The Role of Ubiquitination and the E3 Ligase Nedd4 in Regulating Corneal Epithelial Wound Healing

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Purpose. Ubiquitination serves as a fundamental post-translational modification in numerous cellular events. Yet, its role in regulating corneal epithelial wound healing (CEWH) remains elusive. This study endeavored to determine the function and mechanism of ubiquitination in CEWH.

Methods. Western blot and immunoprecipitation were used to discern ubiquitination alterations during CEWH in mice. Interventions, including neuronally expressed developmentally downregulated 4 (Nedd4) siRNA and proteasome/lysosome inhibitor, assessed their impact on CEWH. In vitro analyses, such as the scratch wound assay, MTS assay, and EdU staining, were conducted to gauge cell migration and proliferation in human corneal epithelial cells (HCECs). Moreover, transfection of miR-30/200 coupled with a luciferase activity assay ascertained their regulatory mechanism on Nedd4.

Results. Global ubiquitination levels were markedly increased during the mouse CEWH. Importantly, the application of either proteasomal or lysosomal inhibitors notably impeded the healing process both in vivo and in vitro. Furthermore, Nedd4 was identified as an essential E3 ligase for CEWH. Nedd4 expression was significantly upregulated during CEWH. In vivo studies revealed that downregulation of Nedd4 substantially delayed CEWH, whereas further investigations underscored its role in regulating cell proliferation and migration, through the Stat3 pathway by targeting phosphatase and tensin homolog (PTEN). Notably, our findings pinpointed miR-30/200 family members as direct regulators of Nedd4.

Conclusions. Ubiquitination holds pivotal significance in orchestrating CEWH. The critical E3 ligase Nedd4, under the regulatory purview of miR-30 and miR-200, facilitates CEWH through PTEN-mediated Stat3 signaling. This revelation sheds light on a prospective therapeutic target within the realm of CEWH.

Keywords: Nedd4, corneal epithelial wound healing, ubiquitination, Stat3, PTEN

The corneal epithelium represents the foremost anterior layer of the eye and is essential in shielding the eye from diverse external adversities, such as physical injuries and microbial infections.[1] The preservation of the cornea's integrity and its transparency are dependent on the proficient healing of corneal epithelial wounds. This multifaceted healing process synchronizes and integrates various cellular activities: apoptosis, proliferation, migration, differentiation, stratification, and modifications in the extracellular matrix (ECM).[2,3] Although there have been significant advancements in understanding the intricacies of corneal epithelial wound healing (CEWH) over the years, these undertakings are leading to uncharted territories that warrant further exploration.

In recent times, an emerging body of research has been increasingly focusing on the role of protein post-translational modifications (PTMs) in the context of corneal injuries. Notably, modifications, such as acetylation[4] and methylation,[5] have been implicated in these processes. These observations emphasize the fundamental role that PTMs play in the pathogenesis of corneal injury. Beyond these, there are a plethora of other PTMs, such as phosphorylation, ubiquitination, lactylation, and succinylation. Within the spectrum of known PTMs, ubiquitination stands out as a particularly well-characterized and canonical PTM, integral to a myriad of cellular events, including cell proliferation, cell differentiation, inflammatory responses, and transcription regulation. The molecular machinery governing ubiquitination consists of ubiquitin proteins, the E1 ubiquitin-activating enzyme, the E2 ubiquitin-conjugating enzyme, the E3 ubiquitin ligase, and a large contingent of deubiquitinases.[6] Substrate conjugation with ubiquitin molecules can occur by its amino-terminal methionine M1, or the 7 internal lysines K6, K11, K27, K29, K33, K48, and K63, with each linkage type influencing unique cellular outcomes.[7] Among the various ubiquitin linkages, K48 and K63 are the most extensively studied. K48-linked ubiquitination is traditionally linked to the proteasomal degradation of proteins. Conversely, K63-linked ubiquitination plays a role in non-degradative processes, such as the regu-

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lation of protein activity, protein-protein interactions, and intracellular trafficking, highlighting its diverse functions in maintaining cellular homeostasis.[8] A burgeoning body of evidence elucidates that an intricate relationship exists between the protein ubiquitination pathway and the skin wound healing process. Notably, E3 ligase itch-deficient mice exhibited enhanced cutaneous wound healing coupled with protracted epithelial remodeling.[9] Additionally, in an ex vivo corneal organ culture wound healing model, the deubiquitinase USP10 was observed to have elevated expression levels; suppression of USP10 curtailed the induction of fibrotic markers, thereby augmenting regenerative healing.[10] However, the precise role of ubiquitination in mediating responses underlying CEWH remains an open area of investigation.

In this study, we sought to elucidate the role of ubiquitination in modulating CEWH. Our analyses revealed a marked augmentation of global protein ubiquitination during CEWH, pinpointing neuronally expressed developmentally downregulated 4 (Nedd4) as a crucial E3 ligase implicated in this mechanism. We observed an upregulation in Nedd4 protein levels within the epithelial cells during CEWH and underscored that the diminished expression of miR-30 and miR-200 potentiates Nedd4 protein levels, which in turn favorably modulates Stat3 signaling during CEWH. These findings provide invaluable insights, enriching our comprehension of the ubiquitination function in CEWH.

Materials and Methods

Mouse Corneal Epithelial Wound Healing Model

The corneal epithelial wound healing model was developed in male C57BL6/J mice aged 8 to 10 weeks, as previously described.[11] For the inhibitor experiments in vivo, wounded eyes were made with 2-mm diameter trephine. Following epithelial removal, eye drops containing either a saline solution with 0.0001% DMSO or with the proteasome inhibitor MLN2238 (40 µM) or the lysosome inhibitor Lys05 (50 µM) were administered to the wounds 8 times daily.

For the in vivo Nedd4 siRNA experiment, Nedd4 siRNA or negative control (NC) siRNA were combined with the in vivo-jetPEI DNA and siRNA Delivery Reagent (Polyplus, NY, USA) and subsequently injected beneath the corneal epithelial surface using a 308-beveled needle (Hamilton, Bonaduz, Switzerland). Wounds were examined and imaged at 0 hours and 24 hours using a slit lamp fitted with a digital camera. The wounded areas were quantified using ImageJ software.

For in vivo EdU staining experiment, mice received an intraperitoneal injection of EdU (50 µg/g) for 4 hours following transfection with either NC or Nedd4 siRNA. Subsequently, corneas were excised, fixed, and processed for EdU staining in accordance with the manufacturer's instructions, as described previously (Click-iT EdU Alexa Fluor 594 Imaging Kit; Invitrogen, Carlsbad, CA, USA).[5]

All animal experiments were carried out in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and approved by the Wenzhou Medical University Animal Care and Use Committee. The sequence of Nedd4 siRNA in vivo was as follows: sense 5′-GGGAAAUCGUACGAGAAGATT-3′, antisense 5′-UCUUCUCGUACGAUUUCCCTT-3′.

Co-Immunoprecipitation and Mass Spectrometric Analysis

Corneal epithelium samples from 40 injured or non-injured mouse eyes were obtained and lysed in buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, and 1 mM PMSF. The lysates were incubated at 4°C with Protein A/G Agarose beads coupled to either FK2 antibody (ST1200; Sigma-Aldrich) for 12 hours. Subsequently, the beads were washed thrice with the lysis buffer and eluted in 50 µL of loading buffer at 95°C for 5 minutes. After brief electrophoresis on a 10% SDS-PAGE gel (approximately 10 minutes), we excised the loaded gel segments and then performed in-gel digestion and subsequent mass spectrometric analysis.

Cell Culture

The SV40 immortalized human corneal epithelial cells (HCECs), kindly provided by Araki Sasaki from the Kagoshima Miyata Eye Clinic, Kagoshima, Japan,[12] were cultured in a growth medium (DMEM/F12) supplemented with 10% FBS. The HCECs were maintained at 37°C in a 5% CO2 atmosphere.

Cell Proliferation Assay

HCECs were seeded to achieve approximately 30% confluence. For siRNA transfection experiments, cells were treated with 50 nM siRNA using Lipofectamine RNAi MAX (Invitrogen). In the context of proteasome inhibitor testing, HCECs were exposed to either 10 µM MLN2238 (Selleck) or DMSO for a duration of 5 hours. For lysosome inhibitor evaluation, cells were treated with 10 µM Lys05 (Selleck) or DMSO for 12 hours. In experiments involving the p-Stat3 inhibitor, HCECs were incubated with 40 µM Niclosamide (Selleck) for 5 hours. Following the inhibitor treatments, the cell culture medium was refreshed with a standard growth medium. Cell proliferation rates were detected using the MTS assay (CellTiter 96 Aqueous; Promega, Madison, WI, USA) and EdU staining (Click-iT EdU Alexa Fluor 594 Imaging Kit; Invitrogen), according to the manufacturer's protocols, as previously outlined.[5,11] The siRNA sequences used in these experiments are detailed below: Nedd4 siRNA: sense 5′-GCACAUCUCGGGUGCCUAUTT-3′, antisense 5′-AUAGGCACCCGAGAUGUGCTT-3, PTEN siRNA: sense 5′-GAAGGCGUAUACAGGAACATT-3′, and antisense 5′-UGUUCCUGUAUACGCCUUCTT-3′.

In Vivo Ubiquitination Assay

HCECs were transfected with either an NC or Nedd4 siRNA for 48 hours, followed by a 50 µM MG132 treatment prior to harvesting. Cells were lysed using denaturing buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1% SDS, and 1 mM DTT) and heated at 95°C for 5 minutes. The lysate was then diluted 5-fold with NP40 lysis buffer and incubated overnight at 4°C with Protein A/G Agarose beads pre-bound to PTEN antibody (9188; Cell Signaling Technology). After incubation, the beads were washed 3 times with NP40 lysis buffer and the bound proteins were eluted with 30 µL of loading buffer by heating at 95°C for 5 minutes. For Western blot analysis post-immunoprecipitation (IP), the specific secondary antibody, Mouse anti-rabbit IgG (Conformation Specific) L27A9 mAb (5127; Cell Signaling Technology) was utilized.

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In Vitro Scratch Wound Assay

For experiments concerning Nedd4 and PTEN, HCECs were treated with 50 nM of Nedd4 siRNA, PTEN siRNA, or an NC transfection. After 48 hours post-transfection, a scratch wound was introduced to the HCEC monolayer using a 200 µL pipette tip, and migration assays were conducted as previously described.[13] In the context of the proteasome, lysosome, or p-Stat3 inhibitor experiments, a scratch wound was created in the HCEC monolayer using a 200 µL pipette tip. Following this, cells were incubated with or without 10 µM MLN2238 for 5 hours, 10 µM Lys05 for 12 hours, or 40 µM Niclosamide for 5 hours in a serum-free medium. The progression of wound closure was monitored by capturing images of the wounded region at both the 0-hour mark and 24 hours post-incubation using an Imager Z1 (Zeiss, Jena, Germany). The wound areas were then quantified using the ImageJ software.

Western Blot Analysis

Both HCECs and mouse corneal epithelial cells were lysed with either 1 × Loading buffer (50 mM Tris, pH 7.4, 1% SDS, and 1 mM DTT) or RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, and 1 mM PMSF). The following antibodies were used in the Western blot experiments: Nedd4 (2740, 1:1000; Cell Signaling Technology); FK2 (ST1200, 1:1000; Sigma-Aldrich); P4D1 (3936, 1:1000; Cell Signaling Technology); Lys48 Ub (8081, 1:1000; Cell Signaling Technology); Lys63 Ub (5621, 1:1000; Cell Signaling Technology); Actin (66009-1-Ig, 1:1000; Proteintech); Stat3 (12640, 1:1000; Cell Signaling Technology); p-Stat3 (9145, 1:1000; Cell Signaling Technology); and PTEN (9188, 1:1000; Cell Signaling Technology).

Luciferase Activity Assay

TargetScan was utilized to predict potential miR-30 family and miR-200b binding sites within the 3' untranslated region (3'UTR) of Nedd4, and the seed regions within the 3'UTR were mutated. Both the wild-type and the mutant 3′UTRs were cloned into the Mlu I and Hind III restriction sites of the pMIR-REPORT Luciferase vector for further analysis. The reporter plasmids were synthesized by Obio Technology (Shanghai) Corp., Ltd. HEK293 cells, seeded in 96-well plates, were co-transfected with 1 µg of either the 3′UTR reporter or the mutant reporter, 0.5 µg of pRL-SV40, and 100 nM of various mimics (including NC, miR-30b, miR-30c, miR-30d, and miR-200b) using Lipofectamine 2000 (Invitrogen). Each transfection condition was replicated six times. After a 24-hour incubation period, the activities of Firefly and Renilla luciferase were evaluated using the Dual Luciferase Reporter Assay System (Promega).

Statistical Analysis

Each experiment was replicated at least three times, with the results either presented as representative samples or as an average of the collected data. Data are expressed as the mean ± standard error of the mean (SEM). Statistical significance was determined using 2-tailed *t*-tests, with a *P* value of less than 0.05 considered statistically significant. Statistical significance: $^{*}P < 0.05$; $^{**}P < 0.01$; and $^{***}P < 0.001$.

Results

Defining the Ubiquitination Landscape and Its Involvement in Mouse Corneal Epithelial Wound Healing

To elucidate the role of ubiquitination during mouse corneal epithelial wound healing, we first assessed global ubiquitination levels in CEWH models. It is well-established that P4D1 recognizes the total ubiquitin level, FK2 targets both mono- and poly-ubiquitinated proteins, K48Ub is specific for Lys48-linked ubiquitination, and K63Ub identifies Lys63-specific ubiquitination.[14] Our findings revealed elevated levels of P4D1 (Fig. 1A), FK2 (Fig. 1B), K48Ub (Fig. 1C), and K63Ub (Fig. 1D) at 48 hours post mouse corneal epithelial wound healing, underscoring the pivotal role of the protein ubiquitination pathway in the wound healing process.

Considering the involvement of K48Ub and K63Ub in the ubiquitin-proteasomal and lysosomal degradation pathways, respectively, we subsequently used the proteasomal inhibitor MLN2238 and lysosomal inhibitor Lys05 to discern the contributions of both pathways to CEWH. The proteasomal inhibitor (Figs. 2A, 2B) or lysosomal inhibitor (Figs. 2C, 2D) both delayed the wound closure. These effects substantiate that both the ubiquitin-proteasomal and lysosomal degradation pathways are involved in mediating mouse corneal epithelial wound healing.

Inhibition of Proteasome or Lysosomal Pathways Impairs HCEC Migration and Proliferation

Cell migration and proliferation are pivotal processes in the wound healing cascade.[2,3] To further delineate the functional implications of ubiquitination in CEWH, we exposed HCECs to either MLN2238 or Lys05 inhibitor. Their effects on cell migration and proliferation were quantified using in vitro wound healing assays, MTS assays, and EdU proliferation assays, respectively. Relative to untreated control cells, those treated with the proteasome or lysosomal inhibitors exhibited hindered cell migration (Figs. 3A, 3B) and diminished cell proliferation (Figs. 3C–3E) in HCECs. Collectively, these findings suggest that the ubiquitin-proteasomal and lysosomal pathways potentially modulate epithelial wound healing by influencing cell migration and proliferation dynamics.

Identification of Nedd4 as a Crucial E3 Ligase Involved in CEWH

To pinpoint ubiquitination-associated proteins, including E3 ligases, deubiquitinases, and ubiquitinated proteins involved in CEWH, we used the FK2 antibody to enrich these proteins. Subsequent analysis via liquid-chromatography tandem mass spectrometry (LC-MS/MS) revealed a host of differentially expressed candidates (Fig. 4A). A total of five ubiquitin ligases and six deubiquitinases were identified (Supplementary Table S1). Notably, the content of the E3 ligase Nedd4 was substantial and underwent marked upregulation during CEWH, highlighting its significance in the process (Fig. 4B). To further elucidate its role, we used specific siRNA to knock down Nedd4 in mouse corneal epithelium. Successful knockdown efficiency was validated through RT-qPCR (Fig. 4C). The in vivo downregulation of Nedd4 resulted in a concomitant decrease in ubiquitin markers (Supplementary Fig. S1A), with levels decreasing as

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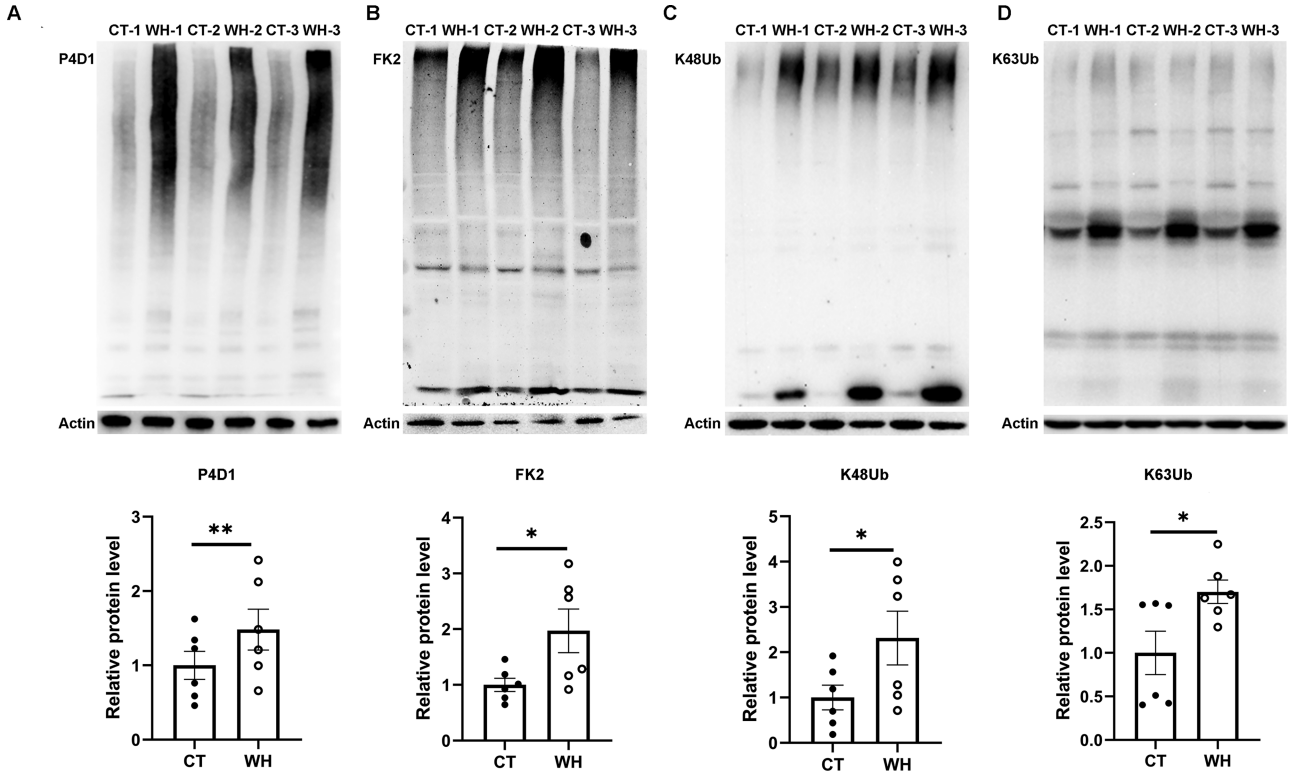

Figure 1. Elevated ubiquitination levels during mouse corneal epithelial wound healing. (**A**) Western blot analysis (*top*) and corresponding densitometric quantification (*bottom*) showing levels of P4D1 in corneal epithelium isolated from either control (CT) or wound healing (WH) eyes at 48 hours post-injury ($n = 6$/group). (**B**) Levels of FK2 determined by Western blot and in corneal epithelium obtained from CT or WH eyes at the 48 hours mark ($n = 6$/group). (**C**) Western blot representation of K48Ub expression in corneal epithelium sampled from CT or WH eyes 48 hours post-injury ($n = 6$/group). (**D**) Display of K63Ub levels via Western blot from corneal epithelium of either CT or WH eyes, evaluated at 48 hours ($n = 6$/group). CT, control; WH, wound healing.

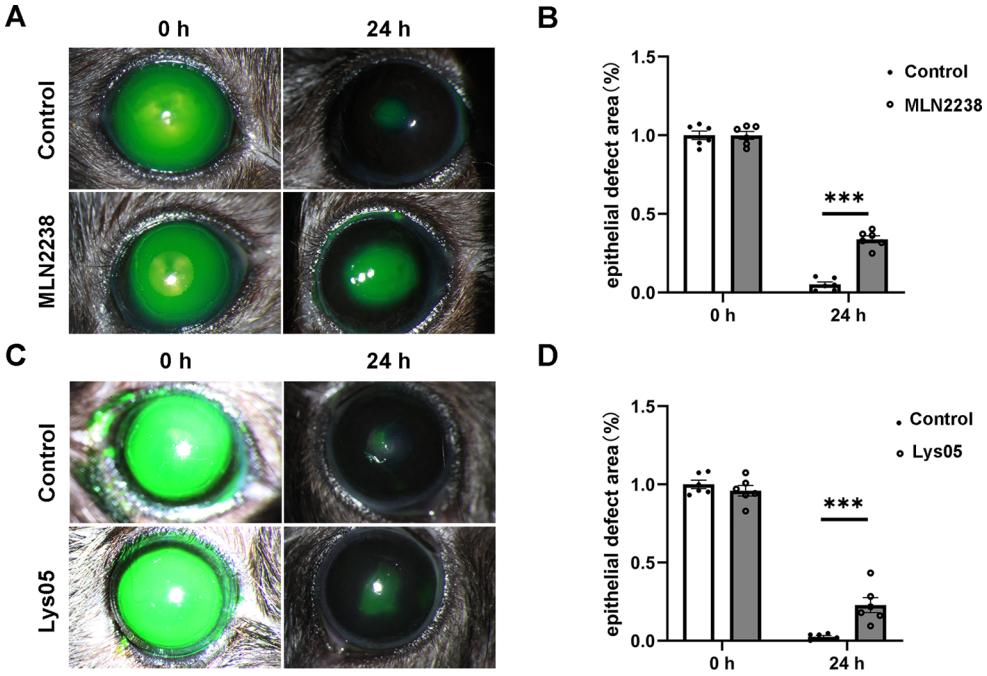

Figure 2. Impact of proteasomal and lysosomal inhibitors on mouse CEWH. (**A, B**) Fluorescein-stained representative images and corresponding quantification of wound defect areas in injured corneas treated with either control (DMSO) or the proteasome inhibitor MLN2238. (**C, D**) Fluorescein-stained representative images and corresponding quantification of wound defect areas in injured corneas treated with either control (DMSO) or the lysosomal inhibitor Lys05. Wound boundaries are delineated by *green markings*. Wound defect areas were quantified using ImageJ software ($n = 6$/group).

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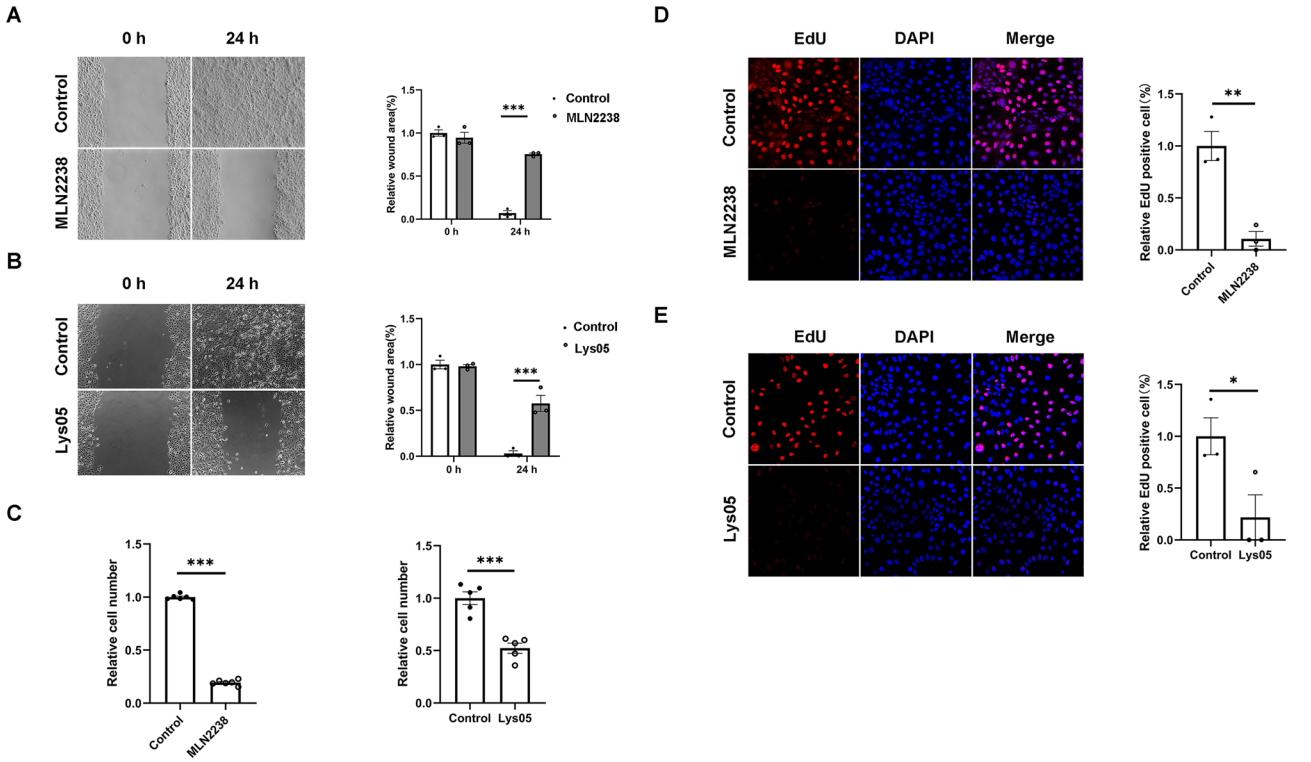

Figure 3. Proteasomal and lysosomal inhibitors impede cell migration and proliferation in HCECs. (**A, B**) In vitro scratch wound assays comparing control HCECs treated with DMSO to those treated with the proteasome inhibitor MLN2238 (**A**) or the lysosomal inhibitor Lys05 (**B**) (n = 3/group). (**C**) Cell proliferation rates of HCECs, assessed via MTS assays, following treatment with DMSO, MLN2238 (n = 6/group), or Lys05 (n = 5/group). (**D, E**) EdU staining comparing HCECs treated with DMSO to those treated with MLN2238 or Lys05 (n = 3/group).

follows: 33% reduction for P4D1, 29% reduction for FK2, 26% reduction for K48Ub, and 18% reduction for K63Ub. Intriguingly, the downregulation of Nedd4 considerably impeded epithelial wound closure (Figs. 4D, 4E), accompanied by the decrease of cell proliferation (Supplementary Fig. S1B), underscoring the pivotal role of Nedd4 in mediating the mouse corneal epithelial wound healing trajectory.

Knockdown of Nedd4 Impedes the Migration and Proliferation of HCECs

To delineate the functional role of Nedd4 in epithelial wound healing, we silenced Nedd4 in HCECs and subsequently assessed its effects on cell migration and proliferation. Our initial investigation assessed the influence of Nedd4 silencing on ubiquitination. In vitro, a complete depletion of Nedd4 led to substantial declines in ubiquitination levels (65% decrease for P4D1, 45% decrease for FK2, 40% decrease for K48Ub, and 66% decrease for K63Ub; Supplementary Fig. S2). The subsequent cellular analysis revealed a marked suppression of both cell migration (Figs. 5A, 5B) and proliferation (Figs. 5C–5E). These observations imply that Nedd4 potentially orchestrates CEWH through modulating cell migration and proliferation dynamics.

Nedd4 Modulates Stat3 Signaling Via PTEN in CEWH

Nedd4 has been documented as an E3 ligase that modulates p-Stat3 signaling.[15] Concurrently, augmented p-Stat3 signaling is identified as a potent facilitator of CEWH in diabetic mice.[16] Consequently, we postulated that Nedd4 might steer the CEWH process by manipulating p-Stat3 formation. In agreement with our hypothesis, knockdown of Nedd4 in mouse corneal epithelium or HCECs conspicuously reduced the protein abundance of p-Stat3, albeit the overall protein concentration of Stat3 remained stable (Figs. 6A, 6B). Reinforcing the notion that changes in the p-Stat3 status underlie Nedd4 control of CEWH, the p-Stat3 expression underwent a robust elevation during CEWH (Figs. 6C, 6D). Furthermore, in vitro assays demonstrated that curbing p-Stat3 signaling via its inhibitor, Niclosamide, hinders both cell migration and proliferation (Figs. 6E–6I).

To elucidate the substrate proteins targeted by Nedd4 within the Stat3 signaling cascade, we embarked on identifying potential substrate candidates. Given that PTEN stands as an established substrate of Nedd4 and is known to temper p-Stat3 expression,[17–19] it emerged as a focal point. Significantly, Nedd4 knockdown, both in vivo and in vitro, led to an upregulation of PTEN levels, as illustrated in Figure 7A. Conversely, a reduced expression of PTEN was observed to enhance cell migration and proliferation rates (Figs. 7B–7D). Co-immunoprecipitation assays have confirmed that Nedd4 is capable of interacting with PTEN (Fig. 7E). Furthermore, the silencing of Nedd4 results in a decreased ubiquitination of PTEN in HCECs (Fig. 7F). This lends credence to the hypothesis that PTEN may serve as a substrate for Nedd4 during the CEWH process, which was further corroborated by the observed downregulation of PTEN amidst CEWH (Figs. 7G, 7H). In summation, our findings attest to the pivotal role of Nedd4

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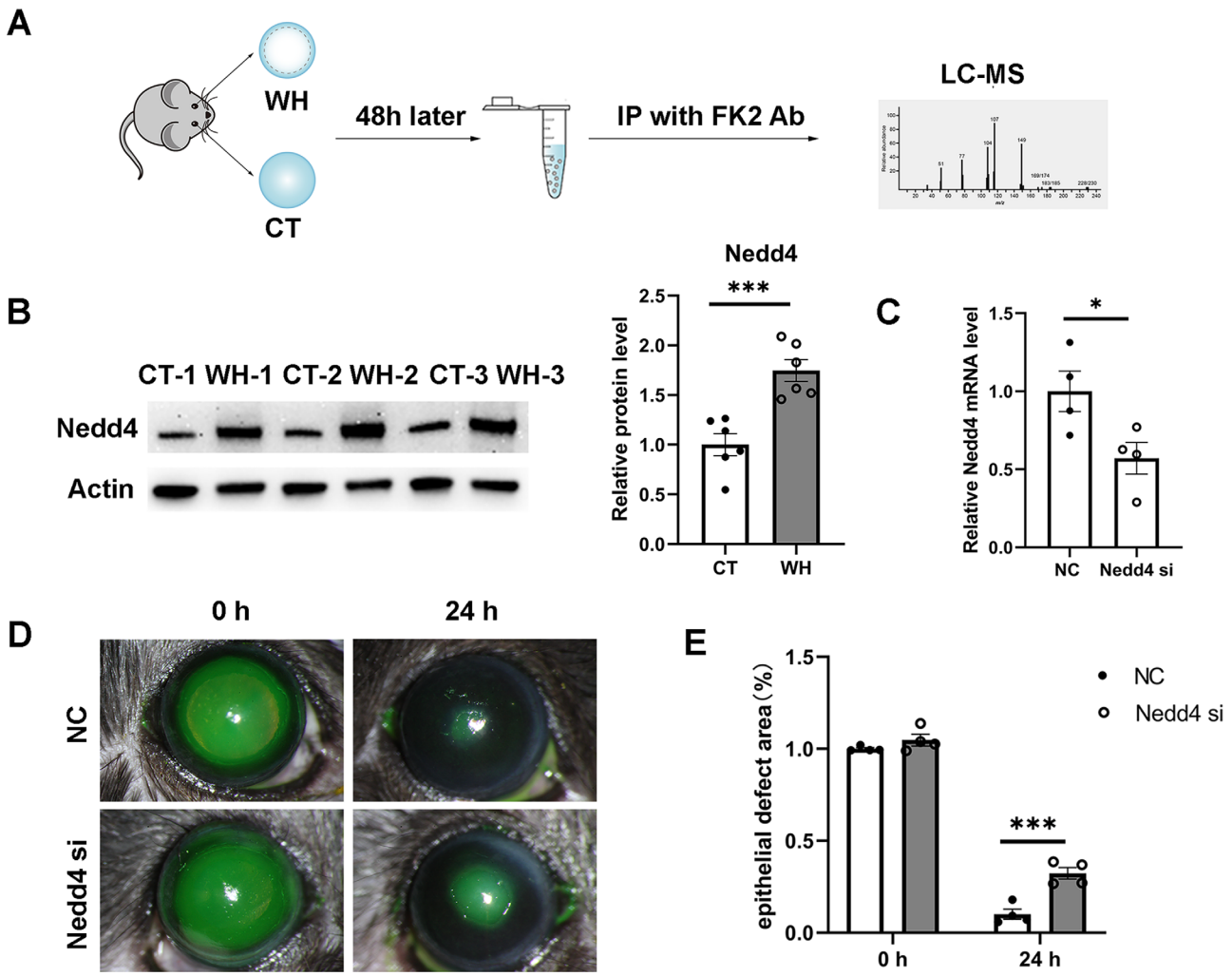

Figure 4. E3 ligase Nedd4 emerges as a crucial regulator in CEWH. (**A**) Schematic representation of the LC-MS/MS analysis, utilizing FK2 antibody to enrich ubiquitination-associated proteins from control (CT) or wound healing (WH) mouse corneal epithelium. (**B**) Western blot analysis highlighting Nedd4 expression in CT and WH mouse corneal epithelium samples (n = 6/group). (**C**) Relative mRNA levels of Nedd4 between the negative control (NC) and Nedd4 siRNA-treated samples. β-actin served as an internal control (n = 4/group). (**D**) Representative fluorescein staining images of injured corneas treated with either negative control (NC) or Nedd4 siRNA. Wound margins are denoted by *green segments*. (**E**) Quantitative analysis of wound defect areas in both NC and Nedd4 siRNA-treated groups (n = 4/group).

in modulating p-Stat3 signaling via PTEN during mouse CEWH.

Direct Regulation of Nedd4 by miR-30 and miR-200 Family Members

Having discerned a correlation between Nedd4 and CEWH, we turned our focus toward uncovering potential upstream regulators of Nedd4 expression. In our prior research, we observed a remarkable decline in the expression of miR-30b, miR-30c, miR-30d, and miR-200b during mouse CEWH.[11] Further bioinformatic analysis using TargetScan suggested that Nedd4 might be a target of miR-30b, miR-30c, miR-30d, and miR-200b.

To validate the direct interaction between the 3'UTR of Nedd4 and the aforementioned miRNAs, we used a luciferase reporter assay. Bioinformatics analysis predicted 4 potential binding sites for the miR-30 family and 2 for miR-200b within the Nedd4 3'UTR (Fig. 8A). Introduction of miR-30b, miR-30c, miR-30d, or miR-200b into HEK293 cells harboring the wild-type 3'UTR construct (pLuc-Nedd4 3'UTR), resulted in significant repression of luciferase activity relative to the NC. However, when these miRNAs were co-transfected with vectors carrying mutated binding sites (pLuc-Nedd4 3'UTR-30Mut or pLuc-Nedd4 3'UTR-200Mut), the luciferase activity remained unaffected (Figs. 8B, 8C).

To further examine if the observed downregulation of these miRNAs could contribute to the elevated Nedd4 levels during wound healing, we transfected HCECs with mimics of miR-30b, miR-30c, miR-30d, or miR-200b and subsequently analyzed Nedd4 protein expression. Strikingly, Nedd4 protein levels were substantially suppressed in the presence of these miRNA mimics compared to the negative control (Figs. 8D, 8E). Collectively, these findings compellingly suggest that miR-30b/c/d and miR-200b are potential upstream regulators of Nedd4 during the wound healing process.

Collectively, these results demonstrate that the downregulation of the miR-30 and miR-200 families leads to an

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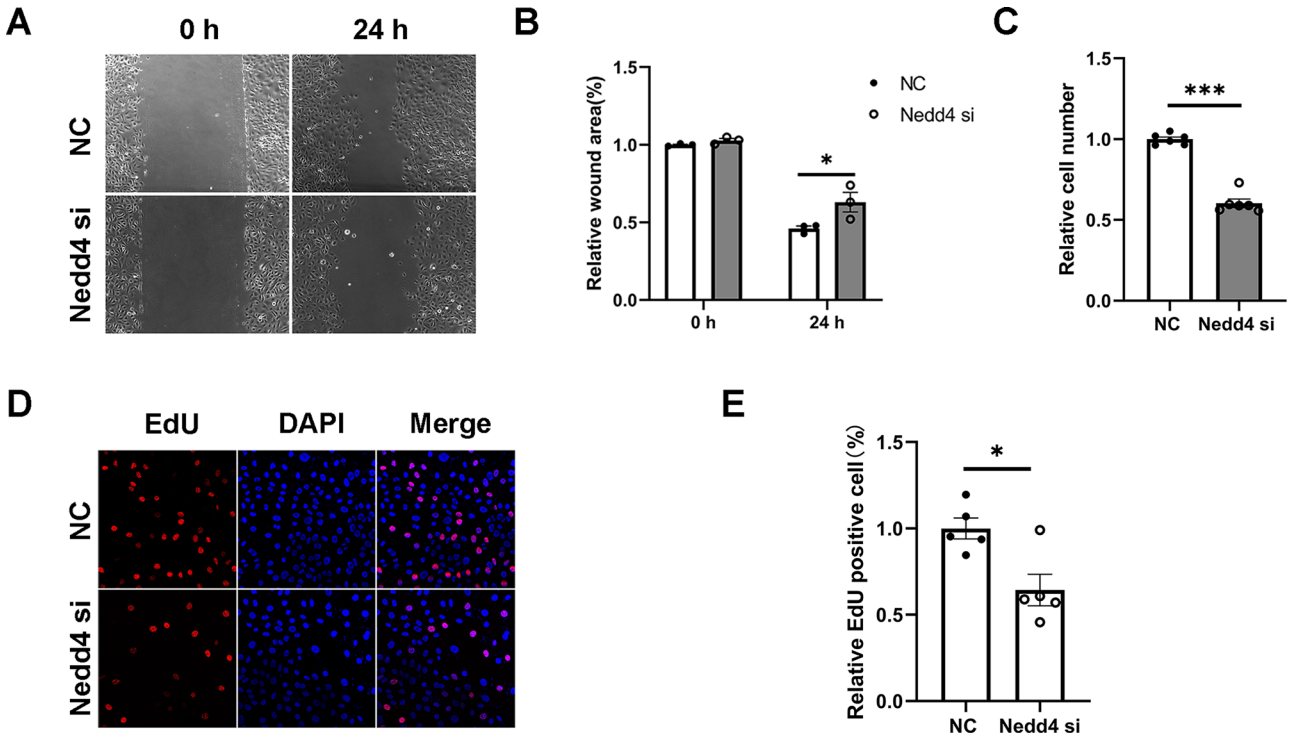

Figure 5. Nedd4 knockdown suppresses cell migration and proliferation in HCECs. (**A**) Representative images from the in vitro scratch wound assay comparing cells treated with either negative control (NC) or Nedd4 siRNA. (**B**) Quantitative analysis of wound closure in HCECs treated with NC or Nedd4 siRNA ($n = 3$/group). (**C**) Cell proliferation rates for NC and Nedd4 siRNA-treated HCECs, as determined by MTS assays ($n = 6$/group). (**D, E**) EdU staining results for cells under NC or Nedd4 siRNA treatment ($n = 5$/group).

increase in Nedd4 protein levels, which in turn promotes p-Stat3 formation by facilitating the degradation of PTEN during CEWH (Fig. 9).

Discussion

Ubiquitination plays a pivotal role in modulating numerous signaling pathways by governing the degradation, localization, and functionality of substrate proteins. Several intricate and diverse signaling pathways are implicated in CEWH, including Wnt,[20,21] p-Akt,[22,23] TGF-β,[24,25] MAPK,[26] and NF-κB.[27] Ubiquitination has been demonstrated to influence the stability and activity of these pathways through key regulators, such as ligands, receptors, kinases, phosphatases, and transcription factors. Although prior studies have confirmed the involvement of several E3 ligases and deubiquitinases in the wound healing processes,[9,10] the potential alterations in the ubiquitination process during CEWH remained ambiguous. In this study, we observed a marked increase in global protein ubiquitination levels during CEWH and a total of five E3s and six deubiquitinases have been identified. Our data unambiguously positions Nedd4 among the top three ubiquitin ligases implicated in CEWH, highlighting its prominent and probable pivotal role. Moreover, our interest extends beyond Nedd4. Parallel to our efforts with Nedd4, we are in the process of expounding on the regulatory functions and mechanisms by which the E3 ligase UBR5, and the deubiquitinase (Otub1 and Usp14) influences CEWH (data not shown). Unraveling the roles and mechanisms of the ubiquitination process within CEWH could offer innovative strategies for wound repair in the cornea and beyond.

Nedd4 was originally identified as a pivotal regulator during the development and differentiation of the central nervous system in the mouse brain.[28] Subsequent research has underscored its crucial roles in diverse cellular processes, including cancer development,[29] neurodevelopment,[30] immunity,[31,32] and lipid transport[33] among others. Nedd4 has been implicated in the modulation of several signaling pathways, such as Wnt signaling,[34] Notch signaling,[35,36] p-Akt signaling,[17] and Stat3 signaling.[15,37] In the present study, we discovered an upregulation of Nedd4 during CEWH. The knockdown of Nedd4 was observed to impede the wound healing process, attenuating both cell migration and proliferation through the Stat3 signaling pathway. In alignment with our observations, Nedd4 has been documented as a cardinal regulator of skin homeostasis and wound repair, with a particular influence on the EGFR/ERK1/2 and Yap signaling pathways.[38] Several in vitro studies further corroborated the role of Nedd4 in promoting cell migration and proliferation.[39–41] Collectively, these findings underscore the significance of Nedd4 as a central player in the wound healing processes, extending beyond the corneal epithelium to other tissue types.

The multifaceted roles of Nedd4 arise from its context-dependent cellular functions and its interaction with a plethora of substrates.[42] Notably, the phosphatase PTEN stands out as one of its significant substrates.[17,43] PTEN has been previously shown to act as a negative regulator of the Stat3 signaling pathway across various models.[18,19] In line with these findings, our study reveals that the knockdown of Nedd4 results in an augmented protein level of PTEN. Interestingly, recent research has unveiled PTEN's involvement in diverse tissue repair mechanisms.[44–47] In studies focusing on

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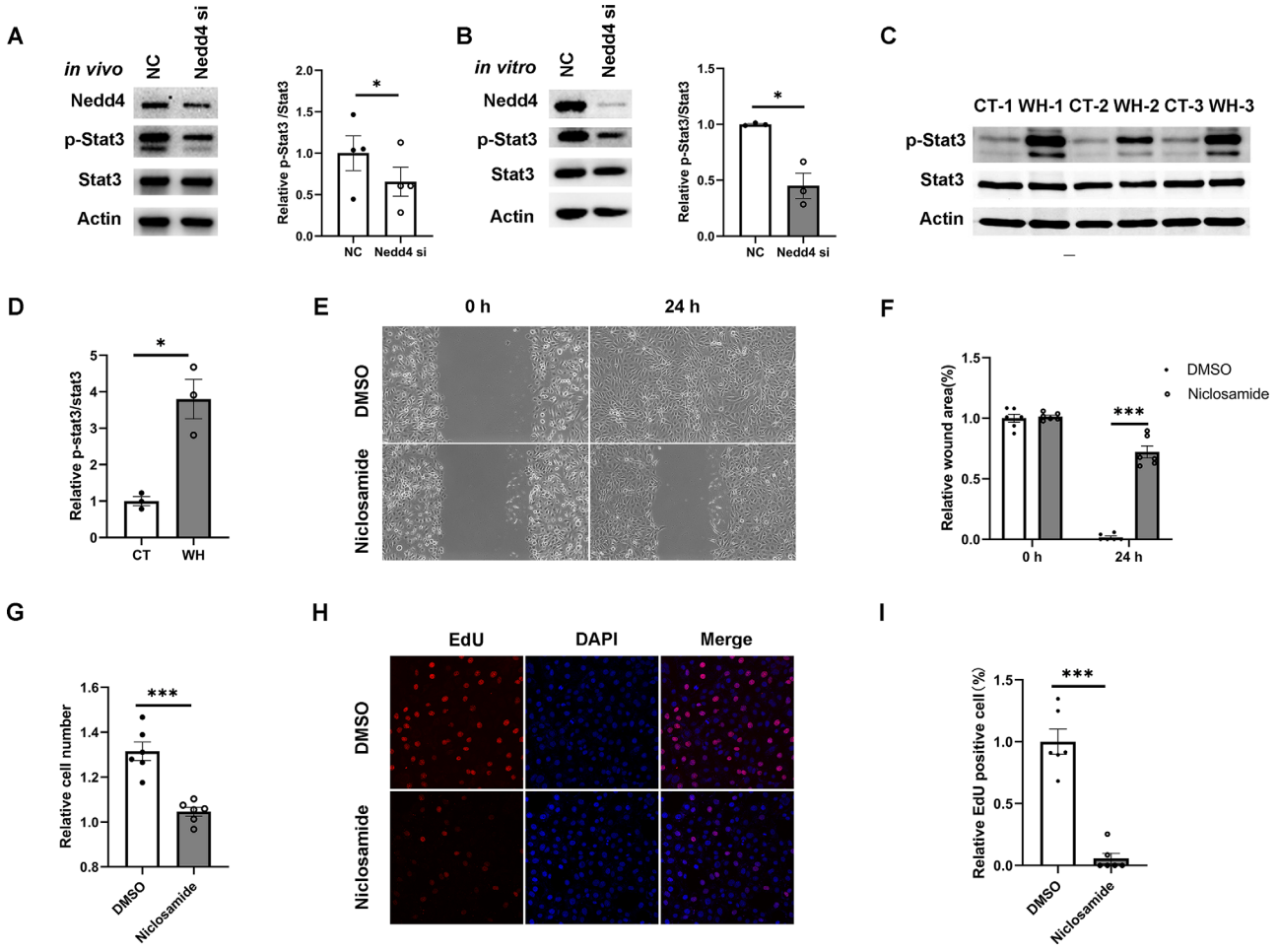

Figure 6. Nedd4 modulates Stat3 signaling, impacting cell migration and proliferation during CEWH. (**A, B**) Western blot analyses and subsequent quantification of Nedd4, Stat3, p-Stat3, and β-actin in mouse corneal epithelium samples (n = 4/group) or HCECs (n = 3/group) transfected with either negative control (NC) or Nedd4 siRNA. (**C, D**) Western blot assessments and quantification of Stat3 and p-Stat3 from corneal epithelium isolated from control (CT) or 48-hour post-wound healing (WH) eyes (n = 3/group). (**E, F**) In vitro scratch wound assays conducted on HCECs treated with either DMSO or Niclosamide (n = 6/group). (**G**) Cell proliferation rates derived from MTS assays comparing DMSO and Niclosamide treated HCECs (n = 6/group). (**H, I**) EdU staining results for HCECs treated with DMSO or Niclosamide (n = 6/group).

corneal epithelial injuries, both under normal and diabetic conditions, a diminished expression of PTEN was observed. Reduction or inhibition of PTEN expedites the wound healing process.[23,48]

A salient point to consider is the nature of ubiquitination: substrates can be conjugated with either single or polymeric ubiquitin chains. Depending on the type of ubiquitin linkage chains, the fate of substrate proteins can vary. It has been identified that substrates targeted by Nedd4 often contain PY motifs, including LPxY and PPxY sequences.[49] Nedd4 modulates its substrates through different ubiquitin linkage types, such as K48, K63, K11, and K27, leading to either degradation of target proteins or alteration in their activity.[42] In this study, although we confirmed that the knockdown of Nedd4 reduced the ubiquitination level of PTEN, the specific type of ubiquitin chains implicated in PTEN's regulation by Nedd4 remains elusive. Recently, in addition to the canonical cytosolic PTEN form, two distinct splice variants of PTEN have been identified in eukaryotes.[50,51] Unlike the well-established tumor-suppressive function of the canonical PTEN, PTENα, and PTENβ can potentially promote tumorigenesis.[52,53] It poses an intriguing question: does Nedd4 influence these alternative splice variants of PTEN? Further exploration in this direction is warranted.

Recently, multiple studies have elucidated the upstream signaling pathways modulating Nedd4 expression. Notably, miR-1, miR-7, and miR-30 have been identified as pivotal upstream regulators of Nedd4.[54–56] Our prior research indicated a marked decrease in the expression levels of miR-30b, miR-30c, miR-30d, and miR-200b during CEWH.[11] However, the downstream target of miR-30b, miR-30c, miR-30d, and miR-200b are not clear in CEWH. In the study, we established that miR-30b, miR-30c, miR-30d, and miR-200b directly modulate Nedd4 expression, offering a novel avenue for targeting Nedd4 in the context of CEWH. It is crucial to recognize that although miRNAs can directly regulate Nedd4, the proposed hypothesis does not assume a simplistic, linear regulatory mechanism from miRNAs to Nedd4. Given that miRNAs often target a multitude of genes, and that Nedd4 regulation may extend beyond miRNAs, any contributions of miR-30 and miR-200 to wound heal-

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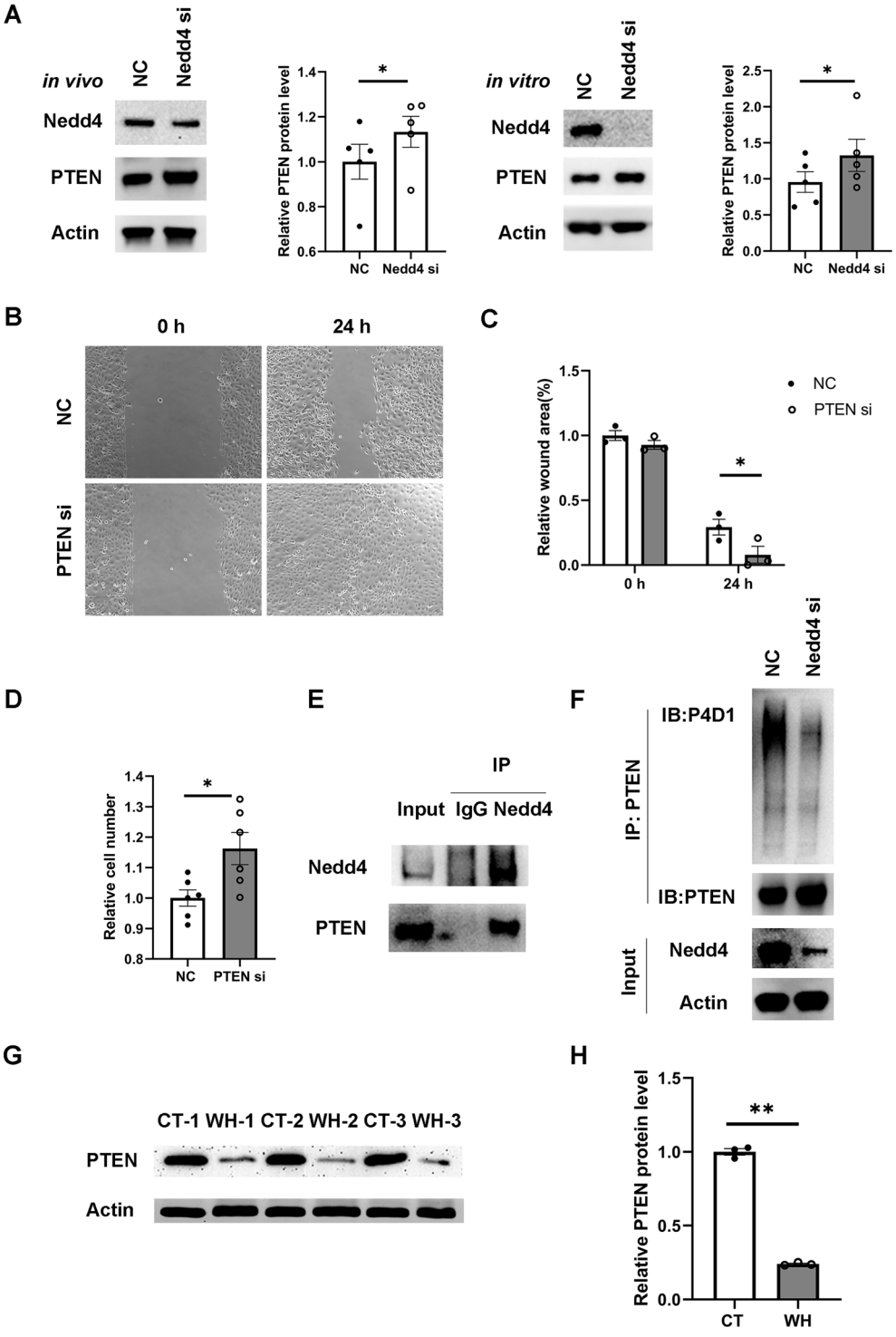

Figure 7. Nedd4 depletion elevates PTEN levels, influencing cell migration and proliferation in CEWH. (**A**) Western blot analyses and subsequent quantification show levels of Nedd4, PTEN, and β-actin in whole cell lysates from mouse corneal epithelium samples or HCECs transfected with either negative control (NC) or Nedd4 siRNA. (**B, C**) Following transfection with NC or PTEN siRNA, in vitro scratch wound assays were conducted on HCECs, and the resulting wound areas were quantified (n = 3/group). (**D**) Cell proliferation rates in HCECs treated with either NC or PTEN siRNA were determined using MTS assays (n = 6/group). (**E**) A co-immunoprecipitation assay was performed to detect the interaction of endogenous Nedd4 and PTEN using an Nedd4 antibody in HCECs. (**F**) To assess the ubiquitination level of PTEN upon Nedd4 knockdown in HCECs, cells transfected with either NC or Nedd4 siRNA were treated with 50 µM MG132 for 6 hours, followed by immunoprecipitation using an anti-PTEN antibody. (**G, H**) Western blotting, complemented by quantification, displays the levels of PTEN and β-actin in corneal epithelium isolated from control (CT) or wound healing (WH) eyes at 48 hours post-injury (n = 3/group).

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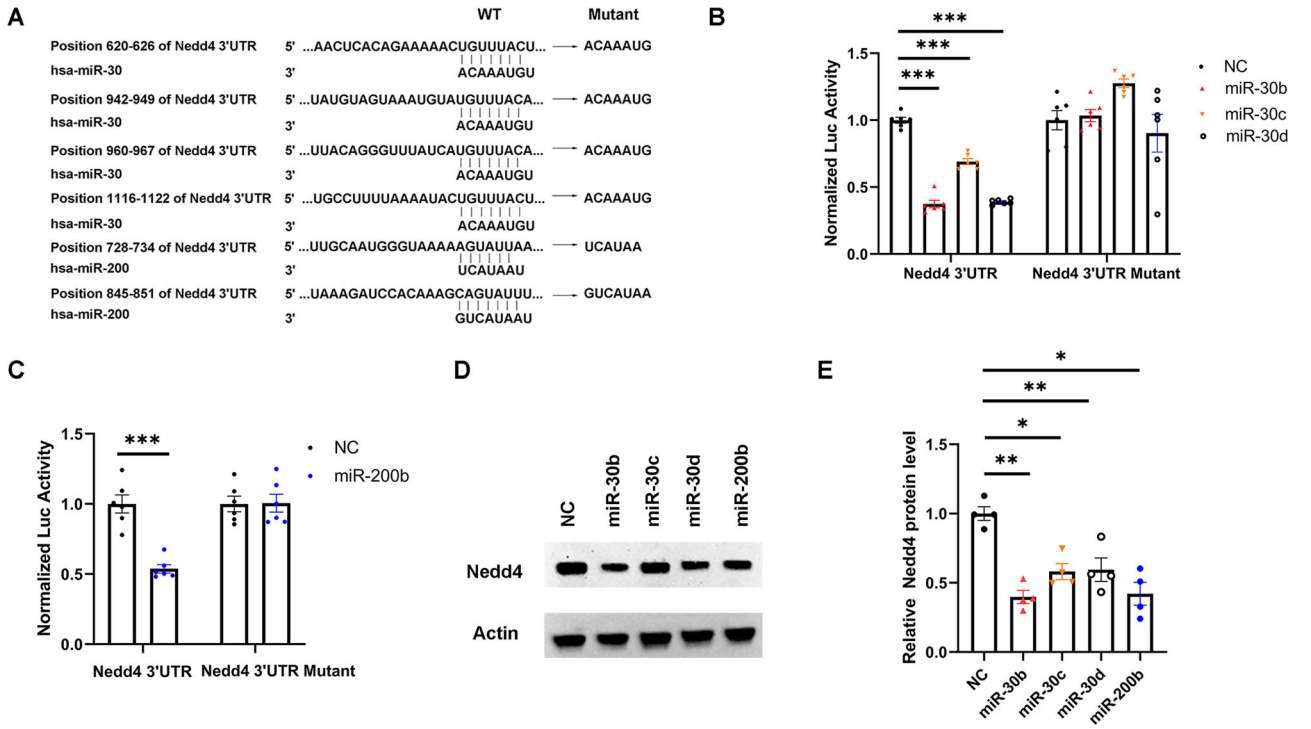

Figure 8. miR-30 and miR-200 directly regulate Nedd4 expression. (**A**) The alignment illustrating the predicted target sites of miR-30 and miR-200 within the 3'UTR of Nedd4 is presented. (**B, C**) HEK293 cells underwent co-transfection with a combination of either NC, miR-30b/c/d, or miR-200b mimics, alongside pLuc-Nedd4 3'UTR, pLuc-Nedd4 3'UTR 30 Mutant, or pLuc-Nedd4 3'UTR 200b Mutant and a pRL-SV40 reporter plasmid. Following a 24-hour incubation, luciferase activity was assessed. Data represent the ratio of luciferase activity to Renilla luciferase activity (n = 6/group). (**D, E**) Western blot analyses reveal Nedd4 levels in HCECs following transfection with either negative control (NC), miR-30b/c/d mimics, or miR-200b mimics.

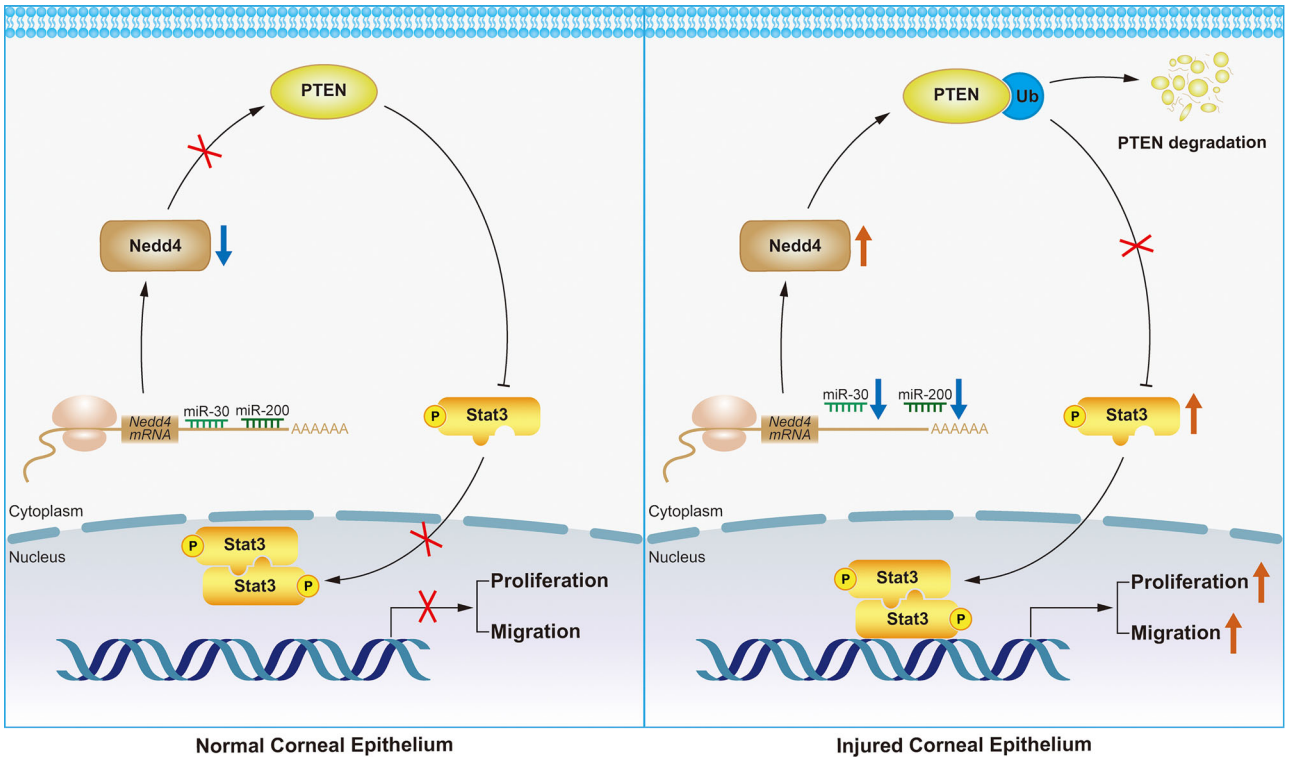

Figure 9. Schematic representation illustrating the involvement of ubiquitination and the pivotal role of the E3 ligase, Nedd4, in CEWH. Under the regulatory influence of miR-30 and miR-200, Nedd4 enhances CEWH progression via the modulation of PTEN and downstream Stat3 signaling.

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ing processes are likely complex and not solely via the Nedd4/PTEN/p-STAT3 axis.

In conclusion, our study underscores the integral role of ubiquitination in corneal epithelial wound healing. We highlight that the E3 ligase Nedd4, under the regulatory purview of miR-30b, miR-30c, miR-30d, and miR-200b, enhances CEWH progression through PTEN-mediated Stat3 signaling. To the best of our knowledge, this represents the inaugural investigation revealing the global ubiquitination dynamics during CEWH. These insights augment our comprehension of the function of ubiquitination in CEWH. Considering the potential involvement of other E3 ligases and DUBs in CEWH, future research should probe the roles of additional ubiquitination-associated proteins in this process.

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