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Characterization of the role of SPINK6 in the epidermis using transgenic models

Charakterizace role SPINK6 v epidermis za použití transgenních modelů

Diplomová práce

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Prohlášení:

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Abstract

Epidermal homeostasis, including proper turnover of keratinocytes, plays important role in the barrier function and serine proteases and their inhibitors are the key players. Activated proteases cleave desmosomes in uppermost layer and thus shed the cells from the epidermal surface. Therefore the serine protease inhibitors are secreted in lower epidermal layers to prevent premature activation of proteases and consequent disruption of epidermal barrier. The most studied inhibitors in epidermis belong to Serine proteases inhibitors Kazal-type family (SPINK).

This diploma thesis is aimed to investigate function of murine SPINK6 in epidermal compartment *in vivo*. To achieve this, the transgenic mice overexpressing mSPINK6 under modified human involucrin promoter was generated. Two of five transgenic lines exhibited higher expression of mSPINK6 at mRNA and protein levels. The mSPINK6 transgenic mice are viable with no apparent phenotype. The small but in most cases not significant differences were observed on microscopic level among mSPINK6 transgenic and wild type animals

In conclusion, this work showed that mSPINK6 does not play major role in skin homeostasis but gains significant importance under specific challenges of epidermal barrier. Therefore mSPINK6 transgenic mice, in combination with other deletion or overexpressing models, represent useful tool for future studies.

Key words: Serine proteases inhibitors Kazal-type (SPINK), Lympho-epithelial inhibitors Kazal-type (LEKTI), epidermis, transgenic mouse, skin, involucrin promoter, Kallikrein

Abstrakt

Epidermis tvoří bariéru mezi vnitřním a vnějším prostředím, přičemž celková homeostáze je závislá mezi jiným i na správné obměně keratinocytů. Podstatnou úlohu hrají v tomto procesu serinové proteázy a jejich inhibitory. Aktivované proteázy štěpí desmosomy ve svrchní vrstvě epidermis a umožňují tak odlupování keratinocytů. V nižších vrstvách epidermis jsou proto vylučovány proteázové inhibitory, jejichž funkcí je zabránit předčasně aktivaci proteáz, a tím rozrušení epidermální bariéry. Mezi nejznámější inhibitory v epidermis patří inhibitory serinových proteáz kazálního typu (SPINK).

Tato práce je orientována na výzkum myšního SPINK6 *in vivo*. K tomu účelu jsme pomocí modifikovaného lidského involukrinového promotoru vytvořili transgenní myši exprimující mSPINK6 ve zvýšené míře, přičemž vyšší expresi mSPINK6 na mRNA a proteinové úrovni RNA vykazovaly dvě z pěti transgenních linií.

Ukázali jsme, že transgenní zvířata jsou životaschopní jedinci bez zjevného fenotypového projevu. Na makroskopické úrovni jsme pak popsali mírný fenotyp, který odlišuje transgenní mSPINK6 myši od normálních jedinců.

Závěrem lze říci, že mSPINK6 hraje v homeostázi kůže nejspíš pouze minoritní roli, naproti tomu však může být velmi důležitý při specifické obměně epidermální bariéry. Nami připravené mSPINK6 transgenní myši mohou být navíc velmi užitečné v kombinaci s jinými typy modelových transgenních organismů.

Klíčová slova: inhibitory serinových proteáz kazálního typu (SPINK), lymfo-epiteliální inhibitory kazálního typu (LEKTI), kůže, epidermis, transgenní myš, involukrinový promotor, kalikrein

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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
Cy3	Cyanine dye 3
D	Domain
DEPC	Diethylpyrocarbonate
dH ₂ O	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
EtBr	Ethidium bromide
Fig	Figure
hINV	Human involucrin promoter
hINVm	Truncated human involucrin promoter
hINVm-int	Truncated human involucrin promoter containing involucrin intron
IgE	Immunoglobulin E
IgG	Immunoglobulin G
Int	Intron
kDa	Kilo Daltons
KLK	Human Kallikrein-related peptidase
LBs	Lamellar bodies
LEKTI	Human Lympho-epithelial Kazal-type inhibitor
mKlk	Murine Kallikrein-related peptidase
mLekti	Murine Lympho-epithelial Kazal-type inhibitor
MuLV	Murine leukemia virus

NS	Netherthon syndrome
OD	Optical density
OCT	Optimal cutting temperature
PAR 2	Protease activated receptor 2
PBS	phosphate buffered saline
PCR	Polymerase chain reaction
PNI	Pronuclear injection
PVDF	Polyvinylidene fluoride membrane
qRT-PCR	Quantitative real-time polymerase chain reaction
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute medium
RT	Room temperature
SB	<i>Stratum basale</i>
SC	<i>Stratum corneum</i>
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecylsulfate polyacrylamide gel electrophoresis
SG	<i>Stratum granulosum</i>
SL	<i>Stratum lucidum</i>
SPINK	Serine protease inhibitor Kazal-type
SS	<i>Stratum spinosum</i>
SPR	Small proline-rich proteins
SV40	Simian vacuolating virus 40
TAE	Tris base/ Acetic acid/ EDTA buffer
TBS	Tris-buffered saline
TG	Transgenic mice
TSLP	Thymic stromal lymphopoetin
U	Unit
WT	Wild type mice

1 Introduction

1.1 The skin

The skin represents mechanical, biochemical and innate immune barrier protecting body against invasion of environmental pathogens. The other functions that skin can provide are: regulation of body temperature, secretion of salts, synthesis of vitamin D, protection against UV light and water loss.

The skin consists of three layers that are tightly connected to each other (Fig. 1). The outermost layer is called epidermis. This layer fits tightly to the other layer – dermis. Dermis is subdivided into two compartments. The first one is called stratum papillare (papillary dermis) and is formed by tenuousligament with high amount of fibroblasts. The next layer beneath is stratum reticulare (reticular dermis) of dense amorphous ligament. The dermis is highly penetrated by blood, lymph vessels and nerves. The third layer is hypodermis or subcutaneous layer that mainly consists of adipocytes and connects the epidermis and dermis to adjacent tissues.

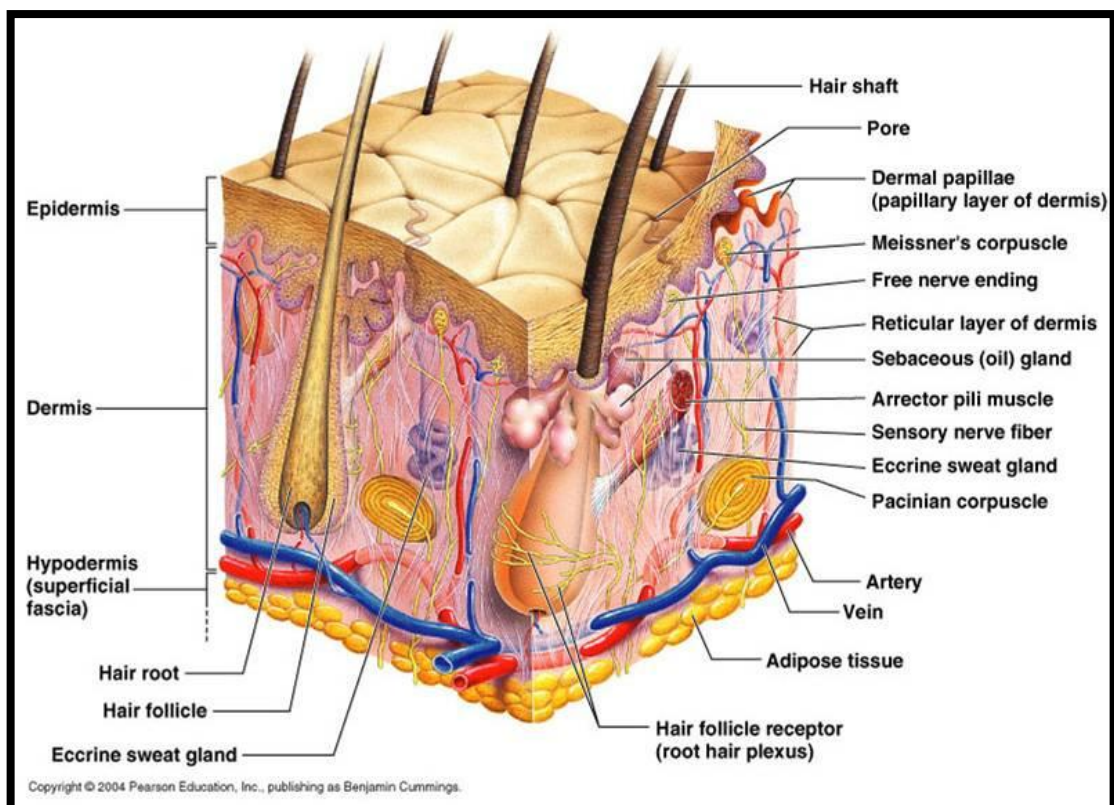


Figure 1. Schematic skin diagram (taken from <http://www.osovo.com/diagram/skindiagram.htm>)

1.2 The epidermis

The epidermis is formed as stratified squamous epithelial layer. The predominant cell type of epidermis is the keratinocyte, the major producer of keratin, an intermediate filament protein. The other cell type characteristic for this layer is the melanocyte, which is responsible for the production of melanin and therefore for the color of the skin. Further specialized cells in the epidermis are Langerhans cells (epidermal dendritic cells), and Merkel cells, which are involved in tactile sensation.

Epidermis can be divided in five sublayers or strata according to structure, function and shape of keratinocytes (Fig. 2):

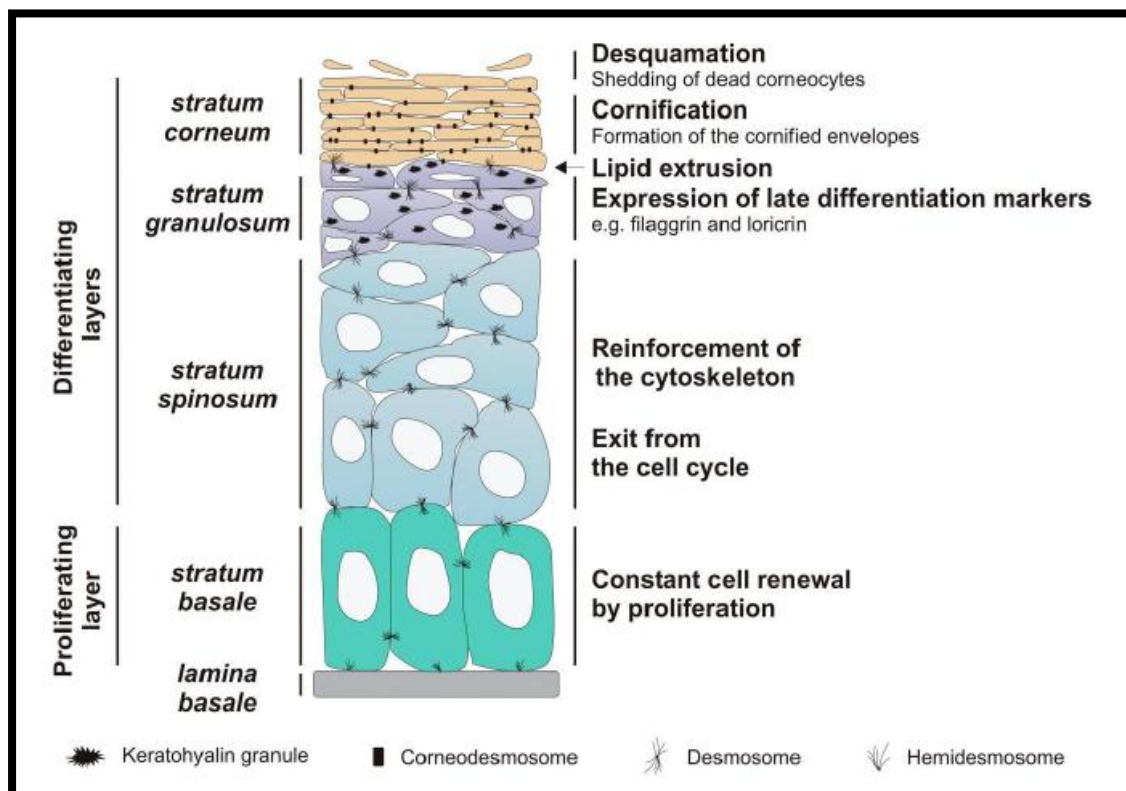


Figure 2. Schematic structure of epidermis. *Stratum basale*, *stratum spinosum*, *stratum granulosum* and *stratum corneum* are four main layers in epidermis. The fifth layer, *stratum lucidum*, is present only on human soles and palms. Each layer contains keratinocytes of distinctive differentiation state. (Denecker *et al.*, 2008)

Stratum basale (SB) consists of single layer of columnar or cubical keratinocytes attached by hemidesmosomes to basement membrane. The cytoplasm contains intermediates filaments called tonofilaments that are associated with desmosomes. The stratum basale contains the population of stem cells, which

undergo mitosis and produce new cells that steadily migrate to epidermal surface and differentiate.

Stratum spinosum (SS) involves the keratinocytes of polygonal shape. In their cytoplasm small granules with lamellar core are formed. They are described as lamellar bodies (LBs). The main function of these cells is to produce the tonofilaments to increase mechanical stamina of epidermis. This layer can be very thick especially on mechanically stressed places (skin on palms and soles).

Stratum granulosum (SG) is formed by three to five layers of flattened polygonal cells characterized by small nuclei, basophilic keratohyalin granules, increased number of LBs, and formation of tight junctions. The lamellar bodies fuse with plasma membrane and release the glycolipid acylglucosylceramid to the extracellular space.

Stratum lucidum (SL) is a thin layer consisting of flattened keratinocytes without nuclei and organelles. This layer is more evident in thick skin, which is located on human soles and palms.

The uppermost layer of epidermis is called *stratum corneum (SC)*. The keratinocytes (also known as corneocytes) of SC are totally flattened, without nuclei and metabolic activity. They are bound to each other by modified desmosomes, called corneodesmosomes. The cytoplasm of corneocytes is filled by aggregated keratin filaments cross-linked by filaggrin. This reaction is catalyzed by several transglutaminases. The keratin–filaggrin complex is attached to additional proteins: loricrin, involucrin, and small proline–rich proteins (SPRs) that are also covalently linked. Additional proteins reinforce the keratin–filaggrin complex and form together the structure called cornified cell envelope (CE) (reviewed in Candi *et al.*, 2005). Outside of the cell is the lipid layer (lipid envelope) linked to involucrin. This lipid layer consists of ceramids, cholesterol and fatty acid; all released by LBs (Marekov and Steinert, 1998). All this components make the cells and therefore also epidermis resistant to mechanical stress and penetration of fluids (permeability barrier).

The epidermis is also characteristic by specific gradients. The first one is pH gradient ranging from neutral pH = 7 in *stratum granulosum* to acidic pH = 4.5 in the upper layers of *stratum corneum*. The main factors responsible for acidity in SC are free fatty acids. Local changes in pH activate different classes of extracellular proteases (Elias, 2004). The other important gradient includes Ca²⁺ ions. Ca²⁺ influx regulates the protein synthesis, differentiation of keratinocytes and formation of

intracellular junctions. The low concentration of calcium ions is typical for stratum basale and is gradually increasing towards SC (Menon *et al.*, 1985).

The *stratum corneum* is also characterized by sequential shedding of corneocytes from the surface of the epidermis. This process is known as desquamation or exfoliation. Desquamation represents fine balance between renewal of epidermal cells and conservation of intact barrier protecting organism against penetration of allergens, pathogens or irritants into deeper epidermal layers. The current model of desquamation suggests that an extracellular network of serine, cysteine and aspartic proteases, regulated by their inhibitors is present in SC. This proteolytic network is responsible for cleavage of corneodesmosomal proteins such as corneodesmosin (CDSN), desmoglein (Dsg) and desmocollin (Dsc). Once the corneodesmosomes are cleaved the cell is released, i.e. shed from the surface of epidermis (Elias, 2004; Lundstrom and Egelrud, 1990). Recently, it was proposed that Kazal-type serine proteases inhibitors (SPINK) may play important role in desquamation and maintain the permeability barrier (Deraison *et al.*, 2007).

1.3 Serine proteases inhibitors Kazal-type (SPINKs)

This type of inhibitors can be found in all living systems from bacteria to mammals. Kazal-type inhibitors are grouped into the family I1 and were named by Kazal who isolated inhibitor of bovine pancreatic secretory trypsin (also known as SPINK1) (Kazal *et al.*, 1948). Kazal-type inhibitors usually consist of one to seven Kazal-type domains. Each domain is composed of 40–60 amino acid (aa) residues with a conserved motif of six cysteine residues forming three intra-domain disulfide bridges between cysteines numbers 1–5, 2–4, 3–6 (Laskowski and Kato, 1980). Magert *et al.* (1999) described new type of Kazal domain, a Kazal-type related domain, which lacks two of the six cysteines but shows identical pattern of the remaining four cysteines and therefore this type of inhibitors contain only two disulfide bridges (Magert *et al.*, 1999). The amino acid pattern of the Kazal-type domain related is very similar to classical Kazal-type domain (Fig. 3).

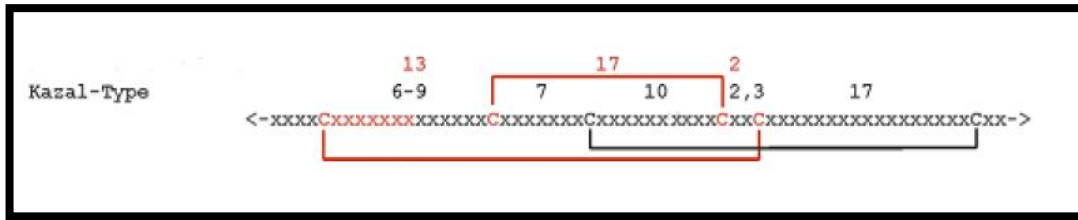


Figure 3. Schematic localization of cysteines in the Kazal domain. The figures indicate the number of amino acid residues among cysteines. Red marked cysteine residues are present in both types of Kazal domain; black marked residues represent disulfide bridge present only in classical six cysteine Kazal domain. (Laubert *et al.*, 2003)

The SPINK family consists of nine genes in the human genome: *SPINK1*, *SPINK2*, *SPINK4*, *SPINK5*, *SPINK5L2*, *SPINK5L3*, *SPINK6*, *SPINK7* and *SPINK9* according to Ensemble genome browser (<http://www.ensembl.org/index.html>). The inhibitors are mainly involved in protection against proteolytic degradation of mucosal and epithelial tissues. *SPINK1* is secreted by pancreatic acinar cells and prevents premature activation of zymogens. Its mutations are associated with hereditary pancreatitis (Pfutzer *et al.*, 2000). *SPINK2* is trypsin/acrosin inhibitor mainly synthesized in the testis, epididymis and seminal vesicle. Expression of this inhibitor is closely related to lymphoma cancer (Rockett *et al.*, 2004). The expression of *SPINK1*, *SPINK5*, *SPINK6*, and *SPINK9* were confirmed in epidermis (Brattsand *et al.*, 2009; Chavanas *et al.*, 2000a; Meyer-Hoffert *et al.*, 2010). *SPINK5* (also known as Lympho-epithelial inhibitor Kazal-type, LEKTI) is the most described SPINK inhibitor, which plays a crucial role in skin homeostasis. It was recently proposed that *SPINK6* and *SPINK9* (LEKTI2) may additionally influence the homeostasis guided by LEKTI.

1.3.1 Serine proteases inhibitors in epidermis

In 1999 Magert *et al.* isolated two unknown polypeptides from human blood filtrate that were parts of a larger precursor protein designated as LEKTI (Magert *et al.*, 1999). Human LEKTI is encoded by *SPINK5* gene that is localized on 5q31-32 chromosome region. LEKTI2 was purified from human skin by using chromatographic methods. The sequence matches to putative *SPINK9* that is located on human chromosome 5q33.1 (Brattsand *et al.*, 2009; Meyer-Hoffert *et al.*, 2009b). *SPINK6* was isolated as a potential partner of one protease playing important role in epidermis and its gene is localized on 5q32 (Meyer-Hoffert *et al.*, 2010).

1.3.2 Structure and expression of SPINKs

The three different N-glykosylated proproteins of human LEKTI are formed. The full-length isoform of 1067 aa residues that form 15 domains (145 kDa), a shorter proprotein (125 kDa) composed of first 13 domains and longer isoform (148 kDa) including a 30 amino acid residues insertion (Bitoun *et al.*, 2003; Tartaglia-Polcini *et al.*, 2006). The murine ortholog (mLekti) was isolated as 1017 aa long precursor of 130 kDa and both orthologes share 60% homology (Galliano *et al.*, 2005; Yang *et al.*, 2004). Two of the fifteen LEKTI domains (the second and the fifteenth) contain three disulfide bridges while the rest of domains forms two bridges (Magert *et al.*, 2002). In trans-Golgi complex of keratinocyte, the proproteins are cleaved by furin, a subtilisin-like proprotein convertase. Furin-dependent processing of LEKTI generates fragments of molecular weight of 90 kDa, 60 kDa, 40 kDa or 37 kDa (Bitoun *et al.*, 2003; Deraison *et al.*, 2007). It was proposed that digestion of N-terminal part of LEKTI results in single domain (D) fragments. In contrast, the digestion of C-terminal part leads to multiple domains fragments (Bitoun *et al.*, 2003). These fragments are secreted via lamellar bodies to *stratum granulosum/stratum corneum* interface (Ishida-Yamamoto *et al.*, 2005).

Human LEKTI2 proprotein has 86 aa residues (7.7 kDa) including 16 residues from the first Met as a signal peptide and the next 55 residues corresponding to the Kazal domain with six cysteine residues (Brattsand *et al.*, 2009; Meyer-Hoffert *et al.*, 2009b). This Kazal domain has 33% concordance to second domain (D2) and 32% concordance to D15 of human LEKTI. The important difference is between reactive centers (P1–P1' sites) that suggesting different affinities of LEKTI/LEKTI2 to proteases (Fig. 4) (Meyer-Hoffert *et al.*, 2009b). Also SPINK6 encodes protein product of 80 aa (6 kDa) containing one typical Kazal domain with six cysteine residues. Comparison of SPINK6 with the Kazal domains of LEKTI and LEKTI2 showed that SPINK6 is highly homologous to Kazal-type domains of LEKTI especially in the reactive centre (Meyer-Hoffert *et al.*, 2010).

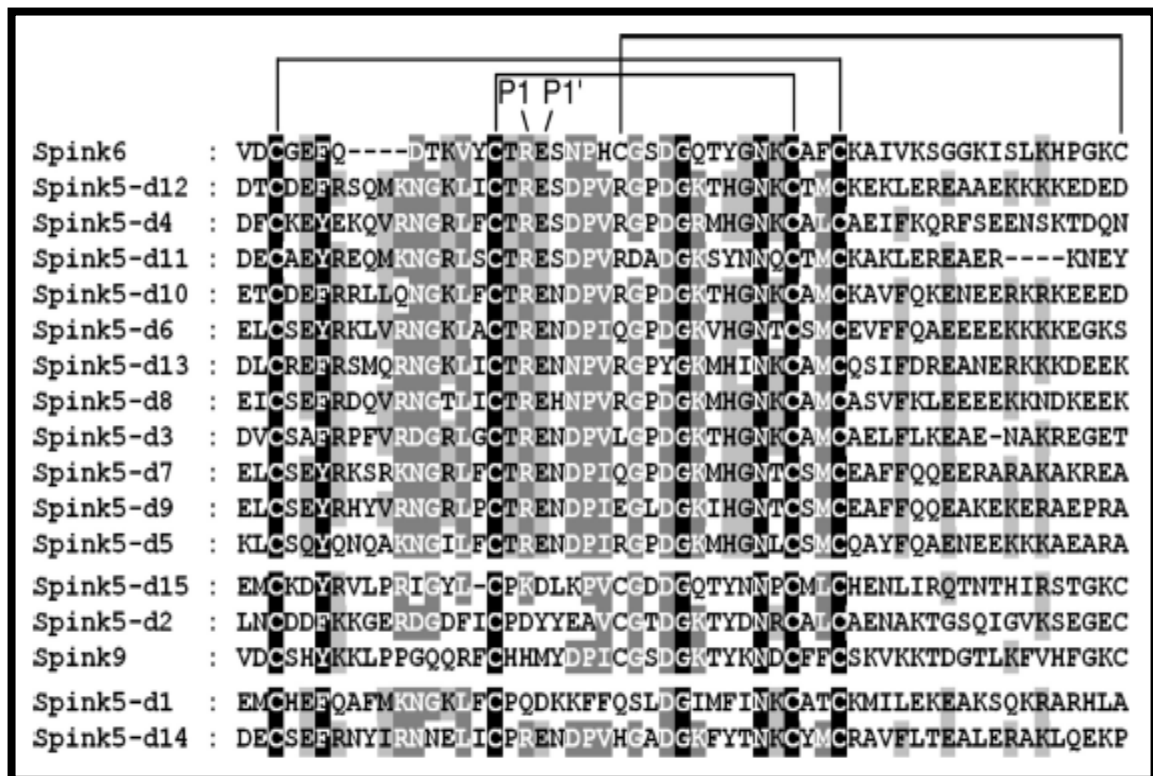


Figure 4. The alignment of amino acids sequences of SPINK6, SPINK9/LEKTI2 and SPINK5/LEKTI. The identical residues are in black boxes while grey boxes indicate partially conserved residues (the more grey the more are amino acids conserved among SPINK family). The P1 and P1' sites are marked. (Meyer-Hoffert *et al.*, 2010)

According to data obtained from *in situ* hybridization, real-time poly chain reaction and immunohistochemistry LEKTI is mainly expressed in epidermal *stratum granulosum*, *spinosum*, *corneum* and in hair follicles. LEKTI was also detected in the oral mucosa (tongue), blood, tonsils, thymus, vaginal epithelium, parathyroid gland and at very low levels in the lung, kidney, prostate, duodenum, bladder, rectum, stomach and colon (Bitoun *et al.*, 2003; Hachem *et al.*, 2006b; Komatsu *et al.*, 2003; gert *et al.*, 1999; Tartaglia-Polcini *et al.*, 2006).

The strong expression of LEKTI 2 and also SPINK6 proteins were detected using immunohistochemistry and mRNA analysis in *stratum granulosum* and *stratum corneum* of palmar epidermis. Furthermore SPINK6 was detected in sebaceous glands and in some sweat glands although LEKTI2 appears to have low or none expression in these epidermal compartments. Intermediate expression of LEKTI 2 was confirmed in thymus, brain, pancreas and liver (Brattsand *et al.*, 2009; Meyer-Hoffert *et al.*, 2010; Meyer-Hoffert *et al.*, 2009b).

1.4 The role of SPINKs in epidermis

1.4.1 Inhibition targets

The fifteen domains of LEKTI and presence of other two inhibitors (LEKTI2 and Spink6) have a great potential for large range of inhibition targets: among them kallikrein related proteases (further described as kallikreins).

Human kallikreins (KLKs) create a large family of trypsin- or chymotrypsin-like secreted serine proteases that are encoded by 15 genes (*KLK1-KLK15*), all located in a cluster on chromosome 19q13.4 (Yousef *et al.*, 2001). The expression of KLK1, KLK4-KLK11, KLK13 and KLK14 was confirmed on the basis of RT-PCR analysis in epidermal *stratum granulosum*, *stratum corneum* in the hair follicle and in sebaceous glands. The expression of kallikreins is colocalized with the expression of LEKTI in *stratum corneum* (Ishida-Yamamoto *et al.*, 2005; Komatsu *et al.*, 2003). Kallikreins are synthesized as pre-proenzymes. The signal peptide of pre-proenzyme is cleaved during transport into endoplasmatic reticulum and the protein as a zymogen (nonactive enzyme) is secreted via lamellar bodies to extracellular space of SC/SG interface. LEKTI and KLKs share the same secretory pathway although they are loaded into distinct LBs to prevent abortive interactions (Ishida-Yamamoto *et al.*, 2005). Kallikreins are activated through cleavage of N-terminal propeptide. The most studied and probably the most important kallikrein in epidermis appears to be KLK5, which is responsible for 50% of trypsin-like activity in upper layers of epidermis (Brattsand *et al.*, 2005). Both KLK7 and KLK5 were isolated from epidermis and marked as stratum corneum tryptic enzyme (KLK5) and stratum corneum chymotryptic enzyme (KLK7). KLK5 is able to cleave PAR 2 (protease-activated receptor 2) that induces expression of TSLP (thymic stromal lymphopoetin) and thus triggers inflammation and immune response (Briot *et al.*, 2009). According to many studies altered expression and/or activity of KLK5 was observed in psoriasis vulgaris (Komatsu *et al.*, 2007b), atopic dermatitis (Komatsu *et al.*, 2007a), rosacea (Yamasaki *et al.*, 2007) and Netherton syndrome (Komatsu *et al.*, 2002).

Studies based on *in vitro* experiments showed that most of KLKs (KLK5, KLK6, KLK7, KLK13 and KLK14) are inhibited by recombinant LEKTI fragments composed of domains 1–6, 6–8, 8–11 and 9–12 (Deraison *et al.*, 2007; Schechter *et al.*, 2005). Also single domains (D5 and D6) show significant inhibition properties against

KLK5, KLK7 and KLK14 *in vitro* (Deraison *et al.*, 2007). LEKTI2 and SPINK6 exhibit more specific affinities for inhibition targets. Similarly to D5 and D6 of LEKTI SPINK6 inhibits KLK5, KLK7 and KLK14 (Meyer-Hoffert *et al.*, 2010). In contrast, LEKTI2 appears to inhibit only KLK5 but not KLK7 or KLK14 (Brattsand *et al.*, 2009; Meyer-Hoffert *et al.*, 2009a). This indicates that each fragment or domain of these inhibitors has one or more favored protease targets *in vivo*.

Kallikreins are not the only possible targets of SPINKs. Several authors described that a recombinant LEKTI fragment containing D6–D8 is able to inhibit trypsin, subtilisin A and chymotrypsin *in vitro* (Jayakumar *et al.*, 2004; Schechter *et al.*, 2005). Single domains of LEKTI D5, D6 and D15 inhibit trypsin and in the case of D15 plasmin (Egelrud *et al.*, 2005). Recently it was published that recombinant LEKTI fragments D1–D6, D6–D8 and D9–D12 inhibit caspase 14, which is cysteine protease that possibly plays a role in terminal differentiation of keratinocytes. This finding is quite surprising considering that LEKTI is classified as serine protease inhibitor Kazal-type. On the other side the domain 2 of LEKTI contains at the P1 site aspartic acid residue which is preferred by cystein proteases (Bennett *et al.*, 2010). These results suggest that LEKTI serves as multifunctional proteases inhibitor playing a role not only in the skin homeostasis but also in other compartments of human body.

1.4.2 Role of SPINKs in desquamation

Both kallikreins and their inhibitors play important role in the desquamation process. The ability of kallikreins to cleave specific products is pH-dependent and also the inhibition by serine proteases inhibitors is efficient at neutral but not at acidic pH. This observation lead to establishment of four-step hypothesis (Hachem *et al.*, 2006a). First, the KLKs are secreted into *stratum granulosum* extracellular space where are proteolytically activated by other protease (for example matriptase) or by their own autoproteolytic activities (in the case of KLK5, KLK11) (Brattsand *et al.*, 2005; Yoon *et al.*, 2007). KLK5 is able to proteolytically activate zymogens of other kallikreins present in epidermis and therefore it can be on the top of kallikrein signaling cascade (Yoon *et al.*, 2007). In addition, matriptase, a transmembrane serine protease, is able to efficiently activate KLK5, KLK7 *in vitro* (Sales *et al.*, 2010). Second, activated kallikreins but not matriptase are inhibited by LEKTI, LEKTI2 or SPINK6 to prevent premature cleavage of corneodesmosomes in *stratum granulosum* and lower layers of

stratum corneum. Third the acidic pH in the upper layers of *stratum corneum* triggers off dissociation of the inhibitor-kallikrein complexes. Fourth, the free KLKs cleave the corneodesmosomal proteins and thus release of corneocytes from the outmost layer of *stratum corneum*. This was proved by *in vitro* experiments where KLK5 was able to cleave CDSN, Dsg1, and Dsc1 at pH 5, 6. KLK7 cleaves CDSN and Dsc1 and other epidermal kallikreins KLK1, KLK6, KLK13 and KLK14 are able to cleave Dsg1 (Borgo *et al.*, 2007; Caubet *et al.*, 2004)

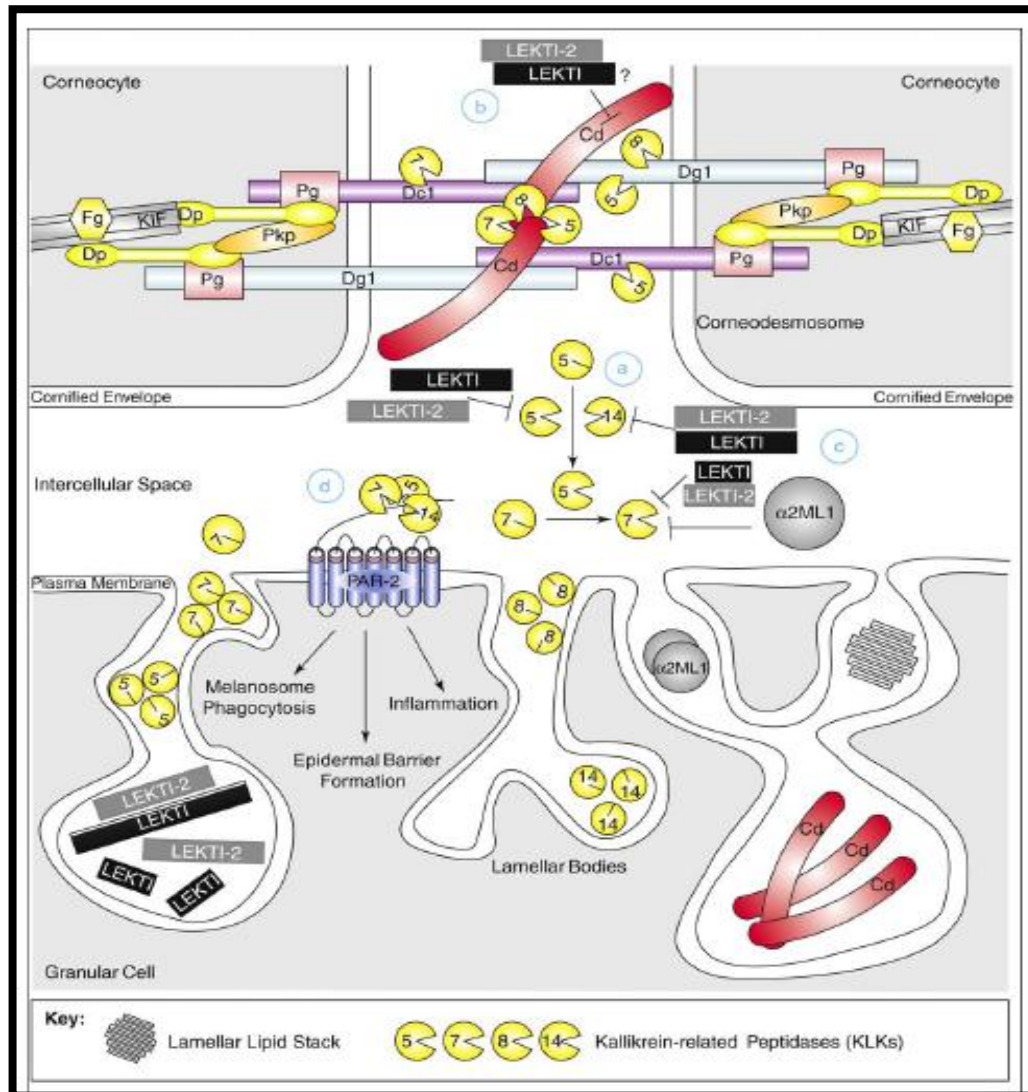


Figure 5. Hypothetical model of epidermal desquamation processes. The keratinocytes of *stratum granulosum* produce high amount of lamellar bodies that include serine proteases (such as kallikrein-related proteases), serine proteases inhibitors (such as LEKTI and α2ML1), lamellar lipids and components of corneodesmosomes (corneodesmosin). (a) KLK5 is auto-activated or is activated by KLK14. Activated KLK5 cleaves KLK7 proenzyme and activates it this way. (b) Corneodesmosomes maintain structural integrity of lower epidermal layers. The main components of corneodesmosomes are corneodesmosins (Cd), desmoglein (Dg), desmocollin (Dc), desmoplakin (Dp), plakoglobin (Pk), filaggrin (Fg) and keratin intermediate filaments (KIFs). Kallikreins are able to cleave extracellular corneodesmosomal proteins Dg1, Cd and Dc1. (c) KLKs secreted into intercellular space are prevented from premature activation by inhibition of serine proteases inhibitors, mainly LEKTI, LEKTI-2 and alpha-2 macroglobulin-like 1(α2ML1). (d) KLK5, KLK7 and KLK14 activate PAR-2 receptor that triggers inflammation, melanosome phagocytosis and/or epidermal barrier formation. (Taken from Ovaere *et al.*, 2009)

1.4.3 Role of SPINKs in skin immunology and physiology

The important role of LEKTI in skin desquamation and in forming the permeability barrier has been shown in the case of Netherton syndrome (NS). This autosomal recessive genetic disorder is caused by mutation in *SPINK5* gene leading to reduction or loss of LEKTI (Chavanas *et al.*, 1999; Sprecher *et al.*, 2001). The infants with NS are characterized at macroscopic level by erythroderma, a specific defect in hair structure described as bamboo hairs. They also exhibit a wide range of allergic manifestations such as atopic dermatitis, elevated serum IgE and chronic inflammation (Chavanas *et al.*, 2000b; Comel, 1949; Netherton, 1958). Bacterial infections, robust weight loss, decreased mental development and heat intolerance are common findings (reviewed in Sun and Linden, 2006). The microscopic manifestations are defined by thin *stratum corneum* often detached from lower layers of epidermis, frequently absent *stratum granulosum* and premature fused lamellar bodies with atypical morphology (Fartasch *et al.*, 1999; Muller *et al.*, 2002).

Spink5 knockout mice (*Spink5*^{-/-}) reproduce features of NS and have been used for identification of key pathophysiological pathways of this disease (Descargues *et al.*, 2005; Hewett *et al.*, 2005; Yang *et al.*, 2004). The *Spink5*^{-/-} newborns died within a few hours after birth. Histological analysis revealed detached *stratum corneum* from *stratum granulosum*, hair malformation and a skin barrier defect. Also increased proteolytic activity (especially KLK5) was observed in the epidermis of *Spink5*^{-/-} animals (Descargues *et al.*, 2005; Hewett *et al.*, 2005). These observations lead to hypothesis that absence of LEKTI causes increased activity of kallikreins that are known to cleave desmosomal proteins already in *stratum granulosum* and are responsible for SG/SC detachment (Fig.5). Impaired barrier function induced by SG/SC detachment facilitates easier access of pathogens to human body (Descargues *et al.*, 2005).

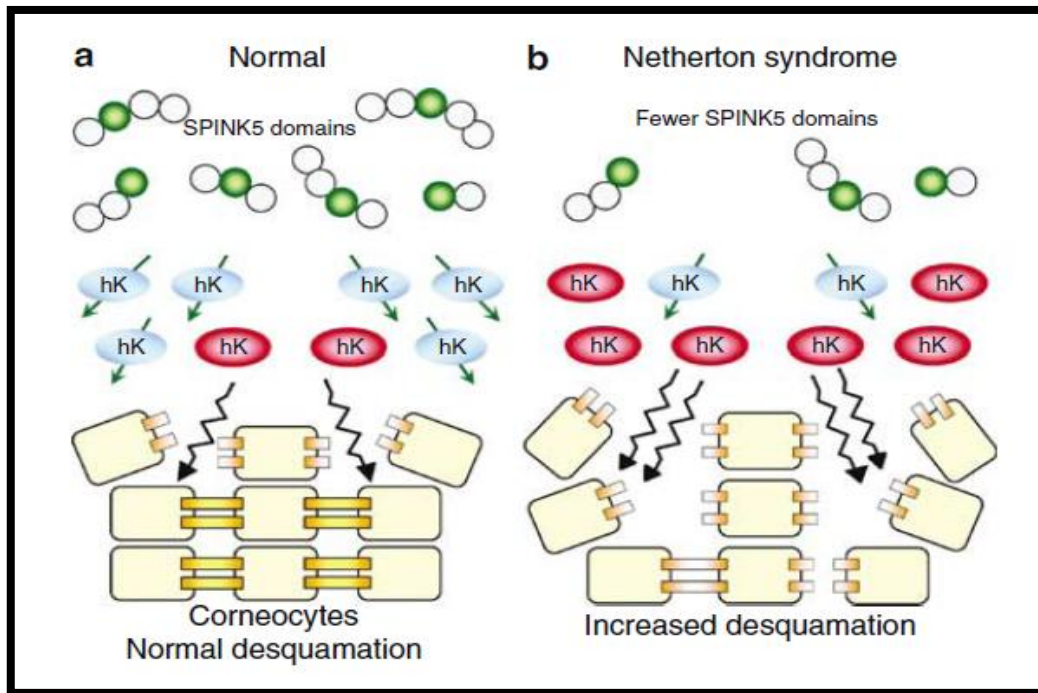


Figure 6. Schematic model of interaction between LEKTI and KLKs. (a) Under normal condition LEKTI domains prevent premature cleavage of corneodesmosomes. (b) In the case of Netherton syndrome depletion of LEKTI cause increased activity of kallikreins which triggers increased desquamation. (Komatsu *et al.*, 2006)

1.5 Summary

LEKTI is multidomain inhibitor with a wide range of possible inhibition targets. In epidermis these targets are KLK5, KLK7 and KLK14. All of these serine proteases belong to kallikreins proteolytic cascade, which is responsible for cleavage of desmosomal proteins, activation of immune responses through PAR2 – TSLP pathway and formation of epidermal barrier. Decrease of LEKTI in epidermis leads to enhanced KLKs activity and impaired epidermal barrier function with clinical manifestation described as Netherton syndrome. For better understanding of the role of LEKTI in the epidermis, *Spink5* knockout mice were generated (Descargues *et al.*, 2005; Yang *et al.*, 2004). This model facilitates description of processes causing pathophysiology of the Netherton syndrome.

LEKTI2 and SPINK6 are one domain inhibitors with probably slightly different range of inhibition targets and expression pattern from LEKTI. Therefore it is possible that LEKTI2 and SPINK6 modulate processes occurring in epidermis. Until now, roles of LEKTI2 and SPINK6 remained unchallenged in mouse models.

Creating of transgenic mice is useful approach to investigate physiological function of proteins *in vivo*. To avoid unspecific effects of transgene it is possible to target expression of protein into specific tissue using tissue specific promoter. Involucrin promoter has been investigated as a useful tool for specific targeting to epidermis. Recently mouse expressing Elastase 2 from involucrin promoter was described. This promoter specifically targets expression of gene into uppermost epidermal layers (Bonnart *et al.*, 2010). In presented diploma thesis the similar transgenic mice was used to investigate the role of murine SPINK6 in skin physiology.

2 Materials and methods

2.1 DNA cloning techniques

2.1.1 Polymerase chain reaction (PCR)

PCR per one reaction was prepared as follows:

Component	Volume
DNA template	5 ng
Pfu polymerase	0.4 μ l (1U)
10x Pfu buffer with MgSO ₄	5 μ l
dNTP's (2.5 mM)	4 μ l
Forward primer (100 μ M)	0.5 μ l
Reverse primer (100 μ M)	0.5 μ l
dH ₂ O	to 50 μ l
Total volume	50 μl

All reagents were obtained from Fermentas, Canada.

The oligonucleotide sequences are as follows:

mSpink6EcoRI-F	5'-TAAGAATTCCACCATGAAAGTAGCAGGTGTCTTT-3'
mSpink6FLAG-NotI-R	5'-TTAGCGGCCGCTCACTTGTCATCGTCGTCCTTGTAAATC GCATTTCCCACGATGCTTCAGGTTG-3'

The thermal profile of PCR:

Temperature	Duration
95 °C	3 min
95 °C	30s (25x)
55 °C	30s (25x)
72 °C	2 min (25x)
72 °C	5 min

2.1.2 Preparation of competent cells by calcium chloride method

E. Coli XL1 Blue MRF supercompetent cells (Stratagene, Cambridge) were used to prepare competent cells. A frozen stock of cells (50 μ l) was thawed on ice, transferred into 3 ml of LB media and placed in a shaking incubator (37 °C, 250 rpm) overnight

(16 hours). To the Erlenmeyer flask with 200 ml of LB media 2 ml of the culture was added and the bacteria were grown until $OD_{600} = 0.4$. Bacterial suspension was centrifuged at 5000 rpm, 4 °C for 10 minutes (valid also for all consequent centrifugations). The bacterial pellet was resuspended in 50 ml of 100 mM calcium chloride. Cells were incubated on ice for 5 minutes and then centrifuged. Afterward the pellet was resuspended in 10 ml of 100 mM calcium chloride and again centrifuged after 15 min incubation on ice. The pellet was resuspended in 4 ml of 100 mM calcium chloride (85% v/v) and glycerol (15% v/v), and the competent cells were immediately distributed into 50 µl aliquots and stored at -80 °C.

2.1.3 Transformation of bacterial cells

An aliquot of E.coli XL1 Blue competent cells was used for each transformation. The cells were thawed on ice for 5 minutes and then mixed with 1 µl of plasmid DNA (concentration ranging 0.1–0.5 µg/µl) or 5 µl of inactivated ligation mixture (65 °C, 10 min) following another incubation on ice for 20 minutes. The heat shock was performed at 42 °C for 1.5 min. An aliquot of 800 µl of LB media was immediately added to the transformation mixture and the cells were incubated for 1 hour at 37 °C. The mixture (200 µl aliquot) was placed on LB agar plate containing appropriate antibiotic resistance and the plates were incubated overnight at 37 °C.

2.1.4 Isolation of plasmid DNA

Single colony was picked from the LB agar plate using sterile pipette tip and inoculated in 3 ml of LB media supplemented with antibiotic in appropriate concentration. Tubes were incubated in the shaking incubator for 20 hours (37 °C, 250 rpm).

GeneJET Plasmid Miniprep Kit (Fermentas, Canada) was used for isolation of plasmid DNA from bacteria. 2 ml of bacterial culture ($OD_{600} = 0.4$) gain from single colony was centrifuged for 10 minutes (3000 g, 4°C). The isolation steps were processed according GeneJET Plasmid Miniprep Kit protocol (accessible on www.Fermentas.com). Plasmid DNA was eluted by 35 µl of Elution buffer. The flow through containing plasmid DNA was stored at -20 °C.

2.1.5 Restriction of DNA

Generally plasmid DNA (1–4 µg) was mixed with 0.5 µl (5 U) of restriction enzyme, 2 µl of corresponding 10x buffer and dH₂O to the final volume of 20 µl. The mixture was incubated at 37 °C for 2 hours.

2.1.6 Ligation of DNA fragments

DNA fragments of linear vector (100 ng) and insert (in amount corresponding to molar ratio 1:3) were mixed with 0.5 µl of T4 ligase (Fermentas, Canada), 1 µl of 10x T4 ligase buffers and dH₂O to the final volume of 10 µl. The mixture was incubated overnight at 16°C. 5 µl of the mixture was used for transformation of competent bacterial cells.

2.1.7 Agarose gel electrophoresis

Agarose powder was dissolved in 1x TAE buffer to the concentration of 0.01 g/ml was completely melted in microwave for 2–3 min. The mixture was left to cool down and then ethidium bromide (EtBr) was added in the final concentration of 0.5 µg/ml. In the case of isolation of DNA from gel the concentration of EtBr was 0.2 µg/ml. Solidified gel was inserted into electrophoresis tank filled with 1x TAE buffer. Running conditions of electrophoresis were set on 4,5 V/cm for 40 min. UV trans-illumination at wavelength of 312 nm was used for the visualization of DNA.

2.1.8 Isolation of DNA fragments from agarose gel

Band of proper size was excised by a sterile scalpel from gel that was placed under UV light. DNA from gel slice was isolated according steps in PCR kombi kit protocol (SeqLab, Germany, www.seqlab.com). DNA fragments were incubated in 30 µl of the Elution buffer for 10 min. The DNA sample was stored in -20 °C.

2.1.9 Preparation of DNA fragments for pronuclear microinjection

Plasmid DNA was digested using specific restriction enzymes. The digested plasmid was separated on 1% agarose gel and DNA fragment containing promoter and transgene was excised. DNA fragment was isolated from a gel slice using the PCR kombi kit (SeqLab, Germany) and eluted in 50 µl of elution buffer. Another purification step was required therefore the DNA fragment was purified by Illustra Nick Column (GE Healthcare, UK). The 1x microinjection buffer (400 µl) was used for elution and was captured in four fractions. The fraction with highest concentration of desired

DNA fragment was filtered through 0.22 micron pore membrane and used for pronuclear injection (PNI).

2.2 DNA analysis

2.2.1 Genomic DNA isolation

Tail biopsies (0.5–1 cm) from four-week-old mice were placed into a 2 ml tube containing 1ml lysis buffer and 30 μ l of Proteinase K (10 mg/ml). The tube was incubated until complete dissolving of tail at 55 °C (approximately 16 hours). 1 ml of 1:1 phenol/chloroform was added to the mixture and tube was repeatedly mixed by inverting up and down. For the separation of phases, the tube was centrifuged at 12 000 g for 2 min (valid for all consequent centrifugations). The upper phase containing DNA was transferred into fresh tube, mixed with 1 ml of chloroform and centrifuged. The chloroform was separated from the water phase that was transferred into new 2 ml tube. This upper aqueous phase was mixed with 1 ml of 96% ethanol and 100 μ l of sodium acetate (3 M, pH = 6) and the mixture was centrifuged. The supernatant was discarded, the pellet was washed by 1 ml of 75% ethanol and tube was centrifuged again. Afterwards the supernatant was carefully removed and tube with pellet was dried completely. The pellet was dissolved in 100 μ l dH₂O. The genomic DNA samples were stored at 4 °C.

2.2.2 Genotyping

The genomic DNA obtained from murine tails was diluted in dH₂O (1:30). This diluted DNA was used as a template for PCR reaction. As a positive control was used DNA fragment from microinjections or DNA from positive specimens. Reaction mixture per one reaction was prepared as below:

Components	Volume
DreamTaq polymerase (1 U/ μ l)	0.1 μ l
DreamTaq buffer 10x	1.5 μ l
dNTP (2.5 mM)	1.2 μ l
F primer (100 μ M)	0.08 μ l
R primer (100 μ M)	0.08 μ l
Genomic DNA	4 μ l
H ₂ O	8.04 μ l
Total volume	15 μl

The sequences of primers used for genotyping are described at table below as well as the thermal conditions of PCR reaction.

Primer's name	Sequence
Seq INV prom mid-F	5'-GGGGAATAGCATTCAACTTC-3'
F-genSPinINV	5'-CATGCCAGAAACACACAGC-3'
R-genSP6	5'-CCTTAAGACACCTTCCCTG-3'

Temperature	Duration
95 °C	3 min
95 °C	40s (34x)
60 °C	40s (34x)
72 °C	40s (34x)
72 °C	3 min

2.3 RNA analysis

2.3.1 RNA isolation

Skin sample of 50 mg was frozen in liquid nitrogen and homogenized using mortar and pestle. The crushed sample was resuspended in 1 ml of TRIzol Reagent (Invitrogen, USA) and then was incubated for 5 min in 30 °C. 200 µl of chloroform was added to the sample and tube was shaking by hand for 15 s. The mixture was incubated at 25 °C for 2 min and then centrifuged at 12 000g for 15 min, 4 °C. Aqueous phase containing RNA was transferred into new tube and mixed with 0.5 ml of isopropanol. The mixture was incubated for 10 min at 25 °C and then centrifuged for 10 min at 12 000g, 4 °C. The supernatant was removed and the pellet was washed by 1 ml of 75% ethanol. The sample was briefly mixed by vortexing and centrifugated for 5 min at 7500g, 4 °C. Afterwards the supernatant was discarded and the tube with pellet was place into fume hood to dry out. The sample was dissolved in 50 µl of RNase-free water and stored at -20 °C.

2.3.2 cDNA synthesis

First Strand cDNA Synthesis kit (Fermentas, Canada) was used to prepare cDNA from the isolated RNA. Per one sample 250 ng of RNA was mixed with 1 µl of oligo (dT)₁₈ primer and diluted by DEPC- treated water to the final volume of 10 µl. Mixture was incubated at 70 °C for 10 min, then spinned down and cooled on

ice for 2–3 min. The mixture of 4 µl of 5x Reaction buffer, 2 µl of 10 mM dNTP mix, 1 µl of RiboLock RNase Inhibitor (20 U/µl) was added to each sample. Afterwards the 2 µl of M-MuLV Reverse Transcriptase (20 U/µl) was added and the mixture was incubated at 37 °C for 60 min. The reaction was terminated by incubation at 70 °C for 5 minutes and cDNA was stored at -20 °C.

2.3.3 Quantitative real-time poly-chain reaction (qRT PCR)

The SYBR Green JumpStart *Taq* Ready Mix (Sigma-Aldrich, USA) was used for qRT-PCR reaction according to manufacturer's protocol and the result of reaction was detected by CFX96 real-time PCR detection system (Biorad, Canada). The cycling program used for the amplification reaction is following:

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

The amount of mSPINK6 mRNA was quantified with these sets of primers:

Primers for qRT-PCR	Sequence
mGAPDH-F	5'-GCAGTGGCAAAGTGGAGATT-3'
mGAPDH-R	5'-TTGGCTCCACCCTTCAAG T-3'
mSPINK6-F	5'-CAAGGAGGACAGATTAATTGT-3'
mSPINK6-R	5'-ATCAGAGCCGCAGAGTGG-3'

2.4 Protein analysis

2.4.1 Protein isolation from tissue biopsies

The ear biopsies were frozen in -80°C and crushed by mortar and pestle into small pieces. To the crushed sample 600 µl of TRIzol reagent were added and incubate 5 min at RT. The mixture was centrifuged for 10 min at 12 000 g, 4°C. The supernatant were transferred into fresh tube and 120 µl of chloroform was added, the sample was incubated for 3 min at RT and again centrifuged for 15 min at 12 000 g, 4 °C. After centrifugation the upper aqueous phase and interphase were removed. The DNA was precipitated by 180 µl of 100% ethanol. The samples were mixed by inversion, incubated for 3 min and centrifuged for 10 min at 2 000 g, 4 °C. The supernatant was

transferred into fresh tube and the proteins were precipitated by 900 μ l of isopropanol. The samples with isopropanol were stored for 10 min at RT and the proteins were centrifuged for 10 min at 12 000 g, 4 °C. The protein pellet was 3 times washed by 1.2 ml of 0.3 M guanidine hydrochloride in 95% ethanol and stored for 20 min at RT. The mixture was centrifuged at 7 500 g for 5 min, 4 °C after each washing step. After final washing step the pellet mixed by vortexing with 100% ethanol, incubated for 20 min, RT and then centrifugated at 7 500 g for 5 min, 4 °C. The pellet was drain for 10 min, RT and dissolved in 100 μ l of 1% SDS with 1x protease inhibitors (Sigma-Aldrich, USA).

2.4.2 BCA protein assay

BCA Protein Assay kit (Pierce, USA) was used to determine the protein concentration from cell/tissue lysates. The protein samples were 10 times diluted in dH₂O, standards and working reagent were prepared according to manufacturer's protocol. To the 25 μ l of the sample or standard was added 200 μ l of working reagent. Mixture was incubated at 37 °C for 30 minutes and then the absorbance of standards or samples was measured at 562 nm by Tecan Reader (Tecan group). The concentration of samples was estimated from the standard curve.

2.4.3 Preparation of protein samples for SDS-PAGE

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

2.4.4 Western blotting

The polyacrylamide gel (7–15%) of width 1.5 mm was used for separation of proteins. The gel was run at 100 V for 90 minutes. Polyacrylamide gel was washed in Transfer buffer for 15 minutes. PVDF membrane (0.45 μ m) was activated by incubation in methanol for 30 s, then washed in distilled water and incubated in Transfer buffer for 10 minutes. Mini Trans-Blot apparatus (BioRad, USA) was assembled according manufacturer's protocol. Blotting was performed at 0.35A for 1 hour. PVDF membrane with transferred proteins was blocked in 10% milk TBS-0.1% Tween20 for 1 hour at RT. Membrane was 3x washed by TBS-0.1% Tween20 between each step. Primary antibody was dissolved in appropriate concentration (Table below) in 2% milk TBS-0.1% Tween20 and incubated with membrane overnight

at 4 °C. PVDF membrane was incubated with secondary antibody (see list of antibodies below) dissolved in 2% milk TBS-0.1% Tween20 for 1 hour, RT. After washing the membrane was incubated with ECL plus Western blotting system kit to detected proteins recognized by antibodies. 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).

	Name of antibody (host animal)	Dilution
Primary antibodies		
	Flag (rabbit)	1 : 1000
	Dsg1 (rabbit)	1 : 6000
	mLekti3 (goat)	1 : 1.500
	Keratine 6 (rabbit)	1 : 6000
	Keratine 14 (rabbit)	1: 10 000
	KLK5 (rabbit)	1 : 1000
Secondary antibodies		
	Anti-goat HRP conjugated	1 : 10 000
	goat anti-rabbit HRP conjugated	1 : 10 000

2.5 Cell culture techniques

2.5.1 Cell culture

The Pam 212 cells were cultivated on tissue culture treated plates in RPMI culture medium with supplements (10% FBS, 1% Penicilin/Streptomycin). When the cells reached 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 until the cells detached from the plate (approximately 10 min.). 5 ml of RPMI medium was added to the cells, in order to neutralize the trypsin. Resuspended cells were transferred into a 15 ml falcon tube and centrifuged for 3 minutes at 1000 rpm. The supernatant was discarded and the cells were resuspended at 1 ml of culture medium. 100 µl of the suspension was added in a fresh culture plate containing 10 ml of medium. The cells were incubated at 37 °C, 5% CO₂.

2.5.2 Transfection of cultured cells

The Pam 212 cells were cultivated on 24-well-plate (50 000 cell per well) for 24 hours at 37 °C, 5% CO₂. The culture medium was discarded and fresh medium was added. The cells were transfected using Fugene HD (Roche, Switzerland). The transfection mixture consists of 1 µg of plasmid DNA (concentration 0.1-2 µg/µl), diluted in RPMI medium (no supplements) to the total volume of 22 µl and 3 µl of transfection reagent Fugen HD. Mixture was vortexed, incubated for 15 min at RT and added to the cell by dropwise manner. The transfected cells were incubated at 37 °C, 5% CO₂. The transfection efficiency was checked 24 and 48 hours after transfection.

2.5.3 Immunofluorescence staining of the cells

The Pam 212 transfected cells in 24-well-plate were rinsed with 1x PBS and were fixated in 0.8 ml of 3.7% paraformaldehyde/PBS for 10 min. The plate was washed 3x with PBS and the cells were permeabilized by 0.8 ml of ice cold methanol for 4 min. Then the cells were washed again with PBS three times. For blocking was used 1% BSA diluted in PBS and the plate was incubated for 45 minutes at 37 °C. Murine SPINK6 antibody was diluted 1:300 (kindly provided by Prof. Schröder, Kiel, Germany) in 0.5% BSA/PBS and 150 µl was added to the cells. The Pam212 were incubated with primary antibody for 1hour at RT and that the cells were washed with PBS, five times. Anti-goat IgG biotinylated was used as a secondary antibody in dilution 1:1000 and the cells were incubated for 30 min, RT. The cells were afterwards washed again with PBS, five times and the Extra avidin Cy3 conjugated (Sigma-Aldrich, USA) antibodies in dilution 1:700 were added to the cells for 30 min, RT. The cells were washed with PBS and visualized by fluorescent microscopy

2.6 Histological analysis techniques

2.6.1 Hematoxilin-eosin staining

The tissue was fixed in neutral buffered 10% formalin and embedded in paraffin. Paraffinized piece of tissue was cut by microtome (Leica, Germany) into sections of 4 µm. The sections were deparaffinized by washing twice with xylene for 20 min. Descending alcohol set (96–70% ethanol, 10 min each incubation) was used for rehydration. The sections were rinsed in distilled water for 5 min, stained by Harris hematoxillin (Sigma - Aldrich, USA) for 1min and differentiated by running tap

water for 3 min. Then the sections were counterstained by Eosin-Y for 30s and rinsed in tap water for 1 min. Dehydration was performed by ascending alcohol set and the sections were incubated with xylene for 10 min. DPX Mountant was used as a mounting reagent for histology (Sigma-Aldrich, USA).

2.6.2 Immunohistological staining of tissue samples

Skin samples were frozen in OCT Compound TissueTek (Sakura, USA) and sectioned by cryostat Leica CM 1950 (Leica, Germany). The 5 µm thick sections were washed by acetone for 5 minutes, RT and then the samples were washed by 1x PBS for 10 min. The sections were incubated with 5% BSA in PBS for 1hour at RT. Primary antibody was diluted in 1% BSA/PBS and incubated with the sections for 1 hour, RT. Then the samples were washed 3 times by 1% PBS. The sections were incubated with secondary antibody diluted in 1% BSA/PBS for 40 minutes, RT (for dilutions see Table below). Alternatively the sections were stained with Hoechst 33258 (Invitrogen, USA) for 10 minutes. Stained sections were visualized by fluorescent microscopy.

	Name of antibody (host animal)	Dilution
Primary antibodies		
	Dsg1 (rabbit)	1 : 600
	Filaggrin (rabbit)	1 : 700
	Keratine 6 (rabbit)	1 : 1000
	Keratine 14 (rabbit)	1 : 10 000
	KLK5 (rabbit)	1 : 800
Secondary antibodies		
	Anti-rabbit Alexa Fluor 488	1: 10 000

Skin samples embedded in paraffin were sectioned by microtome RM 2255 (Leica, Germany). The 4 µm thick sections were rehydrated according protocol described at HE staining and were treated with 0.01% proteinase K (20 mM Tris, 1% NaN₃, pH = 7.4) for 5min. The sections were washed with 1x PBS and blocked in 2% BSA/ PBS for 1 hour, RT. In next step the sections were washed again and incubated with mSPINK6 antibodies diluted 1:300 in PBS overnight at 4 °C. Next day the sections were washed, incubated with secondary anti-goat IgG biotin conjugated antibodies in dilution 1:10 000 (Santa Cruz, USA), again washed and incubated with streptavidin-horsereadish peroxidase conjugate (BD Bioscience, USA). The sections

were visualised by DAB+ substrate chromogen system kit (DAKO Cytomation, USA) and the reaction were stop after 1 min in tap water. The sections were dehydrated according protocol described in HE staining.

2.7 Mouse experiments

2.7.1 Model of irritant dermatitis

The model of irritant dermatitis is commonly used and was performed according to protocol is described at Swingle (Swingle *et al.*, 1981). Six mSPINK6 transgenic mice and six negative controls eight-week-old were used for experiment and two TG and two WT for western blotting. The dermatitis was induced by 0.8% or 0.25% croton oil dissolved at acetone. 50 μ l of croton oil solution was applied epicutaneously on both sides of one ear (25 μ l + 25 μ l). Ear thickness was measured with micrometer Toym IP25 (Mitutoyo, USA) before and 24, 48 and 72 after application. Differences in the thickness above the basal values were calculated for each mouse at individual time points.

To analyze the data from model of 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.

2.7.2 Barrier recovery model

The model of disruption of barrier recovery is commonly used and well described at many publications (Ghadially *et al.*, 1995). The basic transepidermal water loss (TEWL) was measured by special instrument Tewameter TM 300 (CK electronic, Germany). The water evaporation assessment is based on diffusion principle in an open chamber according equation:

$$\text{---} \qquad \qquad \qquad \text{---}$$

Where m = water transported (in g), t = time (h), D = diffusion constant (= 0.0877 g/m/h (mm Hg)), A = surface (m^2), p = vapor pressure of atmosphere (mm Hg), x = distance from skin surface to the point of measurement (m). Therefore the value of measured TEWL is formulated in g/h/m^2 . The experiment was arranged as follows: Six WT and six mSPINK6 transgenic mice at the age between 9–10 weeks

were used for this experiment. The mice were shaved and depilated by Opilca cream one day before assay induction. Firstly, the basic TEWL was measured, then the barrier was disrupted by tape-stripping to reach the TEWL value = 40 g/h/m² and was noticed as time-point 0. The TEWL values were measured at two and four hours after tape-stripping. All measurements and tape stripping were done under anesthesia (Zoletil mix-50, 2 µl/g). The ratio of barrier recovery was counted according equation:

The barrier recovery ratio in % indicates the velocity of the barrier renewal. All data was statistically analyzed by Mann Whitney test.

3 Aims of diploma thesis

The ability of human SPINK6 to inhibit several KLKs suggests its possible role in epidermal homeostasis; however, the exact mechanism of inhibition, interactions with other inhibitors and proteases, and also certain functional substitution of LEKTI remain unclear. To better understand the role of SPINK6 in epidermis a transgenic mouse model with targeted expression of SPINK6 in the epidermis should be studied. The first aim of this diploma thesis was generation of transgenic mice expressing murine SPINK6 specifically directed into upper epidermal layers. For this purpose the truncated human involucrin promoter (hINVm) modified by insertion of involucrin intron (int) between hINVm promoter and translational start was used. This construct was used to generate a transgenic mouse expressing reporter gene (TdTomato) in epidermis to confirm *in vivo* that involucrin promoter specifically targets the protein expression into uppermost layers of epidermis (Kasperek, 2010 : diploma work). The second part of this work has aimed to characterize the transgenic mSPINK6 mice using histological, proteomic, molecular, and experimental tools.

4 Results

4.1 Generation of transgenic construct

Mouse cDNA encoding SPINK6 was obtained from Prof. Schröder (Kiel, Germany) and was amplified by PCR using Pfu polymerase to add the Flag-tag to the 3' end of the cDNA as well as restriction sites for EcoRI and NotI at each end. C-terminally fused Flag-tag allows immunodetection of mSPINK6. The fragment of a 380 bp was obtained and purified by PCR kombi kit. To gain variable multiple cloning site, the mSPINK6 construct was introduced into pBluescript II SK+ using EcoRI, NotI restriction sites. The sequencing revealed that the mSPINK6 cDNA lacks the third exon and therefore the mSPINK6 cDNA had to be synthesized. The synthetic cDNA was then purchased from Genscript (USA). mSPINK6 cDNA sequence fused with Flag-tag was delivered in pUC57 and had to be subcloned. Modified mSPINK6 sequence was excised by restriction enzyme EcoRI, NotI and, thus, two fragments were obtained: a 2710 bp fragment (pUC57) and a 380 bp fragment containing mSPINK6 cDNA. This fragment was cloned into pBluescript II SK+ using EcoRI, NotI restriction sites. Obtained clones were confirmed by restriction analysis (Fig.7)

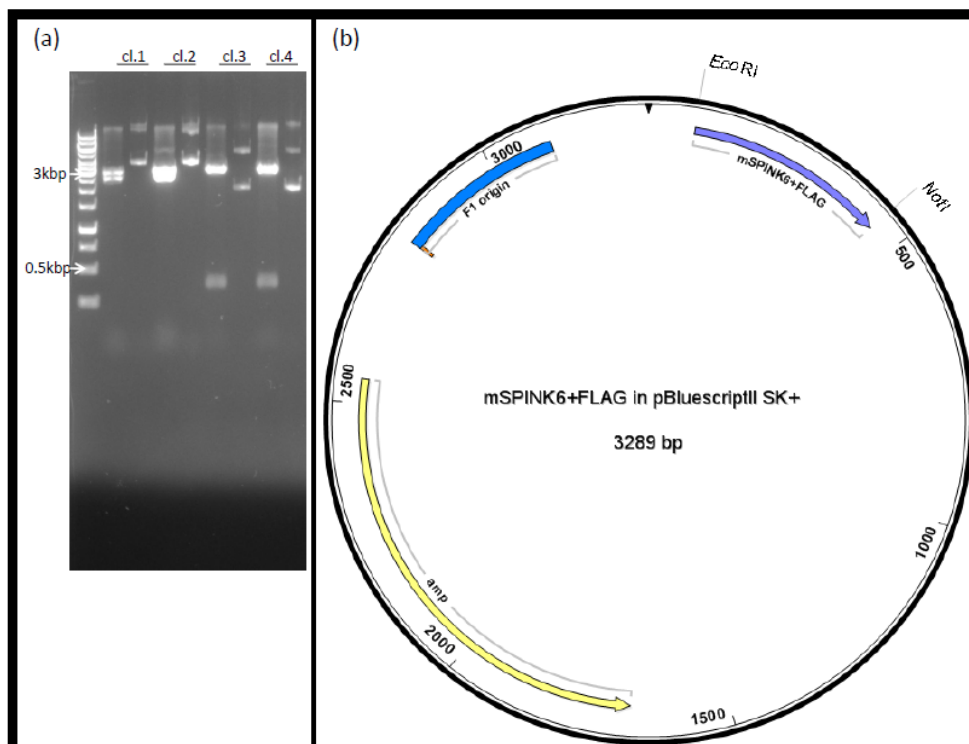


Figure 7. Cloning of mSPINK6-Flag into pBluescript II SK. (a) Restriction analysis of pBluescript II SK(+)-mSPINK6-Flag. 4 clones and control pBluescript II SK+ were digested by EcoRI and NotI. All four clones were positive and contained band of 500 bp. (b) pBluescript II SK(+)-mSPINK6-Flag vector map with restriction sites used in cloning. DNA star software was

In order to prepare pBroad-hINVm-int-mSPINK6-Flag the pBroad-hINVm-int-tdTomato was used as a vector source. pBroad-hINVm-int-tdTomato was cleaved by BamHI and XbaI to replace tdTomato gene by mouse SPINK6-Flag from pBluescript II SK+. The final construct consists of a modified human involucrin promoter (hINVm) with intron (int) following mSPINK6-Flag and beta-globin polyA. Clones obtained from ligation of mSPINK6-Flag with pBroad-hINVm-int were analyzed by restriction with EcoRI and NotI restriction enzymes (Fig. 8).

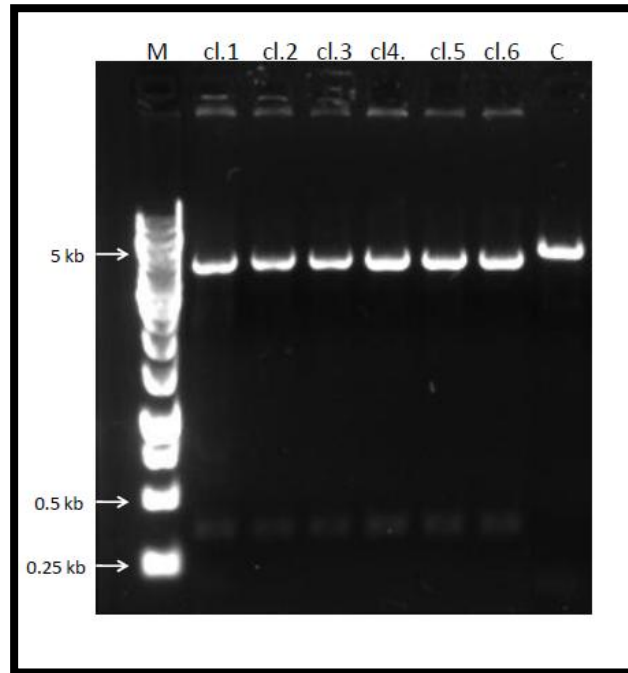


Figure 8. Restriction analysis of pBroad-hINVm-int-mSPINK6-Flag. Clones 1–6 (cl.1–cl.6) were cleaved by EcoRI and NotI resulting in two DNA fragments of size 380 bp (containing mSPINK6-Flag) and 4300 bp (the rest of vector). pBroad-hINVm-int- tdTomato was used as a negative control.

According to restriction analysis six positive clones were obtained. The clone no.1 was further analyzed by sequencing to confirm the correct preparation of pBroad-hINVm-int-mSPINK6-Flag (Fig. 9).

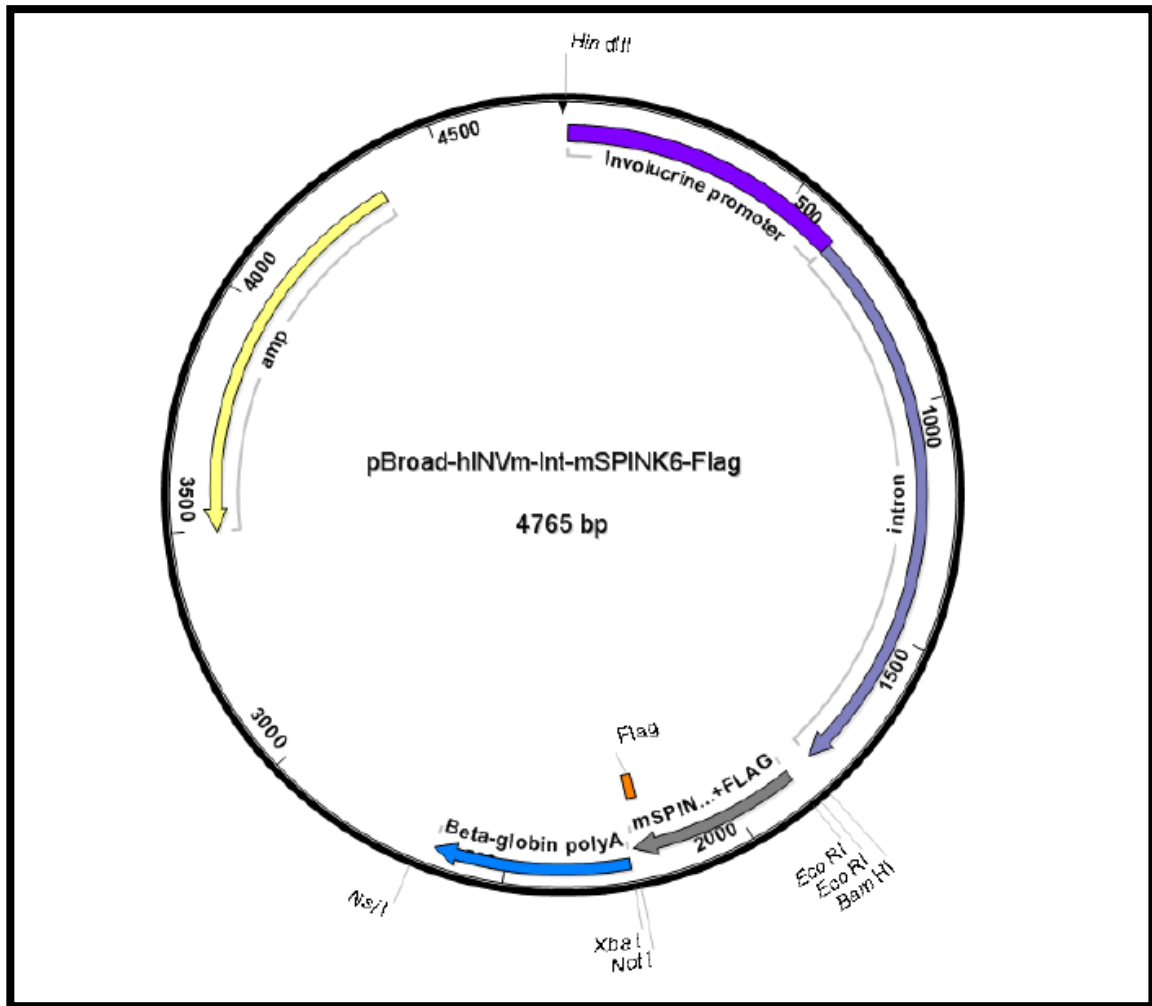


Figure 9. pBroad-hINVm-int-mSPINK6-Flag vector map. The scheme shows truncated human involucrin promoter including intron sequence with mouse mSPINK6 cDNA fused with Flag-tag and beta-globin polyA. The restriction sites used for generating desired vector and for transgene preparation are shown. The vector map was prepared in DNA star software.

4.2 Preparation of mSPINK6-Flag fragment for PNI

pBroad-hINVm-int-mSPINK6-Flag fragment was excised with HindIII and NsiI from pBroad-hINVm-int-mSPINK6-Flag vector and was purified by Nick Column (Fig. 10). The construct was then injected into oocytes pronuclei (pronuclear injection, PNI) in transgenic core facilities of *MPI-CBG* (Dresden) and *IMG* (Prague) to speed up the generation of transgenic mice. Injected oocytes were then implanted in pseudopregnant C57BL/6 females.

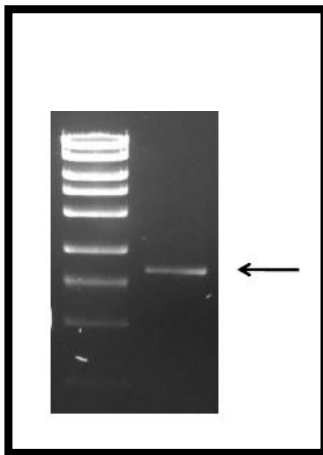


Figure 10. pBroad-hINVm-int-mSPINK6-Flag fragment used for PNI. The 2640 bp long DNA fragment marked by arrow was excised by HindIII and NsiI from pBroad-hINVm-int-mSPINK6-Flag. The concentration of DNA was 10 ng/ μ l.

4.3 Genotyping of mSPINK6 transgenic mice

Altogether, five founders were generated by PNI. These founders were further crossed with C57BL/6 animals and the offspring was genotyped using genomic DNA isolated from tail biopsies of three-week-old animals. To distinguish wild type animals (WT) from transgenic mice (TG) two sets of specific primers were designed. The forward primers (INV prom mid-F, F-genSPinINV) are specific for modified human involucrin promoter and the reverse primers are designed to recognize the end of mSPINK6 gen (R-genSP6) (Fig.11). All founders showed positive signals for the above PCR reactions and the transgenes were also transferred to their offspring. Thus, five transgenic lines were established.

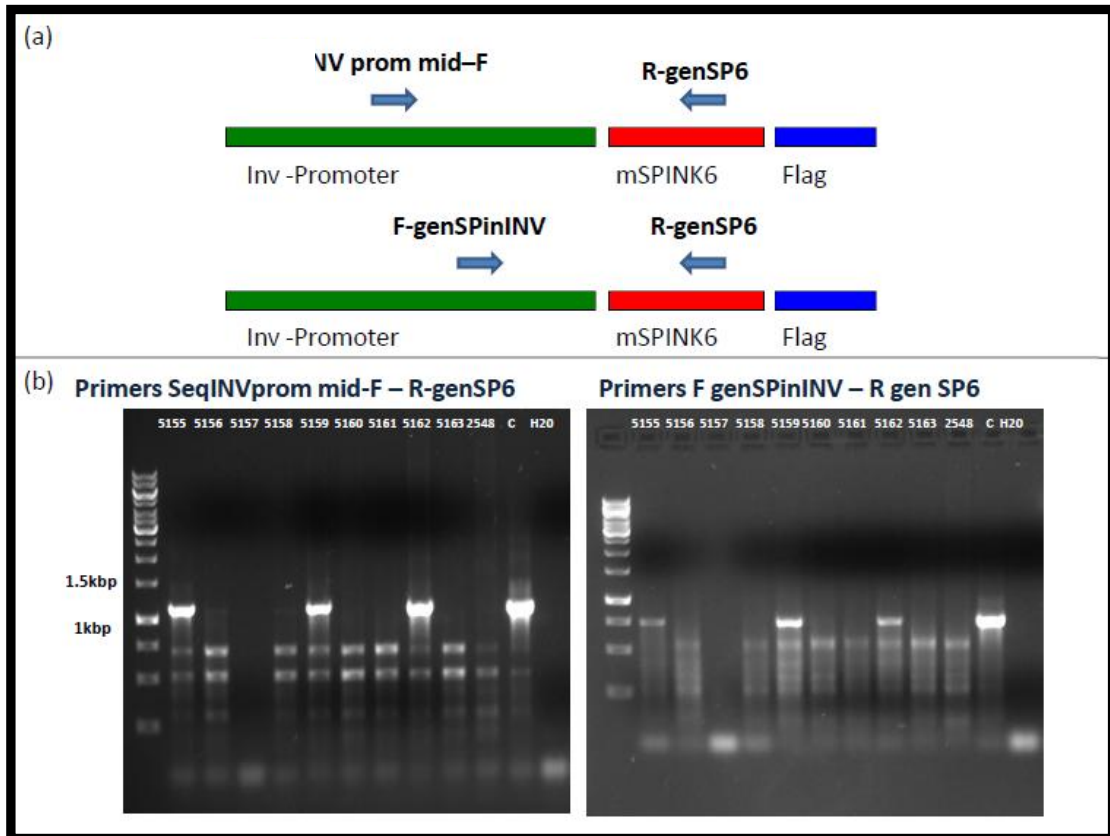


Figure 11. Genotyping of pBroad-hINVm-int-mSPINK6-Flag mice. (a) Schematic representation of transgene with the location of transgene specific primers used for PCR. (b) INV prom mid-F and R-genSP6 primers produce the 1200 bp PCR product in positive mice, F-genSPinINV and R-genSP6 amplify the 750 bp specific fragment. As a positive control (marked as c) was used genomic DNA from positive mice previously confirmed by genotyping, the water control is shown.

4.4 Characterization of mSPINK6 transgenic mice

4.4.1 The mSPINK6 expression level among the five transgenic lines

All founders and their transgenic progeny were viable. The transgenic lines were further analyzed regarding the expression of the transgene at the mRNA and protein levels to select lines with high expression of mSPINK6 for further experiments

To examine the mRNA level of mSPINK6 in the transgenic lines the mRNA was isolated from ears biopsies of twelve 6-week-old animals (2–3 mice per each line). The selection involved the littermates without transgene, which were taken as a control. The expression level was examined by qRT-PCR using mSPINK6 specific primers and revealed that the transgenic line with highest expression is derived from founder 4998 (470–480 fold expression in mRNA level). Also the transgenic line Inv-mSPINK6⁵⁰⁰³ exhibited increased level of mSPINK6 mRNA (30 fold upregulation).

The other lines (Inv-mSPINK6⁵⁰⁰⁰, Inv-mSPINK6⁵⁰⁰¹, and Inv-mSPINK6⁵⁰⁰⁴) showed similar levels of mSPINK6 expression comparable to negative animals (Fig.12).

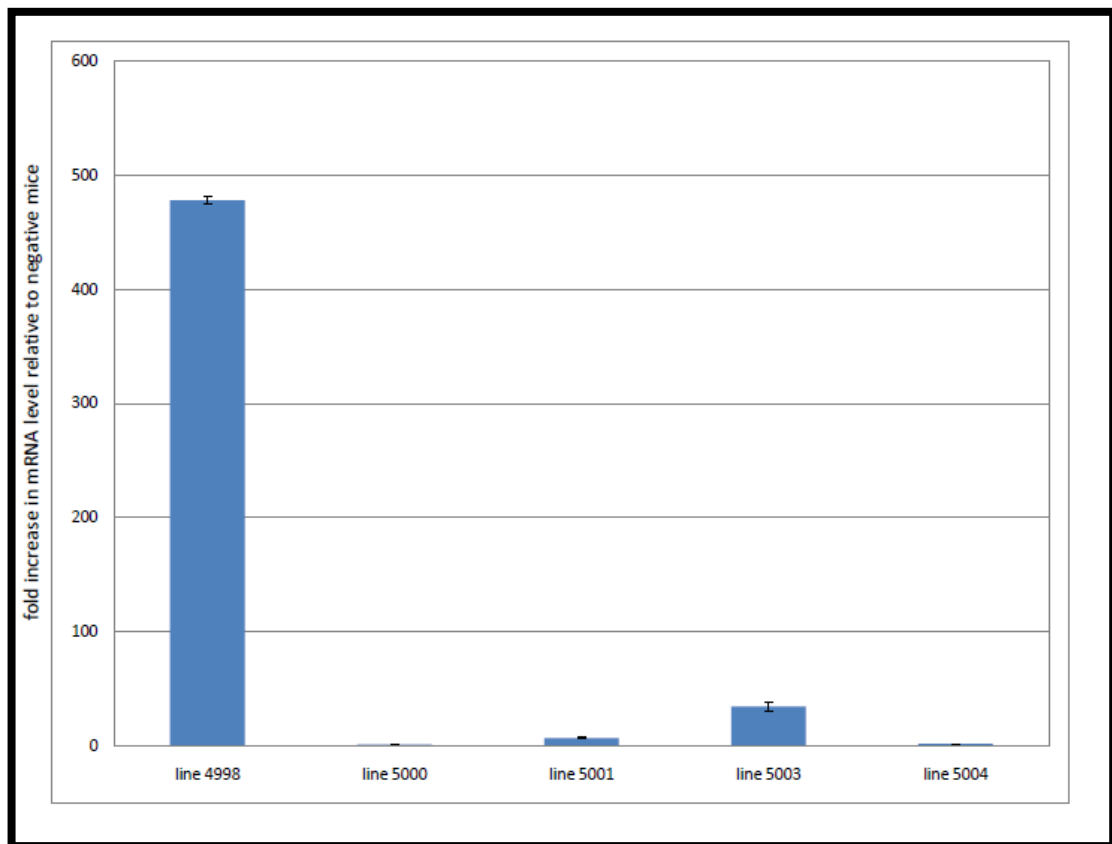


Figure 12. mRNA expression analysis of mSPINK6 transgenic lines. The amount of mRNA is expressed as fold increase in mRNA level relative to negative mice. The transgenic line with highest mRNA expression (470–480 fold) is Inv-mSPINK6⁴⁹⁹⁸ line, the second one is line 5003 (30 fold upregulation). The mRNA expression in other lines is not notably changed from negative animals. At least two animals from each line were taken for analysis. Error bars means standard deviation.

Transgenic line Inv-mSPINK6⁴⁹⁹⁸ was selected for further experiments but also Inv-mSPINK6⁵⁰⁰³ line was bred in the order to serve as a source for control, negative animals.

To investigate protein expression in transgenic mice the mSPINK6 antibodies generated in the laboratory of Prof. Schröder (Kiel, Germany) were used. The specificity of antibody was demonstrated by immunofluorescence experiment on PAM 212 cells. The cells were transfected with pTracer vector including mSPINK6 and the original pTracer vector was used as a mock control. Enhanced green fluorescent protein (EGFP) under independent CMV promoter is expressed from pTracer and therefore it was possible to distinguish transfected from non-transfected cells. This experiment proved that mSPINK6 is expressed in transfected cells and that the antibody is able to detect mSPINK6 protein (Fig.13).

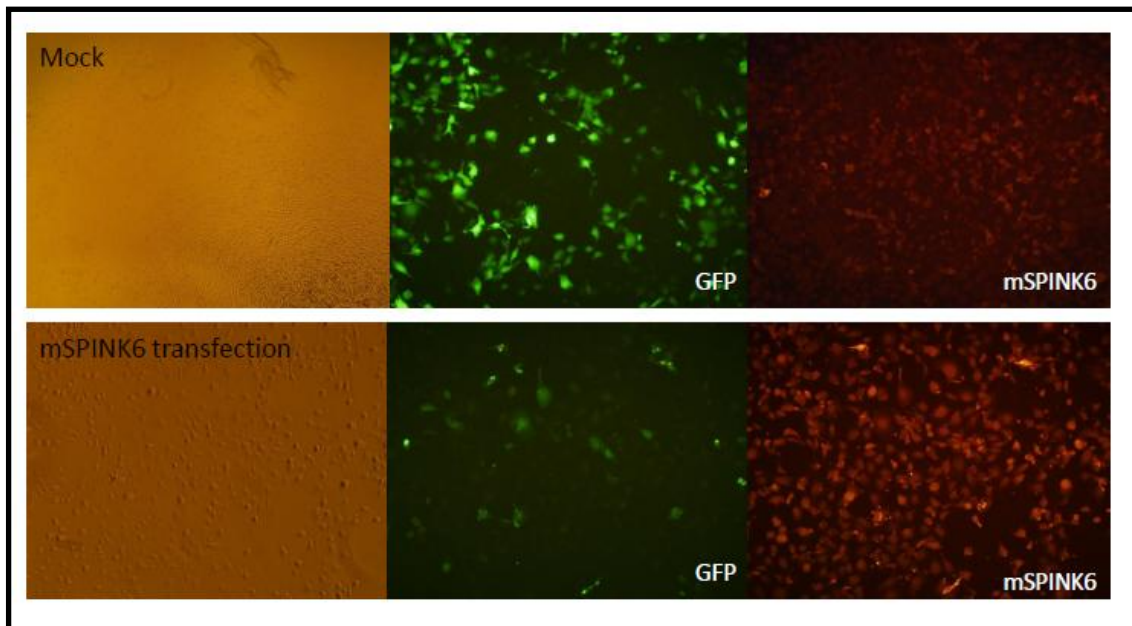


Figure 13. The specificity of mSPINK6 antibody - immunofluorescence. PAM 212 cells were transfected by pTracer (mock) and pTracer-mSPINK6 vector. Transfected cells that produce GFP (*green fluorescence*) were stained for mSPINK6 (*red fluorescence*). 200x magnification.

The protein lysates derived from skin of WT and transgenic animals and from primary WT and transgenic keratinocytes were tested for specificity of the mSPINK6 antibody using immunoblotting; however, no mSPINK6 protein of 11.8 kDa could be detected. Together four WT and four TG mice were used. The antibody generated against Flag-tag was also used in immunoblotting and immunofluorescence analysis. As in the case of mSPINK6 antibodies the Flag-tag antibodies could not detect mSPINK6 in tissues or cells derived from transgenic animals although the positive control (Tace Flag-tag) was detected (Fig.14).

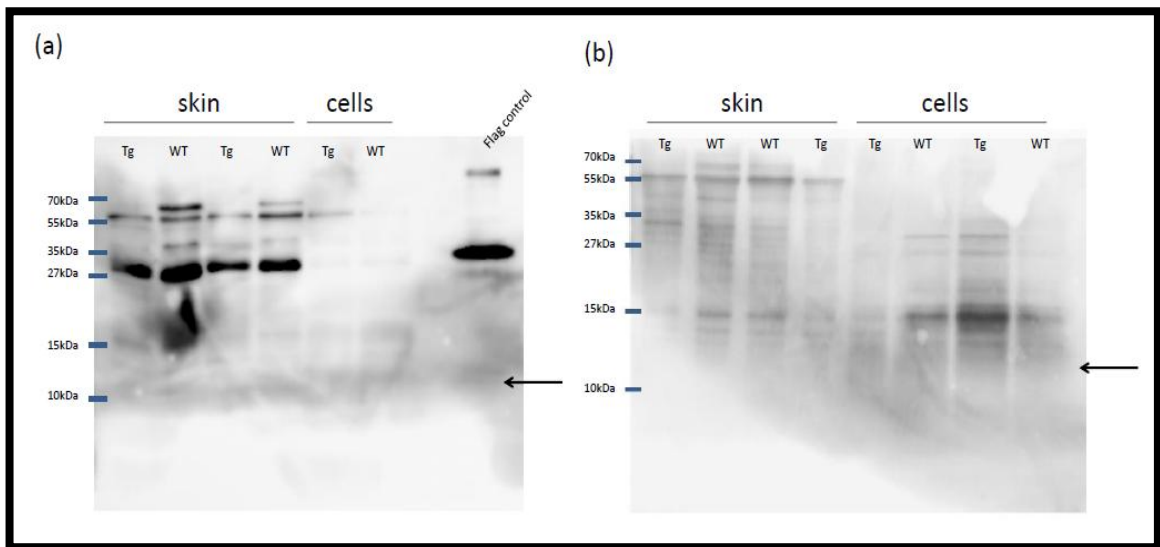


Figure 14. The detection of mSPINK6 by immunoblotting. Protein lysates for expression analysis were prepared from skin of two WT and two mSPINK6 transgenic animals. The primary keratinocytes were also isolated from two negative and two mSPINK6 transgenic animals. Tace fused with Flag tag of 130kDa was used as a control. The predicted mSPINK6 molecular weight is 11,6kDa, marked by arrow. (a)The blot membrane was incubated with Flag-tag antibodies. (b) The blot membrane was incubated with mSPINK6 antibody (a-mSPINK6). None of used antibodies were able to detect mSPINK6.

Therefore mSPINK6 antibodies were further used in immunohistochemistry analysis to characterize the mSPINK6 expression in transgenic mice (Fig.15). The dorsal skin of three WT and three mSPINK6 transgenic animals was stained for the immunoreactivity and mSpink6 was detected in *stratum spinosum/stratum granulosum* and also upper layers of *stratum corneum* as expected from the targeting specificity of the Inv-promoter (Kasperek, 2010 : diploma thesis). The staining also showed that mSPINK6 protein is elevated in transgenic mice. More mice have to be investigated to obtain really solid result.

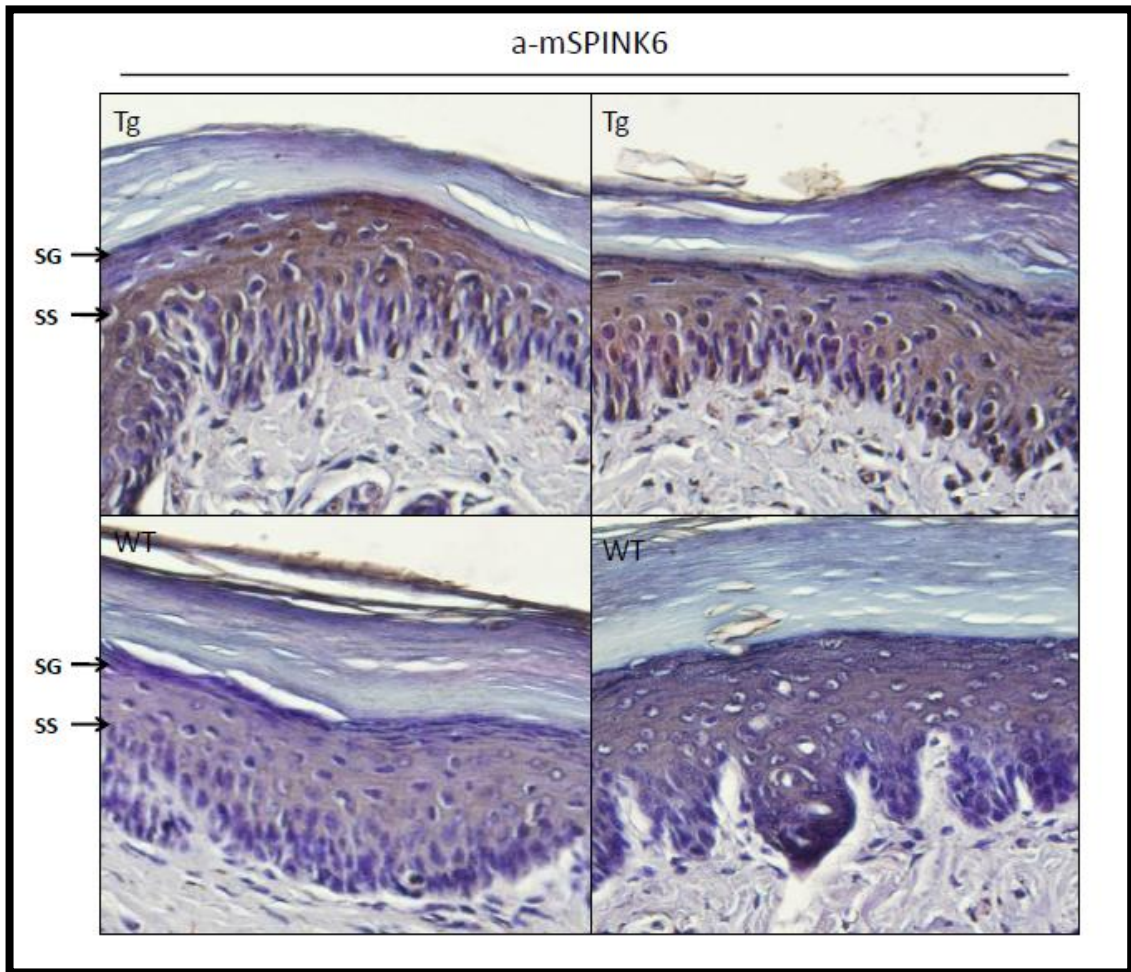


Figure 15. Expression of mSPINK6 in epidermis of transgenic and wild type animals. Skin sections from footpad of WT and TG animals were analyzed by immunohistochemistry. mSPINK6 was mainly detected in *stratum spinosum/stratum granulosum* and in upper layer of *stratum corneum* (marked by arrow). Immunohistochemistry showed differences in staining intensity among mSPINK6 transgenic and negative mice. 400x magnification.

4.4.2 Phenotype analysis of mSPINK6 transgenic mice

The construct with involucrin promoter was proved to specifically target transgene into upper layers of epidermis. The possible phenotype, caused by higher expression of mSPINK6, could be then visible at the macroscopic level. Therefore, TG mice were observed daily from newborns until the age of 50 weeks. However, transgenic animals did not show any gross phenotype and were indistinguishable from WT animals (Fig. 16).



Figure 16. Macroscopic examination of mSPINK6 transgenic mice. (a) 1 day old pups of positive line derived from founder 4998. (b) Pups 5 days old and (c) two weeks old mice of transgenic line Inv-mSPINK6⁴⁹⁹⁸.

Despite no evident macroscopic phenotype was observed, the skin of twenty-week-old animals was further analyzed using hematoxylin and eosin (HE) staining and immunofluorescence staining for markers of proliferation or differentiation. The paraffin embedded sections of skin back, ear, and footpad obtained from six mSPINK6 transgenic and six WT specimens were stained by HE. The analysis revealed that all epidermal layers were properly developed and the *stratum corneum* was attached to the *stratum granulosum*. Hairs exhibited normal development. The architecture of epidermal and dermal layers was similar among WT and TG animals (Fig. 17). Altogether, the HE staining revealed that the epidermis of mSPINK6 transgenic mice is intact under normal physiologic conditions.

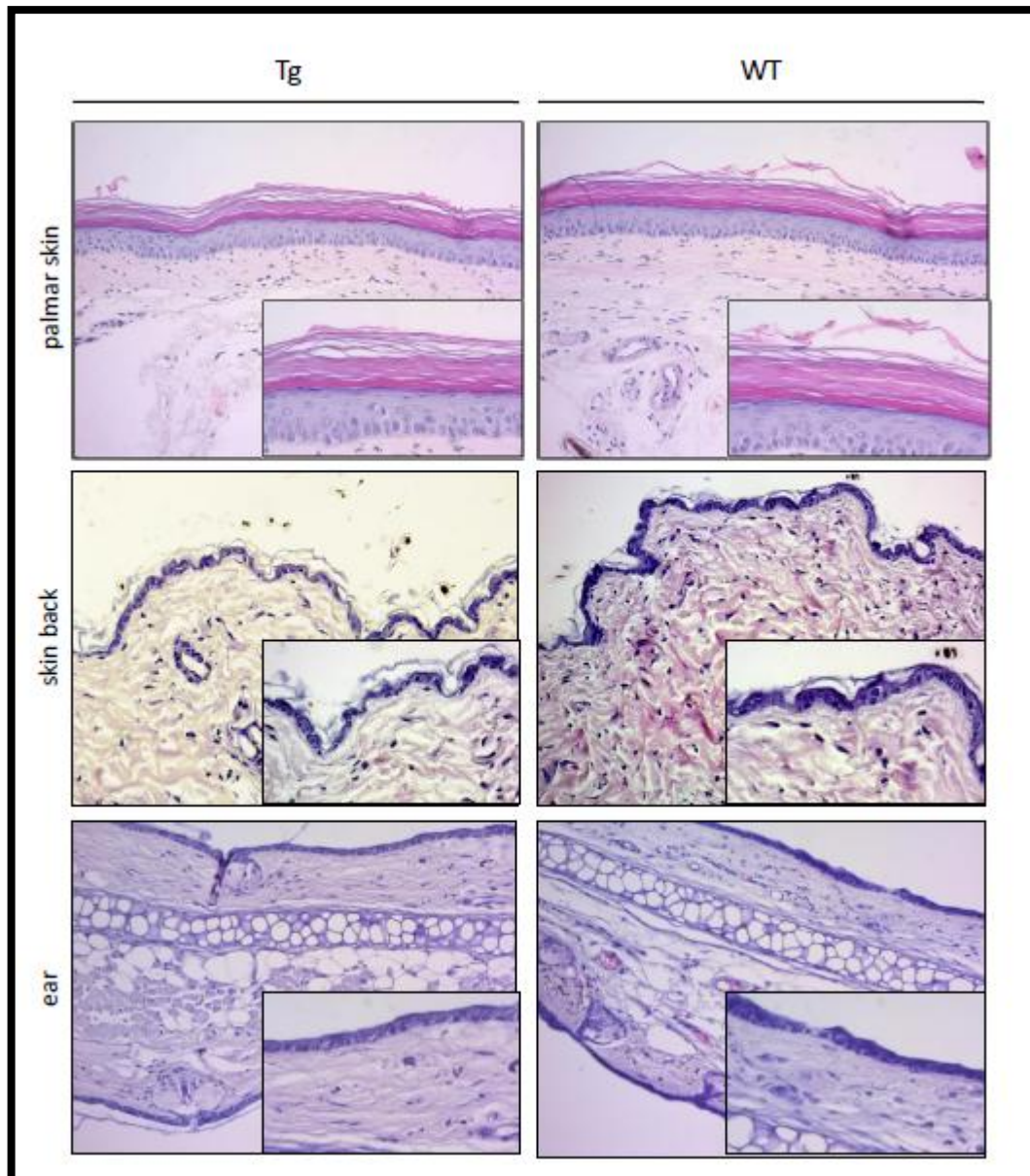


Figure 17. HE staining of WT and -mSPINK6 transgenic mice. Paraffin section of skin back, footpad skin, and ear was stained by HE. The large pictures, 200x magnifications, higher magnifications (400x) of each panel are enclosed.

To investigate proliferation or differentiation in the transgenic mice, keratin 6, keratin 14, filaggrin, and desmoglein1 were chosen for immunofluorescence analysis. Two isoforms of keratin 6 are present in mouse and are expressed in proliferating keratinocytes, for instance, in the hair follicle, tongue, and in the footpad skin (Rothnagel *et al.*, 1999). Keratin 6 is upregulated under pathological and hyperproliferative conditions including psoriasis and wound healing (Reviewed in Chu and Weiss, 2002). Keratin 14 is major product of basal keratinocytes and therefore serves as a marker of keratinocytes in *stratum basale*. The filaggrin is necessary for

cornified cell envelope formation and desmoglein 1 (Dsg1) is part of desmosomes. The expression of murine Klk5, as a potential inhibition target, was also investigated to examine whether the desquamation process was changed. Skin in ears, skin back, and footpad skin of three wild type and three transgenic mice were analyzed using cryosection. The expression level and localization of keratin 14, filaggrin, Dsg1, and Klk5 were not changed (Fig. 18 and Fig 19), which revealed together with the histological analysis that epidermal barrier of mSPINK6 transgenic mice was not altered or disrupted under normal conditions. On the other side the expression of keratin 6 was slightly elevated in transgenic mice (Fig. 18, Fig. 19). This could indicate an altered proliferation in transgenic mice, which might play a certain role under stress conditions.

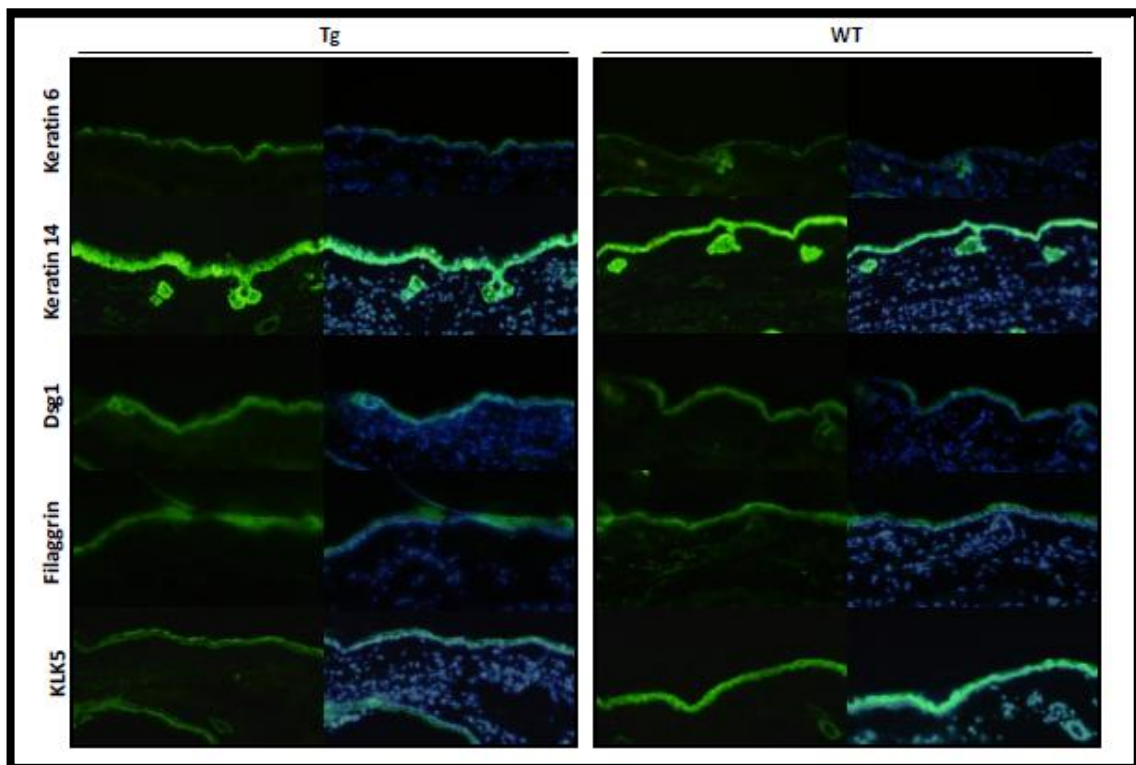


Figure 18. Investigation of epidermal markers in transgenic animals. The cryosections: of ear were stained for keratin 6, keratin14, Dsg1, Filaggrin, Klk5. 200x magnification.

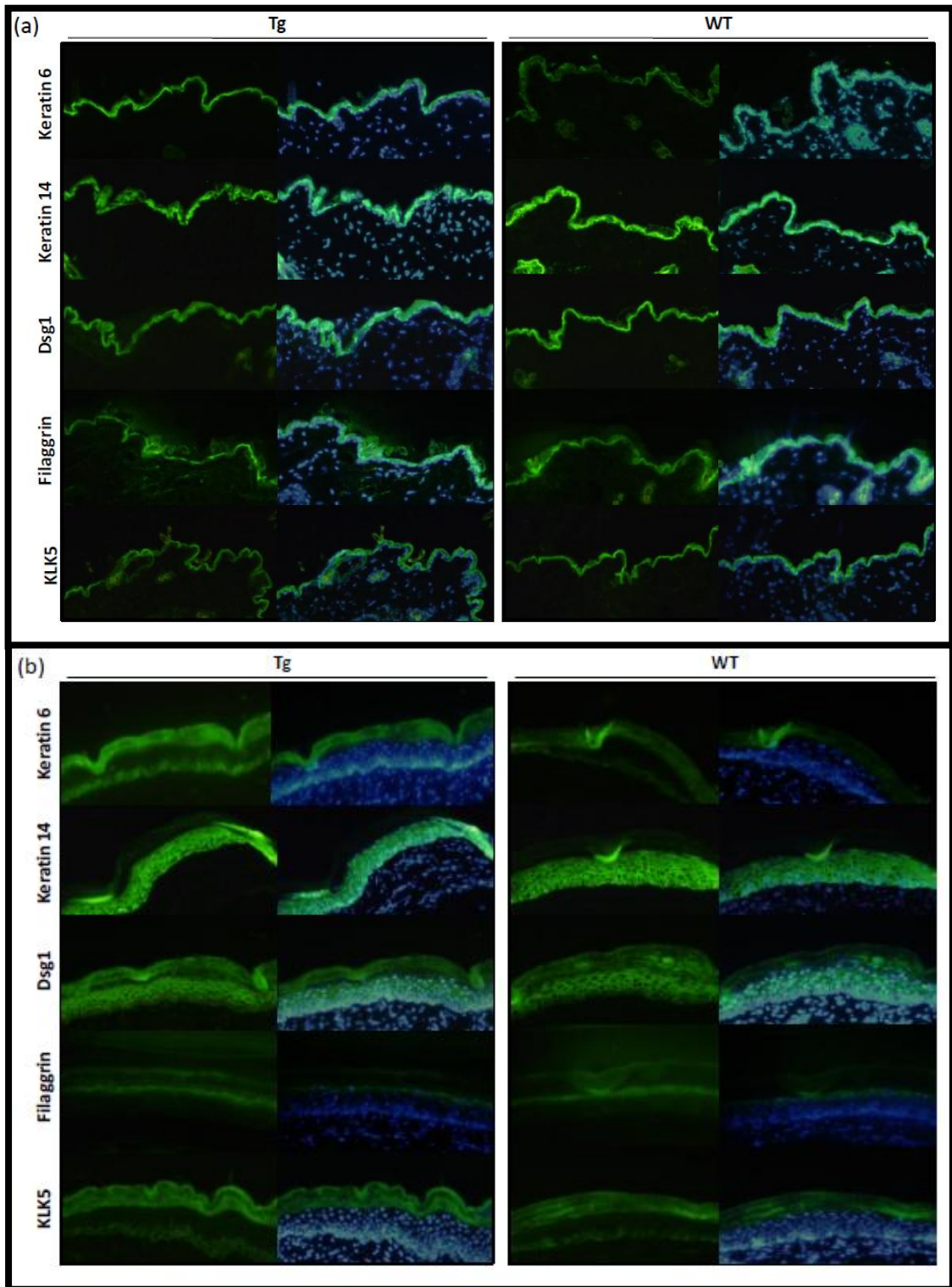


Figure 19. Investigation of epidermal markers in transgenic animals. The cryosections: of skin back (a) and footpad (b) were stained for keratin 6, keratin14, Dsg1, Filaggrin, Klk5. 200x magnification.

4.4.3 Investigation of skin homeostasis in transgenic mice

The results from experiments under normal, non-challenging conditions showed that epidermal barrier function of mSPINK6 transgenic animals is not altered except for changes in the expression of keratin 6. This might point to possibly disturbed keratinocytes turnover or protective function of epidermal barrier.

Nevertheless, the exposure to challenging condition, such as infliction of wound or disruption of the epidermal barrier could reveal some specific phenotype of transgenic animals. Therefore, additional assays were carried out. In the first attempt the upper stratified layers were removed by tape stripping and the transepidermal water loss (TEWL) was measured. Second, to trigger inflammatory response and hyperkeratosis the croton oil was used to induce acute irritant dermatitis.

4.4.3.1 The barrier recovery model

Elevated water loss is one of the factors indicating disruption of epidermal barrier, therefore the transepidermal water loss (TEWL) is important parameter to investigate. The water changes were measured by specialized instrument, Tewameter on six transgenic and seven WT animals in two independent experiments. The basic TEWL value was measured at time point 0 thus before assay induction. The values for basic TEWL was ranging between 2–7 g/h/m² and also the values measured after two and four hours were not uniformed. The calculated ratio for all WT and TG animals showed that the barrier recovery is slightly improved in mSPINK6 transgenic mice although the difference was not statistically significant (Fig. 20).

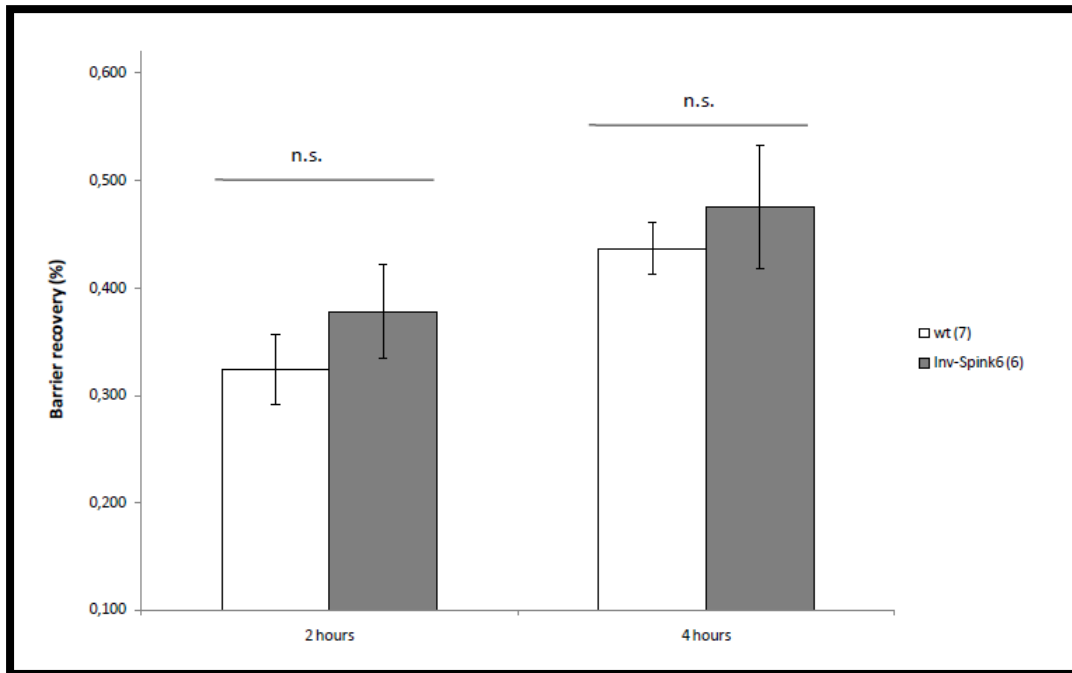


Figure 20. Barrier recovery model. Six mSPINK6 transgenic mice and seven WT mice were analyzed by TEWL assay before and 2 and 4 hours after tape-stripping, when the epidermal barrier was disrupted. The barrier recovery in % was calculated as a ratio of TEWL measured at 2 and 4 h after to the TEWL value reached by tape-tripping. The error bars indicate standard mean error. The differences between cohort of WT and transgenic animals were not significant according to Mann-Whitney test.

4.4.3.2 The irritant dermatitis model

The skin homeostasis can be easily impaired by inflammation and consequent hyperproliferation. Acute irritant contact dermatitis induced by croton oil can serve as a suitable model to introduce these conditions. The local inflammatory reaction triggers upregulation of proteolytic activity that could be decreased by high expression of an inhibitor, for instance by mSPINK6. The kinetics of inflammation is measured by ear thickness. The croton oil in two different concentrations was applied on the ear of six TG and six WT mice. The ear thickness was measured before and 24, 48, and 72 hours after croton oil application. No significant differences between WT and mSPINK6 transgenic animals were observed (Fig.21).

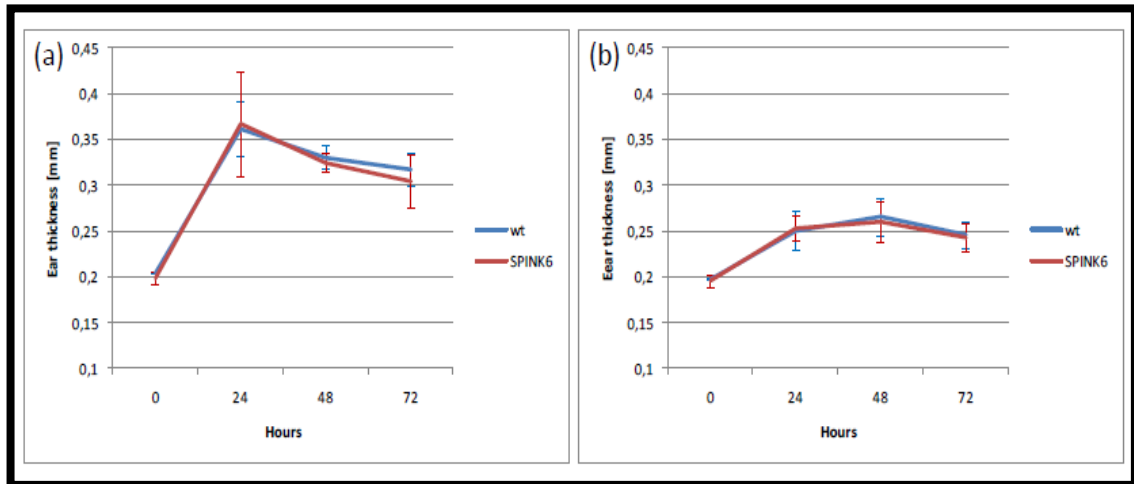


Figure 21. Acute Irritant dermatitis model. The six WT and six Tg animals were treated with 0.8 % (a) or 0.27 % (b) croton oil. The ear thickness was measured before treatment and 24h, 48h, and 72h after croton oil application. The errors bars indicate standard deviation.

Ear biopsies were taken before and 24 hour after from croton oil application. The protein lysates from the biopsies were analyzed using Western blotting for keratin 6, keratin 14, Klk5, and Dsg1 markers (Fig. 22). The Western blot analysis from untreated ears revealed that keratin 6 expression is slightly elevated in mSPINK6 mice (Fig. 21) After croton oil treatment, the level of keratin 6 was elevated but no differences in expression were observed among WT and TG animals. In the case of keratin 14, Klk5 and Dsg1 no differences before and after treatment were distinguished. The result for keratin 6 displays possible direction in future research and therefore the other mice cohort have to be investigated to confirm this finding.

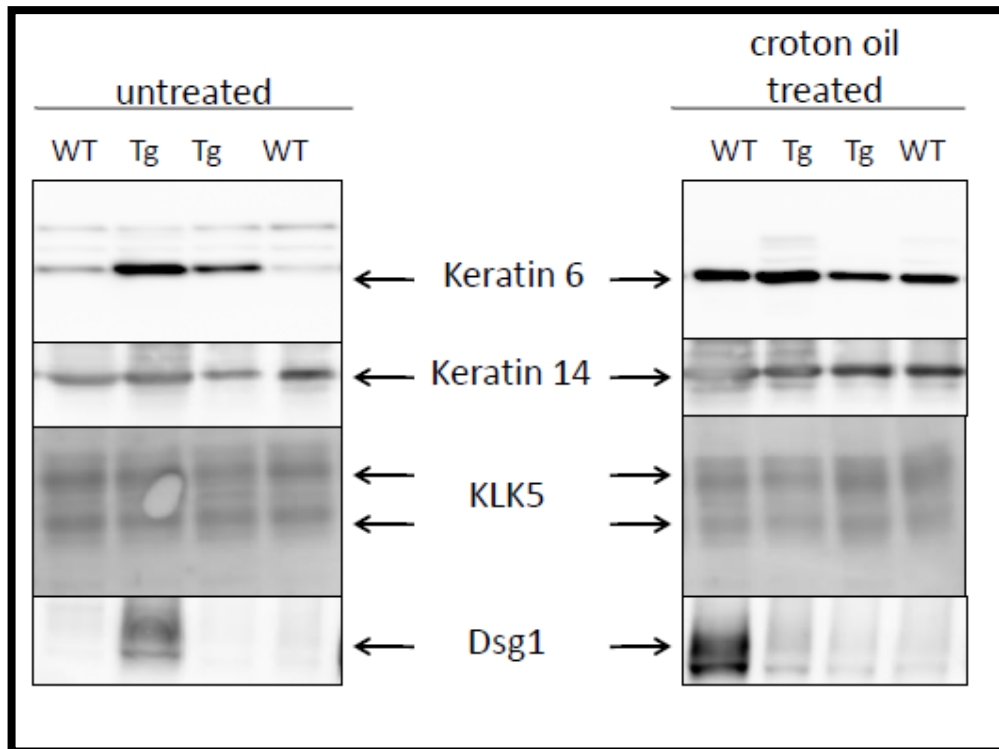


Figure 22. Induced irritant dermatitis model. Ear biopsies were taken before treatment with croton oil and 24 hours after treatment. The expression of keratin 6, keratin 14, Klk5 and Dsg1 was analyzed using Western blotting of a lysate prepared from mouse ear. The specific fragment of molecular weight 56kDa was detected by keratin 6 antibodies (marked by arrow). Also fragments corresponding 34 kDa of pro-mKlk5 and 28,5 kDa of active mKlk5 were identified (marked by arrow). Keratin 14t of molecular weight 56kDa and fragment of 114kDa of Dsg1 was detected.

5 Discussion

Recently published papers about SPINK6 in human shed new light on regulation of skin homeostasis. It was suggested that not only LEKTI is important player in skin desquamation but also LEKTI2 and SPINK6 has similar inhibitory potential. Therefore we decided to investigate the function of mouse SPINK6 *in vivo*. The expression of SPINK6 in human was described in *stratum corneum* and *stratum granulosum* of palmar skin. Thus, for generating transgenic mouse, we had to find a promoter with higher expression rate whose activity is not restricted to one specific skin area. On the other side chosen promoter had to be specific enough to direct expression only to upper epidermal layers. Human involucrin (INV) was originally described by Rice and Green (Rice and Green, 1979). Involucrin is expressed in *stratum spinosum* and *stratum granulosum* and is one of the essential proteins of cornified cell envelope that serves as a an initial scaffold component for other proteins (Steinert and Marekov, 1997). It was showed that truncated promoter containing only distal regulatory region (DRR) and proximal regulatory region (PRR) exhibits the same expression specificity as the native human involucrin promoter (Ghazizadeh *et al.*, 2002). This sequence was further modified by insertion of involucrin intron (int) between hINVm promoter and translational start and was used in our laboratory to generate a transgenic mouse expressing reporter gene (TdTomato) in the epidermis. This hINVm-TdTomato mouse confirmed *in vivo* that involucrin promoter specifically targets the protein expression into upper layers of epidermis. Strong fluorescence was also found in ears, the weak fluorescence signal was observed in tongue and bladder of positives mice. No fluorescence was detected in other organs Based on these results mSPINK6 transgenic mice were prepared with confirmed expression specifically targeted to the *stratum spinosum/stratums granulosum*.

The fragment including hINVm with intron, mSPINK6, and globin polyA was successfully prepared and injected to pronucleus. We obtained five transgenic lines. The transgenic animals were analyzed on mRNA level by qRT-PCR. The analysis revealed two transgenic lines with higher mRNA level of mSPINK6: Inv-mSPINK6⁴⁹⁹⁸ and Inv-mSPINK6⁵⁰⁰³ line. The line derived from 4998 founder with 470–480 fold mSPINK6 mRNA expression was used for further experiments and analyses.

On the protein level, the mSPINK6 was detected with mSPINK6 antibodies by immunohistochemistry *in vivo* and by immunofluorescence *in vitro*. Immunohistological analysis confirms higher expression level of mSPINK6 protein in transgenic mice in *stratum spinosum* and *stratum granulosum*. However, the protein was not detected with the same antibodies on Western blots and therefore mSPINK6 expression could not be quantified. Also, the Flag-tag antibody did not recognize the Flag-tagged mSPINK6 protein. The possible explanation is that in this case one copy of the Flag-tag is not sufficient for protein detection (Hernan *et al.*, 2000). Thus, the high mRNA expression of the transgene appeared not to correlate with the protein levels or the polyclonal mSPINK6 antibodies prepared against the selected epitope of mSPINK6 were not able to detect the form on the blotting membrane. It is also possible that large part of epidermal proteins was cross-linked and during preparation of the lysate removed by centrifugation. This could also explain why the mSPINK6 antibodies detected the protein expression on the paraffin and cryo-sections, where the natural folding, structure and localization was preserved.

All mice born in two selected lines were viable, did not show any gross malformations, and during mice adolescence and adulthood exhibited no obvious morphological changes in the epidermis. Histological analysis revealed that morphologic organization of transgenic mice is indistinguishable from WT animals. All epidermal layers were connected and no differences in exfoliation of upper cornified layers were observed. Also the structure of the hair sheath and hair itself were normally developed. These results altogether indicate that desquamation of mSPINK6-transgenic mice is not changed and that the enhanced expression of mSPINK6 does not significantly deregulate the equilibrium among epidermal inhibitors and proteases. However, small changes in epidermal homeostasis or inhibitors/proteases equilibrium would not be detectable by histological analysis.

To describe properly the situation in epidermis of the transgenic mice, the markers of differentiation and proliferation were investigated by immunofluorescence. The markers playing important role in epidermal permeability barrier formation, i.e. filaggrin, Dsg1, and Klk5, were tested. Filaggrin is synthesized as a large phosphorylated precursor by keratinocytes of granular layer. During formation of cornified cell envelope, the profilaggrin is cleaved by serine proteases or matriptases and then the filaggrin fragments bind to keratin filaments to form complexes that reinforce cornified cell envelope. The altered processing of filaggrin caused by mutation

in filaggrin gene or by enhanced proteolytic activity leads to common disorder of keratinization, ichthyosis vulgaris (type IV) or/and eczema (Reviewed in O'Regan *et al.*, 2008). Moreover, increased profilaggrin processing was also described in *Spink5*^{-/-} mice suggesting its important role in permeability barrier function (Descargues *et al.*, 2005; Hewett *et al.*, 2005). The murine model with deficient *Spink5* showed that Klk5 without specific inhibition by *Spink5* is hyperactivated. Higher proteolytic activity of Klk5 results in earlier degradation of Dsg 1 (Descargues *et al.*, 2005). Therefore it is possible that higher expression of Klk5 potential inhibitor, i.e. mSPINK6, could result in reduced proteolytic activity and, thus, in reduced degradation of desmosomal components. However, based on immunofluorescence and also on immunoblotting analysis of ears from irritant dermatitis model, no changes in expression or distribution of Klk5, Dsg1 or filaggrin were observed. These results suggest that Klk5 may not be the main inhibition target of mSPINK6. Another possible explanation is that, the murine orthologues of either SPINK6 or KLK5 exerts distinct functions from those known for the human one. As the filaggrin processing was not investigated by western blot analysis, it is possible that mSPINK6 specifically inhibits other proteases such as caspase 14.

Keratin 14 and keratin 6 are additional markers of proliferation which were investigated. Keratin 14 is constitutively produced by keratinocytes of epidermal *stratum basale* and therefore serves as a good marker of basal layer or lower epidermal layers under deregulated epidermal homeostasis (Reviewed in Chu and Weiss, 2002; Coulombe *et al.*, 1989). The keratin 6 is constitutively expressed in footpad skin and in hair shaft (Rothnagel *et al.*, 1999) and its expression is induced under pathological and hyperproliferative conditions such as wound healing, psoriasis, and by proliferation inducing agents such as retinoic acid (Mansbridge and Knapp, 1987; Schweizer *et al.*, 1987; Weiss *et al.*, 1984). Keratin 14 expression was not changed based on immunofluorescence experiment. However, immunofluorescence and western blot analysis of untreated ears from irritant dermatitis model showed slightly elevated expression of keratin 6. Therefore it is possible that murine SPINK6 plays some, so far unspecified, role in proliferation of keratinocytes or could influence proteolytic processes essential for wound healing or other conditions of barrier disruption. Also inhibition of transglutaminases results in higher expression of keratin 6 (Harrison *et al.*, 2007) and since transglutaminases deficiency was described in Netherton syndrome (Raghunath *et al.*, 2004), the mSPINK6 might be involved in transglutaminase processing.

On the other hand, changes in transglutaminase activity could result in the alternation in epidermal permeability barrier that should be revealed by shift in barrier recovery ratio. The differences between WT and mSPINK6 transgenic animals observed in barrier recovery model were very mild although there was an obvious tendency of improved barrier recovery in transgenic animals. Also other markers that were not investigated in this thesis could be altered.

To reveal the role of mSPINK6 under conditions disregulating the epidermal barrier, mice were challenged using models of irritant dermatitis and disruption of permeability barrier. These models are often used to disclose a role of a protein studied under challenging condition. However, these models do not address specifically the upper epidermal layers and, thus, these conditions could be suboptimal for proteins as mSPINK6, that should exert its function in the uppermost layers.

The model of induced dermatitis did not show any differences between the animal groups. It is possible that dermatitis induced by croton oil is not sufficient to reveal any phenotype and therefore usage a different chemical irritant such as sodium lauryl sulfate (SLS) could activate different responses (Willis *et al.*, 1989, 1990). There is also possibility that LEKTI with its 15 Kazal-type inhibitor domains and multiple combinations of domain fragments plays major role in skin homeostasis and thus, SPINK6 can fulfill only specific role or has to interact with other member of SPINK family or even with other inhibitors. It is also possible that SPINK6 could exhibit important impact in organs, in which the SPINK5 is not that abundant.

In summary, transgenic mice expressing mSPINK6 under the truncated human involucrin promoter that targeted its expression into upper layers of epidermis was generated. Although the transgenic mice exhibited vast increase of mRNA level of mSPINK6 the upregulation at the protein level was mild. Thus, the transgenic mice did exhibit obvious phenotype neither under normal unchallenged conditions nor in the models of simple barrier disruption or irritant dermatitis. Nevertheless, the barrier disruption showed clear tendency for improved barrier recovery in the transgenic mSPINK6 mice. Only obvious change in comparison to control mice was elevated expression of keratin 6, which might be important in some types of epidermal diseases. Although the initial investigation have not revealed any gross effects of mSPINK6 its function should be studied; for instance in combination with other SPINK inhibitors or in additional specific models of epidermal disregulation. Despite mild phenotype, the

mSPINK6 transgenic mouse is useful model to study inhibition mechanism *in vivo*, especially when used in combination with protease overexpressing or deficient models.

6 Summary

Serine proteases inhibitors Kazal-type play important role in epidermal processes. To support *in vitro* results and studies on human we aimed to generate the mouse model overexpressing mSPINK6 in the uppermost epidermal layer. Modified human involucrin promoter was used as a tissue specific promoter with confirmed expression in *stratum spinosum* and *stratum granulosum*.

Transgenic mice with elevated mRNA and protein level of mSPINK6 have been successfully generated for first time. The mice were viable, however no obvious phenotype induced by mSPINK6 overexpression was observed. Further investigations of mSPINK6 transgenic mice revealed mild changes in expression of keratin 6 and slightly but not significantly enhanced barrier recovery.

The analysis of mSPINK6 transgenic mice showed that mSPINK6 probably modulates the role of LEKTI in skin homeostasis. In summary the mSPINK6 transgenic mice could be useful tool in combination with deletion or transgenic mouse model

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8 Appendix

Antibodies

Primary antibodies

Anti-Dsg1 rabbit polyclonal	<i>Santa cruz, USA</i>
anti-Cytokeratin 6 rabbit polyclonal	<i>Abcam, UK</i>
anti-Cytokeratin 14 rabbit polyclonal	<i>Abcam, UK</i>
anti-Filaggrin rabbit polyclonal	<i>Abcam, UK</i>
anti-FLAG monoclonal	<i>Sigma-Aldrich, USA</i>
anti-mKlk5 rabbit-Ab SN	provided by M. Brattsand
anti-mSPINK6 goat-Ab	provided by Prof. Schröder

Secondary antibodies

Extravidin Cy3 conjugated	<i>Sigma-Aldrich, USA</i>
IgG Alexa fluor 488–goat anti-rabbit	<i>Molecular probes, USA</i>
IgG Biotin conjugated– sheep anti-goat	<i>Santa Cruz, USA</i>
IgG HRP conjugated – goat anti-rabbit	<i>Pierce, USA</i>

Bacterial strains

Escherichia Coli XL-1 Blue MRF^c: $\Delta(\text{mrcA})183 \Delta(\text{mrcCB-hsdSMR-mrr})173 \text{ endA1 supE44 thi-1recA1 gyrA96 relA1 lac}[F^c \text{ proAB lacI}^q\text{Z}\Delta\text{M15 Tn10(Tet}^r\text{)}]$, *Stratagene, USA*

Chemicals

Acetic acid glacial, 100%	<i>Roth, Germany</i>
Acrylamid Rotiphorese Gel 30	<i>Roth, Germany</i>
Agarose Seakem LE	<i>Cambrex, USA</i>
Ammonium persulphate (APS)	<i>Sigma-Aldrich, USA</i>
Ampicillin	<i>Biotika, Slovakia</i>
Bovine serum albumin (BSA)	<i>Sigma-Aldrich, USA</i>
Complete protease inhibitor EDTA free	<i>Roche, Switzerland</i>
Dithiotreitol (DTT)	<i>Sigma-Aldrich, USA</i>

EDTA	<i>Roth, Karlsruhe, Germany</i>
dNTP mix (10mM each)	<i>Fermentas, Canada</i>
Ethanol	<i>Penta, Czech republic</i>
Ethidium bromide	<i>Sigma-Aldrich, USA</i>
Fetal Bovine serum	<i>PAA Laboratories, Austria</i>
Formalin	<i>Walter CMP, Germany</i>
FUGENE HD	<i>Roche, Switzerland</i>
Glycerol	<i>Penta, Czech republic</i>
Hydrochloric acid	<i>Roth, Germany</i>
Hoechst 33258	<i>Invitrogen, USA</i>
Chlorophofm	<i>Lachema, Czech republic</i>
Isopropanol	<i>Lachema, Czech republic</i>
Liquid nitrogen	<i>IMG media facility, Czech republic</i>
Magnesium chloride	<i>Fermentas, Canada</i>
2-Mercaptoethanol	<i>Merk, Germany</i>
Methanol	<i>Penta, Czech republic</i>
Milk powder	<i>Roth, Germany</i>
Nonidetp40	<i>Sigma-Aldrich,USA</i>
Penicillin/Streptomycin	<i>PAA Laboratories, Austria</i>
Phenol	<i>Roth, Germany</i>
Potassium acetate	<i>Roth, Germany</i>
Sodium acetate	<i>Sigma-Aldrich, USA</i>
Sodium dodecyl sulfate (SDS pellets)	<i>Sigma-Aldrich, USA</i>
Sodium chloride	<i>Lachema, Czech Republic</i>
Sodium hydroxide	<i>Lachema, Czech Republic</i>
Straptavidine-Horsereadish peroxidase conjugate	<i>BD Bioscience, USA</i>
TissueTek OCT Compound	<i>Sakura, USA</i>
Tris	<i>Roth, Germany</i>
Tris hydrochloride	<i>Roth, Germany</i>
Triton X-100	<i>Sigma-Aldrich, USA</i>
TRIzol	<i>Invitrogen, USA</i>
Trypan Blue	<i>Gibco, Invitrogen, USA</i>
Trypsin/EDTA	<i>PAA Laboratories, Austria</i>
Tween-20	<i>Riedel-de Haën, Germany</i>

Commercial kits

BCA protein Assay kit	<i>Pierce, USA</i>
DAB+ Substrate chromogen system kit	<i>DAKO Cytomation, USA</i>
ECL Plus Western Blot detection reagents	<i>GE Healthcare, UK</i>
GeneJET Plasmid Miniprep Kit	<i>Fermentas, Canada</i>
Illustra Nick columns, Sephadex G-50	<i>GE Healthcare, UK</i>
PCR-Combi-Kit	<i>Seqlab, Germany</i>
First strand cDNA	<i>Fermentas, Canada</i>

Enzymes

BamHI	<i>Fermentas, Canada</i>
BglII	<i>Fermentas, Canada</i>
DNaseI (RNase-free)	<i>Fermentas, Canada</i>
Dream Taq DNA polymerase	<i>Fermentas, Canada</i>
EcoRI	<i>Fermentas, Canada</i>
HindIII	<i>Fermentas, Canada</i>
NotI	<i>Fermentas, Canada</i>
NsiI	<i>Fermentas, Canada</i>
Proteinase K	<i>Sigma-Aldrich, USA</i>
Pfu polymerase	<i>Fermentas, Canada</i>
T4 DNA ligase	<i>Fermentas, Canada</i>
XbaI	<i>Fermentas, Canada</i>

Instruments and equipment

AccuBlock digital dry bath	<i>Labnet, USA</i>
Balance Kern 440-35N	<i>Kern, Germany</i>
Blot paper Protean xi and XL size	<i>Bio-Rad, Canada</i>
Centrifuge 5415 R	<i>Eppendorf, Germany</i>
Centrifuge 5810 R	<i>Eppendorf, Germany</i>
Electroforetic chamber Wide-Sub Cell GT	<i>Bio-Rad, Canada</i>
Electroforetic chambre Mini-Sub Cell GT	<i>Bio-Rad, Canada</i>
EnVision® Multilabel Reader 2104	<i>PerkinElmer, USA</i>
Flowbox Bio-II-A Biological Safety Cabinets	<i>Telstar, Spain</i>

Freezer and fridge Comfort	<i>Liebherr, Austria</i>
Freezer and fridge Profi line	<i>Liebherr, Austria</i>
Fridge A class	<i>Whirlpool, USA</i>
Freezer (-80°C)	<i>Sanyo, Japan</i>
Gel imager Universal Hood II	<i>Bio-Rad, Canada</i>
Hotplate and Stirrer Jenway 1000	<i>Jenway, UK</i>
Incubator Forma DH CO2 Hepa Class 100	<i>Thermo Fisher Scientific, USA</i>
Incubator NB-205	<i>N-biotek, Korea</i>
Leica CM-1950	<i>Leica, Germany</i>
Luminiscent image analyser LAS – 3000	<i>Fujifilm Life Science, USA</i>
Microscope DMI 4000B inverted	<i>Leica, Germany</i>
Microscope IX51, inverted	<i>Olympus, USA</i>
Microscope TIRF	<i>Leica, Germany</i>
Mixing Block MB-102	<i>BioER Technology, China</i>
Nanodrop 1000	<i>Thermo Fisher Scientific, USA</i>
Orbit 100 shaker	<i>Labnet, USA</i>
pH meter W3B	<i>BEL Engineering, Italy</i>
Pipette set BioPette	<i>Labnet, USA</i>
Stuart roller mixer SRT6	<i>Barloworld Scientific, UK</i>
Syringe 2 ml	<i>Braun, Germany</i>
Syringe Omifix-F 1ml	<i>Braun, Germany</i>
Tecan Reader Infinite M200	<i>Tecan group, Switzerland</i>
Tewameter TM300	<i>CK electronic, Germany</i>
Trans-Blot SD	<i>Bio-Rad, Canada</i>
Tissue culture plastic	<i>Greiner, Germany</i>
	<i>Corning, USA</i>
Vortex mixer VX – 100	<i>Labnet, USA</i>
Water bath Nb9	<i>Nüve, Turkey</i>
Western Blot PVDF Transfer Hybond-P	<i>GE Healthcare, UK</i>

Media

LB medium	<i>IMG media facility, Czech republic</i>
Amp LB agar plates	<i>IMG media facility, Czech republic</i>
RPMI-1640	<i>IMG media facility, Czech republic</i>

Mouse strains

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

Solutions and buffers

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

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

Vectors

pBluescriptII-SK+-mSPINK6-FLAG	generated by Halka Buryová
pBroad-hINVm-int-mSPINK6-Flag	generated by Halka Buryová
pBroad-tdTomato	generated by Branislav Slavik
pTracer-mSPINK6	generated by Halka Buryová
pTracer	<i>Invitrogen, USA</i>