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Generation of a transgenic mouse model to study biological role of KLK5 in epidermis

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Abstract

A number of studies provide strong evidence that KLK5 is one of the most important serine proteases in the epidermis and is involved in processes such as desquamation, processing of antimicrobial peptides or induction of inflammatory reaction. The role of KLK5 has been deduced from *in vitro* experiments and thus its functions should be verified *in vivo*.

This work aimed to develop a specific tissue targeting strategy to study the role of murine kallikreins in the epidermal compartment *in vivo* and to generate a transgenic model overexpressing mKlk5 in the mouse epidermis.

Using the modified promoter of human involucrin, transgenic mice expressing the fluorescent marker, tdTomato, were generated in the first step. This transgenic reporter mouse showed specific targeting pattern of the reporter in the upper epidermal layers and, thus, the modified involucrin promoter could be employed for targeting further gene of interest in the differentiated epidermal compartment.

In the second step transgenic mouse lines expressing murine kallikrein 5 were successfully generated. Among them two lines exhibited approximately 6-9 fold overexpression at the mRNA and protein levels, however it appeared that the immature protease was not activated under normal healthy conditions. Therefore two models of epidermal diseases were performed to disbalance the epidermal barrier, activate the protease and reveal its impact on the targeted compartment. Nevertheless, the transgenic mice did not show any differences in comparison to the wild-type mice in the model of irritant dermatitis and wound healing.

Altogether, we have successfully generated two transgenic mice and developed a targeting strategy for upper layers of epidermis although to target an active protease into these layers will necessitate additional technical modifications.

Key words: kallikrein, transgenic mouse, skin, epidermis, involucrin promoter

Contents

List of Abbreviations

aa	Amino acid
Ab	Antibody
AD	Atopic dermatitis
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
bp	Base pair
cDNA	Complementary DNA
CDSN	Corneodesmosin
CE	Cornified Cell Envelope
DEPC	Diethylpyrocarbonate
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotides
DSC1	Desmocollin 1
DSG1	Desmoglein 1
DTT	Dithiothreitol
ECL	Electrochemical luminescence
EDTA	Ethylenediaminetetraacetic acid
EGFP	Enhanced Green fluorescent protein
Fig.	Figure
hINV	Human involucrin promoter
hINVm	Truncated human involucrin promoter
hINVm-int	Truncated human involucrin promoter containing involucrin intron
ICL	Intercellular lipid matrix
KLK	Human Kallikrein-related peptidase
mKlk	Murine Kallikrein-related peptidase
LB	Lamellar bodies
LEKTI	Lympho-epithelial Kazal-type inhibitor
NS	Netherthon syndrome
PAR	Protease activated receptor
PBS	phosphate buffered saline
PCR	Polymerase chain reaction
pro-KLK	Proform of human Kallikrein-related peptidase
pro-mKLK	Proform of murine Kallikrein-related peptidase
PSA	Prostate Specific Antigen
PVDF	Membrane polyvinylidene fluoride membrane
qRT-PCR	Quantitative real-time polymerase chain reaction
RNA	Ribonucleic acid
RT	Room temperature
SB	Stratum basale
SC	Stratum corneum
SCTE	Stratum corneum tryptic enzyme
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecylsulfate polyacrylamide gel electrophoresis
SG	Stratum granulosum
SL	Stratum lucidum

SS	Stratum spinosum
SV40	simian vacuolating virus 40
TAE	Tris base/ Acetic acid/ EDTA buffer
TBST	Tris-buffered saline Tween-20
TBSTM	Tris-buffered saline Tween-20 Milk
TEMED	N, N, N´, N´ tetramethylethylenediamine

1 Introduction

1.1 The skin

The skin is the largest organ of the body and has many functions essential for the organism´s survival and comfort. It helps us to regulate the body temperature, interact with the environment by touching and feeling and also store and synthesize some important substances.

Primary and the most important function of the skin is forming a barrier between the organism and the environment. It provides effective protection from the pathogens, UV light, physical or chemical damage, as well as the unregulated loss of water and solutes. Maintenance of the skin barrier is highly regulated process and any disbalance can lead to severe diseases and disorders dramatically reducing the quality of life.

Mammalian skin is formed by three main layers, the hypodermis, the dermis and the superficial layer epidermis (Fig. 1).

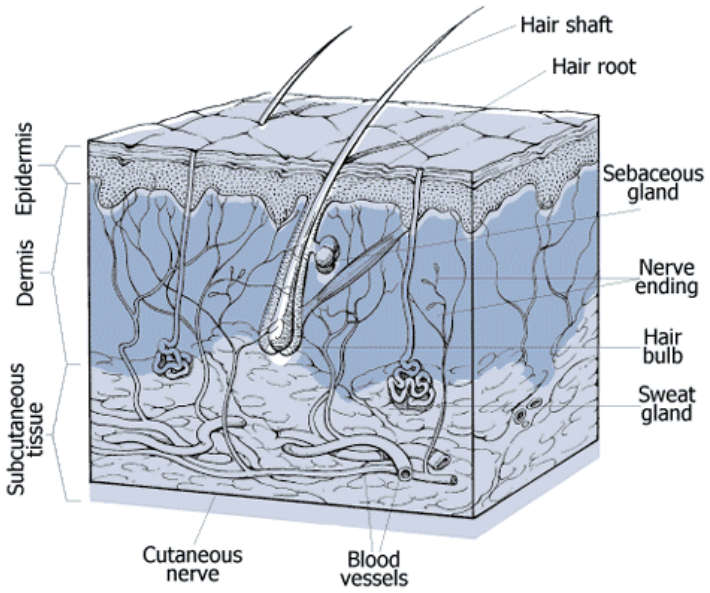


Figure 1. Schematic representation of the skin structure (taken from www.elizspa.com)

Hypodermis (subcutaneous tissue) is the lowermost layer. It is important for thermal and mechanical protection of the body and also serves as storage of the fatty tissue. The main cell type in hypodermis is the adipocyte.

Next skin layer, the dermis, is mainly composed of collagen and elastic fibers that are necessary for its strength and flexibility. It contains several cell types. Most common are fibroblasts, followed by mast cells, macrophages and lymphocytes that are important in skin immunology. A lot of specialized structures, such as nerve fibers, hair follicles, sweat and sebaceous glands and a network of blood vessels, are present in the dermis. These are necessary for sensation, thermoregulation and also to support the outmost skin layer - epidermis.

1.1.1 Epidermis

In terms of the barrier function of the skin, epidermis is the most important layer as it is in direct contact with the environment. Epidermal thickness ranges from 0.05 to 1.5 mm, depending on body location. Also epidermis contains several different types of cells essential for its structure and function. Melanocytes, located at basal layer of epidermis, are cells producing melanin - pigment responsible for the color of skin. Merkel cells, also present in the basal layer, act as specialized touch receptors. Langerhans cells are dendritic cells in epidermis playing an important role in skin immunology (reviewed in Merad et al., 2008).

The most predominant epidermal cells are the keratinocytes. Their name is derived from keratins, a family of fibrous proteins that are major products and also structural components of these cells. Keratinocytes are generated in basal layer of epidermis and then migrate to the surface. On their journey, these cells undergo a process of differentiation affecting their shape and physiology. Epidermis can be divided into 5 sublayers, mostly defined by presence of keratinocytes of particular differentiated state. These layers are called *stratum basale (SB) stratum spinosum (SS), stratum luicidum (SL), stratum granulosum SG)* and superficial *stratum corneum (SC)* (Fig. 2).

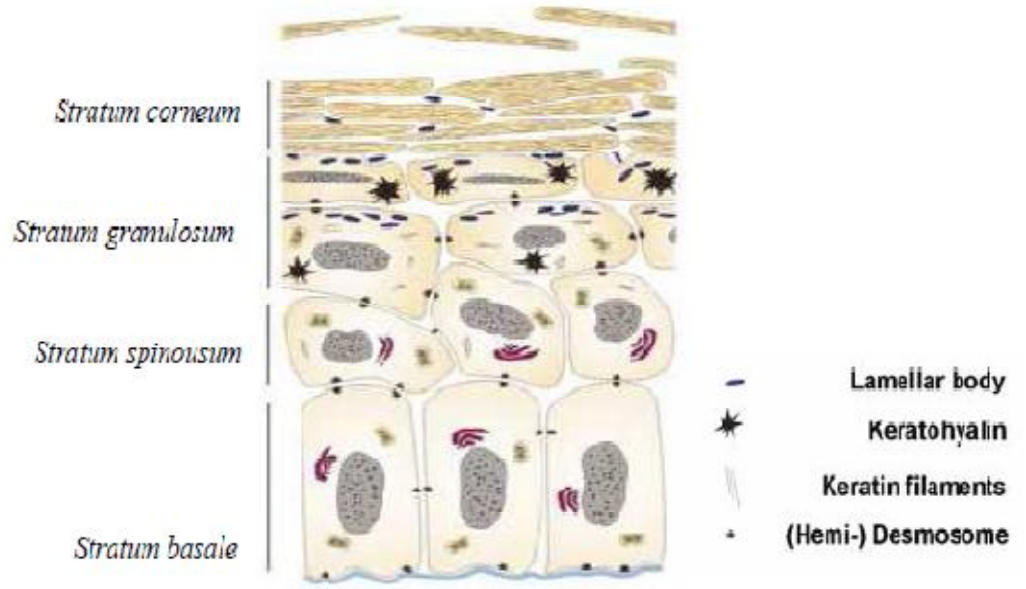


Figure 2. Schematic cross section of epidermis
Stratum basale, stratum spinosum, stratum granulosum and *stratum corneum* are four main layers of epidermis. Thick skin, located on human soles and palms, contains one additional epidermal layer - *stratum lucidum* (not depicted). (taken from Lippens et al., 2005)

Stratum basale, the innermost epidermal layer is formed by a single row of cuboidal or cylindrical cells attached to basal lamina. The cells are connected to each other via cell-to-cell junctions called desmosomes, whereas attachment of cells to basal lamina is mediated via similar structures - hemidesmosomes. Epidermal stem cells, that are present in *stratum basale*, continually divide and new cells push the older ones to the surface.

Stratum spinosum is the thickest layer of epidermis. It is made up of large polyhedral keratinocytes joined by desmosoms. Cells in *stratum spinosum* produce cytokeratin, which aggregates to form intracellular filaments called tonofibrils. These structures act as a part of cytoskeleton and are important to stabilize the cells and provide protection from mechanical stress.

Stratum granulosum consists of 2 - 4 layers of flattened keratinocytes and it is the last epidermal layer with cells exhibiting metabolic activity. Keratinocytes in upper layers of SG lose most of their organelles including the nucleus. Name of this layer is derived from basofil *keratohyalin granules* that are accumulated in cytoplasm of the cells.

Other important structures in *stratum granulosum* are lamellar bodies. These secretory organelles originate from Golgi aparatus and may contain hydrolytic enzymes, inhibitors or other important substances that are necessary in upper epidermal layers. After

fusion with keratinocyte plasma membrane, the contain of lamellar bodies is released into extracellular matrix (Ishida-Yamamoto et al., 2004).

The *stratum lucidum* is present only in "thick skin", located on human soles and palms. This layer is made up of dead, flat cells and is important to the skin barrier function in locations subjected to tension and mechanical stress.

Stratum corneum is the most superficial layer of epidermis. Keratinocytes in SC (also known as corneocytes) are metabolically dead, flattened cells filled with keratin filaments. Adjacent corneocytes are linked together by adhesive structures called corneodesmosomes. Cell membrane of corneocytes is associated with cornified cell envelope (CE). This insoluble structure is generated during terminal differentiation of keratinocytes and it is made up of proteins covalently linked together by transglutaminases. Cornified cell envelope act as a rigid scaffolding, that is important for cohesion and barrier function of SC (Candi et al., 2005). Corneocytes are embedded in an intercellular lipid matrix (ICL), which is mainly composed of ceramids, cholesterol and free fatty acids (Weerheim et al., 2001). ICL is a primary barrier to water and solutes. Fatty acids present in startum corneum are responsible for acidic pH at the surface of epidermis. This pH gradient, ranging from pH = 6.5 at SG/SC border to pH = 4.5 at the skin surface, seems to play an important role in regulation of many physiological processes in SC. Since the *stratum corneum* is in direct contact with harmful environment, all its components need to be continually replaced to maintain structural intactness and integrity of SC. Recent publications suggest that proteolytic enzymes, such as kallikreins, may play an important role in this process.

1.2 Kallikrein-related peptidases

Kallikrein-related peptidases (further kallikreins, KLKs) form a large family of serine proteases with diverse physiological and pathophysiological functions. The name "kallikrein" is derived from "kallikreas", a Greek expression for pancreas, from where was the first kallikrein (later KLK1) isolated. The human kallikrein family consists of 15 members (KLK1-KLK15), which are encoded by a gene cluster located on chromosome 19q13.4 (Yousef and Diamondis, 2001). Most of kallikrein genes are conserved in mammalian species and mouse paralogs (mKlks) have been reported to be located on chromosome 7 in a cluster formed by at least 24 genes (Evans et al., 1987).

1.2.1 Structure and function of kallikreins

All kallikreins are synthesized as inactive preproenzymes (Fig. 3). N-terminal prepeptide (16 - 34 amino-acids) acts as a signal sequence targeting the protein into endoplasmatic reticulum, where is this sequence removed. After the proenzyme is secreted into extracellular space, also the propeptide (3 - 37 amino-acids) is usually proteolytically processed. This leads to conformational changes resulting in formation of active site and activation of the enzyme (reviewed in Lundwall and Brattsand, 2008). Active site of kallikreins contains three conserved amino-acids, typical for many other serine proteases - Asp102 - His57 - Ser195. This catalytic triad, in combination with other specific amino-acid residues present in active site, is mainly responsible for the substrate specificity of these proteases. Most kallikreins exhibit trypsine-like substrate specificity, thus, they prefere to cleave the substrates after lysine or arginine aminoacid residues. The specificity of KLK3, KLK7 and KLK9 is chymotrypsin-like, with substrate cleavage preference after tryptophan, tyrosine, or phenylalanine (reviewed in Lundwall and Brattsand, 2008).

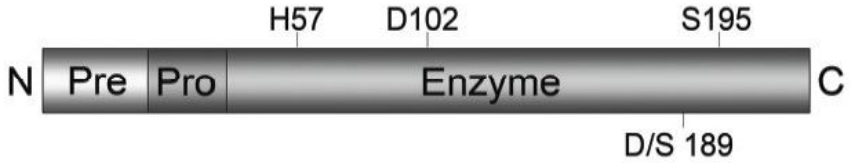


Figure 3. KLK preproenzyme (taken from Steffanson, 2008)

Despite high degree of homology between individual KLKs, there is a big diversity in physiological functions of these proteases. KLK1 is a member of signaling pathway involved in regulation of blood pressure (Chao et al., 2006). KLK3, enzyme secreted in prostate gland, seems to be one of key components in a process of semen liquefaction, which is necessary for proper function of male reproductive system (Lilja H., 1985). KLK3 is also an important marker for prostate malfunctions. Elevated serum levels of KLK3 are typical for prostate cancer patients (Wang et al., 1981) and KLK3 is commonly known by an alternative name "Prostate Specific Antigen" (PSA). KLK8 appears to be to be involved in a process of brain development and memory acquisition, possibly via processing of extracellular matrix proteins (Tamura et al., 2006). KLK8 and KLK6 also have been reported to be deregulated in many neurodegenerative diseases, such as Alzheimer´s disease or multiple sclerosis (Terayama et al, 2007; Little et al., 1997).

Most of kallikrein genes are expressed in skin. *Klk1, Klk4* - *Klk11* and *Klk13* - *Klk14* mRNA was identified in epidermis by RT-PCR analysis (Komatsu et al., 2003) and KLK5, 7 and 14 proteins have been isolated from epidermal extracts (Brattsand et al., 2005; Ekholm et al., 2000). However, function of KLKs in skin is still not well understood. One of the most intensively studied kallikreins in skin is KLK5. According to some studies, this enzyme may account for as much as 50% of the overall trypsin-like activity in superficial epidermal layers (Brattsand et al., 2005). Recent publications show involvement of KLK5 in many physiological processes in epidermis, as well as in skin pathophysiology.

1.2.2 KLK5

1.2.2.1 Expression and regulation of KLK5 in epidermis

The preproenzyme of human KLK5 consists of 293 amino acid residues (aa), with 30 aa signal sequence and 37 aa propeptide (Debela et al., 2008). Its mouse orthologue (mKlk5) has a correspondent lenght of 293 aa and both enzymes share 68% homology. Based on high degree of active-site homology and similar expression profile (R. Sedlacek, unbublished data), the same activity and physiological functions can be assumed.

KLK5 has been found to be expressed in a wide range of tissues, such as lymph nodes, stomach, kidney, heart, ovaries and uterus (reviewed in Lundwall and Brattsand, 2008). However, most experimental data refer to its expression and function in epidermis.

Expression of KLK5 was detected by RT-PCR and immunohistochemistry in superficial epidermal layers - *stratum corneum* and *stratum basale* (Brattsand and Egelrud, 1999). This specific localization gives KLK5 also an alternative name - Stratum Corneum Tryptic Enzyme (SCTE). KLK5 has been found also in hair follicles, sweat glands and sebaceous glands (Ekholm et al., 2000, Komatsu et al., 2003).

It is assumed that pro-KLK5 is synthesized by keratinocytes in *stratum granulosum* and is transported via lamellar bodies towards the *stratum granulosum/stratum corneum* interface, where is the enzyme released into extracellular matrix of SC (Ishida-Yamamoto et al., 2005). Once the proenzyme is present in *stratum corneum*, it can be activated by proteolytic cleavage of the propeptide sequence. It has been shown, that active form of KLK5 is able to activate its own pro-form (Brattsand et al., 2005) and also pro-forms of other KLKs present in epidermis - KLK1, KLK6, KLK7, KLK11, KLK12 and KLK14 (Yoon et al., 2007). Furthermore, pro-KLK5 can be activated by KLK14 and by a transmembrane proteolytic enzyme, matriptase (Brattsand et al., 2005; Sales et al., 2010).

This suggests, that skin kallikreins may form complex activation cascades and altered activity of one enzyme can up- or down-regulate a big variety of other proteases. Among proteases expressed in epidermis, KLK5 has the widest range of pro-KLK substrates (Yoon et al., 2007). Thus, KLK5 was postulated to play a key role in activation of other KLKs and initiation of epidermal proteolytic cascades (Brattsand et al., 2005). However, biological role of these cascades is yet to be determined, as well as the role of majority of participating enzymes.

Activation of KLK5 is not the only way, how to control its enzymatic activity. Once the mature enzyme is generated, its activity may be regulated by several inhibitors. It was reported, that even low concentration of Zn^{2+} ions can lead to effective inhibition of KLK5 (Debela et al., 2007). However, no data have supported relevance of this mechanism in epidermis.

Another potential inhibitor of KLK5 in epidermis may be cholesterol sulfate. This lipid, naturally present in *stratum granulosum* and *stratum corneum* (Elias et al., 1984), has been reported to inhibit trypsin-like activity in epidermis (Sato et al., 1998). On the other hand, there is no direct evidence that cholesterol sulfate inhibits specifically KLK5.

Until now, the most intensively studied inhibitor of KLK5 in epidermis has been *Lympho-epithelial Kazal-type inhibitor* (LEKTI). This multidomain protein, encoded by gene SPINK5, has been found to be present in *stratum spinosum, stratum granulosum* and *stratum corneum* (Bitoun et al., 2003; Komatsu et al., 2002). LEKTI is synthesized as a large protein consisting of 15 inhibitory domains (D1 - D15). Intracellular processing of this protein gives rise to single or multidomain fragments that are secreted to extracellular spaces (Bitoun et al., 2003). Similarly to KLK5, also LEKTI fragments are transported to superficial epidermal layers by system of lamellar bodies (Ishida-Yamamoto et al., 2005).*In vitro* studies have shown that all LEKTI fragments D8-D11 and D12-15 exhibit strong and specific inhibitory effect towards recombinant KLK5 (Deraison et al., 2007, Borgono et al., 2007). Additionally, affinity of LEKTI fragments to several skin proteases has been compared and it has been suggested that KLK5 is the major target of LEKTI (Deraison et al., 2007).

It seems that inhibition of KLK5 by LEKTI fragments is a process tightly controlled by pH gradient in *stratum corneum*. The complex of KLK5 and D8-D11 shows high stability at pH 7.5 that would be found in *stratum corneum/stratum granulosum* interface. Decreasing pH at *stratum corneum* (to the values of pH = 4 at the surface of epidermis) supports dissociation of KLK5 and LEKTI fragments, which results in higher

proteolytic activity of KLK5 in superficial layers (Deraison et al., 2007). Indeed, presumed biological functions of KLK5, such as degradation of corneodosmosomes or processing of antimicrobial peptides, mainly take place in upper layers of *stratum corneum*.

1.2.2.2 The role of KLK5 in epidermis

1.2.2.2.1 Desquamation

As new keratinocytes are continuously being generated in *stratum basale*, old cells are shed from the surface of epidermis. This process is denoted by the term "desquamation". It has been demonstrated that shedding of corneocytes from the skin surface occurs through degradation of corneodesmosomes - modified desmosomes present in *stratum corneum* (Lundström et al., 1994). Similarly to desmosomes, the function of corneodesmosomes is to maintain adhesion between corneocytes in *stratum corneum*. Once the corneodemosomes are degraded, cells can be released from the skin surface. It has been proved, that degradation of corneodesmosomes is mediated via proteolytic cleavage of their structural proteins (Suzuki et al., 1994). Corneodesmosomes are composed of two main proteins: desmoglein 1 (DSG1) and desmocollin 1 (DSC1). Both proteins are members of the cadherin family and form the transmembrane part of the corneodesmosome. Furthermore, corneodesmosomes contain a glycoprotein, corneodesmosin (CDSN) (reviewed in Kowalczyk et al., 1999).

In vitro experiments have shown, that KLK5 is able to digest proteins DSG1, DSC1 and CDSN (Caubet et al., 2004). CDSN and DSC1 can be also digested by KLK7 (Caubet et al., 2004), an epidermal protease activated exclusively by KLK5 (Brattsand et al., 2005). Role of KLK5 in desquamation is further emphasized by phenotype of mouse deficient in LEKTI - the KLK5 inhibitor. LEKTI deficient mice exhibit hyperactivity of KLK5 and KLK7 in epidermis resulting in increased degradation of CDSN and premature desquamation (Yang et al., 2004). It is assumed that in healthy skin LEKTI regulates KLK5-mediated degradation of corneodesmosomals proteins in pH-dependent manner (Fig 4.) (Deraison et al., 2007).

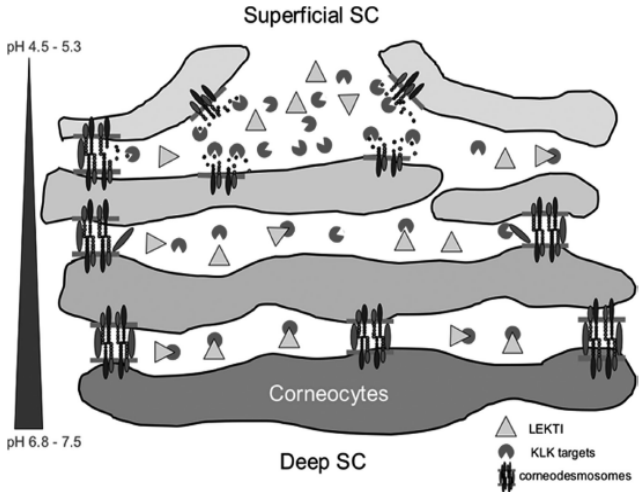


Figure 4. Model of desquamation
LEKTI forms stable complex with KLK5 at the pH at SG/SC interface. Acidic pH at the skin surface supports dissociation of KLK5 and LEKTI. Active enzyme may take part in degradation of corneodesmosomal proteins that leads to desquamation of keratinocytes. (taken from Deraison et al., 2007)

1.2.2.2.2 Processing of cathelicidins

Cathelicidins are proteins with an antimicrobial effect expressed in many tissues including epidermis. Human cathelicidins are generated from the precursor protein hCAP18, which is proteolytically processed into a antimicrobial peptide LL-37 (Sorensen et al., 2001). This can be further cleaved into smaller antimicrobial peptides. It has been proved, that KLK5 is able to digest hCAP18 giving a rise to LL-37. KLK5 can also generate peptides KS-22, KS-29 and KS-30 by processing of LL-37 (Yamasaki et al., 2006). Cathelicidins have been found in superficial layers of *stratum corneum*, as well as at the surface of the epidermis (Yamasaki et al., 2006), which corresponds to the locations with increased proteolytic activity of KLK5. Therefore, it has been suggested, that KLK5 may be the key activator of epidermal cathelicidin (Yamasaki et al., 2006).

1.2.2.2.3 Activation of PAR2

Proteinase-activated receptors (PARs) are G-protein coupled receptors (PAR-1 - PAR-4), that can be activated by proteolytic cleveage of their N-terminal extracellular domain (Fig. 5). PAR-2 is expressed by keratinocytes of *stratum granulosum* and *stratum spinosum* (Santulli et al., 1995) and can be found also in hair follicles, sweat glands, sebaceous glands and sensory neurons (Steinhoff et al., 1999; Steinhoff et al., 2003).

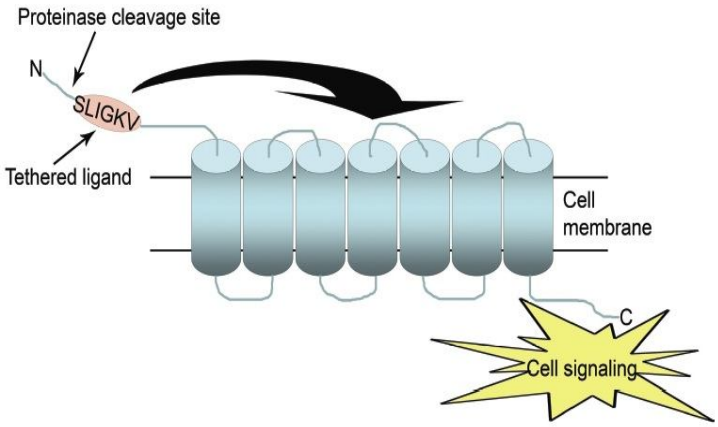


Figure 5. PAR-mediated signaling
Cleavage of the N-terminal extracellular domain leads to the release of tethered ligand, which binds to the receptor. This leads to a conformational change of the receptor, resulting in intracellular signaling. (taken from Steinhoff et al., 2005)

In vitro, PAR-2 can be activated by trypsin, mast cell tryptase (Steinhoff et al., 2005) and recent discoveries show that also by KLK5 and KLK14 (Stefansson et al., 2008). Endogenous activators of PAR-2 have not been determined *in vivo*. However, coexpression of KLK5 and PAR-2 in epidermis suggests that KLK5 may be a potential candidate.

Research has shown that activation of PAR-2 in epidermis results in inhibition of lamellar bodies secretion (Hachem et al., 2006). Hachem et al. further demonstrated that this process is induced by increased activity of serine proteases. LB secretion is required to transport many structural components of *stratum corneum*, such as glucosylceramids and phospholipids. Processing of these molecules give rise to ceramids and free fatty acids that are necessary in formation of ICL, preventing water and electrolytes loss (Harris et al., 2007). Indeed, upregulated expression of KLK5 in skin disorders, such as atopic dermatitis or rosacea, is accompanied by defects of epidermal barrier and higher transepidermal water losses (Yamasaki et al., 2007, Ong et al., 2002).

Activation of PAR-2 may also induce inflammation and immune response in skin (Coughlin and Camerer, 2003). *In vivo* experiments have proved that wild type mice show edema and granulocyte infiltration upon injection of trypsin IV into a paw. The same experiment with PAR-2 deficient mice does not trigger the immune response (Knecht et al., 2007). Similarly to defects of epidermal barrier, many inflammatory skin disorders are associated with KLK5 overexpression (Komatsu et al., 2007; Yamasaki et al., 2007).

Furthermore, PAR-2 may be involved in skin pigmentation (Seiberg et al., 2000; Paine et al., 2001), wound healing and scar formation (Materazzi et al., 2007).

1.2.2.2.4 KLK5 in skin pathophysiology

Altered expression or activity of KLK5 has been observed in many skin diseases, such as atopic dermatitis, psoriasis vulgaris, rosacea and Netherton syndrom.

Atopic dermatitis (AD) is a chronic inflammatory skin disorder manifested by dry skin, long-term itch and enhanced susceptibility to microbial infections. Skin of AD patients exhibits impaired differentiation and cornification of keratinocytes, increase of trans-epidermal water losses and abnormal expression and processing of antimicrobial peptides (Ong et al., 2002; Palmer et al., 2006).

Defective processing of cathelicidins and chronic inflammation are symptomatic for another skin disease – rosacea (Yamasaki et al., 2007).

Psoriasis is an autoimmune disease that can appear on skin as inflamed, scaly patches that may itch. It is accompanied by dysfunctions of the keratinization process and hyperproliferation of epidermal cells (Van Erp et al., 1989).

All of these pathological conditions are to some extent associated with overexpression of multiple skin KLKs including KLK5 (Komatsu et al., 2007; Yamasaki et al., 2007). However, increased proteolytic activity of KLKs has not been detected in AD. Psoriatic skin exhibits hyperactivity of KLK7, which is dependent on activation by KLK5 (Komatsu et al.,2007; Brattsand et al., 2005). Furthermore, disruption of epidermal barrier, induced inflammation and impaired processing of antimicrobial peptides associated with these skin diseases, correspond to presumed biological effects of KLK5 and suggest a possible linkage to this enzyme.

Involvement of KLK5 in skin pathophysiology is most obvious in Netherton syndrom (NS). This autosomal recessive genetic skin disorder is caused by a mutation in the *SPINK5* gene resulting in deficiency of KLK5-inhibitor - LEKTI. Newborn children suffering from NS exhibit thin, scaly skin susceptible to trans-epidermal water losses. Increased activity of KLK5 and KLK7 results in premature desquamation, severely disrupting the epidermal barrier (Descargues et al., 2005; Yang et al., 2004). Phenotype of LEKTI deficient mice mimics symptoms of Netherton syndrome (Descargues et al., 2005).

1.2.2.3 KLK5 in epidermis – summary

Physiological function of KLK5 in skin is not fully understood. Emerging evidence suggests its involvement in many processes that are crucial for a proper barrier function of epidermis, such as desquamation of keratinocytes and protection from pathogens. Through activation of PAR-2, KLK5 can possibly regulate epidermal barrier maintenance and be involved in induction of inflammatory reaction. Additionally, KLK5 may be a key player of epidermal proteolytic cascades, which could further broaden its potential role in regulation of skin homeostasis.

However, most data referring to KLK5´s role in epidermis are based on using *in vitro* techniques. Experiments with keratinocyte cell lines and recombinant proteins helped to reveal a wide range of potential substrates and inhibitors. Yet, to understand the role of KLK5 in tightly regulated and complex skin physiology, *in vivo* model is necessary.

Transgenic mouse models overexpressing epidermal proteases proved to be useful tools to determine physiological functions of these enzymes (Hansson et al., 2002; Bonnart et al., 2010). Mouse expressing human KLK7 from SV40 promoter generated by Hansson et al. helped to link KLK7 to several skin disorders and to reveal its role in desquamation. However, SV40 as ubiquitous promoter could possibly lead to unspecific effects of the transgene. More suitable seems to be a model of Bonnart et al, who prepared transgenic mouse expressing Elastase2 from involucrin promoter. This limited the expression of transgene specifically to epidermis (Bonnart et al., 2010). Similarly, this diploma thesis aims to prepare a transgenic mouse overexpressing mKlk5 targeted to superficial epidermal layers.

2 Materials and methods

2.1 Bacteria and DNA techniques

2.1.1 Preparation of competent cells

E. Coli XL1 Blue cells (Stargene, Cedar Creek, USA) were used to prepare competent cells by calcium chloride method. A frozen stock of cells (50 µl) was kept on ice for approximately 20 minutes and once the cells were thawed, the stock was resuspended at 3 ml of LB media. The cells were kept in a shaking incubator (37 °C, 250 rpm) for 16 hours. 2 ml of the culture was added into Erlemayer flask with 200 ml of LB media and the bacteria were grown until OD_{600} = 0,4. Bacterial suspension was distributed into four 50 ml centrifugation tubes and centrifuged at 1500 g, 4 °C for 10 minutes. After the supernatant was discarded, the cells were placed on ice and resuspended in 50 ml of 100 mM calcium chloride. Cells were incubated on ice for 5 minutes and then centrifuged (1500 g, 4 °C) for 10 minutes. The supernatant was discarded, 10 ml of 100 mM calcium chloride was added to the pellet and after 15 min incubation on ice, the cells centrifuged for 10 minutes (1500 g, 4 °C). The pellet was resuspended in 3 ml of 100 mM calcium chloride and kept on ice. After 3 hours of incubation, 1 ml of glycerol was added to the cells. Competent cells were distributed in 50 µl aliquotes and stored at -80 °C.

2.1.2 Transformation of bacteria

A 50 µl aliquot of competent cells (E. coli XL-1 Blue) was used for a single transformation. After 10 minutes of incubation on ice the cells were mixed with 1 µl of plasmid DNA (concentration ranging from 0.1 – 0.5 µg/µl) or 5 µl of ligation mixture and incubated on ice for another 10 minutes. The cells were heated to 42 °C for 90 s. 800 µl of LB media was added to the transformation mixture and the cells were incubated for 1 hour at 37 °C. 100 µl of the mixture was plated on LB agar plate containing ampicillin (concentration 25 µg/ml) and the plates were incubated for 20 hours at 37 °C.

2.1.3 Colony picking and inoculation

Single colony was picked from the LB agar plate using sterile pipette tip and resuspended in 15ml Falcon tube containing 5 ml of LB media supplemented with

ampicillin (1 µg/ml). Tubes were incubated in the shaking incubator for 20 hours (37 °C, 250 rpm).

2.1.4 Isolation of plasmid DNA

GeneJet Plasmid Miniprep Kit was used to isolate plasmid DNA from bacteria. 3 ml of bacterial culture (OD_{600} = 0,4) was centrifuged for 2 minutes (10000 g, 4°C). After discarding of the supernatant, the pellet was resuspended in 250 µl of Resuspension buffer (A1) by pipetting up and down. 250 µl of the Lysis buffer (A2) was added to the suspension and the tube was inverted 6 times in order to mix the solutions. 350 µl of Neutralization buffer (A3) was added, and the content of the tube was mixed by brief vortexing. After 5 minutes of centrifugation (11000 g, RT), the supernatant was transferred onto the provided spin column. Column was briefly centrifuged (5000 g, RT, 1 minute) and the flow-through was discarded. 500 µl of Wash solution was loaded onto the column. The tube was centrifuged for 1 minute (11000 g, RT) and the flow-through was discarded. Washing of the column was repeated with 500 µl of Wash solution. After discarding of the washing buffer, the column was centrifuged for 1 minute (11000 g, RT) in order to remove residual Wash solution. The spin column was transferred into a sterile 1.5 ml tube and 50 µl of the Elution buffer was loaded on the spin column membrane. The tube was incubated for 2 minutes at RT and then centrifuged for 1 minute (11000 g, RT). The flow through containing plasmid DNA was stored at -20°C.

2.1.5 Agarose gel electrophoresis

Agarose gel electrophoresis was used for visualisation, separation and purification of DNA fragments. Agarose powder was mixed with TAE buffer to the concentration of 0,01 g/ml, then heated in a microwave oven until the agarose was completely melted (2-3 minutes). The solution was left to cool down for 2-3 minutes at room temperature. Ethidium bromide was added to the solution to the final concentration of 0.5 µg/ml. The solution was poured into a casting tray and a sample comb was inserted. After the gel had solidified (20 - 25 minutes), the sample comb was carefully removed and the casting tray was inserted into an electrophoresis tank, which was filled with TAE buffer. Samples were mixed with Loading dye (1:1) and 7 µl of the sample was pipetted into the sample well. 5 µl of the DNA ladder was loaded next to the samples in order to determine size of the

DNA fragments. The electrophoresis was run at 4,5 V/cm for 35 minutes. UV trans-illumination at wavelenght of 312 nm was used for the visualisation of DNA.

2.1.6 Restriction of DNA

Plasmid DNA (1-4 µg) was was mixed with 1 µl (10 U) of restriction enzyme, corresponding buffer and dH_2O to the final volume of 20 µl. The mixture was incubated at 37°C for 1 hour.

2.1.7 Ligation of DNA fragments

Both DNA fragments (5 -10 µl of linear vector DNA (50-400 ng) and DNA insert in amount corresponding to molar ratio 1:3) were mixed with 1µl of T4 ligase (Fermentas, Canada), 2 µl of T4 ligase buffer (10x) and dH_2O to the final volume of 20 µl. The mixture was incubated for 1 hour at room temperature. 5 µl of the mixture was used for transformation of competent bacterial cells.

2.1.8 Isolation of DNA fragments from agarose gel

Agarose gels used for separation and isolation of DNA fragments were prepared as above, with lower concentration of ethidium bromide (0.2 µg/ml). After electrophoresis (4,5 V/cm, 60 minutes), the agarose gel was placed under UV light to visualize the bands and desired DNA fragment was excised with a sterile disposable scalpel.The gel slice was placed into 2 ml tube. PCR-Combi-Kit (Seqlab, Germany) was used for isolation of DNA from the gel. 1 ml of Gel solubilizer was added to a gel slice and the sample was incubated at 50 °C until the gel slice was completely melted. 500 µl of Binding buffer was added to the sample and the mixture was mixed by vortexing. Filter cartridge was assambled with 2 ml receiver vessel and 750 µl of the sample was loaded onto the filter. The ensemble was centrifuged for 1 minute at 10000 g. Flow through was discarded and the binding step was repeated with residual solution. Afterwards, the cartridge was placed into anew receiver vessel. 500 µl of Washing solution HS was pipetted into the cartridge and the ensemble was centrifuged for 1 minute at 10000 g. Flow through was discarded and the washing step was repeated with 750 µl of Washing solution MS. Flow through was discarded, the cartridge was reassambled with the receiver vessel and centrifuged for 2 minutes at 10000 g to remove the remnants of ethanol. Filter cartridge was assambled with 1.5 ml Elution

vessel. 50 µl of Elution buffer was loaded on the filter membrane. The ensemble was incubated for 5 minutes at room temperature and then centrifuged for 1 minute at 6000 g. The flow through contained purified DNA. The sample was stored at -20 °C.

2.1.9 Preparation of DNA fragments for pronuclear microinjection

Restriction enzymes were used to release a linear DNA fragment from the plasmid. The reaction mixture was loaded on 1% agarose gel and after electrophoresis (4,5 V/cm, 60 minutes), the DNA fragment containing promoter sequence and transgene was excised. Desired DNA fragment was purified by PCR-Combi-Kit and diluted at 50 µl of destilled water. Illustra Nick Column (GE Healthcare, UK) was used for further purification of the DNA fragment. The purificication procedure was performed according to manufacturer´s instruction protocol. 400 µl of the microinjjection buffer was used to elute the DNA fragment from the column. Five fractions containing the desired DNA were collected. Two fractions with highest concentration of DNA were pooled together and filtered through 0.22 micron pore membrane.

2.1.10 Genomic DNA isolation

Tail biopsies (0.5 - 1 cm) were obtained from 3-4 weeks old mice. Prior to the DNA isolation, tails from Inv_tdTomato mice were analyzed for tdTomato expression by fluorescent microscope. Subsequently, the tail was placed into a 2 ml tube containing 1 ml of Mouse lysis buffer and 30 µl of Proteinase K (10 mg/ml). The tube was incubated at 55 °C until the tail was completely dissolved (usually 16 hours). 1 ml of 1:1 phenol/chloroform solution was added and the content of the tubes was mixed by inverting it up and down. To separate the phases, tube was centrifuged for 2 minutes at 12000 g. The upper phase containing genomic DNA was transferred into a fresh 2 ml tube, mixed with 1 ml of chloroform and centrifuged again for 2 minutes at 12000 g. The upper aqueous supernatant was transferred into 2 ml tube and mixed with 1 ml of 96% ethanol and 100 µl of sodium acetate (3 M, pH = 6). The mixture was centrifuged as previously (12000 g, 2 minutes). The supernatant was carefully removed and 1 ml of 75% ethanol was added to the pellet. Tube was centrifuged for 2 minutes at 12000 g, the supernatant was carfully removed at the tubes were placed into a fume hood in order to completely dry the pellets. After 1-2 hours, the pellet was dissolved in 100 µl of destilled water and the tubes were stored at 4 °C.

2.1.11 Genotyping

The DNA isolated from tail biospies was diluted in destilled water (1:30) and was used as a template for PCR reaction. DNA fragment used for microinjections and DNA from positive animals (if available) were used as positive controls. DNA isolated from a non-transgenic mouse was used as a negative control. Reaction mixture was prepared as described in the Table 1. Primers used for individual trasgenic lines are described in the Table 2. Thermal profile of PCR reaction is described in the Table 3.

Table 1. PCR reaction mix for genotyping

Components	Volume
DreamTaq polymerase (1 U/µl)	0.1 µl
DremTaq buffer 10x	1.5 µl
dNTP (2.5 mM)	1.2 µl
F primer (100 µM)	0.15 µl
R primer (100 µM)	0.15 µl
Genomic DNA	3.6 µl
H_2O	8.4 µl
Total volume	15 µl

Table 2. Primers used for genotyping

	Inv-tdTomato	Inv-Klk5
Forward primer	Inv-fwd	Inv-fwd
Reverse primer	Tom_rew	FLAG-rew

Table 3. PCR program used for genotyping

Temperature	Duration
95 °C	3 min
95 °C	40s (30x)
60 °C	40s (30x)
72 °C	90s (30x)
72 °C	3 min

2.2 Cell Culture techniques

2.2.1 Cell culture maintenance

Cells were cultivated on tissue culture treated plates in appropriate culture medium with supplements (Table 4). Upon 70-80% confluence, the medium was discarded and the cells were rinsed with 5 ml of PBS. 0.5 ml of 0.25% Trypsin/ 0.53 mM EDTA was added to the cells and the plate was incubated at 37 °C for 10 - 15 minutes (until the cells detached from the plate. 5 ml of appropriate medium was added to the cells, in order to neutralize the trypsin. The cells were resuspended in the medium, transfered into a 15 ml falcon tube and centrifuged for 3 minutes at 400 g. The supernatant was discarded and the cells were resuspended at 1 ml of culture medium. 0.1 ml of the suspension was added in a fresh tissue culture plate containing 10 ml of medium. The cells were incubated at 37 °C, 5% CO_2.

Table 4. Media and supplements used for cell culture

Cell line	Medium	Supplements
PAM212	RPMI	10% FBS, 1%Pen/Strep
HaCaT	DMEM	10% FBS, 1%Pen/Strep
Cos7	DMEM	10% FBS, 1%Pen/Strep
HepG2	EMEM	10% FBS, 1%Pen/Strep

2.2.2 Transfection of cultured cells

Cells were plated on 24-well plate (50000 cells/well) and incubated for 24 hours at 37 °C, 5% CO_2. The culture medium was aspirated and 0.5 ml of fresh medium was added to the cells. Transfection mixture was prepared in 1.5 ml tube by mixing 1 µg of plasmid DNA (concentration 0.1 - 2 µg/µl) with appropriate culture medium (no supplements added) to the finale volume of 22 µl. 3 µl of transfection reagent Fugene HD (Roche, Switzerland) was added into the tube. Mixture was vortexed for 5 s, incubated for 15 minutes at room temperature and then added dropwise to the cells. Transfected cells were incubated at 37 °C, 5% CO_2. The cullture medium was changed for a fresh one 24 hours post-transfection.

2.2.3 Immunofluorescence staining of the cells

Transfected cells cultured in 24-well plate were rinsed with PBS and 0.8 ml of 3.7% paraformaldehyde in PBS was added. 3.7% paraformaldehyde was aspirated after 10 minutes of incubation and the plate was washed 3x with PBS. To permeabilize the cells, 0.8 ml of ice cold methanol was added. After 4 minutes of incubation with methanol, the cells were washed with PBS and 1% BSA diluted in PBS was added. The plate was incubated for 45 minutes at 37 °C. 1 µl of anti-FLAG mAb (Sigma-Aldrich, USA) was diluted in 500 µl of 0.5% BSA/PBS and 150 µl was added to the cells. After 40 minutes, the cells were washed with PBS. 1 µl IgG TRITC – goat anti-rabbit (*Sigma-Aldrich*, USA) was diluted in 200 µl of 0.5% BSA/PBS and applied on the sample. The sections were incubated for 40 minutes at RT and washed with PBS. Samples were visualised by fuorescent microscopy.

2.2.4 Preparation of cell lysates from tissue cultured cells

Cells cultured on 10 cm Petri dish were washed with 5 ml of PBS. After removal of the PBS, 475 µl of RIPA buffer and 25 µl of Complete protease inhibitor – EDTA free (Roche, Germany) was added to the cells. Plate was incubated at 4 °C on a plate shaker for 60 minutes. Cells were scraped from the surface of the plate and the suspension was transferred into a 1.5 ml tube. Suspension was centrifuged at 15000 g at 4 °C for 15 minutes. Supernatant was pipetted into a fresh 1.5 ml tube. The sample was stored at -20 °C.

2.3 RNA analysis

2.3.1 RNA isolation

50 mg of skin sample was frozen in liquid nitrogen, homogenized using mortar and pestle and resuspended in 1 ml of TRIzol Reagent (Invitrogen, USA). The sample was incubated for 5 minutes at 30 °C. 200 µl of chloroform was added and the tube was inverted 15 times to mix the content. After 2 minutes of incubation at 25 °C, the sample was centrifuged (12000 g, 4 °C, 15 minutes). Aqueous phase containing the RNA was transfered into a fresh tube, mixed with 0.5 ml of isopropanol and incubated for 10 minutes at 25 °C. After centrifugation (12000 g, 4 °C, 10 minutes), the supernatant was removed and the pellet was washed with 1 ml of 75% ethanol. The sample was briefly vortexed and

centrifuged (7500 g, 4 °C, 5 minutes). The supernatant was discarded and the tube was placed into a fume hood in order to dry the pellet. The pellet was dissolved in 50 µl of RNAse free water. Sample was stored at -20 °C.

2.3.2 cDNA synthesis

First Strand cDNA Synthesis kit (Fermentas, Canada) was used to prepare the cDNA. RNA (concentration 50 - 200 ng/µl) was mixed with 1 µl of oligo $(dT)_{18}$primer and DEPC-treated water to the final volume of 11 µl. Mixture was incubated at 65 °C for 5 minutes, cooled on ice and spinned down in a microcentrifuge. 4 µl of 5x Reaction buffer, 1 µl of RiboLock RNase Inhibitor (20 U/µl), 2 µl of 10 mM dNTP mix and 2 µl of M-MuLV Reverse Transcriptase (20 U/µl) were added to the tube. The mixture was incubated at 37 °C. After 60 minutes of incubation, the reaction was terminated by heating the tube at 70 °C for 5 minutes. cDNA was stored at -20 °C.

2.3.3 qRT-PCR

Expression of *mKLK5* in individual transgenic lines was examined by qPCR with the Klk5 gene specific primer. The PCR reaction was performed using SYBR Green JumpStart *Taq* Ready Mix (*Sigma-Aldrich,* USA) and CFX96 real-time PCR detection system (*Biorad*, Canada). Experiment was repeated 3 times with duplicate reactions for all samples. The expression of mKLK5 analyzed in ears was normalized to the expression of mGAPDH. The mKLK5 expression in transgenic animals was further normalized to the averadge expression of mKLK5 in wt mice that was set to one.

Table 3. PCR program used for qRT-PCR

Temperature	Duration
95 °C	2 min
95 °C	15s (39x)
60 °C	20s (39x)
72 °C	30s (39x)
65 °C	5s
95°C	2 min

2.4 Protein techniques

2.4.1 BCA protein assay

BCA Protein Assay kit (Pierce, USA) was used to determine the protein concentration in a cell lysate. Standard samples of known concentration were prepared according to manufacturer´s instructions. Protein samples were diluted in PBS (1:1) and 25 µl of the sample was pipetted into 96 well microplate. Working reagent was prepared by mixing of solutions A and B (50:1). 200 µl of working reagent was added to the samples and standards in the microplate. The plate was covered and incubated at 37 °C for 30 minutes. Absorbance was measured at 562 nm by Tecan Reader (Tecan Group, Switzerland). The protein concentration was estimated from the standard calibration curve.

2.4.2 Preparation of protein samples for SDS-PAGE

75 µl of the cell lysate was mixed with 4x Protein loading buffer with DTT. Sasmple was mixed by vortexing and incubated at 95 °C for 5 minutes. 50 or µg of protein was loaded on polyacrylamide gel.

2.4.3 SDS-PAGE separation of proteins

12.5% polyacrylamide resolving gel of width 1.5 mm was used to for SDS-PAGE separation of proteins. Solution was pipetted into assembled gel plates. In order to produce straight top of the resolving gel, isopropanol was pipetted on top of the gel. After the gel polymerized (usually 60 minutes), isopropanol was discarded and 4% stacking gel was prepared, pipetted on top of the resolving gel and the comb was inserted. After the stacking gel polymerized (30 minutes), the comb was carefully removed and the samples were loaded into the wells. Prestained protein molecular weight marker (*Fermentas*, Canada) was used as a standard. The gel was run at 100 V for 90 minutes.

2.4.4 Western blotting

Mini Trans-Blot aparature (BioRad, USA) was used for western blotting. Polyacrylamide gel used in SDS-PAGE was washed in Transfer buffer for 15 minutes. PVDF membrane was activated by incubation in methanol for 30 s, washed in destilled water and incubated in transfer buffer for 10 minutes. Blotting sandwich was assambled

according to manufacturers instructions, placed into the buffer tank together with he cooling unit and the tank was filled with transfer buffer. Blotting was performed for 1 hour at 100 V. PVDF membrane was washed in TBST and placed into a 50 ml falcon tube with 10% TBSTM. After 1 hour of incubation a roller, the membrane was washed 3x with TBST and 6 ml of primary antibody diluted in 2% TBSTM (1:600) was added (rabbit anti-mKlk5 SN Ab, kindly provided by M. Brattsand). Falcon tube with the membrane was placed for 2 hours on a roller. Membrane was washed 3x with TBST, 6 ml of secondary antibody (anti-rabbit POD) diluted in 2% TBSTM (dilution 1:10000) was added and the tube was incubated for 1 hour on a roller.After washing of the membrane with TBST, ECL Plus Western Blot reagents (GE Healthcare, UK) were used to detect the proteins by chemiluminiscence. 1 ml of detection solution A was mixed with 25 µl of detection solution B and 1 ml of the mixture was pipetted on the membrane. The mixture of detection solutions was aspirated from the membrane after 5 minutes of incubation. Membrane was covered by Saran wrap foil and analyzed with LAS-3000 (Fujifilm Life Science, USA).

2.4.5 Determination of proteolytic activity in tg mice

In order to analyse trypsin-like activity in the skin of Inv-Klk5 mice, 20 whole ear biopsies were taken from 5 transgenic and 5 wild type adult mice. Immediately after collection, samples were frozen with dry ice and stored in -80°C until further processing. Frozen ear tissue was disintegrated with hammer blows and further disrupted in the V-shape 2 ml tube with polypropylene pestle matching the tube shape. Soluble proteins were released from the disrupted sample using modified RIPA buffer (Tris-HCl [ph 8,0]; 150mM NaCL; 1% [v/v] Nonidet P40, 0,5% [w/v] sodium deoxycholate) supplied with 1x PhosSTOP (Roche, USA). The extracts of 5 ears (collected from 5 different transgenic mice) and 5 ears (collected from 5 different wild type mice), respectively, were pooled within both group of animals and repeatedly passaged through 21G needle until homogeneous fluid was obtained. Following homogenization, combined samples were incubated with shaking (150 rpm) in 4°C for 35 minutes and further centrifuged twice at 16,000 g for 15 min at 4°C. The protein concentration of the clarified lysates was determined with Bradford protein assay kit (Bio-Rad, USA) according to the manufacturer instructions.

Immediately after protein quantification, combined extracts were diluted to the concentration of 1600ng/µl and to the 90 µl of each sample 10 µl of 10 mM chromogenic

substrate S-2288 (Chromogenix, GB) was added. Mixtures containing substrate and extract or lysis buffer, were incubated in duplicate at 37°C overnight. The increase in released chromophor concentration was monitored as increase in absorbance at 405nm with 2104 EnVision® Multilabel Reader (PerkinElmer, USA). Substrate S-2288 is sensitive for hKLK5 and potentially to other trypsin-like serine proteases (Brattsand et al. 2005, Stefanson et al.2006), therefore enzyme activity measured is referred here as a trypsin-like activity.

2.5 Immunohistology

2.5.1 Cryosectioning of tissue samples

Skin samples frozen in OCT Compound TissueTek (Sakura, USA) were sectioned by cryostat Leica CM 1950 (*Leica*, Germany). 7 micron sections were cut at -22 °C. The sections were dried on the slide at room temperature. The slides were stored at -20 °C.

2.5.2 Immunohistological staining of tissue samples

The sections were fixed by incubation with 4% paraformaldehyfe/PBS for 20 minutes at room temperature. After washing with PBS, the samples were incubated with 0.2% Triton X-100 in PBS for two minutes on ice. Samples were washed with PBS and blocked with 10% BS/PBS for 20 minutes at RT. 1 µl of anti-mKlk5 Ab SN (provided by M. Brattsand) was diluted in 400 µl of 25% BSA/PBS and pipetted on the sections. After 40 minutes, the cells were washed with PBS. 3 µl of IgG TRITC – goat anti-rabbit (*Sigma-Aldrich*, USA) was diluted in 500 µl of 0.5% BSA/PBS and 150 µl was added to the slides. The samples were incubated for 40 minutes, washed with PBS and 0.5 ml of PBS was added into the well. Alternatively the section were stained with Hoechst 33258 (*Invitrogen*, USA) by adding of 1 µM solution diluted in PBS to the samples (15 minute incubation) Stained sections were visualised by fuorescent microscopy.

2.6 Mouse experiments

2.6.1 Model of irritant dermatitis

The model of croton oil induced dermatitis is commonly used and was performed accroding to protocol described formerly (Swingle et al., 1981). Five positive Inv-Klk5

males and five negative controls at the age of 8 weeks were used for the experiment. Croton oil was diluted in acetone (1:125) and 50 µl of croton oil solution was applied epicutaneously on both sides of one ear (25 µl + 25 µl). Before and 24, 48 and 72 hours after application, ear thickness was measured with a micrometer Toym IP25 (*Mitutoyo*, USA) to follow up edema formation, i.e. the inflammatory reaction. Difference in thickness above the basal values were calculated for each mouse at individual time points.

2.6.2 Wound Healing model

Five positive Inv-Klk5 males and five negative controls at the age of 8 weeks were used for the experiment. The procedure was performed as described in Schunck et al, 2005. Briefly, at day 0, circular skin wound (150 mm^2) was intrudced by excisition of dorsal skin of depilated mouse. The wound was covered with semi-occlusive trasnparent dressing Comfeel Plus (Coloplast, Denmark). Detail photographs of the wound were taken at days 2, 4, 6, 8, 10, 12 and 14). Reepithelization of the wound [mm^2] was measured using computer software ImageJ (Wayne Rasband, USA). The kinetics of wound healing was quantified as relative reepithelization of the wound area (reepithelization at day X [mm^2]/original wound area [mm^2]).

2.7 Statistical analyses

To analyze the data from models of wound-healing and irritant dermatitis , SigmaPlot (Systat Software, USA) was used. To analyze the differences between two or more independent groups, One-way Anova test was performed. Compared data with $p<0.05$ were assumed to show statistically significant difference.

3 Results

3.1 Aims of diploma thesis

The primary objective of this diploma thesis was to generate a transgenic mouse overexpressing mKlk5 in superficial epidermal layers and investigate its impact on skin physiology. In order to target the gene expression specifically into upper layers of epidermis, modified involucrin promoter (truncated involucrin promoter with intron sequence, hINVm-int) was developed in laboratory of R. Sedláček, IMG. However, its specificity and strenght remained to be evaluated *in vivo*. To achieve this task, transgenic mouse expressing a fluorescent reporter protein under the control of hINVm-int promoter was prepared.First part of this chapter presents development and analysis of the reporter mouse. Second part describes generation and characterization of transgenic mouse overexpressing mKlk5.

3.2 Generation and analysis of Inv-tdTomato mouse

Involucrin is one of the components of cornified cell envelope in stratum corneum and is specifically expressed in stratum spinosum and stratum granulosum (Rice and Green, 1979). This expression profile mimics the physiological expression pattern of mKlk5, thus, human involucrin promoter (hINV) appears to be suitable to drive the transgene expression in mKlk5 overexpressing mice.

Two regulatory regions are responsible for tissue-specific activity of human involucrin promoter – a distal element (-2473/-2088), containing AP-1 and Sp1 binding sites, and a proximal region (-185/-1) (reviewed in Ghazizadeh et al., 2002). It has been shown that reduction of full lenght hINV (approximately 3700 bp) to 754 bp fragment containing both regulatory regions (truncated human involucrin promoter, hINVm) does not affect tissue-specificity of the promoter (Ghazizadeh et al., 2002). Minimizing the promoter size facilitates molecular cloning and furthermore, shorter promoter sequences are more suitable for transgenesis due to limited capacity of some gene delivery systems, such as viral-mediated transgenesis.

Truncated involucrin promoter developed by Ghazizadeh et al., has been recently tested in our laboratory and *in vitro* experiments did not confirm tissue-specificity of the

promoter. The expression of reporter gene driven by hINVm was visible in all cell types tested; i.e., hepatocyte (HepG2), kidney (Cos7) and keratinocyte (HaCaT) cell lines (R. Sedlacek, personal communication). To restore tissue-specific expression from hINVm, viral vector R60-hINVm-EGFP (kindly provided by S. Ghazizadeh), expressing EGFP protein under the control of hINVm promoter, was modified by insertion of involucrin intron between hINVm promoter and translational start of EGFP. As it has been reported, involucrin intron sequence has regulatory effect and contributes to specific expression in keratinocytes (Carrol and Taichman, 1992). Indeed, R60-hINVm-int-EGFP containing involucrin intron was able to drive the gene expression exclusively in keratinocyte cell line, whereas infected hepatocyte and kidney cell lines did not show any expression of EGFP (R. Sedlacek, personal communication). In order to evaluate the expression from hINVm-int promoter *in vivo*, we decided to generate a transgenic mouse expressing red fluorescent protein tdTomato under the control of hINVm-int.

3.2.1 Development of targeting vector pBroad-hINVm-int-tdTomato

In order to prepare a transgenic mice expressing tdTomato driven by hINVm-int promoter, vector pBroad-hINVm-int-tdTomato was prepared as a source of DNA for pronuclear microinjections. pBroad-hINVm-int-tdTomato (Fig. 6) was derived from pBroad_tdTomato, a plasmid previously constructed in laboratory of Radislav Sedláček, IMG. Plasmid pBroad-tdTomato is based on pBroad3 mcs (Invivogen, USA), which was specifically designed for mouse and rat transgenesis. pBroad3-mcs contains murine ROSA promoter, human beta-globin derived polyadenylation sequence that allows effective arrest of the transgene transcription and polyadenylation reaction resulting in high levels of mRNA (Yu and Russel, 2001) and suitable restriction sites that enable easy excision of DNA fragment for transgenesis. Furthermore, the plasmid contains the ampicillin resistence, gene that allows the selection of transformed bacteria carrying the pBroad3-mcs plasmid.

In order to excise murine ROSA promoter from pBroad-tdTomato, the plasmid was restricted with restriction enzymes EcoRI and HindIII. Two DNA fragments were obtained, a 1930 bp fragment representing murine ROSA promoter and 3994 bp fragment representing pBroad3-mcs with tdTomato. The 3994 bp fragment was isolated from the gel slice and used for further ligation with hINVm-int promoter. hINVm-int was obtained by restriction digestion of R60hINVm-int-EGFP with enzymes EcoRI and HindIII. The 1824

bp fragment representing hINVm-int promoter was excised from the gel slice and ligated with 3994 bp fragment originated from pBroad-tdTomato.

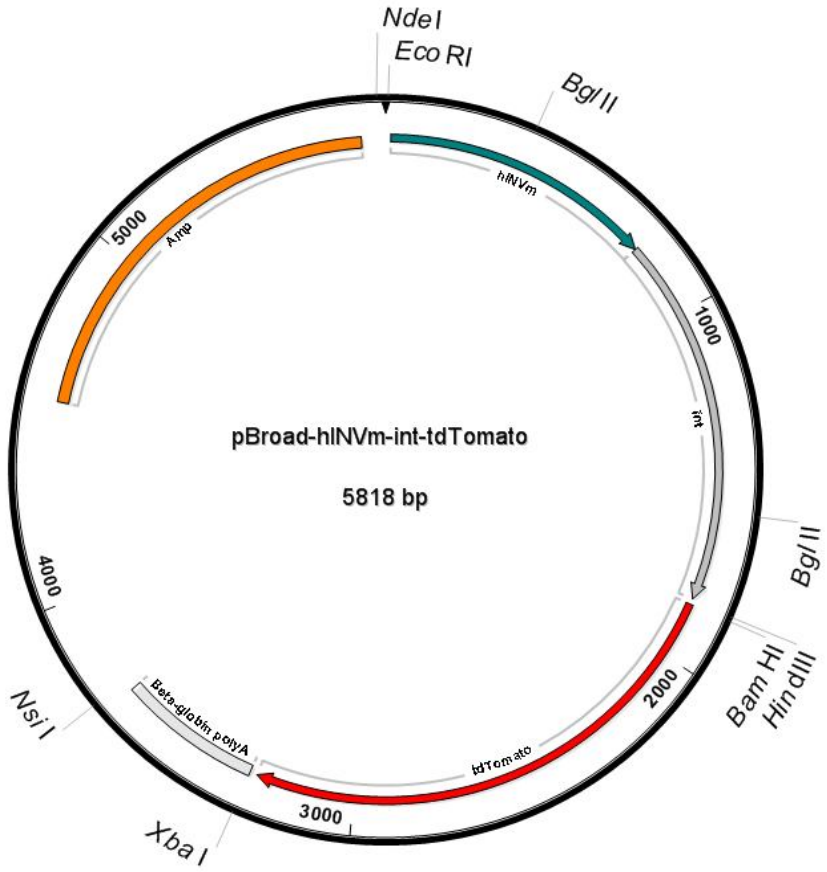


Figure 6. A map of targeting vector pBroad-hINVm-int-tdTomato
estriction enzymes EcoRI and HindIII were used for cloning of hINVm-int into the plasmid pBroad-tdTomato. BamHI and BglII were used for restriction analyses of plasmid DNA. Enzymes NdeI and NsiI allow exciscion of a DNA fragment for pronuclear microinjections.

After ligation, competent bacterial cells were transformed with ligation mixture and plated on LB agar plate containing ampicillin. Ten bacterial colonies were selected for plasmid DNA isolation and restriction analysis. To analyze the size of plasmid DNA, samples were digested with enzyme BamHI, which cuts the vector DNA at one site, and visualized by agarose gel electrophoresis. DNA isolated from clones 2, 4, 5, 7, 8, 9 and 10 corresponded to the expected size of pBroad-hINVm-int-tdTomato (5818 bp). DNA from clones 9 and 10 was further analyzed by restriction with Bgl II. DNA fragments of

expected size (4628 bp and 1190 bp) were obtained after digestion (Fig. 7) and the vector was used for further experiments.

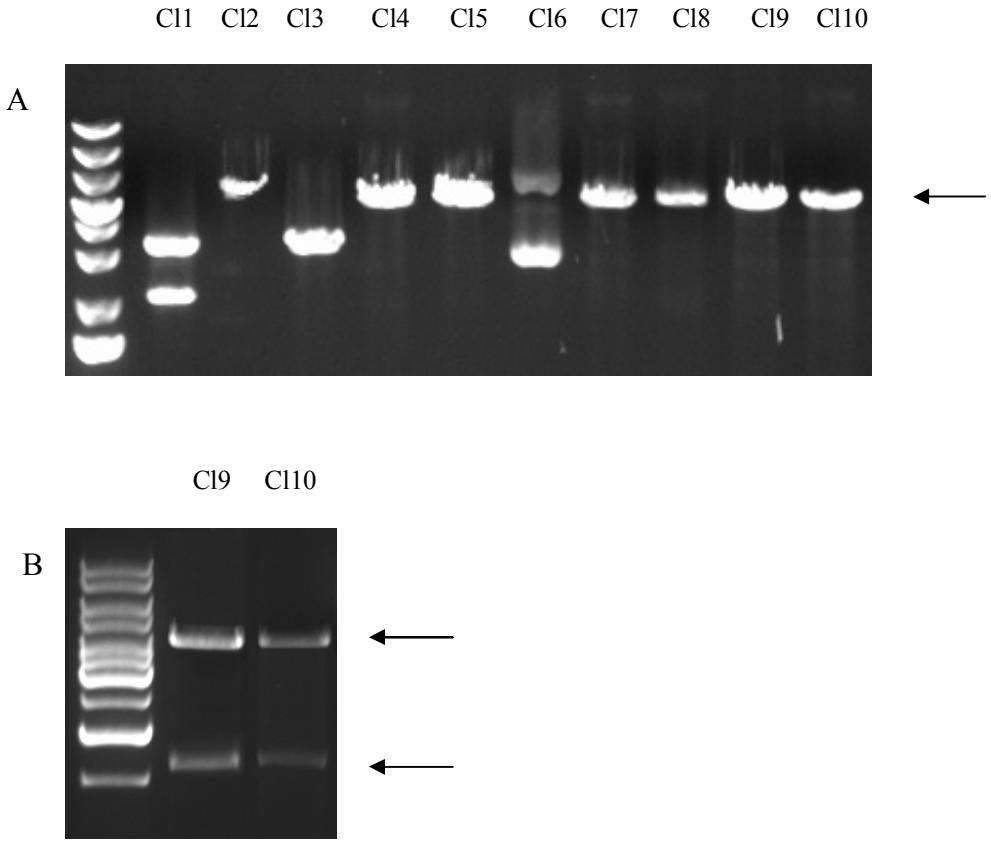


Figure 7. Restriction analyis of the vector pBroad-hINVm-int-tdTomato

A. The arrow indicates the desired 5818bp band in clones 2, 4, 5, 7, 8, 9 and 10. The size corresponds to pBroad-hINVm-int-tdTomato restricted with BamHI.

B. Two bands of expected size 4628bp and 1190bp (indicated by arrows) were obtained after restriction of pBroad-hINVm-int-tdTomato with BglII.

3.2.2 Analysis of the vector pBroad-hINVm-int-tdTomato in cell culture

To test the functionality of the targeting vector pBroad-hINVm-int-tdTomato *in vitro*, keratinocyte-derived cells HaCaT and PAM212 were transiently transfected with the plasmid. Furthermore, HepG2 cells (derived from liver cells) were used for the transfection, in order to confirm tissue-specificity of hINVm-int promoter. At 48 hours post-transfection, the cells were examined by fluorescent microscopy for tdTomato expression. tdTomato-positive cells were identified in both keratinocyte cell lines, HaCaT and PAM212. No expression of tdTomato was observed in transfected HepG2 cells (Fig. 8). These data indicated that the targeting vector pBroad-hINVm-int-tdTomato was

functional and could be used for transgnesis. Additionaly, keratinocyte-specific expression directed by hINVm-int promoter was confirmed.

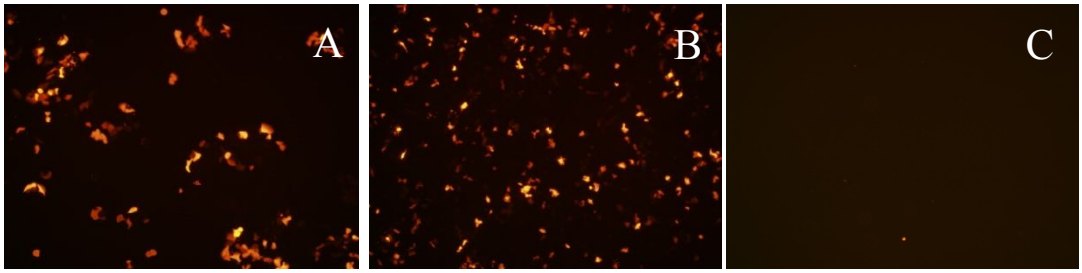


Figure 8. Expression of tdTomato from the hINVm-int promoter in various cell lines
HaCaT (A), PAM212 (B) and HepG2 (C) cells were transiently transfected with the vector pBroad-hINVm-int-tdTomato. tdTomato was expressed in both keratinocyte-derived cell lines (A,B). No positive cells were found in cells derived from hepatocytes (C).

3.2.3 Preparation of the DNA fragment for pronuclear microinjection

Plasmid pBroad-hINVm-int-tdTomato was digested with restriction enzymes NdeI and NsiI to release a DNA fragment containing hINVm-int, tdTomato gene and human beta-globin derived polyadenylation sequence. The desired DNA fragment (3756 bp) was excised from the gel slice and purified as described in 2.1.9. The purified DNA (Fig. 9) was delivered to Transgenic Unit, IMG, to be injected into the male pronculeus of fertilized oocytes from C57Bl/6N mice. After the microinjections, one- or two- stage embryos were transferred in the oviducts of foster mothers.

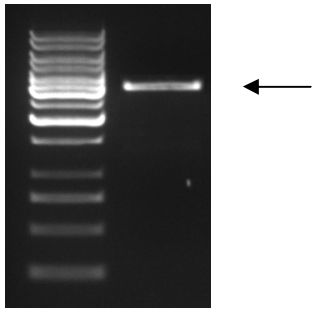


Figure 9. A DNA for pronuclear microinjections derived from pBroad-hINVm-int-tdTomato
The desired DNA fragment is indicated by arrow. The DNA was delivered to Transgenic unit, IMG at the concentration of 17,3 ng/ µl

3.2.4 Genotyping of newborn mice

Overall, 74 mice were born from microinjected zygotes. Tail biopsies were obtained from the mice at the age of 3 weeks and the tails were screened for tdTomato expression under the fluorescent microscope. Tails obtained from mice No. 4775 and 4780 were identified as positive (Fig. 10). Tails from other 72 mice appeared to be negative.

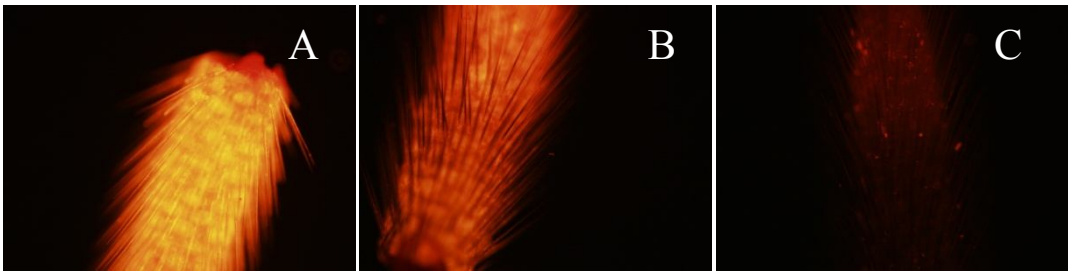


Figure 10. Tails from mice 4775 (A), 4780 (B) and WT mouse (C) visualized by fluorescent microscopy

However, transgenic mice generated by pronuclear microinjections may exhibit mosaic expression of the transgene and the expression may not be apparent in F0-generation animals. To investigated, whether the transgenic DNA was incorporated into mouse genome, genomic DNA was isolated from the tails and genotyping by PCR was performed using transgene-specific Tom_rew and Inv_fwd primers (Fig. 11). Genotyping confirmed the presence of transgene in mice 4775 and 4780. Furthermore, mice 4849, 4869 and 4870 were identified as positive.

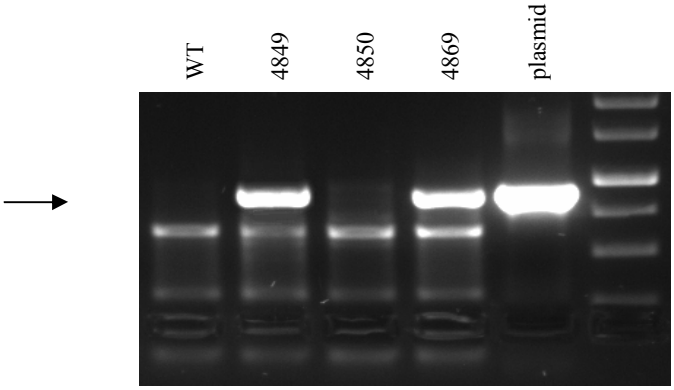


Figure 11. PCR analysis of genomic DNA using transgene specific primers
The arrow indicates the presence of a specific 858bp band in transgenic animals. Plasmid pBroad-hINVm-int-tdTomato was used as a positive control. DNA isolated from WT animal was used as negative control.

All 5 mice carrying the sequence of hINVm-int-tdTomato in the genome (transgenic line Inv-tdTomato) were used for further breeding. However, only F1-generation mice derived from founders 4775, 4780 and 4849 expressed high levels of tdTomato. Among these, transgenic line derived from founder 4775 exhibited the strongest expression of the transgene, thus, this line was selected for further experiments.

3.2.5 Analysis of tdTomato expression in Inv-tdTomato mice

Whole-body imaging was employed to overview the gross expression pattern of tdTomato in the skin of positive mice (Fig. 12).The fluorescent signal was clearly distinguishable in all transgenic mice analyzed although the expression appeared not to be uniform in all sites but exhibited a spotty character. Notably, the tdTomato was upregulated in the ear that was previously partially cut to obtain a skin sample for analyses. This suggests that activity of hINVm-int promoter can be increased by disruption of epidermal barrier or wounding the skin.

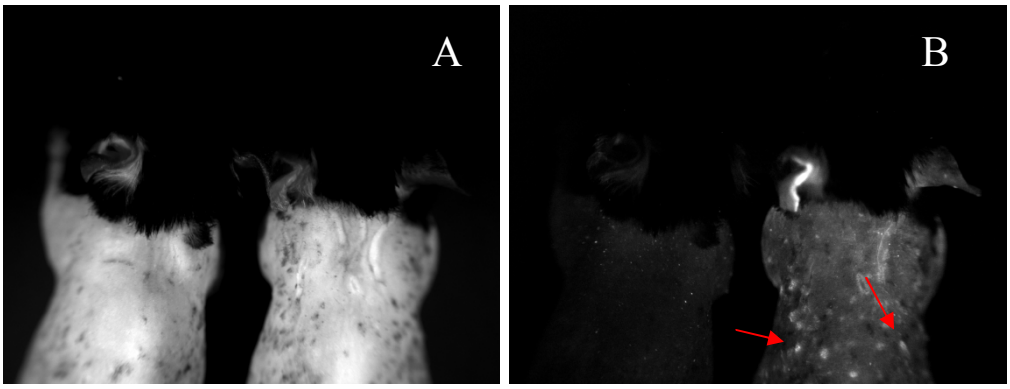


Figure 12. WT (left side) and Inv-tdTomato mouse visualized by whole-body imaging in bright field (A) and under fluorescent light (B)
Sites with higher expression of tdTomato are indicated with arrows. High expression in the left ear of positive mouse may be caused by previous disruprion of epidermis.

To analyze the expression in detail, samples from ear and back were examined using cryosections and fluorescence microscopy. Samples from negative littermates were used as a control. The results showed that tdTomato is expressed exclusively in epidermis, with the strongest expression in superficial layers (Fig. 13).

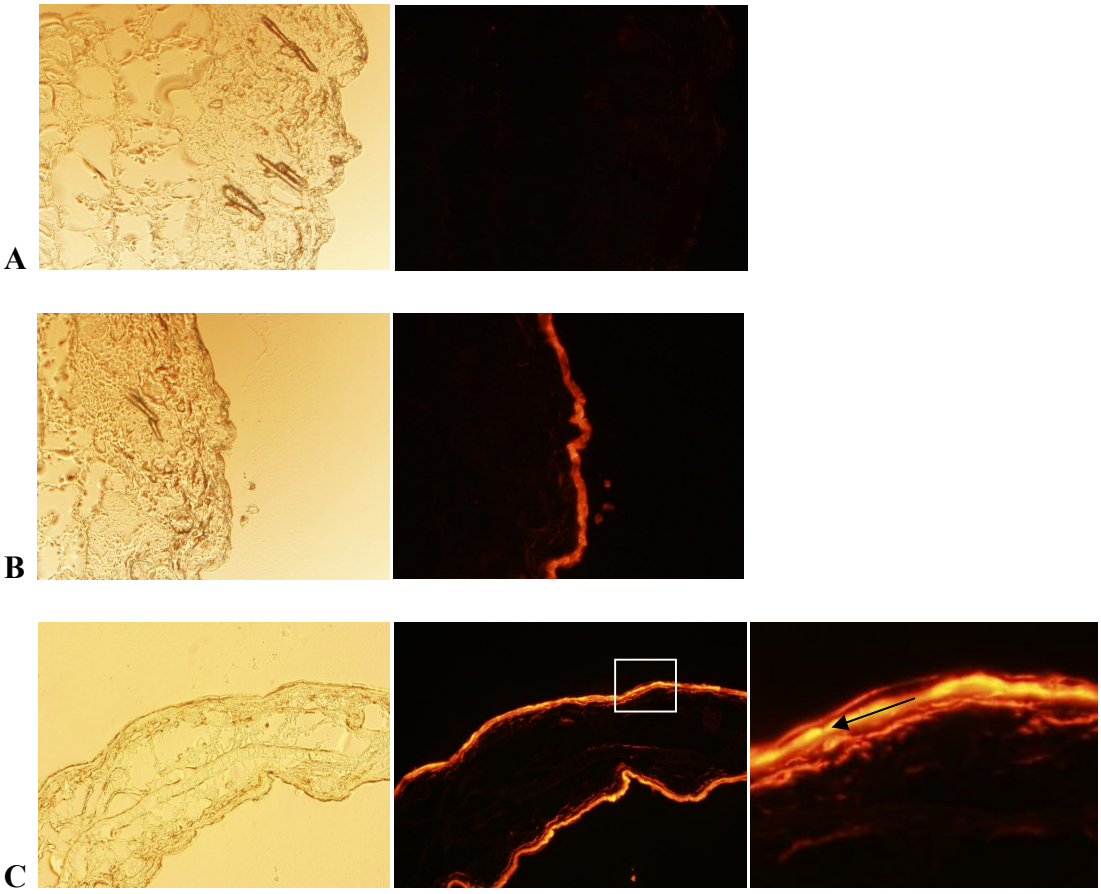


Figure 13. Cryosections from Inv-tdTomato mouse and WT control
Back skin isolated from WT (A) and tdTomato mouse (B) visualized by fluorescent microscopy. Ear from Inv-tdTomato mouse (C). Expression of the transgene was obvious in epidermis of positive animals. Detail of the ear from tdTomato mouse reveals the strongest expression in the upper layers of epidermis (indicated by arrow).

In order to evaluate the specificity of tdTomato expression, multiple organs were isolated from positive mice and examined by fluorescent microscopy for tdTomato expression. Organs isolated from negative littermates were used as negative controls. Strong expression of tdTomato was observed in skin isolated from positive mice. Furthermore, tdTomato was found to be weakly expressed in tongue and bladder. No fluorescence signal was detected in heart, skeletal muscle, kidney, liver, brain, colon and spleen harvested from Inv-tdTomato mice (Fig. 14).

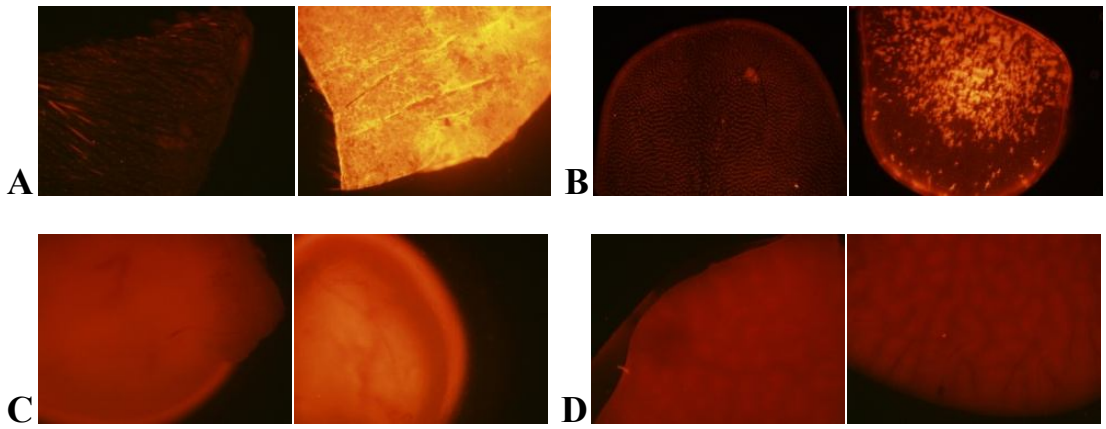


Figure 14. Organs isolated from wt (left) and Inv-tdTomato mouse (right) visualized by fluorescence microscopy
Among organs examined, the strongest expression of tdTomato was observed in skin (A). Spotty expression was found in the tongue of positive mice (C) and weak fluorescent signal was detected in the bladder (C). No expression of the transgene was found in liver (D), heart, skeletal muscle, kidney, brain, colon and in the spleen of positive mice (data not shown).

3.3 Generation and analysis of Inv-Klk5 mouse

After the tissue-specific expression directed by hINVm-int promoter has been verified in Inv-tdTomato mice, the project could proceed to the primary task - generation and analysis of transgenic mouse overexpressing mKlk5 in superficial epidermal layers.

3.3.1 Development of targeting vector pBroad-hINVm-int-Klk5-FLAG

Since the vector pBroad-hINVm-int-tdTomato proved to be an efficient tool for targeting the gene of interest into upper layers of epidermis, we decided to derive mKlk5-expression vector from this plasmid. mKlk5 cDNA fused with FLAG tag was obtained from pcDNA-Klk5-FLAG, a plasmid previously developed in laboratory of Radislav Sedlacek, IMG. C-terminal fusion with FLAG tag allows immunodetection of mKlk5. In order to prepare the vector DNA fragment, pBroad-hINVm-int-tdTomato was restricted with enzymes BamHI and XbaI. Two DNA fragments were obtained, a 1457 bp fragment representing tdTomato gene and 4361 bp fragment (pBroad-hINVm-int) that was used for further ligation with mKlk5 cDNA. To release Klk5-FLAG from pcDNA-Klk5-FLAG, the plasmid was restricted with BamHI and XbaI. 1013 bp fragment representing KLK5-FLAG was ligated with pBroad-hINVm-int and competent bacterial cells were transformed

with the ligation mixture. Plasmid DNA isolated from bacterial colonies was analyzed by restriction with BamHI (Fig 15).

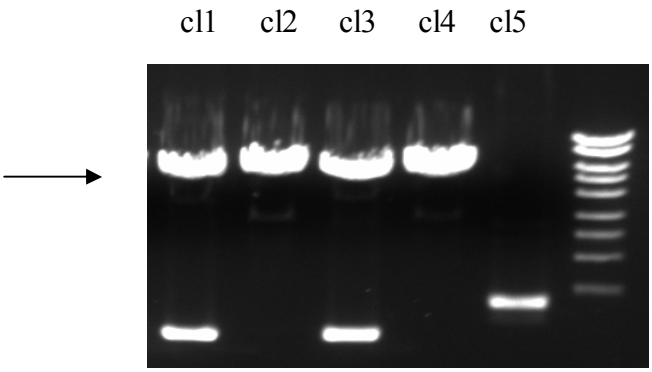


Figure 15. Restriction analyis of the vector pBroad-hINVm-int-Klk5-FLAG
The band of a size corresponding to pBroad-hINVm-int-Klk5-FLAG (5374 bp, indicated by arrow) was obtained after restriction of plasmid DNA isolated from clones 2 and 4.

The result of restriction analysis showed that clones 2 and 4 contained the DNA of expected size (5374 bp). DNA sequencing further confirmed that clone 2 contained desired vector pBroad-hINVm-int-tdTomato-Klk5-FLAG (Fig. 16).

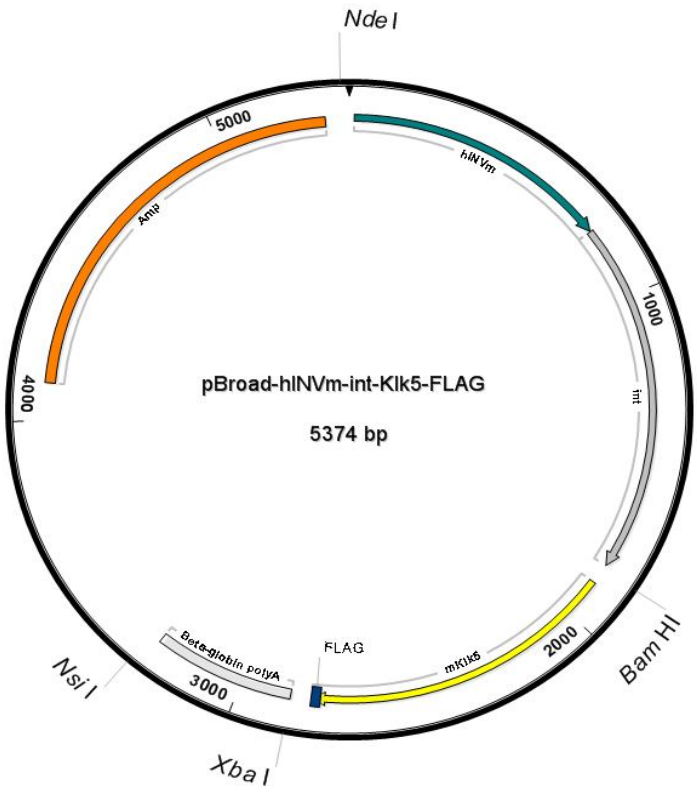


Figure 16. A map of targeting vector pBroad-hINVm-int-Klk5-FLAG

3.3.2 Analysis of the vector pBroad-hINVm-Klk5-FLAG in cell culture

HaCat cells were transiently transfected with pBroad-hINVm-int-Klk5-FLAG to test the expression of mKlk5 from this vector *in vitro*. mKlk5 expression was detected by immunostaining against FLAG tag. Cells transfected with pCF-hTubo-FLAG, a plasmid expressing γ-tubulin fused with FLAG tag, were used as a positive control. Untransfected cells were used as a negative control (Fig. 17). The results showed that mKlk5 was expressed in transfected cells and could be detected with anti-FLAG antibodies.

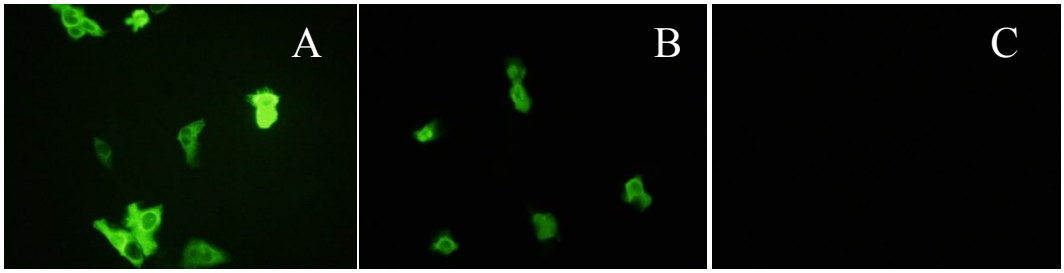


Figure 17. Immunostaining of HaCaT cells transiently transfected with pBroad-hINVm-int-Klk5-FLAG
mKlk5 was detected with mouse monoclonal antinody against FLAG peptide (*Sigma-Aldrich, USA*) (A). Positive control of immunostaining was pCF-hTubo-FLAG, expressing γ-tubulin linked to FLAG tag (B). Untransfected cells were used as a negative control (C).

3.3.3 Preparation of the DNA fragment for pronuclear microinjection

DNA fragment carrying hINVm-int-Klk5-FLAG and human beta-globin polyadenylation sequence was prepared as described for pBroad-hINVm-int-tdTomato in the section 3.1.4. Purified DNA (Fig. 18) was delivered to Transgenic unit, IMG.

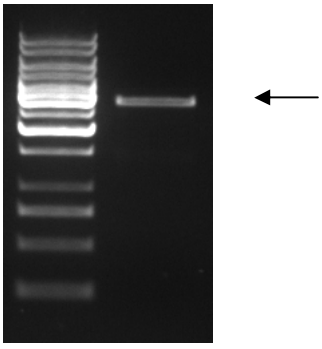


Figure 18. DNA fragment for pronuclear microinjections derived from pBroad-hINVm-int-Klk5-FLAG
The desired DNA fragment is indicated by arrow. The DNA was delivered to Transgenic unit, IMG at the concentration of 20,3 ng/ µl.

3.3.4 Genotyping of newborn mice

We obtained 70 F0-generation mice. Genomic DNA was isolated from 3 weeks old mice and PCR-genotyping was carried out using FLAG-rew and Inv-fwd primers (Fig. 19). Mice No. 4075, 4092, 4137, 4204, 4252 and 4253 were identified as positive. Six transgenic lines (Inv-Klk5^{4075}, Inv-Klk5^{4092}, Inv-Klk5^{4137}, Inv-Klk5^{4204}, Inv-Klk5^{4252}, Inv-Klk5^{4253}) were established by breeding the positive founders with wt Black6 animals.

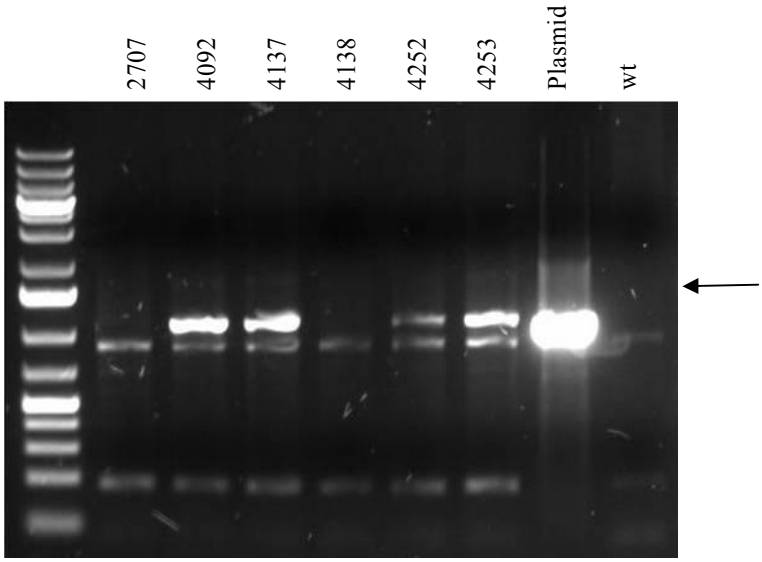


Figure 19. PCR analysis of genomic DNA using transgene specific primers
The arrow indicates the presence of a specific 1109bp band in transgenic animals. Plasmid pBroad-hINVm-int-Klk5-FLAG was used as a positive control. DNA isolated from WT animals was used as negative control.

3.3.5 Characterization of Inv-Klk5 mice

As the mKlk5 was targeted into upper epidermal layers, new born, immature and adult transgenic animals were inspected daily for skin changes until their age of 15 weeks, however no gross abnormalities were found. Thus, the expression level of the transgene was analyzed at the mRNA and protein levels and two models of epidermal barrier disruption were employed to reveal the impact of the mKlk5 in epidermis.

3.3.5.1 The level of Klk5 expression in Inv-Klk5 mice

RNA isolated from ears of F1 generation animals was used to determine the level of transgene expression among individual lines. RNA isolated from negative littermates was used as a control. Analysis by quantitative real-time PCR using mKlk5-specific primers showed the highest expression in transgenic lines Inv-Klk5^{4137} and Inv-Klk5^{4204} with 6-9 fold upregulation of mKlk5 mRNA compared to negative animals. mKlk5 expression in lines Inv-Klk5^{4075}, Inv-Klk5^{4092}, Inv-Klk5^{4252} and Inv-Klk5^{4253} was comparable to negative animals (Fig. 20), thus, we assume that the transgene has been silenced. Lines Inv-Klk5^{4137} and Inv-Klk5^{4204} were selected for further experiments.

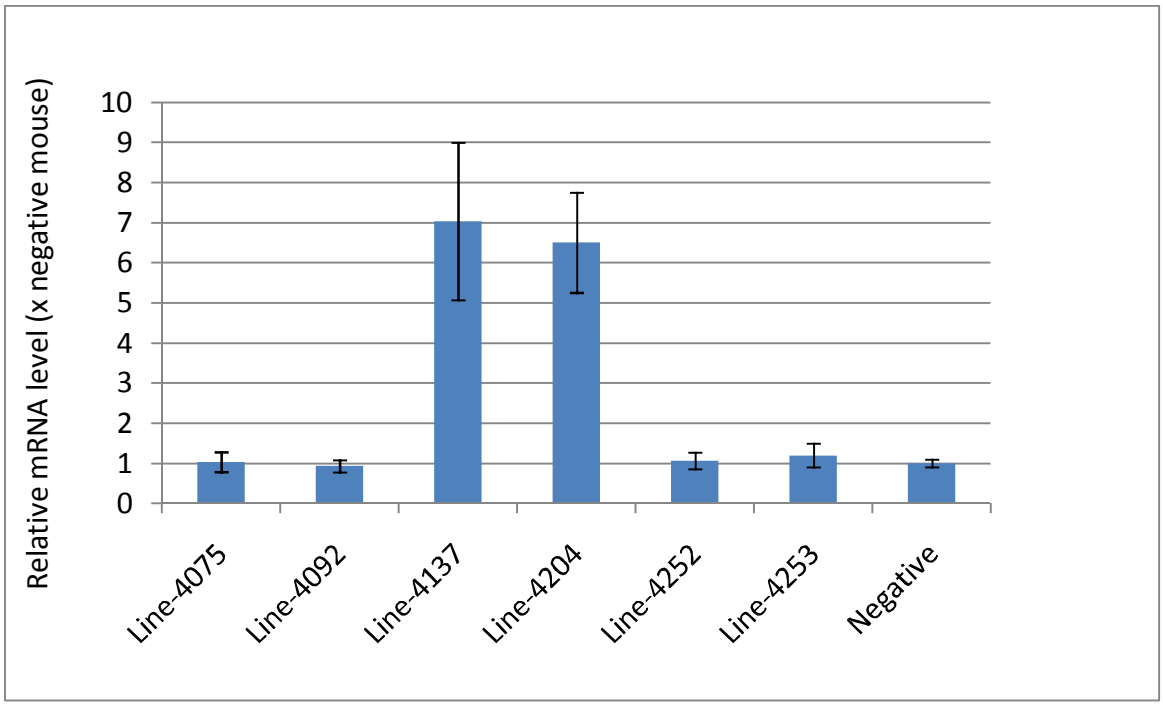


Figure 20. mKlk5 mRNA in transgenic animals and WT controls
Quantitative real-time PCR analysis of mRNA isolated from ears using mKlk5-specific primers. Three animals from each line were used for the experiment. Negative littermates were used as a control. Positive mice from lines INV-Klk5^{4137} and INV-Klk5^{4204} showed 6-9 fold upregulation of mKlk5 mRNA compared to negative animals.

In order to confirm the upregulation of mKlk5 on protein level, ear samples were collected from F1-generation mice and skin extracts were analyzed by western blotting using anti-mKlk5 antibodies (kindly provided by M. Brattsand) (Fig. 21). Altogether, four forms of mKlk5 were present in all mice analyzed. Two forms corresponding to inactive pro-mKlk5 and two forms representing the active enzyme. This heterogenity may be caused by variations in the degree of glycosylation. All forms of mKlk5 show molecular

masses corresponding to those reported for human KLK5 (Brattsand et al., 2005). Experiment confirmed the overexpression of mKlk5 in transgenic lines Inv-Klk5^{4137} and Inv-Klk5^{4204}, however, most of the protein corresponded to inactive pro-mKlk5. The amount of active mKlk5 in positive mice appeared to be similar as for non-transgenic littermates.

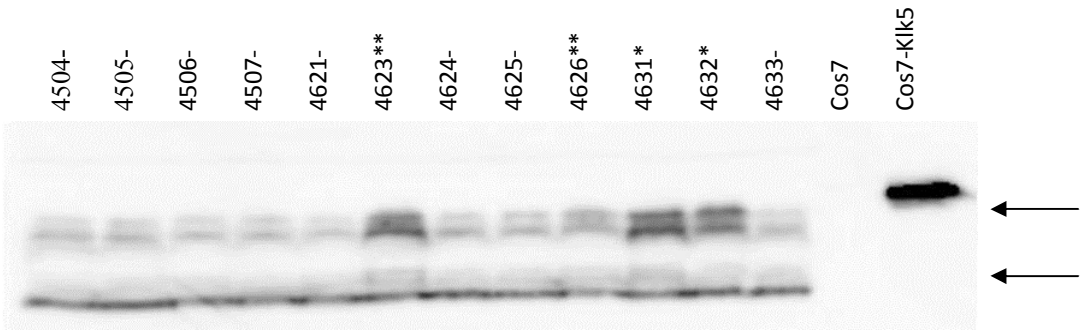


Figure 21. Klk5 in skin extracts of Inv-Klk5 mice
Skin extracts of F1 generation mice (8 wk of age) were analyzed with Western blot using anti-mKlk5 antibodies. Samples of the same concentration were used. The upper arrow indicates the size of 34 kDa corresponding to molecular mass of pro-mKlk5. The lower arrow (28,5 kDa) indicates active mKlk5. Both, pro-mKlk5 and mKlk5 are present in two forms of slightly different size. Cos7 cells transiently transfected with pTracer-KLK5 were used as a control. Only mKlk5 proenzyme is present in cultured cells due to the lack of proteolytic activators in Cos-7 cell line. The molecular mass of pro-mKlk5 expressed in transfected cells is higher because of C-terminal fusion with His-tag and V5 epitope.
* mice from the line Inv-Klk5^{4204}
** mice from the line Inv-Klk5^{4137}
\- negative mice

To analyze the expression of mKlk5 within the skin and epidermal layers, ear samples were cryosectioned and stained with anti-FLAG antibodies, that can discriminate between endogenous and transgenic mKlk5. However, the antibodies did not work with cryosections. Alternatively, we used anti-mKlk5 Ab. These antibodies exhibit relatively large cross-reactivity to a number of skin and epidermal structures, however, they could clearly identify mKlk5 expression pattern in epidermis (Fig. 22).

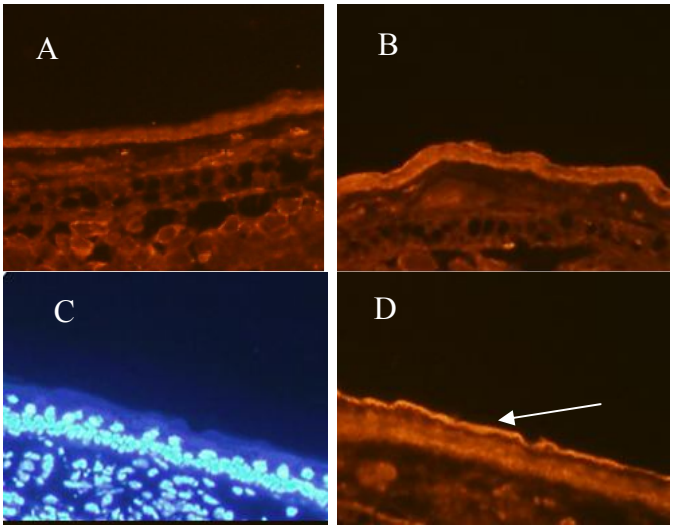


Figure 22. Immunohistology of skin from WT (A) and Inv-Klk5^{4204} (B, C, D) mouse
Cryosection from mouse ear, 8 wk of age. Immunofluorescence staining with anti-mKlk5 antibody (A, B). Expression of mKlk5 was detected in stratum spinosum, stratum granulosum and stratum corneum with the strongest signal in superficial layers (indicated by arrow). To visualize the structure of epidermis, sample C was additionally stained by o.Hoechst staining.

mKlk5 was overexpressed in suprabasal epidermal layers according to our expectations based on tdTomato expression in Inv-tdTomato mice. These results demonstrate that we have successfully targeted mKlk5 expression into epidermal compartments, where is the enzyme active under physiological conditions. The reason, why is majority of the enzyme in immature form is not clear, since the proteases activating mKlk5 should be present in *stratum corneum* and one of these activators, mature Klk5 itself, was detected by western blotting.

3.3.5.2 Proteolytic activity in the skin of Inv-Klk5

Chromogenic substrates were used to analyze the total tryptic activity in epidermal extracts from positive animals. Possible deregulations of epidermal proteolytic cascades caused by mKlk5 overexpression or direct hyperactivity of mKlk5 could be detected by this method. Nevertheless, no difference in proteolytic activity was found between WT and transgenic animals (Fig. 23). This further points to the fact, that mKlk5 is not active in Inv-Klk5 mice.

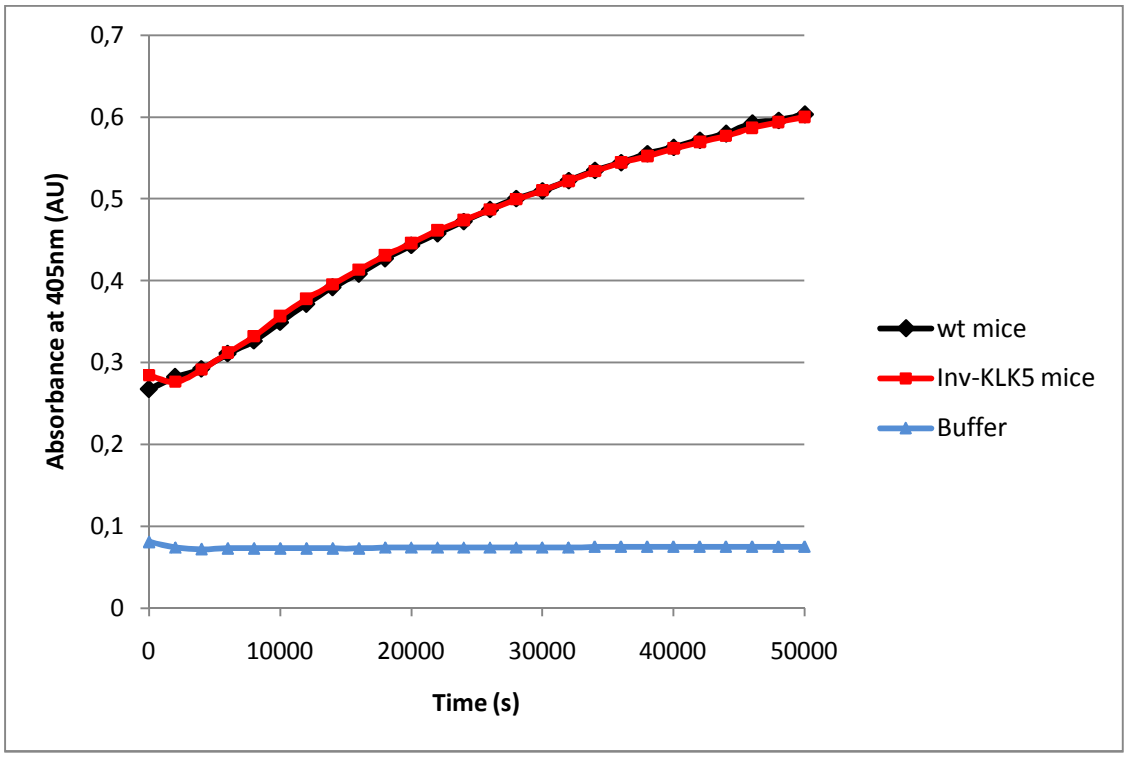


Figure 23. Trypsin-like activity in the skin of Inv-KLK5 and WT mice
Pooled protein ear extracts obtained either from Inv-Klk5 or wt mice were incubated with a chromogenic substrate specific for KLKs with trypsin-like activity. The substrate processing was monitored as an increase in absorbance at 405nm. No obvious difference in trypsin-like KLK activity was observed between transgenic and wildtype animals.

3.3.5.3 Model of irritant dermatitis

As the normal epidermis did not show any defect, possibly due to the lack of mKlk5 proteolytic activators, model of irritant dermatitis was employed to activate pro-mKlk5 and reveal its possible role in skin inflammation. Croton oil was used as irritant inducing inflammation. Application of croton oil on mouse ear results in a local inflammatory reaction measured by an increase in ear thickness. Upregulated proteolytic activity induced by dermatitis can lead to activation of transgenic pro-mKlk5 and altered inflammatory reaction in Inv-Klk5 mice, manifested by different kinetics of ear swelling. However, no significant differences were observed between Inv-Klk5 mice and WT animals (Fig. 24).

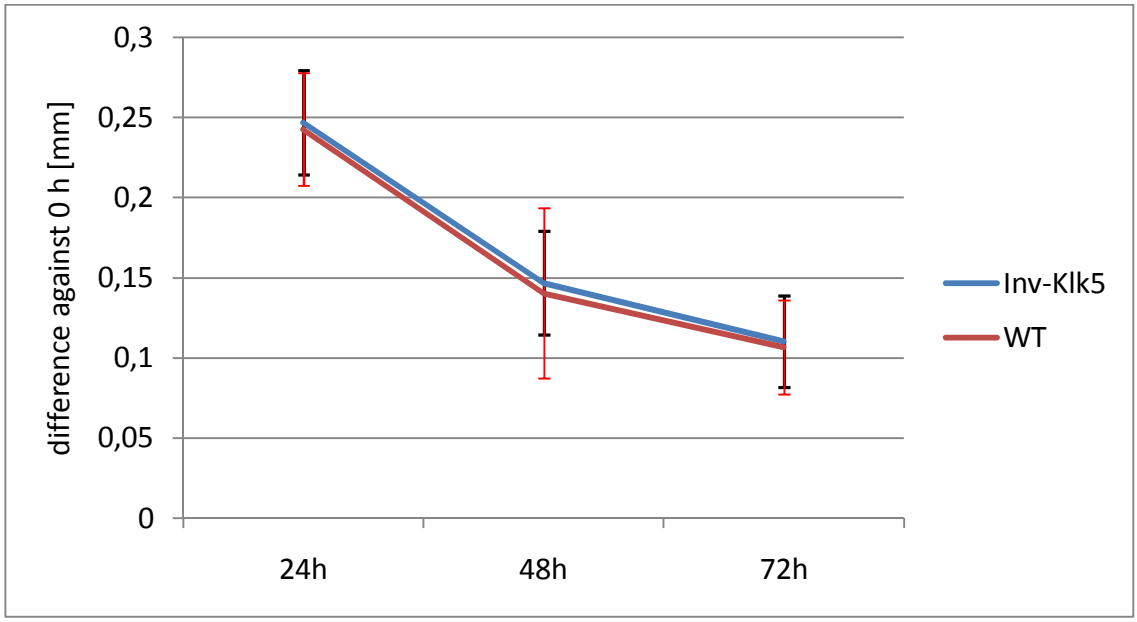
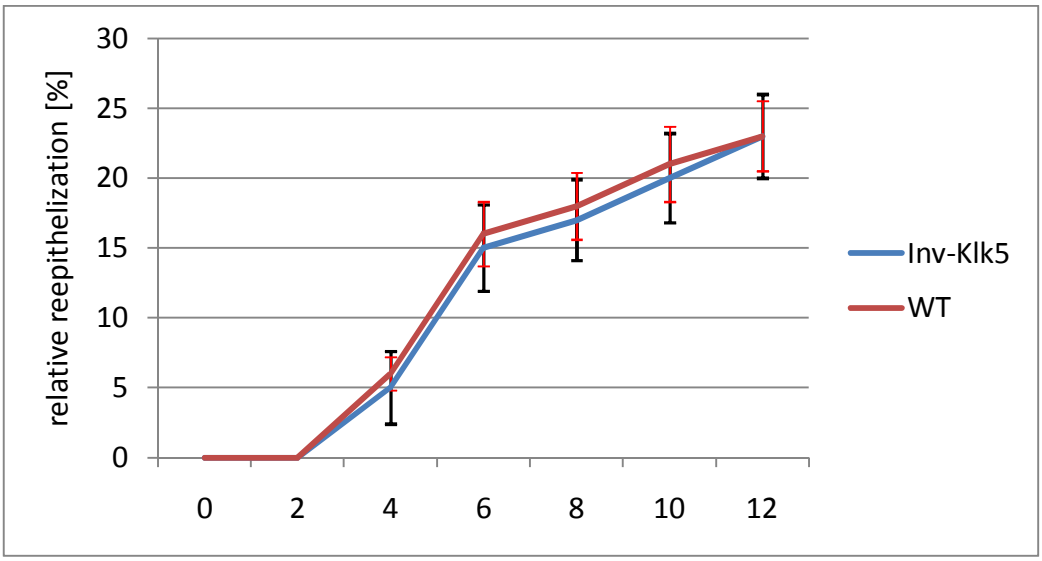


Figure 24. Irritant dermatitis model in Inv-Klk5 mice
After application of croton oil on ears of Inv-KLK5 and WT mice, ear thickness was measured at times indicated. Mean values are given in mm as difference to time point 0 h. The difference in ear swelling observed between Inv-Klk5 and WT mice was not statistically significant ($p>0.05$).

3.3.5.4 Model of wound healing

As observed in Inv-tdTomato mice, expression from hINVm-int promoter can be induced by disruption of the skin (Fig. 25). Thus, employing of wound healing model in Inv-mKlk5 mice can be an option how to further upregulate exogenous mKlk5. Additionally, as it has been reported, KLK5 may be involved in the process of wound-healing through activation of PAR-2 (Materazzi et al., 2007). This suggests that increased activity of mKlk5 might be manifested by different kinectics of wound-healing. Still, our analysis did not confirm any significant difference between Inv-mKlk and WT animals.



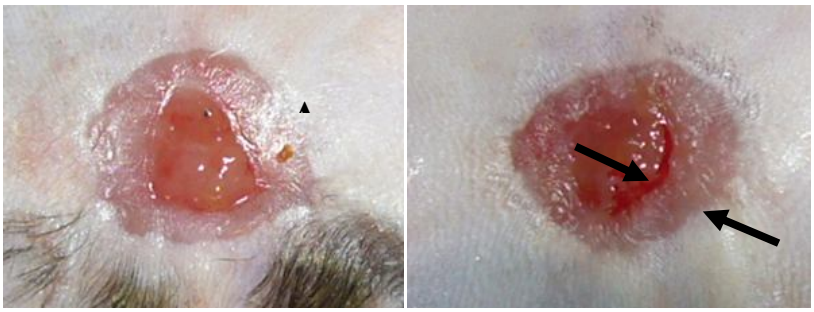


Figure 25. Wound healing model in Inv-Klk5 mice
The kinetics of the wound healing was quantified as relative reepithelization of the wound. The reepithelization was measured at days 2, 4, 6, 8, 10 and 12. Mean values in %. The differnces in wound healing between Inv-KLK5 mice and WT mice were not statistically significant ($p>0.05$). The skin wound of Inv-Klk5 mouse (A) and WT control (B) wound at day 10. Reepithelized area is indicated by arrows.

4 Discussion

4.1 hINVm-int promoter

The truncated human involucrin promoter containing involucrin intron (hINVm-int) has been previously shown to direct the keratinocyte-specific expression *in vitro* (R. Sedláček, oral communication). Here, we present a transgenic mouse expressing red fluorescent protein tdTomato under the control of hINVm-int.

Analysis of the mice revealed high expression of tdTomato in the skin. The detailed examination of the expression pattern in epidermal compartments showed the strongest fluorescence signal in suprabasal layers. Besides the skin, tdTomato was weakly expressed also in the epidermis of tongue and bladder.

These results confirm that hINVm-int promoter targets the gene specifically into epidermal compartments. We estimate that weak expression in the tongue, bladder and possibly other organs with stratified squamous epithelia should not cause any unspecific effects in mice transgenic for mKlk5.

Our further observations suggest that hINVm-int activity may be increased by wounding the skin. This is in agreement with previous reports of involucrin-upregulation in hyperproliferative epidermis of healing wounds (Li et al., 2000). Possibly, this mechanism could be utilized to stimulate the expression of the transgene by disrupting the skin.

4.2 Inv-KLK5 mice

A transgenic mouse overexpressing mKlk5 appears to be a useful tool to study physiological and pathophysiological functions of this enzyme. In this study, we have generated a transgenic mouse expressing mKlk5 under the control of hINVm-int promoter. Altogether, we obtained six transgenic lines although only two lines showed high upregulation of mKlk5 RNA in positive animals. These mice did not exhibit any obvious skin abnormalities during the ontogenesis, immature age or adulthood. Immunohistochemical analysis confirmed that mKlk5 is upregulated in all desired epidermal layers, *stratum spinosum*, *stratum granulosum* and *stratum corneum*, having the

same expression profile as endogenous mKlk5. Nevertheless, further analysis revealed that most of the upregulated enzyme remained in inactive pro-form.

There is no clear explanation for the enzyme inactivity. It has been shown that KLK5 is able to activate its own pro-form (Brattsand et al., 2005). Our results proved that mKlk5 is present in epidermal extracts of Inv-Klk5 mice, thus, it can act as an activator of upregulated pro-mKlk5. In theory, this should increase the proteolytic activity in skin, promoting further activation of the pro-enzyme.

It could be expected from studies with the human orthologue and LEKTI-inhibitors that hyperactivity of mKlk5 might have a dramatic impact on skin physiology. Absence of such dramatic effects may be a further indication of enzyme inactivity in Inv-Klk5 mice. Therefore, it seems that the conversion of immature protein into an active one was disabled by an unknown mechanism.

It has been shown that KLK5 activity may induce PAR-2 mediated signaling (Stefansson et al., 2008). PAR-2 activation can consequently inhibit secretion of lamellar bodies that are necessary to transport KLK5 into *stratum corneum* (Ishida-Yamamoto et al., 2005). Such negative feed-back loop mechanism could possibly explain the lack of mKlk5 activity in Inv-Klk5 mice. On the other hand, this regulatory pathway could lead to accumulation of pro-mKlk5 in *stratum granulosum* and *stratum spinosum,* where the lamellar bodies are formed. Such effect was not observed in mKlk5 mice.

Other mechanism that could lead to disabled activation process of the mKlk5 is unexpected upregulation of proteolytic inhibitors or a generally low increase of mKlk5 expression over the endogenous levels. The lower levels of mKlk5 expression, and thus also its impact, might be then easily outbalanced by available inhibitors that could also inhibit the activators of the kallikrein-like protease.

Possibly, pro-mKlk5 overexpressed in Inv-KLK5 mice is activated to some extent however, the amount of mature enzyme is not sufficient to provide any visible effect. As shown in the mice transgenic for KLK7, the enzyme has been only partially converted into its mature form and vast majority of KLK7 remained inactive (Hansson et al., 2002). Yet, the amount of mature KLK7 was sufficient to induce significant abnormalities in the skin of positive animals. Hansson et al. used SV40e promoter to control the expression of KLK7. Generation of a mouse expressing mKlk5 from a strong, ubiquitous promoter for instance SV40 can be a possible way to obtain sufficient amount of catalytically active enzyme in transgenic animals. However, the expression would not be tissue-specific. Major disadvantage of such approach is the generation of artifact phenotypes due to

targeting the protease to sites, in which it could find potentially cleavable substrates. Thus, the strong expression of a proteolytic enzyme such as mKlk5 would likely lead to a new but wrong phenotype. Moreover, as shown in mice deficient in LEKTI, ubiquitous hyperactivity of mKlk5 leads to neonathal lethality (Descargues et al., 2005).

The activity of mKlk5 can be stimulated indirectly by upregulation of its proteolytic activators, promoting the conversion of pro-mKlk5 to mature enzyme. In this study, we induced irritant dermatitis in the skin of Inv-Klk5 mice. It has been shown that atopic dermatitis is associated with the increase of serine-protease activity in stratum corneum (Voegeli et al., 2009). Serine proteases such as KLK5, KLK14 or matriptase, have been identified as KLK5 activators, thus, possible upregulation of these enzymes in atopic dermatitis may result in pro-KLK5 activation. However, we did not observe any differences in manifestation of the dermatitis between Inv-Klk5 and WT animals.

Alternatively, the levels of mKlk5 activators can be upregulated by overexpression of these enzymes. Double-transgenic mouse overexpressing mKlk5 and one of its activators could be also a useful model to study the role and interactions of these enzymes in epidermal proteolytic cascades. Similarly, Inv-Klk5 can be crossed with mouse deficient in LEKTI.

To circumvent the necessity of pro-enzyme activation, transgenic mouse expressing a mature protease (lacking the pro-domain) can be prepared. However, this strategy would probably result in unspecific phenotype, especially in compartments such as epidermis, where are the enzymes synthesized at sites different from their final destination.

Furthermore, it is possible that we did not find any obvious phenotype simply because the mKlk5 does not play as important role in epidermal compartments as we assumed. A majority of *in vitro* studies are examining the function of human KLK5. Although it is generally believed that both enzymes have the same function in epidermis, it is possible that the physiological role of these proteases slightly differs. Nevertheless, most data obtained in mouse experiments proves the opposite. Upregulation of mKlk5 in LEKTI-deficient mouse is manifested by similar effects that are contributed to human KLK5, such as abnormal desquamation or disruption of epidermal barrier (Descargues et al., 2005). Recent studies show, that skin abnormalities in LEKTI-deficient mouse can be eliminated by ablation of KLK5´s activator – the matriptase (Sales et al., 2010). Based on these data, it is legitimate to conclude, that murine and human kallikrein 5 share the same regulatory and activatory mechanisms.

In summary, we have successfully generated a transgenic mouse expressing mKlk5 in suprabasal layers of epidermis. No phenotypic changes in mouse skin or other effects of the transgene were observed. This may be caused by insufficient expression of the transgene or by an unknown mechanism that disables the activation of the enzyme. However, Inv-Klk5 mice still may be a very useful model to study the activation mechanism of this enzyme.

5 Summary

To support *in vitro* studies pointing to the important role of kallikrein 5 in epidermis, we aimed to generate a transgenic mouse overexpressing mKlk5 in superficial epidermal layers.

To characterize tissue-specific promoter, we prepared a reporter mouse expressing fluorescent marker tdTomato under the control of hINVm-int promoter. The analysis of transgenic animals confirmed keratinocyte-specific expression pattern of tdTomato, suggesting that hINVm-int promoter is suitable to target mKlk5 expression specifically into suprabasal epidermal layers.

Transgenic mice overexpressing mKlk5 have been successfully generated, however no obvious phenotype pointing to the enzyme hyperactivity has been observed. Detail analysis confirmed that mKlk5 expression is upregulated on both, mRNA and protein levels, and is specific for epidermal compartments. Nevertheless, majority of the enzyme remained in inactive pro-form. This may be caused by the lack of proteolytic activators or insufficient expression of the transgene. However, the exact cause is not known. The activation of mKlk5 pro-form should be the topic of following studies and Inv-Klk5 mice still have the potential to become a new valid model of skin diseases.

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7 Appendix

Instruments and equipment

AccuBlock digital dry bath	*Labnet*, USA
Biophotometer	*Eppendorf*, Germany
Balance Kern 440-35N	*Kern*, Germany
Barrier wrap Saran	*Dow*, USA
Blot paper Protean xi and XL size	*Bio-Rad*, Canada
Centrifuge 5415 R	*Eppendorf*, Germany
Centrifuge 5810 R	*Eppendorf*, Germany
Electroforetic chamber Wide-Sub Cell GT	*Bio-Rad*, Canada
Electroforetic chambre Mini-Sub Cell GT	*Bio-Rad*, Canada
EnVision® Multilabel Reader 2104	*PerkinElmer*, USA
Flowbox Bio-II-A Biological Safety Cabinets	*Telstar*, Spain
Freezer and fridge Comfort	*Liebherr*, Austria
Freezer and fridge Profi line	*Liebherr*, Austria
Fridge A class	*Whirlpool*, USA
Freezer (-80°C)	*Sanyo*, Japan
Gel imager Universal Hood II	*Bio-Rad*, Canada
Hotplate and Stirrer Jenway 1000	*Jenway*, UK
Incubator Forma DH CO2 Hepa Class 100	*Thermo Fisher Scientific*, USA
Incubator NB-205	*N-biotek*, Korea
Leica CM-1950	*Leica*, Germany
Luminiscent image analyser LAS – 3000	*Fujifilm Life Science*, USA
Microscope DMI 4000B inverted	*Leica*, Germany
Microscope IX51, inverted	*Olympus*, USA
Microscope TIRF	*Leica*, Germany
Mixing Block MB-102	*BioER Technology*, China
Nanodrop 1000	*Thermo Fisher Scientific*, USA
Orbit 100 shaker	*Labnet*, USA
OV100 small animal imaging system	*Olympus*, Japan
pH meter W3B	*BEL Engineering*, Italy
Pipette set BioPette	*Labnet*, USA

Stuart roller mixer SRT6	*Barloworld Scientific*, UK
Syringe 2 ml	*Braun*, Germany
Syringe Omifix-F 1ml	*Braun*, Germany
Tecan Reader Infinite M200	*Tecan group*, Switzerland
Toym IP25	*Mitutoyo*, USA
Trans-Blot SD	*Bio-Rad*, Canada
Tissue culture plastic	*Greiner*, Germany
	Corning, USA
Vortex mixer VX – 100	*Labnet*, USA
Water bath Nb9	*Nüve*, Turkey
Western Blot PVDF Transfer Hybond-P	*GE Healthcare*, UK

Bacterial strains

Escherichia Coli XL-1 Blue MRF‘: Δ(mrcA)183 Δ(mrcCB-hsdSMR-mrr)173 endA1 supE44 thi-1recA1 gyrA96 relA1 lac[F‘ proAB lacl^{q}ZΔM15 Tn10(Tet^{r})], *Stratagene*, USA

Primers

Primers for genotyping of INV-tdTomato mice

Inv_fwd: 5‘-AGGGAAGAGGGGATGCTAA-3’

Tom_rew: 5‘-tctcggtctcttcctcctca-3’

Primers for genotyping of Inv-KLK5 mice

Inv_fwd: 5‘-AGGGAAGAGGGGATGCTAA-3’

FLAG-rew: 5‘-CTTGTCATCGTCGTCCTTGT-3’

Primers for qRT-PCR (mKlk5)

Fwd: 5′-CTG GTT CTG GGG GTC TCA-3′

Rew: 5′-ATCCGTGCTGAGGTCTCTGT-3′

Primers for qRT-PCR (mGAPDH)

Fwd: 5′-GCA GTG GCA AAGTGGAGATT-3′

Rew: 5′-TTG GCT CCA CCC TTC AAG T-3′

Vectors

pBroad-tdTomato	generated by Branislav Slavik
pcDNA-Klk5-FLAG	generated by Petr Kašpárek
pTracer-Klk5	generated by Petr Kašpárek
pCF-hTubo-FLAG	generated by S.Vinopal
R60-hINVm-EGFP	provided by S. Ghazizadeh
R60-hINVm-int-EGFP	generated by Halka Buryová

Cell lines

Cos-7	*ATCC*, USA
HaCaT	Institute of Biochemistry, Univesity of Kiel,Germany
HepG2	*ATCC*, USA
PAM212	Institute of Biochemistry, Univesity of Kiel, Germany

Mouse strains

C57Bl/6N strain	*IMG animal house*, Czech republic

Media

DMEM	*IMG media facility*, Czech republic
EMEM	*IMG media facility*, Czech republic
LB medium	*IMG media facility*, Czech republic
Amp LB agar plates	*IMG media facility*, Czech republic
RPMI-1640	*IMG media facility*, Czech republic

Commercial kits

BCA protein Assay kit	*Pierce*, USA
ECL Plus Western Blot detection reagents	*GE Healthcare*, UK
GeneJET Plasmid Miniprep Kit	*Fermentas*, Canada
Illustra Nick columns, Sephadex G-50	*GE Healthcare*, UK
PCR-Combi-Kit	*Seqlab*, Germany
First strand cDNA	*Fermentas,* Canada

Chemicals

Acetic acid glacial, 100%	*Roth*, Germany
Acrylamid Rotiphorese Gel 30	*Roth*, Germany
Agarose Seakem LE	*Cambrex*, USA
Ammonium persulphate (APS)	*Sigma-Aldrich*, USA
Ampicillin	*Biotika*, Slovakia
Bovine serum albumin (BSA)	*Sigma-Aldrich*, USA
Calcium chloride	*Roth*, Germany
Complete protease inhibitor EDTA free	*Roche*, Switzerland
Dithiotreitol (DTT)	*Sigma-Aldrich*, USA
EDTA	*Roth*, Karlsruhe, Germany
dNTP mix (10mM each)	*Fermentas*, Canada
Ethanol	*Penta*, Czech republic
Ethidium bromide	*Sigma-Aldrich*, USA
Foetal Bovine serum	*PAA Laboratories*, Austria
Formalin	*Walter CMP*, Germany
FUGENE HD	*Roche*, Switzerland
Glycerol	*Penta*, Czech republic
Hydrochloric acid	*Roth*, Germany
Hoechst 33258	*Invitrogen*, USA
Chlorophofm	*Lachema*, Czech republic
Isopropanol	*Lachema*, Czech republic
Liquid nitrogen	*IMG media facility*, Czech republic
Magnesium chloride	*Fermentas,* Canada
2-Mercaptoethanol	*Merk*, Germany
Methanol	*Penta*, Czech republic
Milk powder	*Roth*, Germany
Nonidetp40	*Sigma-Aldrich*,USA
Penicillin/Streptomycin	*PAA Laboratories*, Austria
Phenol	*Roth*, Germany
Potassium acetate	*Roth*, Germany
Sodium acetate	*Sigma-Aldrich*, USA
Sodium dodecyl sulfate (SDS pellets)	*Sigma-Aldrich*, USA
Sodium chloride	*Lachema*, Czech Republic

Sodium hydroxide	*Lachema*, Czech Republic
TissueTek OCT Compound	*Sakura*, USA
Tris	*Roth*, Germany
Tris hydrochloride	*Roth*, Germany
Triton X-100	*Sigma-Aldrich*, USA
TRIZOL	*Invitrogen,* USA
Trypan Blue	*Gibco*, *Invitrogen*, USA
Trypsin/EDTA	*PAA Laboratories*, Austria
Tween-20	*Riedel-de Haën*, Germany

Solutions and buffers

BamHI buffer, 10x	*Fermentas*, Canada
BSA, 10x	*New England Biolabs*, USA
Buffer R, 10x	*Fermentas*, Canada
DNA Ladder GeneRuler 1kb	*Fermentas*, Canada
DNA Ladder GeneRuler 1kb plus	*Fermentas,* Canada
DNA Loading Dye, 2x	*Fermentas*, Canada
DNA Loading Dye, 10x	*Fermentas*, Canada
DreamTaq buffer, 10x	*Fermentas*, Canada
EcoRI buffer, 10x	*Fermentas*, Canada
PBS 1x (Phosphate buffer)	*IMG media facility*, Czech Republic
Prestained protein molecular weight marker	*Fermentas*, Canada
Stripping buffer	*Pierce*, USA
T4 DNA ligase buffer, 10x	*Fermentas*, Canada
Tango buffer, 10x	*Fermentas*, Canada

Microinjection buffer: 10 mM Tris-HCl; 0.1 mM EDTA,pH=7.4.

Mouse lysis buffer: 200 mM NaCl; 100 mM Tris; 5 mM EDTA; 0.2% SDS; pH=8.0

TAE buffer, 1x: 10 mM Tris; 20 mM Acetic acid; 1 mM EDTA; pH=8,0

TBS 10x: 200mM Tris; 5 M NaCl; pH=7.5

Tissue lysis buffer: 50 mM Tris, 0.15 M NaCl; 1% SDS; 10 mM EDTA

Transfer buffer: 3.03 g Tris; 14.27 g Glycine; 200 ml methanol; ddH2O to 1l

Enzymes

BamHI	*Fermentas*, Canada
BglII	*Fermentas*, Canada
DNaseI (RNase-free)	*Fermentas*, Canada
Dream Taq DNA polymerase	*Fermentas*, Canada
EcoRI	*Fermentas*, Canada
HindIII	*Fermentas*, Canada
NsiI	*Fermentas*, Canada
NdeI	*Fermentas*, Canada
Proteinase K	*Sigma-Aldrich*, USA
T4 DNA ligase	*Fermentas*, Canada
T4 DNA polymerase	*Fermentas*, Canada
Taq DNA polymerase LC	*Fermentas*, Canada
XbaI	*Fermentas*, Canada

Primary antibodies

anti-FLAG monoclonal	*Sigma-Aldrich*, USA
anti-mKlk5 rabbit-Ab SN	provided by M. Brattsand

Secondary antibodies

IgG Alexa fluor 488 – goat anti-mouse	*Molecular probes*, USA
IgG TRITC – goat anti-rabbit	*Sigma-Aldrich*, USA
IgG HRP conjugated – goat anti-rabbit	*Pierce*, USA