

Gapmer Antisense Oligonucleotide Targeting E-Cadherin Rescues Abnormal Keratinization in X-Linked Ichthyosis Models

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Abstract

X-linked ichthyosis (XLI) is an inherited disorder of keratinization resulting from a deficiency of steroid sulfatase (STS), for which no effective therapy is currently available. E-cadherin, a key upstream regulator of keratinocyte differentiation, has been found to be markedly overexpressed in STS-deficient HaCaT cells, suggesting its potential as a therapeutic target in XLI. To investigate the functional role of E-cadherin and explore its therapeutic potential, we introduced mutations into the N-terminal region of E-cadherin and examined the resulting effects on keratinocyte differentiation. In addition, a microRNA (miR-6766) and a rationally designed gapmer antisense oligonucleotide (gASO) targeting the same E-cadherin mRNA sequence were employed to modulate E-cadherin expression in HaCaT cells. Mutations within the N-terminal region of E-cadherin significantly reduced keratin 1 expression, underscoring the critical role of this domain in regulating keratinocyte differentiation. Treatment with miR-6766 led to downregulation of both early and terminal differentiation markers. Building on this, the gASO modified with 2'-O-methoxyethyl and phosphorothioate linkages exhibited enhanced potency and stability, resulting in stronger suppression of E-cadherin and keratin 1 expression compared with miR-6766 (maintained 37.7% greater inhibition of E-cadherin at 96 h and 35.7% greater inhibition of keratin 1 at 96 h). Furthermore, gASO treatment induced a concentration-dependent reduction in early (keratin 1 and keratin 10) and terminal (transglutaminase 1, involucrin, and loricrin) differentiation markers. These findings demonstrate that an E-cadherin-targeting gASO effectively suppresses abnormal keratinocyte differentiation and may serve as a promising therapeutic strategy for X-linked ichthyosis.

Key Words: Steroid sulfatase, X-linked ichthyosis, E-cadherin, Gapmer antisense oligonucleotide, Differentiation marker

INTRODUCTION

X-linked ichthyosis (XLI) is a genetic disorder affecting approximately 1 in 2,000-6,000 individuals worldwide, with a significantly higher prevalence in males (Ingordo *et al.*, 2003). This condition results from mutations or deletions in the steroid sulfatase (STS) gene (Fernandes *et al.*, 2010). Loss of STS function causes accumulation of cholesterol sulfate, decreasing cholesterol levels and subsequently inducing excessive keratinocyte differentiation and stratification (Williams and Elias, 1981). Consequently, STS gene deficiency causes thick, rigid, and scaly skin barriers that define the characteristic XLI phenotype (Wells and Kerr, 1966). Currently, no specific treatment exists for XLI, and existing therapeutic approaches are limited to topical moisturizers and keratolytic agents that offer only temporary relief for the impaired skin barrier (Vahlquist *et*

al., 2008). Thus, novel therapeutic strategies addressing the underlying pathology of XLI are urgently required.

Epidermal keratinocytes detect external signals from the extracellular matrix via integrins and transmit intercellular messages through cell-cell junctions (Watt *et al.*, 1993). Differentiated keratinocytes increase the formation of cell-cell junctions (Charest *et al.*, 2009), highlighting the critical role of intercellular junctions in the differentiation process. Adherens junctions, a class of intercellular junctions predominantly regulated by E-cadherin, play a crucial role in keratinocyte differentiation by signaling through catenins and inositol 1,4,5-trisphosphate (IP₃) (Shrestha *et al.*, 2016; Tu and Bikle, 2013). In human keratinocytes, blocking E-cadherin impairs calcium-induced activation of phosphoinositide 3-kinase (PI3K) and phospholipase C-γ1 (PLC-γ1), ultimately disrupting keratinocyte differentiation (Lau *et al.*, 2011; Xie and Bikle, 2007). Thus, E-cadherin

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plays a pivotal role in keratinocyte differentiation and serves as a potential therapeutic target for XLI.

Keratinocytes undergo a stepwise differentiation process through the epidermal layers, culminating in cornification. This progression starts in the basal layer, advances through the spinous and granular layers, and ends in the corneous layer, with distinct protein expression and structural modifications at each stage (Tarshish *et al.*, 2023). During early differentiation, basal keratins, such as keratin 5 and keratin 14, decrease in expression. As differentiation progresses, keratin 1 and keratin 10 dominate expression in the spinous and granular layers (Alam *et al.*, 2011). Throughout this process, keratinocytes alter their adhesion and cytoskeletal organization (Gutowska-Owsiak *et al.*, 2020). In late differentiation, keratin-forming proteins mature within the granular layer (Holthaus and Eckhart, 2024). Transglutaminase 1 (TGM1) cross-links structural proteins, including involucrin, loricrin, and filaggrin, to form the insoluble cornified envelope (CE) (Inada *et al.*, 2000). This TGM1-mediated cross-linking strengthens barrier integrity in the corneous layer, enhancing its resilience over earlier differentiation stages (Surbek *et al.*, 2023). Thus, fully differentiated keratinocytes predominantly express early differentiation markers, including keratin 1 and keratin 10, alongside terminal differentiation markers such as TGM1, involucrin, and loricrin, ultimately reinforcing the barrier (Pondeljak *et al.*, 2023).

Gapmer antisense oligonucleotides (gASOs) exhibit enhanced target specificity and molecular stability compared with miRNA- or siRNA-based modalities (Liang *et al.*, 2013; Kim, 2023; Kim *et al.*, 2023). miRNAs are susceptible to non-specific binding and degradation by endogenous nucleases due to their long sequences (Bravo *et al.*, 2007), whereas gASOs offer a promising alternative to overcome these limitations. gASOs incorporate phosphorothioate (PS) linkages, substituting the bridging oxygen of the phosphate group with sulfur to prevent recognition and degradation by endogenous nucleases. This modification disperses the negative charge on the PS backbone at physiological pH more than phosphodiester (PO), enhancing ASO lipophilicity, promoting plasma protein binding, and reducing rapid renal clearance (Chen *et al.*, 2024; Mekonnen *et al.*, 2025). PS linkages serve as fundamental backbone modification in several approved ASOs (Crooke *et al.*, 2021). Additionally, modifications to the sugar moiety of ASOs critically influence binding affinity, nuclease resistance, and *in vivo* performance (Crooke *et al.*, 2020). The 2'-O-methoxyethyl (2'-MOE) modification enhances efficacy, extends tissue half-life, and mitigates inflammation, thereby reducing potential side effects (Crooke *et al.*, 2021; Evers *et al.*, 2015). gASOs hybridize with target mRNA to form a DNA/RNA duplex, which RNase H subsequently recognizes and degrades (Crooke *et al.*, 2021; Roberts *et al.*, 2020). Several ASO-based therapeutics are undergoing clinical trials, with some already approved and commercialized (Crooke *et al.*, 2021).

Despite advances in understanding XLI pathogenesis, there is still no targeted treatment that directly addresses the abnormal keratinocyte differentiation caused by STS deficiency. Although E-cadherin is known to play a key role in keratinization, its therapeutic targeting remains underexplored. Therefore, this study aims to design a gASO targeting the E-cadherin gene (CDH1) and evaluate its efficacy in suppressing keratinocyte differentiation markers (keratin 1, keratin 10, TGM1, involucrin, and loricrin), offering a novel therapeutic

approach for XLI.

MATERIALS AND METHODS

Reagents

DMEM was purchased from HyClone (Logan, UT, USA) and FBS from Tissue Culture Biologicals (Long Beach, CA, USA). The Neon™ Transfection System was supplied by Thermo Fisher Scientific (Waltham, MA, USA), and the D-Plus™ ECL solution from Dongin LS (Seoul, Korea). Antibodies used in this study included anti-STS polyclonal antibody (ab62219), anti-involucrin (ab53112), and anti-loricrin (ab85679) from Abcam (Cambridge, MA, USA); anti-TGM 1 (12912-3-AP), anti-cytokeratin 1 (16848-1-AP), and anti-cytokeratin 10 (18341-1-AP) from Proteintech (Rosemont, IL, USA); anti-E-cadherin (07-697) from Merck Millipore (Burlington, MA, USA); and goat anti-rabbit IgG-Texas Red (sc-2780) from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture

The HaCaT cells were sourced from CLS Cell Lines Service (Germany). Cas9-control HaCaT and Cas9-STS^{-/-} HaCaT cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 20% (v/v) fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂.

Transient and stable transfection

For transient transfection, the STS coding sequence was cloned into the pcDNA3.1/Zeo+ vector. Cells were transfected using the Neon™ Transfection System (Thermo Fisher Scientific, CA, USA) with either 0 or 50 nM siRNA, 0, 25, 50, or 100 nM miRNA, or 3 µg of plasmid DNA. E-cadherin-targeting siRNA was purchased from Qiagen (Netherlands), and hsa-miR-6766 miRNA was from Bioneer (Seoul, Korea). The following plasmids gifted by Barry Gumbiner were acquired from Addgene: hE-cadherin-pcDNA3 (Addgene plasmid #45769), hE-cadherin/Δp120 (Addgene plasmid #45770), hE-cadherin/α-catenin fusion-pcDNA3 (Addgene plasmid #45771), hE-cadherin/Δβ-catenin-pcDNA3 (Addgene plasmid #45772), and IL2R/hE-cadherin-cytotail (Addgene plasmid #45773).

Generation of Cas9-STS^{-/-} HaCaT cells

The plasmid vectors pLentiCas9-T2A-GFP (Addgene plasmid #78548, gifted by Roderic Guigo and Rory Johnson) and tet-pLKO-sgRNA-puro (Addgene plasmid #104321, gifted by Nathanael Gray) were obtained from Addgene. Lentiviral supernatants carrying either the Cas9 gene or STS-targeting sgRNA were generated using HEK293T packaging cells. Cas9-STS^{-/-} HaCaT cells were subsequently generated through single-cell cloning in 96-well plates following lentiviral transduction.

Synthesis of gASO

Gapmer ASOs were synthesized by QMINE Inc. (Seoul, Korea) using a standard solid-phase phosphoramidite method on a controlled-pore glass support equipped with a universal linker. The synthesis process involved sequential washes with acetonitrile, detritylation using 3% trichloroacetic acid in dichloromethane, coupling with phosphoramidite reagents, and

oxidation with iodine in pyridine/THF. Capping was performed using acetic anhydride and N-methylimidazole. To incorporate phosphorothioate linkages, the oxidation step was substituted with either a 0.1 M 3-[(dimethylamino)methyleneamino]-3H-1,2,4-dithiazole-5-thione (DDTT) solution in pyridine or a 0.05 M DDTT mixture in pyridine/acetonitrile (1:1). Upon completion of the synthesis, oligonucleotides were cleaved from the solid support and deprotected using concentrated ammonia at 60°C for 12-18 h. The crude product was then desalted via Sephadex G-25 resin, lyophilized, and either used directly or further purified through preparative HPLC, followed by ethanol precipitation from a 0.3 M NaCl or NaOAc solution.

Quantitative RT-PCR

Quantitative RT-PCR (qRT-PCR) was performed using a Rotor-Gene Q instrument (Qiagen, Netherlands), and data analysis was performed with Rotor-Gene Q Series software (Qiagen). Each reaction mixture contained 10 µL of Q Green 2× qPCR Master Mix, 1 µM of each forward and reverse primer and 20 ng of cDNA in a final volume of 20 µL. The thermal cycling protocol included an initial denaturation step at 95°C for 5 min, followed by 40 amplification cycles of 15 s at 95°C (denaturation) and 45 s at 56°C (annealing/extension). The following primer sets were utilized for qRT-PCR analysis: 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'; Keratin 1: 5'- CAG CAT CAT TGC TGA GGT CAA GG - 3' and 5' - CAT GTC TGC CAG CAG TGA TCT G - 3'; Keratin 10: 5' - CCT GCT TCA GAT CGA CAA TGC C - 3' and 5' - ATC TCC AGG TCA GCC TTG GTC A - 3'; Transglutaminase 1: 5'- GCA CCA CAC AGA CGA GTA TGA - 3' and 5' - GGT GAT GCG ATC AGA GGA TTC - 3'; hsa-miR-6766-3p: 5' - TGA TTG TCT TCC CCC ACC CTC A - 3'.

Western blotting analysis

Cells were lysed in ice-cold PE buffer containing 50 mM NaF. Total protein extracts were separated via SDS-PAGE on 8%, 10%, or 12% polyacrylamide gels and transferred onto 0.45 µm PVDF membranes via electrophoresis. Membranes were blocked for 2 h at 4°C in 5% (w/v) BSA prepared in Tris-buffered saline with 0.1% Tween-20 (TBST), then incubated with primary antibodies diluted 1:1000 in TBST. Membranes were then incubated with appropriate secondary antibodies for 2 h. Protein signals were detected using D-Plus™ ECL solution (Dongin LS) and visualized with a ChemiDoc XRS imaging system (Bio-Rad, CA, USA).

Immunofluorescence

Cells were fixed in 10% neutral buffered formalin for 30 min at room temperature (24°C), then blocked for 5 min in PBS containing 10% goat serum and 0.2% Triton X-100. Following

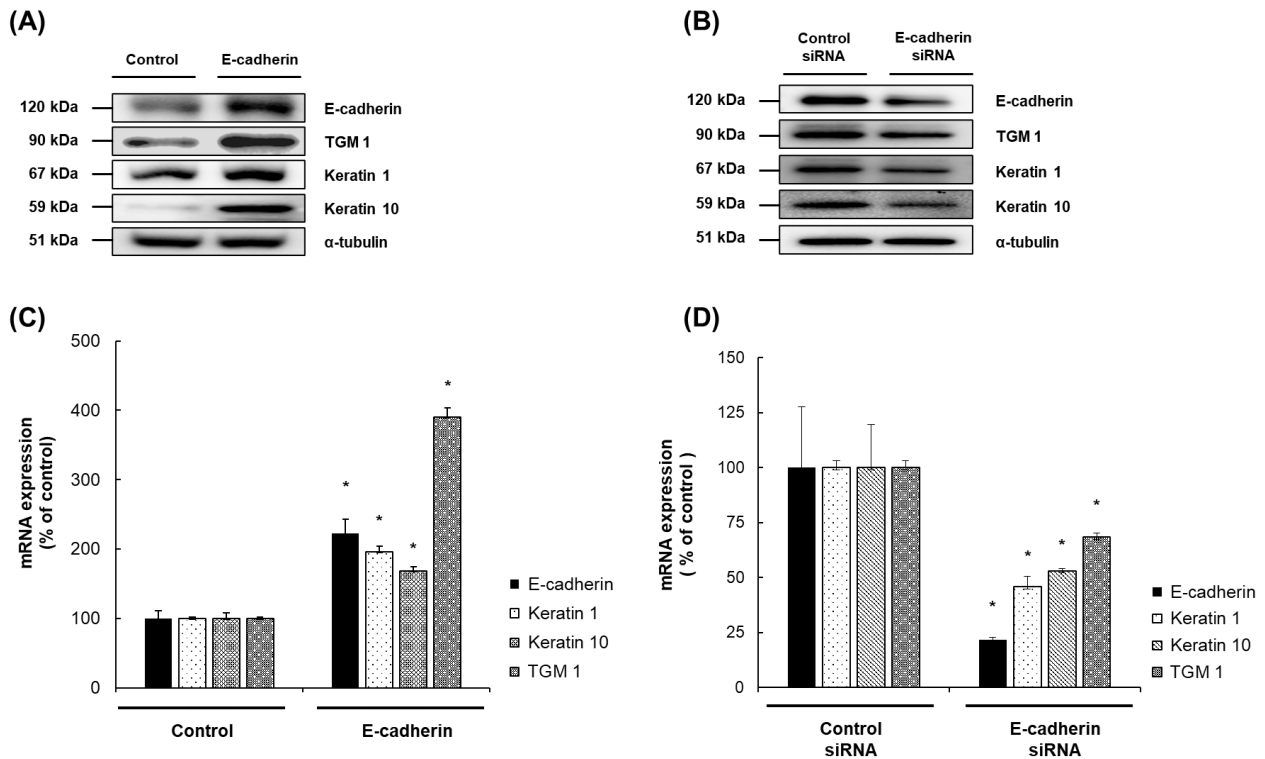


Fig. 1. Reduced E-cadherin expression via siRNA decreases keratinocyte differentiation marker levels in HaCaT cells. (A-D) HaCaT cells were transfected with pcDNA3.1-E-cadherin or siE-cadherin for 48 h. (A, B) Total cellular protein (10 µg) was subjected to western blot analysis using E-cadherin, TGM 1, keratin 1, and keratin 10 antibodies. α-tubulin served as a loading control. (C, D) RT-qPCR was performed to detect E-cadherin, TGM 1, keratin 1, and keratin 10 mRNA expressions in cells. Each data point represents the mean ± SEM of three experiments. **p*<0.05 compared to the control. HaCaT, human keratinocyte cell.

blocking, cells were incubated overnight at 4°C with a primary antibody diluted 1:200, then incubated overnight with goat anti-rabbit IgG-Alexa Fluor 594 (1:200). After three washes with PBS, coverslips were mounted onto glass slides using Ultra Cruz™ mounting medium. Fluorescence images were captured using an LSM 800 confocal laser scanning microscope (Carl Zeiss, Germany).

Statistical analysis

Statistical analysis was performed using Dunnett's pairwise multiple comparison t-test in GraphPad Prism 7 (GraphPad Software, CA, USA). A *p*-value of less than 0.05 (**p*<0.05) was considered statistically significant.

RESULTS

Reduced E-cadherin expression decreases the levels of keratinocyte differentiation markers in HaCaT cells

Based on previous studies showing that STS depletion elevates E-cadherin expression (Kwon *et al.*, 2025a, 2025b; Shin *et al.*, 2017). We investigated how alterations in E-cadherin levels influence the expression of keratinocyte differentiation markers. E-cadherin overexpression markedly upregulated the early differentiation markers keratin 1 and keratin 10, along with the terminal differentiation marker TGM1, whereas siRNA-mediated E-cadherin knockdown reduced their expression (Fig. 1A, 1B). To assess whether E-cadherin modulation influences keratinocyte differentiation at the mRNA level, cells

were treated for 48 h with either an E-cadherin expression vector or siRNA. The result demonstrated that the upregulation of E-cadherin was associated with increased mRNA levels of keratin 1, keratin 10, and TGM1 (Fig. 1C). In contrast, E-cadherin downregulation significantly reduced these markers, with keratin 1 and keratin 10 exhibiting approximately a 50% decrease in expression (Fig. 1D). These findings indicate that E-cadherin regulation modulates keratinocyte differentiation, establishing it as a key regulator in this process.

N-terminal domain of E-cadherin plays a vital role in regulating keratin 1 expression in keratinocytes

To investigate the role of E-cadherin in regulating keratinocyte differentiation markers, we transfected human keratinocyte (HaCaT) cells with vectors expressing wild-type or structurally mutated E-cadherin and analyzed the expression patterns of keratin 1, keratin 10, and TGM1. The mutations introduced into the vectors can be broadly classified into two groups: those targeting the N-terminus of E-cadherin, including mutants lacking the N-terminal domain, dominant-negative N-terminal mutants, and transmembrane mutants, and those affecting the C-terminus, such as mutants with a fused α -catenin binding domain, removed β -catenin binding domain, or blocked interaction with the p120-catenin binding domain (Kwon *et al.*, 2025b).

Our results showed a reduced expression of keratin 1, keratin 10, and TGM 1 in cells transfected with mutant vectors. Mutations in the N-terminal region of E-cadherin, essential for cell adhesion, significantly decreased keratin 1 expression.

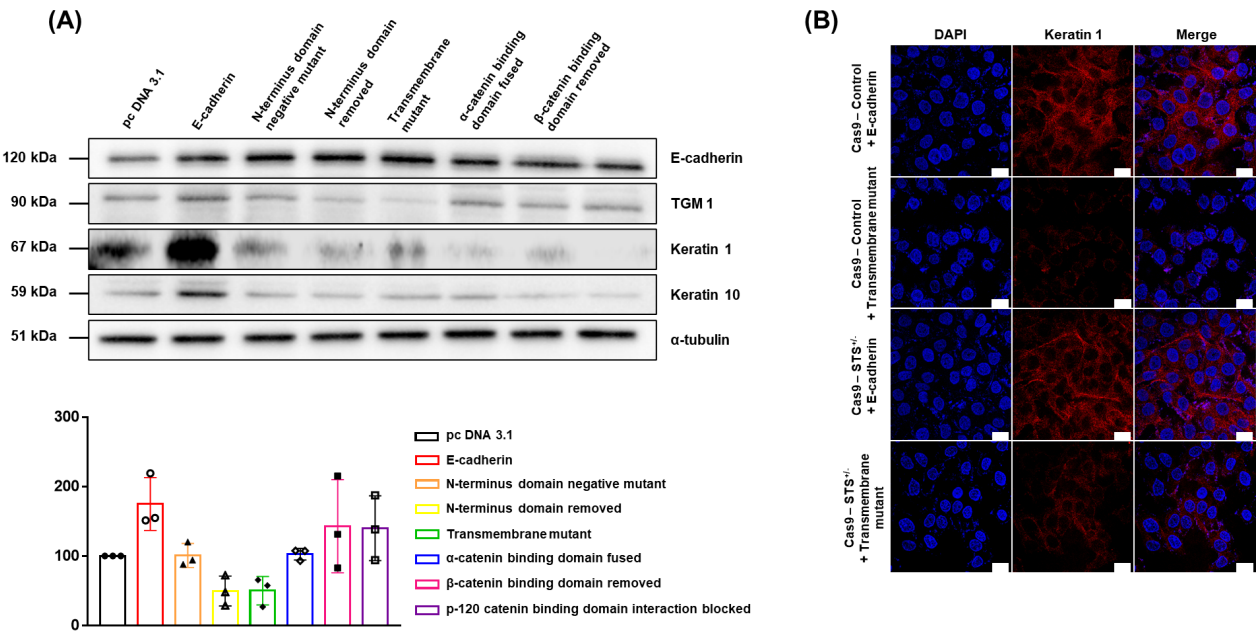


Fig. 2. N-terminal domain of E-cadherin is essential for regulating keratin 1 expression in HaCaT cells. (A) HaCaT cells were transfected for 48 h with vectors encoding wild-type E-cadherin, N-terminal deletion mutant, N-terminal dominant negative mutant, transmembrane mutant, α -catenin binding domain fusion mutant, β -catenin binding domain deletion mutant, or p120-catenin binding domain interaction blocked mutant. Total cellular protein (10 μ g) was subjected to western blot analysis with E-cadherin, TGM 1, keratin 1, and keratin 10 antibodies. α -tubulin served as a loading control. (B) Cas9-STS^{-/-} HaCaT cells were transfected with vectors for wild-type E-cadherin or transmembrane mutant for 48 h. Immunofluorescence staining was performed. Cells were fixed, incubated with keratin 1 antibody, and then stained with Alexa Fluor 594-labeled secondary antibody. Images were captured using confocal fluorescence microscopy. Blue=DAPI; Microscopy scale bar=20 μ m. HaCaT, human keratinocyte cell.

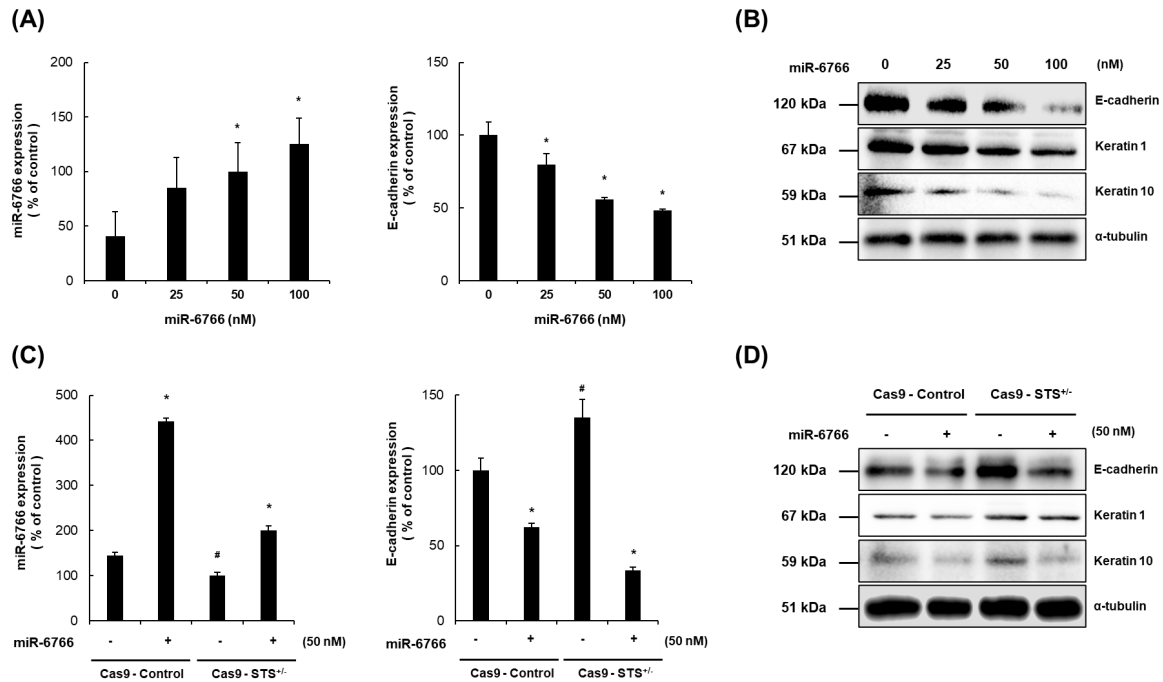


Fig. 3. Downregulation of E-cadherin by miR-6766 suppresses the expression of keratin 1 and keratin 10, key early differentiation markers in HaCaT cells. (A, B) HaCaT cells were transfected with miR-6766 (25, 50, or 100 nM) for 48 h. (A, C) qRT-qPCR was performed to detect miR-6766 or E-cadherin mRNA expressions in miR-6766-transfected HaCaT cells or Cas9-STS^{-/-} HaCaT cells. Each data point represents the mean ± SEM of three experiments. * $p < 0.05$ compared to the control miRNA or # $p < 0.05$ compared to the Cas9-control HaCaT cells. (B, D) Total cellular protein (10 µg) was subjected to western blot analysis with E-cadherin, keratin 1, and keratin 10 antibodies. α-tubulin served as a loading control. (C, D) Cas9-STS^{-/-} HaCaT cells were transfected with miR-6766 (0 or 50 nM) for 48 h. HaCaT, human keratinocyte cell; Quantitative RT-PCR, qRT-PCR.

Conversely, mutations in the C-terminal region of E-cadherin decreased the expression of TGM 1 and keratin 10 (Fig. 2A). These findings indicate that loss-of-function mutations in the N-terminal and C-terminal domains of E-cadherin disrupt signaling pathways associated with keratinization. Our findings emphasize the critical role of the E-cadherin N-terminal region in regulating keratin 1 expression. We performed confocal microscopy to directly assess the effect of the E-cadherin N-terminal deletion mutation on keratin 1 expression. Our results showed that cells expressing the E-cadherin transmembrane mutant exhibited significantly reduced keratin 1 expression levels (Fig. 2B). These findings indicate that E-cadherin expression closely links to keratin 1 levels, emphasizing the importance of the N-terminal domain of E-cadherin in regulating keratin 1 expression.

Treatment with miR-6766, which targets E-cadherin mRNA, suppresses the expression of early keratinocyte differentiation markers

Fig. 2 illustrates a robust link between E-cadherin and keratin 1 expression. Based on this, we hypothesized that decreasing keratinocyte differentiation markers through E-cadherin knockdown could offer a therapeutic approach for XLI. To further investigate this possibility, we employed an alternative genetic strategy to target E-cadherin expression.

Previous studies reported that knockdown of E-cadherin using miR-6766 in keratinocytes significantly reduced the expression of terminal differentiation markers, such as involucrin and loricrin (Kwon *et al.*, 2025b). In this study, we investigated

the effect of miR-6766 on early keratinocyte differentiation markers. HaCaT cells were treated with various concentrations of miR-6766 (0, 25, 50, or 100 nM), and the expression levels of keratin 1 and keratin 10 were assessed. Our results showed that miR-6766 treatment reduced keratin 1 and keratin 10 mRNA levels in a concentration-dependent manner (Fig. 3A), which was further confirmed at the protein level (Fig. 3B).

In addition, we investigated whether miR-6766 could regulate early differentiation markers under STS-deficient conditions. Partial STS-knockout cells generated via the CRISPR/Cas9 system were transfected with miR-6766, and the expression levels of keratin 1 and keratin 10 were subsequently measured. In STS^{-/-} HaCaT cells, keratin 1 and keratin 10 mRNA levels were significantly elevated. However, miR-6766 treatment effectively reduced the expression of these differentiation markers (Fig. 3C). Similar results were also observed at the protein level, where miR-6766 treatment significantly decreased differentiation markers (Fig. 3D).

Design of gapmer antisense oligonucleotides

miR-6766 treatment markedly reduced the expression of early differentiation markers in HaCaT cells (Fig. 3). However, these results prompted us to investigate a more stable and specific approach to targeting the E-cadherin gene. Therefore, we designed a gASO targeting the same sequence in the E-cadherin gene recognized by miR-6766. To optimize the gASO sequence, we utilized the miRNA database (miRDB; <http://www.mirdb.org/>). Fig. 4A illustrates the sequences of miR-6766 and its target site within the CDH1 gene.

(A)

miRNA Name : hsa-miR-6766-3p

5' - ugauugucuucccccacccuca - 3' (length = 22)

3' UTR Sequence

```

1681 tggaggttag gggtagtag ggaatcttga ttgatctct tttatttaa atgaattt
1741 caactttttg caatcaaa aagacttttt attaaatag cttactatt tctcaagt
1801 ttggagaaa aaactcaacc ctgaactcac tttttgaa ttcttaatt ttccagat
1861 tcaagctata tgaatataa tctatgttag agaatgtcac tgaatttta agtatata
1921 tttgtgggtg ctgaatata ttattttct ttgggttag aaaaagaaa caattcaagc
1981 taagaaagt attctcaag atocattttt ataatttta ttaacaaat ttgttaaac
2041 attaaaaaaa aaaaaaaa aaaaaaaa

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(B)

Antisense Oligonucleotide (18-mer: 5-8-5)

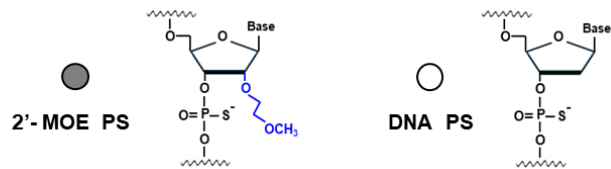
5' -  - 3'

Fig. 4. Schematic structure of the designed gASO specific to E-cadherin. (A) Information on miR-6766, the microRNA used for the synthesis of gASO, and the 3' UTR sequence of CDH1 targeted by miR-6766. (B) The designed gASO was synthesized as an 18-mer, with 2'-MOE (2'-O-methoxyethyl) modifications on both wings, providing minimal side effects, excellent clinical efficacy, and stability. All nucleotide linkages were made with PS bonds. gASO, gapmer antisense oligonucleotide; PS, phosphorothioate.

We synthesized gASO as an 18-mer with 2'-MOE (2'-O-methoxyethyl) modifications on both flanking regions, which reportedly enhance stability and clinical efficacy and reduce side effects (Crooke *et al.*, 2021). Additionally, all nucleotide linkages incorporated PS bonds, which enhanced cellular stability compared to PO bonds (Fig. 4B).

gASO demonstrates more pronounced and sustained suppression of E-cadherin and early keratinocyte differentiation markers compared to treatment with miR-6766

We studied the inhibition and duration of the synthesized gASO by directly comparing its effect on E-cadherin and keratin 1 expression with those of miR-6766. HaCaT cells were transfected with either gASO or miR-6766 at the same concentrations (0 or 50 nM), harvested at 24, 48, 72, and 96 h, and subjected to Western blot to evaluate changes in the protein levels of E-cadherin and keratin 1. Our result showed that gASO treatment more effectively suppressed the expression of E-cadherin and keratin 1 compared to treatment with miR-6766 across all examined time points, and recovery of expression occurred faster in cells treated with miR-6766 (Fig. 5A). Furthermore, differences in protein expression between treatments persisted or intensified over time. Specifically, Fig. 5B illustrates that gASO maintained approximately 30% greater inhibition of E-cadherin at 72 h, increasing to 37.7% at 96 h. For keratin 1, inhibition by gASO exceeded miR-6766 by 19.9% at 24 h, increasing to 34.5% by 72 h. At 96 h, this difference remained substantial at 35.7%, confirming the enhanced inhibition capacity of gASO. Overall, these findings indicate that gASO exerts stronger and more prolonged suppression of E-cadherin and keratin 1 expression than that of miR-6766.

gASO targeting E-cadherin efficiently suppresses the expression of early and terminal keratinocyte differentiation markers

Previously, Fig. 5 depicts that gASO downregulated E-cadherin and the early differentiation markers keratin 1 and keratin 10 more effectively and persistently. To further investigate whether gASO influences overall keratinocyte differentiation, HaCaT cells were treated with increasing gASO concentrations (0, 25, 50, or 100 nM) and analyzed the expression lev-

els of differentiation markers, including keratin 1, keratin 10, TGM 1, involucrin, and loricrin. Our results showed that gASO markedly suppressed the expression of E-cadherin, early differentiation markers (keratin 1 and keratin 10), and terminal differentiation markers (TGM 1, involucrin, and loricrin) in a concentration-dependent manner, as evidenced by the mRNA and protein levels (Fig. 6A, 6B).

Additionally, to evaluate the efficacy of gASO under STS-deficient conditions, we transfected partial STS-knockout HaCaT cells with gASO and analyzed the expression of keratinocyte differentiation markers. Our results showed that gASO markedly suppressed the elevated E-cadherin expression caused by STS deficiency and effectively decreased early differentiation markers (keratin 1 and keratin 10) and terminal differentiation markers (TGM 1, involucrin, and loricrin) at the mRNA and protein levels (Fig. 6C, 6D). Notably, gASO treatment led to a pronounced reduction in the expression of all examined keratinocyte differentiation markers in STS^{-/-} HaCaT cells.

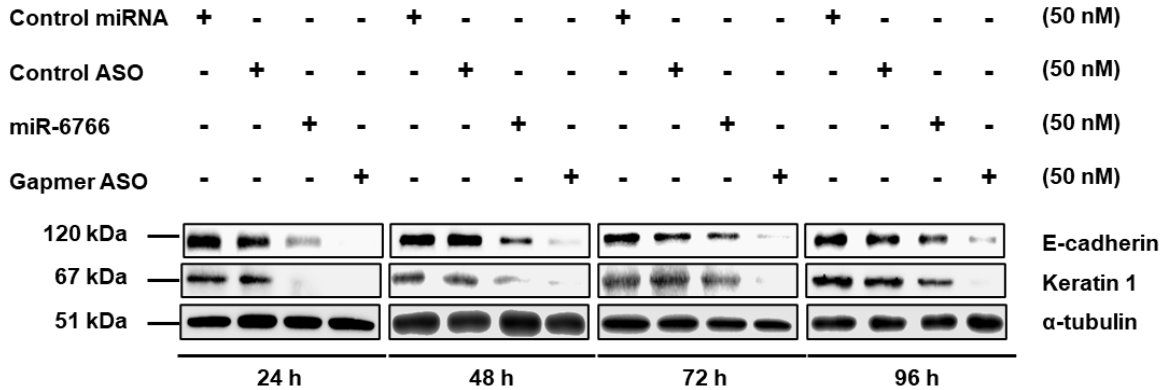
To further confirm the Western blot results, we conducted confocal microscopy analysis to assess the expression of keratinocyte differentiation markers. STS^{-/-} HaCaT cells treated with gASO (0 or 50 nM) exhibited substantially reduced levels of keratin 1, keratin 10, and TGM1, with these proteins nearly undetectable (Fig. 6E).

In conclusion, our results showed that gASO targeting E-cadherin effectively suppressed the expression of early and terminal differentiation markers in keratinocytes under STS-deficient conditions. These findings indicate that gASO may represent a promising therapeutic approach for XLI treatment (Fig. 7).

DISCUSSION

In this study, we investigated the essential function of E-cadherin in regulating keratinocyte differentiation and evaluated its therapeutic relevance for XLI treatment. Using HaCaT cells under normal and STS-deficient conditions, E-cadherin significantly modulates keratinization markers and differentiation-related signaling pathways. Additionally, our findings indicate E-cadherin-targeting agents, such as gASO, as promising therapeutic candidates for alleviating abnormal ke-

(A)



(B)

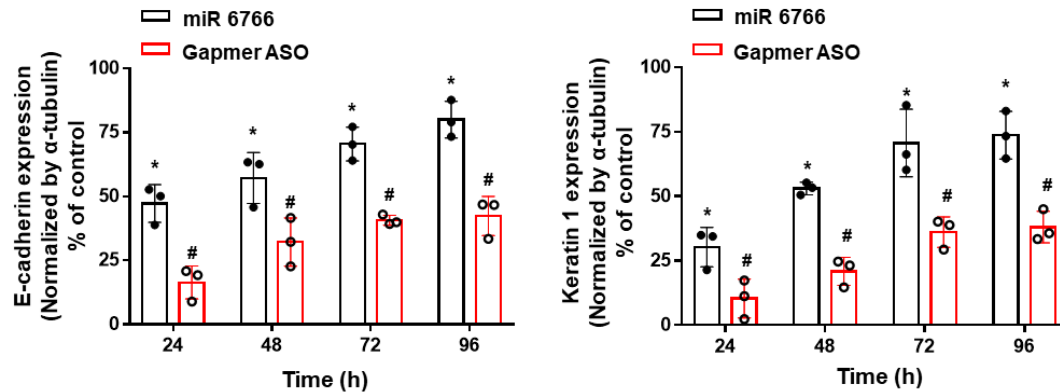


Fig. 5. gASO suppresses E-cadherin and keratin 1 expression more efficiently and for a longer duration than treatment with miR-6766. The gASO was designed based on the sequence of miR-6766. (A, B) HaCaT cells were transfected with miR-6766 or gASO for 24, 48, 72, or 96 h. (A) Total cellular protein (10 μ g) was subjected to western blot analysis with E-cadherin and keratin 1 antibodies. α -tubulin served as a loading control. Representative western blot image displays E-cadherin and keratin 1 protein levels in HaCaT cells. (B) Each data point represents the mean $\pm$ SEM of three experiments. * p <0.05 compared to the control miRNA or # p <0.05 compared to the control ASO. HaCaT, human keratinocyte cell; gASO, gapmer antisense oligonucleotide.

ratinoocyte differentiation associated with XLI.

Our findings suggest E-cadherin as a critical regulator of early and terminal keratinocyte differentiation markers. HaCaT cells overexpressing E-cadherin significantly upregulated keratin 1, keratin 10, and TGM 1 expression (Fig. 1A), whereas knockdown of E-cadherin using siRNA markedly decreased their mRNA and protein levels (Fig. 1B, 1C). These findings support that of a previous study identifying E-cadherin as an essential mediator of calcium-driven keratinocyte differentiation (Tu *et al.*, 2008), reinforcing its pivotal role in keratinocyte maturation.

Structurally, E-cadherin contains a conserved extracellular N-terminal region with multiple cadherin repeats that mediate Ca^{2+} binding and a cytoplasmic C-terminal region that associates with catenins (Coopman and Djiane, 2016). Calcium signals received by the N-terminal domain of E-cadherin are transmitted intracellularly through catenin interactions at its C-terminal domain, ultimately promoting keratinocyte differentiation (Nagar *et al.*, 1996; Takeichi, 1990). The C-terminal tail of E-cadherin interacts with p120-catenin and β -catenin, while

α -catenin links β -catenin to the actin cytoskeleton (Qian *et al.*, 2002). Based on these structural insights, we used domain-specific E-cadherin deletion mutants to examine how alterations in distinct regions of the protein affect the expression of keratinocyte differentiation markers (Liang *et al.*, 2013).

The results showed that the N-terminal domain of E-cadherin is essential for regulating the expression of the early differentiation marker keratin 1 (Fig. 2). Mutations introduced into this domain significantly reduced keratin 1 levels, highlighting the crucial role of Ca^{2+} -dependent cell-cell adhesion mediated by the N-terminal region (Fig. 2A). Confocal microscopy further validated these findings, indicating a pronounced decrease in keratin 1 expression in cells transfected with N-terminal mutant vectors compared to those expressing wild-type E-cadherin (Fig. 2B).

Interestingly, subsequent experiments revealed that both the N-terminal and C-terminal domains of E-cadherin are required for the proper expression of keratin 10 and TGM1 (Fig. 2A). Mutations in either domain reduced the expression of these markers, suggesting that both regions may regulate

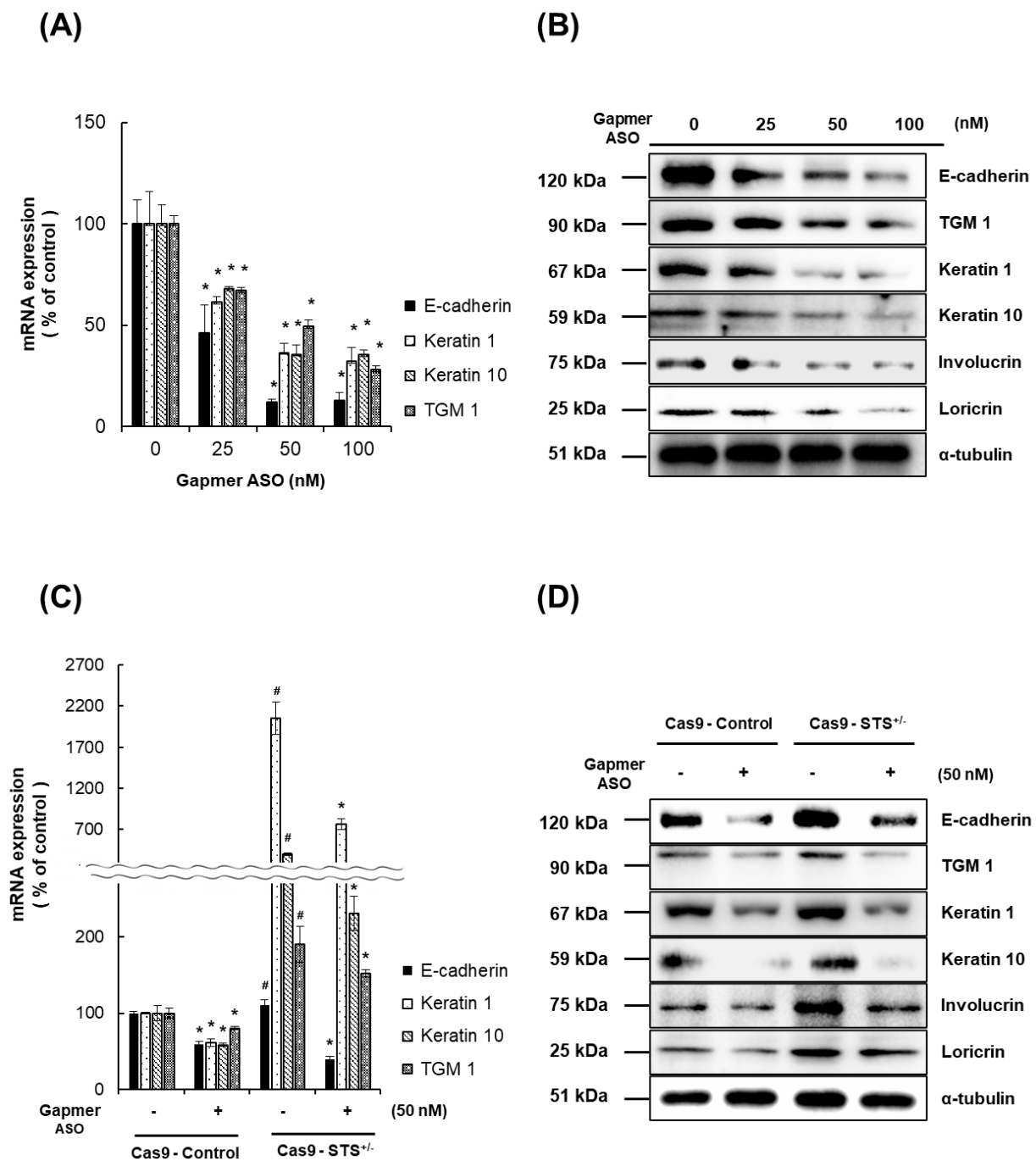


Fig. 6. Suppression of E-cadherin by gASO downregulates the expression of early and terminal keratinocyte differentiation markers. (A, B) HaCaT cells were transfected with gASO (0, 25, 50, or 100 nM) for 48 h. (C-E) Cas9-STS^{-/-} HaCaT cells were transfected with gASO (50 nM) for 48 h. (A, C) RT-qPCR was performed to detect E-cadherin, TGM 1, keratin 1, and keratin 10 mRNA expressions in gASO-treated HaCaT cells or Cas9-STS^{-/-} HaCaT cells. Each data point represents the mean $\pm$ SEM of three experiments. * p <0.05 compared to the control ASO or # p <0.05 compared to the Cas9-control HaCaT cells. (B, D) Total cellular protein (10 μ g) was subjected to western blot analysis with E-cadherin, TGM 1, keratin 1, keratin 10, involucrin, and loricrin antibodies. α -tubulin was used as a loading control. (C-E) Cas9-STS^{-/-} HaCaT cells were transfected with gASO (50 nM) for 48 h. (E) Immunofluorescence staining was performed. Cells were fixed and incubated with E-cadherin, keratin 1, keratin 10, and TGM 1 antibodies. After cells were stained with Alexa Fluor 594-labeled secondary antibody, the image was analyzed using confocal fluorescence microscopy. Immunofluorescence staining was performed. Blue=DAPI; Microscopy scale bar=20 μ m. HaCaT, human keratinocyte cell; gASO, gapmer antisense oligonucleotide; qRT-PCR, Quantitative RT-PCR.

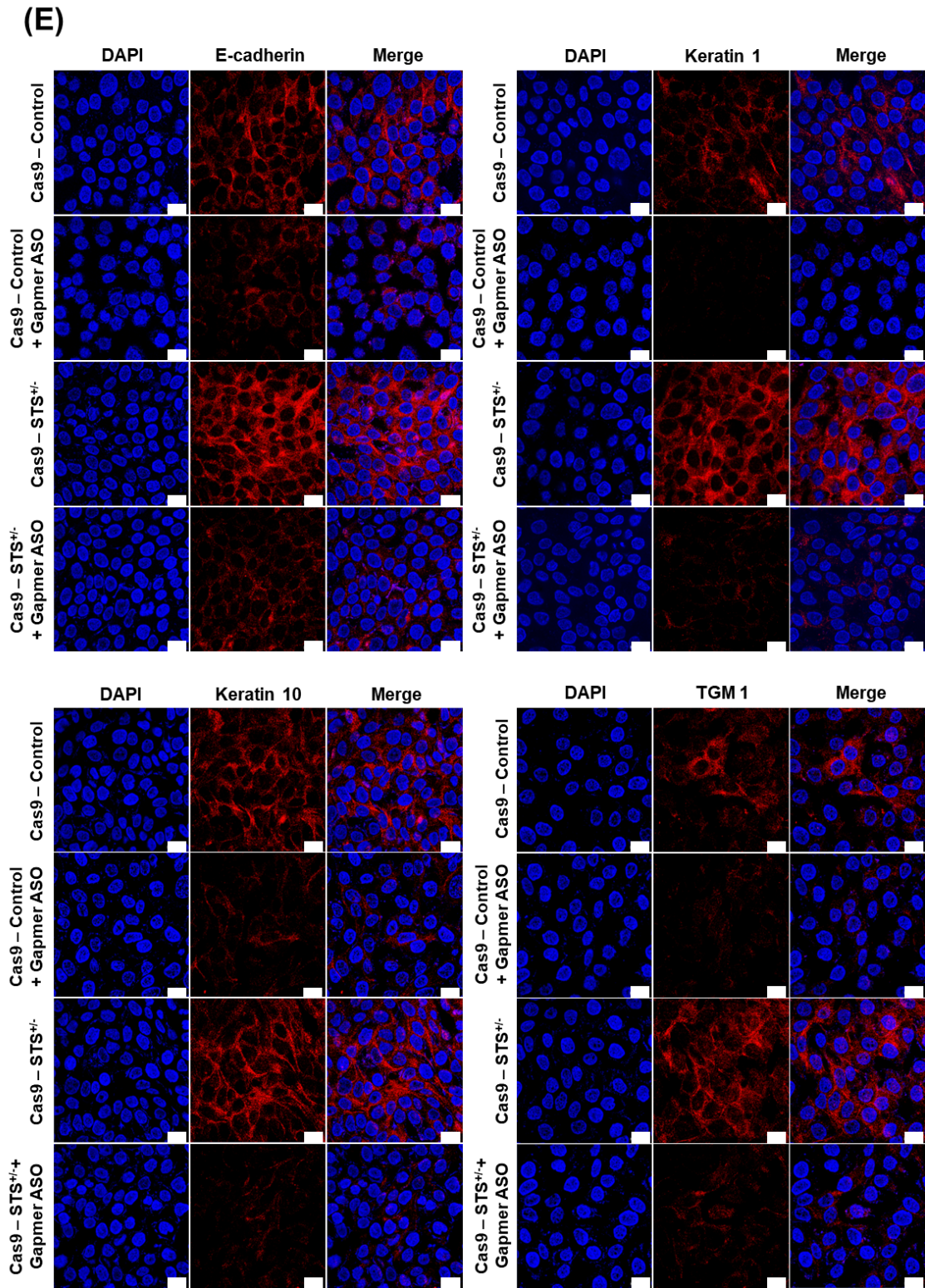


Fig. 6. Continued.

keratin 10 and TGM1 expression through distinct but complementary mechanisms. These findings suggest that coordinated activity between the N-terminal and C-terminal domains is

essential for proper keratinocyte differentiation, underscoring the importance of the structural integrity of E-cadherin. Collectively, our findings highlight the cooperative roles of these do-

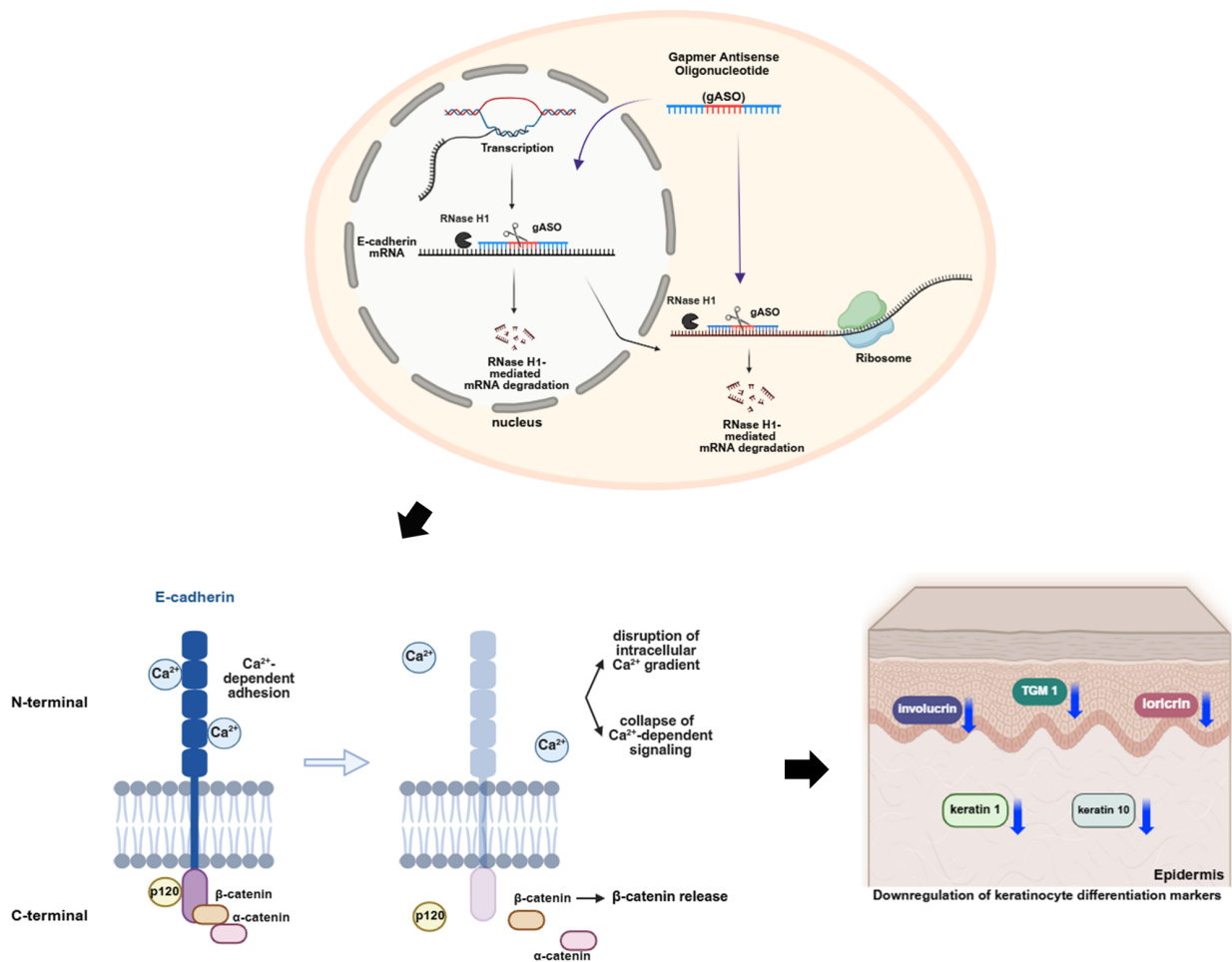


Fig. 7. Mechanism of gASO-mediated inhibition of E-cadherin expression and subsequent downregulation of keratinocyte differentiation markers. The gASO transfected into keratinocytes forms a DNA/RNA hybrid by binding to the E-cadherin mRNA. This hybrid is recognized and degraded by RNase H1. The gASO functions within the nucleus or cytoplasm. Degradation of E-cadherin mRNA and downregulation of E-cadherin protein by gASO may cause suppression of early keratinocyte differentiation markers (keratin 1 and keratin 10) and terminal differentiation markers (TGM 1, involucrin, and loricrin). gASO, gapmer antisense oligonucleotide; TGM 1, Transglutaminase 1.

mains and offer deeper insight into how E-cadherin regulates the expression of keratin 10 and TGM1.

To investigate the therapeutic potential of targeting E-cadherin in XLI, we evaluated the effects of miR-6766, a miRNA that downregulates E-cadherin expression. Transfecting HaCaT cells with increasing concentrations of miR-6766 led to a dose-dependent decrease in keratin 1 and keratin 10 expressions at the mRNA and protein levels (Fig. 3A, 3B). Under STS-deficient conditions, miR-6766 effectively counteracted the elevated expression of these markers, demonstrating its ability to modulate keratinocyte differentiation in a pathological context (Fig. 3C, 3D). These findings align with those of previous reports highlighting the role of miRNAs in regulating E-cadherin expression and influencing keratinocyte differentiation (Kwon *et al.*, 2025b).

Building on the findings from miR-6766 treatment, we designed a gASO to target the E-cadherin gene for enhanced and prolonged regulatory effects. Compared to miR-6766, gASO induced a stronger and more sustained suppression of E-cadherin and keratin 1 expression over a 72 h period,

with minimal recovery observed (Fig. 5A, 5B). This prolonged inhibition highlights the potential of gASO as a more effective therapeutic strategy for XLI.

A key advantage of gASO is its ability to modulate early and terminal keratinocyte differentiation markers. Concentration-dependent experiments revealed that gASO significantly downregulated the expression of E-cadherin, early differentiation markers (keratin 1 and keratin 10), and terminal markers (TGM1, involucrin, and loricrin) in HaCaT cells (Fig. 6A, 6B). Even under STS-deficient conditions, gASO effectively suppressed the upregulation of these differentiation markers, further supporting its therapeutic potential for XLI (Fig. 6C, 6D). Confocal microscopy analysis corroborated these findings, revealing significant reductions in keratin 1, keratin 10, and TGM1 expression in STS^{-/-} cells treated with gASO, with protein levels nearly undetectable (Fig. 6E). Collectively, these findings indicate the broad efficacy of gASO in modulating keratinocyte differentiation pathways and highlight its potential as a comprehensive therapeutic strategy for managing hyperkeratosis in XLI.

XLI, caused by STS deficiency, results in the accumulation of cholesterol sulfate and impaired keratinocyte differentiation (Baek *et al.*, 2021; Zhou *et al.*, 2025). In this study, we identified E-cadherin as a key regulator in the pathophysiological mechanism, acting through calcium-mediated signaling pathways to control the expression of differentiation markers. By specifically targeting E-cadherin, gASO provides a novel therapeutic strategy to counteract excessive keratinocyte differentiation observed in XLI (Fig. 7).

Concerns may arise regarding the potential cancer risk associated with targeting E-cadherin using gASO. However, XLI is a genetic disorder characterized by the chronic upregulation of E-cadherin expression at the genetic level due to STS deficiency. In this context, appropriately controlled gASO administration may normalize E-cadherin expression without reducing it below physiological levels. To minimize potential cancer-related adverse effects, it is essential to adopt a cautious approach during drug development, including restricting administration to skin regions exhibiting XLI-associated pathological features such as hyperkeratosis, and selecting topical formulations that limit unintended systemic exposure. Nevertheless, as this study was limited to *in vitro* experiments, further *in vivo* validation is needed to confirm the therapeutic potential and safety of gASO in clinical settings.

In conclusion, this study highlights the essential function of E-cadherin in keratinocyte differentiation and introduces gASO as a promising therapeutic approach for treating XLI. In the future, our efforts should aim to focus on optimizing gASO delivery methods and systematically assessing its long-term therapeutic efficacy and safety in preclinical and clinical models. Due to the absence of experimental evidence demonstrating the correction of XLI-like barrier defects by gASO in this study, additional investigations are also required to substantiate its therapeutic efficacy. Advancing these strategies may significantly drive the development of targeted treatments for XLI and other skin disorders, ultimately enhancing patient care and clinical outcomes.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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