Biol. Chem., Vol. 389, pp. 99–109, February 2008 • Copyright © by Walter de Gruyter • Berlin • New York. DOI 10.1515/BC.2008.013

Tissue-specific promoter utilisation of the kallikrein-related peptidase genes, *KLK5* and *KLK7*, and cellular localisation of the encoded proteins suggest roles in exocrine pancreatic function

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Abstract

Tissue kallikrein (kallikrein 1) was first identified in pancreas and is the namesake of the kallikrein-related peptidase (KLK) family. KLK1 and the other 14 members of the human KLK family are encoded by 15 serine protease genes clustered at chromosome 19q13.4. Our Northern blot analysis of 19 normal human tissues for expression of *KLK4* to *KLK15* identified pancreas as a common expression site for the gene cluster spanning *KLK5* to *KLK13*, as well as for *KLK15* which is located adjacent to *KLK1*. Consistent with previous reports detailing the ability of *KLK* genes to generate organ- and disease-specific transcripts, detailed molecular and *in silico* analyses indicated that *KLK5* and *KLK7* generate transcripts in pancreas variant from those in skin or ovary. Consistently, we identified in the promoters of these *KLK* genes motifs which conform with consensus binding sites for transcription factors conferring pancreatic expression. In addition, immunohistochemical analysis revealed predominant localisation of KLK5 and KLK7 in acinar cells of the exocrine pancreas, suggesting roles for these enzymes in digestion. Our data also support expression patterns derived from gene duplication events in the human *KLK* cluster. These findings suggest that, in addition to KLK1, other related KLK enzymes will function in the exocrine pancreas.

Keywords: kallikrein; mRNA splicing; pancreas; promoter analysis; tissue specific; transcription factors.

Introduction

Tissue kallikrein or kallikrein 1 (*KLK1*/KLK1) was the first described member and namesake of a gene family, encoding 15 serine proteases, localized in a discrete gene cluster on chromosome 19q13.4 (Gan et al., 2000; Harvey et al., 2000; Yousef and Diamandis, 2001). Tissue kallikrein was first identified in pancreatic extract (Kraut et al., 1930) and pancreas is still recognised as a site of most abundant *KLK1* expression (Fukushima et al., 1985; Clements, 1997). In this and other organs, KLK1, principally via kinin generation, is involved in the regulation of local blood flow and pressure, vascular permeability and inflammation and has been implicated in pathophysiological processes in the cardiovascular, respiratory, renal, gastrointestinal, reproductive and central nervous systems (Clements, 1997). Similar to KLK1, the other 14 KLK proteases function in post-translational modification of polypeptides, including activation of other KLKs and growth factors and degradation/remodelling of the extracellular matrix. These proteolytic events regulate diverse cellular processes, including proliferation differentiation, and angiogenesis, as well as a variety of pathophysiological processes in organs, such as the prostate, ovary, brain and skin (Borgono and Diamandis, 2004; Clements et al., 2004). However, little attention has been focussed on the potential function and/or expression of the more recently identified KLKs (KLK4–KLK15) in the pancreas.

A feature of the *KLK* gene family is the discrepancy apparent in expression patterns when comparing data generated by Northern blot and reverse transcription-polymerase chain reaction (RT-PCR) analysis; tight tissue-restricted expression is generally observed by Northern blot analysis, whereas broader expression is often observed by RT-PCR analysis (Harvey et al., 2000). Additionally, these analyses have indicated that clusters of *KLK* locus genes show common sites of expression. For instance, the contiguous *KLK15-KLK3-KLK2-KLK4* genes have a common prostatic expression (Harvey et al., 2000). Similarly, the contiguous *KLK4–KLK11* and *KLK13–14* genes are highly expressed in ovarian cancer (Yousef and Diamandis, 2001; Clements et al., 2004). Furthermore, *KLK6*, *KLK8* and *KLK15* are highly expressed in the central nervous system (Yousef and Diamandis, 2001; Clements et al., 2004). These data generally support the notion of gene duplication within this locus (Lundwall et al., 2006) and common DNA elements regulating tissue-specific expression. In this regard, we have previously shown using mRNA dot blot analysis that the *KLK6–KLK13* cluster of genes exhibit high pancreatic expression (Harvey et al., 2000). Surprisingly, using the

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more sensitive RT-PCR approach, only *KLK10* and *KLK12* showed expression in the pancreas (Gan et al., 2000; Harvey et al., 2000). The reason for this discrepancy remains unknown.

A second feature of the *KLK* family is the extraordinary number of mRNA splice variants. To date, more than 80 splice variants have been described for the 15 *KLK* genes, with only a small number of these characterised to any extent (Kurlender et al., 2005; Tan et al., 2006). The tissue- and disease-specific expression of these characterised transcripts has led to the proposal that *KLK* splice variants may provide useful biomarkers for several diseases (Tan et al., 2006). Interestingly, most of these splice variants have been discovered by analysis of expressed sequence tag (EST) databases, and thus do not have complete 5′-sequences. Therefore, differences between the 5′-untranslated regions (UTRs) or proximal promoters of the majority of *KLK* variant transcripts are unknown. In this respect, we have previously described novel *KLK5* and *KLK7* transcripts in ovarian cancer cells that have different 5′-sequences to those first described in skin (Dong et al., 2003). This potentially indicates that these variant transcripts or the encoded proteins will have tissue- or disease-specific roles.

In this study, using Northern blot analysis, we have identified a cluster of *KLK* genes with high pancreatic expression. We have further investigated the *KLK5* and *KLK7* transcripts expressed in the pancreas comparing these to transcripts generated in skin or ovary. We also provide evidence for tissue- and disease-specific transcriptional regulation of *KLK* expression. Finally, we have determined the cellular localisation of KLK5 and KLK7 in pancreatic tissue samples, providing for the first time insight into potential roles for these proteases in the pancreas.

Results

High expression of *KLK* gene clusters in the pancreas

To identify common sites of mRNA expression of *KLK4–15*, 19 normal human tissues were examined by Northern blot analysis using probes specific to each KLK. The autoradiographs obtained are shown in Figure 1, with a summary and comparison with *KLK1–KLK3* in Table 1. Strong hybridisation was clearly observed in the pancreas for *KLK5*, *KLK8* and *KLK10*, and at a moderate level for *KLK7*, *KLK9*, *KLK11–13* and *KLK15*, suggesting that this is the major site of expression for these *KLK* genes. Faint hybridisation was also observed for *KLK6*. A strong *KLK4* mRNA band was only detected in the prostate, indicating that similar to *KLK2* and *KLK3*, *KLK4* expression is prostate restricted. In addition, *KLK8*, *KLK10*, *KLK11*, *KLK14* and *KLK15* hybridisation was also observed in the prostate. Testis showed strong *KLK5* hybridisation, while a faint *KLK5* band was observed in intestine and uterus. Similarly, a strong *KLK6* hybridisation was observed in the prostate, and less intense bands for *KLK6* were observed in heart and kidney, pancreas, spleen and ovary. *KLK10* was additionally detected in thymus, testis and ovary, whereas *KLK11* and

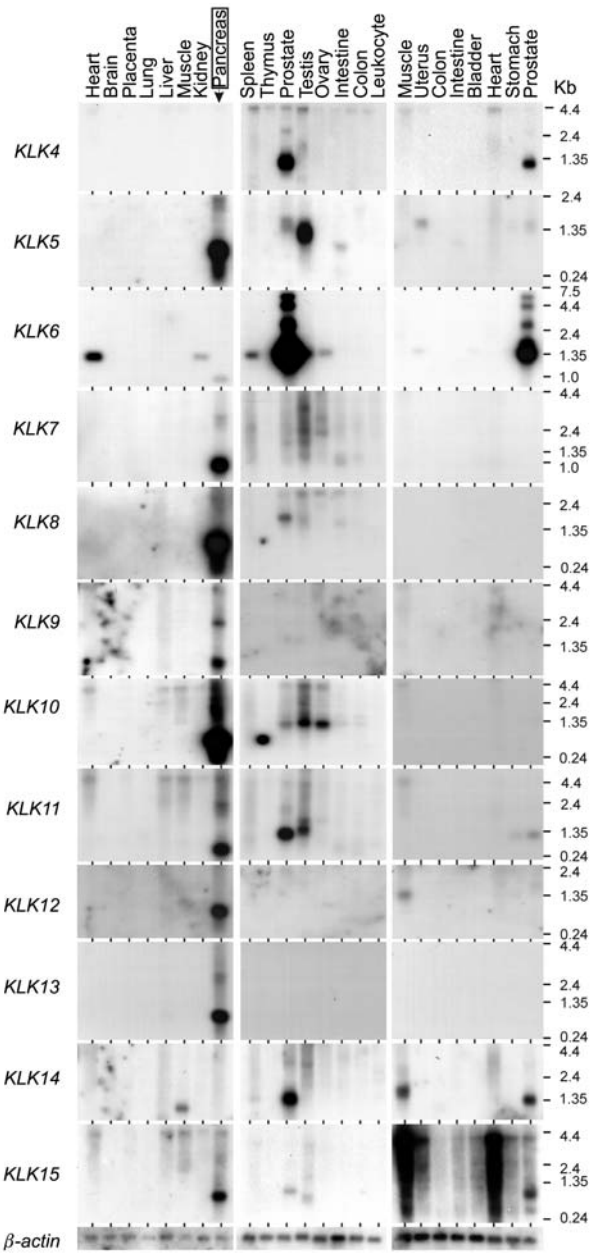

Figure 1 Expression of *KLKs* in normal human tissues by Northern blot analysis.
Human multiple tissue mRNA blots were hybridised with [^{32}P]-labelled cRNA probes for *KLK4–15* as shown on the left-hand side of the panel, or a [^{32}P]-random-labelled β-actin cRNA probe. The transcript lengths are indicated on the right.

KLK12 were also expressed in testis and muscle, respectively. Consistently, serial analysis of gene expression (SAGE; SAGEmap database), virtual Northern blotting and EST database searches detected pancreatic transcripts for *KLK5*, *6*, and *11* (GenBank: AF168768, AF013988 and AB012917; SAGEmap database), and *KLK1, KLK7* and *KLK10* (GenBank: BC005313, L33404 and BC002710; both SAGEmap database and EST database) (Table 2). Taken together with published data (Fukushima et al., 1985; Harvey et al., 2000), the pancreas showed common expression through the contiguous gene clusters of *KLK1–KLK15* and *KLK5–13*, with the pancreas being the most abundant site of expression for *KLK5*, *KLK7–KLK10*, *KLK12*, *KLK13* and

Table 1 *KLK* expression levels from Northern blot analysis.

Genes	Tissues with **intense** or faint hybridisation bands	Approximate transcript size (kb)	References
KLK1	**Pancreas**, kidney, salivary gland	0.9	Fukushima et al., 1985
KLK2	**Prostate**	1.2, 1.5	Riegman et al., 1991
KLK3	**Prostate**	1.6, 2.1, 3.1, 5.2, 6.1	Heuze-Vourc'h et al., 2003
KLK4	**Prostate**	1.35	
KLK5	**Pancreas**, **testis**, prostate, intestine, uterus	1.0, 1.35	
KLK6	**Heart, prostate**, kidney, pancreas, spleen, ovary	1.0, 1.35, 3.0, 4.4, 6.0	
KLK7	**Pancreas**	1.0	
KLK8	**Pancreas**, prostate	1.2, 2.0	
KLK9	Pancreas	1.0, 2.5	
KLK10	**Pancreas, thymus**, prostate, testis, ovary	1.0, 1.35	
KLK11	**Pancreas, prostate**, testis	1.0, 1.35	
KLK12	**Pancreas**, muscle	1.0, 1.6	
KLK13	**Pancreas**	1.0	
KLK14	**Prostate**, **skeletal muscle**	2.0, 1.35	
KLK15	**Pancreas**, prostate, testis	1.0, 1.35	

KLK15. Notably, expression of *KLK7* and *KLK13* was restricted to the pancreas.

It was of interest to note that multiple bands were observed in certain tissues, such as *KLK6* in prostate (1.35, 3.0, 4.4, 6.0 kb) and *KLK9* in pancreas (1.0, 2.5 kb) (Figure 1), suggesting the existence of multiple *KLK* mRNA transcripts. Additionally, mRNA transcripts with different sizes were observed between the pancreas and other tissues for *KLK5*, *KLK6*, *KLK8* and *KLK10–12* (Figure 1). For instance, all pancreatic transcripts (*KLK5–13* and *KLK15*) are approximately 1.0 kb and similar in size to the *KLK10* transcript in thymus and *KLK15* in testis. However, the transcripts expressed in other tissues appear larger – notably approximately 1.35 kb for prostatic *KLK4*, *KLK6*, *KLK10*, *KLK11*, *KLK14* and *KLK15*, testicular *KLK5*, *KLK10* and *KLK11*, ovarian *KLK6* and *KLK10*, and heart, kidney and spleen *KLK6*, with a ca. 2.0 kb *KLK8* transcript also observed in the prostate. Taken together with previous studies (Harvey et al., 2000), the various mRNA transcript sizes from different tissues for each *KLK* gene indicated the existence of tissue-specific *KLK* mRNA transcripts.

Identification of pancreas-specific *KLK5* and *KLK7* transcripts

Owing to differences in the size of *KLK5* and *KLK7* transcripts in pancreas compared with transcripts from other tissues, we obtained the complete cDNA sequence of the pancreatic transcripts produced from each of these genes by transcription initiation site (TIS) identification (through RNA ligase-mediated rapid amplification of cDNA ends, RLM-RACE) and 3′-RACE, as well as sequencing of a pancreatic EST clone.

Table 2 *In silico* analysis of *KLK* expression in the pancreas using SAGEmap and EST databases.

Kallikrein	SAGEmap		EST database	
	Normal (3[a])	Carcinoma (6[a])	Normal (9[a])	Carcinoma (10[a])
KLK1	1/3	2/6	2/9	2/10
KLK5	1/3	0/6	0/9	0/10
KLK6	1/3	4/6	0/9	0/10
KLK7	0/3	2/6	0/9	1/10
KLK10	0/3	4/6	0/9	3/10
KLK11	0/3	3/6	0/9	0/10

[a]Total clone number in the NCBI database (http://www.ncbi.nlm.nih.gov).

Figure 2A,B shows the 5′- and 3′-RACE products, respectively, for *KLK5* in the pancreas, with control reactions on RNA from HaCat and OVCAR-3 cells. The regions amplified for each tissue-specific product (bands 1–5) are indicated schematically in Figure 2C. Sequencing of 5′-RACE products indicted that the transcription of *KLK5* was initiated from the same site in pancreas (band 1), HaCat cells (short form, band 3) and ovary (short form, AF435981, Figure 2C). In addition, our sequencing data showed that the HaCat long and OVCAR-3 transcripts (Figure 2A, bands 2 and 4; AF435980) have an additional 40 bp at the 5′-end (exon 1b) compared with the previously published skin *KLK5* transcript (exon 1c, NM_012427). Using the 3′-primer set (exons 4–5), indicated in Figure 2C, *KLK5* 3′-UTR bands of the same size were observed from pancreas and the cell lines HaCat and OVCAR-3 (band 5, Figure 2B), which gave an equivalent 3′-UTR sequence to that identified in skin (NM_012427) (Brattsand and Egelrud, 1999). No band was observed in the negative (without cDNA) control (Figure 2A,B). Of note, the *KLK5* transcripts in different tissues have the same coding region which encodes pre-pro-KLK5, though alternative 5′-UTRs are clearly utilised, giving rise to exons 1a, 1b and 1c that are specifically expressed in pancreas (1a), skin (1a, 1b and 1d) or ovary (1b and 1c).

The comparison of 5′- and 3′-UTR for *KLK7* in the pancreas and HaCat and OVCAR-3 cells are shown in Figure 3. Sequencing of 5′-RLM-RACE products revealed that pancreatic *KLK7* 5′-UTR (band 1) is 121 bp shorter (exon 1a) than that of HaCat (bands 2 and 3) and OVCAR-3 cells (band 4, exon 1c), but has 110 bp of further 5′-UTR sequence upstream of the reported skin transcript (L33404, Figure 3C). The pancreatic *KLK7* 5′-UTR sequence also has an additional 19 bp in comparison to the sequence reported for the pancreatic EST clone (GenBank AA101043, data not shown). Two pancreatic *KLK7* 3′-bands (bands 5 and 6) of 1300 bp and 1050 bp,

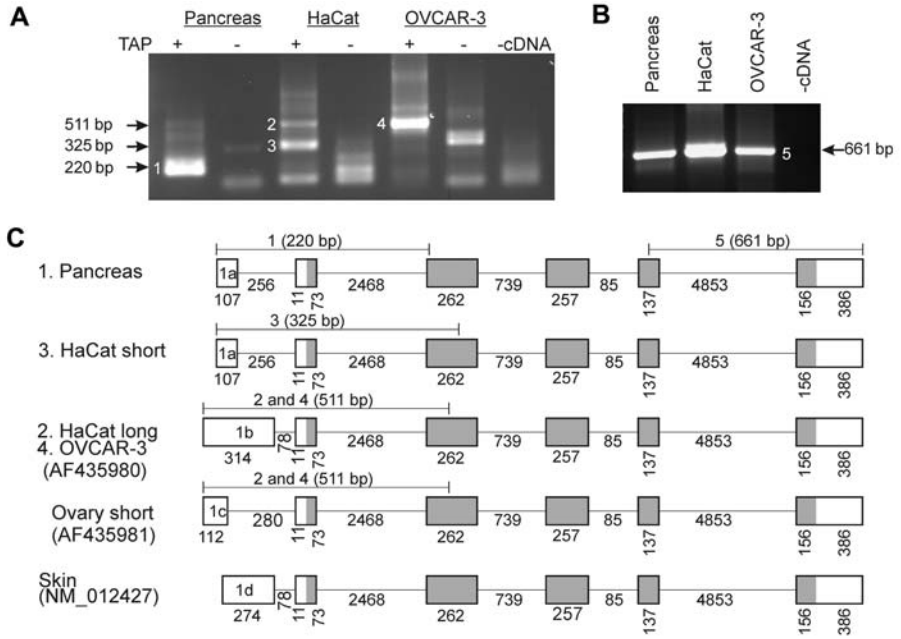

Figure 2 Identification of 5′- and 3′-sequences of tissue-specific *KLK5* transcripts.
(A) 5′-RLM-RACE products from pancreatic, HaCat and OVCAR-3 RNA with (+) or without (-) tobacco alkaline pyrophosphatase (TAP) treatment as indicated. (B) 3′-RACE products from the above RNA. (C) Schematic representation of *KLK5* transcripts from the above tissues in comparison to transcripts from skin (NM_012427). Shaded boxes, coding exons; open boxes, non-coding exons. Introns are shown as lines. Size of exons and introns with nucleotide number is indicated. AF435980, AF435981 and NM_012427, GenBank accession number. The primer sets (1–5) used to generate the PCR products (1–5) in panels (A) and (B) and the product sizes are indicated.

respectively, were amplified. The no cDNA control was appropriately negative (Figure 3A,B). The schematic alignment of sequences obtained from the pancreas, HaCat and OVCAR-3 cells and skin (L33404) (Hansson et al., 1994) compared to ovary and skin (exon 1c) is shown in Figure 3C. These data show that different

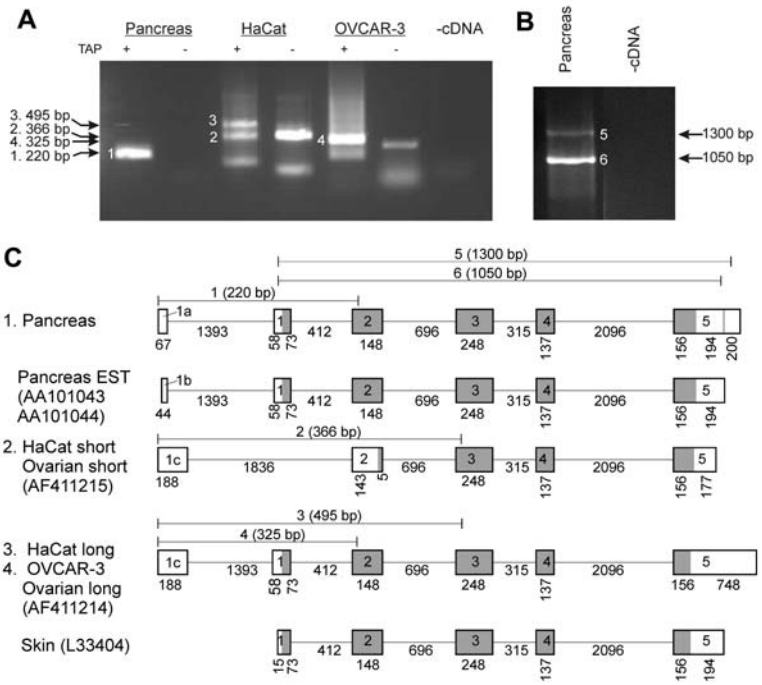

Figure 3 Identification of 5′- and 3′-sequences of tissue-specific *KLK7* transcripts.
(A) 5′-RLM-RACE products from the pancreatic, HaCat and OVCAR-3 RNA with (+) or without (-) TAP treatment as indicated, with no TAP treated RNA as a control reaction. (B) 3′-RACE products from pancreas RNA. (C) Schematic representation of *KLK7* transcripts from the above tissues in comparison to transcripts from ovary (AF411214 and AF411215) and skin (L33404). Pancreatic EST entries (AA101043 and AA101044) are shown for comparison. Shaded boxes, coding exons; open boxes, non-coding exons. Introns are shown as lines. Size of exons and introns with nucleotide number is indicated. GenBank accession numbers are indicated in brackets. The primer sets (1–6) used in the panels (A) and (B) and the product size are indicated.

5′ *KLK7* exons (exons 1a, 1b or 1c) are utilised in the pancreas (exons 1a and 1b). As shown in Figure 3C, the pancreatic *KLK7* mRNA transcripts have the same coding region as skin and ovarian long transcripts, which would be translated into full-length *KLK7*, but is different from the ovarian short *KLK7* transcript (AF414215) which encodes an N-terminal truncated protein, because exon 1 is missing. On the other hand, the pancreatic transcripts have different 5′-UTR and 3′-UTR from ovary and skin, suggesting the usage of alternative promoters and different transcriptional regulation in these organs.

Pancreas-specific transcription factor binding sites in *KLK5* and *KLK7* promoters

We also examined the genomic nucleotide sequence of the proximal promoter region of *KLK5* (Figure 4) and *KLK7* (Figure 5) to identify consensus basal and tissue-specific regulatory elements. A potential TATA box was localised 29 bp upstream from the identified pancreatic TIS for *KLK5* (ttataa, Figure 4A) and *KLK7* (atataa, Figure 5A) obtained from 5′-RLM-RACE approaches. Using the MatInspector program, which has been used for *KLK6* promoter region analysis (Christophi et al., 2004), *in silico* analysis of approximately 2 kb immediately 5′ to the TIS of *KLK5* (GenBank: AF135028) and *KLK7* (AF166330) revealed a number of pancreatic-specific transcription factor binding sites in this region. For instance, the sequences of binding sites for the PBC family of homeodomain protein PBX1, three amino acid loop extension (TALE) family member MEIS1, B-cell-specific activating protein PAX-5 and HOX protein were found in the *KLK5* promoter (Figure 4). In the *KLK7* promoter region, transcription factor binding sites were also found for PDX1, PBX1, MEIS2, PAX-5 and HOX (Figure 5). In addition, nuclear receptor regulatory binding sites for the progesterone receptor (PR) were found in the *KLK5* promoter region (Figure 4), while androgen receptor (AR) and glu-

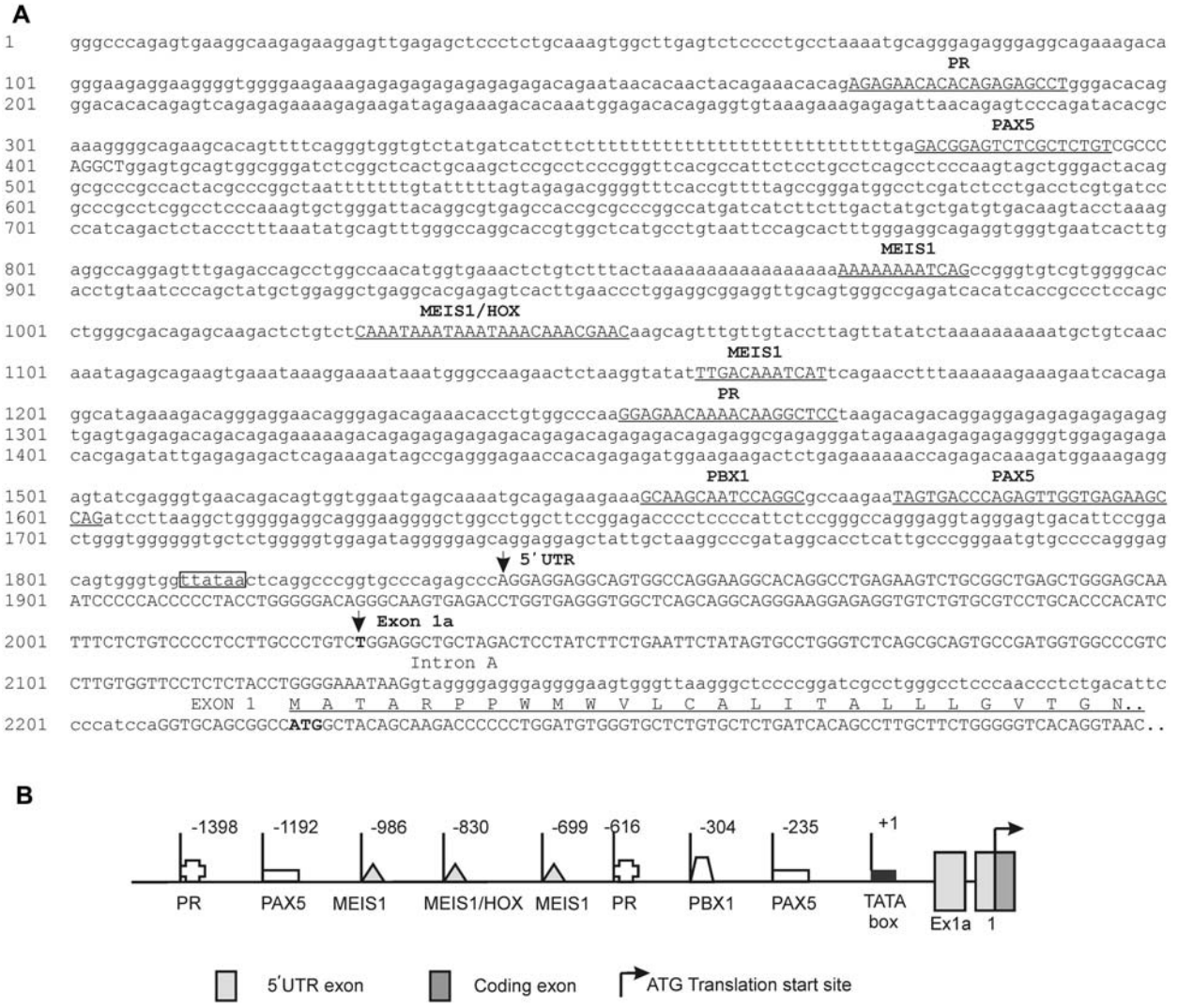

Figure 4 Sequence analysis of the human *KLK5* promoter.
(A) Nucleotide sequence of the proximal promoter and 5′-flanking region of *KLK5*. The TIS is indicated by the arrow and a putative TATA box sequence 29 bp upstream is boxed. The uppercase sequence represents the transcribed region. The translation start codon ATG is in bold font. The intron and exons 1/1a are as indicated. The partial translated amino acid sequence is in uppercase and underlined. Potential transcription factor binding sites are in uppercase and underlined with the name above. PAX-5, B-cell-specific activating protein; HOX, PBX1 and MEIS1, family of homeodomain-containing transcription factors; PR, progesterone receptor binding site. '↓' indicates the start site of 5′-UTR and exon 1a. (B) Schematic diagram for transcription factor binding sites in the *KLK5* promoter region.

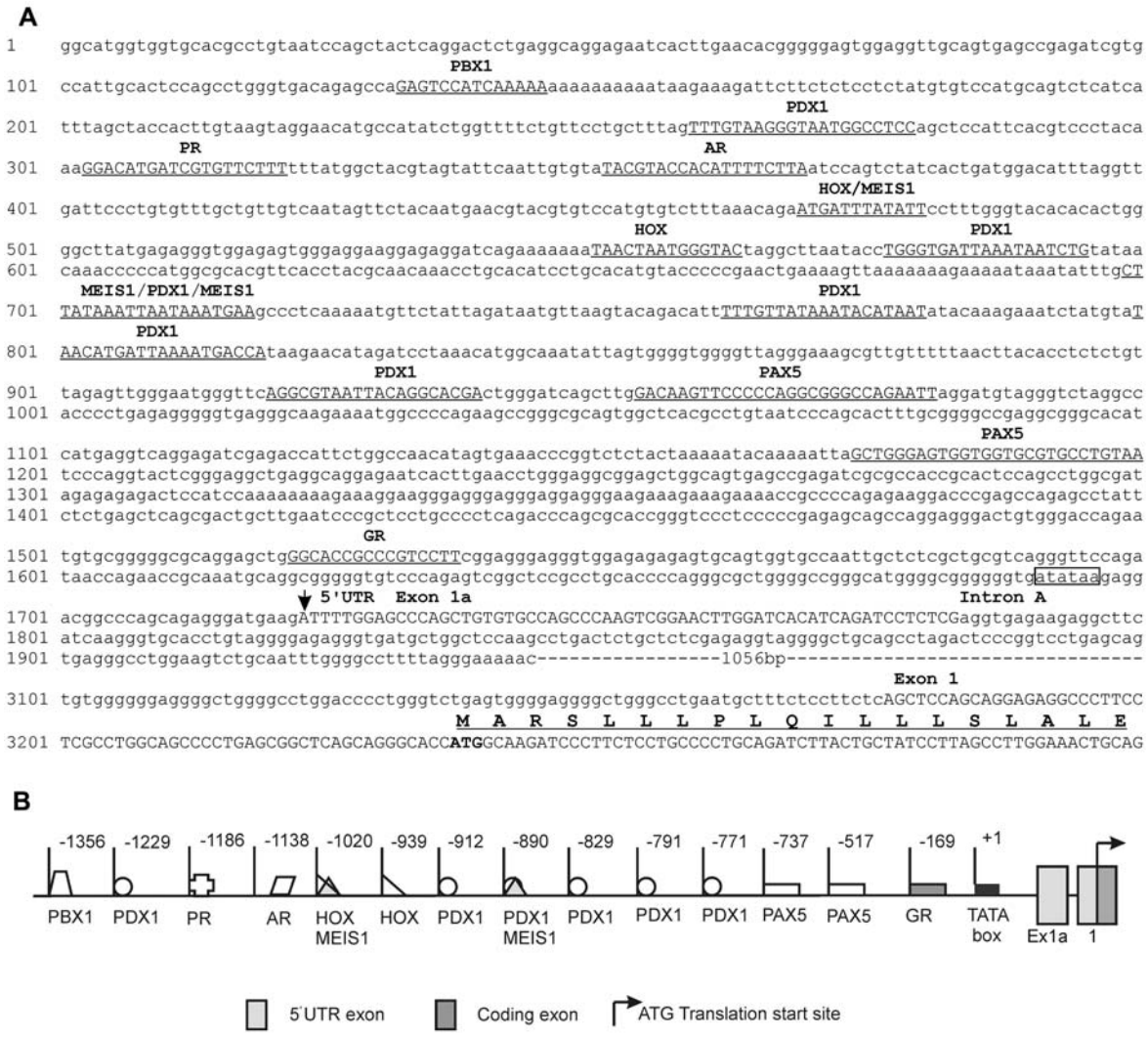

Figure 5 Sequence analysis of the human *KLK7* promoter.
(A) Nucleotide sequence of the proximal promoter and 5′-flanking region of *KLK7.* The TIS is indicated by the arrow and a putative TATA box sequence 29 bp upstream is boxed. The uppercase sequence represents the transcribed region. The translation start codon ATG is in bold font. The intron and exon 1/1a are as indicated. The partial translated amino acid sequence is in uppercase and underlined. Potential transcription factor binding sites are in uppercases and underlined with the name above. PDX1, pancreatic LIM-homeodomain transcription factor; PAX-5, B-cell-specific activating protein; HOX and MEIS1, family of homeodomain-containing transcription factors homeobox protein; PR, progesterone receptor binding site; AR, androgen receptor binding site; GR, glucocorticoid receptor. '↓' indicates start site of 5′-UTR and exon 1a. (B) Schematic diagram for transcription factor binding sites in the *KLK7* promoter region.

cocorticoid receptor (GR) binding elements were identified in the *KLK7* promoter region (Figure 5).

KLK5 and KLK7 are expressed in pancreatic tissues by acinar cells

Because KLK5 can activate KLK7 *in vitro* (Brattsand et al., 2005) and the co-localisation of these proteins in skin and ovary has been reported (Dong et al., 2003), immunohistochemical staining was performed to examine the cellular localisation of these proteins in pancreatic tissues (Figure 6). Both KLK5 and KLK7 staining was confined to acinar cells (open arrows in Figure 6A–C for KLK5, open arrows in Figure 6D,E for KLK7), where KLK5 and KLK7 staining can be observed in the cytoplasm only (Figure 6A–E). KLK5 staining was essentially homogenous throughout the cytoplasm of acinar cells (open arrows, Figure 6A), whereas KLK7 displayed granular staining of these cells (open arrows, Figure 6E). KLK7 also displayed clear staining in ducts (arrowhead in Figure 6D), indicating secretion from the pancreas, whereas only faint ductal staining was observed for KLK5 (arrowheads, Figure 6B,C). Faint staining on the islet of Langerhans (black arrows in Figure 6B for KLK5; Figure 6D for KLK7), but no stromal cell staining (open arrowheads in Figure 6B,C for KLK5; open arrowhead in Figure 6D for KLK7) was observed. No staining was observed in the negative controls with primary antibody omitted (Figure 6F).

Discussion

We have demonstrated by Northern blot analysis that specific clusters of *KLK* genes (*KLK5–13* and

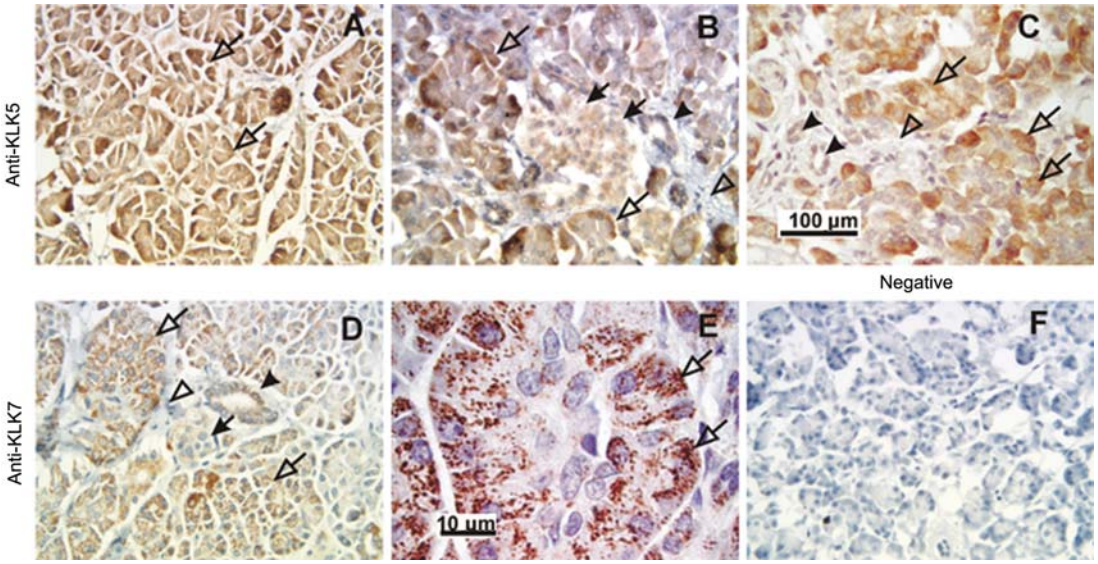

Figure 6 Localisation of KLK5 and KLK7 in human pancreas tissues by immunohistochemical staining.
KLK5 expression was detected with the anti-active-KLK5 antibody and KLK7 expression was detected with anti-recombinant KLK7 antibody as described in the materials and methods section. (A) Acinar cells with cytoplasmic KLK5 staining (open arrows). (B) Cytoplasmic staining of KLK5 in acinar cells (open arrows), but faint staining of KLK5 in the islet of Langerhans (black arrows), a small duct (arrow head) and stromal tissue (open arrowhead). (C) Another tissue section showing KLK5 expression in acinar cells (open arrows), with faint staining of small ducts (arrowheads) and stromal tissue (open arrowhead). (D) Granular cytoplasmic KLK7 staining in acinar cells (open arrows) and the ductal epithelium (arrowhead), faint staining of islet of Langerhans (arrow), but no staining on the surrounding stromal tissue (open arrowhead). (E) Another tissue section displaying secretory granular cytoplasmic staining (open arrows). (F) Negative control (10% normal goat serum instead of primary antibody) showing no staining. Panels (A–D) and (F) have the same magnification as indicated in panel (C).

KLK1:KLK15) display a common pancreatic expression. Although the size of the pancreatic transcripts (1.0 kb) was generally comparable for all these genes, several different transcript lengths were observed in other tissues. *KLK5* and *KLK7* transcripts from the pancreas were also compared with transcripts from keratinocyte and ovarian cancer cell lines. Consistent with abundant expression only in pancreas, a number of consensus pancreatic-specific transcription factor binding sites were detected in the *KLK5* and *KLK7* promoters. We have additionally shown for the first time that the KLK5 and KLK7 proteins are expressed in pancreatic tissues by acinar cells, suggesting a specific role in these cells.

The expression patterns of the *KLK* gene family have been extensively examined using the sensitive RT-PCR and ELISA methods with varying degrees of expression reported for all 15 family members in a wide range of tissues and cell lines (Gan et al., 2000; Harvey et al., 2000; Borgono et al., 2004; Clements et al., 2004; Shaw and Diamandis, 2007). However, it has also been reported that Northern blot and mRNA dot blot analyses indicated that abundant expression levels for the *KLKs* is restricted to a limited number of tissues (Harvey et al., 2000; Clements et al., 2004). Our present Northern blot analysis data show a similar restricted expression profile for several *KLKs* (*KLK5–KLK13*, *KLK15*) and in particular in the pancreas. The prostate is also a major site of expression for *KLK4*, *KLK6*, *KLK8*, *KLK10*, *KLK11*, *KLK14* and *KLK15*. High expression for *KLK5*, *KLK10* and *KLK11* was also observed in testis, for *KLK10* in ovary, *KLK6* in heart and *KLK10* in thymus. The expression of these and other *KLKs* in various human tissues and associations with human diseases, including cancer have been well reviewed (Borgono et al., 2004; Clements et al., 2004). These results suggest two major clusters of *KLK* abundant expression in the pancreas and prostate, implying the function of KLK enzymes in these normal glands, as well as potentially tissue associated pathological conditions, such as diabetes and cancer. Furthermore, high expression of *KLK5*/KLK5 and *KLK7*/KLK7 has also been found in ovarian cancer (Tanimoto et al., 1999; Yousef et al., 2002; Dong et al., 2003), suggesting that these two enzymes may play roles in cancer progression, perhaps by enabling shedding of cancer cells into the peritoneum and facilitating local invasion.

Our data also add weight to the proposition that the *KLK* locus has arisen as a result of gene duplication. Indeed, intriguingly, a recent phylogenetic study of the *KLK* family suggested that gene duplication occurred more randomly than previously proposed and links *KLK8* most closely with *KLK1* (Elliott et al., 2006). *KLK9*, *KLK11* and *KLK15* are linked in one tree, *KLK10* and *KLK12* are suggested as duplicates of *KLK9* and *KLK11*, and *KLK7* and *KLK13* as sister taxa (Elliott et al., 2006). *KLK4*, *KLK5* and *KLK14* are aligned on one tree, with *KLK4* and *KLK5* suggested as direct duplicates, while *KLK6* is thought to be the gene which diverged first. The expression profiles of these genes may also be useful indicators of sister taxa relationships, depending on the extent to which gene flanking sequences that harbour expression regulatory elements have also been duplicated. In this case, our observation of high expression of *KLK8* in pancreas, and to a lesser extent in prostate, would support linkage to the classical pancreatic *KLK1* gene and the prostate-restricted *KLK2* and *KLK3* genes. Similarly, *KLK15* and *KLK11* have identical expression profiles (pancreas,

prostate and testis) as do *KLK7* and *KLK13* (pancreatic-restricted). Less clear is the suggested duplication of *KLK4* and *KLK5*, given the prostate restricted expression of *KLK4*, yet strong pancreatic and testicular expression of *KLK5*, but no prostatic expression. Nonetheless, the inclusion of expression profiles when attempting to determine which genes are sister taxa and the result of gene duplication may help unravel the precise evolutionary events that occurred.

The *KLK* family of 15 genes is a multi-transcript rich family. Multiple transcripts arising from alternate splicing retained intronic segments, exon deletion or utilisation of alternate TISs have all been described (Kurlender et al., 2005; Tan et al., 2006). To date, more than 80 transcripts have been described (Kurlender et al., 2005; Tan et al., 2006), with at least two transcripts (*KLK10* and *KLK14*) (Hooper et al., 2001; Kurlender et al., 2005; Tan et al., 2006) for each gene and up to 11 variants for the PSA/*KLK3* gene (as reviewed by Kurlender et al., 2005; Tan et al., 2006). Indeed, the multiple bands of different sizes observed from our present Northern blot data (Figure 1) clearly reflect these findings. For instance, there are at least four *KLK6* transcripts expressed in prostate and three *KLK9* transcripts in pancreas. Of interest, the size of the pancreatic transcripts for all *KLK* genes expressed in this tissue (*KLK5*, *KLK6*, *KLK8*, *KLK10* and *KLK11*) is smaller than that observed in other tissues, but comparable to that reported for *KLK1*, suggesting that there is specific transcriptional machinery utilised for all these genes when expressed in the pancreas.

The *KLK7* transcript identified by RLM-RACE from pancreas here further confirmed expression of this gene in the pancreas and tissue-specific differences from that expressed in skin or ovary. However, as the only difference is in the 5′-UTR with no alteration in coding region, these *KLK7* transcripts would encode the same full-length KLK7 enzyme, suggesting its potential serine protease function in pancreas as well as skin and ovary. Similar tissue specific transcripts with varying 5′-UTRs have been previously reported for *KLK11* between prostate and brain tissues (Mitsui et al., 2000). We and others have previously reported that distinct forms of *KLK3*, *KLK4*, *KLK5*, *KLK7*, and *KLK11*, each the result of alternate mRNA splicing, are expressed in malignant tumour cells (reviewed in Tan et al., 2006). In the case of *KLK5*, the presence of different 5′-UTRs in ovarian cancer cells indicates selective use of different promoters in a tumour-specific fashion (Dong et al., 2003). Another unique 5′-UTR in a *KLK6* transcript was recently found to be expressed in demyelination and inflammatory diseases of the central nervous system (Christophi et al., 2004). Taken together, these findings suggest that tissue- or disease-specific transcripts use alternate promoters and that these regions likely harbour tissue-specific elements directing their expression.

Indeed, our *in silico* analyses revealed that consensus transcription factor binding sites involved in the regulation of the endocrine and exocrine pancreas, such as PBX and MEIS (Swift et al., 1998), are localised in the 5′-flanking region of both *KLK5* and *KLK7*. Interestingly, in the *KLK7* promoter, we found a putative binding site for the crucial developmental regulator PDX1 (Hale et al., 2005), which in other genes functions by binding the co-regulators PBX and MEIS. An example is the elastase 1 gene (*ELA1*), where the PDX1/PBX1/MEIS2 complex binds to enhancer components of the gene to direct its acinar-specific expression (Swift et al., 1998). Furthermore, PBX1 and PDX1 are required not only for early pancreatic development but also for the genesis of acinar tissue, the compartment of the pancreas that produces digestive enzymes (Kim and MacDonald, 2002; Muharram et al., 2005). Of note, MEIS1, not MEIS2 consensus elements were found in both *KLK5* and *KLK7* promoter regions, and therefore this homologue may play a similar role (Geerts et al., 2005), although its function and that of these other pancreatic transcription factors in *KLK* gene regulation needs to be experimentally confirmed.

Consistent with these findings, our immunohistochemistry (IHC) staining showed that the expression of KLK5 and KLK7 was predominantly localised in the acini of the exocrine pancreas, with less in islets of Langerhans. These data suggest the involvement of KLK5 and KLK7 in the digestive function of the pancreas, perhaps in the activation of other secreted enzymes in the digestive pathway (Borgono and Diamandis, 2004; Clements et al., 2004). Indeed, the KLK7 staining pattern was indicative of secretory granules in the acini, which function to secrete the digestive enzymes (Wasle and Edwardson, 2002). In contrast, KLK5 showed predominant uniform staining on acinar cells with faint staining throughout the rest of the cells, suggesting a different cellular function. Furthermore, a protective role in the pancreas is possible for KLK5, as this enzyme is a potential activator for protease activated receptors 1 and 2 (Oikonomopoulou et al., 2006), which play important roles in protection against pancreatitis by stimulating exocrine secretion (Singh et al., 2007). However, direct or indirect roles of KLKs in exocrine or endocrine pancreas function remain to be investigated. Of note, KLK5 can activate KLK7 in skin and both are involved in the degradation of extra cellular matrix and play important roles in the desquamation of skin (Brattsand et al., 2005). Whether these proteases will have similar roles in pancreatic matrix remodelling or simply act as activators of other pancreatic proteins is yet to be established.

KLK1, also the first-named pancreatic kallikrein, has been described in the β-cells of the pancreas (ole-MoiYoi et al., 1979), but also has a strong exocrine cell localisation (Chao et al., 1980). Interestingly, KLK6, KLK10 and KLK13 were reported to be localised to the islets of Langerhans (Petraki et al., 2002, 2003), suggesting different roles from KLK5 or KLK7 and providing further evidence of the diverse functions of these proteases. KLK6 and KLK10 were also detected in endocrine-related neoplasms, including tumours producing glucagon or somatostatin, and foci of endocrine dysplasia (Petraki et al., 2002). It would also be interesting to examine the expression of KLK5 and KLK7 in exocrine- or endocrine-derived pancreatic tumours. The different localisation observed between the above kallikrein-related peptidases may result from the regulation of exocrine and endocrine expression by similar promoter specific elements. Thus, the potential transcription factor binding sites that we identified in the *KLK5* and *KLK7* promoters may effect acinar cell expression in the pancreas. Furthermore, promoter regions of other *KLK* genes remain to be invest-

igated for the presence of motifs directing endocrine and exocrine expression in the pancreas.

We have demonstrated strong expression of *KLK5* and *KLK7* mRNA and protein in normal pancreas. In contrast, a recent report did not detect *KLK5* or *KLK7* mRNA or protein in this tissue (Shaw and Diamandis, 2007), while another report detected only low levels of *KLK7* mRNA and protein in normal pancreas (Johnson et al., 2007). These differences are likely due to variations in the employed approaches. Of particular relevance, in contrast to our TIS identification, 3′-RACE and Northern blot analyses, which employed pancreatic poly(A)$^+$ RNA, Shaw and Diamandis (2007) and Johnson et al. (2007) used total RNA in their respective semi-quantitative RT-PCR protocols. Furthermore, although different primer sets were used by each research group, this is unlikely to explain the differences in observed expression levels, as overlapping sequences were amplified by all three groups. In addition, the protocol employed by us to analyse KLK5 and KLK7 protein levels in pancreas differed from those employed by Johnson et al. (2007) and Shaw and Diamandis (2007). Although the former group also used IHC to detect KLK7 in pancreas, these authors employed citric acid antigen retrieval, whereas we used urea in this step of the protocol. In contrast, Shaw and Diamandis (2007) used capture ELISA protocols to analyse the levels of kallikreins in embryonic and adult tissue extracts. It is not clear why this group failed to detect KLK5 and KLK7 in pancreas extracts. However, it is interesting to note that the goat anti-KLK7 antibody used by Johnson et al. (2007) was generated against the same antigen (recombinant KLK7 residues 23–252) as the monoclonal antibody used by Shaw and Diamandis (2007); although, the goat antibody detected KLK7 in pancreas using IHC, the monoclonal antibody failed to detect KLK7 in pancreas tissue.

In summary, using Northern blot analysis, we have demonstrated a common and high expression of *KLK5–13* and *KLK15* (which is contiguous to the first described pancreatic *KLK1* gene) in the pancreas. These data provide further evidence supporting the gene duplication evolution of this family and suggest common regulatory elements or locus control regions that confer tissue-specific expression. We have also identified specific pancreatic *KLK5* and *KLK7* transcripts which, in comparison with those in skin (HaCat) and ovarian (OVCAR-3) cell lines show the presence of tissue-specific TIS and 5′-UTR sequences. We have shown the localisation of KLK5 and KLK7 in the acinar cells of pancreatic tissues consistent with our *in silico* analysis, which located several potential pancreatic exocrine regulatory elements in the promoter regions of these genes. This study has provided the basis for further investigation into the molecular mechanisms underlying *KLK5* and *KLK7* regulation in different tissues and especially in the pancreas.

Materials and methods

Cell lines, RNA and tissue samples

Both the keratinocyte cell line HaCat and ovarian cancer cell line OVCAR-3 were from the American Tissue Culture Collection (ATCC, Manassas, VA, USA). All cell lines were cultured in RPMI-1640 medium (Invitrogen, Mount Waverley, Australia) with 10% foetal calf serum supplemented with 1% penicillin and streptomycin (CSL Biosciences, Parkville, Australia) and incubated at 37°C with 5% CO_2. Poly(A)$^+$ RNA was purchased as a pooled sampled from pancreatic tissues of 15 males and females who died suddenly (Clontech, Mountain View, CA, USA). Mycoplasma testing for cultured cells was performed every 4–6 weeks using a Mycoplasma Detection kit (Roche, Brisbane, Australia).

Northern blot analysis

Human multiple tissue Northern blots containing poly(A)$^+$ RNA were purchased (Clontech) and hybridised as described previously (Harvey et al., 2000).

Transcription initiation site and cDNA 3′-end identification

Transcription initiation site identification was performed by 5′-RNA ligase mediated rapid amplification of cDNA ends (RLM-RACE) using a FirstChoice™ RLM-RACE kit (Ambion, Austin, TX, USA), according to the manufacturer's instructions. Briefly, after treatment of calf intestinal phosphatase and tobacco acid pyrophosphatase (TAP), an RNA oligonucleotide was ligated to full-length, decapped mRNAs, and then reverse transcription was performed using random primers supplied from the kit. Using this approach, incomplete mRNAs are eliminated using alkaline phosphatase and the mRNA cap structure is removed using TAP to ensure only full-length transcripts are amplified (Maruyama and Sugano, 1994). PCR was performed using the 5′-outer primer from the RLM-RACE kit and a KLK5 (pancreas, 5′-GAG AAC ATG CTC TGT GAC CCC C-3′; HaCat and OVCAR-3, 5′-AGA CGG ACT CTG AAA ACT TTC TT-3′) or KLK7 specific primer (pancreas, 5′-ATG AGG TCA TTA ACA TGG GTC T-3′; HaCat and OVCAR-3, 5′-CAA AGG CTA AGG ATA GCA GTA AGA TCT-3′). A second round of PCR was then performed using the 5′-inner primer from the kit and a nested KLK5 (pancreas, 5′-TCT GAT CAC AGC CTT GCT TCT GGG GGT CA-3′; HaCat and OVCAR-3, 5′-GAG CTG GGG CCG GGG AAG AC-3′) or KLK7 specific primer (pancreas, 5′-AGA TCT TAC TGC TAT CCT TAG CCT TG-3′; HaCat, 5′-CAT TAA CAT GGG TCT GTG TGG AGT AGC C-3′; OVCAR-3, 5′-CAG TGG AGC TGA TTG CCA CTG A-3′). Amplification conditions were 35 or 45 cycles for 1 min with annealing temperatures of 55°C for the first round and 62°C for the second round PCR. For cDNA 3′-end identification, 3′-RACE was employed using the universal 3′-UTR primer (Promega, Madison, WI, USA) followed by PCR using 1 μl of the cDNA reaction and KLK5- or KLK7-specific primers as described previously (Dong et al., 2003).

In silico analysis of KLK genes using serial analysis gene expression (SAGE; SAGEmap) and expression sequence tag (EST) databases

The 15 human KLK mRNA sequences were used to identify unique sequence tags of UniGene clusters for each *KLK*. The respective GenBank entries for *KLK1–15* were M33105, M18157, M27274, AF148532, NM_012427, AF013988, L33404, AB009849, AF135026, BC002710/NM_002776, AB012917, NM_145894, BC069334, AF283669 and NM_023006. The sequence tags anchored by restriction digestion enzymes NlaIII and Sau3A were used to determine the expression of different *KLKs* in pancreatic libraries (3 from normal and 6 from cancer) and a list of all libraries were found at http://www.ncbi.nlm.nlh.gov/geo/gds/.

The full-length mRNA sequence of each KLK was also compared against the human EST databases of the National Centre for Biotechnology Information (NCBI). At the time of the study, these databases contained 8 normal and 10 carcinoma pancre-

atic libraries. Expression was determined by identification of positive clones with more than 95% homology to each *KLK* in these libraries.

DNA sequence analysis of EST clones and PCR products

DNA of two KLK pancreatic EST clones (GenBank AA101043 and AA101044; Invitrogen) with >95% homology to *KLK7* (GenBank: NM_005046) was sequenced at the Australian Genome Research Facility (Brisbane, Australia) and analysed using tBLASTN (www.ncbi.nlm.nih.gov/blast).

Identification of consensus transcription factor binding sites

The sequence upstream of the TIS for both *KLK5* (GenBank: AF135028) and *KLK7* (GenBank: AF166330) was analysed for transcription factor binding sites using the MatInspector program (www.gene-regulation.com/cgi-bin/pub/programs/tfblast/tfblast.cgi), and confirmed using the rVista2.0 algorithm (www.dcode.org).

Immunohistochemistry (IHC)

Institutional ethics committee approval was obtained for the use of normal pancreatic tissues collected at autopsy. Six paraffin-embedded pancreatic tissue blocks were sectioned (4 μm), deparaffinised and rehydrated. IHC was performed as described previously (Dong et al., 2003), using an affinity purified anti-KLK5 peptide antibody (3 μg/ml) (Dong et al., 2003) or an affinity purified anti-recombinant KLK7 antibody (3 μg/ml) (Ekholm and Egelrud, 1998; Ekholm et al., 2000; Dong et al., 2003). Antigen retrieval was performed by microwave heat treatment in 5% urea in 0.1 M Tris buffer (pH 9.5). Normal goat serum (10%) instead of primary antibody was used for negative controls.

Acknowledgements

This work was supported by the National Health and Medical Research Council of Australia (Project grants 101202, 242220 and 390123 for J.A.C. and Y.D.; R.D. Wright Fellowship 339732 for J.D.H.) and the Queensland University of Technology (N.M.). We thank Dr. Maria Brattsand from Umeå University, Umeå, Sweden, for generous gifts of anti-KLK5 and anti-KLK7 antibodies.

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Received June 20, 2007; accepted October 12, 2007