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Transgenic kallikrein 14 mice display major hair shaft defects associated with desmoglein 3 and 4 degradation, abnormal epidermal differentiation and Il-36 signature

Olivier Gouin^{1,2}, Claire Barbieux^{1,2}, Florent Leturcq^{1,2}, Mathilde Bonnet des Claustres^{1,2}, Evgeniya Petrova¹, Alain Hovnanian^{1,2,3}.

¹INSERM UMR 1163, Laboratory of Genetic Skin Diseases, Imagine Institute, 75015 Paris, France

²University Paris Descartes-Sorbonne Paris Cité, 75006 Paris, France

³Department of Genetics, Necker Hospital, 75015 Paris, France

Corresponding Author:

Alain Hovnanian, MD, PhD,

Imagine Institute, INSERM UMR1163

Genetic Skin Diseases: From Disease Mechanisms to Therapies,

24, Boulevard du Montparnasse,

75015 Paris, France

mail: alain.hovnanian@inserm.fr

tel: +33 1 42 75 42 89

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Abstract (Word: 200/200)

Netherthron syndrome (NS) is a rare autosomal recessive skin disease caused by loss-of-function mutations in *SPINK5* encoding Lymphoepithelial Kazal-Type-related Inhibitor (LEKTI) that results in unopposed activity of epidermal kallikrein-related peptidases (KLKs), mainly KLK5, KLK7 and KLK14. While the function of KLK5 and KLK7 has been previously studied, the role of KLK14 in skin homeostasis and its contribution to NS pathogenesis remain unknown. We generated a transgenic murine model overexpressing human *KLK14* (*TghKLK14*) in *stratum granulosum*. *TghKLK14* mice showed increased proteolytic activity in the granular layers and in hair follicles. Their hair did not grow and displayed major defects with hyperplastic hair follicles when hKLK14 was overexpressed. *TghKLK14* mice displayed abnormal epidermal hyperproliferation and differentiation. Ultrastructural analysis revealed cell separation in the hair cortex and increased thickness of Huxley's layer. Dsg2 staining was increased while Dsg3 and Dsg4 were markedly reduced. *In vitro* studies showed that hKLK14 directly cleaves rhDSG3 and rhDSG4, suggesting that their degradation contributes to hair abnormalities. Their skin showed an inflammatory signature, with enhanced expression of IL-36 family members and their downstream targets involved in innate immunity. This *in vivo* study identifies KLK14 as an important contributor to hair abnormalities and skin inflammation seen in NS.

Introduction

Netherthor syndrome (NS) is a rare autosomal recessive skin disease that affects approximately one in 200,000 persons and can be life threatening in infants (Hovnanian, 2013). Newborns affected with NS exhibit congenital scaling erythroderma, develop a hair shaft defect and atopic manifestations with elevated serum IgE levels (Hannula-Jouppi et al., 2014). NS is caused by loss-of-function mutations in *SPINK5* encoding lymphoepithelial Kazal-type-related inhibitor (LEKTI). LEKTI deficiency results in unopposed protease activity in the epidermis (Chavanas et al., 2000; Descargues et al., 2005; Kasperek et al., 2016). The main targets of LEKTI are the kallikrein-related peptidases (KLKs), mainly KLK5, KLK7 and KLK14 (Deraison et al., 2007; Egelrud et al., 2005; Hovnanian, 2013; Meyer-Hoffert et al., 2009). Unrestrained KLK5 and KLK7 proteolytic activities are currently considered as playing a major role in NS pathogenesis (Furio et al., 2014, 2015; Kasperek et al., 2017).

Spink5 knock out (*Spink5*KO) mice were the first *in vivo* model that faithfully reproduced all clinical features of NS patients, but these animals died from dehydration only a few hours after birth (Descargues et al., 2005; Hewett et al., 2005; Kasperek et al., 2016, 2017; Yang et al., 2004). Despite neonatal *Spink5*KO lethality, this model was instrumental in disclosing the involvement of KLKs in NS pathogenesis. *Spink5*KO pups revealed unopposed KLK5 proteolytic activity and its downstream targets (KLK7, KLK14, and elastase 2), resulting in the degradation of desmosomal proteins, increased profilaggrin processing (Flg) and lipid anomalies, leading to *stratum corneum* (SC) detachment and a profound skin barrier defect with severe skin inflammation (Bonnart et al., 2010; Descargues et al., 2005; Furio et al., 2014, 2015). While KLK5 can cleave desmoglein 1 (DSG1), desmocollin 1 (DSC1) and corneodesmosin (CDSN), KLK7 and KLK14 were shown to cleave CDSN and DSG1, respectively (Caubet et al., 2004; Borgoño et al., 2007). To gain further insight into the role of KLK5 in NS, several murine models were developed including a transgenic mouse overexpressing human KLK5 (*TghKLK5*) in the *stratum granulosum* (SG) and a double knockout mouse for *Spink5* and *Klk5* (*Spink5/Klk5*KO) (Furio et al., 2014, 2015). *TghKLK5* mice display major NS clinical features including SC detachment, scaling, skin inflammation, hair shaft defects and

exhibit a prolonged survival (Furio et al., 2014). Conversely, *Klk5* deletion in *Spink5/Klk5*KO mice normalizes major hallmarks of NS in newborn pups, confirming the predominant role of KLK5 in NS pathophysiology. Nevertheless, KLK5 deficiency does not rescue the lethality of *Spink5*KO mice, suggesting that other actors directly or indirectly cause NS (Furio et al., 2015). KLK7 and KLK14 proteolytic activities are increased in the epidermis of *Spink5*KO and *TghKLK5*, and are normalized in *Spink5Klk5*KO mice, supporting the notion that KLK5 activates KLK7 and KLK14 *in vivo* (Furio et al., 2014, 2015; Prassas et al., 2015). Double knockout mice for *Spink5* and *Klk7* (*Spink5/Klk7*KO) displayed numerous epidermal lesions and died within 12 hours after birth, indicating that lack of *Klk7* does not prevent the development of the NS phenotype (Kasperek et al., 2017). In contrast, triple knockout mice for *Spink5*, *Klk5* and *Klk7* (*Spink5/Klk5/Klk7*KO) fully rescued lethality after birth and the animals survived until adulthood with no major skin alteration, although they displayed hair abnormalities until 20 days after birth (Kasperek et al., 2017). While the involvement of both KLK5 and KLK7 in the epidermis has been extensively studied in these murine models, the role of KLK14 in skin homeostasis and its involvement in NS pathogenesis remained relatively unexplored probably due to the lack of animal model available.

In this study, we developed and characterized a transgenic murine model overexpressing human KLK14 (*TghKLK14*) in the SG of the epidermis in order to gain further insight into the role of KLK14 in NS pathogenesis. This study demonstrates that KLK14, to our knowledge previously unreported, plays a major causal role in hair shaft defects seen in NS murine models and also contributes to skin inflammation.

Results

hKLK14 is overexpressed and active in the *stratum granulosum* of *TghKLK14* mice

To analyse the consequences of excessive KLK14 proteolytic activity, a transgenic mouse model overexpressing hKLK14 under the control of the human involucrin (IVL) promoter was generated to target KLK14 expression in the SG of murine epidermis. Animals carrying the full-length cDNA encoding hKLK14 (Figure 1A) were identified using a qPCR genotyping strategy targeting the

hKLK14 transgene and the *IVL* promoter. Using a *hKLK14*-specific antibody, the correct localisation of *hKLK14* in the SG of interfollicular epidermis and in hair follicles of *TghKLK14* mice was confirmed (Figure 1B). Western-blot analysis of skin protein lysates from *TghKLK14* mice showed that *hKLK14* was overexpressed, while *hKLK14* remained undetectable in WT mice (Figure 1C). *In situ* zymography of skin cryosections from *TghKLK14* mice using casein-BODIPY-FL substrate revealed increased proteolytic activity mainly localized in the SG and in hair follicles (Figure 1D and E).

***TghKLK14* mice display major hair abnormalities and a skin barrier defect**

TghKLK14 newborns were indistinguishable from WT controls. Before the 5th day after birth, while hair growth led to skin coloration in WT pups, the skin of *TghKLK14* pups remained pink without visible hair (Figure 2A). At this age, no significant difference in transepidermal water loss (TEWL) was observed (Figure 2A). From day 5 to 20, *TghKLK14* mice displayed no visible hair except on their face (Figure 2B), where whiskers and hair around their eyes and muzzle were observed (Figure S1A). At the same time, TEWL was increased, although their skin showed no visible redness or scaling (Figure 2B). *TghKLK14* mice between 20 and 30 days of age showed progressive recovery of hair growth on their entire body, although their fur remained sparse throughout their body (Figure 2C). TEWL of residual nude skin was still significantly increased in comparison to WT skin, whereas hairy skin areas trended to present increased TEWL values with no statistical significance (Figure 2C). From 30 days of life, no difference could be observed in the hair shaft structure, including the hair tip, between *TghKLK14* and WT mice (Figure S1B). Analysis of transgene expression showed that reversion of hair abnormalities was associated with loss of *hKLK14* protein expression in the skin from *TghKLK14* older than 30 days. (Figure S1C).

Histological analysis was performed on skin from *TghKLK14* mice at age 1 day, 20 days, 30 days and 50 days (Figure 2A-C, Figure S1D). The epidermis of *TghKLK14* mice between 1 and 20 days of age was hyperkeratotic and acanthotic with a 2-fold increase in epidermal thickness compared to WT mice, while at 30 days of age the epidermal thickness was similar to WT mice (Figure 2D). Between 1

and 20 days of age, the SC was disrupted and detached from the epidermis. Mice younger than 5 days displayed comparable hair follicle numbers than WT mice but hair shafts were fragmented in approximately 50% of the hair follicles. At the age of 20 days, hair follicles became hyperplastic although their number was not significantly decreased compared to WT mice. At the age of 20 days, hair follicles in the anagen phase were hyperkeratotic with ingrown hair shafts, which were abnormally structured, curled up, broken and fragmented without normal alternation of black and white bands (Figure 2B). In contrast, *TghKLK14* mice older than 30 days showed normal hair growth although their fur remained sparse. Histological analysis showed hair follicles with normal appearance but reduced in number (Figure 2C, Figure S1D).

Histological analysis of *TghKLK14* skin discloses hair shaft defects and epidermal abnormalities

In order to analyse the impact of hKLK14 overexpression on skin homeostasis, mice at 20 days of age, prior to the loss of transgene expression, were further investigated. Ki67 and keratin 14 (Krt14) immunostaining was increased in the *stratum basal* (SB) of *TghKLK14* mice compared to WT controls (Figure 3A), indicating epidermal hyperproliferation. Increased protein expression of Krt14 was confirmed by Western blotting of skin extracts from *TghKLK14* mice (Figure 3B and C). Expression of terminal epidermal differentiation markers such as Ivl and Flg, was strongly increased in the SG of *TghKLK14* mice compared to WT mice, as determined by immunofluorescence staining of skin sections, Western blotting of skin extracts (Figure 3) and by RT-qPCR (Figure S2A). Assessment of Flg expression by immuno-blotting revealed an increase of the dimer form with no significant variation in trimer and monomer forms in comparison to WT mice. Flg fragments with a molecular weight between 15kDa and 25kDa, were enhanced in *TghKLK14* skin extracts (Figure3B).

hKLK14 hyperactivity leads to Dsg3 and Dsg4 degradation associated with hair shaft defects

Transmission electron microscopy of hair follicle cross sections was performed on 20-day-old *TghKLK14* mice (Figure 4A). The hair shaft structure was abnormal as evidenced by cortex alterations. Cells from the hair shaft cortex were separated from each other, leading to gap formation where melanin granules could be seen in the cell interspace in the cortex (Figure 4A). While the

thickness of Henle's layer (He) of the inner root sheath (IRS) and the outer root sheath (ORS) remained comparable to WT mice, the Huxley's layer (Hx) thickness of IRS was increased by 2-fold in *TghKLK14* mice (Figure 4A and B). Since desmoglein (Dsg) and Cdsn proteins are particularly important in hair shaft structure and organisation, Dsg and Cdsn abnormalities were investigated in the skin of *TghKLK14* mice.

Here, we hypothesized that KLK14 could cleave Dsg and Cdsn, involved in hair formation. The pattern of Dsg1 expression in *TghKLK14* skin was comparable to that of WT mice and was localised in the inter-follicular epidermis and the ORS/IRS of hair follicles (Figure 4C). Dsg2 staining displayed a similar distribution but was increased in *TghKLK14* mice (Figure 4C), as confirmed by immunoblotting (Figure 4C). In contrast, Dsg3 and Dsg4, which localized to hair follicles and to inter-follicular epidermis in WT mice, were drastically reduced in *TghKLK14* mice compared to WT controls (Figure 4C). Immunoblotting of skin extracts confirmed that Dsg3 and Dsg4 protein levels were reduced in comparison with WT mice (Figure 4D and E). Increased Dsg4 and Dsg2 transcript levels were detected in skin lysates from *TghKLK14* mice, while those of Dsg1 and Dsg3 remained unchanged (Figure S2B). Digestion time-course analysis provided evidence for direct *in vitro* proteolytic degradation of rhDSG1, rhDSG2, rhCDSN (Figure S2C), rhDSG3, rhDSG4, and rmCdsn by thermolysin-activated rhKLK14 (Figure 4F, Figure S2D).

hKLK14 hyperactivity triggers skin inflammation and immune cell infiltration

To address the potential role of KLK14 in impacting gene expression in the skin, mRNA expression levels of several pro-inflammatory interleukins and chemokines (*Il-36a*, *Il-36b*, *Il-36g*, *Il-17c*, chemokine C-X-C motif ligand 20; *Ccl20* and *interferon-γ*; *Ifn-g*), antimicrobial peptides (*S100a8*, *S100a9* and *Defensin b4*; *Defb4*) and receptors triggering neutrophil- and monocyte-mediated inflammatory responses (*Triggering Receptor Expressed On Myeloid Cells 1*; *Trem1*) were analysed in skin extracts from *TghKLK14* mice and compared to WT mice (Figure 5A). The expression of *Il-36* family members was up-regulated in skin extracts from *TghKLK14* mice by 23-, 3- and 2-fold-change (FC) for *Il-36a*, *Il-36b* and *Il-36g*, respectively. *Ccl20* (3.5-FC) and *Ifn-g* (12.5-FC) were also

significantly increased in *TghKLK14* mice. Enhanced expression of *Trem1* (17-FC) and antimicrobial and cytotoxic peptides such as *S100a8* (12-FC), *S100a9* (43-FC), and *Defb4* (9-FC) indicated that innate immunity against viral and bacterial infections was stimulated in *TghKLK14* (Figure 5A).

Immunofluorescence staining of Il-36a and Il-17c showed a significant increase in the epidermis of *TghKLK14* mice compared to WT mice confirming the differences seen at the transcript level. Increased expression of Il-36a and Il-17c was more pronounced in the SG but also extended across most epidermal layers (Figure 5B). The increased protein level of Il-17c and Il-36a was further validated by immunoblotting of skin extracts from *TghKLK14* and WT mice (Figure 5C). Il-17c and Il-36 family cytokines are known to be involved in immune cell chemo-attraction. HES and toluidine blue coloration analyses revealed an increased number of eosinophils and mast cells, respectively (Figure 5D and F). Immunofluorescence staining showed a strong infiltrate of Cd3⁺ T cells in the epidermis of *TghKLK14* mice, while neutrophils (NIMP-R14) could not be detected neither in *TghKLK14* skin sections nor in WT (Figure 5E and F). Immunostaining for Cd4 and Cd8 revealed that Cd4⁺ cells were mainly localised in the dermis whereas Cd8⁺ cells were found in the epidermis, similarly to Cd3⁺ cells (Figure 5E). The immune cell infiltrates were quantified by counting the amount of positive cells in 2 different fields of view (Figure 5F).

Discussion

While the function of KLK5 and KLK7 in NS pathophysiology has been previously studied in several *in vivo* murine models (Furio et al., 2014, 2015; Kasperek et al., 2016, 2017), the role of KLK14 remains poorly understood. In this study, we developed and characterized a transgenic NS model overexpressing *hKLK14* in the granular layers of the epidermis in order to investigate the contribution of KLK14 hyperactivity in NS pathogenesis *in vivo*.

Lekti deficiency in *Spink5*KO mice or KLK5 overexpression in *TghKLK5* mice results in unopposed serine protease activity leading to increased proteolytic activity in the SG and SC (Descargues et al., 2005; Furio et al., 2014, 2015; Kasperek et al., 2016). In this study, KLK14 overexpression in the epidermis led to enhanced proteolytic activity that mainly co-localized with hKLK14 detection in the

SG and hair follicles. Despite the improvement of NS clinical features in *Spink5/Klk5*KO and prolonged survival of *Spink5/Klk5/Klk7*KO mice, KLK5 and/or KLK7 deletion does not prevent hair abnormalities to develop as shown by delayed hair growth up to 20 days after birth in both murine models (Furio et al., 2015; Kasperek et al., 2017). While *TghKLK14* mice did not display clinical skin inflammation, they exhibited major hair anomalies when the transgene was expressed, supporting the notion that KLK14 could be directly involved in hair defects in NS. Between 20 and 30 days of age, the fur of *TghKLK14* mice grew progressively and hair had normal appearance but remained sparse over the whole body. Reversal of hair shaft anomalies was associated with loss of epidermal hKLK14 expression, likely resulting from silencing of the *hKLK14* transgene. Histological analysis of skin from 20-day-old *TghKLK14* mice showed hyperplastic hair follicles and hair shaft degeneration, confirming that KLK14 plays a central role in hair integrity. These mice also exhibited acanthosis and hyperkeratosis with increased expression of proliferation markers (Ki67, Krt14) and epidermal differentiation markers (Ivl, Flg), as seen in *TghKLK5* mice (Furio et al., 2014). Unlike *Spink5*KO mice which show Ivl and Flg over-degradation, *TghKLK14* mice display increased expression of both markers (Descargues et al., 2005; Furio et al., 2015), suggesting that increased degradation of Ivl and Flg in NS involves additional proteases and/or factors. In *TghKLK14*, the dimer form of Flg and Flg fragments were increased at the protein level, suggesting that in contrast to caspase-14, KLK14 is likely to be involved in the proteolytic processing of Flg but not in Flg fragment degradation (Hoste et al., 2011). Previous works have shown that deregulated expression of proliferation and differentiation markers was normalized in *Spink5/Klk5*KO and *Spink5/Klk5/Klk7*KO mice and that epidermal thickness was strongly improved, indicating that *Spink5*KO-mediated epidermal abnormalities are KLK5- and KLK7-dependent (Furio et al., 2014; Kasperek et al., 2017). Here we provide evidence that KLK14 is also an inducer of acanthosis and hyperkeratosis.

KLK14 is involved in KLK proteolytic cascades in the skin by cleaving and activating pro-KLK1, pro-KLK3, pro-KLK5 and pro-KLK11, leading to global enhancement of KLKs proteolytic activities (Emami and Diamandis, 2008). It is therefore likely that KLK14 overexpression in *TghKLK14* mice activates pro-KLK5, leading to a phenotype, which overlaps with *TghKLK5* mice. Another feature of

NS is SC detachment caused by KLK5- and KLK7-mediated desmosomal cleavage through Dsg1, Dsc1, CDSN degradation (Caubet et al., 2004; Descargues et al., 2005, 2006; Furio et al., 2014). Except for DSG1 (Borgoño et al., 2007), it was unknown whether KLK14 could directly cleave other DSG family members. DSGs are distributed in a specific manner through the hair follicle and epidermal structures : DSG1 and 4 are expressed in suprabasal layers, while DSG2 and 3 localize in basal layers (Wu et al., 2003). Of all DSGs members, Dsg4 is the only one expressed in the hair shaft cortex where hair abnormalities predominate in *TghKLK14* mice (Bazzi et al., 2006, 2009). Increased IRS thickness and gaps in the hair shaft cortex observed in *TghKLK14* mice suggest alterations in Dsg3 and/or Dsg4. In support of this possibility, the expression level of Dsg3 and Dsg4 was drastically reduced in *TghKLK14* skin and we provided evidence that KLK14 can directly cleave DSG3 and DSG4. Besides the degradation of Dsg family members, SC detachment observed in *TghKLK14* could also partly result from the ability of KLK14 to cleave Cdsn, although no evidence for Cdsn degradation *in vivo* could be provided in the absence of specific antibodies to murine Cdsn available. Cdsn deficient mice exhibit not only inflammatory features but also a skin barrier defect leading to postnatal death. In addition, grafts of skin from *Cdsn*^{-/-} mice develop sparse hair in the first weeks of the graft, and hair is completely lost after 9 weeks of grafting (Leclerc et al., 2009). It is therefore possible that the combined proteolytic degradation of Dsg4, Dsg3 and Cdsn contributes to hair loss seen in *TghKLK14*, although complete and specific lack of each desmosomal component separately leads to different hair abnormalities.

Indeed, loss-of-function mutations in *DSG4* cause autosomal recessive hypotrichosis (LAH) in humans and the *lanceolate hair* (*lah*) phenotype in rodents, which are characterized by abnormal epidermal hyperproliferation, impaired hair shaft differentiation, and hair loss (Kljuic et al., 2003; Montagutelli et al., 1996). *TgKLK14* mice share several epidermal and hair abnormalities with the lanceolate hair mice, but they did not develop the specific lance-head shaped hair shaft abnormality (Jahoda et al., 2004; Kljuic et al., 2003). *Dsg3*KO mice also display a hair loss phenotype resulting from defective anchorage of telogen hairs to the follicular epithelium which was not seen in *TgKLK14* mice (Koch et al., 1998). Finally, dominant negative mutations in *CDSN* lead to hypotrichosis simplex

type 2, revealing the role of CDSN in the maintenance of hair in humans (Levy-Nissenbaum et al., 2003).

In vitro enzymatic digestion of rhDSG1 by rhKLK14 is consistent with previous data showing that KLK14 mediates DSG1 degradation (Borgoño et al., 2007; Emami and Diamandis, 2008). However, neither Dsg1 protein nor transcript expression were increased in *TghKLK14*, suggesting that additional mechanisms prevent over-degradation of Dsg1 by hKLK14 *in vivo*. *TghKLK14* mice display increased Dsg2 transcript and protein levels in their skin. Transgenic mice overexpressing *Dsg2* in SG show a histological signature similar to *TghKLK14* mice, with acanthosis and keratinocyte hyperproliferation associated with *Krt14*, *Ivl* and *Flg* overexpression (Brennan et al., 2007). *Dsg2* overexpression could therefore contribute to epidermal deregulation seen in *TghKLK14* mice. The mechanism by which KLK14 hyperactivity leads to increased *Dsg2* remains uncertain. However, a previous study reported that loss of *Dsg3* is compensated by enhanced *Dsg2* expression in human keratinocytes (Hartlieb et al., 2014), suggesting that increased *Dsg2* expression in *TghKLK14* could be a compensatory mechanism to extensive degradation of *Dsg3*.

Recent transcriptomic profiling studies in skin extracts from NS patients revealed an increase of both IL-36A and IL-17C (Malik et al., 2018; Paller et al., 2017). Besides affecting genes involved in innate immunity (*S100a8*, *S100a9*, *Defb4*, and *Trem1*) previously identified as deregulated in NS (Furio et al., 2014, 2015), KLK14 overexpression also deregulated inflammatory cytokines such as *Ifn-g*, *Il-17c*, *Ccl20* and *Il-36* family members. Interestingly, *TghKLK14* mice and transgenic mice overexpressing *Il-36a* (Blumberg et al., 2007, 2010) or *Il-17c* (Johnston et al., 2013) share histological skin features including acanthosis with increased expression of inflammatory cytokines and chemokines (*Cxcl2*, *Cxcl8* and *Ccl20*) and antimicrobial peptides (*Defb4*, *S100a7*, *S100a8* and *S100a9*). This is consistent with the notion that IL-36 and IL-17 family members are powerful inducers of anti-microbial peptides such as *DEFb4* and *S100* peptides, but also of inflammatory cytokines such as *IFN-g* (Carrier et al., 2011). *CCL20* is also an important chemokine induced by inflammatory cytokines and IL-17 pathway activation (Harper et al., 2009). The expression of IL-36,

IL-17c and CCL20 is triggered by Toll like Receptor (TLR) activation, including TLR-3, -4, -5 (Boutet et al., 2019; Johnston et al., 2013; Lebre et al., 2007). Therefore, increased expression of these genes is likely to be initiated by the skin barrier observed in *TghKLK14* mice.

The cytokine expression profile related with the analysis of immune cells infiltrates. Transgenic mice overexpressing *Il-36a* (Blumberg et al., 2007, 2010) and *Il-17c* (Johnston et al., 2013) displayed eosinophil and mast cell infiltrates as well as Cd3⁺ T cell and Cd4⁺ cell infiltration, which were also detected in *TghKLK14* skin. In *TghKLK14*, Cd4⁺ cells were exclusively observed in the dermis whereas Cd3⁺ Cd8⁺ T cells were detected only in the epidermis, suggesting that Cd4⁺ cells were plasmacytoid-like dendritic cells (pDC) and not T lymphocytes (Palamara et al., 2004). The cytotoxic T cell infiltration might result from enhanced expression of IFN- γ since this cytokine activates CD8⁺ T cells motility (Bhat et al., 2017). In psoriasis, IL-36 activates pDC that play a critical role in the initiation of skin inflammation by producing type-I IFN (Catapano et al., 2019). Therefore, infiltration of pDC-like in the skin from *TghKLK14* mice could be an early sign of skin inflammation. Taken together, this study identifies KLK14 as a potential inducer of skin inflammation and immune cell infiltration through the production of epidermal cytokines such as Il-36a and Il-17c, which might also contribute to hair follicle abnormalities.

In summary, overexpression of KLK14 in the epidermis results in major hair defects and epidermal abnormalities. Marked decreased expression of Dsg3 and Dsg4 together with the demonstration that rhKLK14 degrades these desmosomal components *in vitro*, support the possibility that KLK14-mediated Dsg3 and Dsg4 degradation contributes to the NS phenotype in *TghKLK14* mice. Furthermore, these results establish a role of KLK14 in inducing expression of Il-36a and Il-17c and identify KLK14 as a potential therapeutic target to improve skin and hair abnormalities in NS. This study provides evidence that KLK14 overexpression in the epidermis elicits major hair shaft defects, but also skin barrier anomalies, skin inflammation and immune cell infiltration, establishing KLK14 as an important player in NS pathogenesis.

Materials and methods

Transgenic mouse line establishment

The IVL-*TghKLK14* mouse lines were established at the Mouse Clinical Institute - (Illkirch, France). The full length cDNA encoding human KLK14 (OriGene clone SC317386; NM_022046) was cloned into the human IVL promoter vector, pH3700-pL2 (Carroll and Taichman, 1992) (Figure S3). The transgene (comprising the IVL promoter, IVL exon 1, intron 1, a SV40 intron and human KLK14 cDNA followed by a SV40 pA sequence) was excised from pH3700-pL2 by digestion with SalI and injected into the pronuclei of C57BL/6N fertilized oocytes, and finally implanted into pseudo-pregnant females. Founder mice were genotyped and crossed with wild-type C57BL/6N animals to establish the transgenic line. Genotyping was performed using the listed primer sequences in Table S1. Experiments were carried out according to Institution guidelines and EU legislations (agreement #15833-2018011017165115).

Transepidermal Water Loss measurement.

TEWL was measured using Tewameter TM300 apparatus (Courage + Khazaka, Cologne, Germany).

RNA isolation and Quantitative polymerase chain reaction

Total mRNA was extracted using RNeasy® Fibrous Tissue Mini Kit (Qiagen, Redwood, CA, USA). Reverse transcription was carried out with 100 ng of total RNA using Super Script IV Reverse transcription kit (Life Technologies, Carlsbad, CA, USA). cDNA was amplified using Mesa Green qPCR kits for SYBR® Assay (Eurogentec, Liège, Belgium) and Applied Biosystems 7300 Real Time PCR System (Life Technologies, Carlsbad, CA, USA). Primers for each target gene are listed in Table S1. Relative gene expression was normalized to *Hprt* and calculated using the $2^{-\Delta\Delta C_t}$ method.

Immunofluorescence staining

Formalin-fixed paraffin or snap frozen O.C.T.-embedded skin sections (5- μ m thickness) from *TghKLK14* and WT mice were used. Hematoxylin/eosin/safranin staining and immunofluorescence staining were performed on paraffin-embedded skin sections using standard histological procedures.

Dsg3, Dsg4, Cd3, Cd4, Cd8 and NIMP-R14 stainings were performed on skin cryosections. Antibodies used in this study are listed in Table S2. Isotype controls were used for each experiment. Nuclei were stained in blue with 1 µg/ml DAPI. Image acquisition was performed using Leica TCS SP8 SMD confocal microscope and pictures were further processed using ImageJ software.

Immunoblotting

Tissue samples were lysed in RIPA buffer (Life Technologies, Carlsbad, CA, USA) containing Protease Inhibitor Cocktail Tablets (Roche, Basel, Switzerland). Proteins were loaded on a 4-12% precast gel (Invitrogen, Carlsbad, CA, USA) for SDS-PAGE separation. Proteins were transferred to nitrocellulose membranes using Trans-Blot Turbo system (Bio-Rad, Hercules, CA, USA). The antibodies used are listed in Table S2.

Proteolytic degradation of DSG1, 2, 3 and 4 and CDSN by KLK14

10 ng rhKLK14 were activated with 1 ng thermolysin during 1 hour at 37°C and rhKLK14 activity was tested using 100 µM Boc-VPR-AMC Fluorogenic peptide substrate, following the manufacturer's recommendations (R&D systems, Lille, France) (Figure S2E). 10 ng activated rhKLK14 was incubated with 1.5 µg rhDSG1, 2, 3 or 4 or rh or rmCDSN during 5, 15, 30, 60, 120 and 180 min at 37°C (R&D systems, Lille, France). Incubation with 1.5 µg pro-HGF was used as a positive control (Reid et al., 2016) (Figure S2F). Digestion products of DSG 1, 2, 3 and 4 were revealed by immunoblotting whereas CDSN digestion gel were silver-stained according to manufacturer's instructions (Pierce Silver Stain Kit, ThermoFisher).

In situ zymography

Skin cryosections (5 µm thickness) were washed with 2% Tween 20 in PBS and incubated overnight at 37°C with 100 µg/ml BODIPY FL casein (Life Technologies, Carlsbad, CA, USA). Proteolytic activity was visualized and analyzed using Leica SP8 SMD confocal microscope and ImageJ software, respectively.

Transmission electron microscopy

Skin samples were fixed in 4% paraformaldehyde and 2% glutaraldehyde, washed in Sorensen buffer, post fixed in 2% OsO₄, dehydrated in graded ethanol solution and embedded in epon. Skin sections were counterstained with uranyl acetate and lead citrate before imaging using a 1011 transmission electron microscope (Jeol Inc., Japan) coupled to an Erlangshen CCD camera and a Digital Micrograph software (Gatan Inc.)

Statistical analysis

Experiments were performed three times using at least 4 mice. Significance level was assessed using a Student t test, or Mann-Whitney test: * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, **** $p < 0.0001$.

Competing interests

The authors declare no conflict of interest.

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Author Contributions

Conceptualization: OG, AH; Formal analysis: OG; Funding Acquisition: AH; Investigation: OG, CB, MBDC, FL; Methodology: OG, CB; Project Administration: AH; Supervision: AH; Validation: OG,

CB, FL, MBDC, EP, AH; Visualization: OG, CB; Writing-Original Draft preparation: OG, CB; Writing- Review and Editing: OG, CB, EP, AH.

Data Availability Statement

Data sharing is not applicable to this article as no datasets were generated or analysed during the current study. However, all data can be shared upon request to the authors.

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Figure 1: Generation of Tg*KLK14* mice.

(A) Schematic representation of the human full-length KLK14 cDNA transgene under the control of the human involucrin promoter. (B) Confirmation of hKLK14 protein *in situ* localisation and expression by immunostaining of skin sections, and in skin lysates (C) by western blot analysis in 20-day-old Tg*KLK14* mice (Tg) using a hKLK14-specific antibody. Scale bar, 50 μ m; Dapi, blue; hKLK14, red; the dotted white line delineates the dermal-epidermal junction. The experiment shown is representative of three experiments performed with at least three animals per genotype. (D) Fluorescence microscopy image of *in situ* zymography with casein-BODIPY-FL substrate on skin cryosections from 20-day-old Tg*KLK14* mice (scale bar, 50 μ m). (E) The intensity of the proteolytic activity in the epidermis was measured using ImageJ software and normalized to the WT condition. Results are mean values \pm SEM from at least 4 mice of each genotype. P-values are calculated using the Mann-Whitney t test. *, $P < 0.05$.

Figure 2: *TghKLK14* mice display hair abnormalities and a skin barrier defect.

Clinical features, Transepidermal water loss (TEWL), Hematoxylin/eosin/safranin (HES) skin staining and hair follicle counts per mm² in HES skin sections of *TghKLK14* mice (Tg) at the age of (A) < 5 days, (B) 20 days and (C) 30 days in comparison to WT mice. The yellow arrowhead shows the presence of a curled ingrown hair shaft in an anagen hair follicle. Data represent mean values \pm SEM from at least 7 mice. norHF, Normal Hair Follicles; abnHF, Abnormal Hair Follicles. (D) Epidermal thickness measurements from HES staining of skin sections from <5-, 20- and 30-day-old *TghKLK14* and WT mice. Data are mean values \pm SEM from 20 measurements per skin sections of at least 4 mice. P-values are calculated using Mann-Whitney test. *, P < 0.05; **, P < 0,01.

Figure 3: *TghKLK14* mice show major hair shaft defects and epidermal anomalies.

(A) Immunofluorescence staining of Ki67, keratin 14 (Krt14), involucrin (Ivl) and filaggrin (Flg) on skin sections from *TghKLK14* and WT mice. Pictures are representative of 3 independent experiments performed on skin sections from at least 4 mice. Scale bar, 50 μ m; blue, Dapi; red, targets; the dotted white line delineates the dermal-epidermal junction (B) Immunodetection of Krt14, Ivl and Flg proteins in skin extracts from 20-day-old *TghKLK14* and WT mice. (C) Protein level measurement of Krt14, Ivl and Flg normalized to Hsc70 and expressed as fold change relative to WT control. Data are mean values \pm SEM from 3 experiments with at least 4 mice. P-values are calculated using the Mann-Whitney test. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.

Figure 4: *TghKLK14* mice display defective hair shaft structure with Dsg3 and Dsg4 drastic reduction.

(A) Ultrastructure analysis of a skin section from 20-day-old *TghKLK14* (Tg) and WT mice. Red arrow heads show gaps in the hair shaft structure and yellow scale bars indicate inner root sheath (IRS) thickness. M, medulla; Cx, cortex; Ce, hair cuticle; Hx, Huxley's layer; He, Henle's layer, ORS, outer root sheath; Mg, melanin granules. Scale bar, 1 μ m. (B) IRS thickness measurements from transmission electron microscopy pictures of skin sections from 20-day-old *TghKLK14* and WT mice. Data are mean values \pm SEM from 20 measurements per skin sections of at least 4 mice. (C) Immunostaining of Dsg1, Dsg2, Dsg3 and Dsg4 on skin sections from 20-day-old *TghKLK14* and WT mice. Pictures are representative of 3 independent experiments performed on skin sections from at least 4 mice. Scale bar, 50 μ m; blue, Dapi; red, Dsg; the dotted white line delineates the dermal-epidermal junction. (D) Immunodetection of Dsg1, Dsg2, Dsg3 and Dsg4 protein in skin extracts from 20-day-old *TghKLK14* and WT mice. (E) Protein level measurement of Dsg1, Dsg2, Dsg3 and Dsg4 normalized by Hsc70. (F) Digestion time-course of 1.5 μ g rhDSG3, rhDSG4 and rmCdsn by 10 ng rhKLK14 activated by 1 ng thermolysin at 37°C. Data are mean values \pm SEM from 3 experiments on at least 3 mice. P-values are calculated using the Mann-Whitney test. *, $P < 0.05$; **, $P < 0.01$.

Figure 5: *TghKLK14* display skin inflammation with increased expression of Il-36a and Il-17c in the epidermis and immune cell infiltration.

(A) mRNA expression of *Il-36a*, *Il-36b*, *Il-36g*, *Il-17c*, *Trem1*, *Defb4*, *S100a8*, *S100a9*, *Ccl20* and *Ifn-g* normalized to *Hprt* in skin extracts from *TghKLK14* (Tg) and WT mice at the age of 20 days. (C) Immuno-detection of Il-36a and Il-17c on skin sections and skin lysates from 20-day-old *TghKLK14* and WT mice. Protein level measurement of Il-36a and Il-17c by immunoblotting was normalized to Hsc70 and expressed as fold change relative to WT controls. (D) Eosinophil and Mast cells detection in Hematoxylin/eosin/safranin (HES) and toluidine blue stained skin section from *TghKLK14* mice and WT at the age of 20 days, respectively. (E) Immunostaining of CD3⁺ cells, CD4⁺ cells, CD8a⁺ cells and neutrophils (NIMP-R14) on skin sections from 20-day-old *TghKLK14* and WT mice. (F) Eosinophil and Mast cell counts per mm² of skin section as well as CD3⁺ cells, CD4⁺ cells, CD8a⁺ cell and neutrophil counts per field in the skin. For immunostaining, pictures are representative of 3 independent experiments performed on skin sections from at least 4 mice. Scale bar, 50 μ m; blue, Dapi; red, targets; the white line delineates the dermal-epidermal junction. Data are mean values \pm SEM from skin extracts or lysates of at least 4 mice. P-values are calculated using Mann-Whitney test. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.

Figure S1: Loss of transgene expression leads to normalization of skin anomalies with a decrease in the number of hair follicles.

(A) Whiskers' aspects of *TghKLK14* and WT mice at the age of 20 days. (B) Microscopic phenotype of hair shaft from *TghKLK14* and WT mice at 30 days of age. (C) Kinetics of hKLK14 protein and transcript expression in skin lysates from 1-, 10-, 20-, 30 and 50-day-old *TghKLK14* mice (Tg). (D) Hematoxylin/eosin/safranin (HES) staining and epidermal thickness of the skin from *TghKLK14* and WT mice at the age of 50 days. Scale bar, 100 μ m. Data are mean values \pm SEM from 20 measurements per skin sections of 4 mice. P-values are calculated using Mann-Whitney test. *, $P < 0.05$; **, $P < 0.01$.

Figure S2: Validation of rhDSGs proteolytic degradation by rhKLK14.

mRNA expression of (A) *Ivl*, *Flg*, (B) *Dsg1*, *Dsg2*, *Dsg3* and *Dsg4* normalized to *Hprt* in skin extracts from *TghKLK14* (Tg) and WT mice. Data are mean values \pm SEM from skin extracts of at least 4 mice. P-values are calculated using Mann-Whitney test. *, $P < 0.05$. (C) rhDSG1, rhDSG2 and rhCDSN were cleaved by rhKLK14. 1.5 μ g of rhDSG1, rhDSG2 or rhCDSN were incubated with 10 ng of activated rhKLK14 during 0, 5, 15, 30, 60 120, and 180 min at 37°C. Assay buffer and 1 ng thermolysin alone were used as negative control. (D) Negative control showed that assay buffer and thermolysin alone did not digest rhDSG4, rhDSG3 and rmCdsn. 1.5 μ g of rhDSG3, rhDSG4 and rmCdsn were incubated with assay buffer or 1 ng thermolysin or 10 ng of rhKLK14 activated with 1 ng thermolysin during 180 min at 37°C. (E) Kinetics of rhKLK14 proteolytic activity showed that rhKLK14 was biologically active. 10 ng activated rhKLK14 by 1 ng thermolysin was incubated with 100 μ M substrate Boc-VPR-AMC substrate. Thermolysin or Boc-VPR-AMC substrate alone was used as a negative control. (F) Positive control for rhKLK14 activity showed that pro-HGF was cleaved by rhKLK14 into HGF α - and β - chains. 1.5 μ g of rhpro-HGF were incubated with 10 ng of activated rhKLK14 during 0, 5, 15, 30, 60 120, and 180 min at 37°C. Negative control showed that assay buffer and 1 ng thermolysin alone did not digest pro-HGF. Data are representative at least from 3 independent experiments.

Figure S3: Sequence of the *IVL-hKLK14* transgene

aagcttctccatgtgcatgggatatgagctccttattatgttgggtgggggtggacagttaccagacttgcattggacctggagcttatgag
gtcattcacataggcagtgaagaacctctccatatacgtgaatgctgtctcccaaatggggcaacctgtgggcagaataaggacttctagcc
ctagaatgttgagggttcccaacccctcccttcacacacacacacacacacactccctcagctgtatccactgccctcttccacacccctagcttg
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KLK14 cDNA in red

SV40 pA in light blue

SV40 intron green

IVL EXON 1 in uppercase

IVL partial EXON2 in uppercase

In black in lowercase: promoter and intron 1









