ISOLATION, CHARACTERIZATION, MAPPING AND EXPRESSION ANALYSIS OF PORCINE TISSUE KALLIKREINS

By

SAMODHA CHARAKA FERNANDO

Bachelor of Science

University of Kelaniya

Sri Lanka

2002

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
December, 2004

ISOLATION, CHARACTERIZATION,

MAPPING AND EXPRESSION

ANALYSIS OF

PORCINE TISSUE

KALLIKREINS

Thesis Approved:

Dr. Udaya DeSilva

Thesis Adviser

Dr. Rodney Geisert

Dr. David S. Buchanan

Dr. Gordon Emslie

Dean of the Graduate College

Acknowledgements

The time I spent at Oklahoma State University has been a learning experience for me as a person and as a professional. I consider it a privilege to acknowledge many people who have encouraged, inspired, and guided me throughout my stay at the Oklahoma State University.

First, I would like to extend my sincere thanks to my adviser Dr. Udaya DeSilva for giving me this opportunity to further my education. Dr. Desilva you have been an inspiration to me. Thank you for all the advice, guidance and encouragement. I would also like to thank my thesis committee members, Dr. Rodney Geisert and Dr. David S Buchanan for all the guidance, advice, and motivation given to me.

I am thankful to Dr. Bruce Roe and his lab for helping with sequencing the BAC clone, Dr. Eric Green and Shish-Queen Lee Lin for helping with the BAC fingerprinting and the Porcine Genome consortium for all their help.

I would also like to extend a special thanks to Xiaoping Guo and Asitha Vasudevan pillai for all their help, friendship and advice given to me during my day-to-day activities in the lab.

I also wish to thank the Faculty and Staff of the Department of Animal Science for making my learning experience more memorable.

Finally, I wish to thank the people who are most responsible for my achievements, my parents, Ajantha and Leslie Fernando for providing me with the basis from which to

succeed, I extend my sincere thanks and love to you both. I also thank my brothers Sudham and Dhanushka for all the help and support given to me. Thanks guys.

Now, I thank the most wonderful person in my life, my wife Anju. Thank you for the love, support and encouragement; you have been a tower of strength to me. Thank you.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figure Page

CHAPTER I

Literature Review

Introduction

Since domestication of pigs over 5000 years ago in China (Darwin 1868), the modern day domestic pig has come a long way. Today, there are over 350 breeds of domestic pigs in more than 180 countries (United Nations Environmental Program: http://www.unep.org/). During the last three decades many modern reproductive technologies in research such as gamete and embryo preservation, in-vitro fertilization, culturing and manipulation of embryos, and embryo transfer has boosted the swine industry and helped increase it's productivity (Rothschild and Bidanel 1998). From an economic stand point; increasing productivity by manipulation of embryos is not desirable. Even though selection for reproductive traits has increased conception rates, 35%-45% of the embryos are lost during the first 2-3 week of pregnancy (Pope and First 1985). Therefore, one of the primary objectives in the swine industry today is increasing litter size by overcoming early embryonic loss during the first 2 – 3 weeks of pregnancy. By increasing litter size the incremental cost related to the production of additional pigs are minimal, thus substantial gains can be achieved.

This literature review presents the mapping and expression patterns of human, rat, mouse, and pig tissue kallikrein genes. First, the literature review describes what kallikreins are and presents the organization and function of tissue kallikreins in humans and other animals. Then presents the tissue expression of kallikrein genes, with special emphasis on the kallikreins expressed in reproductive tissue.

Development in the Pig

During early embryonic development in the pig, initial control of the conceptus occurs through maternally derived messages and transcripts until the late four-cell stage of development. At the four-cell stage, the embryo takes over development by synthesizing its own RNA transcripts while slowly degrading the maternal messages (Tomanek et al. 1989). By day 10, the embryo forms a blastocyst and starts a unique cellular remodeling process leading to conceptus expansion and elongation. At this time the conceptus secretes estrogen as the maternal recognition signal of pregnancy and attaches to the uterine epithelium forming an epitheliochorial placenta (Geisert et al. 1982a,b & 1997). Throughout the 114 day gestation period in the pig, the greatest embryonic loss occurs prior to day 25 of pregnancy (Pope and First 1985). This period of embryonic development corresponds to critical events involving, conceptus intrauterine migration, trophoblastic elongation, establishment of pregnancy, and placental attachment (Geisert et al. 1990).

Unfortunately, little information exists regarding the identity of the gene products and the genetic and physiological mechanisms underlying early embryonic development in the pig. Understanding and identifying the expression of genes involved with cellular growth, differentiation, immuno-protection, and morphological change during conceptus development and placental attachment will provide an opportunity to regulate embryo development and to possibly improve embryonic survival in the pig.

Gene Maps in the Pig

Over the past decade an increasing interest in mapping genes of economic importance in farm animals has led to the construction of a fairly dense genetic and physical maps. Presently, the porcine genome is among the most well studied farm animal genomes and ranks high among mammalian gene maps available today.

Pig chromosomes generate a special interest to many farm animal cytogeneticists as having relatively fewer chromosomes (2n=38) when compared with other farm animal species such as cattle (2n=60), sheep (2n=54), goat (2n=60), horse (2n=64), and chicken (2n=78) (Chowdhary 1998). Interest in pig cytogenetics has led to unprecedented progress in the physical mapping in the pig during the past 3-4 years enabling broader understanding of the genome. During the 1990s concerted efforts have helped develop comprehensive linkage maps of the porcine genome with contribution from, PiGMaP consortium, Nordic collaboration, and the USDA Meat Animal Research Center. These combined efforts have placed more than 1500 polymorphic genetic markers on the porcine linkage map. These resources have helped animal geneticists and breeders to better understand the genetic control of traits critical to agricultural performance such as growth, litter size and disease resistance and also to identify individual genes with effects on reproductive traits (Chowdhary 1998).

With the genetic and physical maps showing an almost uniform coverage of the porcine genome (Chowdhary 1998) attention is now shifting towards further development of gene maps to answer problems of economic significance.

What are Kallikreins?

The term "kallikrein" was first introduced in the 1930's by Werle and colleagues who found that high concentrations of kallikrein in the pancreas (in Greek, the "kallikreas") (Yousef and Diamandis 2001, 2003). Since then, the interest in the kallikrein gene family has increased tremendously. During the early 1980s extensive research on tissue kallikrein gene families of humans and rodents were carried out (Evans et al. 1987), these studies have helped us to better understand the structural and functional similarities among tissue kallikrein gene family members (See Table 1).

Table 1. Structural similarities among human kallikrein gene family members

Structural similarity	Reference
All kallikrein genes code for a putative serine proteases with conserved catalytic triad (Histidine, Aspartic acid and Serine in appropriate positions)	(Yousef and Diamandis 2001)
All kallikrein genes have five coding exons (some members may contain one or more 5'-untranslated exons)	(Yousef and Diamandis 2001)
The intron-exons boundaries are conserved with the exception of human KLK10	(Yousef and Diamandis 2001)
All genes show 40-80% sequence homologies at the DNA and amino acid level	(Wines et al. 1989; Yousef and Diamandis 2001),
Several kallikreins are regulated by steroid hormones	(Yousef and Diamandis 2001)
Coding exon sizes are similar or identical	(Yousef and Diamandis 2001)

Kallikreins are members of a multigene family of serine proteases that are widespread throughout living organisms (Gan et al. 2000). They show high degree of substrate specificity and have highly diverse physiological functions (Evans et al. 1987).

Kallikreins are seen expressed in various tissues and biological fluids (Southardsmith et al. 1994; Yousef and Diamandis 2001, 2003) and are involved in functions such as fertilization, digestion, regulation of blood flow, blood coagulation, inflammatory responses, endothelial cell migration, leukocyte aggregation, tissue remodeling, tumor-cell invasion and programmed cell death as well as disease related specialized enzymatic activities (Southardsmith et al. 1994; Gan et al. 2000; Cassim et al. 2002; Yousef and Diamandis 2003).

The term "tissue kallikrein" is not restricted to describe enzymes that release kinins (bioactive peptides) from precursor molecules, but is used to describe a group of enzymes that show high degree of conservation at both gene and protein structure level, and co-localize or organize into the same chromosomal locus (Evans et al. 1987; Yousef and Diamandis 2001 & 2003). Therefore, the term "kallikrein" does not necessarily indicate that all family members have the kininogenase activity (Yousef and Diamandis 2003). Kallikreins demonstrate similarity at gene and protein level and to some extent at the tertiary structure level. Based on the conservation of the primary and tertiary structures and the enzymatic activities among the serine proteases, it is hypothesized that all known serine proteases arose from a common ancestor through gene and/or chromosomal duplication during the course of evolution, which may have caused the serine protease gene family to be clustered in the genome (Gan et al. 2000; Yousef and Diamandis 2001). The relatively similar sizes of rodent kallikrein gene families suggest that these two families of serine proteases may have amplified in the rodent lineage before the divergence of rat and the mouse. While the differences in numbers of gene family

members among rodents may be due to close linkage of the family, allowing expansion and contraction of the gene families leading to unequal crossing over (Wines et al. 1991). Comparisons of the kallikrein family members among species reveal that there is a greater level of conservation within species than among orthologous genes across species, which suggests that recombination has occurred within a species leading to concerted evolution (Wines et al. 1991). Nucleotide differences among kallikreins within a species generally exist in single or double nucleotide substitutions, insertions or deletions, but in the coding regions, these differences are mostly clustered in short segments of 20bp - 30bp in length that accommodate up to 12 nucleotide differences in pair-wise comparison (Southardsmith et al. 1994).

Presently the kallikrein gene family is divided into two major subgroups of kallikreins, plasma kallikrein which includes just one family member and a larger group of kallikreins called the tissue or glandular kallikreins (Gan et al. 2000; Yousef and Diamandis 2001 & 2003; Cassim et al, 2002). Plasma and tissue kallikreins differ from each other with respect to their molecular weight, isoelectric point, substrate specificity, immunological profile, gene structure, expression pattern and type of bioactive peptides released (Cassim et al. 2002; Yousef and Diamandis 2001, 2003).

Plasma kallikrein

Plasma kallikrein is secreted by the liver as plasma prekallikrein. In humans, plasma kallikrein is encoded by a single gene located on human chromosome 4q34-q35 (Cassim et al. 2002; Yu et al. 1998) that is ~22 kb in length (Beaubien et al. 1991). The gene coding for plasma kallikrein is composed of 15 exons (Yousef and Diamandis 2001;

Cassim et al. 2002) and encodes an enzyme that releases the nano-peptide bradykinin upon acting on high molecular weight kininogen (HMWK) (Bhoola et al. 1992; Cassim et al. 2002).

Plasma prekallikrein is a single chain glycoprotein that is released into the circulatory system (Cassim et al. 2002). This single chain glycoprotein is found in two forms in plasma, as 85 kDa and 88 kDa peptide fragments (Hojima et al. 1985; Veloso and Colman 1991). This inactive plasma prekallikrein forms a heterodimer with factor XI to its substrate high molecular weight kininogen (Cassim et al. 2002). Following vascular damage, HMW kininogen chaperones preplasma kallikrein and Factor XI towards factor XII (Hageman factor). Subsequently, Factor XII activates preplasma kallikrein to its activated form of plasma kallikrein. Activated plasma kallikrein hydrolyses the Lys-Arg and Arg-Ser bonds in high molecular weight kininogen to produce the nano-peptide bradykinin (Bhoola et al. 1992; Cassim et al. 2002).

Tissue kallikreins

Tissue kallikreins are acidic glycoproteins with isoelectric points ranging from 3.5 to 4.4 (Cassim et al. 2002). Their molecular weights range from 25 kDa to 40 kDa (Evans et al. 1987). Kallikreins show different substrate specificities (Evans et al. 1987). Tissue kallikrein 1, which contains the true kininogenase activity, hydrolyzes low molecular weight kininogen (LMWK) at the Met-Lys bond at the amino terminal and the Arg-Ser bond at the carboxy terminal to release the deca-peptide Lys-bradykinin (Kallidin) (Fiedler 1979; Cassim et al. 2002). Later, this deca-peptide Lys-bradykinin is converted into bradykinin by amino peptidase (Guimaraes et al 1973).Although LMW-kininogen is

the preferred substrate of tissue kallikrein, it can also cleave HMWK to give rise to kinin (Carretero and Scicli 1980).

Kallikrein gene families in humans and other animals

Human Kallikrein Gene Family

The human kallikrein gene family was first discovered in 1980s. At the time it was believed that the human kallikrein gene family was composed of only 3 members, KLK1 encoding pancreatic/renal kallikrein (Evans et al. 1988), KLK2 encoding human prostate glandular kallikrein 2 and KLK3 encoding PSA (Prostate Specific Antigen) (Riegman et al. 1989; Yousef and Diamandis 2001; Gan et al. 2000).

Since then extensive research has been carried out on the kallikrein gene family with emphasis on the tissue expression of kallikrein 2 (KLK2) and kallikrein 3 (KLK3), which shows potential as candidate biomarkers for tumors and other prostatic diseases (Rittenhouse et al. 1998). Currently 15 kallikrein genes have been identified from diverse tissues, which co-localize to the same kallikrein gene locus on chromosome 19q13.3 – q13.4 (Yousef and Diamandis 2001). These newly identified kallikrein genes share high degree of structural similarity. Several of these genes appear to be tumor suppressor genes, whereas others are implicated to breast, ovarian and other human cancers (Yousef and Diamandis 2001).

Sequence similarity studies carried out among classical kallikreins (KLK1, KLK2, and KLK3) show high similarity (~80%) at protein level, whereas the newly identified kallikreins only share 30-50% similarity with the classical kallikreins. Furthermore, the distinct “kallikrein loop” that is present in the classical kallikreins is not found in the newly identified non-classical human kallikreins (Yousef and Diamandis 2003). Differences among human kallikrein gene family members together with the fact that only KLK2 and KLK3 of the human kallikrein family does not have any rodent orthologs

(Figure 3) suggests that, the newly identified kallikrein genes in the human may have diverged after the rodent-primate split between 65 and 85 million years ago (Yousef and Diamandis 2003). It is believed that the human kallikrein gene family arose due to gene duplication and exon shuffling which might have occurred during unequal crossing over of sister chromatids in meiotic recombination (Yousef and Diamandis 2003).

The human kallikrein gene family can be divided into two main evolutionary families, the trypsin-like serine proteases which may have evolved from a single ancestral gene through gene duplication to give rise to other genes that gradually mutated with time to produce related proteases and protease subfamilies with new functions, and the subtilisin-like pro-protein convertases, which are assumed to be a product of convergent evolution (Yousef and Diamandis 2001). Trypsin-like serine proteases have an aspartic acid residue in their binding pocket, which form strong electrostatic bonds with arginine and lysine residues. Therefore, this aspartic acid residue is partly responsible for the diverse substrate specificity among kallikreins. Among the 15 known human kallikreins, 11 have aspartic acid in the binding pocket indicating that these kallikreins may have trypsin-like activity. The remaining four kallikreins, KLK3 (serine), KlK7 (asparagines), KLK9 (glycine), and KLK15 (glutamic acid) are expected to have chymotrypsin-like or other specific activity (Yousef and Diamandis 2001).

Table 2. Classification of kallikreins based on functional similarity. The rat, mouse and human tissue kallikrein gene family members are grouped based on their functional similarity (Evens et al. 1987; Wines et al. 1989; Yousef and Diamandis 2001).

Mouse kallikreins	Rat kallikreins	Human Kallikreins	FUNCTION
mGK6	rKLK1 or rGK1	KLK1	True kininogenase activity (cleavage of LMW-kininogen to Lys-bradykinin
	rKLK2 or rGK2		Tonin- converts angiotensinogen to angiotensin
mGK3			Nerve growth factor binding and processing enzyme
mGK4			Nerve growth factor binding and processing enzyme
	rGK10		Kininogenase activity which cleaves T-kininogen to T-kinin
mGK9			Epidermal growth factor binding protein
mGK13			Epidermal growth factor binding protein
mGK16			γ- renin
mGK22			Nerve growth factor-inactivating enzyme
mGK26			Prorenin-converting enzyme-2
mGK1,mGK11 mGK14,mGK21, mGK23,mGK25	rGK3,rGK4, rGK5,rGK6, rGK7,rGK8	KLK2,KLK3, KLK4,KLK5, KLK6,KLK7, KLK8,KLK9, KLK10,KLK11, KLK12,KLK13, KLK14,KLK15	Unknown
mGK2,mGK7, mGK10,mGK12, mGK15,mGK17, mGK18,mGK19, mGK23,mGK25			Pseudogenes

Gene Organization of human kallikreins

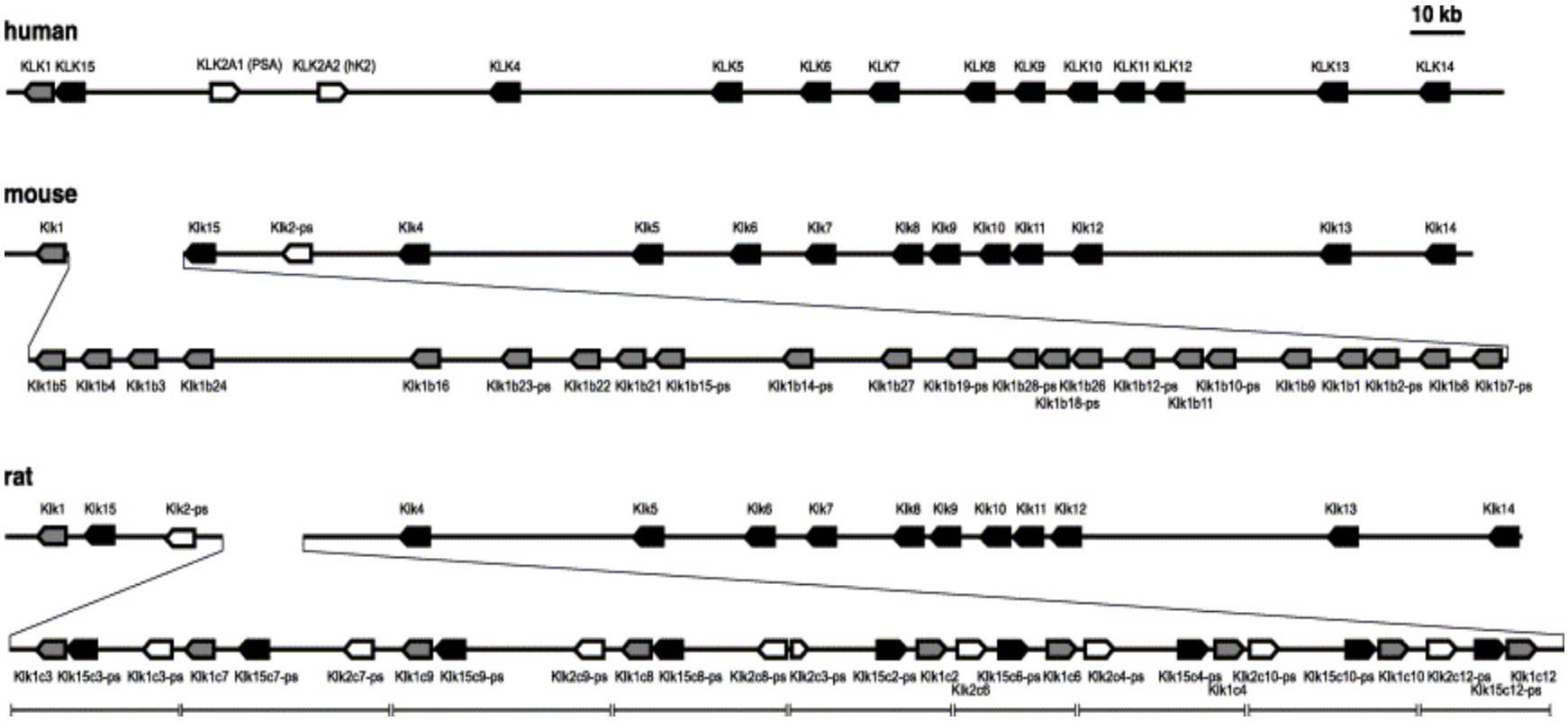
The sequence information generated around chromosome 19q13.3-q13.4 from the human genome project has allowed precise localization of the human kallikrein gene family members. This shows that the three classical kallikreins (KLK1, KLK2, and KLK3) are clustered within a 60 kb region whereas the newly discovered kallikrein gene, KLK15, maps in-between KLK1 and KLK2 (Yousef and Diamandis 2003; Diamandis et al. 2004). The remaining kallikreins are localized as shown in figure 1 without any intervention by other genes. The direction of transcription of kallikreins is from telomere to centromere with the exception of KLK2 and KLK3, which are transcribed from centromere to telomere (Yousef and Diamandis 2001).

All known human kallikreins consists of 5 coding exons and show similar organization (Yousef and Diamandis 2001). Unlike classical kallikreins, all other human kallikreins have 1-2 exon 5'-untranslated regions. Similarly, 3'-untranslated region among kallikreins are variable and give rise to the variants of different mRNA lengths. The catalytic triad characteristic to all serine proteases is found in a similar pattern among all kallikrein family members, with the second exon harboring histidine, the third exon harboring aspartic acid and the fifth exon harboring the serine residue. The protein alignment among tissue kallikreins ranges from 40-80% and show the highest similarity at the catalytic amino acids histidine, aspartic acid, and serine (Yousef and Diamandis 2001).

Figure 1

Schematic representation of human, mouse and rat tissue kallikrein gene loci. Arrows represent genes and point towards the direction of transcription. The three classical human glandular kallikrein genes are represented in grey (KLK1) and white (KLK2, KLK3). The newly identified human glandular kallikrein genes are represented in black (KLK4-15). The paralogs of KLK1 are represented in grey, KLK2 paralogs in white and paralogs of KLK4-15 in black. The boundaries of duplicated regions of the rat and mouse are shown at the bottom.

Reproduced from (Olsson et al. 2004).


human
10 kb
KLK1 KLK15
KLK2A1 (PSA)
KLK2A2 (hK2)
KLK4
KLK5
KLK6
KLK7
KLK8
KLK9
KLK10
KLK11
KLK12
KLK13
KLK14
mouse
Klk1
Klk15
Klk2-ps
Klk4
Klk5
Klk6
Klk7
Klk8
Klk9
Klk10
Klk11
Klk12
Klk13
Klk14
Klk1b5
Klk1b4
Klk1b3
Klk1b24
Klk1b16
Klk1b23-ps
Klk1b22
Klk1b21
Klk1b15-ps
Klk1b14-ps
Klk1b27
Klk1b19-ps
Klk1b28-ps
Klk1b18-ps
Klk1b26
Klk1b12-ps
Klk1b11
Klk1b10-ps
Klk1b9
Klk1b1
Klk1b2-ps
Klk1b8
Klk1b7-ps
rat
Klk1
Klk15
Klk2-ps
Klk4
Klk5
Klk6
Klk7
Klk8
Klk9
Klk10
Klk11
Klk12
Klk13
Klk14
Klk1c3
Klk15c3-ps
Klk1c3-ps
Klk1c7
Klk15c7-ps
Klk2c7-ps
Klk1c9
Klk15c9-ps
Klk2c9-ps
Klk1c8
Klk15c8-ps
Klk2c8-ps
Klk2c3-ps
Klk15c2-ps
Klk1c2
Klk2c6
Klk15c6-ps
Klk1c6
Klk2c4-ps
Klk15c4-ps
Klk1c4
Klk2c10-ps
Klk15c10-ps
Klk1c10
Klk2c12-ps
Klk1c12
Klk15c12-ps

Mouse Kallikrein Gene Family

The mouse kallikrein gene family is the largest family of kallikreins known to date. It consists of 24 members among which, 14 encode for active proteins, while the remaining are pseudogenes (Evans et al. 1987; Yousef and Diamandis 2001, 2003). The data generated by the Mouse Genome Project (http://www.ncbi.nlm.nih.gov/genome/guide/mouse) suggests that this number could increase up to 37 family members (Yousef and Diamandis 2003). This large family of serine proteases in the mouse is localized on chromosome 7, in a ~310 kb region corresponding to the Tam-1, Prt-4, and Prt-5 loci (Fig. 2). The human - mouse homology maps at NCBI (http://www.ncbi.nlm.nih.gov/Homology) show that this ~310 kb region of the mouse kallikrein gene family has conserved synteny to human chromosome 19q13.4 which harbors the human kallikrein gene family (Yousef and Diamandis 2003). All described mouse kallikrein genes share high degree of sequence similarity at mRNA and protein level ranging form 70%-90% (Yousef and Diamandis 2003), yet, these family members share a great diversity in their function. Among known mouse kallikrein genes, only one member (mKLK-1 or mGK6), has true kininogenase activity enabling it to cleave LMWK to release bradykinin (Vanleeuwen et al. 1986), while all other known kallikrein family members show, diverse physiological functions (see Table 3).

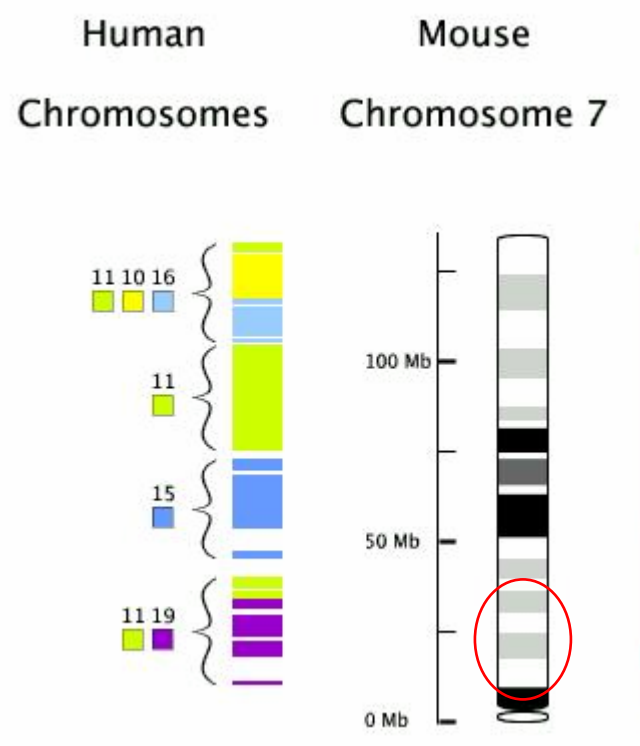
The mouse kallikrein genes are transcribed in the same direction and share the same genomic organization, consisting of five coding exons and four introns, with conserved intron-exon splice sites (Yousef and Diamandis 2003). All these kallikreins code for prekallikreins that are 261 amino acids long which contain a 18-amino acid signal peptide (Yousef and Diamandis 2003).

Table 3. Functions of kallikrein family members in the mouse

GENE	FUNCTION	REFERENCE
mGK1	Unknown	(Evans et al. 1987)
mGK2	Pseudogene	(Evans et al. 1987)
mGK3	Nerve growth factor binding and processing enzyme	(Yousef and Diamandis 2003)
mGK4	Nerve growth factor binding and processing enzyme	(Yousef and Diamandis 2003)
mGK5	Unknown	(Evans et al. 1987)
mGK6	True kininogenase activity (cleavage of LMW-kininogen to Lys-bradykinin	(Yousef and Diamandis 2003), (Vanleeuwen et al. 1986)
mGK7	Pseudogene	(Evans et al. 1987)
mGK8	Unknown	(Evans et al. 1987)
mGK9	Epidermal growth factor binding protein	(Evans et al. 1987)
mGK10	Pseudogene	(Evans et al. 1987)
mGK11	Unknown	(Evans et al. 1987)
mGK12	Pseudogene	(Evans et al. 1987)
mGK13	Epidermal growth factor binding protein	(Yousef and Diamandis 2003)
mGK14	Unknown	(Evans et al. 1987)
mGK15	Pseudogene	(Evans et al. 1987)
mGK16	γ- renin	(Fahnestock et al. 1991)
mGK17	Pseudogene	(Evans et al. 1987)
mGK18	Pseudogene	(Evans et al. 1987)
mGK19	Pseudogene	(Evans et al. 1987)
mGK21	Unknown	(Evans et al. 1987)
mGK22	Nerve growth factor-inactivating enzyme	(Yousef and Diamandis 2003)
mGK23	Pseudogene	(Evans et al. 1987)
mGK24	Unknown	(Evans et al. 1987)
mGK25	Pseudogene	(Evans et al. 1987)
mGK26	Prorenin-converting enzyme-2	(Yousef and Diamandis 2003)

Figure 2

The Human – Mouse homology map of mouse chromosome 7. Mouse chromosome 7 shows conserved synteny to several human chromosomes. Parts of human chromosome 10 (yellow), 11 (green), 15 (dark-blue), 16 (light-blue), and 19 (purple) shows conserved synteny to mouse chromosome 7. Mouse kallikrein gene region is circled in red. Reproduced from www.genboree.org


Human
Chromosomes
Mouse
Chromosome 7
11 10 16
11
15
11 19
100 Mb
50 Mb
0 Mb

Rat Kallikrein Gene Family

The third largest family of kallikreins next to the mouse and the human is the rat kallikrein gene family. This gene family consists of 13 family members, among which 10 code for active genes, while the remaining 3 genes are pseudogenes (Yousef and Diamandis 2003; Southardsmith et al. 1994; Wines et al. 1989). But, the data generated from the Rat Genome Database (http://ratmap.gen.gu.SE/) suggest that the rat kallikrein gene family may be larger (Yousef and Diamandis 2003).

As in the mouse, the rat kallikrein gene family is clustered together within a single 440 kb chromosomal region in two clusters. These two kallikrein gene clusters in the rat are named as the odd gene cluster and the even gene cluster because all the odd kallikreins (rKLK1, rKLK3, rKLK7, rKLK9) are localized in one cluster while all the even kallikreins (rKLK8, rKLK2, rKLK6, rKLK4, rKLK10) are found in one cluster (Southardsmith et al. 1994). The rat –mouse homology map suggests that the rat kallikrein gene family may be clustered in chromosome 1 (Fig 3).

Like in humans and mouse, the rat kallikrein gene family shares high degree of sequence homology (>80%) among family members (Wines et al. 1989; Southardsmith et al. 1994) and codes for active serine proteases which are 261 amino acids in length (see table 2). As in the mouse only one rat kallikrein (rKLK1) has the true kininogenase activity (Yousef and Diamandis 2003; Ashley and Macdonald 1985) while all other rat kallikrein genes are shown to have diverse physiological functions (see table 4).

Figure 3

Mouse –Rat homology map of mouse chromosome 7. Mouse chromosome 7 shows high conserved synteny to rat chromosome 1 (shown in brown) suggesting that the rat kallikrein genes may be clustered in chromosome 1.

Reproduced from www.genboree.org

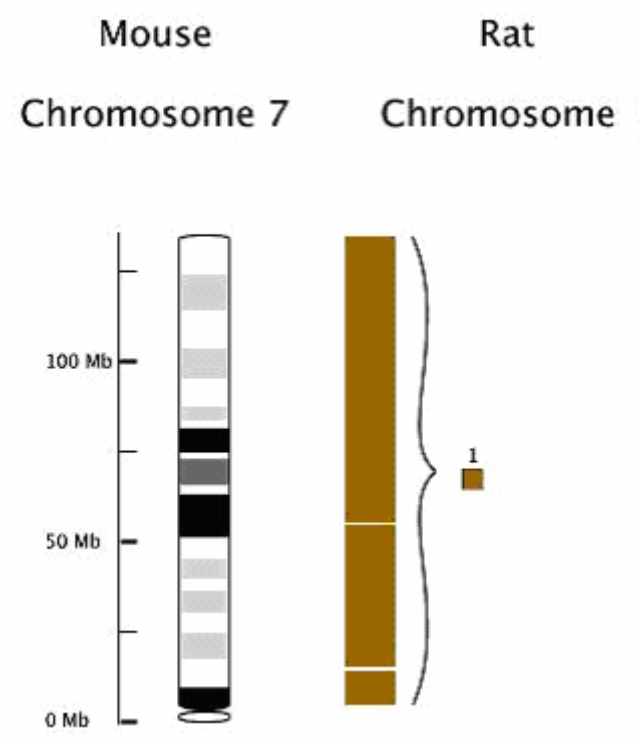

Mouse
Chromosome 7
Rat
Chromosome
100 Mb
50 Mb
0 Mb
1

Table 4. Functions of kallikrein family members in the rat

GENE	FUNCTION	REFERENCE
rKLK1 or rGK1	True kininogenase activity. Also known to be up-regulated by estrogen.	(Yousef and Diamandis 2003)
rKLK2 or rGK2	Tonin- converts angiotensinogen to angiotensin	(Xiong et al. 1990)
rGK3	Unknown	(Wines et al. 1989)
rGK4	Unknown	(Wines et al. 1989)
rGK5	Unknown	(Wines et al. 1989)
rGK6	Unknown	(Wines et al. 1989)
rGK7	Unknown	(Wines et al. 1989)
rGK8	Unknown	(Wines et al. 1989)
rGK10	Kininogenase activity which cleaves T-kininogen to T-kinin.	(Yousef and Diamandis 2003)

rGK - rat glandular kallikreins

rKLK – rat kallikrein

Kallikrein gene families in other species

Two kallikreins have been identified in the dog. Where dKLK1 has the true kininogenase activity and encodes a 261 amino acid prepropeptide that contains a conserved catalytic triad of serine proteases, and a tissue kallikrein substrate binding pocket, while the second, (dKLK2), codes for a canine arginine esterase (Chapdelaine et al. 1991). This dKLK2 serine protease shows a diverse pattern of tissue expression and only shows about 50% sequence conservation when compared to kallikreins of other species (Yousef and Diamandis 2003).

Kallikrein genes of the African rodent Mastomys has been studied because of its androgen responsive prostate in females (Yousef and Diamandis 2003). The cloning of

the kallikreins in Mastomys was first reported by Fahnestock in 1994, where he described three kallikreins, two of which are expressed in the kidney and sub-mandibular gland, while the 3rd was only expressed in the sub-mandibular gland (Fahnestock et al. 1991). Among the 3 kallikreins described one of the two expressed in the kidney is believed to code for a true tissue kallikrein which has the kininogenase activity (Fahnestock et al. 1991).

A few kallikrein genes in the cynomolgus monkey has been identified by screening a kidney cDNA library. These genes show 90% similarity at the nucleotide level and 93% similarity at the amino acid level to humans (Lin et al. 1993). A 515 bp long preproenzyme KLK3 (rmKLK3) has been cloned from a rhesus monkey prostatic cDNA (Gauthier et al. 1993). This KLK3 shows 89% homology to human kallikrein 3 (KLK3) and 71% homology to human kallikrein 2 (KLK2) at the amino acid level. The rmKLK3 lacks the true kininogenase activity as the Tyr93 residue which is essential to the kininogenase activity is substituted by a serine residue (Yousef and Diamandis 2003).

Why study kallikreins

With the development of estrous synchronization, superovulation, artificial insemination and embryo transfer techniques, attention is now being directed towards understanding the mechanisms and regulatory processes that control embryonic survival in the pig. As a model species, the pig has many advantages such as a high fertilization rate and relatively long gestation period compared to other model species such as the mouse (21 days) which provides a better opportunity to study different regulatory mechanisms affecting embryonic survival, but having a 35-45 % natural prenatal mortality (Pope and First 1985) makes pigs an ideal model to study the regulatory mechanisms present during early embryonic development to understand the processes that cause early embryonic loss. Pregnancy in swine is, typically, initiated with the presence of 14 to 16 viable embryos, but only 9 to 10 of these embryos are carried to term. Therefore, understanding the mechanisms involved in early embryonic mortality in the pig has an additional economic significance, as increasing the litter size by overcoming early embryonic loss would be more profitable than improving rate of gain, feed conversion, or lean growth rate (Pope and First 1985).

Tissue expression and Functions of Kallikreins

Highly sensitive immunological techniques and RT-PCR has identified kallikrein gene expression in a diverse array of tissues. The expression of KLK1 has been shown to be high in kidney, pancreas and salivary glands (Yousef and Diamandis 2001). Whereas, KLK2 and KLK3 genes are expressed at low concentrations in a wide array of tissues. The functional diversity among kallikreins is emphasized by their diverse, tissue specific expression patterns. Tissue kallikreins through their multifunctional roles are involved in (patho) physiology of brain, kidney, respiratory, gastrointestinal, and reproductive system (Bhoola et al. 1992). Table 5 depicts the expression patterns of human tissue kallikreins and their proposed functions.

Until recently, the human kallikrein gene family consisted of only 3 genes (KLK1-3), but has now been extended to 15 family members (Yousef and Diamandis 2001). Among the various kallikrein gene family members, only KLK1 has the true kininogenase activity to produce bradykinin from the proteolytic cleavage of low molecular weight kininogen (Guimaraes et al. 1973; Fiedler 1979) which binds to the G-protein coupled kinin receptor B to modulate a variety of physiological processors (Luo et al. 2003). This release of kinins through the proteolytic cleavage of low molecular weight kininogen has recently received increased attention in therapeutic angiogenesis (Emanueli and Madeddu 2001) suggesting that kinins induce increased microvascular permeability, increased blood flow, contraction of smooth muscle, release of calcium and prostaglandins, and vascular growth (Bhoola et al. 1992; Fabre et al. 1999), which are many of the alterations that occur in the porcine uterus during establishment of pregnancy (Geisert et al. 1997).

However, the diverse expression pattern of human kallikrein 1(hK1) has led to the suggestion that the functional role of this enzyme is dependent upon cell type (Yousef and Diamandis 2001) indicating that apart from its kininogenase activity, hK1 may have the ability to process and activate growth factors and peptide hormones (Yousef and Diamandis 2001). Also, hK1 is implied as one of the proteinases that participate in the process of degrading the extracellular matrix and assisting in the attachment of the conceptus to the luminal epithelium (Valdes et al. 2001).

Lee et al. (1998) reported the presence of an unknown serine protease in the pig uterine lumen prior to conceptus elongation which degrades several insulin-like growth factor binding proteins (IGFBP). Through its serine protease activity, kallikrein could function directly or indirectly through activation of several metalloproteinases to degrade IGFBPs (Lee et al. 1998; Vonnahme et al. 1999). Cleavage of IGFBPs stimulate the release of IGF enabling enhanced conceptus growth and estrogen release to establish pregnancy in the pig (Vonnahme et al. 1999; Gerisert et al. 2001).

Human kallikrein 2 (KLK2) and 3 (KLK3/PSA) are two kallikreins predominantly expressed in the prostate, which are used as potential biomarkers for prostate cancer (Luo et al. 2003). KLK2 which has trypsin like enzymatic activity, is known to activate PSA/KLK3 (Yousef and Diamandis 2001; Luo et al. 2003). Furthermore, KLK2 is suggested to play a role in regulating growth factors through IGFBP-3 proteolysis (Yousef and Diamandis 2001). Recently, KLK2 was found to activate urokinase plasminogen activator (uPA). Since, uPA is involved in the propagation of cancer metastasis, KLK2 may be involved in prostate cancer (Yousef and Diamandis 2001). KLK3/PSA is the best prostate cancer biomarker discovered thus far, and is widely used

in prostate cancer diagnosis (Luo et al. 2003). KLK3 is highly expressed in the seminal plasma and has a chymotripsin-like substrate specificity, it cleaves seminogelin I and II , as well as fibronectin to liquefy semen clots (Luo et al. 2003 ; Yousef and Diamandis 2001) in addition to chymotrypsin several other potential substrates such as, IGFBP-3, TGFβ, and plasminogen has been identified for KLK3.

Although enzymatic activities and functional roles for rest of the kallikreins are yet to be identified, preliminary studies show that KLK6, KLK8, and KLK11 have trypsin like enzymatic activities and KLK7 has chymotrypsin-like enzymatic activity (Luo et al. 2003). KLK6 is highly expressed in the brain and is implicated to cleave amyloid precursor protein indicating it's involvement in Alzheimer's disease (Luo et al. 2003). KLK7 is shown to be abundantly expressed in the skin, and catalyzes the degradation of intracellular cohesive structures in the skin and contribute to the cell shredding process. Among the many other known kallikreins, KLK4, KLK5, KLK10, KLK12, KLK13, and KLK14 are predicted to have trypsin-like activity, while KLK9 and KLK15 may possess chymotrypsin like activity (Yousef and Diamandis 2001; Luo et al. 2003).

Most of the kallikreins identified today have a potential of being biomarkers for diagnosis of various malignancies. KLK8, KLK9, and KLK14 were found to be over-expressed in ovarian cancer, while KLK13 was over-expressed in breast cancer indicating that these kallikreins may be used as biomarkers for detection of breast and ovarian cancers. Furthermore, KLK10 has been shown to be down-regulated in breast and prostate cancer suggesting that KLK10 may function as a tumor suppressor gene (Luo et al. 2003). Kallikrein 4 (KLK4), which is also known as prostase, KLK-L1 and PRSS17, has recently received increase attention as a potential biomarker in endometrial cancer, the

fourth most common malignancy in women in the western world (Mayers and Clements 2001). KLK 4 is thought to be regulated by estrogen and progesterone individually and synergistically (Mayers and Clements 2001). Interestingly, KLK4 is expressed in the KLE cell line which has a defective estrogen receptor (ER) (Mayers and Clements 2001). This suggests that an indirect mechanism of response, or a non-genomic steroid response through a membrane bound receptor may be present for kallikrein 4 stimulation (Mayers and Clements 2001). The biological function of KLK4 is yet to be identified; however human kallikrein 4 shares a 78 % homology at the amino acid level with pig enamel matrix serine protease (EMSP1/KLK4) which is involved in the degradation of the enamel protein matrix allowing the maturation of the dental enamel, suggesting that KLK4 may play a role in extracellular matrix remodeling in the uterus (Mayers and Clements 2001).

Table 5. Expression patterns of the human kallikrein gene family. The highly expressed tissues are in bold. Reproduced from (Yousef and Diamandis 2001).

GENE	TISSUE EXPRESSION	FUNCTION /DISEASE
KLK1	**Pancreas, kidney, salivary glands**, central nervous system (CNS), uterus, prostate, testis, breast, placenta	Inflammation, renal disease, and cancer, mediates, bradykinin
KLK2	**Prostate**, breast, thyroid, salivary glands	Biomarker for prostate cancer
KLK3	**Prostate**, breast, thyroid, salivary glands, lung, trachea	Favorable biomarker for prostate and breast cancer
KLK4	**Prostate**, breast, thyroid, testis, uterus, adrenal, colon, spinal cord	Over expressed in ovarian cancer
KLK5	**Breast, brain, testis, skin**, salivary glands, thymus, CNS, prostate, thyroid, trachea	Over expressed in ovarian cancer
KLK6	**CNS, breast, kidney, uterus**, salivary glands, spleen, testis	Down-regulated in breast cancer
KLK7	**Skin, CNS, kidney, breast**, salivary glands, thymus, uterus, thyroid, placenta, trachea, testis, ovary	Over expressed in pathological keratinization, psoriasis and ovarian cancer
KLK8	**CNS, skin, ovary**	Over expressed in CNS injury
KLK9	**Thymus, testis, CNS, trachea**, breast, prostate, salivary glands, ovary, skin	Unknown
KLK10	**Breast, ovary, testis, prostate**, small intestine, lung, colon, pancreas, uterus, CNS, salivary glands, trachea	Down regulated in breast and prostate cancer
KLK11	**Brain, skin, salivary glands, stomach, uterus, lung, thymus, prostate, spleen liver, small intestine, trachea**, heart, fetal liver, breast, thyroid, skeletal muscle	Unknown
KLK12	**Salivary glands, stomach, uterus, trachea, prostate, thymus, lung, colon, brain, breast thyroid**, testis, pancreas, small intestine, spinal cord	Down-regulated in breast cancer
KLK13	**Breast, prostate, salivary glands, testis**, lung, heart, thymus, adrenal, colon, thyroid, trachea	Down-regulated in breast cancer
KLK14	**CNS**, breast, thyroid, uterus, thymus colon, spleen, placenta, small intestine, kidney, bone marrow	Down-regulated in breast cancer
KLK15	**Thyroid, salivary glands, prostate**, adrenal, colon, testis, kidney	Over expressed in prostate cancer

Kallikreins in Reproduction

Previous studies show that the kallikrein-kininogen-kinin system is involved during mediation of reproductive events in the rat (Gao et al. 1992). In the rat, the kallikrein-kininogen-kinin system is activated in the ovary during the time of ovulation (Brann et al. 1995). Activation of the kallikrein-kininogen-kinin system in the rat ovary is physiologically important as the administration of kinin antagonists have shown to block ovulation (Hellberg et al. 1991; Brann et al. 1995) indicating that the kinin system plays an important role during ovulation in the rat (Valdes et al. 1996). Also, "tissue kallikrein mRNA and the corresponding protein (rK1), as well as the type 2 bradykinin receptor, has been immunolocalized in luminal and glandular epithelium, implantation node, deciduomata, and sub-placental sinusoids" in the rat indicating that the kallikrein-kininogen-kinin system is active during early embryonic development in the rat (Valdes et al. 2001).

In the mouse, 5 kallikreins (KLK1, KLK3, KLK5, KLK9, KLK21) are implicated to play a major role in uterine physiological functions and during embryo implantation (Valdes et al. 1996; Corthorn et al. 1997; Chan et al. 1999) where the function of KLK5 and KLK21 are unknown, and KLK1, KLK3 and KLK9 encodes for tissue kallikrein, γ-nerve growth factor binding protein (NGF-BP) and epidermal growth factor binding protein (EGF-BP) respectively (Chan et al. 1999).

It is believed that embryo implantation is tightly regulated by the release of proteinases such as matrix metalloproteinases (MMPs) and urokinase plasminogen activator (Behrendtsen et al. 1992; Harvey et al. 1995) that degrade the extra cellular matrix, enabling conceptus invasion into the uterine stroma. Kallikreins have been implicated to

code for proteins that activate a wide range of substrates including extracellular matrix proteinases and growth factors (Chan et al. 1999), suggesting that kallikreins are involved in many integral processors of early embryonic development such as regulation of local blood flow, angiogenesis, tissue invasion, and mitogenesis (Bhoola et al. 1992).

In humans, the uterine localization of tissue kallikrein mRNA and protein as well as type 2 bradykinin receptor to key sites of embryo attachment, implantation and placentation, supports the notion that the kallikrein-kininogen-kinin system plays a role during pregnancy, probably through vasodilation, increased vasopermeability, enhanced matrix degradation, stimulation of cell proliferation, and myometrial contractility. The higher expression of tissue kallikrein 1 mRNA and protein in the trophoblast and fetal endometrium during early pregnancy indicates that like in the rat and mouse the kallikrein-kininogen- kinin plays a major role during early embryonic development in humans (Valdes et al. 2001).

In the pig, tissue kallikrein enzymatic activity, protein, and gene expression has been detected during the estrous cycle and early conceptus development. An increase in kallikrein enzymatic activity on day 12 of pregnancy in the pig has been detected (Vonnahme et al. 1999). This increase in enzymatic activity occurs simultaneously with the loss of uterine epithelial progesterone receptor, rapid trophoblastic elongation and conceptus estrogen release indicating that the kallikrein release may assist in conceptus attachment through possibly degradation of IαIH4 (Vonnahme et al. 1999). It is hypothesized that, following down-regulation of uterine epithelial progesterone receptor, MUC-1 gene expression is reduced (Bowen et al. 1996), enabling the conceptus to interact with integrins, proteoglycans and heparin, while the cleavage of IαIH4 by

kallikreins may assist in conceptus attachment (Vonnahme et al. 1999). Porcine kallikrein 1 cleaves IαIH4 into 100 kDa and 35 kDa fragments, where the 100 kDa fragment is further cleaved into a 70 kDa fragment (Vonnahme et al. 1999). During conceptus elongation and attachment a 30 kDa fragment corresponding to the C-terminal of IαIH4 has been detected within the uterine lumen suggesting that kallikreins may help regulate alterations in IαIH4 necessary for conceptus attachment and placental development (Geisert et al. 1995; Vonnahme et al. 1999) in the pig.

The localization of bradykinin β_2 receptor in the rat (Murone et al. 1999; Figueroa et al. 2001) sheep (Murone et al. 1996), human (Valdes et al. 2001; Hecquet et al. 2000) and the pig (Allen et al. 2002) uterus and the detection of kallikrein activity also suggests that the kallikrein-kininogen-kallikrein system is functional during pregnancy. In addition, the expression of low molecular weight kininogen (Vonnahme et al. 2003), and pregnancy specific increase of bradykinin (Allen et al.2002) in the porcine uterus during early gestation further suggests that the kallikrein-kininogen-kallikrein system is active in the pig.

Although previous studies have shown tissue kallikrein gene expression in cyclic and pregnant gilts (Vonnahme et al. 1999), it is not clear whether kallikrein gene expression is due to activation of prekallikrein or due to altered gene expression. Therefore, further studies are necessary to determine whether kallikrein gene expression is altered during the estrous cycle and early pregnancy or if the changes in kallikrein gene expression is due to activation of kallikrein activity from prekallikrein (Vonnahme et al. 1999).

Even though preliminary data demonstrates the involvement of kallikreins in uterine events during early embryonic development, expression of specific members of this large

family has not been fully characterized, only porcine kallikrein 4 KLK4 which was isolated from the dental enamel is known (Ryu et al. 2002). Therefore as a first step towards understanding the functional role of tissue kallikreins during early embryonic development in the pig, we have constructed a BAC clone based physical map of the greater kallikrein region in the pig and have mapped the kallikrein region to porcine chromosome 6q1.2 with high confidence. We have also initiated the sequence analysis of the whole kallikrein gene region in the pig and have isolated several new porcine tissue kallikreins. Further more, we have analyzed the differential expression of 3 tissue kallikreins (KLK1, KLK4 and KLK14) during early embryogenesis in both maternal and fetal tissue to better understand the functional role of tissue kallikreins during early embryonic development in the pig.

Reference List

Allen, M.R., B.R. Zhang, A.M. Hettinger, D.W. Goad, J.R. Malayer, and R.D. Geisert. 2002. Detection of bradykinin and bradykinin-β2 receptors in the porcine endometrium during the estrous cycle and early pregnancy. Biology of Reproduction **66**: 574-579.

Ashley, P.L. and R.J. Macdonald. 1985. Tissue-specific expression of kallikrein-related genes in the rat. Biochemistry **24**: 4520-4527.

Beaubien, G., I. Rosinskichupin, M. G.Mattei, M. Mbikay, M. Chretien, and N.G. Seidah. 1991. Gene structure and chromosomal localization of plasma kallikrein. Biochemistry **30**: 1628-1635.

Behrendtsen, O., C.M. Alexander, and Z. Werb. 1992. Metalloproteinases mediate extracellular-matrix degradation by cells from mouse blastocyst outgrowths. Development **114**: 447-456.

Bhoola, K.D., C.D. Figueroa, and K. Worthy. 1992. Bioregulation of kinins - Kallikreins, Kininogens, and Kininases. Pharmacological Reviews **44**: 1-80.

Brann, D.W., L.M. Greenbaum, V.B. Mahesh, and X. Gao. 1995. Changes in kininogens and kallikrein in the plasma, brain, and uterus during pregnancy in the rat. Endocrinology **136**: 46-51.

Bowen, J.A., F.W. Bazer, R.C. Burghardt. 1996. Spatial and temporal analyses of integrin and Muc-1 expression in porcine uterine epithelium and trophectoderm in-vivo. Biology of Reproduction **55**: 1098-1106.

Carretero, O.A. and A.G. Scicli. 1980. The renal kallikrein kinin system. American Journal of Physiology **238**: 247-255.

Cassim, B., G. Mody, and K.D. Bhoola. 2002. Kallikrein cascade and cytokines in inflamed joints. Pharmacology & Therapeutics **94**: 1-34.

Chan, C.S., M.B. Harvey, and J.A. Clements. 1999. Temporal and tissue-specific expression of kallikrein (klk) genes and identification of a novel klk messenger ribonucleic acid transcript during early development in the mouse. Biology of Reproduction **61**: 621-628.

Chapdelaine, P., E. Gauthier, M.A. Hokim, L. Bissonnette, R.R. Tremblay, and J.Y. Dube. 1991. Characterization and expression of the prostatic arginine esterase gene, a canine glandular kallikrein. DNA and Cell Biology **10**: 49-59.

Chowdhary, B.P. 1998. Cytogenetics and Physical chromosome maps. In *The genetics of the pig.* (ed. M.F. Rothschild and A. Ruvinsky), pp. 199-264. CAB International, New York.

Corthorn, J., C. Figueroa, and G. Valdes. 1997. Estrogen and luminal stimulation of rat uterine kallikrein. Biology of Reproduction **56**: 1432-1438.

Darwin, C. 1868. In *The variation of animals and plants under domestication.* John Murray, London.

Diamandis, E.P., G.M. Yousef, and A.Y. Olsson. 2004. An update on human and mouse glandular kallikreins. Clinical Biochemistry **37**: 258-260.

Emanueli, C. and P. Madeddu. 2001. Targeting kinin receptors for the treatment of tissue ischaemia. Trends in Pharmacological Sciences **22**: 478-484.

Evans, B.A., C.C. Drinkwater, and R.I. Richards. 1987. Mouse glandular kallikrein genes: Structure and partial sequence-analysis of the kallikrein gene locus. Journal of Biological Chemistry **262**: 8027-8034.

Evans, B.A., Z.X. Yun, J.A. Close, G.W. Tregear, N. Kitamura, S. Nakanishi, D.F. Callen, E. Baker, V.J. Hyland, G.R. Sutherland, and R.I. Richards. 1988. Structure and chromosomal localization of the human renal kallikrein gene. Biochemistry **27**: 3124-3129.

Fahnestock, M., J.E. Woo, G.A. Lopez, J. Snow, D.A. Walz, M.J. Arici, and W.C. Mobley. 1991. Beta-NGF-endopeptidase: Structure and activity of a kallikrein encoded by the gene mGK-22. Biochemistry **30**: 3443-3450.

Fabre, J.E., A. Rivard, M. Magner, M. Silver, and J.M. Isner. 1999. Tissue inhibition of angiotensin-converting enzyme activity stimulates angiogenesis in vivo. Circulation **99**: 3043-3049.

Fiedler, F. 1979. Enzymology of glandular kallikreins. In *Handbook of experimental, Pharmacology, Supplement, Bradykinin, Kallidin, and Kallikrein* pp. 103-161. New York: Springer-verlag.

Figueroa, C.D., C. Chacon, J. Corthorn, P. Ehrenfeld, W. Muller-Esterl, and G. Valdes. 2001. Temporospatial changes of kinin B2 receptors during the estrous cycle and pregnancy in the rat uterus. Biology of Reproduction **64**: 1590-1599.

Gan, L., I. Lee, R. Smith, R. Argonza-Barrett, H. Lei, J. McCuaig, P. Moss, B. Paeper, and K. Wang. 2000. Sequencing and expression analysis of the serine protease gene cluster located in chromosome 19q13 region. Gene **257**: 119-130.

Gao, X.X., L.M. Greenbaum, V.B. Mahesh, and D.W. Brann. 1992. Characterization of the kinin system in the ovary during ovulation in the rat. Biology of Reproduction **47**: 945-951.

Gauthier, E.R., P. Chapdelaine, R.R. Tremblay, and J.Y. Dube. 1993. Characterization of rhesus-monkey prostate-specific antigen cDNA. Biochimica et Biophysica Acta **1174**: 207-210.

Geisert, R.D., J.W. Brookbank, R.M. Roberts, and F.W. Bazer. 1982a. Establishment of pregnancy in the pig . 2. Cellular remodeling of the porcine blastocyst during elongation on day-12 of pregnancy. Biology of Reproduction **27**: 941-955.

Geisert, R.D., M.J. Dixon, T. Pratt, R.A.M. Schmitt, B.A. Lessley, and J.P. Mcann. 1995. Isolation and characterization of a 30-kDa endometrial glycoprotein synthesized during the estrous cycle and early pregnancy of the pig. Biology of Reproduction **53**: 1038-1050.

Geisert, R.D., R.H. Renegar, W.W. Thatcher, R.M. Roberts and F.W. Bazer 1982b. Establishment of pregnancy in the pig. I. Interrelationships between peri-implantation development of the pig blastocyst and the uterine endometrial secretions. Biology of reproduction **27**: 925-939

Geisert, R.D., C.S. Chamberlain, K.A. Vonnahme, J.R. Malayer, and L.J. Spicer. 2001. Possible role of kallikrein in proteolysis of insulin-like growth factor binding proteins during the oestrous cycle and early pregnancy in pigs. Reproduction **121**: 719-728.

Geisert, R.D. and J.V. Yelich. 1997. Regulation of conceptus development and attachment in pigs. Journal of Reproduction and Fertility **52**: 133-149.

Geisert, R.D., J.V. Yelich, T. Pratt, and D. Pomp. 1998. Expression of an inter-alpha-trypsin inhibitor heavy chain-like protein in the pig endometrium during the estrous cycle and early pregnancy. Journal of Reproduction and Fertility **114**: 35-43.

Geisert, R.D., M.T. Zavy, R.J. Moffatt, and Blair R.M. 1990. Embryonic steroids and the establishment of pregnancy in pigs. Journal of Reproduction and Fertility Supplement **40**: 293-305.

Guimaraes, J.A., D.R. Borges, E.S. Prado, and J.L. Prado. 1973. Kinin converting aminopeptidase from human serum. Biochem Pharmacol **22**: 3157-3172.

Harvey, M.B., K.J. Leco, M.Y. Arcellanapanlilio, X. Zhang, D.R. Edwards, and G.A. Schultz. 1995. Proteinase expression in early mouse embryos is regulated by leukemia inhibitory factor and epidermal growth-factor. Development **121**: 1005-1014.

Hecquet, C., F.L. Tan, B.M. Marcic, and E.G. Erdos. 2000. Human bradykinin β2 receptor is activated by kallikrein and other serine proteases. Molecular Pharmacology **58**: 828-836.

Hellberg, P., L. Larson, J. Olofsson, L. Hedin, and M. Brannstrom. 1991. Stimulatory effects of bradykinin on the ovulatory process in the in-vitro perfused rat ovary. Biology of Reproduction **44**: 269-274.

Hojima, Y., J.V. Pierce, and J.J. Pisona. 1985. Purification and characterization of multiple forms of human-plasma prekallikrein. Journal of Biological Chemistry **260**: 400-406.

Lee, C.Y., M.L. Green, R.C.M. Simmen, and F.A. Simmen. 1998. Proteolysis of insulin-like growth factor-binding proteins (IGFBPs) within the pig uterine lumen associated with pre-implantation conceptus development. Journal Of Reproduction And Fertility **112**: 369-377.

Lin, F.K., C.H. Lin, C.C. Chou, K. Chen, H.S. Lu, and W. Bacheller. 1993. Molecular cloning and sequence analysis of the monkey and human tissue kallikrein genes. Biochimica et Biophysica Acta (BBA) – Bioenergetics **1173**: 325-328.

Luo, L., G. Yousef, and E.P. Diamandis. 2003. Human tissue kallikreins and testicular cancer. APMIS **111**: 225-233.

Mayers, S.A. and J.A. Clements. 2001. Kallikrein 4 (KLK4), A new member of the human kallikrein gene family is upregulated by estrogen and progesterone in the human endometrial cancer cell line, KLE. Journal of Clinical Endocrinology and Metabolism **86**: 2323-2326.

Murone, C., S.Y. Chai, W. Muller-Esterl, F.A.O. Mendelsohn, and J. Clements. 1999. Localization of bradykinin β2 receptors in the endometrium and myometrium of rat uterus and the effects of estrogen and progesterone. Endocrinology **140**: 3372-3382.

Murone, C., R.B. Perich, I. Schlawe, W. MullerEsterl, F.A.O. Mendelsohn, and S.Y. Chai. 1996. Localization of bradykinin B-2 receptors in sheep uterus. Immunopharmacology **33**: 108-112.

Olsson, A.Y., Lilja, H., and Lundwall, A. 2004. Taxon specific evolution of glandular kallikrein genes and identification of progenitor of prostate-specific antigen. Genomics **84**: 147-156.

Pope, W.F. and N.L. First. 1985. Factors affecting the survival of pig embryos. Theriogenology **23**: 91-105.

Riegman, P.H.J., R.J. Vlietstra, P. Klaassen, J.A.G.M. Vanderkorput, A.G. Vankessel, J.C. Romijn, and J. Trapman. 1989. The prostate-specific antigen gene and the human glandular kallikrein-1 gene are tandemly located on chromosome-19. Febs Letters **247**: 123-126.

Rittenhouse, H.G., J.A. Finlay, S.D. Mikolajczyk, and A.W. Partin. 1998. Human kallikrein 2 (hK2) and prostate-specific antigen (PSA): Two closely related, but distinct, kallikreins in the prostate. Critical Reviews in Clinical Laboratory Sciences **35**: 275-368.

Rothschild, M.F. and J.P. Bidanel. 1998. Biology of genetics and reproduction. In *The genetics of the pig.* (ed. M.F. Rothschild and A. Ruvinsky), pp. 313-344. CAB International, New York.

Ryu, O., J.C.C. Hu, Y. Yamakoshi, J.L. Villemain, X. H.Cao, C.H. Zhang, J.D. Bartlett, and J.P. Simmer. 2002. Porcine kallikrein-4 activation, glycosylation, activity, and expression in prokaryotic and eukaryotic hosts. European Journal of Oral Sciences **110**: 358-365.

Southardsmith, M., J.C. Pierce, and R.J. Macdonald. 1994. Physical mapping of the rat-Tissue kallikrein family in 2 gene clusters by analysis of P1-bacteriophage clones. Genomics **22**: 404-417.

Tomanek, M., V. Kopency, and J. Kanka. 1989. Genome reactivation in developing early pig embryos: an ultrastructural and autoradiographic analysis. Anatomy and Embryology **180**: 309-316

Valdes, G., C.D. Figueroa, and J. Corthorn. 1996. Temporospatial changes of kallikrein-like enzymes during the estrous cycle and pregnancy in the rat uterus. Biology of Reproduction **55**: 236-245.

Valdes, G., A.M. Germain, J. Corthorn, C. Chacon, C.D. Figueroa, and W. Muller-Esterl. 2001. Tissue kallikrein and bradykinin B2 receptor in human uterus in luteal phase and in early and late gestation. Endocrine **16**: 207-215.

Vanleeuwen, B.H., B.A. Evans, G.W. Tregear, and R.I. Richards. 1986. Mouse glandular kallikrein genes - identification, structure, and expression of the renal kallikrein gene. Journal of Biological Chemistry **261**: 5529-5535.

Veloso, D. and R.W. Colman. 1991. Western-blot analyses of prekallikrein and its activation products in human plasma. Thrombosis and Haemostasis **65**: 382-388.

Vonnahme, K.A., S.C. Fernando, J.W. Ross, M.D. Ashworth, U. DeSilva, J.R. Malayer, and R.D. Geisert. 2003. Porcine endometrial expression of kininogen, factor XII, and plasma kallikrein in cyclic and pregnant gilts. Biology of Reproduction **70**: 132-138.

Vonnahme, K.A., J.R. Malayer, H.O. Spivey, S.P. Ford, A. Clutter, and R.D. Geisert. 1999. Detection of kallikrein gene expression and enzymatic activity in porcine endometrium during the estrous cycle and early pregnancy. Biology of Reproduction **61**: 1235-1241.

Wines, D.R., J.M. Brady, D.B. Pritchett, J.L. Roberts, and R.J. Macdonald. 1989. Organization and expression of the rat kallikrein gene family. Journal of Biological Chemistry **264**: 7653-7662.

Wines, D.R., J.M. Brady, E.M. Southard, and R.J. Macdonald. 1991. Evolution of the rat kallikrein gene family - gene conversion leads to functional diversity. Journal of Molecular Evolution **32**: 476-492.

Xiong, W., L.M. Chen, and J. Chao. 1990. Purification and characterization of a kallikrein-like T-kininogenase. Journal of Biological Chemistry **265**: 2822-2827.

Yousef, G.M. and E.P. Diamandis. 2001. The new human tissue kallikrein gene family: Structure, function, and association to disease. Endocrine Reviews **22**: 184-204.

Yousef, G.M. and E.P. Diamandis. 2003. An overview of the kallikrein gene families in humans and other species: Emerging candidate tumor markers. Clinical Biochemistry **36**: 443-452.

Yu, H.R., D.W. Bowden, B.J. Spray, S.S. Rich, and B.I. Freedman. 1998. Identification of human plasma kallikrein gene polymorphisms and evaluation of their role in end-stage renal disease. Hypertension **31**: 906-911.

CHAPTER II

Physical and Radiation Hybrid mapping of Porcine Tissue Kallikreins

Introduction

During early pregnancy in the pig, the conceptus undergoes rapid trophoblastic elongation from a 10 mm sphere to a long filamentous thread like structure (Geisert et al. 1982a,b). This morphological alteration on Day 12 occurs concurrently with the establishment of pregnancy in the pig (Bazer et al. 1982; Geisert and Yelich 1997). On day 12 of pregnancy, the conceptus synthesizes and releases estradiol-17β during the rapid trophoblastic elongation phase as the maternal recognition signal for establishment and maintenance of pregnancy (Geisert 1982, 1990 & 1997; Vonnahme et al. 1999). This estrogen release from the conceptus regulates the release of pregnancy associated protein secretions from the uterus (Roberts et al. 1993), prostaglandin synthesis and release (Bazer and Thatcher 1977; Vonnahme et al. 1999), uterine blood flow (Ford et al. 1982; Vonnahme et al. 1999), and uterine cellular morphology (Geisert et al. 1982; Keys and King 1990). The mechanism by which estradiol-17β regulates endometrial secretory activity and the factor or factors induced by conceptus estradiol-17β to orchestrate these uterine changes conductive to conceptus development and survival have not been fully elucidated (Vonnahme et al. 1999).

Pig conceptuses form a noninvasive, diffuse, epitheliochorial placenta (King et al. 1982; Keys and King 1990). During placentation the glycoproteins present within the uterine microvillus glycocalyx aid in the attachment of the conceptus to the uterine surface (Dantzer, 1985). Geisert, et al. (1998) isolated and characterized a glycoprotein homologous to inter-α-trypsin inhibitor heavy chain 4 (IαIH4) (Geisert et al. 1998). It is believed that IαIH4 and other IαI family members play a role in stabilization of extracellular matrix (Bost et al. 1998) where heavy chains of the IαI family members

contain a Von Willebrand type A domain that functions as a target for adhesion molecules such as integrins, collagen, and heparin (Salier et al. 1996). IαIH4 is unique in relation to other known IαI family members as IαIH4 contains a cleavage site for the serine protease kallikrein (Hashimoto et al. 1996). Tissue kallikreins are serine proteases (Gan et al. 2000). Some of which are active members of the kallikrein-kininogen-kinin system which cleaves kininogen to release the vasoactive peptide, bradykinin (Bhoola et al. 1992). Kinins released from kininogen are normally involved in many inflammatory-associated effects such as prostaglandin synthesis and release, smooth muscle contraction, histamine release and increased blood flow (Bhoola et al. 1992) which are similar to the uterine alterations that occur in the pig during establishment of pregnancy (Stroband and Van der Lende 1990; Geisert et al. 1990).

Previous studies have shown that the kallikrein -kininogen-kinin system to be involved with ovulation and implantation in the rat (Gao et al. 1992; Valdes et al. 1996). Vonnahme and coworkers (1999) have demonstrated that endometrial tissue kallikrein enzymatic activity, protein and gene expression is present during the porcine estrous cycle and early pregnancy. Presence of kallikrein suggests that the kallikrein-kininogen-kinin system is active in the pig uterus and could play a role in conceptus trophoblast attachment through cleavage of IαIH4. Tissue kallikrein cleaves porcine IαIH4 into 100 kDa and 35 kDa fragments, where the 100 kDa fragment is further cleaved into a 70 kDa fragment (Hashimoto et al. 1996). During conceptus trophoblastic elongation and attachment, a 30 kDa fragment corresponding to the C-terminal of IαIH4 has been detected within the uterine lumen (Geisert et al. 1995) suggesting that tissue kallikreins may play a role within the uterine lumen of the pig to help regulate alterations in IαIH4

necessary for conceptus attachment and placental development (Vonnahme et al. 1999). In addition, the expression of low molecular weight kininogen in the porcine uterus (Vonnahme et al. 2004) during early gestation further suggests that the K-K-K system is active in the pig.

Although previous studies have shown tissue kallikrein gene expression in cyclic and pregnant gilts, it is not clear whether kallikrein gene expression is due to activation of prekallikrein or due to altered gene expression. Therefore, further studies are necessary to determine whether kallikrein gene expression is altered during the estrus cycle and early pregnancy or if the changes in kallikrein gene expression is due to activation of kallikrein activity from prekallikrein (Vonnahme et al. 1999). Unfortunately, little is known about the kallikrein gene family in the pig. Except for the kallikrein 4 sequence (Ryu et al. 2002) isolated from pig dentine (genbank accession # U76256), no other porcine tissue kallikreins have been identified thus far. Therefore, as first step towards isolation, characterization and functional analysis of the porcine kallikrein gene family, a BAC clone-based physical map of the greater kallikrein gene region of the pig was constructed and was mapped to porcine chromosome 6q1.2 2 with high confidence. We have also initiated the sequence analysis of the whole kallikrein gene region in the pig and have isolated several new porcine tissue kallikreins.

Materials and Methods

Overgo Probe Design and Labeling

Overgo probes were designed for all known human kallikrein exonic sequences (except kallikrein 11) using publicly available sequence information. Care was taken to avoid interspersed repeats and low complexity DNA sequences by analyzing human kallikrein gene sequences using RepeatMasker (http://www.repeatmasker.org/). The resulting sequences were used to design overgo probes using "Overgo Maker" available at http://www.genome.wustl.edu/tools/?overgo=1. Each Overgo probe generated was 40 bp in length and contained two 24-mer oligonucleotides with an 8 bp overlap at their 3' ends (Sambrook and Russel 2001). Overgo probes had a 50% GC content and were highly specific to kallikrein gene targets.

Overgo probes were synthesized (Integrated DNA Technologies, Coralville, IA, USA) and a 10 pmol/µl stock solution was prepared. The 3' ends of the two oligonucleotides were annealed by mixing the two complementary 24-mer oligonucleotides and heating the mixed oligonucleotides at 80ºC for 5 minutes, incubating at 37ºC for 10 min and placing on ice. Annealed oligonucleotides were then subjected to radioactive primer extension to produce a 40 bp double-stranded radiolabeled probe (Sambrook and Russel 2001).

The overgo labeling reaction for radioactive primer extension contained, 1 pmol/µl annealed oligonucleotides, 100 µg/ml BSA, 2 µl OLB [prepared by mixing solution A, B and C in 1:2.5:1.5 ratio. (Solution A: 1 ml of solution O, 18 µl of β-mercaptoethanol, 5 µl of 100 mM dTTP, and 5 µl of 100 mM dGTP); (Solution B: 2M HEPES pH 6.6); (Solution C: 3 mM Tris-Cl, 0.2 mM EDTA); (Solution O: 1.25 mM Tris-Cl, 125 mM

$MgCl_2$)], 0.5 µl of 10mCi/ml [α-^{32}P]dATP, 0.5 µl of 10mCi/ml [α-^{32}P]dCTP and 2U klenow fragment (Sambrook and Russel 2001). Reaction mix was incubated at room temperature for 1 hour and unincorporated nucleotides were removed using Sephadex-G50 columns (Amersham, Piscataway, NJ, USA) by centrifuging the labeling reaction in Sephadex- G50 columns for 2 min at 3000 rpm according to manufacture's protocol.

Southern Blotting

The specificity and sensitivity of the overgo probes developed was evaluated using Southern blots. Briefly, porcine genomic DNA was extracted and digested with EcoR I and Hind III (Promega, Madison, WI, USA) by incubating at 37ºC for 6 hrs. Digest was electrophorized on a 0.85% agarose gel and was washed in 0.25 M HCl for 20 min, twice in denaturation solution (1.5 M NaCl and 0.5M NaOH) for 30 min each, twice in neutralization solution (1.0 M Tris pH-8, 1.5 M NaCl), and in 10X SSC for 30 min. DNA was transferred to a hybond® nylon membrane (Amersham, Piscataway, NJ, USA) over a period of 24 hrs using the downward blotting technique. After transfer, the membrane was UV cross-linked to immobilize the DNA.

Membrane strips were used to evaluate the specificity and sensitivity of the overgo probes by hybridization. Briefly, the membranes were prewetted in 2X SSC and prehybridized with 10 ml of hybridization solution (1% BSA, 1 mM EDTA, 7% SDS, 0.5 M $NaPO_4$) for 4 hrs. After adequate prehybridization, denatured radiolabeled overgo probes (denatured by incubating at 90ºC for 10 min) were individually hybridized to pig genomic DNA containing membrane strips. The hybridization was carried out overnight at 60ºC. Following hybridization overnight at 60ºC, the filters were washed in 1.5X SSC/

0.1% SDS at 58ºC for 30 min and then in 1.0X SSC/ 0.1% SDS at 58ºC for 30 min. hybridization of the probes were detected by exposure of the filter to Bio-Max max efficiency X-ray film (Invitrogen, Carlsbad, CA, USA) for 10-12 hrs at -80ºC.

Porcine BAC Library Screening

High-density gridded membranes containing 6.3X coverage of porcine genome (Pieter de Jong; http://www.chori.org/bacpac/) were screened using protocols previously described by Sambrook and Russel, (2001). Briefly, the BAC filters were prewetted in 2X SSC and prehybridized with 50 ml of hybridization solution (1% BSA, 1 mM EDTA, 7% SDS, 0.5 M $NaPO_4$) for 4 hrs. After prehybridization, radiolabeled overgo probes were pooled denatured and used for hybridization. Hybridization was carried out overnight at 60ºC. Following hybridization, filters were washed in 1.5X SSC/ 0.1% SDS at 58ºC for 30 min followed by 1.0X SSC/ 0.1% SDS at 58ºC for 30 min. Signals were detected by exposure of the filter to Bio-Max max efficiency X-ray film for 10-12 hrs at -80ºC. Positive clones isolated by this screen were further evaluated by hybridization experiments using individual probes. Table 6 lists the Overgo probe sequences used for BAC library screening.

Table 6. Overgo probes used to screen the high-density porcine BAC library. The different kallikrkeins are given as KLK1-KLK15. The two complementry 24 bp long overgo probes are given as Ova & Ovb. The overgo probes developed for porcine KLK4 is given as Sus-Ova & Sus-Ovb.

Probe	Genbank Accession	Sequence 5'-3'
KLK1-Ova	M25629	CGCCACAACTTGTTTGACGACGAA
KLK1-Ovb		CAAACTGGGCTGTGTTTTCGTCGT
KLK2-Ova	M18157	TGTACACCAAGGTGGTGCATTACC
KLK2-Ovb		TCCTTGATCCACTTCCGGTAATGC
KLK3-Ova	M27274	TGGTGTGCTTCAAGGTATCACGTC
KLK3-Ovb		TGGTTCACTGCCCCATGACGTGAT
KLK4-Ova	AF113141	ACGGATTCGTCCAACTTGATGAGC
KLK4-Ovb		TCGCTAACGACCTCATGCTCATCA
KLK5-Ova	AF135028	CAACATCTCTGGGAAGGAATGAGG
KLK5-Ovb		GACACTCCTTTCAGACCCTCATTC
KLK6-Ova	AF013988	TGGATCCAGTTCGTGTATCTGCAG
KLK6-Ovb		GAGTCTACACCAACGTCTGCAGAT
KLK7-Ova	AF166330	CCACTTGGTGAACTTGCACACTTG
KLK7-Ovb		CCCAGGAGTCTACACTCAAGTGTG
KLK8-Ova	AF095743	GTCACGCAGTTGAAGAAGCATCAG
KLK8-Ovb		GGACCACAACCATGATCTGATGCT
KLK9-Ova	AF135026	TTGGATCCAGTCAAGGTAGTGGCA
KLK9-Ovb		AGTCTACACCAGCGTATGCCACTA
KLK10-Ova	NM_002776	CCACTTCCTGCATTTCAGCTTCAG
KLK10-Ovb		CCATTCTCTGCCTGTACTGAAGCT
KLK12-Ova	AF135025	CTGAGACGTGGCACTCGGTGCCAG
KLK12-Ovb		AATGACTGTGCAACCGCTGGCACC
KLK13-Ova	AF135024	ACTGGGAATAAGGAGCAGAGAAGG
KLK13-Ovb		ACATGGTCTGCTTAGCCCTTCTCT
KLK14-Ova	AF161221	CACTGGGTTGAAGTCTGCTCATAG
KLK14-Ovb		CTAGTCTATAGCTCCGCTATGAGC
KLK15-Ova	AF242195	TGCTGATGTTGGCACAATGCAACG
KLK15-Ovb		GTGAGTCTCCCAGATACGTTGCAT
Sus-Ova	U76256	ACACCAACCTCTGCAAATTCACGG
Sus-Ovb		GTCGTCTGTATCCAGTCCGTGAAT

Individual Clone Lysis and Hybridization

BAC clones isolated in the previous experiment were tested for the presence of individual kallikrein genes using a protocol previously described (DeSilva 1998). Clones were grown overnight in a 96 well plate containing 2X YT broth and 20 μg/ml of chloramphenicol (Fischer, Pittsburgh, PA, USA). Clones were spotted on hybond® nylon membrane (Amersham, Piscataway, NJ, USA) using a plate replicator and were grown on the membrane by placing the membrane on a plate containing 2X YT agar and 20 μg/ml of chloramphenicol overnight. Bacterial cells were lysed by placing the filter colony side up on Whatman 3MM paper saturated with 10% SDS for 5 min, 0.5 M NaOH / 1.5 M NaCl for 5 min, twice with 0.5 M Tris pH 8 / 1.5 M NaCl for 5 min, 2X SSC / 0.1% SDS for 5 min, and 2X SSC for 5 min. The filters were dried and UV cross-linked before use. Filters were hybridized using individual overgo probes as described previously.

Pig Genomic DNA Isolation

Genomic DNA was isolated from porcine blood. Blood was collected from gilts by venipuncture and was collected in heparinized vaccutainer tubes. Twenty milliliters of chilled 2x sucrose solution (0.637 M sucrose, 20 mM Tris-base, 10 mM $MgCl_2.6H_2O$, 2% Triton X-100, pH 7.6) was added to 20 ml of porcine blood and centrifuged at 2500 rpm at 4ºC for 30 min. Resulting pellet was resuspended in 1 volume of 1X sucrose solution and centrifuged at 2500 rpm at 4ºC for 30 min. Pellet was resuspended in 0.25 volume of sucrose/proteinase K cell lysis buffer (27% sucrose, 1X SSC, 1mM EDTA, 1% SDS, 200 μg/ml proteinase K) and incubated at 37ºC overnight. The lysate obtained was extracted

using 10 ml of phenol (saturated with 1 M Tris-Cl): chloroform: isoamyl alcohol (25:24:1). The resulting DNA was precipitated in the aqueous phase with 1 volume of isopropyl alcohol. DNA was spooled on a pasture pipette and was washed with 70% ethanol, air dried and resuspended in water (Sambrook and Russel 2001).

BAC DNA Isolation

Individual BAC clones were inoculated into 2 ml of 2X YT broth containing 20 μg/ml of chloramphenicol (Fischer, Pittsburgh, PA, USA) and incubated in a shaking incubator (225 rpm) at 37^0C for 4-5 hrs. Following incubation, the culture was added to 50 ml of 2X YT broth containing 20 μg/ml chloramphenicol (Fischer, Pittsburgh, PA, USA) and was incubated in a shaking incubator (225 rpm) at 37ºC for 16-22 hrs. The total 52 ml volume was used to isolate BAC DNA (Sambrook and Russel 2001). Briefly, the overnight cultures were centrifuged at 4500 rpm at 4ºC for 30 min. The resulting pellet was resuspended in 0.1X volume of chilled GET buffer (50 mM glucose, 25 mM Tris-HCl, pH 8.0, and 10 mM EDTA, pH 8) and 60 μl of 20 mg/ml RNAseA. Five milileters of fresh solution I (0.2N NaOH, 1% SDS), and 5 ml of 3M KOAc pH 5.5 was added to the lysate which was then incubated on ice for 10 min, and was centrifuged at 15,000 rpm at 4ºC for 30 min. Isopropanol (0.67 volume) was added to the supernatant and incubated at room temperature for 15 min and centrifuged at 15,000 rpm at room temperature for 30 min. DNA precipitated was washed with 70% ethanol, air dried and resuspended in water.

Kallikrein 4 PCR assay

Porcine kallikrein 4 forward (5' CTGGGTCTGCACAATCTTGA 3') and reverse (5' AGACAGCGACACCGATTCTT 3') primers were developed (within an exon) for PCR amplification from the known porcine kallikrein 4 sequence (GenBank accession # U76256) using Primer3 software (Rozen and Skaletsky 2000). The PCR product size was 147 bp.

The PCR reactions were performed in a MJ Dyad thermal cycler (MJ Research Inc., Waltham, MA, USA). 20 µl reaction mix contained 1 µl DNA, 1X PCR buffer (Applied Biosystems, Foster City, CA, USA) 1.5 mM $MgCl_2$, 25 pmol of each primer, 200 µM dNTPs and 3 U Taq DNA Polymerase (Promega, Madison, WI, USA). Cycling conditions were: one cycle of 1 min at 95ºC, 30 sec at 60ºC, 15 sec at 72ºC followed by 34 additional cycles of 30 sec at 94ºC, 30 sec at 62ºC, 15 sec at 72ºC and a final extension of 3 min at 72^0C. The resulting PCR products were electrophorized in a 1.5% agarose gel and stained with 5 µl of ethidium bromide (10 mg/ml).

BAC End Isolation

The end fragments of the BAC clones were isolated by Vectorette PCR end rescue approach (Thomas, J.W. Personal Communication). Briefly, 5 µg of BAC DNA was digested with either Hae III or Pvu II (Promega, Madison, WI, USA) by incubating at 37ºC for 2 hrs and heating to 65ºC for 30 min. The digestion contained 5 µg of DNA, 1X buffer, 4U restriction enzyme). A vectorette duplex linker (2 µM of BPB I and BPH II oligos heated to 68^0C for 15 min and slowly cooled to room temperature over 30 min to form the duplex linker) was ligated to the digested DNA by incubating at 37ºC for 2 hrs.

The ligation reaction contained 10 µl of restriction digest, 1X ligase buffer, 2U T4 ligase (Invitrogen, Carlsbad, CA, USA), and 7 nM/ µl vectorette duplex linker.

The ligated product was PCR amplified using T7BACF/ SP6 BACR primers and 224 universal primer. PCRs were performed in a MJ Dyad thermal cycler. The 20 µl reaction mix contained 1 µl DNA, 1X PCR buffer (Applied Biosystems, Foster City, CA, USA) 1.5 mM $MgCl_2$, 25 pmol of each primer, 200 µM dNTPs and 3 U Taq DNA Polymerase (Promega, Madison, WI, USA). Cycling conditions were one cycle of 1 min at 95ºC, 30 sec at 55ºC, 1 min at 72 ºC followed by 34 additional cycles of 30 sec at 94ºC, 30 sec at 55ºC, 1 min at 72ºC and a final extension of 3 min at 72ºC. Single-copy BAC ends obtained were TA cloned and sequenced.

The primer and vectorette oligonucleotide linker sequences are:

```
BPB I:   5′ CAAGAAGAGGACGCTGTCTGTCGAAGGTAAGGAACGGACGAGAGAAGGGAGAG 3′
BPH II:  5′ CTCTCCCTTCTCGAATCGTAACCGTTCGTACGAGAATCGCTGTCCTCTCCTTG 3′
224:     5′ CGAATCGTAACCGTTCGTACGAGAATCGCT 3′
T7BACF:  5′ CCGCTAATACGACTCACTATAGGG 3′
SP6BACR: 5′ GTCGACATTTAGGTGACACTATAG 3′
```

TA Cloning

The BAC ends isolated by vectorette PCR end rescue approach were TA cloned using TOPO® TA cloning kit according to manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). Briefly, fresh PCR products were ligated to vector pCR®2.1-TOPO® by incubating at room temperature for 20 min. The 6 µl ligation mix contained, 2 µl of fresh PCR product, 1 µl of pCR®2.1-TOPO® vector, 1 µl of salt solution, and 2 µl of water. The ligated PCR product (2 µl) was added to 25 µl vial of chemically competent cells and

incubated on ice for 30 min. The cells were heat-shocked for 30 sec at 42ºC without shaking. SOC medium (250 µl) was added to the heat-shocked cells and were incubated at 37ºC for 1 h in a shaking incubator at 225 rpm. Each transformation was spreaded on YT plates containing X-gal (40 mg/ml) and ampicillin (50 µg/ml). The plates were incubated at 37ºC overnight. Positive white clones were selected and further analyzed using cell burst PCR.

"Cell Burst" PCR

A small sample of a bacterial colony was collected on a toothpick, mixed in 30 µl of water and heated at 95ºC for 5 min to lyse cells. One microliter of this cell lysis was used for PCR using specific BAC end primers to select positive clones.

BAC End Sequencing

The PCR products generated through vectorette-PCR were treated with shrimp alkaline phosphatase (1u μl^{-1}) and Exonuclease III (10u μl^{-1}) at a volume equaling 5% of the PCR product and was incubated at 37°C for 30 min followed by 80°C for 10 min. Sequencing reactions were performed in a 10 µl reaction mix containing 2 µl PCR product, 1 mM sequencing primer, 2 µl of BigDye dye terminator mix (Applied Biosystems, Foster City, CA, USA) and 1µl buffer (400 mM Tris, pH 9, 10 mM $MgCl_2$). Cycling conditions were: 60 cycles of 30 sec at 95°C, 20 sec at 50°C, and 4 min at 60°C. Sequencing reactions were cleaned using DTR Gel Filtration columns (Edge Biosystems, Gaithersburg, MD, USA) and analyzed on an ABI 3700 DNA analyzer (Applied Biosystems, Foster City, CA, USA).

Development of a BAC Insert End-Specific PCR Assay

Sequences derived from BAC insert ends were analyzed, and BAC vector sequences were removed manually. The resulting sequences were analyzed for human and other mammalian repeats using RepeatMasker web server (http://www.repeatmasker.org/cgi-bin/WEBRepeatMasker), and PCR primers were designed from non-repetitive sequences using Primer 3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi/) (Rozen and Skaletsky, 2000). Primer sequences developed are listed in Table 7.

The PCR reactions were performed in a MJ Dyad thermal cycler (MJ Research Inc., Waltham, MA, USA). 20 µl reaction mix contained 1 µl DNA, 1X PCR buffer (Applied Biosystems, Foster City, CA, USA) 1.5 mM $MgCl_2$, 25 pmol of each primer, 200 µM dNTPs and 3 U Taq DNA Polymerase (Promega, Madison, WI, USA). Cycling conditions were: one cycle of 1 min at 95ºC, 30 sec at 60ºC, 15 sec at 72ºC followed by 34 additional cycles of 30 sec at 94ºC, 30 sec at 62ºC, 15 sec at 72ºC and a final extension of 3 min at 72ºC.

BAC Fingerprinting Analysis

The positive BAC clones isolated through BAC library screening were subjected to restriction enzyme digest based fingerprinting analysis as described previously by Marra et al. (1997). Briefly, purified BAC DNA was digested with Hind III and electrophorized in a 1% agarose gel at 45V for 16 hrs, and stained with SYBR green (Applied Biosystems, Foster City, CA, USA). The gel was imaged using a molecular Dynamics Fluorimager (Amersham, Piscataway, NJ, USA). The resulting image was then transferred to a UNIX workstation for band calling and contig assembly. Restriction

fragment bands were identified using the program Image 3.3 and contigs were constructed using FPC (Finger Printed Contigs).

Table 7. Primer sequences used for BAC end-specific PCR assay.

Primer	Sequence 5'-3'	Length (bp)	PCR Product Size (bp)	Tm Used
329R-F	TTTCATTGCTTTTGCCTCCT	20	213	62°C
329R-R	GAACCCAAGGCTGGTGACT	19		62°C
069F-F	CCCAAGGTCATGGTCTGATT	20	103	62°C
069F-R	GGAACGTGACATTGCAGGTA	20		62°C
117F-F	CCTCCTTCTTAGCTGGGTTG	20	79	62°C
117F-R	GAAACTTGCTCGAGCCTCAT	20		62°C
117R-F	ACGTGCGGTCCTAAAAAGAC	20	85	60°C
117R-R	CCCCCAATAAACAAAAGTCA	20		60°C
370F-F	TTACTGCTGAGCCACACTGG	20	93	62°C
370F-R	CATGAAGCCATCACCACAAT	20		62°C
329F-F	GAACGTACGCCAGGCACT	18	109	62°C
329F-R	CCGTACTGGAGAAGCCAGAC	20		62°C
370R-R	CCTGGGGCTCTATCCTCTCT	20	150	62°C
370R-F	GGCCTTTCCTGTCATCCAG	18		62°C

Radiation Hybrid (RH) mapping

Radiation hybrid mapping was performed using INRA-Minnesota 7000 rads radiation hybrid panel (IMpRH), which consisted 118 hamster-porcine hybrid cell lines (Hawken et al. 1999; Yerle et al. 1998). The previously described kallikrein 4 primers were used to score the porcine RH map.

Each of the 118 clones in the RH panel was used as a template in PCR. The entire RH panel was scored twice by PCR twice for the kallikrein 4 primer pair. The PCR reactions were performed in a MJ Dyad thermal cycler. A 20µl reaction mix contained 1µl DNA,

1X PCR buffer (Applied Biosystems, Foster City, CA, USA) 1.5 mM $MgCl_2$, 25 pmol of each primer, 200 μM dNTPs and 3 U Taq DNA Polymerase (Promega, Madison, WI, USA). Cycling conditions were one cycle of 1 min at 95ºC, 30 sec at 62ºC, 15 sec at 72ºC followed by 34 additional cycles of 30 sec at 94ºC, 30sec at 62ºC, 15 sec at 72ºC and a final extension of 3 min at 72ºC according to the INRA protocols (http://www.toulouse.inra.fr/lgc/lgc.htm). PCR products were separated on 2% agarose gels with 500 ng/ml of ethidium bromide. No PCR product was obtained for the rodent genomic DNA. Data analysis was performed using software available at IMpRH database (http://imprh.toulouse.inra.fr) for chromosome assignment. Briefly, the radiation hybrid map is constructed using RHMAP 3.0 package. A multipoint analysis and ordering of the markers is performed on the positive clones submitted using the various ordering options of the RHAMXLIK program of RHMAP 3.0 (Hawken et al. 1999).

Results

No pre-existing mapping information was available for the pig kallikrein region. Except for KLK4 (isolated from pig dentine, Genbank accession # U76256) and porcine plasma kallikrein (Genbank accession # NM_214074) no other porcine kallikreins had been identified thus far. As a first step towards isolation, characterization and functional analysis of the porcine kallikrein gene family, overgo probes were developed for all known human kallikrein exonic sequences except kallikrein 11, using sequence information available at NCBI. Overgo probes were used to screen CHORI-242 porcine BAC library. Library screening identified 8 positive clones that contained putative kallikrein gene fragments (clones: 003F18, 069G11, 117C13, 266E15, 329F12, 338F22, 366F08, and 370C15) while clone 051O23 was selected in random as a negative control. Figure 4 depicts a representative BAC filter which contains 2 positive clones. The resulting clones selected were further confirmed by PCR using primers developed for pig kallikrein 4, which confirmed that out of the 8 positive clones selected, seven to have porcine kallikrein 4. (Figure 5). Insert ends of the selected BAC clones were isolated using vectorette PCR end rescue approach (Figure 6) and sequenced. Sequence Tagged Sites (STS) were developed from clone insert ends and were used to assemble the BAC clones into a contig (Figure 7). Hybridization experiments were carried out on individual BAC clones using individual overgo probes to identify the number of kallikreins on each clone (Figure 8). Subsequent sequence analysis of BAC insert ends and overgo-based hybridization experiments with human kallikreins resulted in a BAC clone-based physical map of the porcine greater kallikrein region (see Figure 9). The assembled contig of the pig kallikrein region containing 8 BAC clones provides ordering information for 12

STSs. Of the 12 STSs, seven correspond to BAC insert ends, five new tissue kallikrein genes in the pig and one to the previously identified pig kallikrein 4 gene.

To further confirm the relationship between individual clones in the contig and to identify a minimal tiling set of clones for sequencing the greater kallikrein region of the pig, all 8 BAC clones that represent the kallikrein region in the pig were subjected to restriction enzyme-based fingerprint analysis using a method developed at the Washington University Genome Sequencing Center (Marra et al. 1997). The resulting data were analyzed by FPC (Soderlund et al. 1997), which yielded one contig. The relative order and orientation of the FPC-generated, fingerprint-based contig and the PCR assay and hybridization based contig were identical. Based on the length of all non-redundant restriction fragments the total length of the contig is ~375 kb.

Based on the Human–Pig comparative map (Fronicke et al. 1996) we assumed that the pig kallikrein region may map to porcine chromosome 6. A PCR assay was developed for porcine kallikrein 4 (KLK4) and a pig-hamster radiation hybrid (RH) panel was scored twice to map the kallikrein region in the porcine genome. A representative PCR panel is shown in figure 10. PCR amplification can be seen in hybrid clones that contain kallikrein DNA. The results (Table 8) revealed that KLK4 marker was linked to first-generation markers of *Sus-scrofa* Chromosome 6 (SSC6) (Hawken et al. 1999) with LOD scores of greater than 7 (Table 9) by two point analysis confirming that the pig kallikrein region is clustered within pig chromosome 6q12-q21 (see Figure 11).

Figure 4

A representative BAC filter (#8) containing two positive clones (circled in red). The BAC filter only 22 X 22 cm in size contains 18,432 individual clones which are gridded in duplicate in specific patters to prevent calling of false positives and to help identify the clone address.

The 6.3X deep porcine BAC library was screened using overgo probes (table 4). The overgo probes were developed using human tissue kallikrein exonic sequences.

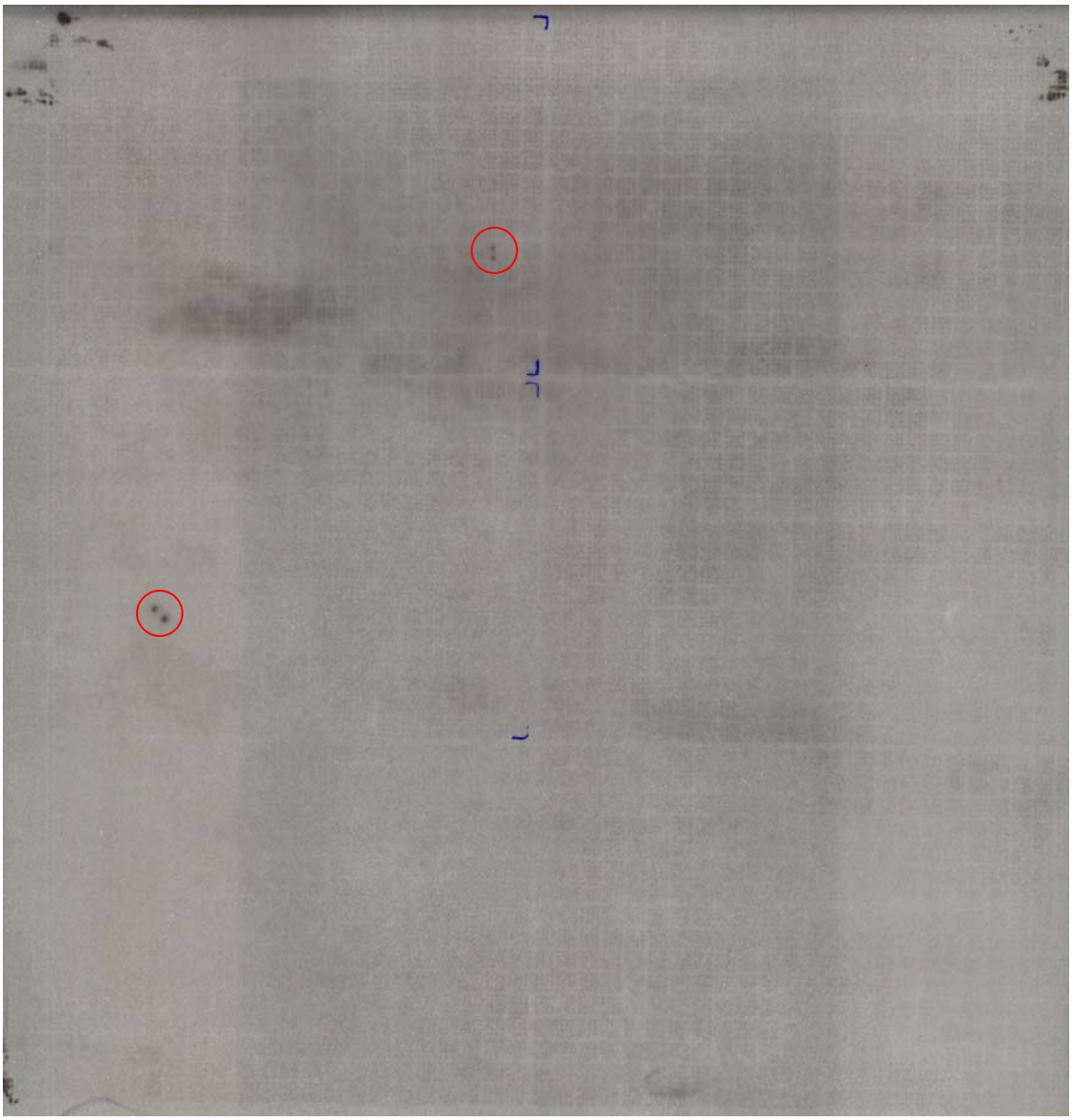


Figure 5

Ethidium bromide stained gel confirming that the positive BAC clones isolated to have porcine tissue kallikreins. DNA was extracted from the BAC clones selected and was PCR amplified using primers specific to pig kallikrein 4. PCR products were separated in a 2% agarose gel in TBE for 30 min at 90V. Name of each clone are given at the top of the figure. BAC clones identified from screening high-density BAC filters were tested by PCR for the presence of porcine kallikrein 4. kallikrein 4 was detected in clones 003F18, 069G11, 117C13, 266E15, 329F12, 338F22, 366F08, and absent in 370C15 and the negative control 051O23.

gDNA= genomic DNA.

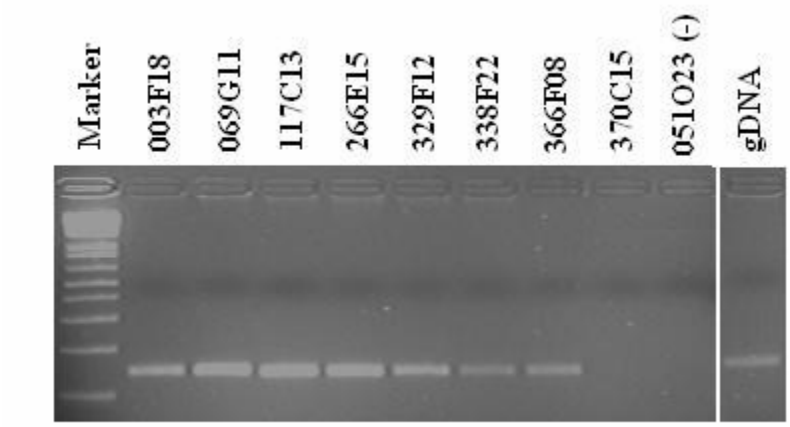

Marker
003F18
069G11
117C13
266E15
329F12
338F22
366F08
370C15
051O23 (-)
gDNA

Figure 6

Ethidium bromide statined gel depicting BAC clone ends isolated by vectorette PCR end rescue approach. BAC DNA was digested with Hae III or Pvu II and a vectorette duplex linker (Bubble) was ligated and PCR amplified using a vector based primer and universal primer developed for the ds DNA linker. PCR products were separated in a 1.2% agarose gel. Single-copy BAC ends obtained were TA cloned and sequenced. Name of each clone is given at the top of the figure. The sizes of the BAC ends isolated ranged from ~400 bp to ~1.2 kb.

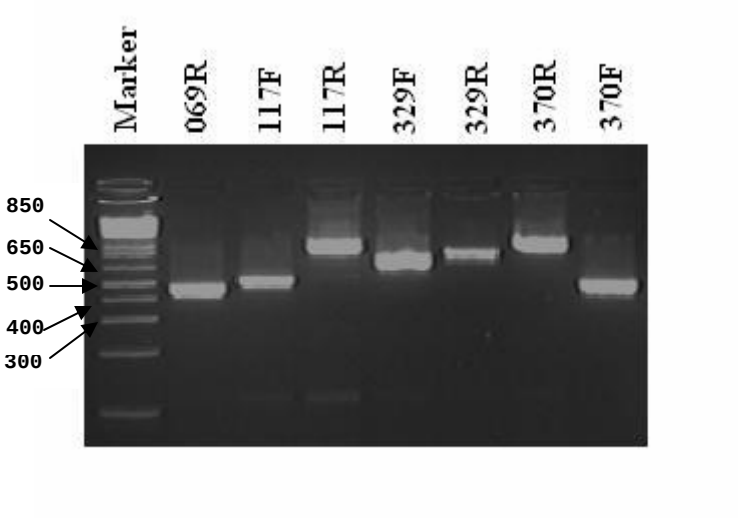

Marker
069R
117F
117R
329F
329R
370R
370F
850
650
500
400
300

Figure 7

PCR panel of the BAC end specific PCR assay. Sequence Tagged Sites (STS) were developed from BAC clone insert end sequences and PCR was performed on each BAC clone using all STS to assemble the BAC clones in to a contig. PCR products were separated in a 2% agarose gel in TBE for 30 min at 90V. The name of each BAC clone in the contig is given on the top of the panel, and the name of each BAC insert end specific STS is given in the vertical axis. “F” and “R” are arbitrarily assigned letters.
The negative control (51O23) did not show any amplification. The clone from which the STS was developed and genomic DNA was used as positive controls.

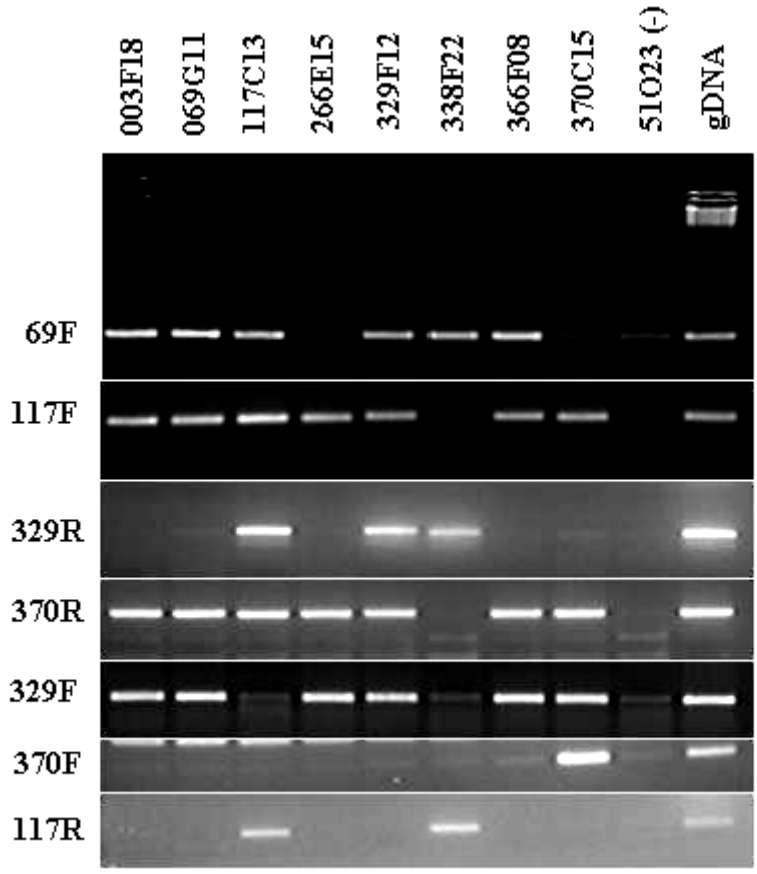
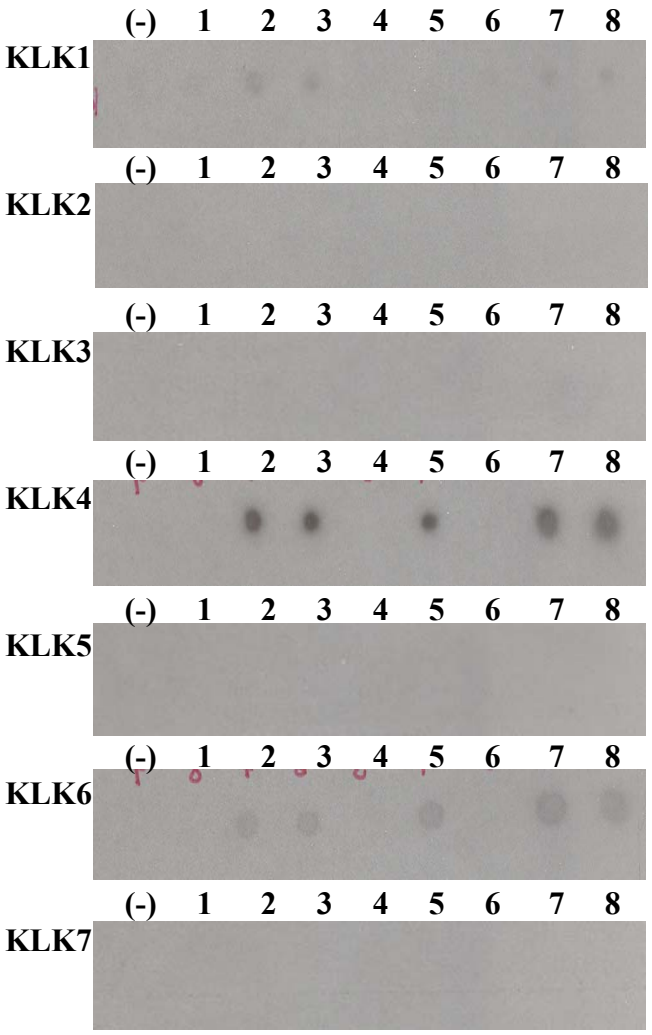

003F18
069G11
117C13
266E15
329F12
338F22
366F08
370C15
51O23 (-)
gDNA
69F
117F
329R
370R
329F
370F
117R

Figure 8

Hybridization experiments carried out on each BAC clone using individual overgo probes to identify the number of kallikreins on each clone (1-370C15, 2-366F08, 3-329F12, 4-306H02, 5-266E15, 6-256O23, 7-117C13, 8-069G11). The BAC clones were grown on a hybond nylon filter, lysed and were hybridized with individual overgo probes. The filter strips were washed and signals were detected by exposing the filter to Bio-Max max efficiency X-ray film for 10-12 hrs at -80^0C. Clones 003F18 and 338F22 were not tested as these two clones were identified in the second screening.

Signals were detected for KLK1, KLK4, KLK6, KLK9, KLK12 and KLK15. The presence of KLK2, KLK3, KLK5, KLK7, KLK8, KLK10, KLK13 and KLK14 could not be detected with the overgo probes we used. This may be because we developed probes from human kallikrein sequences and screened across species. Therefore the overgo probes that we designed for some of the kallikreins were not homologous to screen across species.

The negative clone 51O23 did not hybridize to any probe.


(-) 1 2 3 4 5 6 7 8
KLK1
(-) 1 2 3 4 5 6 7 8
KLK2
(-) 1 2 3 4 5 6 7 8
KLK3
(-) 1 2 3 4 5 6 7 8
KLK4
(-) 1 2 3 4 5 6 7 8
KLK5
(-) 1 2 3 4 5 6 7 8
KLK6
(-) 1 2 3 4 5 6 7 8
KLK7

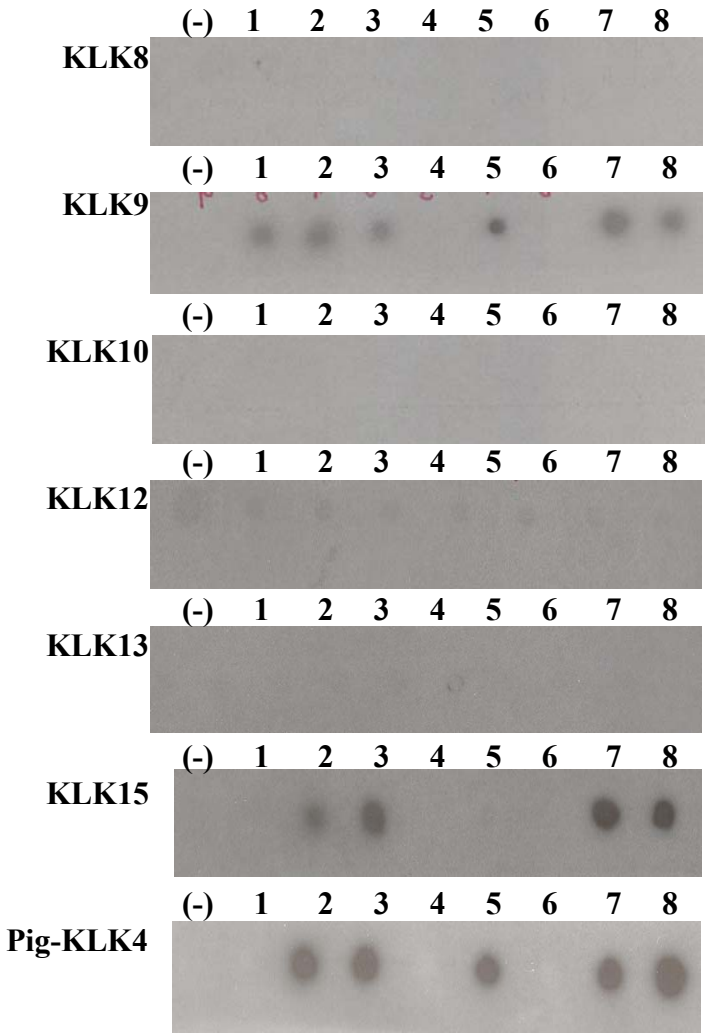

(-) 1 2 3 4 5 6 7 8
KLK8
(-) 1 2 3 4 5 6 7 8
KLK9
(-) 1 2 3 4 5 6 7 8
KLK10
(-) 1 2 3 4 5 6 7 8
KLK12
(-) 1 2 3 4 5 6 7 8
KLK13
(-) 1 2 3 4 5 6 7 8
KLK15
(-) 1 2 3 4 5 6 7 8
Pig-KLK4

Figure 9

BAC clone based physical map of the pig kallikrein region based on sequence analysis of BAC insert ends and overgo-based hybridization experiments with human kallikreins. Clones are depicted as horizontal lines. STSs are listed vertically along the top, with those depicted in red derived from clone insert ends, those in blue are new porcine tissue kallikrein genes. All STSs are not to scale. The size of each BAC clone is given in parenthesis next to the clone name. Unfilled squares on clones 003F18 and 338F22 show untested STSs.

Based on the physical map generated, clone 69G11 was selected to have all porcine tissue kallikreins genes.

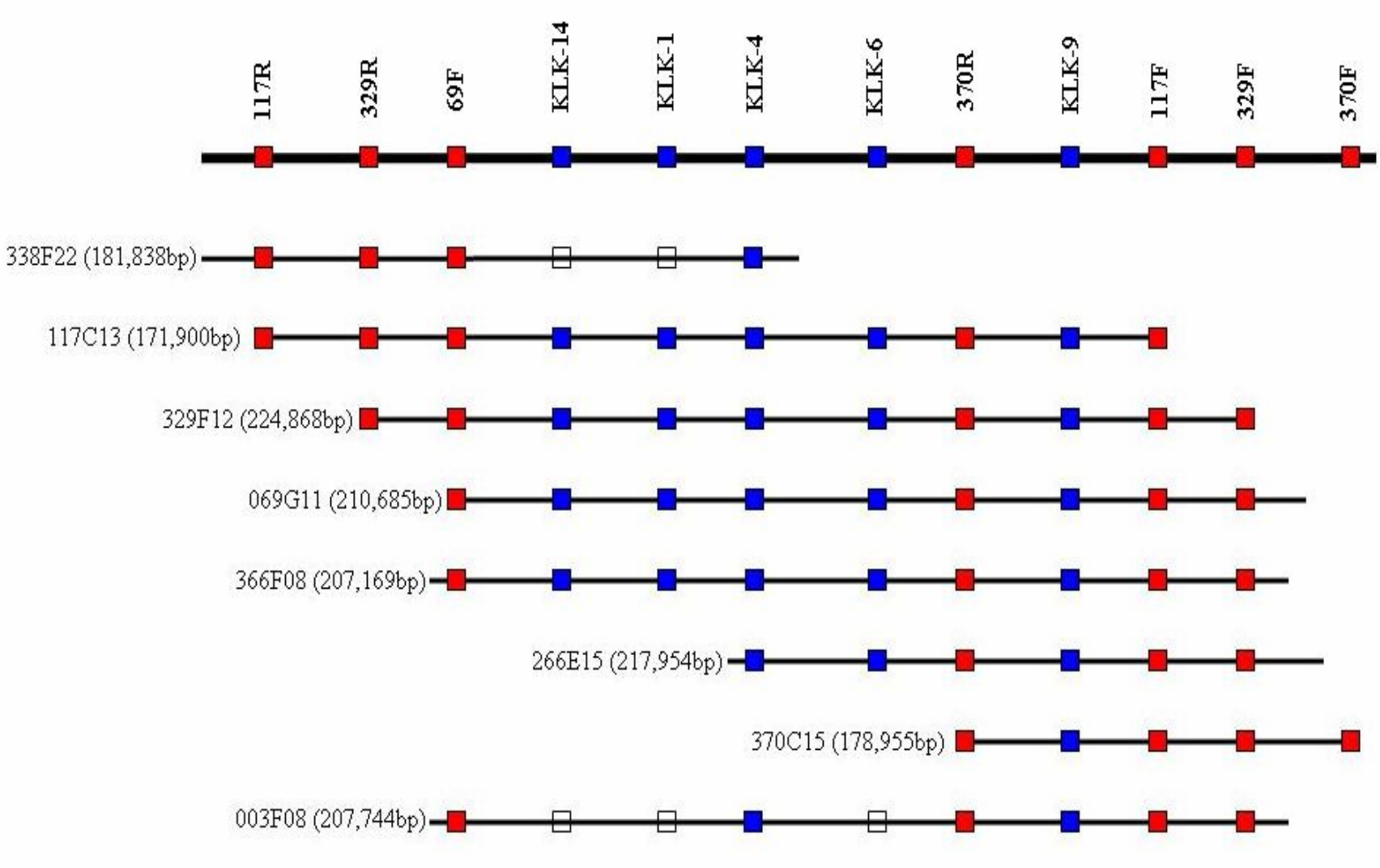

117R
329R
69F
KLK-14
KLK-1
KLK-4
KLK-6
370R
KLK-9
117F
329F
370F
338F22 (181,838bp)
117C13 (171,900bp)
329F12 (224,868bp)
069G11 (210,685bp)
366F08 (207,169bp)
266E15 (217,954bp)
370C15 (178,955bp)
003F08 (207,744bp)

Figure 10

A representative PCR panel showing results obtained by scoring the Pig-hamster radiation hybrid (RH) panel using porcine KLK4 primers. A PCR assay was developed for porcine kallikrein 4 (KLK4) and was used to analyze the pig-hamster radiation hybrid (RH) panel to map the kallikrein region in the pig. Each clone was scored twice using PCR. PCR amplification can be seen in hybrid clones that contain kallikrein DNA. The positive clones for both rounds of PCR (Table 6) were further analyzed using mapping software available at IMpRH database (http://imprh.toulouse.inra.fr) for chromosome assignment.

PLATE 1

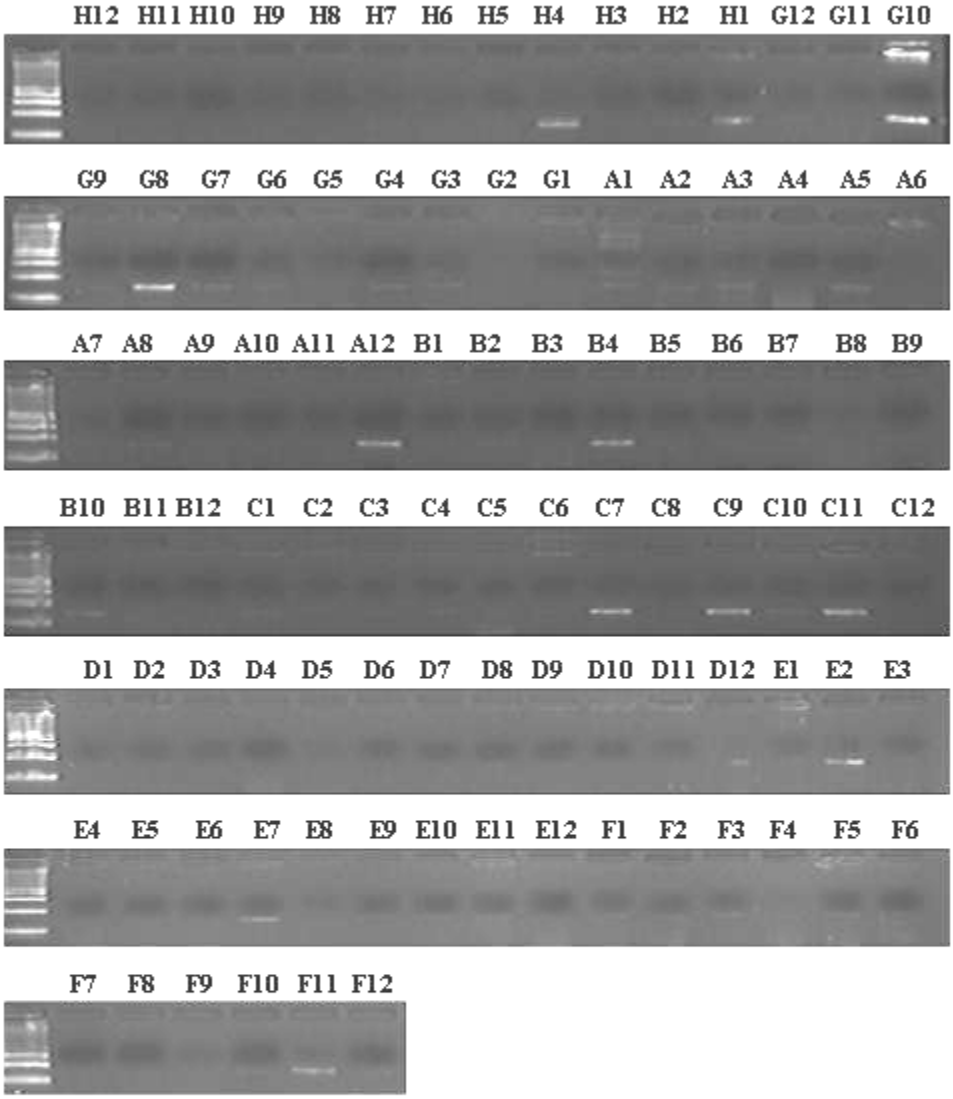


PLATE 2

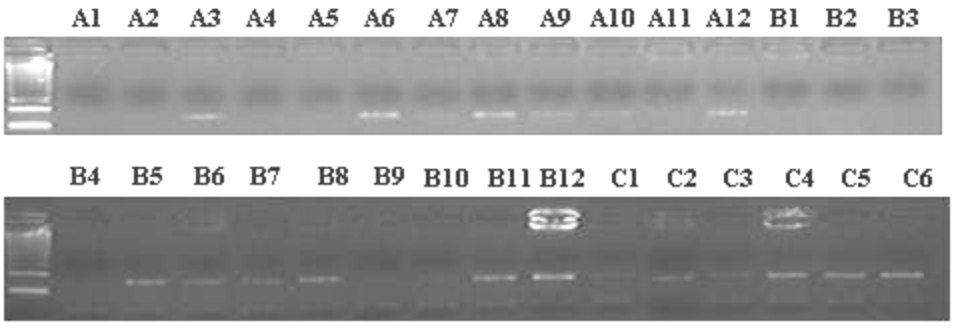


Table 8. Radiation Hybrid Mapping results. Each clone was scored twice using primers developed for porcine KLK4. The clones that were positive for both rounds of screening is shown with a "+" symbol.

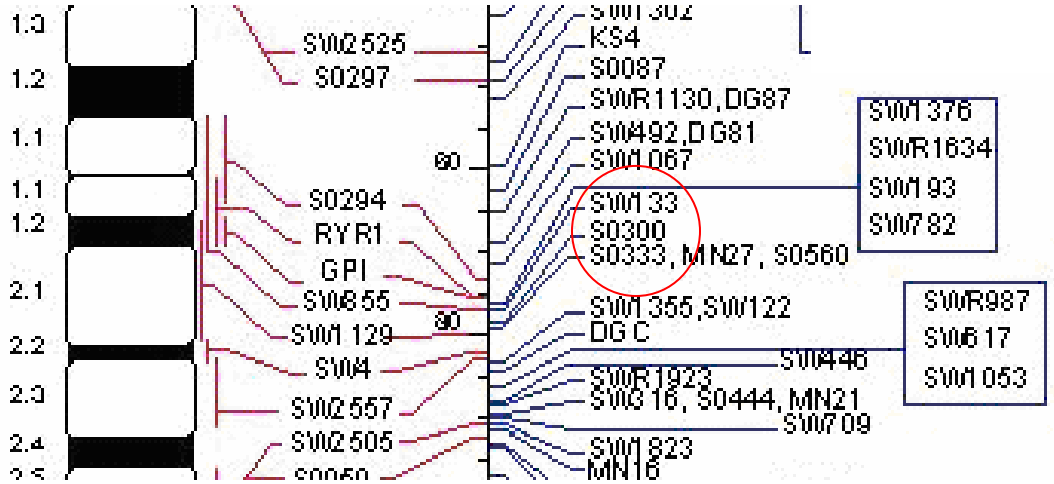
PLATE	WELL	IMpRH #	Result	PLATE	WELL	IMpRH #	Result	PLATE	WELL	IMpRH #	Result
1	A1	L1		1	D7	L43		1	H1	L84	+
1	A2	L2		1	D8	L44		1	H2	L85	
1	A3	L3		1	D9	L45		1	H3	L86	
1	A4	L4		1	D10	L46		1	H4	L87	+
1	A5	L5		1	D11	L47		1	H5	L88	
1	A6	L6		1	D12	L48	+	1	H6	L89	
1	A7	L7		1	E1	L49		1	H7	L90	
1	A8	L8		1	E2	L50	+	1	H8	L91	
1	A9	L9		1	E3	L51		1	H9	L92	
1	A10	L10		1	E4	L52		1	H10	L93	
1	A11	L11		1	E5	L53		1	H11	L94	
1	A12	L12	+	1	E6	L54		1	H12	L95	
1	B1	L13		1	E7	L55	+	2	A1	L96	
1	B2	L14		1	E8	L56		2	A2	L97	
1	B3	L15		1	E9	L57		2	A3	L98	+
1	B4	L16	+	1	E10	L58		2	A4	L99	
1	B5	L17		1	E11	L59		2	A5	F100	
1	B6	L18		1	E12	L60		2	A6	F101	+
1	B7	L19		1	F1	L61		2	A7	F102	
1	B8	L20		1	F2	L62		2	A8	F103	+
1	B9	L21		1	F3	L63		2	A9	F104	
1	B10	L22	+	1	F4	L64		2	A10	F105	
1	B11	L23		1	F5	L65		2	A11	F106	
1	B12	L24		1	F6	L66		2	A12	F107	+
1	C1	L25		1	F7	L67		2	B1	F108	
1	C2	L26		1	F8	L68		2	B2	F109	
1	C3	L27		1	F9	L69		2	B3	F110	
1	C4	L28		1	F10	L70		2	B4	F111	
1	C5	L29		1	F11	L71	+	2	B5	F112	+
1	C6	L30		1	F12	L72		2	B6	F113	+
1	C7	L31	+	1	G1	L73		2	B7	F114	+
1	C8	L32		1	G2	L74		2	B8	F115	+
1	C9	L33	+	1	G3	L75		2	B9	F116	
1	C10	L34		1	G4	L76		2	B10	F117	
1	C11	L35	+	1	G5	L77		2	B11	F118	+
1	C12	L36		1	G6	L78		2	B12	Positive	+
1	D1	L37		1	G7	L79		2	C1	Negative	
1	D2	L38		1	G8	L80	+	2	C2	Parent	+
1	D3	L39		1	G9	L81		2	C3	Parent	+
1	D4	L40		1	G10	Positive	+	2	C4	Parent	+
1	D5	L41		1	G11	L82		2	C5	Parent	+
1	D6	L42		1	G12	L83		2	C6	Parent	+

Table 9. The results obtained from Radiation Hybrid panel mapping software available at IMpRH database (http://imprh.toulouse.inra.fr). The results suggests that the porcine tissue kallikrein gene region shows high linkage to several markers on porcine chromosome 6 with LOD scores of 7 and higher.

Order	Chromosome	Marker	Retention fraction	Distance (ray)	LOD Score
1	6	S0333	22	0.24	11.61
2	6	S0300	20	0.22	11.89
3	6	SW782	16	0.28	10.18
4	6	S0220	20	0.33	9.37
5	6	SSC8E02	17	0.40	7.89
6	6	SW133	19	0.43	7.29
7	6	SE1129	25	0.45	7.06

Figure 11

Localization of the porcine kallikrein region to chromosome 6q1.2. KLK4 marker is linked to first-generation markers of *Sus-scrofa* Chromosome 6 (SSC6) (Hawken et al. 1999) with LOD scores of greater than 7 by two point analysis confirming that the pig kallikrein region is clustered within pig chromosome 6q1.2. The Radiation Hybrid (RH) map is compared with *Suc-scrofa* chromosome 6 (SSC6) Cytogenetic map. The kallikrein region is circled in red.



Cytogenetic map Radiation Hybrid map

Discussion

No pre-existing physical mapping data was available for the pig kallikrein gene region, and except for kallikrein 4 and porcine plasma kallikrein no other porcine kallikreins had been identified thus far.

The deduced physical map of the pig kallikrein region has an estimated length of 375 kb which is covered by the two clones 003F18 and 338F22 (Figure 8). This is comparable to the human kallikrein region which is estimated to be 300 kb (Yousef and Diamandis 2001). Based on the physical map and repeated hybridization studies, we have identified clone 069G11 (Figure 8) to contain all the kallikrein genes identified by us so far. Systematic and complete sequence analysis of this clone is underway. This clone is in the 2^{nd} phase of sequencing; based on the sequence information available clone 069G11 has 13 kallikrein genes (KLK1, KLK4, KLK5, KLK6, KLK7, KLK8, KLK9, KLK10, KLK11, KLK12, KLK13, KLK14, and KLK15) among which 12 are new tissue kallikreins in the pig.

The Radiation Hybrid mapping information indicates that kallikrein gene region has conserved synteny between human chromosome 19 (HSA19) and porcine chromosome 6 (SSC 6), and is consistent with the comparative mapping results obtained by bi-directional chromosome painting (Goureau et al. 1996). We have so far used only one marker to map the kallikrein gene region in the RH panel resulting in fairly low resolution. We are in the process of adding more kallikrein markers to the panel as sequencing results become available.

The physical map generated in this study would be an invaluable resource in further understanding of the role of kallikreins in the pig. Novel porcine tissue kallikrein genes

identified will help us to evaluate the expression of tissue kallikreins in the porcine endometrium and conceptus during the estrus cycle and pregnancy to help us better understand the role of tissue kallikreins in placental development and embryonic survival throughout gestation in the pig.

The tissue kallikrein loci has been extensively investigated in the human, rat and the mouse, which has revealed surprising differences in the number of classical glandular kallikrein genes, where the human has 3 and the mouse has 25, 14 of which are functional and 12 in the rat (Olsson et al. 2004). But, the most striking difference is the size of the region between KLK1 and KLK15, which is only 1.5 kb in humans, but is 290 kb in the mouse and contains 23 classical glandular kallikrein genes, suggesting that the rodent kallikreins have evolved after the human rodent split. Also the phylogenetic analysis of human, mouse and rat classical kallikreins revealed that KLK1 segregated according to species, suggesting that the duplications of KLK1 in the mouse and the rat occurred after the separation of species.

The kallikrein gene family is unique from most other gene families, as in most other gene families the members are spread out throughout the genome, but kallikreins have always been found as cluster in a single chromosome. Gene family expansion is a fundamental process in the evolution of species. On average each gene is thought to be duplicated once every 100 million years, but the half life of a duplicated gene is only 4 million years because of the high rate of silencing (Olsson et al. 2004). “Independent duplication of the same gene has been shown to have evolutionary instability and usually move to the telomere region of different chromosomes, therefore the duplicated gene segments tend to be localized in pericentromeric or subtelomeric regions” (Olsson et al. 2004). However,

that is not the case with the tissue kallikreins as kallikrein genes were formed by tandem duplications and are located in a single chromosomal region. (Olsson et al. 2004). Therefore, the kallikrein gene family is a very interesting gene family that has evolved recently, but what evolutionary pressure keeps the kallikrein family clustered together is still unknown. It is believed that among the human kallikreins KLK4-KLK15 are single copy genes while the classical glandular kallikreins KLK1 and KLK2 display varying number of copies among species. Therefore, the mapping and sequencing data generated for the porcine greater kallikrein gene region should provide insight into the evolutionary origins of this structurally complex genomic region.

Reference List

Bazer, F.W., R.D. Geisert, W.W. Thatcher, and R.M. Roberts. 1982. The establishment and maintenance of pregnancy. In *Control of pig Reproduction* (ed. D.J.A.Cole and G.R.Foxcroft), pp. 227-252. Butterworth Scientific.

Bazer, F.W. and W.W. Thatcher. 1977. Theory of maternal recognition of pregnancy in swine based on estrogen controlled endocrine versus exocrine secretion of prostaglandin $PGF_{2\alpha}$ by the uterine endometrium. Prostaglandins **14**: 397-401.

Bhoola, K.D., C.D. Figueroa, and K. Worthy. 1992. Bioregulation of kinins - Kallikreins, Kininogens, and Kininases. Pharmacological Reviews **44**: 1-80.

Bost, F., M. Diarra-Mehrpour, and J.P. Martin. 1998. Inter-alpha-trypsin inhibitor proteoglycan family - A group of proteins binding and stabilizing the extracellular matrix. European Journal of Biochemistry **252**: 339-346.

Dantzer, V. 1985. Electron-microscopy of the initial-stages of placentation in the pig. Anatomy and Embryology **172**: 281-293.

DeSilva, U. Comparative mapping and sequencing of human chromosome 7 and mouse chromosome 5. 1998. Washington University; Department of genetics. Thesis/Dissertation

Ford, S.P., R.K. Christenson, and J.J. Ford. 1982. Uterine blood-flow and uterine arterial, venous and luminal concentrations of estrogens on days 11, 13 and 15 after estrus in pregnant and nonpregnant sows. Journal of Reproduction and Fertility **64**: 185-190.

Fronicke, L., B.P. Chowdhary, H. Scherthan, and I. Gustavsson. 1996. A comparative map of the porcine and human genomes demonstrates ZOO-FISH and gene mapping-based chromosomal homologies. Mammalian Genome **7**: 285-290.

Gan, L., I. Lee, R. Smith, R. Argonza-Barrett, H. Lei, J. McCuaig, P. Moss, B. Paeper, and K. Wang. 2000. Sequencing and expression analysis of the serine protease gene cluster located in chromosome 19q13 region. Gene **257**: 119-130.

Gao, X.X., L.M. Greenbaum, V.B. Mahesh, and D.W. Brann. 1992. Characterization of the kinin system in the ovary during ovulation in the rat. Biology of Reproduction **47**: 945-951.

Geisert, R.D., J.W. Brookbank, R.M. Roberts, and F.W. Bazer. 1982a. Establishment of pregnancy in the pig . 2. Cellular remodeling of the porcine blastocyst during elongation on day 12 of pregnancy. Biology of Reproduction **27**: 941-955.

Geisert, R.D., M.J. Dixon, T. Pratt, R.A. M.Schmitt, B.A. Lessley, and J.P. Mcann. 1995. Isolation and characterization of a 30-kDa endometrial glycoprotein synthesized during the estrous cycle and early pregnancy of the pig. Biology of Reproduction **53**: 1038-1050.

Geisert, R.D., R.H. Renegar, W.W. Thatcher, R.M. Roberts and F.W. Bazer 1982b. Establishment of pregnancy in the pig. I. Interrelationships between peri-implantation development of the pig blastocyst and the uterine endometrial secretions. Biology of Reproduction **27**: 925-939

Geisert, R.D. and J.V. Yelich. 1997. Regulation of conceptus development and attachment in pigs. Journal of Reproduction and Fertility **52**: 133-149.

Geisert, R.D., J.V. Yelich, T. Pratt, and D. Pomp. 1998. Expression of an inter-alpha-trypsin inhibitor heavy chain-like protein in the pig endometrium during the estrous cycle and early pregnancy. Journal of Reproduction and Fertility **114**: 35-43.

Geisert, R.D., M.T. Zavy, R.J. Moffatt, and Blair R.M. 1990. Embryonic steroids and the establishment of pregnancy in pigs. Journal of Reproduction and Fertility Supplement **40**: 293-305.

Goureau, A., M. Yerle, A. Schmitz, J. Riquet, D. Milan, P. Pinton, G. Frelat, and J. Gellin. 1996. Human and porcine correspondence of chromosome segments using bidirectional chromosome painting. Genomics **36**: 252-262.

Hawken, R.J., J. Murtaugh, G.H. Flickinger, M. Yerle, A. Robic, D. Milan, J. Gellin, C.W. Beattie, L.B. Schook, and L.J. Alexander. 1999. A first-generation porcine whole-genome radiation hybrid map. Mammalian Genome **10**: 824-830.

Hashimoto, K., T. Tabe, J. Sumiya, Y. Sano, N. Chio-Miura, A. Ozawa, H. Yasue, M. Tomita. 1996. Primary structure of the pig homologue of human IHRP: Inter-α-trypsin inhibitor family heavy chain-related protein. Journal of Biochemistry **119**: 577-584.

Keys, J.L. and G.J. King. 1990. Microscopic examination of porcine conceptus-maternal interface between days 10 and 19 of pregnancy. American Journal of Anatomy **188**: 221-238.

King, G.J., B.A. Atkinson, and H.A. Robertson. 1982. Implantation and early placentation in domestic ungulates. Journal of Reproduction and Fertility supplement **31**: 17-30.

Marra, M.A., T.A. Kucaba, N.L. Dietrich, E.D. Green, B. Brownstein, R.K. Wilson, K.M. McDonald, L.W. Hillier, J.D. McPherson, and R.H. Waterston. 1997. High throughput fingerprint analysis of large-insert clones. Genome Research **7**: 1072-1084.

Olsson, A.Y., Lilja, H., and Lundwall, A. 2004. Taxon specific evolution of glandular kallikrein genes and identification of progenitor of prostate-specific antigen. Genomics **84**: 147-156.

Roberts, R.M., W.E. Trout, N. Mathialagan, M. Stallings-Mann, and P. Ling. 1993. Uterine secretory activity and embryonic development. In *Preimplantation Embryo Development* (ed. B.D.Bavister), pp. 229-243. New York:Springer-Verlag.

Rozen, S. and H.J. Skaletsky. 2000. Primer3 on WWW for general users and for biologist programmers. In *Bioinformatics methods and protocols: Methods in molecular biology* (ed. S.Krawetz and S.Misener), pp. 365-386. Humana Press, Totowa, NJ.

Ryu, O., J.C.C.Hu, Y. Yamakoshi, J.L. Villemain, X.H. Cao, C.H. Zhang, J.D. Bartlett, and J.P. Simmer. 2002. Porcine kallikrein-4 activation, glycosylation, activity, and expression in prokaryotic and eukaryotic hosts. European Journal of Oral Sciences **110**: 358-365.

Salier, J.P., P. Rouet, G. Raguenez, and M. Daveau. 1996. The inter-alpha-inhibitor family: From structure to regulation. Biochemical Journal **315**: 1-9.

Sambrook, J. and D.W. Russel. *Molecular cloning: a laboratory manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Soderlund, C., I. Longden, and R. Mott. 1997. FPC: A system for building contigs.

Stroband, H.W.J. and T. Van der Lende. 1990. Embryonic and uterine development during early pregnancy in pigs. Journal of Reproduction and Fertility Supplement **40**: 261-277.

Valdes, G., C.D. Figueroa, and J. Corthorn. 1996. Temporospatial changes of kallikrein-like enzymes during the estrous cycle and pregnancy in the rat uterus. Biology of Reproduction **55**: 236-245.

Vonnahme, K.A., S.C. Fernando, J.W. Ross, M.D. Ashworth, U. DeSilva, J.R. Malayer, and R.D. Geisert. 2004. Porcine endometrial expression of kininogen, factor XII, and plasma kallikrein in cyclic and pregnant gilts. Biology of Reproduction **70**: 132-138.

Vonnahme, K.A., J.R. Malayer, H.O. Spivey, S.P. Ford, A. Clutter, and R.D. Geisert. 1999. Detection of kallikrein gene expression and enzymatic activity in porcine endometrium during the estrous cycle and early pregnancy. Biology of Reproduction **61**: 1235-1241.

Yerle, M., P. Pinton, A. Robic, A. Alfonso, Y. Palvadeau, C. Delcros, R. Hawken, L. Alexander, C. Beattie, L. Schook, D. Milan, and J. Gellin. 1998. Construction of a whole-genome radiation hybrid panel for high-resolution gene mapping in pigs. Cytogenetics and Cell Genetics **82**: 182-188.

Yousef, G.M. and E.P. Diamandis. 2001. The new human tissue kallikrein gene family: Structure, function, and association to disease. Endocrine Reviews **22**: 184-204.

CHAPTER III

Tissue Kallikrein Expression in the Porcine Endometrium and Conceptus

Introduction

During early pregnancy in the pig, the conceptus undergoes a rapid trophoblastic elongation from a 10 mm sphere to a long filamentous thread-like structure (Geisert et al. 1982a,b), and initiates attachment to the uterine epithelium on day 12 of pregnancy (Stroband et al. 1990; Geisert et al. 1997). During attachment of the conceptus to the uterine epithelium a localized increase in transcapillary transport (Keys et al. 1998) and uterine blood flow occurs (Ford et al. 1982) concurrently with the establishment of pregnancy in the pig (Bazer et al. 1982; Geisert et al. 1997).

Many of the endometrial responses that occur during conceptus development resembles the acute-phase responses induced during tissue inflammation (Sailer et al. 1996; Geisert et al. 1997). In the pig, tissue kallikrein enzymatic activity, protein and gene expression has been detected during the estrous cycle and early conceptus development (Vonnahme et al. 1999; Geisert et al. 2001), suggesting that the kallikrein-kininogen-kinin system is active in the pig.

Kallikreins have been implicated to code for proteins that activate a wide range of substrates and growth factors (Chan et al. 1999), suggesting that kallikreins are involved in many integral processes of early embryonic development such as regulation of local blood flow, angiogenesis, tissue invasion, and mitogenesis (Bhoola et al. 1992). In the rat, the kallikrein-kininogen-kinin system is activated in the ovary during ovulation (Brann et al. 1995). In the mouse 5 tissue kallikrein genes are implicated to play a major role in uterine physiological functions and during embryo implantation (Valdes et al. 1996; Corthorn et al. 1997; Chan et al. 1999). The detection of kallikrein enzymatic activity and gene expression in the porcine uterine lumen (Vonnahme et al. 1999), as well

as the detection of the substrate of kallikrein, low molecular weight (LMW) kininogen, suggests the presence of an active kallikrein-kininogen-kinin system in the porcine uterus during early pregnancy.

A clear temporal association between porcine conceptus estrogen release and an increase in uterine blood flow has been detected (Ford. 1995). The bradykinins released through proteolytic cleavage of LMW kininogen during early embryonic development could be involved in the increase of uterine blood flow, vascular permeability, prostaglandin release, and changes to uterine tone (Keys et al. 1990; Ford. 1995; Allen et al. 2002). Recent studies have shown that uterine tissue kallikrein is involved in cleavage of insulin-like growth factor binding proteins on day 12 in cyclic and pregnant gilts (Geisert et al. 2001), suggesting that several kallikreins may be present during early embryonic development in the pig. It is possible that several uterine tissue kallikreins may be involved with extracellular matrix remodeling, insulin-like growth factor binding protein degradation, and bradykinin release during early embryonic development in the pig (Geisert et al. 1998 & 2001).

Therefore, as a first step towards understanding the tissue expression of porcine tissue kallikreins during reproductive events, and their role in placental development and embryonic survival in the pig, we have isolated two novel porcine kallikrein genes using RT-PCR and conserved sequence information from orthologous human kallikrein genes and have carried out quantitative real-time PCR based, expression analysis to evaluate the expression of tissue kallikrein 1, 4 and 14 in the porcine endometrium and conceptus during the estrus cycle and early embryonic development.

Materials and Methods

Sample collection

Cyclic, crossbred gilts of similar age (8-10 mo) and weight (100-130 kg) were checked twice daily for the onset of estrus behavior by intact boars. Onset of estrus was considered day 0 of the estrus cycle. Gilts assigned for mating were bred naturally with fertile boars 12h after the onset of estrus and again 12 hrs later. Cyclic gilts (n=20) were hysterectomized on either day 0, 5, 10, 12, 15 or 18 of the estrous cycle, whereas pregnant gilts (n=16) were hysterectomized on either day 10, 12, 15 or 18 as previously described by Gries et al. (1989). Following initial induction of anesthesia with a 1.5 ml i.m. administration of a cocktail consisting of 2.5 ml Rompum (xylazine; 100 mg/ml; Inc., Shawnee Mission, KS) and 2.5 ml Vetamine (ketamine HCl; 100 mg/ml ; Mallickrodt Veterinary, Mundelein, IL) in 500 mg of Telazol (tiletamine HCl and zolazepam HCl; Fort, Dodge, Syracuse, NE). Anesthesia was maintained with a closed circuit system of halothane (Halocarbon Laboratories, Riveredge, NJ) and oxygen (1.0 L/min). After exposure by midventral laparotomy, the uterine horns and embryos were surgically removed. All animal procedures were in accordance with the International Guiding Principles for biomedical Research Involving Animals as promulgated by the Society for the Study of Reproduction and were approved by the Institutional Animal Care and Use Committee.

Evaluation of kallikrein 4 (KLK4) Gene Expression

RNA Extraction from Endometrium tissue(TRIzol method)

4 ml of TRIzol™ (Invitrogen, Carlsbad, CA, USA) was added to 1 g of tissue and was homogenized using a tissuemizer. This was incubated at room temperature for 5 min. Chloroform (1 ml) was added to the sample and incubated at room temperature for 3 min. After incubation the sample was centrifuged at 4ºC for 30 min at 7000 rpm. The aqueous phase was transferred into a fresh tube, 2.5 ml of isopropyl alcohol was added, plased in a -80ºC freezer for 20 min and centrifuged at 4ºC for 30 min at 12,000 rpm. Supernatant was discarded and the pellet was washed with 3 ml of 75 % ethanol, ethanol was removed and the pellet was air-dried for 5 min and resulting pellet was resuspended in 500 µl of DEPC treated water. This RNA sample was subjected to phenol chloroform extraction. Briefly, phenol (saturated with 1 M Tris-Cl): chloroform: isoamyl alcohol (pH 5.2) 25:24:1 was added at 1:1 ratio to the total RNA and mixed well. Following mixing the sample was centrifuged at 14,000 rpm for 5 min. The supernatant was collected to a fresh tube and 2.5 volumes of 98 % ethanol and 0.1 volume of sodium acetate was added, and placed in a -80ºC freezer for 30 min. The resulting RNA was precipitated by centrifuging at 4ºC for 30 min at 14,000 rpm. Pellet obtained was washed with 70 % ethanol and resuspended in 100 µl of DEPC treated water. The OD of the RNA samples were measured and 5 µg of RNA was run on a 1.25 % denaturation gel .

RNA Extraction from Conceptus tissue (RNAWIZ™)

Ten volumes of RNAWIZ™ (Ambion, Austin, TX, USA) was added to 1 volume of conceptus tissue which was vortexed until the tissue was completely homogenized. This

solution was incubated at room temperature for 5 min, 0.2X starting volume of chloroform was added to the homogenate, vortexed for ~20 sec and then incubated at room temperature for 10 min. After incubation the sample was centrifuged at 4ºC for 30 min at 15,000 rpm. The aqueous phase was transferred into a fresh tube and 0.5X Starting volume of RNase-free water was added and mixed well. To this solution 1X Starting volume of isopropanol was added and was incubated at -80ºC for 30 min and was centrifuged at 4ºC for 15 min at 15,000 rpm. The supernatant was discarded and the pellet was washed with 3 ml of 75 % ethanol. The ethanol was removed and the pellet was air-dried for 5 min. The resulting pellet was resuspended in 50 µl of DEPC treated water.

DNase Treatment

1 µl of 10X DNase I reaction buffer and 2 µl of Amp Grade DNase I (1U/µl) (Invitrogen, Carlsbad, CA, USA) was added to 2 µg of total RNA. The volume was brought up to 10 µl by adding DEPC-treated water and was incubated at room temperature for 15 min. 1 µl of 25 mM EDTA was added to this reaction mix and was heated to 65ºC for 10 min to inactivate the DNase.

cDNA Synthesis

A thin walled PCR tube containing 5 µg of total RNA, 500 ng oligo dT, 750 µM dNTP mix was heated at 65ºC for 5 min and quickly chilled on ice. To this reaction mix, 4 µl of 5X first strand buffer (Invitrogen, Carlsbad, CA, USA), 2 µl of 0.1 M DTT and 40 U RnaseOUT (Invitrogen, Carlsbad, CA, USA) was added and incubated at 42ºC for 2 min. 200 U of SUPERSCRIPT II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) was

added and the resulting reaction mix was incubated at 42ºC for 50 min and was heated to 70ºC for 15 min.

PCR Amplification

Forward (5′ GTGGCTTCTGAGGAGGTCTG 3′) and reverse (5′ GCCTGGATGGTCGTCTGTAT 3′) primers were developed (spanning an intron) for PCR amplification using know porcine kallikrein 4 sequence (GenBank accession # U76256) using Primer3 software (Rozen and Skaletsky 2000). The product size was 245 bp.

The KLK4 primer set developed was optimized and used to evaluate gene expression in pregnant (d10, d12, d15 and d18) and cyclic (d0, d5, d10, d12, d15, and d18) endometrium using RT-PCR. Gene expression of KLK4 was also evaluated in conceptus tissue (d12, d14, and d16) using RT-PCR. The PCR reactions were performed in a MJ Dyad thermal cycler (MJ Research Inc., Waltham, MA, USA). 20 µl reaction mix contained 1 µl cDNA, 1X PCR buffer (Applied Biosystems, Foster City, CA, USA) 1.5 mM $MgCl_2$, 25 pmol of each primer, 200 µM dNTPs and 3 U Taq DNA Polymerase (Promega, Madison, WI, USA). Cycling conditions were: one cycle of 1 min at 95ºC, 30 sec at 63.7ºC, 30 sec at 72ºC followed by 34 additional cycles of 30 sec at 94ºC, 30 sec at 63.7ºC, 30 sec at 72ºC and a final extension of 3 min at 72 ^{0}C.

The resulting PCR products were electrophorized in a 1.5% agarose (Fischer, Pittsburgh, PA, USA) gel and stained with 5 µl of ethidium bromide (10mg/ml). The PCR products visualized on the gel were cut and the DNA was extracted from the gel fragment.

Gel Extraction

The DNA was extracted from the gel fragment using the Qiagen gel extraction kit (Qiagen, Valencia, CA, USA) according to manufacturer's protocols. Briefly, 3 volumes of buffer QG was added to the gel fragment and was incubated at 50ºC for 10 min (until the gel slice was completely dissolved). To the dissolved gel 1 volume of isopropanol was added and the sample was placed in a QIAquick column. The sample was centrifuged at 13,000 rpm for 1 min. The flow-through was discarded and 0.75 ml of buffer PE was added to the sample. The sample was centrifuged twice at 13,000 rpm for 1 min. The column was placed in a fresh tube and the DNA was eluted using 30 µl of dH_2O. The gel purified DNA was used as template for PCR. The PCR product obtained was TA cloned and sequenced to confirm its identity.

TA Cloning

The PCR product was TA cloned using TOPO® TA cloning kit according to manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). Briefly, fresh PCR products were ligated to vector pCR®2.1-TOPO® by incubating at room temperature for 20 min. The 6 µl ligation mix contained 2 µl of fresh PCR product, 1 µl of pCR®2.1-TOPO® vector, 1 µl of salt solution, and 2 µl of water. Two microliters of the ligated PCR product was added to 25 µl vial of chemically competent cells and incubated on ice for 30 min. The cells were heat-shocked for 30 sec at 42ºC without shaking. SOC medium (250 µl) was added to the heat-shocked cells and incubated at 37ºC for an hour on a shaker (225 rpm). Each transformation was spreaded on YT plates containing X-gal (40 mg/ml) and

ampicillin (50 μg/ml). The plates were incubated at 37ºC overnight. Positive white clones were selected and further analyzed using cell burst PCR.

"Cell Burst" PCR

A small sample of a bacterial colony was collected on a toothpick, mixed in 30 μl of water and heated at 95ºC for 5 min to lyse cells. One microliter of this cell lysis was used for PCR using specific primers to select positive clones containing the fragment of interest.

Sequencing Reaction

The PCR products generated through cell burst-PCR were treated with shrimp alkaline phosphatase (1u μl^{-1}) and Exonuclease III (10u μl^{-1}) at a volume equaling 5% of the PCR product and was incubated at 37 °C for 30 min followed by 80°C for 10 min. Sequencing reactions were performed in a 10 μl reaction mix containing 2 μl PCR product, 1 mM sequencing primer, 2 μl of BigDye dye terminator mix (Applied Biosystems, Foster City, CA, USA) and 1μl buffer (400 mM Tris, pH 9, 10 mM $MgCl_2$). Cycling conditions were: 60 cycles of 30 sec at 95°C, 20 sec at 50°C, and 4 min at 60°C. Sequencing reactions were cleaned using DTR Gel Filtration columns (Edge Biosystems, Gaithersburg, MD, USA) and analyzed on an ABI 3700 DNA analyzer (Applied Biosystems, Foster City, CA, USA). The sequences generated were analyzed using Basic Local Alignment Search Tools (BLAST) available at NCBI.

Evaluation of Kallikrein 1 (KLK1) and 14 (KLK14) Gene Expression

As sequence information was not available for pig KLK1 and KLK14, homology searches were carried out at NCBI (http://www.ncbi.nlm.nih.gov) and TIGR (The Institute of Genomic Research; http://www.tigr.org/) database using published pig KLK4 sequence. Homologous sequences identified were used to develop primers based on human-pig homology using primer 3 software (Rozen and Skaletsky 2000). Table 10 shows the primer sequences used for RT-PCR.

Table 10. Primer sequences used for PCR amplification of KLK1 and KLK14 using RT-PCR.

Primer	Sequence 5'-3'	Length (bp)	PCR Product Size (bp)	Tm Used
P KLK-1 F	GGACTACAGCCACGACCTCATGCTGC	26	297	62°C
P KLK-1 R	CACCCATACAGGTGTCCTTGCCACCT	26		
P KLK-14 F	ATGTTCCTCCTGCTGACAGCACTTCA	26	321	60°C
P KLK-14 R	GTGGGTCCGGGAGTTGTACT	20		

The primer sets developed were optimized and used to evaluate gene expression in pregnant (d10, d12, d15 and d18) and cyclic (d0, d5, d10, d12, d15, and d18) endometrium using RT-PCR. Gene expression of KLK1 and KLK14 were also evaluated in conceptus tissue (d12, d14, d16) using RT-PCR. The PCR reactions were performed in a MJ Dyad thermal cycler (MJ Research Inc., Waltham, MA, USA). For KLK1 a 20 µl reaction mix contained 1 µl cDNA, 1X PCR buffer (Applied Biosystems, Foster City, CA, USA) 1.5 mM $MgCl_2$, 25 pmol of each primer, 200 µM dNTPs and 3 U Taq DNA Polymerase (Promega, Madison, WI, USA). Cycling conditions were: one cycle of 1 min

at 95ºC, 30 sec at 62ºC, 30 sec at 72ºC followed by 34 additional cycles of 30 sec at 94ºC, 30 sec at 62ºC, 30 sec at 72ºC and a final extension of 3 min at 72 ^{0}C.

For KLK14 Advantage-GC cDNA PCR amplification kit was used (BD Biosciences, Palo Alto, CA, USA). A 25 µl reaction mix contained 1 µl cDNA, 1X GC cDNA PCR reaction buffer (BD Biosciences, Palo Alto, CA, USA), 1 M GC melt (BD Biosciences, Palo Alto, CA, USA), 25 pmol of each primer, 200 µM dNTPs and 1X Advantage-GC cDNA Pol. mix (BD Biosciences, Palo Alto, CA, USA). Cycling conditions were: one cycle of 1 min at 95ºC, 30 sec at 60ºC, 30 sec at 68ºC followed by 34 additional cycles of 30 sec at 94ºC, 30 sec at 60ºC, 30 sec at 68ºC and a final extension of 3 min at 72 ^{0}C.

The resulting PCR products were electrophorized in a 1.5% agarose (Fischer, Pittsburgh, PA, USA) gels and stained with 5 µl of ethidium bromide (10 mg/ml). The PCR products visualized on the gel were cut and the DNA was extracted from the gel fragment as previously described, PCR amplified and sequenced to confirm its identity.

Quantitative Real Time PCR

Endometrial and conceptus gene expression for KLK1, KLK4, and KLK14 was quantified using one-step reverse transcriptase-polymerase chain reaction (RT-PCR) after DNase treatment. One step qRT-PCR kit (Eurogentec, San Diego, CA, USA) was used for quantitative gene expression analysis. Primers and probes for KLK1, KLK4, and KLK14 were developed using available Genbank sequence for KLK4 and KLK1 and KLK14 sequences identified through RT-PCR using primer 3 software (Rozen and Skaletsky 2000). All dual-labeled probes were designed to have an intron in the middle with a 5' reporter dye (6-FAM) and a 3' quenching dye (TAMRA) (table 11). The qRT-

PCR reactions were performed in an ABI PRISM 7500 sequence detection system (Applied Biosystems, Foster City, CA). Total reaction volume of 25 µl contained 400 nM forward primer, 400 nM reverse primer, 200 nM dual labeled fluorescent probe, 1X Master mix, 12.5 U of reverse transcriptase and 100 ng of total RNA. Thermal cycling conditions were 48°C for 30 min, 95°C for 10 min, followed by 45 additional cycles of 95°C for 15 sec and 60°C for 1 min for KLK1 and 68°C for 1 min for KLK14. Ribosomal 18S RNA (18S, RNA Control Kit (Eurogenetec, Philadelphia, PA, USA) was assayed according to manufacturer's protocol for each sample for normalization. Following qRT-PCR, quantification of gene expression was made by setting the threshold cycle (C_T) in the geometric amplification phase of the plot. Relative quantification of gene expression was evaluated using the comparative C_T method as described previously by Hettinger et al. (2001). The ΔC_T value was determined by subtracting the gene C_T value of each sample from the corresponding sample ribosomal 18S C_T value. Calculation of $\Delta\Delta C_T$ was carried out using the highest mean ΔC_T value as an arbitrary constant to subtract from all other ΔC_T mean values. Fold changes in gene expression are calculated from the $\Delta\Delta$CT values using the formula $2^{-\Delta\Delta Ct}$.

Statistical Analysis

Endometrial gene expression (ΔCT value) was analyzed with least squares using the PROC MIXED of SAS. The statistical model tested the effect of day, reproductive status (cyclic and pregnant), and the day x reproductive status interaction. Results are presented as least square means ± standard error of the mean.

Table 11. Primers and probes used for real-time PCR. Primers and probes were developed with an intron in the middle to prevent genomic DNA amplification using software available at IDT (Integrated DNA Technologies)(http://scitools.idtdna.com/Primerquest/).

Primer	Sequence 5'-3'	Length (bp)	PCR Product Size (bp)
KLK1-F	GGACTACAGCCACGACCTCATGCTGC	26	171
KLK1-R	GTCGGGGAATTCGAAGTCGTCTGG	24	
KLK1-P	TGGAGCTGCCCACCCAGGAACCCGAA	26	
KLK4-F	ATCCGCAATGGGTGCTGTCA	20	92
KLK4-R	GGGTTCTTGTTCGGGCTCAAGATT	24	
KLK4-P	ATTCCTACACCATCGGGCTGGGTC	24	
KLK14-F	ATGTTCCTCCTGCTGACAGCACT	23	155
KLK14-R	CAGAGGAAACGATGCCAGGGACC	23	
KLK14-P	GCCACGGCACAGAGCCAGGGCAATGAGAACAAGATAAT	38	

Results

Kallikrein1 gene expression analysis

RT-PCR amplification of kallikrein 1 using homologous sequence information allowed the amplification of a 297 bp PCR product. This product had an e value of $(P< e^{-143})$ (98% homology) to a recently submitted porcine kallikrein1 mRNA sequence (accession # NM_001001911) and $(P< e^{-16})$ (87% homology) to human kallikrein 1, renal/pancreas/salivary mRNA (accession # BT007253). This primer set also generated a larger fragment of ~450 bp with genomic DNA (Fig. 12A).

RT-PCR amplification of kallikrein 1 in cyclic and pregnant endometrium revealed its expression during days 0, 5, 10, 12, 15 and 18 of the estrous cycle and days 10, 12, 15 and 18 in the pregnant endometrium (Fig.12 A and B) RT-PCR amplification of conceptus RNA indicated that kallikrein 1 was expressed on days 12, 14 and 16 (Fig.13). The endometrial and conceptus gene expression of kallikrein 1 was also quantified using quantitative real-time PCR (qRT-PCR) on days 0, 5, 10, 12, 15, and 18 of the estrous cycle and days 10, 12, 15, and 18 in the pregnant endometrium and days 10-17 of conceptus development. Kallikrein 1 gene expression was detected across all days of the estrous cycle and early pregnancy. No significant day or status difference was detected for endometrial or conceptus kallikrein 1 gene expression (Tables 12 and 13).

Figure 12

Ethidium bromide-stained 1.5% agarose gels of kallikrein 1 expression in cyclic and pregnant endometrium.

A.

A 297 bp PCR product corresponding to the mRNA transcript of kallikrein 1 was detected, while a ~450 bp PCR corresponding to the genomic fragment of kallikrein 1 was also detected. The bands were cut and sequenced to confirm its identity. The expression of kallikrein 1 on days 12 and 18 of the estrous cycle are not visible, but was detected and is shown in fig. 12B.

B.

Kallikrein 1 gene expression after DNase treatment. After DNase treatment the ~450 bp PCR product corresponding to genomic DNA was not present. The expression of kallikrein 1 on day 15 is not visible, but was detected and is shown in figure 12A. Beta-actin was run as a positive control.

Figure 12A

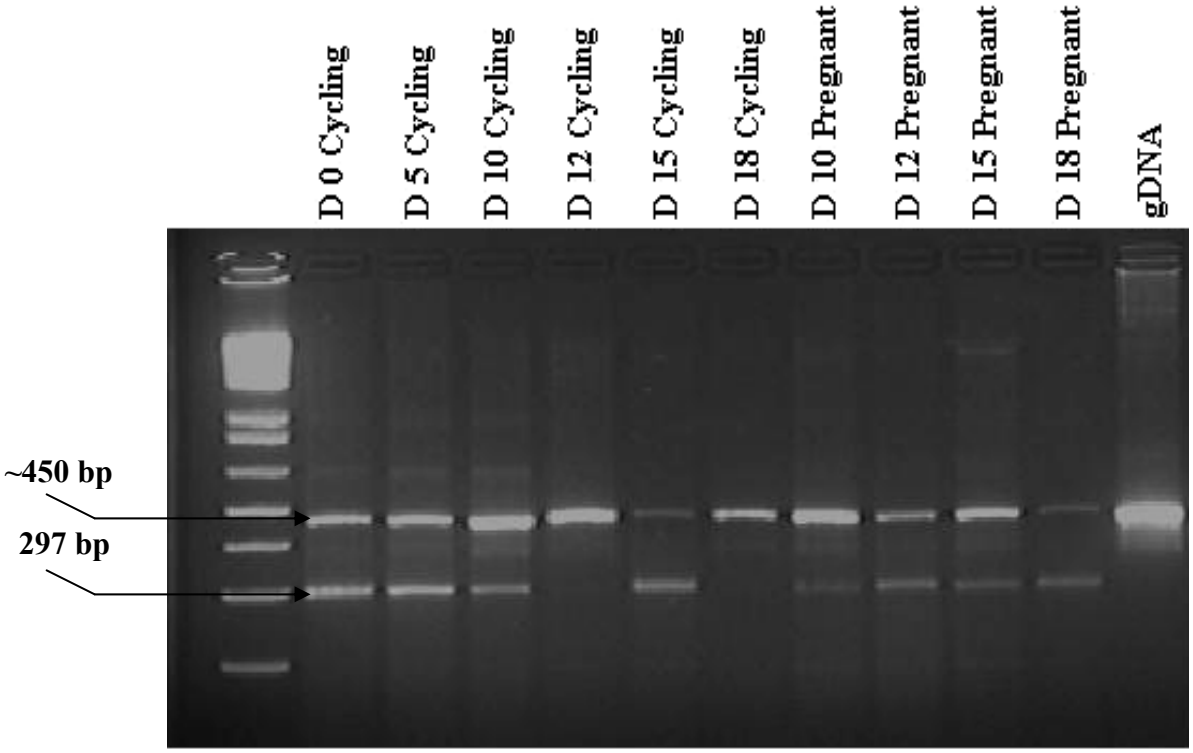


Figure 12B

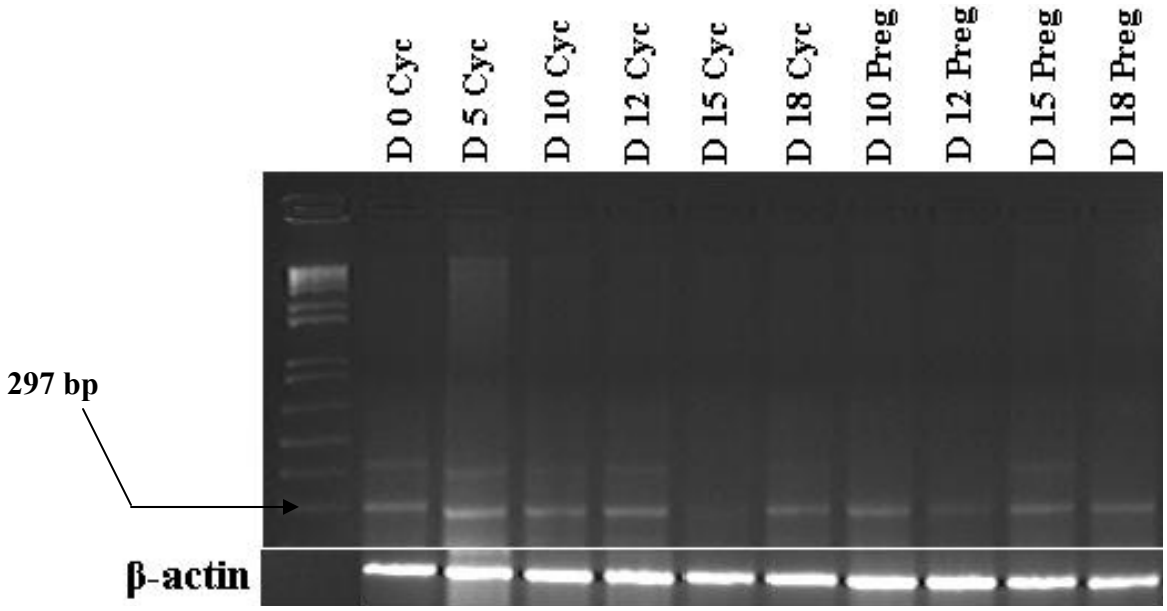


Figure 13

A 297 bp PCR product corresponding to Kallikrein 1 gene expression was detected on days 12, 14 and 16 of conceptus development. The expression of Kallikrein 1 was low during conceptus development. Beta-actin was run as a control.

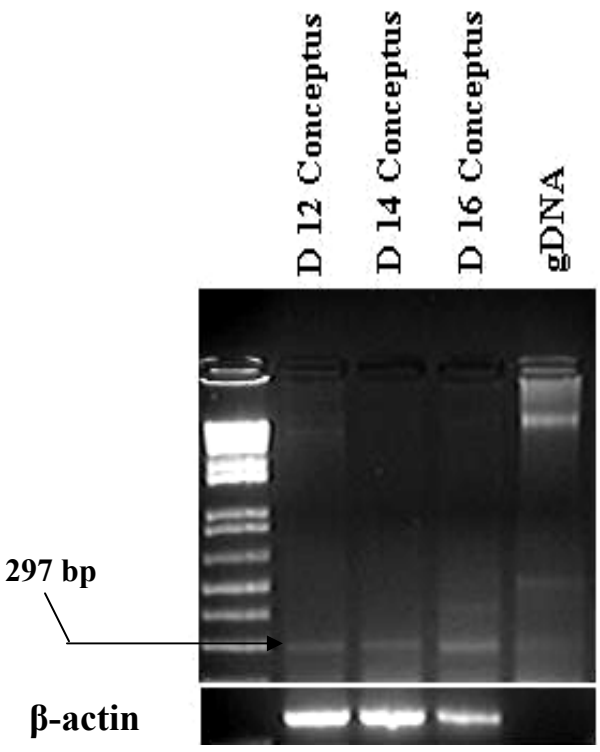

D 12 Conceptus
D 14 Conceptus
D 16 Conceptus
gDNA
297 bp
β-actin

Table 12

Endometrial kallikrein1 gene expression across days of the estrous cycle and early pregnancy using the comparative C_T method.

Day	Status	Kallikrein 1 average C_T*	18S average C_T*	ΔC_T†	$\Delta\Delta C_T$‡
0	Cyclic	32.00 ± 0.62	6.02 ± 0.22	25.97 ± 0.71	0.00
5	Cyclic	29.97 ± 0.62	6.34 ± 0.22	23.63 ± 0.71	-2.34
10	Cyclic	30.82 ± 0.53	6.37 ± 0.19	24.45 ± 0.62	-1.53
	Pregnant	30.29 ± 0.62	5.85 ± 0.22	24.44 ± 0.71	-1.53
12	Cyclic	29.78 ± 0.62	6.43 ± 0.22	23.36 ± 0.71	-2.62
	Pregnant	31.20 ± 0.96	6.19 ± 0.29	25.00 ± 0.62	-0.97
15	Cyclic	31.78 ± 0.53	6.49 ± 0.19	25.29 ± 0.62	-0.68
	Pregnant	32.33 ± 0.62	6.36 ± 0.22	25.97 ± 0.71	0.00
18	Cyclic	31.14 ± 0.53	6.32 ± 0.19	24.82 ± 0.61	-1.15
	Pregnant	30.49 ± 0.53	6.44 ± 0.19	24.04 ± 0.61	-1.93

*C_T = Cycle threshold: Cycle number where amplification crosses the threshold set in the geometric portion of the amplification curve.

†ΔC_T = kallikrein 1 C_T – 18S ribosomal C_T: Normalization of RT-PCR cycles for kallikrein 1 target with 18S ribosomal RNA. No gene expression change was detected (P<0.01).

‡$\Delta\Delta C_T$ = Least square means of ΔC_T – highest Least square mean of ΔC_T value: the least square estimates of day 15 pregnant and day 0 cycling (highest ΔC_T ; lowest expression of target in study) was used as a calibrator to set the baseline to compare mean differences in ΔC_T values across all days.

Table 13

Conceptus kallikrein1 gene expression during early embryonic development using the comparative C_T method.

Day	Status	Kallikrein 1 average C_T*	18S average C_T*	ΔC_T†	$\Delta\Delta C_T$‡
10	Conceptus	30.67 ± 0.34	6.52 ± 0.19	24.15 ± 0.31	-1.19
11	Conceptus	30.27 ± 0.34	5.97 ± 0.19	24.30 ± 0.31	-1.04
12	Conceptus	30.42 ± 0.34	6.12 ± 0.19	24.31 ± 0.31	-1.03
13	Conceptus	30.50 ± 0.45	6.11 ± 0.25	24.38 ± 0.40	-0.95
14	Conceptus	30.96 ± 0.38	5.63 ± 0.22	25.34 ± 0.34	0.00
15	Conceptus	30.87 ± 0.45	5.94 ± 0.25	24.93 ± 0.40	-0.41
16	Conceptus	30.91 ± 0.38	5.69 ± 0.22	25.22 ± 0.34	-0.12
17	Conceptus	30.69 ± 0.38	6.17 ± 0.22	24.53 ± 0.34	-0.81

*C_T = Cycle threshold: Cycle number where amplification crosses the threshold set in the geometric portion of the amplification curve.

†ΔC_T = kallikrein 1 C_T – 18S ribosomal C_T: Normalization of RT-PCR cycles for kallikrein 1 target with 18S ribosomal RNA. No gene expression change was detected (P<0.05).

‡$\Delta\Delta C_T$ = Least square means of ΔC_T – highest Least square mean of ΔC_T value: the least square estimates of day 16 (highest ΔC_T ; lowest expression of target in study) was used as a calibrator to set the baseline to compare mean differences in ΔC_T values across all days.

Kallikrein 4 gene expression analysis

Kallikrein 4 gene expression using RT-PCR detected a 247 bp PCR product. The 247 bp fragment was clearly detected on days 0, 5, 10, 12, 15 and 18 of the estrous cycle and days 10, 12, 15 and 18 in the pregnant endometrium but was not detected with genomic DNA (Fig.14). In the conceptus kallikrein 4 gene expression was detected on day 12 and 14 but was low on day 16 (Fig.15). Quantitative real-time PCR analysis of kallikrein 4 in the endometrium (Table 14) revealed a day effect ($P<0.05$) and status effect ($P<0.01$), but did not detect a day by status interaction. During the estrous cycle, endometrial kallikrein 4 is expressed higher in cyclic animals compared to pregnant animals (Fig.16). Kallikrein 4 expression was elevated in day 0, 5, and 10 of the estrous cycle, while the expression of kallikrein 4 was down-regulated on day 12, 15 and 18 (Fig.16). Quantitative real-time PCR of kallikrein 4 expression in the conceptus (Table 15) detected a day effect ($P<0.01$). Kallikrein 4 expression was 2.5 fold higher on days 10, 11, 12 and 13 compared to day 17. After day 13 there was a gradual decrease of kallikrein 4 expression in the conceptus (Fig. 17). Comparison of kallikrein 4 expression in the conceptus and the pregnant endometrium revealed that the gene is 16-17 fold highly expressed in the conceptus.

Figure 14

Ethidium bromide-stained 1.5% agarose gel of kallikrein 4 expression in cyclic and pregnant endometrium. A 247 bp PCR product corresponding to the mRNA transcript of kallikrein 4 was detected on days 0, 5, 10, 12, 15, and 18 of the estrous cycle and days 10, 12, 15, and 18 of pregnant endometrium. No PCR amplification was detected for genomic DNA. The band corresponding to kallikrein 4 was cut and sequenced to confirm its identity.

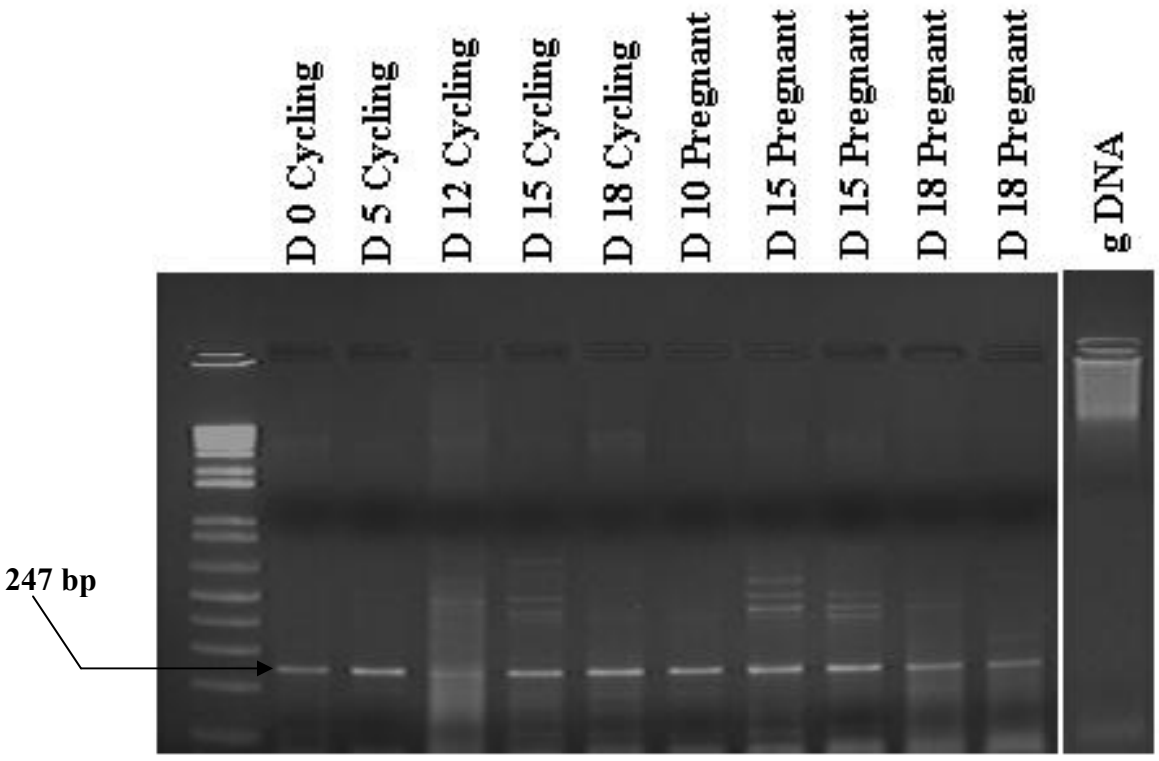

D 0 Cycling
D 5 Cycling
D 12 Cycling
D 15 Cycling
D 18 Cycling
D 10 Pregnant
D 15 Pregnant
D 15 Pregnant
D 18 Pregnant
D 18 Pregnant
g DNA
247 bp

Figure 15

A 247 bp PCR product corresponding to Kallikrein 4 gene expression was detected on days 12, 14 and 16 of conceptus development. The expression of Kallikrein 4 was low on day 16 and was hardly detectable. Beta-actin was run as a control.

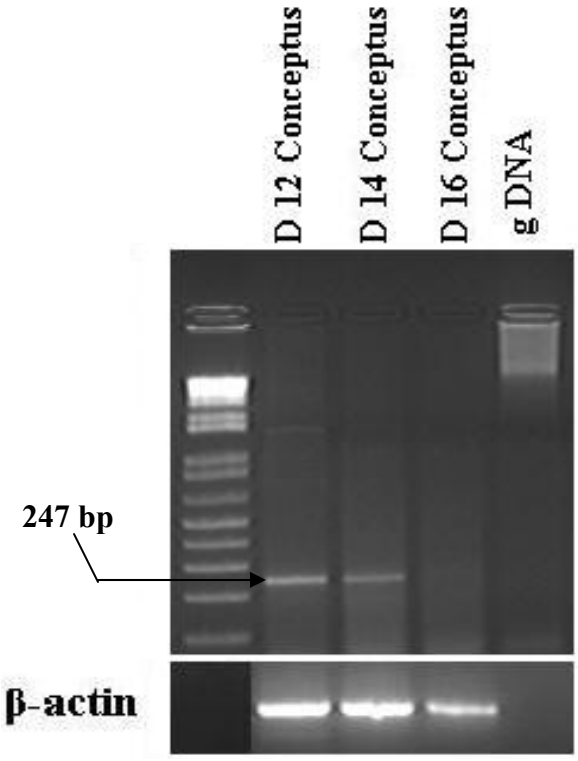

D 12 Conceptus
D 14 Conceptus
D 16 Conceptus
g DNA
247 bp
β-actin

Table 14

Endometrial kallikrein 4 gene expression across days of the estrous cycle and early pregnancy using the comparative C_T method.

Day	Status	Kallikrein 4 average C_T*	18S average C_T*	ΔC_T†	$\Delta\Delta C_T$‡
0	Cyclic	29.52 ± 0.58	18.53 ± 0.33	11.00 ± 0.56	-2.73
5	Cyclic	28.34 ± 0.66	18.42 ± 0.38	9.92 ± 0.65	-3.80
10	Cyclic	28.45 ± 0.58	18.64 ± 0.33	9.82 ± 0.57	-3.90
	Pregnant	30.80 ± 0.67	18.51 ± 0.38	12.30 ± 0.65	-1.42
12	Cyclic	30.45 ± 0.58	19.02 ± 0.33	11.46 ± 0.56	-2.26
	Pregnant	30.68 ± 0.58	18.29 ± 0.33	12.39 ± 0.56	-1.33
15	Cyclic	30.79 ± 0.58	18.34 ± 0.33	12.45 ± 0.56	-1.27
	Pregnant	30.75 ± 0.67	18.44 ± 0.38	12.31 ± 0.65	-1.41
18	Cyclic	30.36 ± 0.58	18.45 ± 0.33	11.90 ± 0..56	-2.63
	Pregnant	32.90 ± 0.58	19.15 ± 0.33	13.72 ± 0.56	0.00

*C_T = Cycle threshold: Cycle number where amplification crosses the threshold set in the geometric portion of the amplification curve.

†ΔC_T = kallikrein 4 C_T – 18S ribosomal C_T: Normalization of RT-PCR cycles for kallikrein 4 target with 18S ribosomal RNA. There was a status effect (P<0.01) and a day effect (P<0.05). No day x status interaction was detected (P<0.05).

‡$\Delta\Delta C_T$ = Least square means of ΔC_T – highest Least square mean of ΔC_T value: the least square estimates of day 18 pregnant (highest ΔC_T ; lowest expression of target in study) was used as a calibrator to set the baseline to compare mean differences in ΔC_T values across all days.

Figure 16

Fold gene expression of kallikrein 4 in endometrium of cyclic and pregnant gilts generated using quantitative one-step RT-PCR.

Quantitative real-time PCR analysis of kallikrein 4 detected a status effect (cyclic or pregnant) ($P<0.01$) in the endometrium. During the estrous cycle, endometrial kallikrein 4 is expressed higher in cyclic animals compared to pregnant animals. The fold changes in expression was calculated from the differences of least squares means of ΔC_T values using the formula $2^{-\Delta\Delta CT}$. Columns with different superscripts represent significant differences ($P<0.01$) in ΔC_T values.

Quantitative real-time PCR analysis of kallikrein 4 also detected a day effect ($P<0.01$). The fold changes in expression was calculated from the differences of least squares means of ΔC_T values using the formula $2^{-\Delta\Delta CT}$. Columns with different superscripts represent significant differences ($P<0.05$) in ΔC_T values for each day.

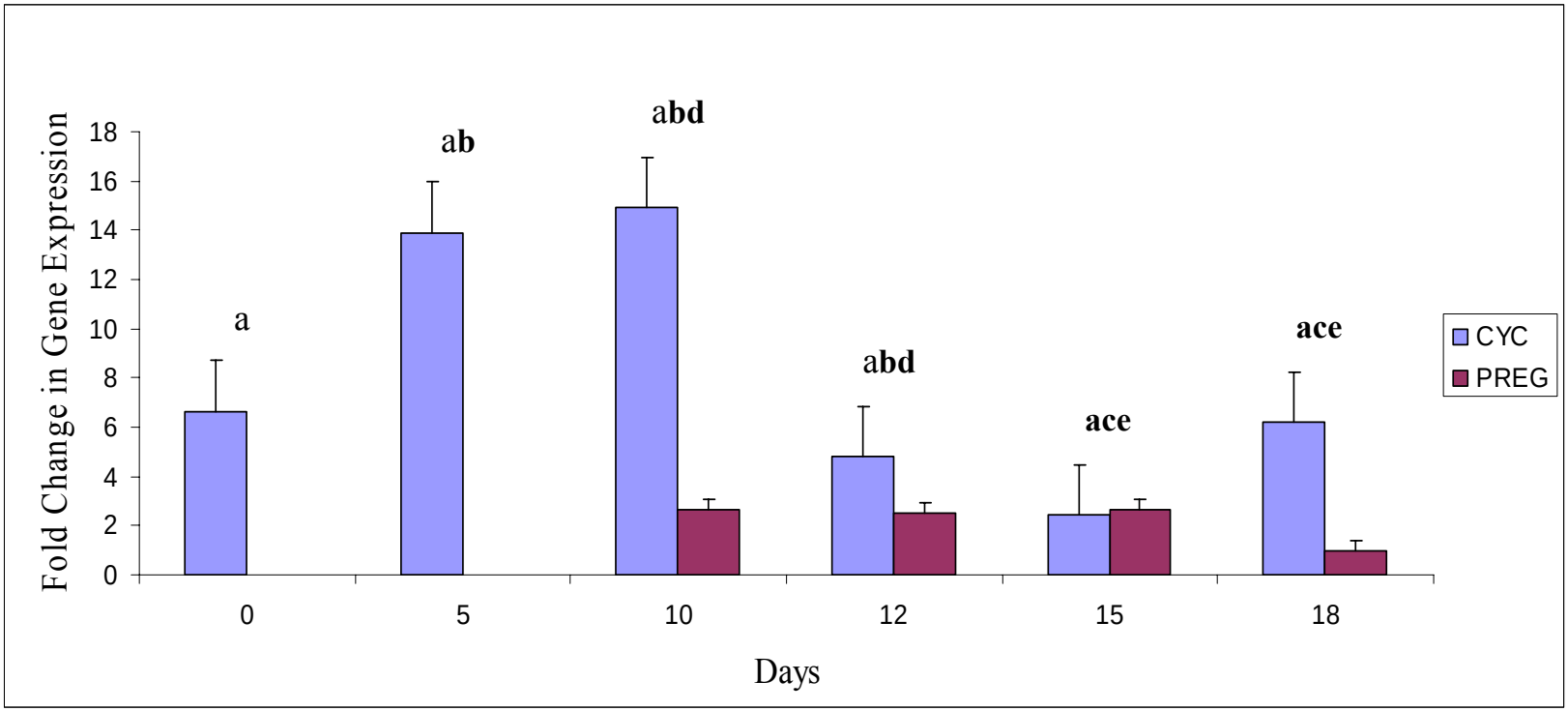

Fold Change in Gene Expression
18
16
14
12
10
8
6
4
2
0
a
ab
abd
abd
ace
ace
0
5
10
12
15
18
Days
CYC
PREG

Table 15

Conceptus kallikrein 4 gene expression across days of embryo development using the comparative C_T method.

Day	Status	Kallikrein 4 average C_T*	18S average C_T*	ΔC_T†	$\Delta\Delta C_T$‡
10	Conceptus	25.45 ± 0.33	6.52 ± 0.19	18.93 ± 0.33	-1.95
11	Conceptus	24.91 ± 0.33	5.97 ± 0.19	18.94 ± 0.33	-1.93
12	Conceptus	25.37 ± 0.33	6.12 ± 0.19	19.26 ± 0.33	-1.62
13	Conceptus	25.33 ± 0.43	6.11 ± 0.25	19.21 ± 0.43	-1.67
14	Conceptus	25.08 ± 0.37	5.63 ± 0.22	19.45 ± 0.37	-1.43
15	Conceptus	26.07 ± 0.43	5.94 ± 0.25	20.12 ± 0.43	-0.76
16	Conceptus	25.86 ± 0.37	5.69 ± 0.22	20.17 ± 0.37	-0.71
17	Conceptus	27.05 ± 0.37	6.17 ± 0.22	20.88 ± 0.37	0.00

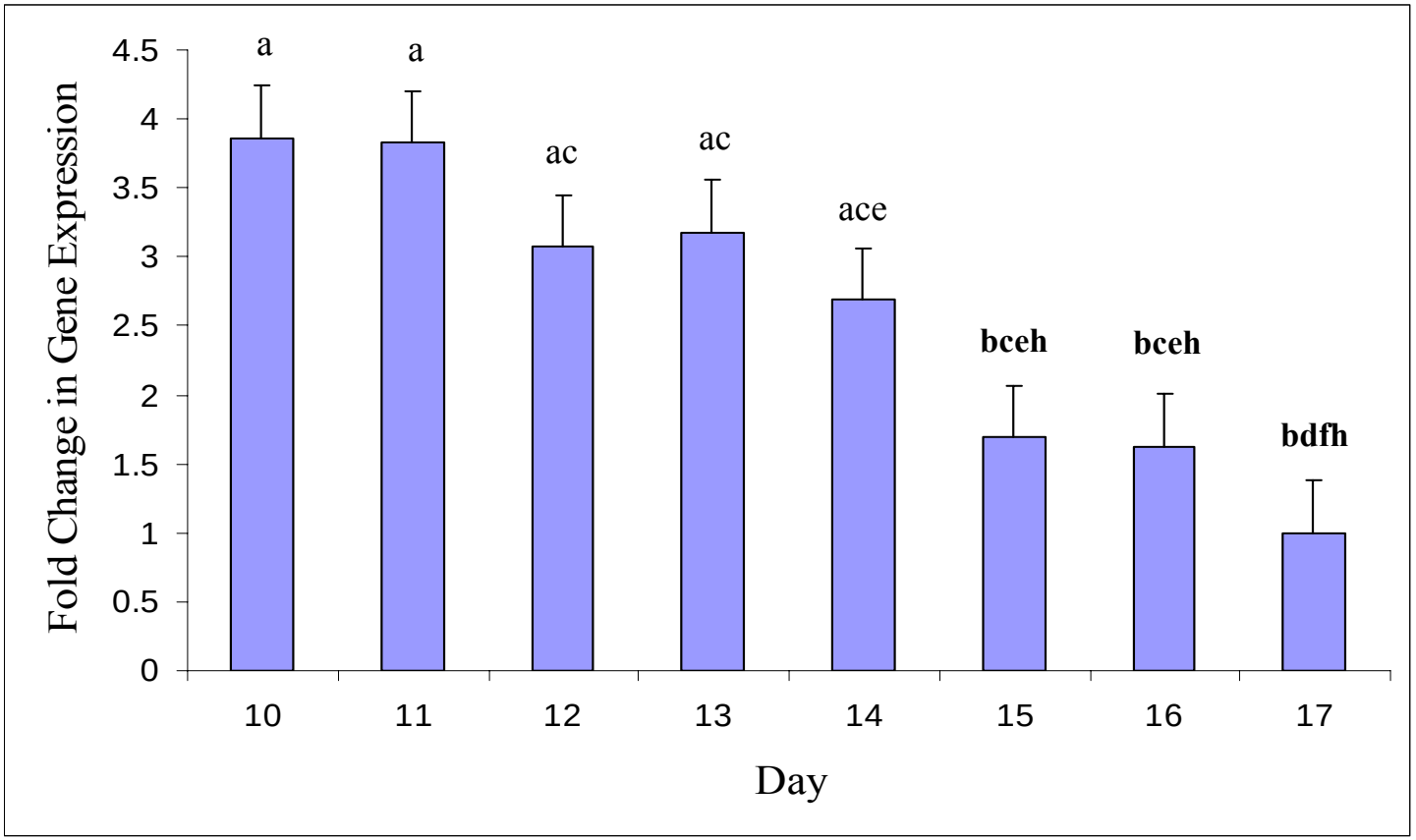
*C_T = Cycle threshold: Cycle number where amplification crosses the threshold set in the geometric portion of the amplification curve.

†ΔC_T = kallikrein 1 C_T – 18S ribosomal C_T: Normalization of RT-PCR cycles for kallikrein 1 target with 18S ribosomal RNA. Day effect (P<0.05) was detected.

‡$\Delta\Delta C_T$ = Least square means of ΔC_T – highest Least square mean of ΔC_T value: the least square estimates of day 17 (highest ΔC_T ; lowest expression of target in study) was used as a calibrator to set the baseline to compare mean differences in ΔC_T values across all days.

Figure 17

Quantitative real-time PCR analysis of kallikrein 4 in the conceptus showing a day effect ($P<0.01$). The fold changes in gene expression was calculated from the $\Delta\Delta C_T$ values using the formula $2^{-\Delta\Delta CT}$. Columns with different superscripts represent significant differences ($P<0.05$) in ΔC_T values.


Fold Change in Gene Expression
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
a
a
ac
ac
ace
bceh
bceh
bdfh
10
11
12
13
14
15
16
17
Day

Kallikrein 14 gene expression analysis

RT-PCR amplification of kallikrein 14 using homologous sequence information allowed the amplification of a 321 bp PCR product. This product has high homology to human kallikrein 14 mRNA sequence (accession # AF283670) *($P< e^{-63}$)* (86% homology). It also has high homology to rat glandular kallikrein 14 (accession # XM_218641) and mouse kallikrein 14 (accession # BC044756) with $P< e^{-18}$ (81% homology) and $P< e^{-13}$ (82% homology) respectively. This primer set also generated a larger fragment of ~400 bp with genomic DNA.

RT-PCR amplification of kallikrein 14 in cyclic and pregnant endometrium indicated that KLK14 was expressed during days 0, 12, 15 and 18 of the estrous cycle and days 12, 15 and 18 of pregnancy (Fig. 18). Only a very faint band was detected on days 5 and 10 of the estrous cycle and day 10 of pregnancy even with greater amounts of cDNA. RT-PCR amplification of kallikrein 14 conceptus gene expression was detected in day 12 and 14 conceptus tissue but was very low in day 16 conceptuses (Fig. 19).

The endometrial and conceptus kallikrein 14 gene expression was analyzed using quantitative real-time PCR. The real-time PCR data confirmed the results of RT-PCR, as no amplification was visible on day 5 and 10 cyclic and day 10 pregnant endometrium after 40 cycles of amplification (Fig. 20). Therefore, for data analyzing purposes we assigned an arbitrary C_T value of 40 cycles to day 5 and 10 cyclic and day 10 pregnant endometrium. A highly significant day effect of (P<0.01) was detected for endometrial kallikrein 14 gene expression during the estrous cycle and pregnant endometrium (Table 16). The quantitative real-time PCR analysis of the conceptus (Table 17) revealed a day

effect ($P<0.05$) where the expression of kallikrein 14 was down-regulated by day 17 (Fig. 21).

Figure 18

Ethidium bromide-stained 1.5% agarose gel of kallikrein 14 expression in cyclic and pregnant endometrium. A 321 bp PCR product corresponding to the mRNA transcript of kallikrein 14 was detected, while a ~420 bp fragment was detected off genomic DNA. The band corresponding to kallikrein 14 mRNA was cut and sequenced to confirm its identity. The RT-PCR amplification detect endometrial kallikrein 14 gene expression at very low levels on days 5 and 10 of estrous cycle and day 10 of pregnancy.

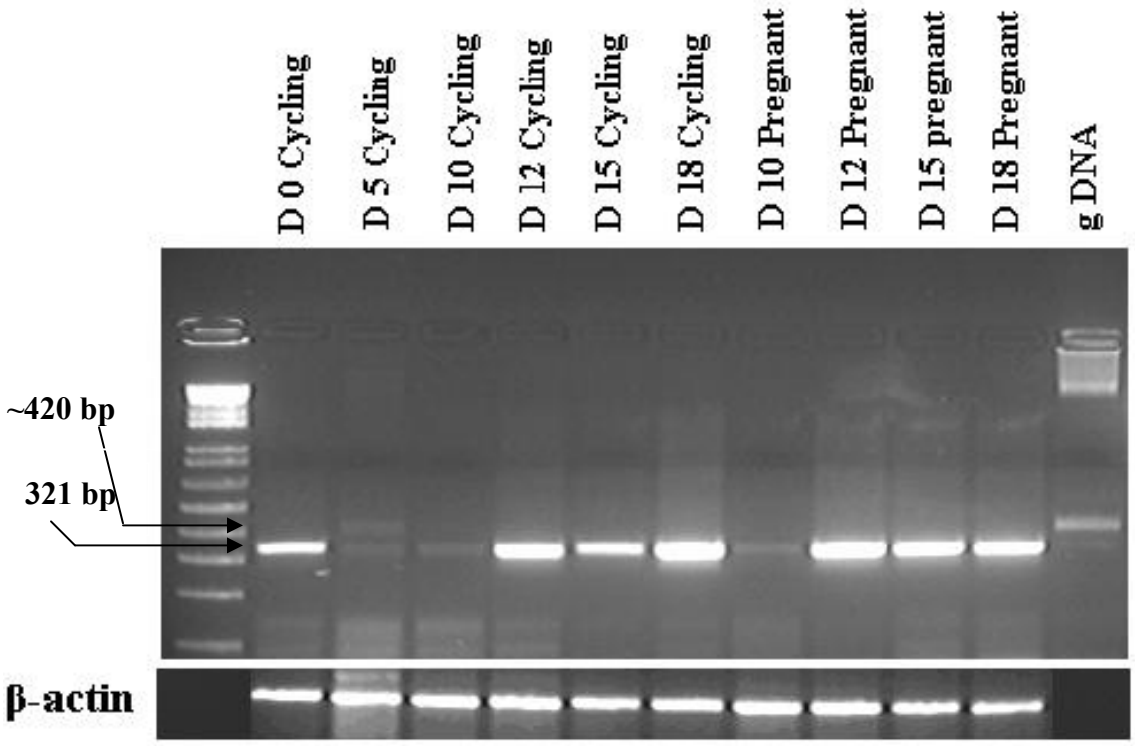

D 0 Cycling
D 5 Cycling
D 10 Cycling
D 12 Cycling
D 15 Cycling
D 18 Cycling
D 10 Pregnant
D 12 Pregnant
D 15 pregnant
D 18 Pregnant
g DNA
~420 bp
321 bp
β-actin

Figure 19

A 321 bp PCR product corresponding to Kallikrein 14 gene expression was detected on day 12 and 14 conceptuses. The expression of kallikrein 16 was undetectable, but the beta-actin shows that the amount of day 16 cDNA used was lower. Therefore the low amounts of kallikrein 14 on day 16 conceptuses may be due to low amounts of cDNA added. Beta-actin was run as a control.

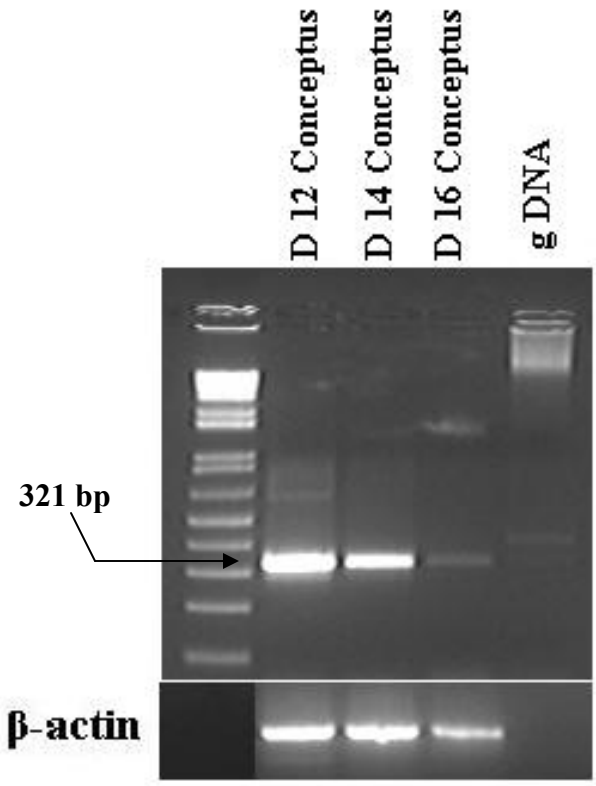

D 12 Conceptus
D 14 Conceptus
D 16 Conceptus
g DNA
321 bp
β-actin

Table 16

Endometrial kallikrein 14 gene expression across days of the estrous cycle and early pregnancy using the comparative C_T method. No amplification was detected on days 5 and 10 of cycling and day 10 of pregnancy after 40 cycles of PCR amplification. Therefore, an arbitrary value of 40 was used as the C_T* value for days 5 and 10 of cycling and day 10 of pregnancy to calculate ΔC_T† and $\Delta\Delta C_T$‡ values.

Day	Status	Kallikrein 14 average C_T*	18S average C_T*	ΔC_T†	$\Delta\Delta C_T$‡
0	Cyclic	26.48 ± 0.72	6.02 ± 0.22	19.81 ± 0.65	-13.85
5	Cyclic	Undetected (40)	6.34 ± 0.22	33.66 ± 0.75	0.00
10	Cyclic	Undetected (40)	6.37 ± 0.19	33.62 ± 0.65	-0.04
	Pregnant	Undetected (40)	5.85 ± 0.22	34.15 ± 0.75	-0.49
12	Cyclic	26.50 ± 0.83	6.43 ± 0.22	20.07 ± 0.75	-13.6
	Pregnant	25.65 ± 0.72	6.19 ± 0.29	19.46 ± 0.65	-14.21
15	Cyclic	25.94 ± 0.72	6.49 ± 0.19	19.45 ± 0.65	-14.21
	Pregnant	25.98 ± 0.83	6.36 ± 0.22	19.62 ± 0.75	-14.04
18	Cyclic	25.65 ± 0.72	6.32 ± 0.19	19.33 ± 0.65	-14.33
	Pregnant	25.02 ± 0.23	6.44 ± 0.19	18.58 ± 0.65	-15.09

*C_T = Cycle threshold: Cycle number where amplification crosses the threshold set in the geometric portion of the amplification curve.

†ΔC_T = kallikrein 1 C_T – 18S ribosomal C_T: Normalization of RT-PCR cycles for kallikrein 1 target with 18S ribosomal RNA. A day effect was detected (P<0.05).

‡$\Delta\Delta C_T$ = Least square means of ΔC_T – highest Least square mean of ΔC_T value: the least square estimates of day 5 cycling (highest ΔC_T ; lowest expression of target in study) was used as a calibrator to set the baseline to compare mean differences in ΔC_T values across all days.

Figure 20

Kallikrein 14 gene expression across days of the estrous cycle and early pregnancy. The fold a changes in gene expression was calculated from the $\Delta\Delta C_T$ values using the formula $2^{-\Delta\Delta CT}$. Kallikrein 14 expression was not detected on days 5 and 10 of the estrous cycle and day 10 of pregnant endometrium. Therefore, an arbitrary value of 40 was used as the C_T* value for days 5 and 10 of cycling and day 10 of pregnancy to calculate changes in gene expression. Columns with different superscripts represent significant differences ($P<0.01$) in ΔC_T values.

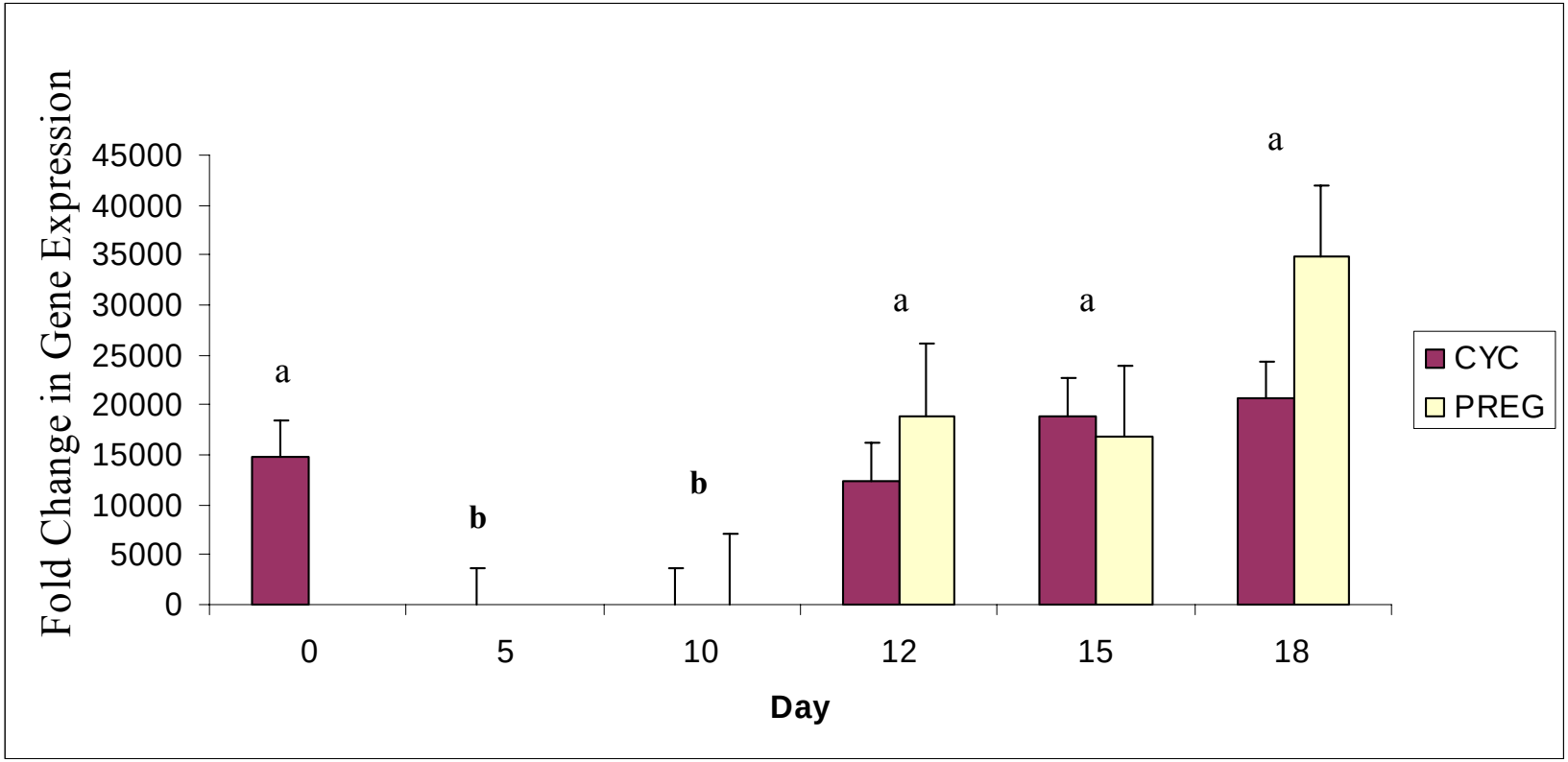

Fold Change in Gene Expression
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
a
b
b
a
a
a
0
5
10
12
15
18
Day
CYC
PREG

Table 17

Conceptus kallikrein 14 gene expression across days of embryo development using the comparative C_T method.

Day	Status	Kallikrein 14 average C_T*	18S average C_T	ΔC_T	$\Delta\Delta C_T$
10	Conceptus	32.39 ± 0.39	6.52 ± 0.19	25.87 ± 0.37	-2.57
11	Conceptus	31.96 ± 0.39	5.97 ± 0.19	25.99 ± 0.37	-2.45
12	Conceptus	31.39 ± 0.44	6.12 ± 0.19	25.45 ± 0.41	-2.99
13	Conceptus	32.29 ± 0.51	6.11 ± 0.25	26.17 ± 0.48	-2.27
14	Conceptus	32.36 ± 0.44	5.63 ± 0.22	26.73 ± 0.41	-1.71
15	Conceptus	32.60 ± 0.51	5.94 ± 0.25	26.66 ± 0.48	-1.78
16	Conceptus	31.89 ± 0.44	5.69 ± 0.22	26.20 ± 0.41	-2.24
17	Conceptus	34.61 ± 0.62	6.17 ± 0.22	28.44 ± 0.59	0.00

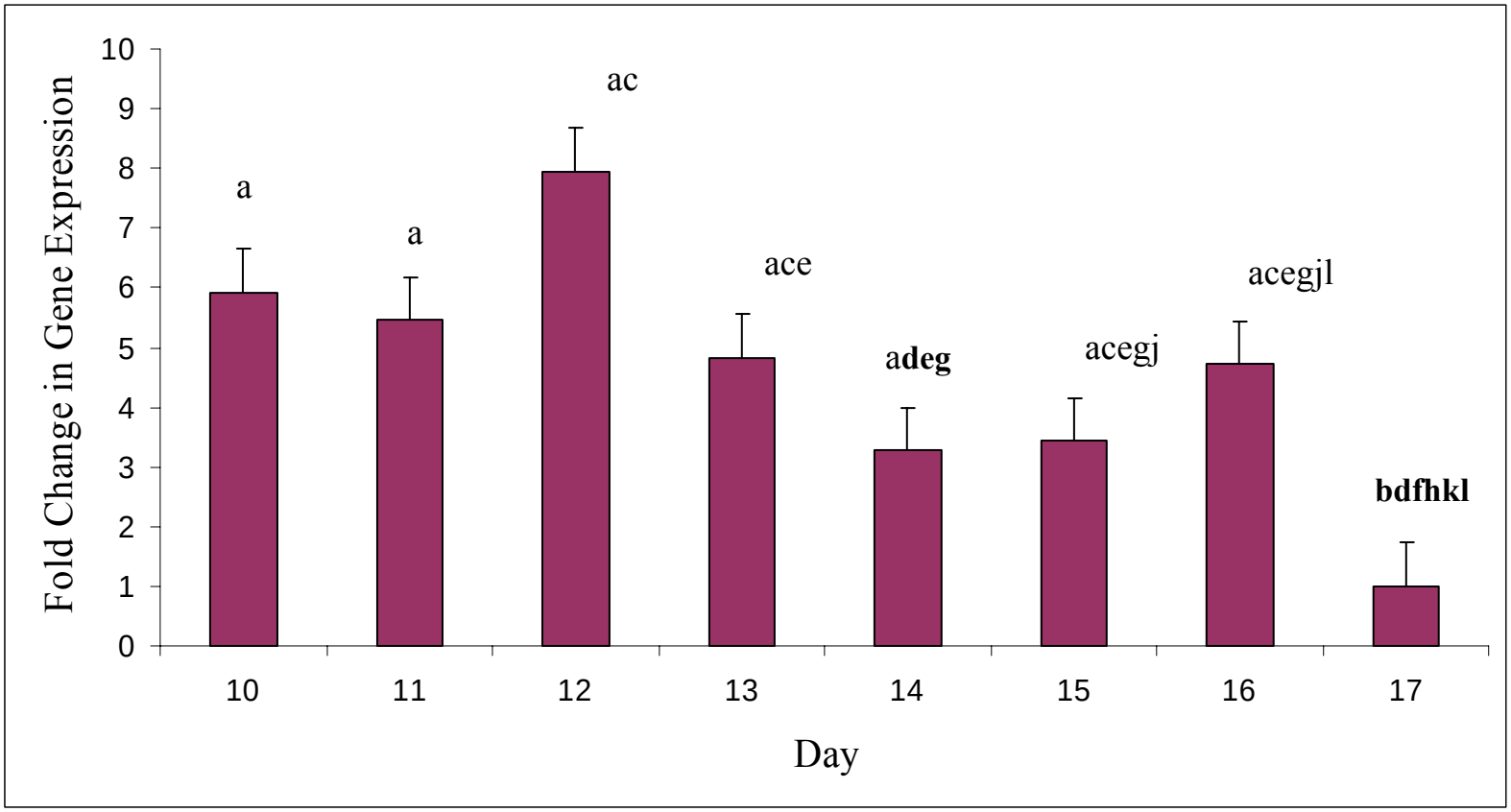
*C_T = Cycle threshold: Cycle number where amplification crosses the threshold set in the geometric portion of the amplification curve.

†ΔC_T = kallikrein 1 C_T – 18S ribosomal C_T: Normalization of RT-PCR cycles for kallikrein 1 target with 18S ribosomal RNA. A day effect was detected ($P<0.05$).

‡$\Delta\Delta C_T$ = Least square means of ΔC_T – highest Least square mean of ΔC_T value: the least square estimates of day 17 (highest ΔC_T ; lowest expression of target in study) was used as a calibrator to set the baseline to compare mean differences in ΔC_T values across all days.

Figure 21

Quantitative real-time PCR analysis of kallikrein 14 in the conceptus showing a day effect ($P<0.05$). The fold changes in gene expression was calculated from the $\Delta\Delta C_T$ values using the formula $2^{-\Delta\Delta CT}$. Columns with different superscripts represent significant differences ($P<0.05$) in ΔC_T values.


Fold Change in Gene Expression
10
9
8
7
6
5
4
3
2
1
0
a
a
ac
ace
adeg
acegj
acegjl
bdfhkl
10
11
12
13
14
15
16
17
Day

Discussion

Many physiological events involved in implantation/placental attachment such as vasodilation, prostaglandin synthesis, and proteolytic tissue remodeling mimic inflammatory responses (Vonnahme et al. 2003). Many of these inflammatory like immunological responses are known to be caused by the kallikrein-kininogen-kinin system. During the time of maternal recognition of pregnancy until the time of conceptus attachment, many alterations occur in the porcine uterine lumen. These changes are similar to an acute inflammatory response, where the rapid elongation and growth of the conceptus, and the estrogen synthesis by the conceptuses can alter uterine secretion of proteins (Roberts et al. 1993), prostaglandins (Bazer and Thatcher 1977), uterine blood flow (Ford et al. 1982) and uterine cellular morphology (Geisert et al. 1982a,b). During the estrous cycle and early pregnancy, LMW and HMW kininogen are present in the porcine endometrium indicating that the kallikrein-kininogen kinin system is active in the pig (Allen et al. 2002; Vonnahme et al. 2003). Tissue kallikreins are known to regulate the activation of many growth factors (Vonnahme et al 1999 & 2003) and to cleave inter-α-trypsin inhibitor heavy chain 4 in the porcine endometrium (Hashimoto et al. 1996; Geisert et al. 1998). Detection of kallikrein 1 which cleaves LMW kininogen to release bradykinins (Allen at al. 2002) in the porcine endometrium and conceptus suggests that this serine protease may be involved in the release of lysl-bradykinin through the proteolytic cleavage of low molecular weight kininogen. The detection of kallikrein both in the conceptus and endometrium also suggests that kallikrein 1 may exert a local effect on the endometrium and conceptus. As no significant gene expression change was detected in cyclic and pregnant endometrium and conceptus, kallikrein 1 may be secreted

as an inactive prekallikrein which needs to be activated to exert its kininogenase activity. Although, the expression of kallikrein 1 was detected during this study, the activity and function of kallikrein 1 needs to be investigated more closely.

In the present study changes in tissue kallikrein 1 expression was not detected, but the detection of tissue kallikrein expression in the conceptus suggests that the conceptus exerts its own kininogenase activity independent of the endometrium, therefore it is possible that the conceptus may be regulating the cleavage of HMW and LMW kininogen to release kinins during early embryonic development.

Tissue kallikrein release of kinins through proteolytic cleavage of low molecular weight kininogen has recently received increased attention in therapeutic angiogenesis. This release of kinins together with the presence of kinin- β type 2 receptor during days 10 and 15 of pregnancy in the pig (Allen et al. 2002) suggests that kallikreins may play an important role in early embryonic development in the pig. The stabilization of the uterine epithelium extracellular matrix through proteolytic cleavage of inter-α-trypsin heavy chain 4 (Geisert et al. 1998) further suggests that tissue kallikrein helps in conceptus attachment and early embryonic survival in the pig by exerting several biologically important processors essential for conceptus elongation and traphoblast attachment in the pig.

Another member of the tissue kallikrein gene family, kallikrein 4, is expressed in an endometrial cancer cell line (Mayers and Clements 2001). Patho-physiology of such cancers are attributed to estrogen and progesterone acting through the regulation of specific genes (Mayers and Clements 2001). Recently, kallikrein 4 gene expression was demonstrated to be increased by progestins in a breast cancer cell line, BT-474 (Mayers

and Clements 2001) indicating that kallikrein 4 may be regulated by progesterone. Also, kallikrein 4 gene expression is increased by estrogen and progesterone alone, and in combination, in KLE cell line which has a defective estrogen receptor (Mayers and Clements 2001). Kallikrein 4 expression was elevated 3-4 fold higher in day 0-12 cyclic and pregnant gilts compared to day 12-18 cyclic and pregnant endometrium in our study, suggesting that kallikrein 4 expression in the porcine endometrium may be regulated by the progesterone receptor which is elevated in the uterine epithelium during this period. Furthermore, potential androgen response elements (AREs) have been identified in the 5' flanking region of kallikrein 4 (Mayers and Clements 2001) . These androgen response elements have not yet been characterized, but as the progesterone receptor can bind to similar androgen response elements and the detection of kallikrein 4 gene regulation in human breast cancer cell line BT-474 (Mayers and Clements 2001) further speculates that the progesterone receptor may bind to these androgen response elements present in the 5' flanking region of kallikrein 4 to regulate KLK4 gene expression.

Furthermore, kallikrein 4 is expressed in the porcine dental enamel (Ryu et al. 2002). In the porcine dental enamel kallikrein 4 is expressed during early transition and maturation stages of enamel formation where kallikrein 4 participates in the degradation of enamel proteins (Ryu et al. 2002). Kallikrein 4, which is expressed in the inactive prekallikrein form needs to be activated to exert its protease activity. MMP-20 is seen to be coexpressed with kallikrein 4 in the odontogenic tissue, suggesting that MMP-20 may be involved with the activation of kallikrein 4 (Ryu et al. 2002). The ability of kallikrein 4 to cleave a broad range of peptides and its higher expression on days 0, 5 and 10 in cyclic

and pregnant endometrium suggests that kallikrein 4 may also play a role in extra-cellular matrix remodeling in the endometrium.

The greater expression of kallikrein 4 in the conceptus with respect to endometrial expression suggests that kallikrein 4 plays a role in early conceptus development. The real-time PCR data suggests a 4 fold increase of kallikrein 4 expression in day 10-13 of conceptus development which gradually decreases from day 15 to 17 of conceptus development. This is the time when the conceptus starts cell proliferation and placental membrane formation occurs, therefore, this suggests that kallikrein 4 may be one of the factors that help conceptus attachment, extracellular matrix degradation and cellular remodeling. More studies need to be carried out on kallikrein 4 to identify the substrate specificity and its biological role during early embryonic development.

The newly identified porcine tissue kallikrein 14 is regulated by androgen receptors. Previous studies have shown that kallikrein 14 is up-regulated in breast, prostate and ovarian cancers and is regulated by androgen and androgen receptor pathways (Yousef et al 2002; & 2003). The expression of kallikrein 14 in the secretory epithelial cells of benign prostate gland (Yousef et al. 2002) and in the prostatic intraepithelial neoplasia (Hooper et al. 2001) and the up-regulation of kallikrein 14 by dihydrotestesterone (4-fold) and estradiol (38-fold) in breast cancer cell lines (Borgono et al. 2003; Yousef et al. 2003) further suggests that kallikrein 14 may be regulated by an androgen receptor. Endometrial kallikrein 14 was up-regulated on day 0 and days 12-18 in cyclic and pregnant gilts suggesting that kallikrein 14 may be regulated by the loss of progesterone receptor in the uterine epithelium.

In 1998, Lee et al. (1998) reported the degradation of several insulin-like growth factor binding proteins by an unknown serine protease. The serine protease degradation of the IGFBP was absent during the spherical stage of conceptus development, but the IGFBP-2 and 3 was completely removed when tubular and filamentous conceptuses were present (Geisert et al. 2001; Vonnahme et al. 1999). Kallikreins are serine proteases that may be involved in the breakdown of IGFBPs directly or indirectly through activation of matrix metalloproteinases. Protease activity towards IGFBPs on day 12 and 15 pregnant and cycling gilt uterine flushings has been detected (Geisert et al. 2001) and specific inhibitors to kallikrein and matrix metalloproteinases has shown to inhibit porcine IGFBP-2, -3, and -5 degradation (Vonnahme et al. 1999; Geisert et al. 2001). The increased expression kallikrein 14 on day 12-18 of pregnant and cyclic endometrium suggests that kallikrein 14 may play a role in the break-down of IGFBPs to stimulate the release of IGFs from their binding proteins to help establish pregnancy and to enhance the growth and estrogen synthesis of the conceptus.

The observation of multiple tissue kallikreins during embryonic development in the pig suggests that kallikreins may participate in a common enzymatic pathway that plays a role in the normal physiology of pregnancy. But, their diverse tissue expression patterns also suggest that the kallikrein gene family members may have different physiological substrates. It is possible that several different uterine kallikrein genes may be involved in alteration of the extracellular matrix, embryo implantation and degradation of insulin like growth factor binding proteins in the porcine uterus. During this study we have established the expression of multiple tissue kallikreins in the porcine endometrium and conceptus suggesting that kallikreins may initiate many biological processors during

early conceptus attachment and survival. The expression of KLK1 during the estrous cycle and pregnancy, and the pregnancy specific expression of kinins in the porcine endometrium (Allen et al. 2002) suggests that KLK1 may be activated by the conceptus during embryonic attachment to the uterine epithelium. The activation of KLK1 will cleave LMW kininogen to release bradykinins, which will increase uterine blood flow, vascular permeability, prostaglandin release during early embryonic development in the pig. Embryo development is dependent upon the efficient transport of nutrients to the developing conceptus across the placenta. Therefore, the increased blood flow by the release of kinins through KLK1 would help conceptus development and embryonic survival in the pig. The greater expression of KLK4 during days 0-10 of the estrous cycle, and the ability of KLK4 to cleave a broad range of peptides suggests that KLK4 may play a role in extracellular matrix remodeling. Also, the greater expression of KLK4 in the conceptus with respect to endometrial expression suggests that kallikrein 4 plays an important role in early conceptus development. The KLK4 expression in the conceptus is high during the time when the conceptus attaches to the uterine epithelium suggesting that KLK4 could play a role in opening sites for conceptus attachment. Therefore, KLK4 may play a role in degrading antiadhesive molecules present in the uterine epithelial surface, allowing the conceptus to interact with adhesion molecules such as integrins, proteoglycans and heparin. These initial interactions may bring the conceptus closer to the uterine epithelium to complete conceptus attachment. The higher expression of KLK14 in cyclic and pregnant endometrium on days 0 and 12-18 is consistent with the time of IGF-BP degradation (Geisert et al. 2001). Therefore KLK14 may be degrading

IGF-BP enabling the release of IGF from it's binding proteins to provide necessary growth factors for embryonic survival in the pig.

Although this preliminary investigation shows the presence of multiple kallikreins during early embryonic development in the pig, a closer investigation of the porcine kallikrein gene family members and their expression patterns need to be carried out, to establish their substrate specificities, methods of activation, and their functional roles during establish pregnancy in the pig.

Reference List

Allen, M.R., B.R. Zhang, A.M. Hettinger, D.W. Goad, J.R. Malayer, and R.D. Geisert. 2002. Detection of bradykinin and bradykinin-β2 receptors in the porcine endometrium during the estrous cycle and early pregnancy. Biology of Reproduction **66**: 574-579.

Bazer, F.W., R.D. Geisert, W.W. Thatcher, and R.M. Roberts. 1982. The establishment and maintenance of pregnancy. In Control of pig Reproduction (ed. D.J.A. Cole and G.R. Foxcroft), pp. 227-252. Butterworth Scientific.

Bazer, F.W. and W.W. Thatcher. 1977. Theory of maternal recognition of pregnancy in swine based on estrogen controlled endocrine versus exocrine secretion of prostaglandin PGF2α by the uterine endometrium. Prostaglandins **14**: 397-401.

Bhoola, K.D., C.D. Figueroa, and K. Worthy. 1992. Bioregulation of kinins - Kallikreins, Kininogens, and Kininases. Pharmacological Reviews **44**: 1-80.

Borgon, C.A., L. Grass, A. Soosaipillai, G.M. Yousef, C.D. Petraki, D.H.C. Howarth, S. Fracchioli, D. Katsaros and E.P. Diamandis. 2003. Human kallikrein 14: A new potential biomarker for Ovarian and breast cancer. Cancer Research **63**: 9032-9041.

Brann, D.W., L.M. Greenbaum, V.B. Mahesh, and X. Gao. 1995. Changes in kininogens and kallikrein in the plasma, brain, and uterus during pregnancy in the rat. Endocrinology **136**: 46-51.

Chan, C.S., M.B. Harvey, and J.A. Clements. 1999. Temporal and tissue-specific expression of kallikrein (klk) genes and identification of a novel klk messenger ribonucleic acid transcript during early development in the mouse. Biology of Reproduction **61**: 621-628.

Corthorn, J., C. Figueroa, and G. Valdes. 1997. Estrogen and luminal stimulation of rat uterine kallikrein. Biology of Reproduction **56**: 1432-1438.

Ford, S.P., R.K. Christenson, and J.J. Ford. 1982. Uterine blood-flow and uterine arterial, venous and luminal concentrations of estrogens on days 11, 13 and 15 after estrus in pregnant and nonpregnant sows. Journal of Reproduction and Fertility **64**: 185-190.

Ford, S.P. 1995. Control of blood flow to the gravid uterus of domestic live stock species. Journal of Animal Science **73**: 1852-1860.

Geisert, R.D., J.W. Brookbank, R.M. Roberts, and F.W. Bazer. 1982. Establishment of pregnancy in the pig: 2. Cellular remodeling of the porcine blastocyst during elongation on day 12 of pregnancy. Biology of Reproduction **27**: 941-955.

Geisert, R.D., C.S. Chamberlain, K.A. Vonnahme, J.R. Malayer, and L.J. Spicer. 2001. Possible role of kallikrein in proteolysis of insulin-like growth factor binding proteins during the estrous cycle and early pregnancy in pigs. Reproduction **121**: 719-728.

Geisert, R.D., R.H. Renegar, W.W. Thatcher, R.M. Roberts, and F.W. Bazer. 1982. Establishment of pregnancy in the pig: I. Interrelationships between peri-implantation development of the pig blastocyst and the uterine endometrial secretions. Biology of Reproduction **27**: 925-939.

Geisert, R.D. and J.V. Yelich. 1997. Regulation of conceptus development and attachment in pigs. Journal of Reproduction and Fertility **52**:133-149.

Geisert, R.D., J.V. Yelich, T. Pratt, and D. Pomp. 1998. Expression of an inter-alpha-trypsin inhibitor heavy chain-like protein in the pig endometrium during the oestrous cycle and early pregnancy. Journal of Reproduction and Fertility **114**: 35-43.

Gries, L.K., R.D. Geisert, M.T. Zavy, J.E. Garrett, G.L. Morgan. 1989. Uterine alterations coincident with embryonic mortality in the gilt after exogenous estrogen administration. Journal of Animal Science **67**: 276-284.

Hashimoto, K., T. Tabe, J. Sumiya, Y. Sano, N. Chio-Miura, A. Ozawa, H. Yasue, M. Tomita. 1996. Primary structure of the pig homologue of human IHRP: Inter-α-trypsin inhibitor family heavy chain-related protein. Journal of Biochemistry **119**: 577-584.

Hooper, J.D., L.T. Bui, F.K. Rae, T.J. Harvey, S.A. Myers, L.K. Ashworth, and J.A. Clements. 2001. Identification and characterization of KLK14, a novel kallikrein serine protease gene located on human chromosome 19q13.4 and expressed in prostate and skeletal muscle. Genomics **73**: 117-122.

Keys, J.L. and G.J. King. 1990. Microscopic examination of porcine conceptus-maternal interface between days 10 and 19 of pregnancy. American Journal of Anatomy **188**: 221-238.

Lee, C.Y., M.L. Green, R.C.M. Simmen, and F.A. Simmen. 1998. Proteolysis of insulin-like growth factor-binding proteins (IGFBPs) within the pig uterine lumen associated with pre-implantation conceptus development. Journal of Reproduction and Fertility **112**: 369-377.

Mayers, S.A. and J.A. Clements. 2001. Kallikrein 4 (KLK4), A new member of the human kallikrein gene family is upregulated by estrogen and progesterone in the human endometrial cancer cell line, KLE. Journal of Clinical Endocrinology and Metabolism **86**: 2323-2326.

Roberts, R.M., W.E. Trout, N. Mathialagan, M. Stallings-Mann, and P. Ling. 1993. Uterine secretory activity and embryonic development. In Preimplantation Embryo Development (ed. B.D. Bavister), pp. 229-243. New York:Springer-Verlag.

Rozen, S. and H.J. Skaletsky. 2000. Primer3 on WWW for general users and for biologist programmers. In Bioinformatics methods and protocols: Methods in molecular biology (ed. S. Krawetz and S. Misener), pp. 365-386. Humana Press, Totowa, NJ.

Ryu, O., J.C.C. Hu, Y. Yamakoshi, J.L. Villemain, X.H. Cao, C.H. Zhang, J.D. Bartlett, and J.P. Simmer. 2002. Porcine kallikrein-4 activation, glycosylation, activity, and expression in prokaryotic and eukaryotic hosts. European Journal of Oral Sciences **110**: 358-365.

Salier, J.P., P. Rouet, G. Raguenez, and M. Daveau. 1996. The inter-alpha-inhibitor family: From structure to regulation. Biochemical Journal **315**: 1-9.

Stroband, H.W.J. and T. Van der Lende. 1990. Embryonic and uterine development during early pregnancy in pigs. Journal of Reproduction and Fertility Suppliment **40**: 261-277.

Vonnahme, K.A., S.C. Fernando, J.W. Ross, M.D. Ashworth, U. DeSilva, J.R. Malayer, and R.D. Geisert. 2003. Porcine endometrial expression of kininogen, factor XII, and plasma kallikrein in cyclic and pregnant gilts. Biology of Reproduction **70**: 132-138.

Vonnahme, K.A., J.R. Malayer, H.O. Spivey, S.P. Ford, A. Clutter, and R.D. Geisert. 1999. Detection of kallikrein gene expression and enzymatic activity in porcine endometrium during the estrous cycle and early pregnancy. Biology of Reproduction **61**: 1235-1241.

Yousef, G.M., C.A. Borgono, A. Scorilas, R. Ponzone, N. Biglia, L. Iskander, M.E. Polymeris, R. Roagna, P. Sismondi, and E.P. Diamandis. 2002. Quantitative analysis of human kallikrein gene 14 expression in breast tumours indicates association with poor prognosis. British Journal of Cancer **87**: 1287-1293.

Yousef, G.M. and E.P. Diamandis. 2003. An overview of the kallikrein gene families in humans and other species: Emerging candidate tumour markers. Clinical Biochemistry **36**: 443-452.

Yousef, G.M., M.J. Francis, A. Scorilas, C.A. Borgono, L. Iskander, M. Puopolo, M. Massobrio, E.P. Diamandis, and S. Katsuma. 2003. Steroid hormone regulation and prognostic value of the human kallikrein gene 14 in ovarian cancer. American Journal of Clinical Pathology **119**: 346-355.

Yousef, G.M., C. Stephan, A. Scorilas, M. Abd Ellatif, K. Jung, G. Kristiansen, M. Jung, M.E. Polymeris, and E.P. Diamandis. 2003. Differential expression of the human kallikrein gene 14 (KLK14) in normal and cancerous prostatic tissues. Prostate **56**: 287-292.

VITA

Samodha Charaka Fernando

Candidate for the Degree of

Master of Science

Thesis: ISOLATION, CHARACTERIZATION, MAPPING AND EXPRESSION ANALYSIS OF PORCINE TISSUE KALLIKREINS

Major Field: Animal Science

Education:

2002 – 2004 **:** Completed the requirements for Master of Science degree with a major in Animal Science at Oklahoma State University in December, 2004.

1998 - 2002 **:** Bachelor of Science Degree in Microbiology with first class Honors University of Kelaniya (Sri Lanka)

Project
Designing a Diagnostic kit for the detection of Citrus Greening Organism *(Liberobacter asiaticum*) using the ELISA technique.

Experience:

2002- 2004 **:** Graduate Assistant, Oklahoma State University, Stillwater, OK

2002 **:** Undergraduate trainee at Unilever Ceylon Limited, Colombo

1997 – 1998 **:** Worked as a Volunteer at the Turtle Conservation Project in school Lecture program, Environmental Education Program, and in day to day *in-situ* nest protection, tagging and monitoring program.

Awards and Honors:

: First prize winner of the Outstanding Research in Biological Sciences, Oral presentation. Organized by the Biochemistry and Molecular Biology Graduate Student Association of Oklahoma State University(2004).

: Phi Kappa Phi National Honor Society (2003).

: First Class Honors Degree in Microbiology (2002).