Transgenic Kallikrein 14 Mice Display Major Hair Shaft Defects Associated with Desmoglein 3 and 4 Degradation, Abnormal Epidermal Differentiation, and IL-36 Signature

Olivier Gouin1,2, Claire Barbieux1,2, Florent Leturcq1,2, Mathilde Bonnet des Claustres1,2, Evgeniya Petrova1,2 and Alain Hovnanian1,2,3

Netherton syndrome is a rare autosomal recessive skin disease caused by loss-of-function mutations in SPINK5 encoding LEKTI protein. LEKTI deficiency results in unopposed activity of epidermal kallikrein-related peptidases (KLKs), mainly KLK5, KLK7, and KLK14. Although the function of KLK5 and KLK7 has been previously studied, the role of KLK14 in skin homeostasis and its contribution to Netherton syndrome pathogenesis remains unknown. We generated a transgenic murine model overexpressing human KLK14 (TgKLK14) in stratum granulosum. TgKLK14 mice revealed 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. TgKLK14 mice displayed abnormal epidermal hyperproliferation and differentiation. Ultrastructural analysis revealed cell separation in the hair cortex and increased thickness of Huxley’s layer. Desmoglein (Dsg) 2 staining was increased, whereas Dsg3 and Dsg4 were markedly reduced. In vitro studies showed that hKLK14 directly cleaves recombinant human DSG3 and recombinant human DSG4, 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 Netherton syndrome.

INTRODUCTION

Netherton syndrome (NS) is a rare autosomal recessive skin disease that affects approximately 1 in 200,000 persons and can be life threatening in infants (Hovnanian, 2013). Newborns affected with NS exhibit congenital scaling erythroderma and develop a hair shaft defect and atomic manifestations with elevated serum IgE levels (Hannula-Jouppi et al., 2014). NS is caused by loss-of-function mutations in SPINK5 encoding LEKTI protein. LEKTI deficiency results in unopposed protease activity in the epidermis (Chavanas et al., 2000; Descargues et al., 2005; Kasparek 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 to a lesser extend KLK7 proteolytic activities are currently considered as playing a major role in NS pathogenesis (Furio et al., 2014, 2015; Kasparek et al., 2017).

Spink5 knockout (Spink5KO) mice were the first in vivo model that faithfully reproduced all clinical features of patients with NS, but these animals died from dehydration only a few hours after birth (Descargues et al., 2005; Hewett et al., 2005; Kasparek et al., 2016, 2017; Yang et al., 2004). Despite neonatal Spink5KO lethality, this model was instrumental in disclosing the involvement of KLKs in NS pathogenesis. Spink5KO pups revealed unopposed KLK5 proteolytic activity and its downstream targets (KLk7, Klk14, and elastase 2), resulting in the degradation of desmosomal proteins, increased profilaggrin (Flg) processing, 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). Although KLK5 can cleave proteins such as desmoglein (DSG) 1, DSC1, and corneodesmosin (CDSN), KLK7 and KLK14 were shown to cleave CDSN and DSG1, respectively (Borgoño et al., 2007; Caubet et al., 2004). To gain further insight into the role of KLK5 in NS, several murine models were developed including a transgenic mouse overexpressing human KLK5 (TgKLK5) in the stratum granulosum (SG) and a double knockout mouse for Spink5 and Klk5 (Spink5/Klk5KO) (Furio et al., 2015,2014). TgKLK5 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 Tg hKLK5 and are normalized in Spink5 Klk5 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 (Kasparek 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 (Kasparek et al., 2017). Although 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 owing to the lack of available animal model.

In this study, we developed and characterized a transgenic murine model overexpressing human KLK14 (Tg hKLK14) in the SG of the epidermis to gain further insight into the role of KLK14 in NS pathogenesis. This study demonstrates that KLK14 overexpression and activity, to our knowledge previously unreported, results in major hair shaft defects as seen in NS murine models and also contributes to skin inflammation.

RESULTS

hKLK14 is overexpressed and active in the stratum granulosum of Tg hKLK14 mice

To analyze 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 an hKLK14-specific antibody, the correct localization of hKLK14 in the SG of interfollicular epidermis and in hair follicles of Tg hKLK14 mice was confirmed (figure 1b). Western blot analysis of skin protein lysates from Tg hKLK14 mice revealed that hKLK14 was overexpressed, whereas hKLK14 remained undetectable in wild-type (WT) mice (figure 1c). In situ zymography of skin cryosections from Tg hKLK14 mice using casein-BODIPY-FL substrate revealed increased proteolytic activity mainly localized in the SG and in hair follicles (figure 1d and e).
**TgKLK14** mice display major hair abnormalities and a skin barrier defect

**TgKLK14** newborns were indistinguishable from WT controls. Before the fifth day after birth, the skin of **TgKLK14** pups remained pink without visible hair, whereas hair growth led to skin coloration in WT pups (Figure 2a). At this age, no significant difference in transepidermal water loss (TEWL) was observed (Figure 2a). From day 5 to day 20, **TgKLK14** mice displayed no visible hair except on their face (Figure 2b), where whiskers and hair around their eyes and muzzle were observed (Supplementary Figure S1a). At the same time, TEWL was increased, although their skin showed no visible redness or scaling (Figure 2b). **TgKLK14** mice between age 20 days and 30 days 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 with WT skin, whereas hairy skin areas tended to present increased TEWL values with no statistical significance (Figure 2c). From 30 days of life, no difference could

![Figure 2. TgKLK14 mice display hair abnormalities and a skin barrier defect.](image)
be observed in the hair shaft structure, including the hair tip, between TgK14 and WT mice (Supplementary Figure S1b). Analysis of transgene expression revealed that reversion of hair abnormalities was associated with loss of hKLK14 protein expression in the skin from TgK14 older than 30 days (Supplementary Figure S1c).

Histologic analysis was performed on skin from TgK14 mice at age 1 day, 20 days, 30 days, and 50 days (Figure 2a-c) (Supplementary Figure S1d). The epidermis of TgK14 mice between 1 and 20 days of age was hyperkeratotic and acanthotic with a 2-fold increase in epidermal thickness compared with WT mice, whereas at age 30 days, the epidermal thickness was similar to that of WT mice (Figure 2d). Between 1 and 20 days of age, the SC was disrupted and detached from the epidermis. Mice younger than five 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 with 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 2d). In contrast, TgK14 mice older than 30 days showed normal hair growth, although their fur remained sparse. Histologic analysis showed hair follicles with normal appearance but reduced in number (Figure 2c) (Supplementary Figure S1d).

Histologic analysis of TgK14 skin discloses hair shaft defects and epidermal abnormalities

To analyze the impact of hKLK14 overexpression on skin homeostasis, mice at age 20 days, before the loss of transgene expression, were further investigated. Ki67 and keratin 14 immunostaining was increased in the stratum basal of TgK14 mice compared with WT controls (Figure 3a), indicating epidermal hyperproliferation. Increased protein expression of keratin 14 was confirmed by Western blotting of skin extracts (Figure 3b and c). Expression of terminal epidermal differentiation markers, such as IvI and Flg, was strongly increased in the SG of TgK14 mice compared with that of WT mice, as determined by immunofluorescence staining of skin sections, Western blotting of skin extracts (Figure 3), and RT-qPCR (Supplementary Figure S2a). Assessment of Flg expression by immunoblotting revealed an increase of the dimer form with no significant variation in trimer and monomer forms in comparison with WT mice. Flg fragments with a molecular weight between 15 kDa and 25 kDa were enhanced in TgK14 mice compared with WT mice (Figure 3a), indicating epidermal hyperproliferation. Increased protein expression of keratin 14 was confirmed by Western blotting of skin extracts (Figure 3b) and RT-qPCR (Supplementary Figure S2a). Assessment of Flg expression by immunoblotting revealed an increase of the dimer form with no significant variation in trimer and monomer forms in comparison with WT mice. Flg fragments with a molecular weight between 15 kDa and 25 kDa were enhanced in TgK14 mice compared with WT mice (Figure 3a), indicating epidermal hyperproliferation. Increased protein expression of keratin 14 was confirmed by Western blotting of skin extracts (Figure 3b). Histologic analysis showed hair follicles with normal appearance but reduced in number (Figure 2c) (Supplementary Figure S1d).
were separated from each other, leading to gap formation where melanin granules could be seen in the cell interspace in the cortex (Figure 4a). Although the thickness of Henle’s layer of the inner root sheath and the outer root sheath remained comparable with WT mice, the Huxley’s layer thickness of inner root sheath was increased by 2-fold in Tg(hKLK14) mice (Figure 4a and b). Because Dsg and Cdsn proteins are particularly important in hair shaft structure and organization, Dsg and Cdsn abnormalities were investigated in the skin of Tg(hKLK14) 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 with that of WT mice and was localized in the interfollicular epidermis and the outer root sheath and/or inner root sheath 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 interfollicular epidermis in WT mice, were drastically reduced in TghKLK14 mice compared with 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, whereas those of Dsg1 and Dsg3 remained unchanged (Supplementary Figure S2b). Digestion time-course analysis provided evidence for direct in vitro proteolytic degradation of recombinant human (rh) DSG1, rhDSC2, rhCDSN (Supplementary Figure S2c), rhDSC3, rhDSC4, and recombinant murine Cdsn by thermolysin-activated rhKLK14 (Figure 4f) (Supplementary Figure S2d).

**DISCUSSION**

Although the function of KLK5 and KLK7 in NS pathophysiology has been previously studied in several in vivo murine models (Furio et al., 2014, 2015; Kasparek 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 to investigate the contribution of KLK14 hyperactivity in NS pathogenesis in vivo.

LEKTI deficiency in Spink5KO 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; Kasparek 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/Klk5KO and prolonged survival of Spink5/ Klk5/Klk7KO 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; Kasparek et al., 2017). Although 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 age 20 days and 30 days, 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. Histologic 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, keratin 14) and epidermal differentiation markers (lsv, Flg), as seen in TghKLK5 mice (Furio et al., 2014). Unlike Spink5KO mice which showed lsv and Flg overdegradation, TghKLK14 mice displayed increased expression of both markers (Descargues et al., 2005; Furio et al., 2015), suggesting that increased degradation of lsv 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/Klk5KO and Spink5/ Klk5/Klk7KO mice and that epidermal thickness was strongly improved, indicating that Spink5KO-mediated epidermal abnormalities are KLK5- and KLK7-dependent (Furio et al., 2014; Kasparek et al., 2017). Here, we provide evidence that KLK14 is also an inducer of acanthosis and hyperkeratosis.

**Transgenic Kallikrein 14 Mice Show Hair Defects**

To address the potential role of KLK14 in impacting gene expression in the skin, mRNA expression levels of several pro-inflammatory IIs and chemokines (Il-36a, Il-36b, Il-36g, and Il-17c; chemokine c-x-c motif ligand 20; Ccl20 and Ifn-γ), antimicrobial peptides (S100a8, S100a9, and defensin b4; Defb4), and receptors triggering neutrophil- and monocyte-mediated inflammatory responses (Trem1) were analyzed in skin extracts from TghKLK14 mice and compared with WT mice (Figure 5a). The expression of Il-36 family members was upregulated 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-γ (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 revealed a significant increase in the epidermis of TghKLK14 mice compared with 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. H&E-safranin and toluidine blue coloration analyses revealed an increased number of eosinophils and mast cells, respectively (Figure 5d–f). Immunofluorescence staining revealed a strong infiltrate of Cd3+ T cells in the epidermis of TghKLK14 mice, whereas 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 localized in the dermis, whereas Cd8+ cells were found in the epidermis, similar to Cd3+ cells (Figure 5e). The immune cell infiltrates were quantified by counting the number of positive cells in two different fields of view (Figure 5f).
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 proteolytic activities of KLKs (Emami and Diamandis, 2008). Therefore, it is likely that KLK14 overexpression in TgKLK14 mice activates pro-Klk5, leading to a phenotype that overlaps with TgKLK5 mice. Another feature of NS is SC detachment caused by KLK5- and KLK7-mediated desmosomal cleavage through DSG1, DSC1, and CDSN degradation (Caubet et al., 2004; Descargues et al., 2005, 2006; Furio et al., 2014). Except for DSG1 (Borgonño 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, whereas DSG2

Figure 5. TgKLK14 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-γ normalized to Hprt in skin extracts from TgKLK14 (Tg) and WT mice at the age of 20 days. (b, c) Immuno-detection of Il-36A and Il-17C on skin sections and skin lysates from 20-day-old TgKLK14 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 HES and toluidine blue stained skin section from TgKLK14 mice and WT at the age of 20 days, respectively. (e) Immunostaining of Cd3+ cells, Cd4+ cells, Cd8a+ cells and neutrophils (NIMPR-R14) on skin sections from 20-day-old TgKLK14 and WT mice. (f) Eosinophil and Mast cell counts per mm² of skin section and Cd3+ cells, Cd4+ cells, Cd8a+ cell and neutrophil counts per field in the skin. For immunostaining, pictures are representative of three independent experiments performed on skin sections from at least four mice. Bar = 50 μm. DAPI, blue; targets, red. The white line delineates the dermo-epidermal junction. Data are mean values ± SEM from skin extracts or lysates of at least four mice. P-values are calculated using Mann-Whitney test. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. HES, H&E-safranin; Hsc, Heat shock cognate protein 70; WT, wild-type.
and 3 are localized in basal layers (Wu et al., 2003). Of all Dsg members, Dsg4 is the only one expressed in the hair shaft cortex where hair abnormalities predominate in TghKLK14 mice (Bazzi et al., 2009, 2006). Increased inner root sheath 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 exhibited not only inflammatory features but also a skin barrier defect leading to postnatal death. In addition, grafts of skin from Cdsn−/− mice developed sparse hair in the first weeks of the graft, and hair was completely lost after nine weeks of grafting (Leclerc et al., 2009). Therefore, it is 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 in humans and the lanceolate hair 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 shared several epidermal and hair abnormalities with the lanceolate hair mice, but they did not develop the specific lance-head shaped hair shaft abnormality (lahoda et al., 2004; Kljuic et al., 2003). Dsg3−/− mice also displayed a hair loss phenotype resulting from defective anchorage of telogen hairs to the follicular epithelium, which was not seen in TghKLK14 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 (Borgno et al., 2007; Emami and Diamandis, 2008). However, neither Dsg1 protein nor transcript expression was increased in TghKLK14, suggesting that additional mechanisms prevent overdegradation of Dsg1 by hKLK14 in vivo. TghKLK14 mice displayed increased Dsg2 transcript and protein levels in their skin. Transgenic mice overexpressing Dsg2 in SG showed a histologic signature similar to TghKLK14 mice, with acanthosis and keratinocyte hyperproliferation associated with keratin 14, 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 patients with NS revealed an increase of both IL-36A and IL-17C transcripts (Malik et al., 2019; 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-γ, IL-17c, Ccl20, and IL-36 family members. Interestingly, TghKL14 mice and transgenic mice overexpressing IL-36A (Blumberg et al., 2007, 2010) or IL-17C (Johnston et al., 2013) share histologic skin features including acanthosis with increased expression of inflammatory cytokines and chemokines (Cxc12, Cxc18, 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 antimicrobial peptides such as Defb4 and S100 peptides, but also of inflammatory cytokines such as IFN-γ (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 activation, including T Toll-like Receptors 3, 4, and 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., 2010;2007) and IL-17C (Johnston et al., 2013) displayed eosinophil and mast cell infiltrates and 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+ and Cd8+ T cells were detected only in the epidermis, suggesting that Cd4+ cells were plasmacytoid-like dendritic cells and not T lymphocytes (Palamara et al., 2004). The cytotoxic T-cell infiltration might result from enhanced expression of Ifn-γ because this cytokine activates Cd8+ T-cell motility (Bhat et al., 2017). In psoriasis, IL-36 activates plasmacytoid-like dendritic cells that play a critical role in the initiation of skin inflammation by producing type-I IFN (Catapano et al., 2019). Therefore, infiltration of plasmacytoid-like dendritic cells-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-Tg(hKLK14 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) (Supplementary 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, injected into the pronuclei of C57BL/6N fertilized oocytes, and finally implanted into pseudo-pregnant females. Founder mice were genotyped and crossed with WT C57BL/6N animals to establish the transgenic line. Genotyping was performed using the listed primer sequences in Supplementary Table S1. Experiments were carried out according to Institution guidelines and European legislations (agreement #15833-2018011017165115).

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

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

Immunofluorescence staining
Formalin-fixed paraffin or snap frozen OCT-embedded skin sections (5-μm thickness) from Tg(hKLK14 and WT mice were used. H&E-staining and immunofluorescence staining were performed on paraffin-embedded skin sections using standard histologic procedures. Dsg3, Dsg4, Cd3, Cd4, Cd8, and NIMP-R14 stainings were performed on skin cryosections. Antibodies used in this study are listed in Supplementary 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 radioimmunoprecipitation assay buffer (Life Technologies) containing Protease Inhibitor Cocktail Tablets (Roche, Basel, Switzerland). Proteins were loaded on a 4–12% precast gel (Invitrogen, Carlsbad, CA) for SDS-PAGE separation. Proteins were transferred to nitrocellulose membranes using Trans-Blot Turbo system (Bio-Rad, Hercules, CA). The antibodies used are listed in Supplementary Table S2.

Proteolytic degradation of DSG1, 2, 3, and 4 and CDSN by KLK14
The 10 ng rhKLK14 were activated with 1 ng thermolysin for 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) (Supplementary Figure S2e). The 10 ng activated rhKLK14 was incubated with 1.5 μg rhDSG1, 2, 3, or 4 or rh or recombinant mouse CDSN during 5, 15, 30, 60, 120, and 180 minutes at 37 °C (R&D systems). Incubation with 1.5 μg pro-HGF was used as a positive control (Reid et al., 2016) (Supplementary Figure S2h). Digestion products of DSG1, 2, 3, and 4 were revealed by immunoblotting, whereas CDSN digestion gel was silver-stained according to manufacturer’s instructions (Pierce Silver Stain Kit, ThermoFisher, Waltham, MA).

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). 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 Sorenesen buffer, post-fixed in 2% osmium tetroxide, dehydrated in graded ethanol solution, and embedded in epon. Skin sections were counterstained with 4% aqueous uranyl acetate and Reynolds lead citrate before imaging using a 1011 transmission electron microscope (Jeol, Tokyo, Japan), coupled to an Erlangshen CCD camera and a Digital Micrograph software (Gatan, Pleasanton, CA).

Statistical analysis
Experiments were performed three times using at least four mice. Significance level was assessed using a Student’s t test or Mann-Whitney test. *P < 0.05, **P < 0.005, ***P < 0.0005, ****P < 0.0001.

Data availability statement
Data sharing is not applicable to this article as no data sets were generated or analyzed during the study. However, all data can be shared on request to the authors.

ORCIDs
Olivier Gouin: https://orcid.org/0000-0002-2649-5844
Claire Barbies: https://orcid.org/0000-0003-1292-588X
Florent Leturcq: https://orcid.org/0000-0002-7819-0428
Mathilde Bonnet des Clausters: https://orcid.org/0000-0002-3609-2942
Evgeniya Petrova: https://orcid.org/0000-0002-7060-1114
Alain Hovnanian: https://orcid.org/0000-0003-3412-7512

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2019.10.026.

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