

Research paper

Steroid sulfatase suppresses keratinization by inducing proteasomal degradation of E-cadherin *via* Hakai regulationTae-Uk Kwon^a, Yeo-Jung Kwon^a, Hyemin Park^a, Hyein Lee^a, Ji-Heung Kwak^a,
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ARTICLE INFO

Keywords:
Steroid sulfatase
E-cadherin
Hakai
Mir-6766
Keratinization

ABSTRACT

X-linked ichthyosis (XLI) is a genetic disorder characterized by a steroid sulfatase (STS) deficiency inducing excessive cholesterol sulfate accumulation and keratinization. Our study utilizes STS knockout mice to reproduce the hyperkeratinization typical of XLI, providing a valuable model for investigating the underlying mechanisms. From the experiment of STS-deficient keratinocytes using the CRISPR/Cas9 system, we observed upregulation of E-cadherin, which is associated with keratinocyte differentiation and stratification. This was accompanied by elevated levels of keratinization markers, including involucrin and loricrin. We also found an increased expression of SULT2B1, which converts cholesterol to cholesterol sulfate, further accelerating cholesterol sulfate accumulation. As a result, STS deficiency and cholesterol sulfate accumulation lead to decreased expression of Hakai, the ubiquitin E3 ligase for E-cadherin. With reduced Hakai, endocytosis and ubiquitin-mediated degradation of E-cadherin are inhibited, resulting in its stabilization. This stabilization of E-cadherin is accompanied by increased expression of involucrin and loricrin, which is suppressed when the N-terminal extracellular domain of E-cadherin, responsible for cell-cell adhesion, is genetically modified. We propose that inhibition of E-cadherin, genetic modification of the N-terminal extracellular domain, and treatment with miR-6766 targeting E-cadherin significantly reduce the expression of keratinization markers, suggesting a potential therapeutic approach. We further suggest that the increased expression of E-cadherin observed in keratinocytes with STS deficiency is regulated by Hakai, underscoring the central role of E-cadherin in the pathogenesis of XLI.

1. Introduction

X-linked ichthyosis (XLI) is a genetic disorder characterized by skin keratinization inherited in an X-linked recessive manner. This disorder primarily affects men and children, making it the second most prevalent type of ichthyosis disease [1,2]. Patients with XLI suffer from hyperkeratinization and exhibit a scaly stratum corneum, where the skin appears thick [3,4]. XLI is predominantly caused by a genetic defect, resulting in a deficit of steroid sulfate (STS) [5–7]. This enzyme is localized within the endoplasmic reticulum (ER) across diverse mammalian tissues [8]. In skin tissue, STS is typically found within the epidermis and participates in active dermal steroid production, lipid regulation, and breaking down steroid sulfates into steroids [9–12]. Therefore, STS deficiency leads to the accumulation of cholesterol sulfate within stratum corneum cell membranes [1,13]. Excess cholesterol

sulfate induces membrane abnormalities in the normal stratum corneum, a prominent indicator of hyperkeratinization in XLI [9,11,12]. Despite these findings, the influence of STS deficiency on hyperkeratinization layer formation in XLI has not been fully understood.

Hyperkeratinization arises from excessive keratinocyte differentiation and stratification, which weakens desquamation due to the increased cohesion among keratinocytes [14–17]. Cadherin protein regulation, especially E-cadherin, a calcium-dependent cell-cell adhesion molecule also known as CDH1, is pivotal during calcium-induced differentiation. E-cadherin maintains cell-cell adhesion and generates secondary messengers that facilitate keratinocyte differentiation by activating various protein kinases and phospholipases [18,19].

E-cadherin is integral for epithelial stratification and polarization [18,19]. Previous studies have highlighted the importance of E-cadherin in mouse skin morphogenesis, confirming that overlying keratinocytes

Abbreviations: CBL1, Cbl proto-oncogene like 1; CHX, Cycloheximide; FAK, Focal adhesion kinase; STS, Steroid sulfatase; XLI, X-linked ichthyosis.

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<https://doi.org/10.1016/j.bbamcr.2025.119898>

Received 10 September 2024; Received in revised form 24 December 2024; Accepted 2 January 2025

Available online 6 January 2025

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depend on E-cadherin for further stratification [17,20,21]. E-cadherin has extracellular cadherin repeats containing Ca^{2+} binding sites that mediate the trans-binding of homologous cadherin molecules among neighboring cells. E-cadherin's highly conserved intracellular tail forms complexes with various cytoplasmic proteins, including α -, β -, and p120-catenins. These structure and binding partners of E-cadherin perform diverse functions, such as maintaining cell-cell adhesion and the cytoskeleton [22]. Enhanced calcium interaction with E-cadherin inhibits its endocytosis, preventing protein ubiquitination. Endocytosis of E-cadherin is mediated through the Src signaling pathway. Activated Src phosphorylates E-cadherin, inducing its ubiquitination by Cbl proto-oncogene like 1 (CBL1 or Hakai), a specific ubiquitin E3 ligase for E-cadherin [23–25].

Differentiation marker synthesis begins in the first suprabasal layer composed of keratinized acanthocytes, whereas involucrin, a precursor of the keratinized integument, is expressed in the upper layer [26,27]. Loricrin is expressed and cross-linked during the final stages of keratinization, in which involucrin is broadly upregulated in the epidermis [28]. Notably, involucrin and loricrin are relatively elevated in the keratinizing cell membranes of differentiated keratinocytes, serving as markers for keratinization [29–31].

This study investigated hyperkeratinization using STS-deficient and overexpressing mouse and keratinocyte models to understand the pathological characteristics and impact of XLI. In our previous work, we found that STS deficiency leads to increased transcription of E-cadherin [32]. In this study, we focused on the proteasomal degradation of E-cadherin and found that STS regulates Hakai, the E3 ligase of E-cadherin, in keratinocytes. This approach allowed us to gain a deeper understanding of how STS affects keratinization by regulating E-cadherin.

2. Materials and methods

2.1. Cell culture

The HaCaT human keratinocyte cell line was obtained from CLS Cell Lines Service (Germany). HaCaT, pLJM1-Control HaCaT, and pLJM1-STS HaCaT cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % (v/v) fetal bovine serum (FBS), 100 U/mL penicillin, and 100 $\mu\text{g}/\text{mL}$ streptomycin. Cas9-HaCaT and Cas9-STS^{+/-} HaCaT cell lines were cultured in DMEM supplemented with 20 % (v/v) FBS, 100 U/mL penicillin, and 100 $\mu\text{g}/\text{mL}$ streptomycin. All cell lines were incubated at 37 °C in a humidified atmosphere with 5 % CO_2 . After 24 h of incubation, the cells were treated with TNF- α or STX-64 for 24 or 48 h.

2.2. Reagents

STX-64 and Ultra Cruz™ mounting medium (sc-24941) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), TNF- α and MG132 from Enzo Biochem (Farmingdale, NY), cholesterol sulfate from Avanti Polar Lipids (Birmingham, AL), and cycloheximide from Sigma-Aldrich (Burlington, MA). All chemicals, except TNF- α (melt into 3DW), were prepared in dimethylsulfoxide (DMSO), stored as small aliquots at -20 °C, and then diluted as needed in cell culture media. DMEM was purchased from HyClone (Logan, UT). FBS and charcoal-stripped FBS were purchased from Tissue Culture Biologicals (Long Beach, CA). The Neon™ Transfection System was obtained from Thermo Fisher Scientific (Waltham, MA), and the D-Plus™ ECL solution was obtained from Dongin LS (Seoul, Korea). The following antibodies were used in our experiments: anti-STS polyclonal antibody (ab62219), anti-involucrin (ab53112), and anti-loricrin (ab85679) from Abcam (Cambridge, MA); anti-HA (sc-7392), anti-FLAG (sc-9996), anti-SULT2B1 (sc-166487), and goat anti-rabbit IgG-Texas Red (sc-2780) from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-E-cadherin (07-697) from Merck Millipore (Burlington, MA); anti-Src (2108S), anti-phospho-Src (Y416) (2101S), anti-FAK (3285S), and anti-phospho-FAK (Y925)

(3284S) from Cell Signaling Technology (Danvers, MA); anti-Hakai (abx231320) from Abxexa (Cambridge, UK); and anti-GAPDH from Cusabio Technology (Houston, TX). All other chemicals were obtained from commercial sources.

2.3. Transient and stable transfection

The STS coding sequence was cloned into the pcDNA3.1/Zeo⁺ vector for transient transfections. The cells were transfected with 50 nM siRNA, 25, 50, 100 nM miRNA or 3 μg plasmid using the Neon Transfection System (Life Technologies, CA). The siRNA E-cadherin (Qiagen, Netherlands) and CBL1 (Bioneer, Korea) were obtained from Qiagen and Bioneer. The miRNA hsa-mir-6766 (Bioneer, Korea) and hsa-mir-6766-3p inhibitor were obtained from Bioneer. The plasmid vectors pLJM1-Empty (a gift from Joshua Mendell, Addgene plasmid # 91980), pLKO.1 puro (a gift from Bob Weinberg, Addgene plasmid #8453), pMD2.G, psPAX2 (gifts from Didier Trono, Addgene plasmids # 12259 and #12260), E-cadherin-GFP (gifts from Jennifer Stow, Addgene plasmid #28009), pWZL Blast DN E-cadherin (a gift from Bob Weinberg, Addgene plasmid #18800), hE-cadherin-pcDNA3 (a gift from Barry Gumbiner, Addgene plasmid #45769), hE-cadherin/ Δ p120 (a gift from Barry Gumbiner, Addgene plasmid #45770), hE-cadherin/ α -catenin fusion-pcDNA3 (a gift from Barry Gumbiner, Addgene plasmid #45771), hE-cadherin/ Δ β -catenin-pcDNA3 (a gift from Barry Gumbiner, Addgene plasmid #45772), IL2R/hE-cadherin-cytotail (a gift from Barry Gumbiner, Addgene plasmid #45773), HA-ubiquitin expression vector (a gift from Edward Yeh, Addgene plasmid #18712). The Human CBL1 gene ORF (Sino biological plasmid, HG20869-UT) was obtained from Sino Biological [33–35]. HEK293T cells were co-transfected with pLJM1-STS, pMD2.G, and psPAX2 vectors. After 48 h, the lentiviral supernatant containing the STS gene was collected and used to treat HaCaT cells along with polybrene (8 $\mu\text{g}/\text{mL}$) for 24 h. HaCaT cells overexpressing STS were selected using puromycin (1 $\mu\text{g}/\text{mL}$).

2.4. Generation of Cas9-STS^{+/-} HaCaT cells

The plasmid vectors pLentiCas9-T2A-GFP (Addgene plasmid #78548, gifted from Roderic Guigo & Rory Johnson) and tet-pLKO-sgRNA-puro (Addgene plasmid #104321, gifted from Nathanael Gray) were obtained from Addgene [35]. Lentiviral supernatants containing the Cas9 gene or STS sgRNA were prepared using HEK293T cells. Cas9-STS^{+/-} HaCaT cells were isolated through single-cell separation using 96-well plates.

2.5. Quantitative PCR (qPCR)

qPCR analysis was performed using a Rotor-Gene Q machine (Qiagen, Netherlands) and analyzed with the QIAGEN Rotor-Gene Q Series software. Each reaction consisted of 10 μL of Q Green 2 $\times$ qPCR Master Mix, 1 μM of oligonucleotide primers, and 20 ng of cDNA in a final volume of 20 μL . The amplification program included one cycle at 95 °C for 5 min, 40 cycles of denaturation at 95 °C for 15 s, and annealing/extension at 56 °C for 45 s. The following primer sets were used for **qPCR**: STS, 5'- CCT CCT ACT GTT CTT TCT GTG GG-3' (forward), and 5'- TGC CAG TIT CTG CAT CTG C-3' (reverse); **E-cadherin**: 5'- GTA CTT GTA ATG ACA CAT CTC-3' and 5'- GGT CGA TAT TGG GAG TCC TGA TA-3'; **18S rRNA**: 5'-GTA ACC CGT TGA ACC CCA TT-3' and 5'-CCA TCC AAT CGG TAG TAG CG-3'; **Involucrin**: 5'- TCC TCC AGT CAA TAC CCA TCA-3' and 5'- CAG CAG TCA TGT GCT TTT CCT -3'; **Loricrin**: 5'- GT GGA CTG CGT GAA GAC CT -3' and 5'- AGC CAG AAC CGC TGC TAC -3'; and **SULT2B1 (mouse)**: 5'- TGG ATG ATT GAG ATC GTC TGC T -3' and 5'- CAT TGA AGG CGC TTA TGA TGG T -3'.

2.6. Western blotting analysis

Cells and mouse skin samples were lysed using ice-cold PE buffer

containing 50 mM NaF. Protein lysates were separated through SDS-PAGE on 8 %, 10 %, or 15 % polyacrylamide gels and electrophoretically transferred to 0.45 μ m PVDF membranes. The membranes were blocked with 5 % (w/v) BSA in Tris-buffered saline containing 0.1 % Tween-20 (TBST) for 2 h at 4 °C, incubated with primary antibodies diluted at a 1:1000 ratio in TBST, and then incubated with secondary antibodies for 2 h. Protein bands were visualized using D-Plus™ ECL solution (Dongin LS, Korea) and analyzed with a ChemiDoc XRS (Bio-Rad, CA).

2.7. Immunoprecipitation (IP)

Immunoprecipitation assays were performed using the Catch and Release® v2.0 (Reversible Immunoprecipitation System) Kit (Millipore, USA) per the manufacturer's instructions. Cultured cells (5×10^6) were washed with ice-cold phosphate-buffered saline, pelleted, and resuspended in ice-cold cell lysis buffer (1 % SDS, 10 mM EDTA, 50 mM Tris-HCl at pH 8.1, containing protease inhibitors). Next, the samples were centrifuged at 15,000 rpm at 4 °C for 10 min. A control aliquot (whole-cell extract) was saved, and supernatants were diluted 10-fold in dilution buffer (16.7 mM Tris-HCl (pH 8.1), 167 mM NaCl, 1.1 % Triton X-100, 0.01 % SDS, 1.2 mM EDTA protease inhibitor). A 2 h incubation on a rotating platform at 4 °C with protein A/G beads was used to remove a nonspecific background. The precleared chromatin material was incubated with anti-FLAG-conjugated beads (~25 μ L slurry per 1×10^6 cells) and anti-Hakai antibody (dilution 1:1000) or anti-Flag antibody (dilution 1:1000) on a rotating platform overnight at 4 °C, followed by magnetic separation. The beads were sequentially washed, and the precipitated material was de-crosslinked and treated, adhering to the instructions. The protein was recovered through column purification and subjected to western blotting.

2.8. Immunofluorescence

Samples were fixed with 10 % neutral formalin for 30 min at 24 °C, blocked for 45 min in PBS buffer containing 10 % goat serum and 0.2 % Triton X-100, incubated with a primary antibody (1:200) overnight at 4 °C, and then stained with goat anti-rabbit IgG- Alexa Fluor 594 (1:200) overnight. After three PBS washes, coverslips were mounted onto glass slides using Ultra Cruz™ mounting medium. Fluorescence was then analyzed using an LSM 800 confocal laser scanning microscope (Carl Zeiss, Germany).

2.9. In vivo delivery of miRNA

MiR-6766 and negative control miRNA (20 μ g) (Bioneer, Korea) were diluted in 100 μ L of a 10 % glucose solution. The volume was adjusted to 200 μ L using RNase/DNase-free water. Solutions were mixed and briefly centrifuged. In a separate tube, 6.4 μ L *in vivo*-jetPEI™ (Polyplus, France) was diluted in 100 μ L of a 10 % glucose solution [36]. The volume was adjusted to 200 μ L using RNase/DNase-free water, and solutions were briefly mixed. The 200 μ L *in vivo*-jetPEI™ solution was then added to the miRNA solution, vortexed, and briefly centrifuged. Solutions were then incubated for 15 min at room temperature to allow complexes to form. MiRNA solution (0.2 μ g/ μ L) was microinjected into the skin of wild-type (WT) male mice and STS KO mice three times at 2-day intervals.

2.10. Generation of STS transgenic (TG) and STS KO mice

A 7088-bp fragment of the human STS gene containing the CMV promoter was synthesized and cloned into the Amp^R site of the pcDNA3.1 vector to generate the STS plasmid for microinjection. The *Pvu*II and *Avr*II restriction enzymes were used to remove the 4.4-kb backbone fragment through gel extraction, leaving behind a 2.2-kb fragment. STS transgenic mice were generated by microinjecting the

STS DNA into the male pronucleus of zygotes and transferring the embryos into the oviducts of pseudopregnant recipient female mice. Once the F₀ generation was born, genotyping tests were performed using tail-cut samples to confirm the presence of the STS gene through PCR analysis with a forward primer for the CMV promoter and a reverse primer for the human STS gene. The Cas9 targeting vector is designed to contain gRNAs targeting the Cas9 gene to induce double-strand breaks (DSBs) in the gene of interest. The vector also contains a selection cassette, such as puromycin resistance genes, to select successfully targeted cells. The Cas9 targeting vector was introduced into ES cells using PEG-mediated transfection. The cells were then cultured under puromycin selection to select cells that had incorporated the targeting vector. PCR and Southern blot analysis were conducted to screen for successful targeting events in the ES cells. PCR primers were designed to amplify the region flanking the Cas9 gene and the targeting vector, whereas the Southern blot probes were designed to hybridize to specific regions within the Cas9 gene. Successfully targeted ES cells were injected into blastocysts and transferred into pseudopregnant females to generate chimeric mice. Chimeric mice comprised a mixture of cells from the original blastocyst and the injected ES cells. All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Chung-Ang, and the STS transgenic mice were kept in pathogen-free conditions at Macrogen, Inc. (Seoul, Korea).

2.11. Measurement of Hakai through ELISA

STS partial knockout or STS overexpression cells were analyzed for Hakai content using an ELISA kit (MyBioSource, Canada) per the manufacturer's instructions. Briefly, 96-well ELISA microplates were coated with specific monoclonal Ab. Samples or standards were added at the appropriate dilution and incubated for 2 h at room temperature. After careful washing, biotinylated goat anti-Hakai was added to each well. Horseradish-peroxidase was used as secondary Ab, and optical density was read at 450 nm.

2.12. Histopathology and immunohistochemistry staining

Mouse tissues were fixed in formalin for at least three days, processed, and embedded in paraffin. Four-micron sections were cut, baked at 60 °C for at least 30 min, and cooled. The sections were then deparaffinized with three changes of xylene for 5 min each and rehydrated using an alcohol and distilled water gradient. If necessary, heat-induced epitope retrieval (HIER) was performed by steaming the slides for 20 min, cooling them for 20 min, and transferring them into a wash buffer for 5 min. Citrate buffer at pH 6.0, Tris-EDTA buffer at pH 9.0, or Trilogy was used as the antigen retrieval buffer. The slides were transferred to TBS-Tween (TBS-T) wash buffer (0.05 M Tris, 0.15 M NaCl, 0.05 % Tween-20, pH 7.6) for 5 min. H&E staining was performed to visualize the skin, epidermis, and stratum corneum. Immunohistochemistry staining for E-cadherin, involucrin, and loricrin was performed to observe keratinization. Antibodies were diluted in TBS containing 1 % BSA at pH 7.5. Endogenous peroxidase was blocked with a 3 % hydrogen peroxide solution for 8 min. When using the biotinylated goat anti-mouse secondary antibody, 15 % normal goat serum in TBS with 1 % BSA was used for 10 min as the protein block. For signal development, two sequential applications of 3,3'-diaminobenzidine were used for 4 min each, followed by a 2-min incubation of hematoxylin as the counterstain. After staining, the slides were dehydrated via an alcohol gradient, cleared in xylene, and coverslipped.

2.13. Statistical analysis

Dunnett's pairwise multiple comparison *t*-test was performed using GraphPad Prism 7 software (GraphPad Software, CA). Differences were considered statistically significant at **p* < 0.05.

3. Results

3.1. STS deficiency leads to the formation of a thicker stratum corneum in mouse skin

STS deficiency influences XLI disease, a hyperkeratinizing skin condition characterized by an abnormally thick stratum corneum [3,4]. Therefore, we investigated how STS affects skin thickness by analyzing skin tissues from 8-week-old male STS knockout (KO) and transgenic (TG) mice (Fig. 1A). Our findings revealed that the overall skin and epidermis thickness in STS KO mice increased by about 18.7 % and 34.1 %, with a significant 54.8 % increase in the stratum corneum compared to WT mice (Fig. 1B).

In contrast, STS TG mice exhibited a decrease in skin, thickness of epidermis, and stratum corneum by approximately 18.3 %, 43.2 %, and 20.7 %, respectively (Fig. 1B). These results suggest that STS plays a crucial role in the excessive keratinization observed in XLI.

3.2. STS inhibition increases protein expression of E-cadherin and the keratinization markers involucrin and loricrin

To elucidate the detailed mechanism of excessive keratinization caused by STS deficiency, we examined the levels of E-cadherin, a

stratification and differentiation promoter in keratinocytes [18,19], and the keratinization markers involucrin and loricrin, components of the keratinization envelope [28–31]. Notably, reduced STS expression in Cas9-STS^{+/-} cells elevated E-cadherin, involucrin, and loricrin protein levels. Conversely, inducing STS expression in STS overexpressing had the opposite effect (Fig. 2A, B).

We also observed that STS regulation significantly affected E-cadherin mRNA expression regardless of its deficiency or overexpression but did not alter the mRNA levels of keratinization markers (Fig. 2C). This finding suggests that STS deficiency primarily impacts involucrin and loricrin expressions at the protein level. Lastly, we analyzed skin samples from STS KO and TG mice to verify if STS deficiency induced E-cadherin and keratinization marker expressions *in vivo*. The results exhibited a significant increase in E-cadherin, involucrin, and loricrin expression in STS KO mice, whereas STS overexpression in TG mice notably decreased these protein levels (Fig. 2D, E).

Delving deeper, STS downregulation in XLI is linked to keratinogenesis due to cholesterol sulfate accumulation [1,13]. STS-deficient mice and cellular models increased SULT2B1 expression, an integral enzyme in cholesterol sulfate synthesis (Fig. 3A, B, and Fig. S1A). Further experiments demonstrated that treating keratinocytes with cholesterol sulfate raised E-cadherin and keratinization marker levels (Fig. 3C, D). Overall, our findings indicate that STS deficiency

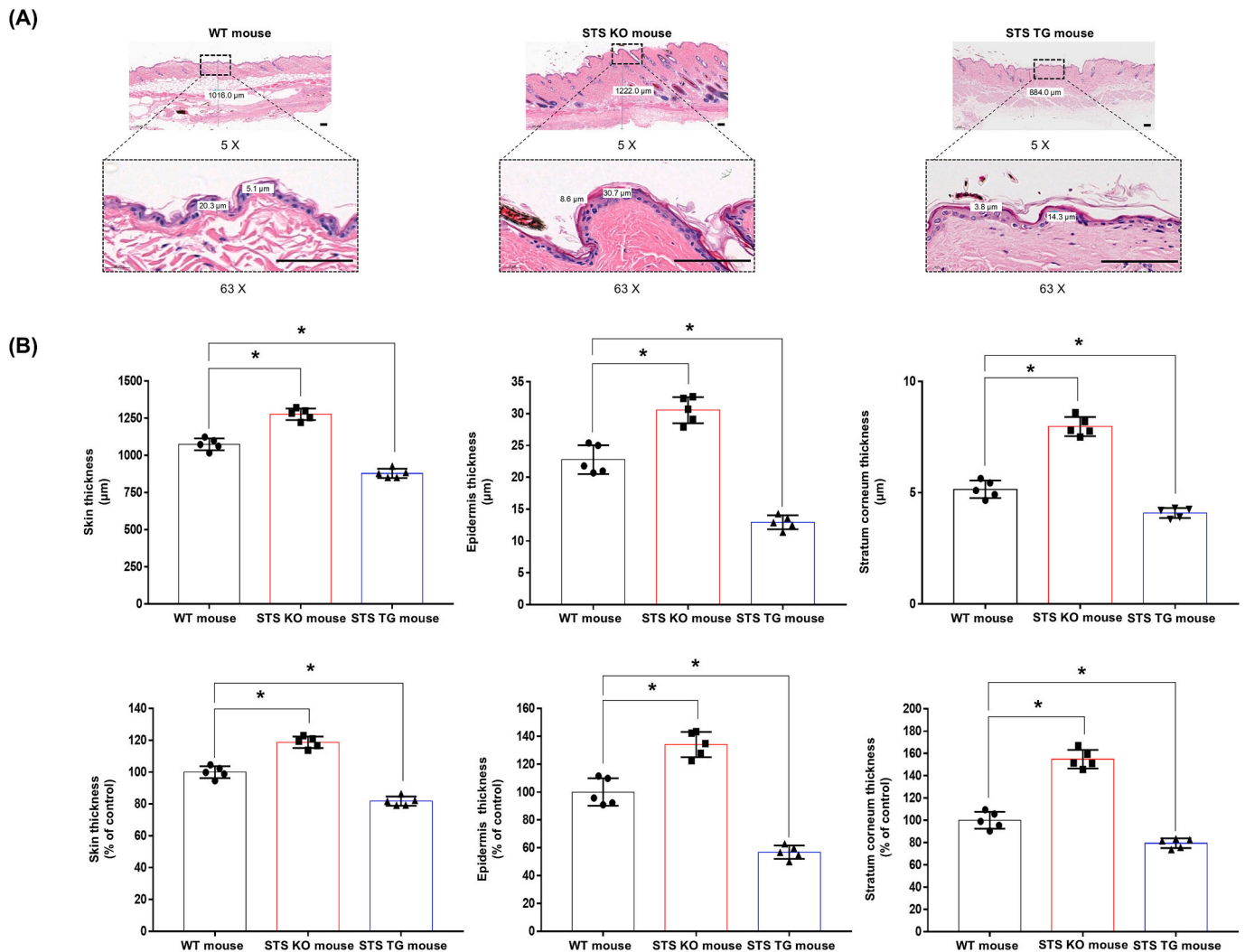
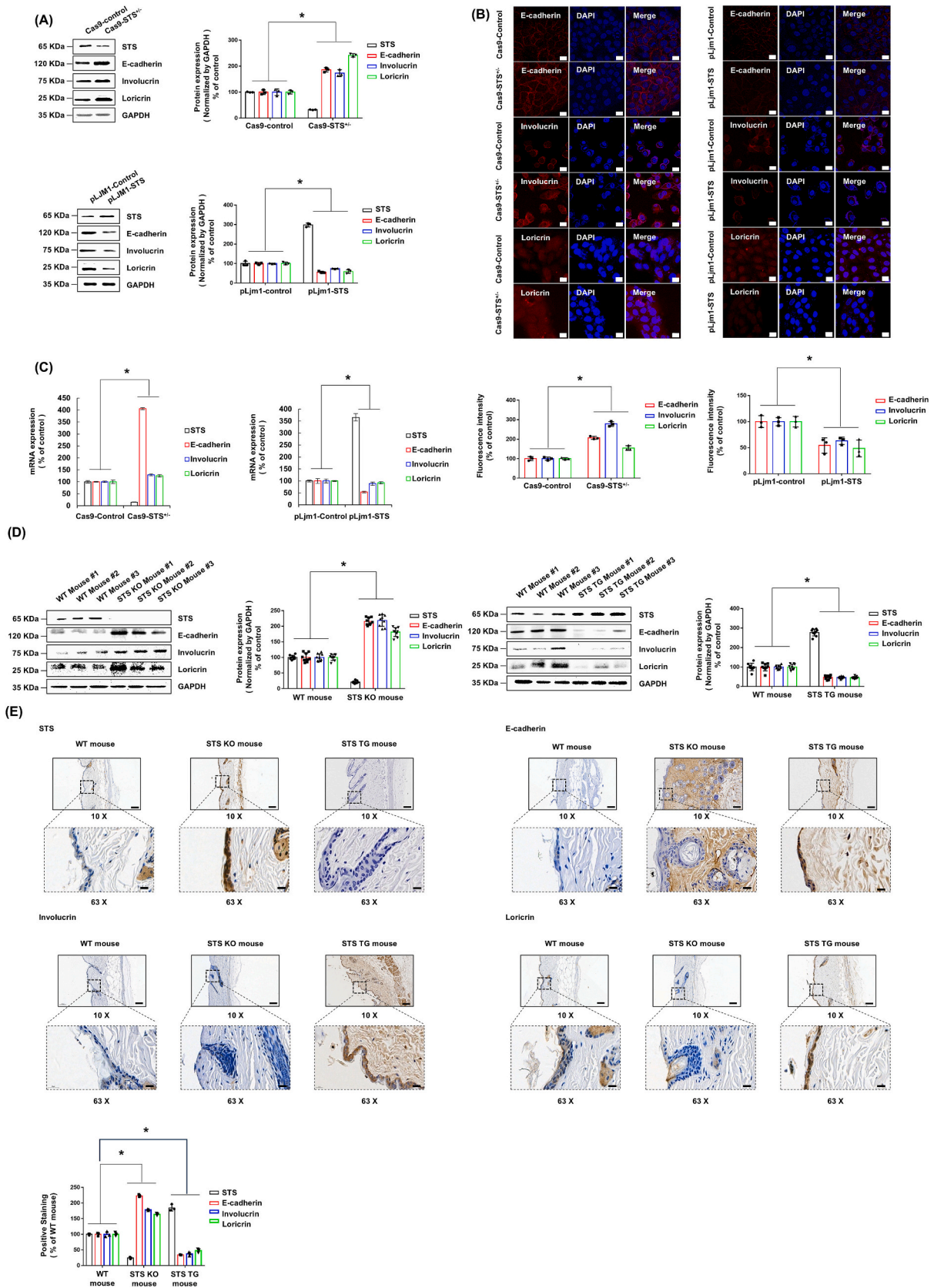


Fig. 1. STS deficiency results in the thick stratum corneum in mouse skin. (A) Representative images of H&E staining indicate differences in WT ($n = 5$), STS KO ($n = 5$), and STS TG mouse skin tissues ($n = 5$). Scale bars, 100µm. (B) Skin, epidermis, and stratum corneum lengths were measured. The data represent the mean $\pm$ SD ($n = 5$). * $p < 0.05$.



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Fig. 2. STS Deficiency induces E-cadherin and keratinization markers, such as involucrin and loricrin, in HaCaT cells. (A) Total cellular protein (20 μ g) was subjected to western blot analysis, and GAPDH was used as a loading control. Representative western blot images of STS, E-cadherin, involucrin, and loricrin protein levels in Cas9-STST^{+/−} or pLjm1-STST HaCaT cells. Each data point represents the mean $\pm$ SEM of three experiments. * p < 0.05 compared to the control. (B) Immunofluorescence staining was performed. Cells were fixed, incubated with E-cadherin, involucrin, and loricrin antibodies, stained with Alexa Fluor 594-labeled secondary antibody; and imaged using fluorescence microscopy. Blue = DAPI; Microscopy scale bar = 20 μ m. Cas9-STST^{+/−} or pLjm1-STST HaCaT cells were used. Representative immunofluorescence images of E-cadherin, involucrin, and loricrin expressions. (C) Real-time qPCR was performed to detect STS, E-cadherin, involucrin, and loricrin mRNA expressions in Cas9-STST^{+/−} HaCaT or pLjm1-STST HaCaT cells. Each data point represents the mean $\pm$ SEM of three experiments. * p < 0.05 compared to the control. (D) Total protein (40 μ g) from the skin tissue of WT, STS KO, and STS TG mice were subjected to western blot analysis, and GAPDH was used as a loading control. Each data point represents the mean $\pm$ SEM of three experiments. * p < 0.05 compared to the control. Representative western blot images of STS, E-cadherin, involucrin, and loricrin protein levels. (E) Immunohistochemistry (IHC) staining was performed on the skin of WT, STS KO, and STS TG mice. Representative IHC staining of STS, E-cadherin, involucrin, and loricrin expressions. Each data point represents the mean $\pm$ SEM of three experiments. * p < 0.05 compared to the control. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

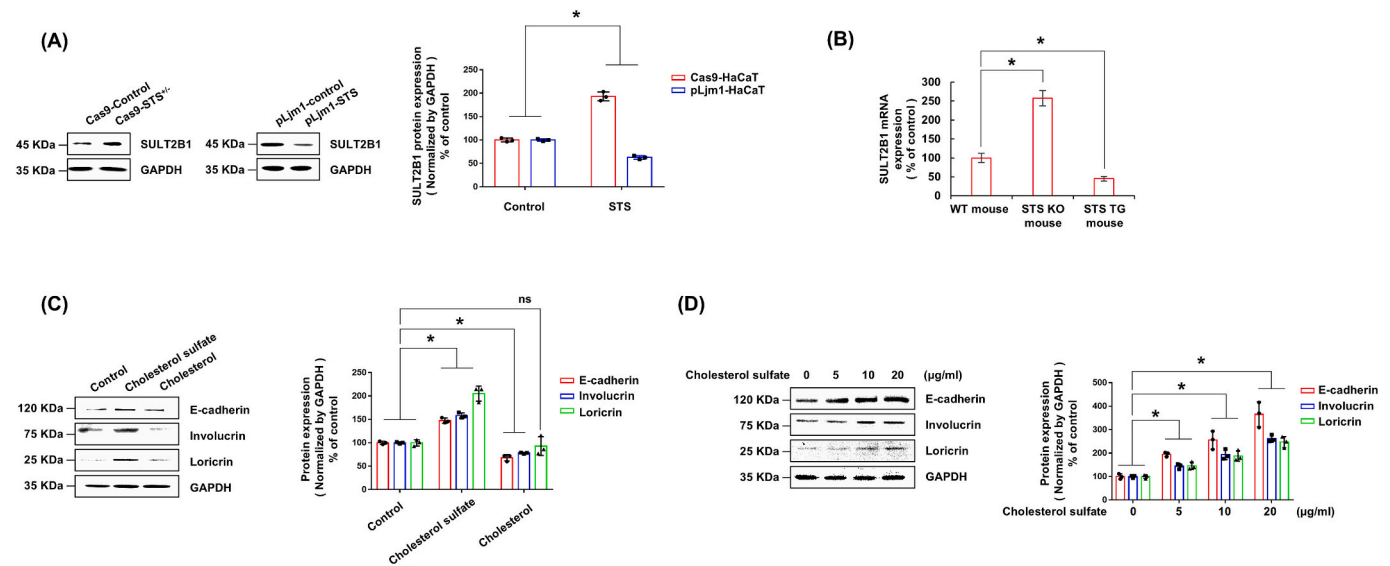


Fig. 3. Reduction of STS increases the expression of SULT2B1, while cholesterol sulfate induces E-cadherin and keratinization markers in keratinocytes. (A) Total cellular protein (20 μ g) was subjected to western blot analysis, and GAPDH was used as a loading control. Representative western blot images of SULT2B1 protein level in Cas9-STST^{+/−} or pLjm1-STST HaCaT cells. Each data point represents the mean $\pm$ SEM of three experiments. * p < 0.05 compared to the control. (B) Real-time qPCR was performed to detect SULT2B1 mRNA expression in the skin tissue of STS KO and STS TG mice. Each data point represents the mean $\pm$ SEM of three experiments. * p < 0.05 compared to the control. (C, D) Total cellular protein (20 μ g) was subjected to western blot analysis, and GAPDH was used as a loading control. Each data point represents the mean $\pm$ SEM of three experiments. * p < 0.05 compared to the control. Representative western blot images of E-cadherin, involucrin, and loricrin protein levels. (C) HaCaT cells were treated with cholesterol (20 ng/mL) or cholesterol-sulfate (20 ng/mL) for 48 h. (D) HaCaT cells were treated with cholesterol-sulfate (0, 5, 10, or 20 ng/mL) for 48 h.

substantially upregulates E-cadherin, involucrin, and loricrin expression in cultured keratinocytes and mouse skin, predominantly at the protein level.

3.3. STS deficiency stabilizes E-cadherin by suppressing Hakai expression, preventing its degradation

As shown in Fig. 2, diminishing STS increased E-cadherin protein expression. Therefore, we measured Hakai expression to determine the detailed mechanisms of E-cadherin protein upregulation [24]. Hakai protein expression was lower in STS-deficient cells and higher in STS-overexpressed cells compared to the control (Fig. 4A, B). Also, Hakai mRNA levels increased 2.2-fold following STS deficiency, whereas STS overexpression decreased them by 53.8 % (Fig. 4C).

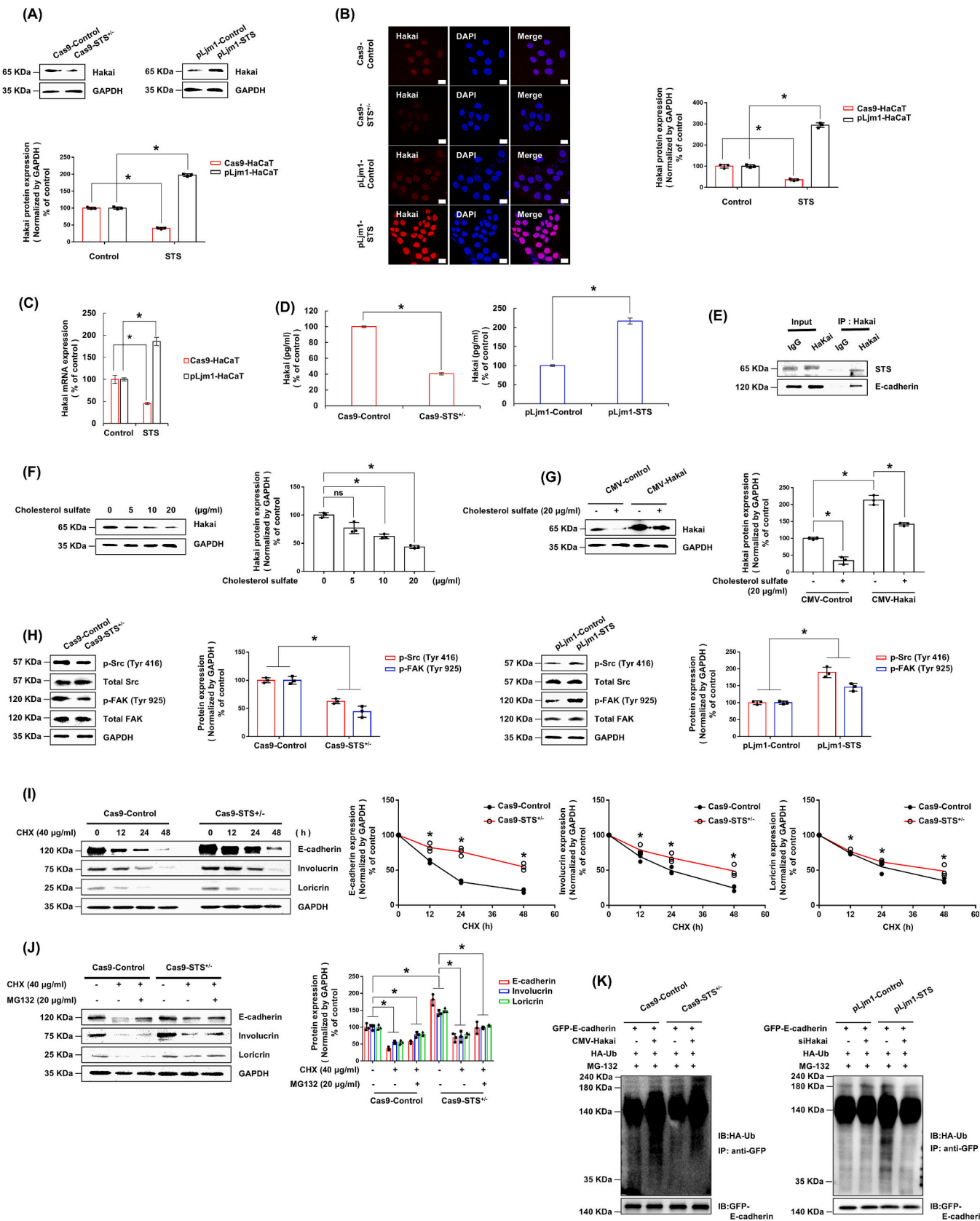
We conducted an enzyme-linked immunosorbent assay (ELISA) to measure Hakai levels and the correlating variations in STS expression to investigate how STS regulates Hakai expression. Consequently, inhibiting STS expression reduced Hakai levels by approximately 59.6 %, whereas overexpressing STS increased them 2.2-fold (Fig. 4D). Additionally, Fig. 4E demonstrates that immunoprecipitation in HaCaT cells confirmed interactions between Hakai and E-cadherin.

Keratinocytes were treated with cholesterol sulfate at different concentrations (0, 5, 10, or 20 ng/mL) to determine whether Hakai

downregulation also occurs due to STS deficiency effectuating cholesterol sulfate accumulation in XLI. We found that as cholesterol sulfate concentration increased, Hakai expression decreased (Fig. 4F). Moreover, cholesterol sulfate treatment following CMV-Hakai vector transfection significantly suppressed elevated Hakai expressions (Fig. 4G). Therefore, our results demonstrate that the cholesterol sulfate accumulation from STS deficiency may downregulate Hakai expression.

E-cadherin forms stabilized complexes with α -, β -, and p120-catenin, which are upregulated under STS-deficient conditions (Fig. S2A, B). Tyrosine must phosphorylate Src to disassemble the E-cadherin complex, enabling Hakai to bind to E-cadherin and its intracellular translocation through endocytosis and subsequent degradation [24]. In particular, the Src (Tyr 416) and focal adhesion kinase (FAK) (Tyr 925) regions are involved in E-cadherin de-regulation, and FAK (Tyr 925) regulates cell-cell junctions on E-cadherin [25].

Therefore, we investigated the tyrosine phosphorylation of Src (Tyr 416) and FAK (Tyr 925). We found that STS deficiency inhibited Tyr 416 and Tyr 925 residues from phosphorylating Src and FAK. On the other hand, STS overexpression augmented phosphorylation (Fig. 4H). Our RNA-seq data indicated no changes in Src and FAK mRNA levels under STS deficiency conditions, which can be interpreted as inhibiting tyrosine phosphorylation activity at the protein level (Fig. S2C). Additionally, Rack1, which suppresses Src-E-cadherin-Hakai interaction, was



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Fig. 4. Downregulation of STS reduces Hakai, subsequently inhibiting E-cadherin degradation in HaCaT cells. (A, F, G, H, I, J) Total cellular protein (20 µg) was subjected to western blot analysis, and GAPDH was used as a loading control. Each data point represents the mean ± SEM of three experiments. **p* < 0.05 compared to the control. (A) Representative western blot images of Hakai protein levels in Cas9-STS^{+/−} and pLJM1-STS HaCaT cells. (B) Immunofluorescence staining was performed. Cells were fixed, incubated with Hakai antibodies, stained with Alexa Fluor 594-labeled secondary antibody, and imaged using fluorescence microscopy. Blue = DAPI; Microscopy scale bar = 20 µm. Representative immunofluorescence images of Hakai expression in Cas9-STS^{+/−} and pLJM1-STS HaCaT cells. (C) Real-time qPCR was performed to detect Hakai mRNA expression in Cas9-STS^{+/−} HaCaT and pLJM1-STS HaCaT cells. Each data point represents the mean ± SEM of three experiments. **p* < 0.05 compared to the control. (D) ELISA was performed to measure Hakai expression in Cas9-STS^{+/−} or pLJM1-STS HaCaT cells. Each data point represents the mean ± SEM of three experiments. **p* < 0.05 compared to the control. (E) Total cellular protein (20 µg) was subjected to western blot analysis. Immunoprecipitation visualizes the interaction between Hakai and STS or E-cadherin in HaCaT cells. (F, G) Representative western blot images of Hakai protein levels in HaCaT cells. (F) HaCaT cells were treated with cholesterol-sulfate (0, 5, 10, or 20 µg/mL) for 48 h. (G) HaCaT cells were transfected with a CMV-Hakai vector for 48 h. (H) Representative western blot of p-Src (Tyr 416), Src, p-FAK (Tyr 925), and FAK protein levels in Cas9-STS^{+/−} and pLJM1-STS HaCaT cells. (I, J) Representative western blot images of E-cadherin, involucrin, and loricrin protein levels in Cas9-STS^{+/−} HaCaT cells. (I) Cas9-STS^{+/−} HaCaT cells were treated with cycloheximide (40 µg/mL) for 0, 12, 24, and 48 h. (J) Cas9-STS^{+/−} HaCaT cells were treated with cycloheximide (40 µg/mL) and MG132 (20 µg/mL) for 24 h. (K) Representative western blot images of E-cadherin degradation levels in Cas9-STS^{+/−} or pLJM1-STS HaCaT cells transfected with HA-ubiquitin, GFP-E-cadherin, and CMV-Hakai or siHakai for 24 h. After 24 h, cells were treated with MG132 (20 µg/mL) for 24 h before harvesting. Immunoprecipitation (IP) was performed using an anti-GFP antibody to detect GFP-E-cadherin (147–152 kDa). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

upregulated (Fig. S2D) [37].

Inhibiting Hakai expression and restricting its binding to the E-cadherin complex prevents E-cadherin degradation and stabilizes the protein. Therefore, we analyzed whether inhibiting E-cadherin stability, which was elevated in STS-deficient cells, affected keratinization. Cells were treated with protein biosynthesis inhibitor cycloheximide (CHX; 40 µg/mL) for 0, 12, 24, and 48 h with or without STS. STS deficient cells exhibited markedly reduced E-cadherin protein degradation compared to control cells. In particular, rapid E-cadherin degradation was observed after 12 h of CHX treatment. Surprisingly, the increase in E-cadherin proteolysis also affected the degradation rates of involucrin and loricrin (Fig. 4I).

Then, cells were treated with the proteasome inhibitor MG132 and CHX to determine whether proteasome inhibition prevents E-cadherin degradation. The degradation of E-cadherin by CHX was restored by MG132 (Fig. 4J). Lastly, to demonstrate that these effects occurred through ubiquitin-proteasome-mediated degradation, we measured E-cadherin ubiquitination levels after transfecting STS-deficient or over-expressing cells with the overexpressed Hakai vector or Hakai siRNA. Cells exhibiting increased STS or Hakai expression demonstrated heightened ubiquitination levels. Conversely, a reduction in ubiquitination was observed in cells where STS or Hakai levels were diminished (Fig. 4K).

Taken together, our data suggest that STS deficiency may down-regulate Hakai expression, potentially influenced by the accumulation of cholesterol sulfate. Furthermore, increased E-cadherin expression and stability may result from reducing Hakai expression and interfering with the binding of the E-cadherin complex to Hakai, potentially through inhibition of specific tyrosine phosphorylation events, such as Src (Tyr 416) and FAK (Tyr 925).

3.4. Mutations in the N-terminal region of E-cadherin disrupted the expression of involucrin and loricrin

Fig. 4I shows that the extent to which CHX degrades E-cadherin in STS-deficient conditions also affects the degradation rates of involucrin and loricrin. As such, we explored the regulatory role of E-cadherin on these protein markers. Our findings revealed that siRNA-mediated E-cadherin inhibition significantly reduced the protein levels of these keratinization markers, while E-cadherin overexpression enhanced their expression (Fig. 5A).

Furthermore, when E-cadherin induction, triggered by STS deficiency, was transiently suppressed using E-cadherin siRNA, involucrin and loricrin expressions notably decreased (Fig. 5B, C). Conversely, using an E-cadherin vector to restore E-cadherin expression in STS-overexpressed cells increased these keratinization markers (Fig. 5D). This pattern was consistent across various treatments, including STX-64, a potent STS inhibitor, TNF-α, known to induce STS, or cholesterol

sulfate (Fig. S3A-C) [7,9,34]. Delving deeper, we examined the effects of E-cadherin degradation on keratinization markers. Hakai over-expression promoted E-cadherin degradation, which subsequently suppressed involucrin and loricrin expression. In contrast, inhibiting Hakai prevented E-cadherin degradation, resulting in an upregulation of these markers (Fig. 5E, F).

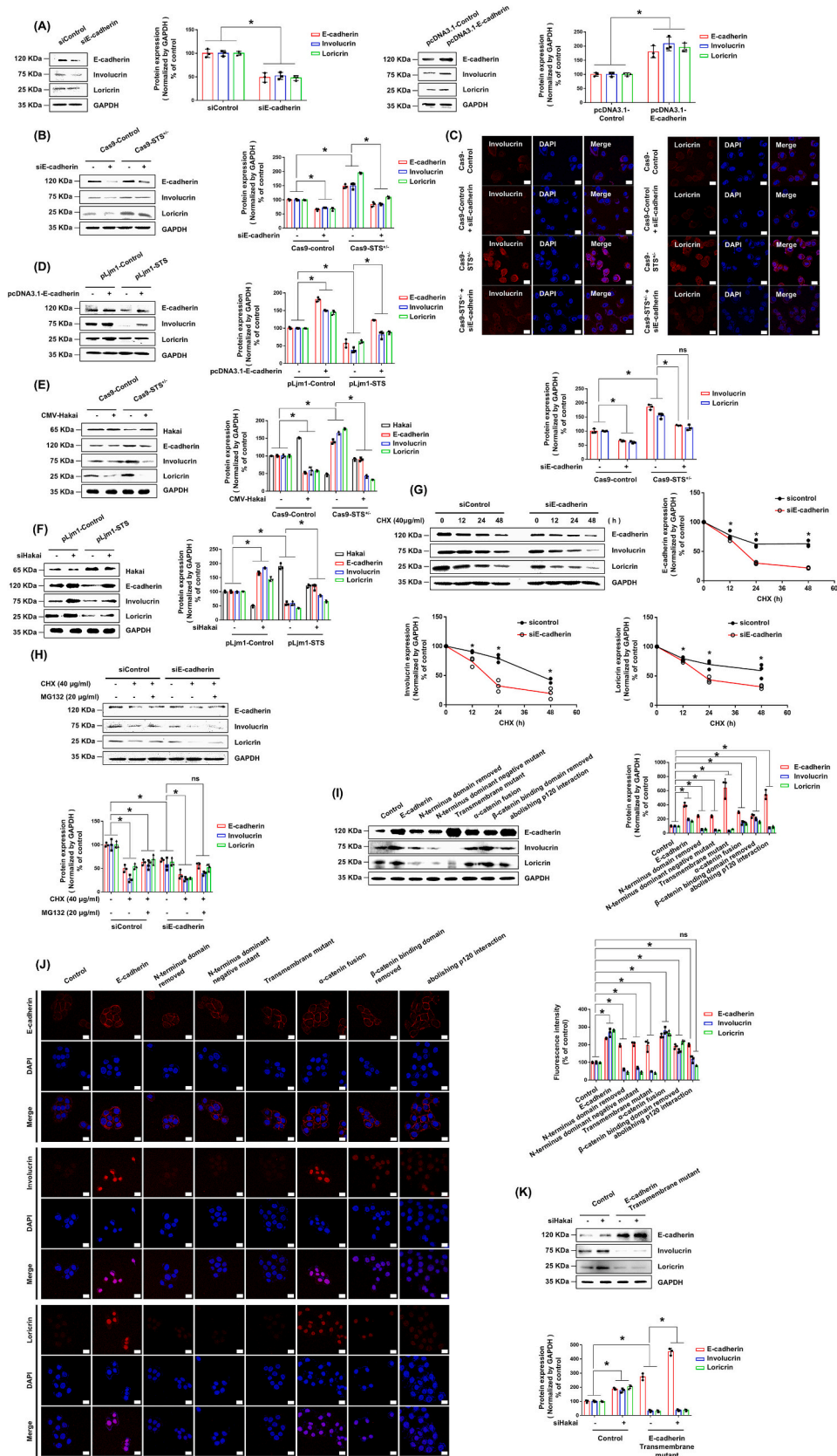
Next, we conducted further experiments to understand how E-cadherin influences keratinization markers. We treated cells with CHX (40 µg/mL) for varying durations (0, 12, 24, and 48 h) and observed their effect on E-cadherin and the keratinization markers involucrin and loricrin. Notably, upon inhibition of protein synthesis and siE-cadherin treatment, these keratinization markers showed decreased stability. (Fig. 5G).

Interestingly, E-cadherin degraded rapidly within 12 h of CHX treatment, a pattern mirrored by the keratinization markers. Thus, we introduced the proteasome inhibitor MG132 and CHX to ascertain if the degradation of these markers could be inhibited by blocking the proteasome pathway. MG132 treatment restored the stability of keratinization markers affected by the degradation caused by E-cadherin inhibition. (Fig. 5H). Our findings indicate that E-cadherin's stability and degradation rate closely correlate with keratinization marker regulation. Notably, E-cadherin overexpression upregulated involucrin and loricrin.

In addition, we explored the impact of structural mutations in E-cadherin for a more comprehensive understanding of the mechanism behind E-cadherin's influence on these markers. Cells were transfected with mutant vectors targeting E-cadherin's extracellular, trans-membrane, and intracellular domains. Some of the vectors we used lost the cell adhesion ability of E-cadherin, and a detailed schematic of the vectors is shown in Fig. S4A [33]. Transfection with all mutation vectors increased E-cadherin expression at the mRNA level (Fig. S4B).

Our results substantiated that these mutations, especially in the N-terminal region of the extracellular domain crucial for calcium interaction and cell adhesion, significantly reduced involucrin and loricrin expressions (Fig. 5I, J). Furthermore, the effects of Hakai on keratinization markers were examined. To minimize the influence of E-cadherin, a transmembrane mutant vector of E-cadherin was used. In the control group, Hakai inhibition led to a significant increase in the expression of keratinization markers. In contrast, this increase was absent in the E-cadherin mutant group (Fig. 5K). These findings suggest that the expression of keratinization markers is more likely regulated by E-cadherin functions, such as cell adhesion and calcium interactions, rather than by the direct effects of Hakai.

This finding suggests that E-cadherin's cell adhesion capability, especially its interaction with calcium, is integral for regulating these keratinization markers. Our study highlights the pivotal role of E-cadherin, particularly its extracellular domain, in modulating the keratinization process under STS deficiency conditions.



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Fig. 5. Upregulation of E-cadherin induces keratinization markers in HaCaT cells. (A, B, D, G-I, K) Total cellular protein (20 μ g) was subjected to western blot analysis, and GAPDH was used as a loading control. Representative western blot images of E-cadherin, involucrin, and loricrin protein levels. Each data point represents the mean $\pm$ SEM of three experiments. * p < 0.05 compared to the control. (A) HaCaT cells were transfected with siE-cadherin or pcDNA3.1-E-cadherin for 24 h. (B, C) Cas9-STST^{+/−} HaCaT cells were transfected with siE-cadherin for 24 h. (C) Immunofluorescence staining was performed. Cells were fixed, incubated with involucrin and loricrin antibodies, stained with Alexa Fluor 594-labeled secondary antibody, and imaged using fluorescence microscopy. Blue = DAPI; Microscopy scale bar = 20 μ m. Representative immunofluorescence images of involucrin and loricrin expression in Cas9-STST^{+/−} HaCaT cells. (D) pLjm1-STST HaCaT cells were transfected with pcDNA3.1-E-cadherin for 24 h. (E, F) Total cellular protein (20 μ g) was subjected to western blot analysis, and GAPDH was used as a loading control. Representative western blot images of Hakai, E-cadherin, involucrin, and loricrin protein levels. Each data point represents the mean $\pm$ SEM of three experiments. * p < 0.05 compared to the control. (E) Cas9-STST^{+/−} HaCaT cells were transfected with CMV-Hakai for 24 h. (F) pLjm1-STST HaCaT cells were transfected with siHakai for 24 h. (G, H) HaCaT cells were transfected with siE-cadherin for 24 h. (G) siE-cadherin HaCaT cells were treated with cycloheximide (40 μ g/mL) for 0, 12, 24, and 48 h. (H) siE-cadherin HaCaT cells were treated with cycloheximide (40 μ g/mL) and MG132 (20 μ g/mL) for 24 h. (I, J) HaCaT cells were transfected with vectors for wild-type E-cadherin, N-terminus domain removed, N-terminus dominant negative mutant, transmembrane mutation, α -catenin fusion, β -catenin binding domain removed, or abolishing p120 interaction for 24 h. (J) Immunofluorescence staining was performed. Cells were fixed; incubated with E-cadherin, involucrin, and loricrin antibodies; stained with Alexa Fluor 594-labeled secondary antibody; and imaged using fluorescence microscopy. Blue = DAPI; Microscopy scale bar = 20 μ m. Representative immunofluorescence images of E-cadherin, involucrin, and loricrin expression in HaCaT cells. (K) HaCaT cells were transfected with vector for transmembrane mutation and siHakai for 24 h. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.5. MiR-6766 treatment reduced E-cadherin and suppressed keratinization marker expression

Our study demonstrated a strong association between STS deficiency-mediated hyperkeratinization of XLI and E-cadherin upregulation by Hakai reduction. We explored potential treatments targeting E-cadherin to address hyperkeratinization. Treatment with E-cadherin antibodies at various concentrations (0, 2.5, 5, or 10 μ g/mL) induced a significant, dose-dependent decrease in the keratinization markers involucrin and loricrin (Fig. 6A). Likewise, keratinization marker expressions that were increased upon STS deficiency were significantly reduced by E-cadherin antibody treatment (Fig. 6B).

We extended our investigation to small molecules, specifically miRNAs. Using a database (miRDB; <http://www.mirdb.org/>), we identified E-cadherin-related miRNAs affected by STS activity (Fig. S5A). After selecting 10 miRNAs by target score, their mRNA levels were evaluated under STS inhibition or overexpression (Fig. S5B). After evaluating several miRNAs, we found that miR-6766 was the most effective for reducing E-cadherin levels, leading to a corresponding decrease in keratinization markers (Fig. S5C, D).

Furthermore, miR-6766 treatment (0, 25, 50, or 100 nM) concentration-dependently decreased E-cadherin expression and effectively inhibited the increase in E-cadherin expression under STS-deficient conditions, thereby suppressing keratinization marker expression (Fig. 6C, D). Additional experiments using the miR-6766 inhibitor demonstrated a dose-dependent elevation in both E-cadherin and keratinization markers (Fig. 6E).

This restoration, especially where STS-induced suppression of E-cadherin transpired, highlights miR-6766's specific impact on this key protein (Fig. 6F). Fig. S5E showcases the mRNA levels of miR-6766-3p miRNA and E-cadherin dependent on miR-6766 or inhibitor concentration. Next, we conducted *in vivo* transfections on STS KO mice to confirm if these results were consistent in living organisms. The miR-6766 mixture was subcutaneously injected three times at 3-day intervals; Fig. S5F presents the possible effects of miR-6766 on mouse E-cadherin sequences.

Fig. S5G displays miRNA and E-cadherin mRNA expression levels by miR-6766 treatment. As a result, this was corroborated by *in vivo* experiments on STS KO mice, where subcutaneous miR-6766 injections reduced elevated E-cadherin levels and associated keratinization markers (Fig. 6G). Overall, our findings highlighted the pivotal role of E-cadherin in the keratinization process associated with XLI. Therefore, modulating E-cadherin expression through targeted small molecule interventions, such as miR-6766, is a promising strategy for managing hyperkeratinization in XLI.

4. Discussion

This study investigated the effects of STS deficiency on skin hyperkeratinization in XLI by examining gene expressions in skin tissues from STS KO and TG mouse models and human keratinocyte HaCaT cells. Our mouse model demonstrated that STS KO mice developed stratum corneum thickening, indicative of XLI disease (Fig. 1). Therefore, our mouse model is suitable for studying keratinization in XLI disease. Our STS mice model allows for genetic manipulation through *in vivo* transfection and complements previous studies of limited analyses, such as metabolite levels or gene expression patterns in the serum of diseased patients with XLI [4,38,39]. Thus, we can provide valuable insights into the mechanisms underlying XLI.

According to the study, XLI patients exhibited high calcium content in the stratum corneum [11]. Importantly, E-cadherin is a calcium-dependent cell-cell adhesion molecule pivotal for keratinocyte differentiation [18,19]. As such, the elevated calcium content in XLI patients further emphasizes the importance of E-cadherin. Additionally, Calcium signaling enhances cholesterol sulfate accumulation. SULT2B1 expression in keratinocytes increased during calcium-induced differentiation [11,40], which indicated enhanced calcium signaling and keratinocyte differentiation due to STS deficiency are expected and warrant further study.

SULT2B1 is responsible for the most cholesterol sulfate production. Previous studies report that SULT2B1 expression elevates protein and mRNA levels in keratinocytes undergoing calcium-induced differentiation [11,40,41]. As shown in Fig. 3A, B, and Fig. S1A, we found that SULT2B1 expression increased under STS-deficient conditions. Therefore, the absence of STS prevents the conversion of cholesterol sulfate to cholesterol, simultaneously escalating cholesterol sulfate production by upregulating SULT2B1. Consequently, this results in a rapid accumulation of cholesterol sulfate, accelerating keratinocyte differentiation. This process compromises the skin barrier, ultimately culminating in hyperkeratinization.

Our study discovered that STS deficiency significantly induces E-cadherin expression. Moreover, E-cadherin regulation by STS is an imperative factor to consider. In our previous study, we established experimental conditions in which STS expression in keratinocyte cells was suppressed. As a result, we found that the expression of EMT factors like Slug and Snail, which suppress E-cadherin transcription [32], were suppressed. We further investigated how E-cadherin is regulated at the protein level. Notably, this upregulation of E-cadherin is linked to reduced levels of Hakai, the E3 ligase responsible for E-cadherin degradation. This down-regulation is predicated upon multiple factors. First, we observed that Hakai associates with STS, indicating that STS may directly influence Hakai activity (Fig. 4A-D). Second, cholesterol sulfate accumulation, a consequence of STS deficiency, further diminishes Hakai expression (Fig. 4F, G). Thus, cholesterol sulfate buildup

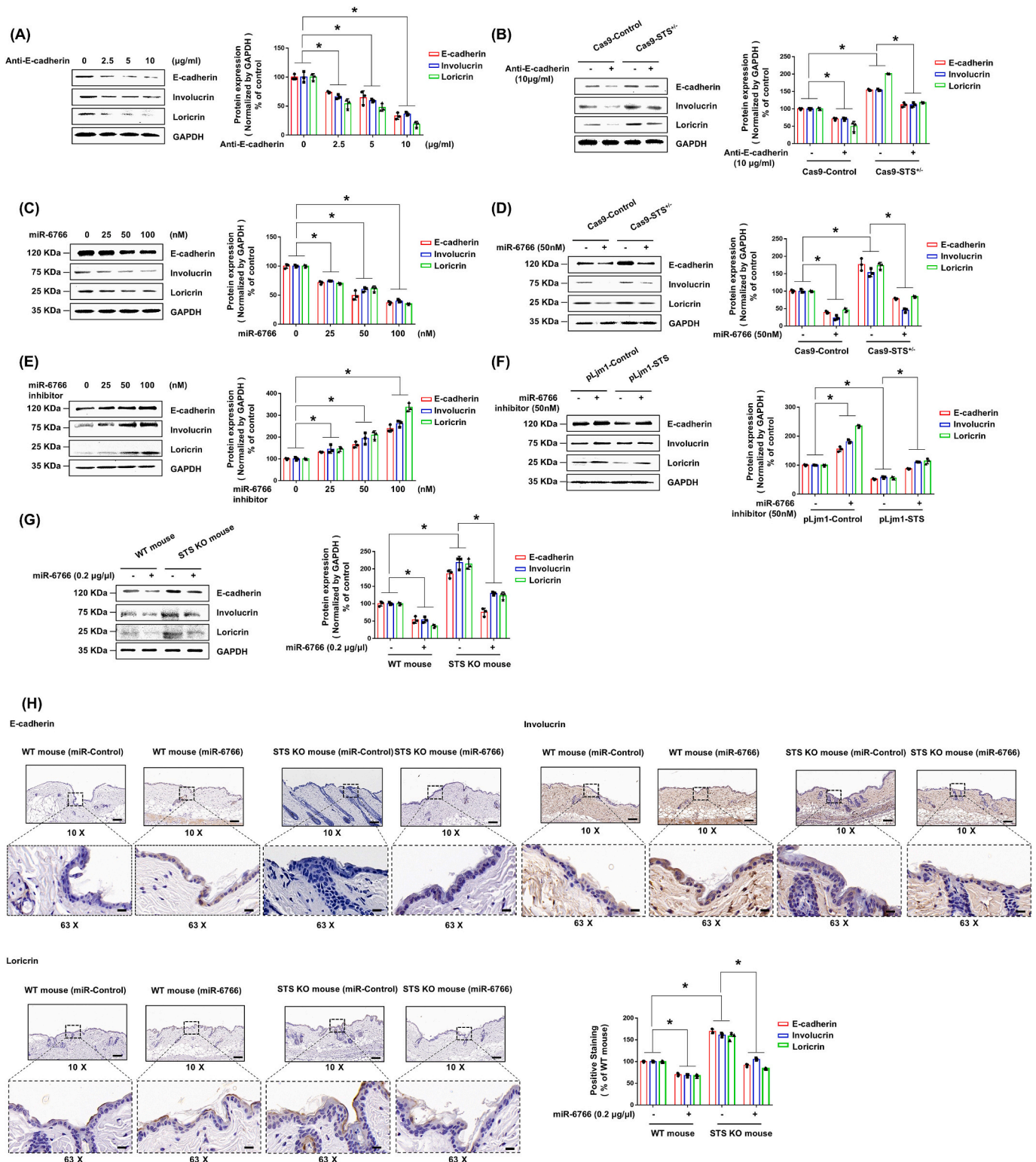


Fig. 6. miR-6766-inhibited E-cadherin downregulates the keratinization markers involucrin and loricrin in HaCaT cells. (A-F) Total cellular protein (20 μ g) was subjected to western blot analysis, and GAPDH was used as a loading control. (A-G) Representative western blot images of E-cadherin, involucrin, and loricrin protein levels. Each data point represents the mean $\pm$ SEM of three experiments. $^*p < 0.05$ compared to the control. (A) HaCaT cells were treated with anti-E-cadherin (0, 2.5, 5, or 10 μ M) for 48 h. (B) Cas9-STS^{+/+} HaCaT cells were treated with anti-E-cadherin (0 or 10 μ M) for 48 h. (C) HaCaT cells were transfected with miR-6766 (0, 25, 50, or 100 nM) for 24 h. (D) Cas9-STS^{-/-} HaCaT cells were transfected with miR-6766 (0 or 50 nM) for 24 h. (E) HaCaT cells were transfected with miR-6766 inhibitor (0, 25, 50, or 100 nM) for 24 h. (F) pLjm1-STS HaCaT cells were transfected with miR-6766 inhibitor (0 or 50 nM) for 24 h. (G) Total protein (40 μ g) from the skin of WT and STS KO mice (miR-6766-injected mice) subjected to western blot analysis. GAPDH was used as a loading control. (H) Immunohistochemistry (IHC) staining was performed on the skin of WT and STS KO mice (miR-6766-injected mice). Representative IHC staining images of E-cadherin, involucrin, and loricrin expressions.

due to a lack of STS significantly impacts Hakai levels. A final factor to consider is TGF- β signaling. TGF- β transcriptionally induces Hakai and, in conjunction, promotes E-cadherin monoubiquitination [42]. Furthermore, our previous research found that STS deficiency curtails TGF- β 1 expression in keratinocytes [32].

E-cadherin degradation initiates with Src kinases phosphorylating tyrosine, resulting in E-cadherin being drawn endocytosis and then marked for ubiquitination by Hakai [37,43]. Previous studies reported that integrins activate Src and FAK. This activation at specific sites drives E-cadherin endocytosis and attracts Hakai to the E-cadherin complex [38,44]. In our previous study, we found that increasing STS levels in cells enhances integrin signaling, prompting the activation of tyrosine phosphorylation in Src (Tyr 416) and FAK (Tyr 925) [45]. However, this phosphorylation did not occur when STS was decreased in keratinocytes, as opposed to conditions with increased STS. Consequently, FAK (Tyr 925) and Src (Tyr 416) phosphorylation were not observed, as demonstrated in Fig. 4H. It is important to emphasize that increased tyrosine phosphorylation is crucial for early keratinocyte differentiation, particularly in calcium signaling [46,47]. Interestingly, the specific tyrosine phosphorylation type associated with cell adhesion tends to decrease during keratinocyte differentiation. This is particularly true for the tyrosine phosphorylation involved in E-cadherin complex formation, which is typically reduced during differentiation [24,46]. Thus, our results suggest that inhibiting specific tyrosine phosphorylation on E-cadherin in STS-deficient conditions stabilizes E-cadherin and reduces its degradation by Hakai.

Remarkably, our findings indicate that stabilizing and enhancing E-cadherin expression increases the stability and expression of the keratinization markers involucrin and loricrin. In particular, previous research has demonstrated that cell adhesion is pivotal in inducing these keratinization markers [48]. Our studies further reveal that STS deficiency significantly impairs cell motility, enhancing cell-cell adhesion [32]. This observation aligns with the upregulation of proteins such as α -, β -, and p120-catenin, which fortify cell-cell junctions by forming complexes with E-cadherin (Fig. S2A, and B). Moreover, the increased expression of Rack1, known to stabilize E-cadherin by disrupting the Src-E-cadherin-Hakai interaction, substantiates this adhesion enhancement in STS-deficient conditions (Fig. S2D) [37]. Therefore, we hypothesize that the heightened cell-cell adhesion mediated by E-cadherin due to STS deficiency significantly contributes to hyperkeratinization.

Our experiments using mutant vectors for E-cadherin's extracellular, transmembrane, and intracellular domains revealed notable patterns. Specifically, the loss of the N-terminal domain in the extracellular domain markedly reduced involucrin and loricrin expression (Fig. 5I, J). This finding suggests that inhibiting cell adhesion or interrupting E-cadherin's interaction with calcium critically influences these keratinization markers. Interestingly, mutations in the α - and β -catenin binding domains increased the expression of these keratinization markers compared to controls. In contrast, mutations that disrupted the binding of p120-catenin to E-cadherin slightly suppressed involucrin and loricrin expression. This effect was likely due to p120-catenin failing to bind E-cadherin and the resulting increased intracellular trafficking of E-cadherin promoted by E-cadherin-Hakai binding. However, the correlation between p120-catenin and keratinization markers requires further study.

XLI is associated with a range of extracutaneous features, including cardiac arrhythmia [49]. Interestingly, evidence suggests that E-cadherin levels are elevated in the cardiac tissue of patients with arrhythmia [50]. In our discussion, we suggest that these findings could apply not only to skin phenotypes and treatments but also to extracutaneous symptoms and therapies. Our study suggests that the increase in E-cadherin protein resulting from STS deficiency may be due to reduced Hakai expression, which potentially limits proteasomal degradation. Interestingly, mutations in the N-terminal region of E-cadherin significantly reduced the expression of keratinization markers, indicating that E-cadherin plays a critical role in the keratinization process in XLI.

Targeting E-cadherin using specific inhibitors, such as anti-E-cadherin antibodies and E-cadherin-targeted microRNA (miR-6766), effectively reduced keratinization marker expression in both cell cultures and mouse models. This approach opens new avenues for developing therapeutic strategies for XLI, laying the foundation for future studies aimed at finding effective treatments for XLI and related skin disorders.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbamcr.2025.119898>.

CRediT authorship contribution statement

Tae-Uk Kwon: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Yeo-Jung Kwon:** Writing – review & editing, Validation, Investigation, Formal analysis. **Hyemin Park:** Investigation, Formal analysis. **Hyein Lee:** Formal analysis. **Ji-Heung Kwak:** Investigation. **Keon Wook Kang:** Writing – review & editing. **Young-Jin Chun:** Writing – review & editing, Validation, Supervision, Resources, Funding acquisition, Conceptualization.

Compliance with ethical standards

All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Chung-Ang University (2017-00096, 2019-00003) and were performed adhering to the approved protocol guidelines by IACUC.

Declaration of competing interest

The authors declare no conflicts of interest.

Acknowledgments

This research was supported by the National Research Foundation of Korea (NRF), funded by the Korean government (MSIP) (NRF-2021R1A2C201239513 and NRF-2022R1A5A600076012) and Chung-Ang University Research Grants in 2023. The funding agency had no role in the study design, data collection, analysis, publication decision, or manuscript preparation.

Data availability

All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials.

References

- [1] N.F. Fernandes, C.K. Janniger, R.A. Schwartz, X-linked ichthyosis: an oculocutaneous genodermatosis, *J. Am. Acad. Dermatol.* 62 (3) (2010) 480–485, <https://doi.org/10.1016/j.jaad.2009.04.028>.
- [2] R.S. Wells, C.B. Kerr, Clinical features of autosomal dominant and sex-linked ichthyosis in an English population, *Br. Med. J.* 1 (5493) (1966) 947–950, <https://doi.org/10.1136/bmj.1.5493.947>.
- [3] H. Chouk, S. Saad, S. Dimassi, N.G. Fetoui, A. Bennour, R. Gammoudi, H. Elmaouk, A. Saad, M. Denguezli, D. H'mida, X-linked recessive ichthyosis in 8 Tunisian patients: awareness of misdiagnosis due to the technical trap of the pseudogene, *BMC Med. Genomics* 15 (1) (2022) 165, <https://doi.org/10.1186/s12920-022-01319-4>.
- [4] Y. Song, J. Chen, Z. Yi, X. Dang, D. Cheng, X. Wu, Y. Tan, Genetic analysis of a 12-year-old boy with X-linked ichthyosis in association with sclerosing glomerulonephritis, *Mol. Med. Rep.* 8 (4) (2013) 1183–1187, <https://doi.org/10.3892/mmr.2013.1625>.
- [5] A. Marinković-Ilsen, J.G. Koppe, A.C. Jöbsis, W.P. de Groot, Enzymatic basis of typical X-linked ichthyosis, *Lancet* 2 (8099) (1978) 1097, [https://doi.org/10.1016/s0140-6736\(78\)91831-7](https://doi.org/10.1016/s0140-6736(78)91831-7).
- [6] L.J. Shapiro, R. Weiss, M.M. Buxman, J. Vidgoff, R.L. Dimond, J.A. Roller, R. S. Wells, Enzymatic basis of typical X-linked ichthyosis, *Lancet* 2 (8093) (1978) 756–757, [https://doi.org/10.1016/s0140-6736\(78\)92646-6](https://doi.org/10.1016/s0140-6736(78)92646-6).
- [7] P. Nussbaumer, A. Billich, Steroid sulfatase inhibitors, *Med. Res. Rev.* 24 (4) (2004) 529–576, <https://doi.org/10.1002/med.20008>.

- [8] T. Sugawara, E. Nomura, N. Hoshi, Both N-terminal and C-terminal regions of steroid sulfatase are important for enzyme activity, *J. Endocrinol.* 188 (2) (2006) 365–374, <https://doi.org/10.1677/joe.1.06417>.
- [9] M.J. Reed, A. Purohit, L.W. Woo, S.P. Newman, B.V. Potter, Steroid sulfatase: molecular biology, regulation, and inhibition, *Endocr. Rev.* 26 (2) (2005) 171–202, <https://doi.org/10.1210/er.2004-0003>.
- [10] J.W. Mueller, L.C. Gilligan, J. Idkowiak, W. Arlt, P.A. Foster, The regulation of steroid action by sulfation and desulfation, *Endocr. Rev.* 36 (5) (2015) 526–563, <https://doi.org/10.1210/er.2015-1036>.
- [11] P.M. Elias, M.L. Williams, E.H. Choi, K.R. Feingold, Role of cholesterol sulfate in epidermal structure and function: lessons from X-linked ichthyosis, *Biochim. Biophys. Acta* 1841 (3) (2014) 353–361, <https://doi.org/10.1016/j.bbap.2013.11.009>.
- [12] E. Zettersten, M.Q. Man, J. Sato, M. Denda, A. Farrell, R. Ghadially, M.L. Williams, K.R. Feingold, P.M. Elias, Recessive x-linked ichthyosis: role of cholesterol-sulfate accumulation in the barrier abnormality, *J. Invest. Dermatol.* 111 (5) (1998) 784–790, <https://doi.org/10.1046/j.1523-1747.1998.00386.x>.
- [13] M.L. Williams, P.M. Elias, Stratum corneum lipids in disorders of cornification: increased cholesterol sulfate content of stratum corneum in recessive x-linked ichthyosis, *J. Clin. Invest.* 68 (6) (1981) 1404–1410, <https://doi.org/10.1172/jci110391>.
- [14] H.H. Bragulla, D.G. Homberger, Structure and functions of keratin proteins in simple, stratified, keratinized and cornified epithelia, *J. Anat.* 214 (4) (2009) 516–559, <https://doi.org/10.1111/j.1469-7580.2009.01066.x>.
- [15] E.J. Van Scott, R.J. Yu, Hyperkeratinization, corneocyte cohesion, and alpha hydroxy acids, *J. Am. Acad. Dermatol.* 11 (5 pt 1) (1984) 867–879, [https://doi.org/10.1016/s0190-9622\(84\)80466-1](https://doi.org/10.1016/s0190-9622(84)80466-1).
- [16] E. Zingkou, G. Pampalakis, G. Sotiropoulou, Keratinocyte differentiation and proteolytic pathways in skin (patho) physiology, *Int. J. Dev. Biol.* 66 (1–2–3) (2022) 269–275, <https://doi.org/10.1387/ijdb.210161gs>.
- [17] X.H. Lian, T. Yang, M.M. Xiang, Roles of tissue plasminogen activator in epidermal stratification, *Colloids Surf. B Biointerfaces* 27 (2–3) (2003) 231–240, [https://doi.org/10.1016/S0927-7765\(02\)00077-2](https://doi.org/10.1016/S0927-7765(02)00077-2).
- [18] M. Takeichi, Cadherins - a molecular family important in selective cell-cell adhesion, *Annu. Rev. Biochem.* 59 (1990) 237–252, <https://doi.org/10.1146/annurev.bi.59.070190.001321>.
- [19] M.E. Bracke, R.F.M. Van, M.M. Mareel, The E-cadherin/catenin complex in invasion and metastasis, *Curr. Top. Microbiol. Immunol.* 213 (Pt 1) (1996) 123–161, https://doi.org/10.1007/978-3-642-61107-0_9.
- [20] I.O. Ellis, M. Galea, N. Broughton, A. Locker, R.W. Blamey, C.W. Elston, Pathological prognostic factors in breast cancer. II. Histological type. Relationship with survival in a large study with long-term follow-up, *Histopathology* 20 (6) (1992) 479–489, <https://doi.org/10.1111/j.1365-2559.1992.tb01032.x>.
- [21] R. Singhai, V.W. Patil, S.R. Jaiswal, S.D. Patil, M.B. Tayade, A.V. Patil, E-cadherin as a diagnostic biomarker in breast cancer, *N. Am. J. Med. Sci.* 3 (5) (2011) 227–233, <https://doi.org/10.4297/najms.2011.3227>.
- [22] P. Coopman, A. Djiane, Adherens junction and E-cadherin complex regulation by epithelial polarity, *Cell. Mol. Life Sci.* 73 (18) (2016) 3535–3553, <https://doi.org/10.1007/s00018-016-2260-8>.
- [23] Y. Shen, D.S. Hirsch, C.A. Sasiela, W.J. Wu, Cdc42 regulates E-cadherin ubiquitination and degradation through an epidermal growth factor receptor to Src-mediated pathway, *J. Biol. Chem.* 283 (8) (2008) 5127–5137, <https://doi.org/10.1074/jbc.M703300200>.
- [24] Y. Fujita, G. Krause, M. Scheffner, D. Zechner, H.E.M. Leddy, J. Behrens, T. Sommer, W. Birchmeier, Hakai, a c-Cbl-like protein, ubiquitinates and induces endocytosis of the E-cadherin complex, *Nat. Cell Biol.* 4 (3) (2002) 222–231, <https://doi.org/10.1038/ncb758>.
- [25] E. Avizienyte, A.W. Wyke, R.J. Jones, G.W. McLean, M.A. Westhoff, V.G. Brunton, M.C. Frame, Src-induced de-regulation of E-cadherin in colon cancer cells requires integrin signalling, *Nat. Cell Biol.* 4 (8) (2002) 632–638, <https://doi.org/10.1038/ncb829>.
- [26] J. Schweizer, H. Winter, Keratin biosynthesis in normal mouse epithelia and in squamous cell carcinomas. mRNA-dependent alterations of the primary structure of distinct keratin subunits in tumors, *J. Biol. Chem.* 258 (21) (1983) 13268–13272.
- [27] R. Eichner, T.T. Sun, U. Aebi, The role of keratin subfamilies and keratin pairs in the formation of human epidermal intermediate filaments, *J. Cell Biol.* 102 (5) (1986) 1767–1777, <https://doi.org/10.1083/jcb.102.5.1767>.
- [28] C.S. Shen, K. Tabata, M. Matsuki, T. Goto, T. Yokochi, K. Yamanishi, Premature apoptosis of keratinocytes and the dysregulation of keratinization in porokeratosis, *Br. J. Dermatol.* 147 (3) (2002) 498–502, <https://doi.org/10.1046/j.1365-2133.2002.04853.x>.
- [29] H.S. Baek, T.U. Kwon, S. Shin, Y.J. Kwon, Y.J. Chun, Steroid sulfatase deficiency causes cellular senescence and abnormal differentiation by inducing yippee-like 3 expression in human keratinocytes, *Sci. Rep.* 11 (1) (2021) 20867, <https://doi.org/10.1038/s41598-021-00051-w>.
- [30] P.M. Steinert, L.N. Marekov, The proteins elafin, filaggrin, keratin intermediate filaments, loricrin, and small proline-rich proteins 1 and 2 are isodipeptide cross-linked components of the human epidermal cornified cell envelope, *J. Biol. Chem.* 270 (30) (1995) 17702–17711, <https://doi.org/10.1074/jbc.270.30.17702>.
- [31] M.H. Jung, S.M. Jung, H.S. Shin, Co-stimulation of HaCaT keratinization with mechanical stress and air-exposure using a novel 3D culture device, *Sci. Rep.* 6 (2016) 33889, <https://doi.org/10.1038/srep33889>.
- [32] T.U. Kwon, Y.J. Kwon, H.S. Baek, H. Park, H. Lee, Y.J. Chun, Unraveling the molecular mechanisms of cell migration impairment and apoptosis associated with steroid sulfatase deficiency: implications for X-linked ichthyosis, *Biochim. Biophys. Acta Mol. basis Dis.* 1870 (3) (2024) 167004, <https://doi.org/10.1016/j.bbdis.2023.167004>.
- [33] C.J. Gottardi, E. Wong, B.M. Gumbiner, E-cadherin suppresses cellular transformation by inhibiting β -catenin signaling in an adhesion-independent manner, *J. Cell Biol.* 153 (5) (2001) 1049–1060, <https://doi.org/10.1083/jcb.153.5.1049>.
- [34] S.A. Stewart, D.M. Dykxhoorn, D. Palliser, H. Mizuno, E.Y. Yu, D.S. An, D. M. Sabatini, I.S.Y. Chen, W.C. Hahn, P.A. Sharp, R.A. Weinberg, C.D. Novina, Lentivirus-delivered stable gene silencing by RNAi in primary cells, *RNA* 9 (4) (2003) 493–501, <https://doi.org/10.1261/rna.2192803>.
- [35] C. Pulido-Quetglas, E. Aparicio-Prat, C. Arnan, T. Polidori, T. Hermoso, E. Palumbo, J. Ponomarenko, R. Guigo, R. Johnson, Scalable design of paired CRISPR guide RNAs for genomic deletion, *PLoS Comput. Biol.* 13 (3) (2017) e1005341, <https://doi.org/10.1371/journal.pcbi.1005341>.
- [36] T. Nagashima, S. Tohyama, K. Mikami, M. Nagase, M. Morishima, A. Kasai, H. Hashimoto, A.M. Watabe, Parabrachial-to-parasubthalamic nucleus pathway mediates fear-induced suppression of feeding in male mice, *Nat. Commun.* 13 (1) (2022) 7913, <https://doi.org/10.1038/s41467-022-35634-2>.
- [37] L.A. Aparicio, M. Valladares, M. Blanco, G. Alonso, A. Figueroa, Biological influence of Hakai in cancer: a 10-year review, *Cancer Metastasis Rev.* 31 (1–2) (2012) 375–386, <https://doi.org/10.1007/s10555-012-9348-x>.
- [38] S. Trent, A. Dennehy, H. Richardson, O.A. Ojarikre, P.S. Burgoyne, T. Humby, W. Davies, Steroid sulfatase-deficient mice exhibit endophenotypes relevant to attention deficit hyperactivity disorder, *Psychoneuroendocrinology* 37 (2) (2012) 221–229, <https://doi.org/10.1016/j.psyneuen.2011.06.006>.
- [39] J. Idkowiak, A.E. Taylor, S. Subtil, D.M. O'Neil, R. Vijzelaar, R.P. Dias, R. Amin, T. G. Barrett, C.H.L. Shackleton, J.M.W. Kirk, C. Moss, W. Arlt, Steroid sulfatase deficiency and androgen activation before and after puberty, *J. Clin. Endocrinol. Metab.* 101 (6) (2016) 2545–2553, <https://doi.org/10.1210/jc.2015-4101>.
- [40] Y. Higashi, H. Fuda, H. Yanai, Y. Lee, T. Fukushige, T. Kanzaki, C.A. Strott, Expression of cholesterol sulfotransferase (SULT2B1b) in human skin and primary cultures of human epidermal keratinocytes, *J. Invest. Dermatol.* 122 (5) (2004) 1207–1213, <https://doi.org/10.1111/j.0022-202X.2004.22416.x>.
- [41] T. Tatsuguchi, T. Uruno, Y. Sugiura, K. Oisaki, D. Takaya, D. Sakata, Y. Izumi, T. Togo, Y. Hattori, K. Kunimura, T. Sakurai, T. Honma, T. Bamba, M. Nakamura, M. Kanai, M. Suematsu, Y. Fukui, pharmacological intervention of cholesterol sulfate-mediated T cell exclusion promotes antitumor immunity, *Biochem. Biophys. Res. Commun.* 609 (2022) 183–188, <https://doi.org/10.1016/j.bbrc.2022.04.035>.
- [42] E. Janda, M. Nevolo, K. Lehmann, J. Downward, H. Beug, M. Grieco, Raf plus TGF β -dependent EMT is initiated by endocytosis and lysosomal degradation of E-cadherin, *Oncogene* 25 (54) (2006) 7117–7130, <https://doi.org/10.1038/sj.onc.1209701>.
- [43] H. Shrestha, T. Ryu, Y.W. Seo, S.Y. Park, Y. He, W. Dai, E. Park, S. Simkhada, H. Kim, K. Lee, K. Kim, Hakai, an E3-ligase for E-cadherin, stabilizes δ -catenin through Src kinase, *Cell. Signal.* 31 (2017) 135–145, <https://doi.org/10.1016/j.cellsig.2017.01.009>.
- [44] W. Guo, F.G. Giancotti, Integrin signalling during tumour progression, *Nat. Rev. Mol. Cell Biol.* 5 (10) (2004) 816–826, <https://doi.org/10.1038/nrm1490>.
- [45] D.J. Ye, Y.J. Kwon, S. Shin, H.S. Baek, D.W. Shin, Y.J. Chun, Induction of integrin signaling by steroid sulfatase in human cervical cancer cells, *Biomol. Ther. (Seoul)* 25 (3) (2017) 321–328, <https://doi.org/10.4062/biomolther.2016.155>.
- [46] S. Cabodi, E. Calautti, C. Talora, T. Kuroki, P.L. Stein, G.P. Dotto, A PKC- η /Fyn-dependent pathway leading to keratinocyte growth arrest and differentiation, *Mol. Cell* 6 (5) (2000) 1121–1129, [https://doi.org/10.1016/S1097-2765\(00\)00110-6](https://doi.org/10.1016/S1097-2765(00)00110-6).
- [47] C.L. Tu, W. Chang, Z. Xie, D.D. Bikle, Inactivation of the calcium sensing receptor inhibits E-cadherin-mediated cell-cell adhesion and calcium-induced differentiation in human epidermal keratinocytes, *J. Biol. Chem.* 283 (6) (2008) 3519–3528, <https://doi.org/10.1074/jbc.M708318200>.
- [48] J.L. Charest, J.M. Jennings, W.P. King, A.P. Kowalczyk, A.J. Garcia, Cadherin-mediated cell-cell contact regulates keratinocyte differentiation, *J. Invest. Dermatol.* 129 (3) (2009) 564–572, <https://doi.org/10.1038/jid.2008.265>.
- [49] G.H. Wren, W. Davies, X-linked ichthyosis: new insights into a multi-system disorder, *Skin Health Dis.* 2 (4) (2022) e179, <https://doi.org/10.1002/ski2.179>.
- [50] Y. Bai, R. Li, H. Jun-Feng, C. Lian-Wan, L. Si-Tong, Z. Xi-Lin, Y.H.L. Gregory, Y. Jin-Kui, Z. Yi-Xi, Hao Wang, Accumulated β -catenin is associated with human atrial fibrosis and atrial fibrillation, *J. Transl. Med.* 22 (1) (2024) 734, <https://doi.org/10.1186/s12967-024-05558-0>.