methods were used. SDS-PAGE fractionation of an individual CVF extract sample and a pooled sample was performed. In the individual sample, I identified 282 unique proteins, 180 (64%) of which were identified by 2 or more unique peptides (Table 6.2). I also identified 181 unique proteins in the pooled CVF extract sample, 130 (72%) of which were identified by 2 or more unique peptides (Table 6.2). SCX liquid chromatography was performed for the pooled CVF extract sample only, and 442 unique proteins were identified. 192 (44%) of these proteins were identified by more than 2 unique peptides (Table 6.2). The reproducibility between duplicates prepared by SCX was 54% (Figure 6.1).

Pre-fractionation of samples by SCX yielded a greater number of proteins and better reproducibility when compared to 1D-SDS-PAGE. This is not surprising, given that SDS-PAGE is known to have a relatively low dynamic range, resulting in the identification of primarily high abundance proteins (218). There are also limitations with respect to the amount of protein that can be loaded onto an SDS-PAGE gel (219). Chromatographic fractionation methods such as SCX have been shown to result in identification of larger numbers and lower abundance proteins (158;220;221).

The study by Dasari et al. (215) employed SCX and SDS-PAGE fractionation of CVF, yielding similar results to our study with respect to the
number of proteins identified using each method. A larger number of proteins were identified as a result of SCX fractionation when compared with SDS-PAGE fractionation. Another recent study (221) used various fractionation approaches to characterize the urinary proteome. Comparing 2D-LC-MS/MS using SCX, with 1D-LC-MS/MS and 1D-SDS-PAGE-LC-MS/MS, this group also identified the largest number of proteins using the 2D-LC-MS/MS (SCX) approach (221). This study also compared reproducibility between experiments using SDS-PAGE separation vs. SCX separation, and found 25% greater reproducibility with SCX fractionation, compared with SDS-PAGE separation (221), in accordance with our own findings (Figure 6.1).

Classification of the identified proteins by Genome Ontology (GO) indicated that 30% of the proteins in CVF are extracellular or membranous (Figure 6.3), as would be expected for an extracellular biological fluid. Analysis of the proteins identified in CVF revealed distinct protein subgroups and identified potential substrates for KLKs in relation to: cell desquamation (desmocollins and desmogleins), cervical mucus (mucins 4 and 5B), and host defense (defensins). Potential KLK functions in relation to these proteins will be discussed in detail in chapter 7.

In addition to the proteins mentioned above, many proteolytic enzymes were identified in CVF extract, in addition to the KLKs, such as: members of the carboxypeptidase family, matrix metalloproteinase-9, myeloperoxidase, neutrophil collagenase and neutrophil gelatinase. Members of the cathepsin family have previously been shown to be present in vaginal fluid; increased cathepsin B levels are associated with cervical carcinoma (222). Matrix
metalloproteinase-9 is known to be present in CVF, and levels have been studied in pregnant women with bacterial vaginosis (223).

Given that many enzymes are present in CVF, it is expected that enzyme inhibitors would also be present, to control enzyme activity. Two main families of inhibitors were identified in CVF extract; inhibitors of serine proteases, and inhibitors of cysteine proteases. Serine protease inhibitors including anti-trypsin, anti-thrombin, elafin, and serpins B3, B4, B9, B12 were identified, as well as the cysteine protease inhibitors cystatin A, and B. Anti-trypsin and cystatin A have recently also been identified in CVF by Di Quinzio et al. (217).
Chapter 7: Potential Roles for KLKs in Cervico-vaginal Physiology

Some of the ideas presented here have been published in the following article,


Copyright permission has been granted.
7.1 **Introduction and Rationale**

7.1.1 **Cell desquamation**

We identified, in CVF extract, several proteins associated with the cytoskeleton, or involved in cell-cell adhesion. Analysis using Ingenuity software revealed that 96 genes were found to be associated with cellular movement, 85 with cell-to-cell signaling and 75 with cellular assembly and organization (Figure 6.4). Cytoskeletal proteins included cornifin B, envoplakin, fibronectin, myosin, and periplakin. I identified several cell-cell adhesion molecules, such as, desmoglein-1 (DSG1), desmocollin-2 (DSC2) and desmocollin-3 (DSC3). These proteins are most likely found in CVF as a result of cyclical changes in the endometrial and vaginal epithelium during the menstrual cycle. The vaginal epithelium undergoes changes throughout the menstrual cycle (224), including keratinization and shedding of epithelial cells upon decreasing estrogen levels and increasing progesterone levels (225). The loss of cell-cell contacts accompanies the shedding of epithelial cells, resulting in enrichment of CVF for these proteins.

Given that KLK levels increase during this period, I hypothesized that KLKs may play a role in vaginal epithelial cell desquamation. KLKs are known to play a role in the desquamation of skin corneocytes through their cleavage of cell-cell adhesion molecules such as desmogleins and desmocollins (74;75). KLKs 5, 6 and 14 and KLKs 5 and 7 have previously been shown to cleave DSG1 (62;74) and desmocollin-1 (DSC1) (74) respectively. Here, I investigated the *in vitro* processing DSG1, DSC2 and DSC3 by active KLKs 5, 6, 11, 12 and 13, which are highly expressed in CVF extract.
7.1.2 A role for KLK in cervical mucus remodelling

We identified the primary cervical mucus proteins, mucin 4 (MUC 4) and mucin 5B (MUC 5B) (136;143) in CVF extract, which was not surprising given that cervical mucus contributes to the CVF milieu. The composition and pH of cervical mucus changes throughout the menstrual cycle in response to changing hormone levels. Around ovulation, the pH rises to approximately 7.8, the volume of mucus increases and it becomes less viscous (150). This highly hydrated mucus allows for migration of sperm through the cervix into the uterus (150).

It has been suggested that proteolytic enzymes may affect the physical properties of mucin proteins causing the changes in mucus observed over the menstrual cycle (163). Treatment of salivary mucus with either trypsin or chymotrypsin was previously shown to cause a loss in mucus viscosity (226). Given that I found KLKs to be expressed by the mucus-secreting epithelial cells of the cervix, it is possible that KLKs are capable of processing mucin proteins and may be involved in remodelling of cervical mucus.

7.1.3 Host defense

As mentioned, a primary function of CVF is host protection from microorganisms (129). Proteins such as haptoglobin (227), neutrophil defensins (139), lysozyme (133) and lactoferrin (134;135) have previously been shown to be present in CVF, contributing to host defense. Analysis by Ingenuity software has shown that 75 of the identified proteins are associated with immune response (Figure 6.4). In addition to the proteins mentioned, several other proteins known to be associated with host defense have been identified in our study (136), such as: azurocidin, dermcidin and galectin-3 binding protein.
KLKs have recently been shown to play a role in host defense in skin and sweat through cleavage of the antimicrobial human cathelicidin protein, hCAP-18 (84). hCAP18 is composed of an N-terminal cathelin domain and C-terminal LL-37 antimicrobial domain; proteolytic cleavage from the cathelin domain liberates the active LL-37 antimicrobial peptide. Specifically, KLK5 has been shown to cleave hCAP18 and liberate active LL-37 (84) and both KLK5 and 7 are capable of further digesting LL-37 into smaller antimicrobial peptides in sweat (84).

We hypothesize that KLKs may also contribute to antimicrobial activity within CVF through processing of antimicrobial proteins found in CVF, such as defensins. I identified several members of the defensin family in CVF extract (136) and speculate that KLKs may be capable of producing antimicrobial peptides through cleavage of defensin proteins.

Defensins are small cationic antimicrobial peptides expressed by epithelial cells and leukocytes (228) that kill gram-negative, gram-positive, fungal and viral pathogens (229). Defensins contain many positively charged and hydrophobic amino acids which help them to interact with the cell membranes of pathogens, causing lysis and death (229). These peptides are also capable of activating adaptive immune responses through their binding to G-protein-coupled receptors (228;229). The human defensin family consists of two members, α-defensins and β-defensins (228); α-defensins are produced by neutrophils and extravasate into mucosal tissues during infection whereas β-defensins are constitutively expressed at many mucosal sites (228).

α-defensins are translated as inactive pre-pro-peptides of approximately 95 amino acids (230). The signal peptide is removed upon secretion from the
secretory pathway leaving the inactive pro-peptide which undergoes further proteolytic processing into the active 29-30 amino acid peptide, once secreted \(^{(230)}\). For example, in the human intestine, pro-\(\alpha\)-defensin 5 is proteolytically processed into the active form by trypsin \(^{(230)}\). \(\beta\)-defensins are translated similarly with a signal peptide, however they contain a short or absent pro-peptide, suggesting that these peptides are secreted in the active form \(^{(230)}\).

Both \(\alpha\) and \(\beta\)-defensins are found in CVF, where these peptides are thought to play an important role in host defense and have been studied particularly for their activity against the human immunodeficiency virus (HIV) \(^{(228)}\). Given the role KLKs have been shown to play in the processing of cathelicidin antimicrobial peptides \(^{(84)}\), I investigated whether KLKs may also be involved in the proteolytic processing of defensins.

### 7.2 Materials and methods

#### 7.2.1 Materials

Recombinant Human Desmoglein-1/Fc Chimera was purchased from R&D systems (Minneapolis, MN). Recombinant human desmocollin-2 (DSC2), desmocollin-3 (DSC3), mucin 4 (MUC4), and mucin 5B (MUC5B) defensin alpha (DEF-\(\alpha\)) and defensin beta (DEF-\(\beta\)) GST fusion proteins were purchased from Abnova Corporation (Taipei, Tiawan). Recombinant KLKs 5, 6, 11 and 13 were produced in our laboratory. These proteins were expressed and purified using the *Pichia pastoris* yeast protein production kit according to the manufacturer’s recommendations (Invitrogen Canada Inc., Burlington, ON). KLK5 was expressed as the proform and activated through autoactivation.
KLK6 was expressed as the proform and activated by thermolysin. Briefly, pro-KLK6 was incubated with thermolysin in a ratio of 115:1 (KLK6 to thermolysin). The reaction was terminated by the addition of 50 µM EDTA followed by flash freezing in liquid N₂.

KLKs 11 and 13 were expressed in the active form. The proform of KLK12 was purchased from R&D Systems (Minneapolis, MN) and was auto-activated according to the manufacturer’s recommendations.

Synthetic substrates, Val-Pro-Arg-amino-4-methylcoumarin (VPR-AMC) and Val-Leu-Lys – thiobenzyl-ester (VLK- SBzl) were purchased from Bachem Bioscience (King of Prussia, PA).

7.2.2 KLK Activity assays for KLKs 5, 6, 11, 12 and 13

Twelve nM (final concentration) of each KLK was incubated with 0.2 mM substrate (VPR-AMC for KLKs 6, 12 and 13, VLK-SBzl for KLK11) diluted in 1X TBS pH 7.6 or pH 6.2 (for KLKs 5 and 12), to a final volume of 100 µL. The entire sample was then pipetted into the well of a white microtiter plate. Fluorescence was measured for KLKs 5, 12 and 13 reactions using an excitation wavelength of 355 nm and emission wavelength of 460 nm using a Wallac Victor Fluorometer (Perkin Elmer, Waltham, MA). The rate of activity is expressed as fluorescence units (FU) released per minute. Absorbance at 420 nm was measured for KLK11 reactions using a Wallac Victor Fluorometer (Perkin Elmer, Waltham, MA) and the rate of activity is expressed as absorbance units (AU) per minute. For all experiments, a negative control reaction containing only the buffer and substrate was performed to determine background fluorescence or absorbance. All experiments were done in triplicate and repeated at least twice.
7.2.3 KLK \textit{in-vitro} cleavage experiments

For analysis of substrate cleavage, 10 ng of KLK was incubated with 100 ng of recombinant protein in 1X TBS (pH 7.6 or pH 6.2) in a final volume of 20 µL at 37°C for 10 minutes, 30 minutes, 2 hours for western blotting (for desmocollins and mucins) and for 30 minutes, 2 hours and 6 hours for silver stain analysis (for desmocollins and mucins). Control reactions containing recombinant protein alone were also performed for 2 hours for western blot analysis (for desmocollins and mucins). KLK alone and recombinant protein alone reactions were carried out for 6 hours for silver stain analysis (for desmocollins and mucins). Cleavage and control reactions were carried out for 1, 2, 4, 8 and 24 hours for desmoglein-1 and for 14 hours for the defensins. After the specified amount of time, samples were flash frozen in liquid nitrogen and stored at -80°C until use. For silver stain analysis, samples were thawed, resolved by SDS-PAGE using the NuPAGE bis-tris gel electrophoresis system and 4-12% gradient gels (Invitrogen Canada Inc., Burlington, ON) under reducing conditions. Protein mixtures were visualized by silver staining using the Silver Xpress ® silver stain kit (Invitrogen Canada Inc., Burlington, ON) according to the manufacturer’s directions.

For western blot analysis, samples were thawed and were resolved by SDS-PAGE as described above, following which they were transferred to Hybond-C blotting membrane (GE Healthcare, Mississauga, ON). Membranes were blocked in 5% milk in Tris-buffered saline-tween (TBST) overnight at 4°C. Membranes were probed for 1 hour at room temperature with an anti-GST antibody (Cell Signaling (Danvers, MA), diluted 1000-fold in 1% milk in TBST). Membranes were then washed 3 times for 15 minutes in TBST after which they
were incubated with alkaline phosphatase conjugated goat-anti-rabbit IgG (Jackson ImmunoResearch, West Grove PA), diluted 5000-fold in 1% milk in TBST) for 1 hour at room temperature. Membranes were washed again, as above, and fluorescence was detected using a chemiluminescent substrate (Diagnostics Products Corp. Los Angeles, CA).

**7.2.4 CVF EX-vivo cleavage experiments**

100 ng of recombinant substrate protein (DSC2, DSC3, MUC4, MUC5B, DEFα or DEFβ) was incubated with a volume of CVF extract (day 22, the sample containing the largest levels of KLKs) enough to make the total reaction volume 20 µL. For DSCs and MUCs, incubations were for 2 hours. For DEFα and DEFβ, samples were incubated for 14 hours. These incubations were performed at 37 °C with constant shaking. Following the specified length of incubation, samples were flash frozen in liquid nitrogen and stored at -80°C until use.

Cleavage analysis was performed by western blotting with a GST antibody as described above.

**7.3 Results**

**7.3.1 Confirmation of KLK enzymatic activity**

Active, recombinant KLKs (5, 6, 11, 12 and 13) were used for the *in vitro* cleavage experiments described below. To confirm that these proteins were active I tested their enzymatic activity against their previously determined preferred substrates. For KLKs 5, 6, 12 and 13 the preferred substrate is valine-proline-arginine and I used the chromogenic substrate VLK-Sbzl. In Figure 7.1 I show that these five KLKs are all active.
against their target substrate in TBS buffer at pH 7.8 (the buffer and pH used for the cleavage experiments described below). KLKs 5 and 12 were found to be active at pH 6.2, albeit at a lower level than at pH 7.8.
Figure 7.1: Enzymatic Activity of KLKs used in in vitro cleavage studies

Enzymatic activity of KLKs was measured using their optimal tripeptide substrates, VPR-AMC (KLK5, 6, 12 and 13) and VLK-Sbzl (KLK11). 12 nM KLK (final concentration) was incubated with 0.2 mM substrate in 100 µL 100 mM Tris, 100 mM NaCl pH 7.6 (and pH 6.2 for KLKs 5 and 12). Fluorescence (VPR-AMC) was measured at an excitation wavelength of 360 nm and emission wavelength of 460 nm and absorbance (VLK-Sbzl) was measured at a wavelength of 405 nm using a Wallac Victor Fluorometer. Regression analysis was performed using Sigma Plot software and activity is shown as fluorescence units released per minute (KLKs 5, 6, 12 and 13) or absorbance units per minute (KLK11).
7.3.2 KLK cleavage of cell-cell adhesion molecules

Here, I investigated the *in vitro* processing of DSG1, DSC2 and DSC3 by active KLKs 5, 6, 11, 12 and 13, which are highly expressed in CVF. Recombinant DSG1/Fc, a chimeric protein comprising the five extracellular domains of DSG1, followed by a peptide linker and the Fc region of human IgG1 was incubated with active KLK5, 6, 11, 12 and 13 in TBS, pH 7.8 at 37 °C for various time points. Cleavage of DSG1 was analyzed by silver staining and as shown in Figure 7.2, KLKs 5, 6 and 12 were able to cleave DSG1. KLK12 was found to process DSG1 quite extensively, compared with the other two KLKs. There was no cleavage by KLKs 11 or 13 (data not shown).

Partial recombinant proteins, DSC2 and DSC3 comprising the extracellular cadherin 5 domain of each protein, linked to a GST tag were incubated with active KLKs 5, 6, 11, 12 and 13 in TBS, pH 7.8 at 37 °C for various time points. Cleavage was monitored by silver staining and western blotting using a GST antibody as shown in Figure 7.3. Both KLK5 and KLK12 were found to process DSC2 and DSC3 extensively after 2 hours of incubation. KLKs 6, 11, and 13 did not cleave DSC2 or DSC3 these two proteins even at higher ratios and after longer time points (data not shown).
Figure 7.2: Silver stains showing cleavage of DSG1 by KLKs 5, 6 and 12

100 ng of recombinant fc-DSG1 was incubated with 10 ng of either active KLK5, 6, 11, 12 or 13 in TBS, pH 7.8 for various time points. Samples were resolved using NuPAGE 4-12% gradient gels (Invitrogen Canada Inc., Burlington, ON) and proteins were stained using the silver Xpress staining kit from Invitrogen. KLKs 5 (A), 6 (B) and 12 (C) found to cleave DSG1. Cleaved fragments are marked with arrowheads.
Figure 7.3: Silver stains and western blots showing cleavage of DSC2 and DSC3 by KLKs 5 and 12

Recominant GST-linked DSC2 and DSC3 were incubated with active KLK5 or KLK12 at a ratio of 1:10 for various time points. The proteins were resolved by NuPAGE Bis-Tris gels (Invitrogen Canada Inc.) and cleavage of DSCs by KLKs was visualized by both silver staining, using the silver Xpress staining kit (Invitrogen) (right panels), and by transferring the proteins to hybond-c blotting paper (GE Healthcare) for western blotting using a GST antibody (Cell Signaling, left panel). Cleavage products are noted with arrowheads, KLK proteins are shown with stars. A) DSC2 cleavage by KLK5, B) DSC2 cleavage by KLK12, C) DSC3 cleavage by KLK5 and D) DSC3 cleavage by KLK12.
7.3.3 KLK cleavage of mucin proteins

To investigate whether KLKs are able to process the primary mucin proteins found in cervical mucus, mucin 4 and mucin 5B, partial recombinant MUC4 (comprising an extracellular portion of the protein) and MUC5B (comprising a C-terminal portion of the protein), both linked to a GST tag, were incubated with active KLKs 5, 6, 11, 12 and 13 in TBS, pH 7.8 at 37°C for various time points. Cleavage of the mucin proteins by the KLKs was monitored by silver staining and western blotting as shown in Figure 7.4. KLKs 5 and 12 were both found to cleave MUC4 and MUC5B extensively by the end of 2 hours incubation, whereas KLKs 6, 11 and 13 did not process either of these proteins even after longer incubation times (data now shown).

Given that the pH of cervical mucus is known to change from 6.2 to approximately 7.8 around the time of ovulation, I investigated both the activity of KLKs 5 and 12 at pH 6.2 and the cleavage of mucin proteins by these two KLKs at pH 6.2. For these experiments, incubations were performed in TBS, pH 6.2 rather than 7.8. As shown in Figure 7.5, KLKs 5 and 12 were active at pH 6.2, albeit at lower levels than at pH 7.8. KLKs 5 and 12 were also able to cleave MUC4 and MUC5B at pH 6.2, as shown by the silver stains in Figure 7.6, however the cleavage was much less pronounced that that at pH 7.8 (Figure 7.4).
Figure 7.4: Silver stains and western blots showing cleavage of MUC4 and MUC5B by KLKs 5 and 12

Recombinant GST-linked MUC4 and MUC5B were incubated with active KLK5 or KLK12 at a ratio of 1:10 for various time points. The proteins were resolved by NuPAGE Bis-Tris gels (Invitrogen Canada Inc.) and cleavage of MUCs by KLKs was visualized by both silver staining, using the silver Xpress staining kit (Invitrogen) (right panels), and by transferring the proteins to hybond c blotting paper (GE Healthcare) for western blotting using a GST antibody (Cell Signaling, left panel). Cleavage products are noted with arrowheads. KLK proteins are shown with stars. A) MUC4 cleavage by KLK5, B) MUC4 cleavage by KLK12, C) MUC5B cleavage by KLK5 and D) MUC5B cleavage by KLK12.
Figure 7.5: Silver stains showing cleavage of MUC4 and MUC5B by KLKs 5 and 12 at pH 8.2

Recominant GST-linked MUC4 and MUC5B were incubated with active KLK5 or KLK12 at a ratio of 1:10 for various time points in TBS, pH 8.2. The proteins were resolved by NuPAGE Bis-Tris gels (Invitrogen Canada Inc.) and cleavage of MUCs by KLKs was visualized by silver staining using the silver Xpress staining kit (Invitrogen). Cleavage products are noted with arrowheads. A) MUC4 cleavage by KLK5 (left panel) and KLK12 (right panel), B) MUC5B cleavage by KLK5 (left panel) and KLK12 (right panel).
7.3.4 KLK cleavage of defensin proteins

Recombinant GST-linked full-length defensin-α (DEFα) and defensin-β (DEFβ) were incubated with active KLKs 5, 6, 11, 12 and 13 for various time points in TBS, pH 7.8. The silver stained gels and western blots in Figure 7.6 show cleavage of DEFα by only KLK5. KLKs 6, 11, 12 and 13 were not found to cleave DEFα (data not shown) and no KLKs were found to cleave DEFβ (Figure 7.6 B). The proposed cleavage sites of defensin-1 alpha are shown in Figure 7.7.

7.3.5 Ex-vivo cleavage of substrates by proteases in CVF

Recombinant GST-like DSC2, DSC3, MUC4, MUC5B, DEFα and DEFβ were incubated with CVF extract (day 22, high KLK content) for 2 hours (DSC2, DSC3, MUC4 and MUC5B) or 14 hours (DEFα and DEFβ). The recombinant proteins were incubated alone for either 2 hours or 14 hours in PBS, pH 7.2, the same buffer the CVF was extracted in, as a control. The western blots in Figure 7.8 show that all of these proteins, with the exception of DEFβ, were cleaved by proteases within CVF.
Figure 7.6: Silver stains and western blots showing cleavage of DEFα and DEFβ by KLKs

Recombinant GST-linked DEFα and DEFβ were incubated with active KLKs at a ratio of 1:10 for various time points. The proteins were resolved by NuPAGE Bis-Tris gels (Invitrogen Canada Inc.) and cleavage of DEFα by KLKs was visualized by both silver staining, using the silver Xpress staining kit (Invitrogen) (left panels), and by transferring the proteins to nitrocellulose blotting paper (GE Healthcare) for western blotting using a GST antibody (Cell Signaling, right panel). Cleavage of DEFβ by KLKs was visualized by western blotting using the above antibody. Cleavage products are noted with arrowheads, A) DEFα cleavage by KLK5, B) DEFβ by KLKs.
Figure 7.7: Diagram of recombinant alpha-1 defensin and proposed cleavage sites for KLK5

A) The amino acid sequence for human alpha-1 defensin (accession # AAH27917) with the proposed KLK5 cleavage sites marked with arrows.

B) Schematic diagram of the recombinant GST-linked alpha-1 defensin protein (Abnova, Taipei, Tiwan) with potential KLK5 cleavage sites marked with arrows. Numbers correspond to the proposed cleavage sites shown in the alpha-1 defensin amino acid sequence (A).
Figure 7.8: *Ex-vivo* cleavage of DSCs, MUCs and DEFα by CVF proteases

100 ng of recombinant DSC, MUC or DEFα was incubated in PBS, pH 7.2 or in CVF (extracted in PBS, pH 7.2) for 2 hours (CSCs and MUCs) or 14 hours (DEFα) at 37 °C. Samples were resolved by SDS-PAGE and transferred to Hybond-C blotting membrane. Cleavage was monitored by blotting membranes with a GST antibody. Cleavage products are marked with arrowheads.
7.4 Discussion

KLKs are known to play a role in the desquamation of skin corneocytes through their cleavage of cell-cell adhesion molecules such as desmogleins (DSG) and desmocollins (DSC) \(^{62,74}\). Specifically KLK5 has been shown to cleave DSC1 \(^{74}\) and KLKs 5, 6 and 14 have been shown to cleave DSG1 \(^{62}\). I confirmed previous findings of DSG1 cleavage by KLKs 5 and 6 and have shown that, in addition, KLK12 is able to process DSG1 (Figure 7.2).

Processing of DSC2 and DSC3 by KLKs has not previously been investigated. I have shown cleavage of these two proteins by KLKs 5 and 12 (Figure 7.3). KLKs 6, 11, and 13 did not cleave these two proteins even at higher ratios and after longer time points.

KLK12 processing of cell-cell adhesion molecules has not previously been investigated. Here I suggest that KLK12 may also play a role in desquamation, through cleavage of DSG1, DSC2 and DSC3 (Figures 7.2 and 7.3), at least in the context of the vaginal epithelium. Examination of DSC2 and DSC3 cleavage by enzymes within CVF extract \textit{ex-vivo} revealed a cleavage pattern similar to that produced by incubation of these proteins with KLK12 (Figure 7.8). These results further implicate KLK12 as playing a role in desquamation of the vaginal epithelium during the menstrual cycle.

It has been suggested that proteolytic enzymes may affect the physical properties of mucin proteins causing the changes in mucus observed over the menstrual cycle \(^{163}\). I found that KLKs 5 and 12 were both able to cleave MUC4 and MUC5B \textit{in vitro} at pH 7.8 and pH 6.2, however the cleavage at pH 6.2 was much less than at pH 7.8, especially in the case of KLK12 (Figure 7.4 and
KLKs 5 and 12 are also found to have increased activity at pH 7.8 compared to 6.2 against their preferred substrate as shown in Figure 7.1. This suggests that the pH change around the time of ovulation may result in the KLKs being increasingly active. KLKs 6, 11 and 13 did not cleave either of these proteins in any conditions. Given that KLKs are expressed by the mucus-secreting columnar epithelial cells of the cervix, these results suggest that KLKs may be involved in the remodelling of cervical mucus at this time. The ex-vivo cleavage pattern of MUC4 by CVF (Figure 7.8) was similar to that of the pattern produced by incubating KLK5 with MUC4 (Figure 7.4), whereas the pattern of MUC5B cleavage by CVF extract (Figure 7.8) was similar to that produced when incubating MUC5B with KLK12 (Figure 7.4). These results further suggest a role for KLKs 5 and 12 in the remodelling of cervical mucus during the menstrual cycle.

Defensin proteins are found abundantly expressed in tissues and biological fluids involved in host defense (230). Human CVF is known to play an important in host defense (129) and I found alpha -1 and beta-1 defensins present in human CVF by proteomic analysis (136).

The alpha-defensins are translated as proteins of approximately 100 amino acids consisting of a signal peptide of approximately 20 amino acids. Proteolytic processing of these proteins results in active antimicrobial peptides around 30 amino acids in length (230). In human intestinal Paneth cells, the enzyme responsible for generating active defensin alpha-5 peptides has been identified as trypsin based on the N-terminal sequences of the active peptides (231).
We analyzed whether members of the KLK family may be involved in the processing of alpha and beta defensin-1 proteins in vitro and found KLK5 able to process alpha-1 defensin (Figure 7.6). Defensin-1 beta was not found to be processed by any of the KLKs tested (Figure 7.6). This is not surprising given that defensin beta is proposed to be translated in the active form and therefore not requiring further processing (230).

Analysis of the defensin-alpha cleavage pattern produced by KLK5 (Figure 7.6) shows that the 33 kDa GST-linked defensin-1 alpha is processed into fragments of approximately 30 kDa, 20 kDa, 10 kDa and 3 kDa evident by silver staining. The 30 kDa and 20 kDa fragments are evident by western blotting with a GST antibody and therefore must contain the GST fusion protein. I speculate that the 30 KDa fragment represents the GST-linked defensin minus the cleaved 3 kDa active peptide with cleavage of alpha-1 defensin after either arginine 62, lysine 63 or arginine 70 as shown in Figure 7.7. I speculate that the 20 kDa and 10 kDa represent cleavage of the full-length defensin-1 alpha protein from the GST link through cleavage after arginine 2 (Figure 7.7). I therefore propose that KLK5 is capable of processing alpha-1 defensin to produce the active 30 amino acid peptide responsible for antimicrobial activity. As mentioned above, KLK5 has been shown previously to perform a similar role in skin physiology where it has been shown to process the human cathelicidin antimicrobial protein (84). These results together with the results presented here suggest that KLK5 is an important regulator of antimicrobial activity. Incubation of defensin-1 alpha with CVF extract containing high levels of KLKs revealed a cleavage pattern containing a 20 kDa band which I propose to be the result of cleavage after
arginine 2, releasing the 10 kDa defensin-1 alpha protein from the GST fusion protein (Figure 7.8). KLK5 produced a similar pattern suggesting that KLK5 in CVF may be responsible for this cleavage.
Chapter 8: Summary of Findings, Overall Conclusions and Future Directions
8.1 Summary

Overall the work presented here has provided insights into potential functions of KLKs in the female reproductive system. Below are a summary of the key findings of this study.

8.2 Key findings

1. KLK Expression in the Female Reproductive System
   a) Many KLKs are co-expressed in tissues of the female reproductive system and in CVF extract, as measured by ELISAs specific for each KLK. In particular, KLKs 5, 6, 7, 10, 11, 12 and 13 were found expressed at relatively high levels.
   b) Within specific tissues of the female reproductive system, KLKs were found localized to the secretory and ciliated epithelial cells of the fallopian tube, the epithelial cells of the endometrium (proliferative and secretory stages), the mucus-secreting epithelium of the cervix and the stratified squamous epithelium of the vagina. I also found large levels of several KLKs in the supernatant of cultured human vaginal epithelial cells. These results explain why large levels of many KLKs are found in the CVF given that CVF is a pool of fluids originating from the fallopian tubes, endometrium, cervix and vagina.

2. KLK Hormonal Regulation in the Female Reproductive System
   a) The expression of several KLKs was found to peak following ovulation in CVF extract and saliva samples collected from a premenopausal woman over the menstrual cycle. Given that progesterone levels rise during the post-ovulatory period, I believe KLKs are regulated by progesterone during the menstrual cycle.
   b) A comparison of KLK levels between CVF extract collected from pregnant women versus non-pregnant women revealed elevated KLK levels in CVF extract.
from pregnant women. During pregnancy, hormone levels increase, particularly progesterone levels, further suggesting KLK regulation by progesterone.

c) Several KLKs were significantly downregulated by the synthetic glucocorticoid, dexamethasone and by estrogen in cultured human vaginal epithelial cells.

3. Potential Physiological Roles for KLKs in the Female Reproductive System

a) KLKs may play a role in the cyclical desquamation of vaginal epithelial cells during the post-ovulatory period of the menstrual cycle. KLKs 5, 6, 11, 12 and 13 were analyzed for the ability to cleave cell-cell adhesion molecules, DSG1, DSC2 and DSC3 in vitro. KLKs 5, 6 and 12 were found to cleave DSG1, and KLKs 5 and 12 were both found to cleave DSC2 and DSC3.

b) KLKs may be involved in the remodelling of cervical mucus which takes place during the menstrual cycle. In vitro, KLKs 5 and 12 were both found to process mucins 4 and 5B, the primary mucins in cervical mucus.

c) KLKs may aid in host defense of the female reproductive system through processing of antimicrobial proteins such as defensins. Defensin-1 alpha was found in CVF by our proteomic analysis and KLK5 was found to process this protein in vitro.

8.3 General conclusions and future directions

8.3.1 KLK co-expression and hormonal regulation in cervico-vaginal physiology

Several members of the KLK family are found to be coexpressed and coregulated in CVF suggesting that KLKs may participate in a proteolytic
cascade in CVF. Proteolytic cascades are important in many biological processes such as in coagulation and complement activation. KLKs have previously been shown to participate in proteolytic cascades in seminal plasma (68;69) and skin (62;70).

As previously mentioned, proteolytic cascades begin with an initiator enzyme which becomes active and subsequently able to cleave and activate other zymogens, to enhance the signal (67). In the case of both the skin and seminal plasma, the initiator KLKs (KLK5 an/or KLK14) auto-activate and are then able to cleave and activate other (inactive) KLKs (68-70). In CVF the major KLKs present are KLKs 6, 11, 12 and 13, with lower levels of KLKs 5, 7, 8 and 10. There are many potential candidates for “initiators” from this pool of enzymes. KLKs 5, 6, and 12, and to a lesser degree KLKs 11 and 14 have all been shown previously to auto-activate (232;233) and may be responsible for the initiation of a proteolytic cascade within CVF. Further experiments should be directed at delineating the KLK cascade within CVF.

We have shown that KLK expression levels in CVF extract and female saliva are altered by hormonal changes occurring during the menstrual cycle (Figures 5.3 and 5.4). The expression levels of several KLKs peak in CVF extract and saliva in the period following ovulation when progesterone levels are known to increase (131;209). We believe that KLKs may play several important roles in cervico-vaginal physiology and pathobiology: 1) in the cyclical desquamation of vaginal epithelial cells during the menstrual cycle, 2) in remodelling of cervical mucus during the menstrual cycle and 3) in host defense.
8.3.2 Desquamation of vaginal epithelial cells

Several KLKs have been previously implicated in the desquamation of skin corneocytes, in particular, KLKs 5, 7 and 14 (62;74). Here I suggest that KLKs may also play a role in desquamation in the context of the vaginal epithelium through their cleavage of desmoglein and desmocollin proteins. In the proposed model outlined in Figure 8.1 I speculate that the increased KLK levels present in the period following ovulation contribute to the desquamation of mature vaginal epithelial cells which is known to occur during this period (131).

8.3.3 KLKs and overdesquamation

As was briefly mentioned in chapter 1, KLKs have been extensively studied with respect to their role in over-desquamation of corneocytes in human skin. Overdesquamation is implicated in many skin disease pathologies such as Netherton syndrome (64;75;81;82), peeling skin syndrome (78), psoriasis (80), and atopic dermatitis (79). The activity of KLKs 5, 7 and 14 has been shown to be controlled by the lympho-epithelial kazal-type-related inhibitor (LEKTI) in skin (62;81;83;234). Mutations in the serine peptidase inhibitor kazal-type 5 (SPINK5) gene, encoding LEKTI, have been shown to result in increased KLK activity and overdesquamation of skin corneocytes, implicated in Netherton syndrome (82).

The levels of kallikrein-related peptidases 5, 6, 7, 8, 10, 11, 13 and 14 are elevated in peeling skin syndrome, psoriasis and atopic dermatitis, resulting in increased trypsin-like and chymotrypsin-like activity and subsequent overdesquamation of corneocytes (78-80).
Figure 8.1: Schematic representation of the changes in the vaginal epithelium under hormonal stimulation throughout the menstrual cycle

During the follicular phase the epithelium consists of non-corroded, stratified squamous cells (A). Estrogen levels increase during the follicular phase and peak at ovulation causing the epithelium to mature and become increasingly corroded (B). Estrogen levels fall after ovulation causing the epithelial cells to shed. Progesterone levels peak and plateau following ovulation. This results in increasing KLK levels which, we propose, cause desquamation of the vaginal epithelium, resulting in the return of the epithelium to its stratified squamous appearance (A).
8.3.4 Desquamative inflammatory vaginitis

Vaginitis defines inflammation of the vagina resulting in itching, and pain. One particular form of vaginitis, referred to as desquamative inflammatory vaginitis, affects mostly pre-menopausal women resulting in discomfort, irritation, increased discharge and painful intercourse \(^{(235)}\). DIV is not caused by infection or estrogen deficiency as some other forms of vaginitis are \(^{(131)}\).

Microscopic analysis reveals increased squamous cell exfoliation, an increased number of immature epithelial cells, a decrease in lactobacilli and an increase in pH from 4.5-5.5 up to 7.4 \(^{(236)}\). DIV is most commonly treated with clindamycin or intravaginal corticosteroids \(^{(131;235;236)}\).

8.3.5 A potential role for KLKs in vaginitis

It is probable that KLKs play a role in the normal desquamation of vaginal epithelial cells similarly to their role in the desquamation of skin corneocytes. In syndromes such as vaginitis, I hypothesize that KLK levels and/or KLK activity are elevated and contribute to over-desquamation, just as in skin pathologies.

I hypothesize that under normal conditions a basal level of KLK activity is required for normal vaginal epithelial cell desquamation. Proteomic analysis of CVF showed the presence of many serine protease inhibitors, including LEKTI \(^{(136)}\), responsible for controlling KLK activity. I speculate that KLK levels and activity are increased during, or contributing to the development of DIV. A recent study showed that in skin KLK activity is increased at lower pH levels compared with higher pH levels because of lower affinity between KLKs and their inhibitor LEKTI at low pH \(^{(63)}\). It is possible in this case that the increased pH associated with vaginitis may play a protective role by encouraging increased association.
between KLK and LEKTI or other inhibitors and therefore reduce KLK activity. Further to this, I have shown that treatment of vaginal epithelial cells with corticosteroids and estrogen reduces KLK expression (Table 5.4). I hypothesize that treatment with corticosteroids and or estrogen helps to reduce KLK levels associated with vaginitis, therefore reducing proteolytic activity and desquamation. A clinical study examining KLK levels and activity in CVF extract of women suffering from vaginitis compared to normal women would be useful in answering the above hypothesis and may reveal the KLKs as potential therapeutic targets for these conditions.

8.3.6 KLKs and periodontal disease

Hormonal changes during the menstrual cycle and during pregnancy have been shown to contribute to the development of periodontal disease and gingivitis in women (237). Women taking oral contraceptives also commonly develop periodontal disease due to the increased hormonal exposure (237). Progesterone, in particular, has been implicated in these affects (237) and has been shown to decrease plasminogen activator inhibitor 2 (PAI-2) in saliva (238). KLKs have been shown to be inhibited by PAI-2 (2); this coupled with our proposal of a progesterone mediated increase in KLK expression and activity suggests that KLKs may contribute to the development of periodontal disease. A clinical study comparing KLK levels in the saliva of multiple women over the menstrual cycle and in women taking oral contraceptives would be useful in delineating a potential role for KLKs in periodontal disease.
8.3.7 Remodelling of cervical mucus

Approximately 200-500 million sperm are deposited onto the cervix during a normal ejaculation episode. For fertilization to take place sperm must migrate through the cervical mucus into the uterus and subsequently into the fallopian tube, where fertilization most often takes place (239). Several factors affect sperm migration through the cervix: the ability of sperm to penetrate mucus, the properties of cervical mucus which assist in sperm transport and the morphology of the cervical crypts (239). The neutral pH and less viscous nature of the cervical mucus at ovulation promotes sperm motility and provides an environment suitable for sperm survival (239). It has been suggested that proteolytic enzymes may affect the physical properties of mucin proteins causing the changes in mucus observed over the menstrual cycle (163). Here I suggest that KLKs may play a role in the remodelling of cervical mucus through their cleavage of mucins 4 and 5B (Figure 7.4), the primary mucins found in cervical mucus (143).

Under the influence of estrogen in the preovulatory phase of the menstrual cycle, the amount of cervical mucus increases and becomes increasingly hydrated (149). After ovulation, rising progesterone levels cause the mucus to become scant and viscous (146;149). These changes in cervical mucus are an important component of the actions of many contraceptives, particularly those which contain progesterone, such as the progestin only pill, depot medroxy-progesterone acetate (DMPA) and the hormonal intrauterine system (containing levonorgestrel) (240). The mechanism of progesterone-only contraceptives is to maintain cervical mucus as scanty, viscous and sperm-hostile (240;241).
Similarly, following conception, increasing progesterone levels cause the mucus to become thick and form a plug which blocks the entrance into the uterus from the vagina (147;152).

Here, I suggest that KLKs may be involved in the remodelling of cervical mucus during the menstrual cycle as outlined in the model I present in Figure 8.2. MUC4 and MUC5B protein levels peak at ovulation and contribute to cervical mucus’ hydrated, elastic properties suitable for sperm infiltration. Increasing progesterone levels stimulate increased expression of KLKs, which process mucin proteins MUC4 and MUC5B within cervical mucus, restoring the cervical mucus to its preovulatory state. It is also possible that KLKs contribute to the action of progesterone-based contraceptives through a similar mechanism. A study analyzing KLK levels in multiple women over the menstrual cycle and in women taking oral contraceptives, particularly progesterone only, would help in delineating the role of KLKs in mucus remodelling. Furthermore, collection of cervical mucus specifically from women for analysis of KLK levels and activity would be useful in determining their role.
Figure 8.2: Schematic diagram illustrating the potential role of KLKs in cervical mucus remodeling

MUC4 and MUC5B protein levels peak at ovulation with rising estrogen levels and cause cervical mucus to become increasingly hydrated and elastic. We propose that increasing progesterone levels, following ovulation, stimulate increased expression of KLKs, which process mucin proteins MUC4 and MUC5B within cervical mucus, restoring the cervical mucus to its preovulatory state.
8.3.8 Processing of antimicrobial peptides

The vagina is open to the outside world and thus exposed to many microorganisms, particularly during sexual intercourse. As such, host defense is an important aspect of vaginal physiology and as previously mentioned, CVF plays an important role in this defense (129). In particular, cationic peptides, such as defensins and the human cathelicidin, found in CVF, have been shown to be fundamental in defending the vagina from infectious agents (129).

The vaginal mucosa and host defense properties play a particularly important role with respect to pathogenesis of and defense against the human immunodeficiency virus (HIV). 50% of those living with HIV/AIDS worldwide are women and natural sexual transmission of the virus occurs through the vaginal mucosa (228). It is becoming increasingly evident that innate defenses of the vaginal epithelium help to protect against invading pathogens, such as HIV.

Members of the defensin family of antimicrobial proteins have been shown to be active against HIV through their ability to inhibit HIV replication and inactivate HIV virions (139;242). Interestingly, defensin levels in CVF have been found to be highest during the secretory, post-ovulatory stage of the menstrual cycle, suggesting that they may be regulated by progesterone (243). The authors of this study suggest that increased defensin presence following ovulation may help to prevent the ascension of pathogens during ovulation (when cervical mucus is thinner and not as protective) therefore maintaining the sterility of the upper reproductive tract.

We found KLK5 able to process defensins known to be expressed in the vagina, in vitro, and found similar processing of defensins by proteases within
CVF ex vivo. Defensins, particularly α-defensin, are cleaved following secretion to yield small, cationic peptides active against many microorganisms (230). Given that KLKs also appear to be regulated by progesterone during the menstrual cycle, I suggest that KLKs may play an antimicrobial role through their processing and activation of active defensin peptides in the vagina during this time.

KLKs may also be important for defensin processing in the cervical mucus plug which is a thick plug formed by cervical mucus during pregnancy, under progesterone control (147;152). The cervical mucus plug acts as a blockade to prevent ascension of microorganisms from the vagina into the uterus and has been shown to have antimicrobial activity (152). Cervical plugs have been found to contain many antimicrobial proteins and peptides, including defensins (147).

Further experimentation including analysis of KLK5-defensin cleavage products by N-terminal sequencing is required to confirm our hypothesis that KLK5 processes defensin-1 alpha into its active form.


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