The Therapeutic Roles of Recombinant Hsp90α on Cornea Epithelial Injury

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Purpose. The purpose of this study was to explore the therapeutic role of heat shock protein 90 (Hsp90) in wound healing of injury cornea epithelium.

Methods. The right eye of C57BL/6N male mice were performed the debridement wounds in the center of the cornea using an algerbrush II blade. The injured area was determined by staining the cornea with fluorescein sodium and measured with image-J. Immunoblotting, ELISA and immunochemistry were used for determining protein expression. The quantitation PCR was performed to measure mRNA expression.

Results. Hsp90α is upregulated at both the mRNA and protein levels, and is secreted extracellularly into the corneal stroma and tear film during the healing process after corneal injury in mice. This upregulation is associated with activation of HSF1. Administration of recombinant exogenous Hsp90α (eHsp90α) speeds up wound healing of injured corneal epithelium. The eHsp90α binds to low-density lipoprotein (LDL)-related protein-1 (LRP-1) on the corneal epithelial cells and increases phosphorylation of AKT at S473, which is associated with proliferation and migration corneal epithelial cells in vitro or vivo. Inhibition of AKT by its inhibitor LY294002 abolishes eHsp90α-induced migration and proliferation of corneal epithelial cells.

Conclusions. Hsp90α is upregulated and secreted after corneal injury and acts to promote the healing process. Recombinant Hsp90α may be a promising therapeutic drug candidate for corneal injury.

Keywords: heat shock protein 90 (Hsp90α), wound healing, proliferation, migration, lipoprotein-related protein-1 (LRP-1)

Heat shock proteins (Hsps) belong to a large family of chaperones that assist in protein folding, trafficking, and degradation in cells under divergent proteotoxic conditions.[1,2] Hsp90 is expressed abundantly in most cells and is involved in regulating cellular proteostasis at both physiological and pathological conditions (e.g., cell proliferation, differentiation, migration, aging, tumorigenesis and metastasis, and neurodegeneration).[3,4] The Hsp90 subfamily consists of Hsp90α and Hsp90β, two isoforms that are encoded by Hsp90AA and Hsp90AB, respectively. Hsp90α shares 89% homology in amino sequences with Hsp90β. Although the expression pattern differs, they compensate each other in chaperoning the intracellular proteostasis.[3] In addition to modulating intracellular proteostasis, both Hsp90α and Hsp90β are secreted extracellularly via a noncanonical pathway and is involved in the regulation of epithelial-mesenchymal transition (EMT), angiogenesis, wound healing, and formation of amyloid-beta fibrillin.[5–7] Preclinical studies suggest that both Hsp90α and Hsp90β are promising drug targets.[6,8]

Extracellular Hsp90 (eHsp90) is associated with many diseases. The eHsp90 exists in the exosome and cross-links with VEGF (90 k), enhancing VEGF activation of neovascularization and attenuating VEGF affinity for bevacizumab, resulting in decreased therapeutic effectiveness of bevacizumab. The eHsp90 inhibitor enhances the therapeutic effect of the anti-VEGF antibody on neovascularization in tumors.[9] The eHsp90/eHsp70-associated extracellular vesicles are associated with tumor-induced muscle wasting.[10] The eHsp90 enhances tumor cell EMT and metastasis by associating with TGF-β1[11] and metalloproteinases (MMP-2 and MMP-9).[12,13] The regulation of eHsp90 on MMP2 protein stabilization and activity is regulated by extracellular cochaperones TRIM and Aha1.[14] The eHsp90 is upregulated and promotes wound healing of skin lesions secondary to mechanical causes, chemical agents and systemic disease.[15–17] The eHsp90 is upregulated and secreted by multiple cell types in injured skin, including keratinocytes, macrophages, and fibroblasts.[18,19] The eHsp90 is secreted through the TMED 10 channel, and

regulated via an unconventional pathway.[20,21] TGF-α triggers eHsp90 expression during dermatologic wound healing,[15] and HIF1α is associated with hypoxia-induced eHsp90 secretion in wounded skin tissues.[7] The eHsp90 promotes wound healing via its M-domain amino acids rather than its ATPase activity.[8,22] The eHsp90 regulates wound healing by binding to and activating its receptor the low-density lipoprotein receptor-related protein-1 (LRP-1) associated pathways, such as activating MAPK1/2 and AKT pathways[22] or upregulating polycomb EZH2 expression. EZH2 binds to and represses E-cadherin promoter activity, resulting in EMT of epithelial cells and tumor cell invasion.[23] In lens tissue, Hsp90 is essential for proliferation, EMT, and migration of capsular residue epithelial cells by interacting with EGFR and TGF-β2 pathways.[24] However, the role of eHsp90 in this lens capsular wound-healing process is not determined yet.

The anterior corneal epithelium is composed of a stratified, squamous, non-keratinized epithelium forming the first barrier against the external environment. Like other epithelial barriers in the human body, the corneal epithelium is a self-renewing tissue with a distinct stem cell niche residing in the limbal basal region to provide an unlimited supply of proliferating cells for epithelial regeneration.[17] The corneal epithelium is continuously exposed to chemical, physical, and biological insults that can result in corneal injury. Corneal epithelial cells respond rapidly to injury, proliferating and migrating to cover the defect and to re-establish its barrier function. This wound-healing process is regulated by coordinated interactions of numerous growth factors and cytokines, including transforming growth factor (TGF-β), platelet derived growth factor (PDGF), fibroblast growth factor (FGF), insulin-like growth factor (IGF-1), etc.[25] In addition, the corneal epithelial cells upregulate the expression of heat shock proteins in response to the injury, which are essential for the survival, proliferation, and migration of corneal epithelial cells during wound healing.[26–28] Hsp27 phosphorylation is induced during mice cornea wound healing.[29,30] Knocking down Hsp27 by siRNA reduces corneal epithelial cell wound healing and upregulates Bax protein expression in the human corneal epithelial cell (HCEC) line in vitro.[31] In canine models, Hsp70 and other heat shock proteins, like Hsp27 and Hsp47, are induced during the re-epithelialization.[27] The inhibition of Hsp70 delays corneal epithelial cell wound healing in vitro.[27] However, the administration of Hsp70 can trigger fibroblast cell migration but not migration of corneal epithelial cells in vitro.[27] In the mouse model, pre-heat shock, which induces Hsp70 in corneal tissue, helps corneal wound healing and protects corneal epithelial cell apoptosis.[32] Like Hsp70, Hsp90α is a stress-inducible chaperone, and under normal conditions, is only weakly expressed in the cornea. Hsp90α is involved in regulating YAP activation and formation of cell-to-cell junction in the corneal epithelium.[33] After injury, Hsp90 is upregulated in corneal stroma keratinocytes and works with TGF-β1 to modulate the transition from keratinocytes to myofibroblast.[34] However, the role of eHsp90α during corneal wound healing still remains unclear.

In this paper, we studied the expression and secretion of Hsp90α during wound healing in injured cornea in mice in vivo and in the HCEC line in vitro. We found that Hsp90α was upregulated both intracellularly and extracellularly during the recovery of injured corneal epithelial cells. The administration of recombinant Hsp90α triggered the healing process of injured cornea by activating the LRP-1-AKT pathway. Accordingly, we proposed that the recombinant Hsp90α may be a promising drug candidate for cornea injury therapy.

Materials and Methods

Antibodies

The anti-Hsp90α antibody was bought from Becton, Dickinson and Company (cat# 610419; Franklin Lakes, NJ, USA), the anti-Hsp70 antibody was from Enzo (cat #ADI-SPA-810-F, New York, NY, USA), the antibodies against Hsp40 (cat #13174-1) and GST (cat #66001-2) were from Proteintech (Wu han, China), the antibodies against Hsp25/27 (cat #95357S), HSF1 (cat #12972S), Ki67 (cat #0129S), and phosphorylated-S473/AKT (cat #4060S) were from CST (Boston, MA, USA), the antibodies to phosphorylated-S326/HSF1 (ab76076) and CK12 (ab185627) were from Abcam (Cambridge Science Park, England), the antibodies against LRP-1 (cat #A0633), AKT (cat #A18675), and β-actin (cat #AC004) were from ABclonal (Wu han, China). The HRP-conjugated goat anti-rabbit IgG (#31460), HRP-conjugated goat anti-mouse IgG (#31430), Alexa Fluor 594-conjugated goat anti-mouse (#A11005), Alexa Fluor 488-conjugated goat anti-rabbit (#A11008), and Alexa Fluor 488-conjugated goat anti-mouse (#A11001) were from Invitrogen (Carlsbad, CA, USA).

Preparation of GST and GST-Hsp90α

The cDNA of Hsp90α was PCR-amplified from human HeLa cells, and subcloned into pGEX6p-1 plasmid at restrictive enzymes SmaI and XhoI, generating PGEX-6p-Hsp90α. The PGEX-6p-1 empty vector and pGEX-6p-Hsp90α were transformed into *E. coli BL21 (DE3) pLysS*. The expression of GST and GST-Hsp90α were induced by IPTG and purified with GST purification column (C600913; BBI Life Sciences, Shanghai, China). The endotoxin was removed from the purified GST and GST-Hsp90α protein by using 1% Triton X-114 treatment. The proteins were filtered with a sterile 0.22 µm filter membrane and stored at −80°C freezer for future use.

Corneal Epithelial Debridement Wounds

The C57BL/6N male mice were bought from Beijing Vital River Laboratory Animal Technology Company at 8 weeks old. All studies were conducted in accordance with the Code of Practice for the Care and Use of Animals for Scientific Purposes. All procedures were approved by the Animal Care and Ethics Committee of Henan University School of Medicine. For wound healing on mouse cornea, mice were anesthetized through intraperitoneal injection of sodium pentobarbital (30–60 mg/kg). The debridement wounds in the center of the cornea were made using an algerbrush II blade (LOT#: 081418C) on the right eye of each mouse. The left eye was left uninjured and acted as the internal control. The injured area was determined by staining the cornea with 5 µL eye drop solution containing 5 µg fluorescein sodium (Tianjin Jingming Technological Development Company). The injured area was observed and photographed under a slit lamp microscope.

For the GST-Hsp90α treatment, the injured right eyes of mice were treated 15 µM GST-Hsp90α 3 times a day using an eye dropper. PBS or equal amounts of GST protein was used as control. For the GST-Hsp90α and LY294002 co-treatment, 15 µM of GST-Hsp90α plus 500 µM LY294002 were used.

The corneal debridement wounds were stained with sodium fluorescein and quantified with image-J. The area healed was normalized to the initial area of the debridement wound. The data shown in the bar graph represent mean ± SD.

To collect tear-film from the injured corneal surface, mice were anesthetized with intraperitoneal injection of sodium pentobarbital. The 20 µL of sterile PBS was dropped on the surface of the right, wounded cornea twice a day. The PBS was then collected and centrifuged. The protein concentration was measured using the BCA kit (CW00145; CWBIO, Beijing, China). The supernatants were subjected to SDS-PAGE electrophoresis for WB, ELISA, or Coomassie brilliant blue staining.

HCEC Line Culture

HCEC (a human corneal epithelial cell line immortalized by SV-40 T-antigen) were maintained in Dulbecco's modified Eagle's medium/Ham's F-12 50/50 Mix (DMEM/F12) supplemented with L-glutamine, 15 mM HEPES (cat: 10-092-CV; Corning, Corning, NY, USA), 10% fetal bovine serum (FBS; cat: 16000-044; Gibco, Grand Island, NY, USA), 5 ug/mL human insulin (cat: I2643; Sigma, St. Louis, MO, USA), and 10 ng/mL human recombinant epidermal growth factor (cat: E9644, Sigma). The cells were grown in a humidified incubator at 5% CO_2 and 37°C.

Enzyme Linked Immunosorbent Assay

The 5 µL of supernatants collected from tear-film of injured cornea was coated on ELISA plates at 4°C overnight. The plates were washed 3 times with PBST buffer and blocked in 5% BSA blocking buffer for 1 hour at room temperature. After washing away the blocking buffer, 100 µL of solution containing mouse anti-Hsp90 antibody (dilution, 1:100) was added to the plates incubated at 4°C overnight. The plates were washed 5 times with PBST to remove the primary antibody and then incubated with the secondary HRP-anti mouse antibody (1:10000) for 1 hour at room temperature. After washing, 100 µL TMB (PR1210; Solarbio, Beijing, China) substrate was added to each well for 20 minutes at 37°C followed by the addition of 50 µL 2M H_2SO_4 to terminate the reaction. The reactions were measured using spectrophotometry at a wavelength of 450 nm (Thermo Fisher Scientific, Waltham, MA, USA).

Western Blot Analysis

Equal amounts of lysates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, and the proteins were transferred to polyvinylidene difluoride membranes (PVDF). After 1 hour of blocking in 5% skim milk/tris-buffered saline (TBS)/0.1% Tween 20, the membranes were incubated with primary antibodies at 4°C overnight. The membranes were washed with TBS/0.1% Tween-20 3 times, then incubated with secondary antibodies conjugated with horseradish peroxidase for 1 hour. The membranes were developed using enhanced chemiluminescence and exposed to X-ray film for signal detection.

RNA Isolation and Quantitative Real-Time PCR

Total RNA was extracted with RNAiso reagent following the manufacturer's protocol (Takara, Beijing, China). One microgram of total RNA was used to synthesize cDNA (Takara). Equal amounts of cDNA were mixed with Faststart Universal SYBR Green Master Mix (Roche, San Francisco, CA, USA). The quantitative real-time PCR (qRT-PCR) was performed using an ABI 7500 system (Applied Biosystems, Foster City, CA, USA). The primer pairs 5′-CGCCACCAGTTCGCCAT-3′ and 5-CCTTCTGACCCATTCCCACC-3 were used for β-actin; primer pairs 5′-AGACGCGCTCCTTTTGATCT-3′ and 5′-TTGTTGCAGCATTTCACGGG-3′ were used for mouse Hsp90α.

Cell Proliferation Assay

The proliferative capacity of the cell was measured by the MTS assay or EdU incorporation staining kits (KGA331-100; KeyGEN BioTECH, Nanjing, China) following the kit's protocols.

For MTS assay, the cells were seeded at 2×10^3 cells/well in 96-well culture plates overnight, and then were replaced with fresh DMEM/F12 media containing GST-Hsp90α and GST at different concentrations for 48 hours. The DMEM/F12 media containing 10% MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)- 2H-tetrazolium; Promega, Madison, WI, USA) were added to cells for 1 to 4 hours. The MTS signal was measured using spectrophotometry at wavelength 570 nm. The results were reported as mean ± SD from three independent experiments. Unpaired 2-tailed *t*-test was used for statistical analysis. $P < 0.05$ was considered statistically significant.

For EdU incorporation staining assay: coverslips were placed in 24-well plates and HCECs were seeded at 1×10^4 cells/well. After the cells attached to the coverslips, the cells were cultured in serum-free DMEM/F12 basal medium alone (sham) or media containing GST, GST-Hsp90α, or GST-Hsp90α plus LY294002 for 24 hours. Then, 10 µM EdU (5-ethynyl-2′-deoxyuridine) was added to the cells for 1 hour, and then removed. Then, 500 µL 4% PFA was then added to the wells for 20 minutes. The 4% PFA was then removed and 500 µL 2 mg/mL glycine solution was added to neutralized the residual 4% PFA. The cells were washed twice using 3% BSA in PBS. It was then incubated with 0.5% Triton X-100 in PBS for 20 minutes and washed twice with 3% BSA in PBS. The 1 mL Click-iT reaction mixture (1 × Click-iT reaction buffer 860 µL, $GuSO_4$ 40 µL, kFluor 488-azide 3 µL, 1 × reaction buffer additive 100 µL) was evenly added to the cells and incubated in the dark at room temperature for 30 minutes. The cells were washed twice in 3% BSA in PBS and once with PBS only. DAPI (1:1000) was added for 5 minutes and the cells were washed twice with PBS. The immunofluorescence signals of coverslips were measured under a confocal microscope (NIKON A1R+STORM). The percentage of EdU positive cells were counted. The data shown represent mean ± SD from five independent experiments. The unpaired 2-tailed *t*-test was used for statistical analysis. $P < 0.05$ was considered statistically significant.

Cell Migration Assay

HCEC lines were seeded into six-well plates and grown overnight to reach complete confluence. The wound was made by scratching the cells with a 200-µL pipette tip. After washing 3 times with PBS to get rid of the suspended cells, the cells were cultured in serum-free DMEM/F-12 medium alone (sham) or media containing 0.625 µg/mL GST, 0.625 µg/mL GST-Hsp90α, 10 µM LY294002, or GST-Hsp90α plus

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LY294002 for 16 hours. Cell migration was photographed and the wound area was quantified with image J. The distance between the cell edges of the wound area at different times points were normalized to the distance at 0 hour. The results represent mean ± SD from three independently repeated experiments.

Immunofluorescence Staining Assay

The cells on coverslips were incubated with serum-free DMEM/F12 basal media containing 2.5 µg/mL GST or 2.5 µg/mL GST-Hsp90α for 30 minutes, after washing 3 times with PBS, the cells were fixed in 4% paraformaldehyde for 30 minutes followed by washing 3 times in PBS buffer. The cells were then incubated with antibodies against GST and LRP-1 at 4°C overnight. After washing 3 times with PBST, the cells were incubated with Alexa Fluor 594-conjugated goat anti-mouse and Alexa Fluor 488-conjugated goat anti-rabbit IgG secondary antibodies for 1 hour at room temperature. After three additional washes with PBST, the samples were counterstained with DAPI. The fluorescent signals were photographed under a confocal microscope (NIKON A1R+STORM).

For cryosection staining, the corneal tissues were fixed in 4% paraformaldehyde for 24 hours and then washed 3 times with PBS. After that, the tissues were dehydrated in 15% and 30% saccharose at 4°C overnight and embedded in OCT. The tissues were sectioned into 7 to 10 µm slices using a cryosection. The sectioned slides were subjected to either hematoxylin and eosin (H&E) staining or immunofluorescence staining.

For the whole mount staining assay, the corneas were fixed in 4% paraformaldehyde and stored at 4°C until further processing. Radical incisions were made to produce six grossly equal pieces under the operating microscope. After washing in PBS, the corneas were incubated with 20 mmol/L EDTA for 30 minutes at 37°C, and then incubated with 0.025% hyaluronidase for 24 hours at 37°C. The corneas were permeabilized in 0.2% Triton X-100 for 20 minutes, and then blocked with 5% BSA for 1 hour at room temperature. The treated samples were subjected to immunofluorescence staining with the proper antibodies.

Statistical Analysis

The statistical analysis was performed with software SPSS version 13.0. All data were expressed as mean ± SD (n = sample size). Comparisons between groups were performed using unpaired 2-tailed t-tests. $P < 0.05$ was considered statistically significant.

Results

Hsp90α is Induced and Secreted Extracellularly During Injured Corneal Epithelium Recovery in Mice

Heat shock proteins (e.g. Hsp70, Hsp47, and Hsp27) have been shown to be upregulated in injured cornea.[27,35] Hsp90α is another stress-inducible chaperone whose expression is upregulated during skin wound healing. However, the expression and biological roles of Hsp90α in the injured corneal epithelium are still unclear. To delineate the role of Hsp90 on corneal injury and repair, we used 2 month old C57BL/6 male mice as a model. Corneal debridement wounds were made using a blade on the right cornea. The injured eye was treated with PBS eye drops three times a day for up to seven days to let the cornea recover. As Figure 1A shows, the epithelial cells covered the defective area within 3 days. We measured the expression of Hsp90α (see Fig. 1) and other heat shock proteins (Supplementary Fig. S1) during corneal re-epithelialization using immunoblot and image J for quantification. The results showed that the expression of HSP90α in the defective cornea 1 day after injury is similar to that of the uninjured cornea. However, HSP90α expression is increased starting on day 2 after injury, and this increase was sustained for at least 7 days after the injury (see Figs. 1B, 1C). We further performed an immunofluorescence assay to test the expression of Hsp90α in normal, recovery day 1 and day 3 corneal tissues postinjury (see Fig. 1D). CK12 was used as epithelial marker. The results showed that Hsp90α was expressed constitutively at a low level in normal, uninjured corneal epithelium and keratinocytes (see Fig. 1D, left panel), and the expression level is similar in the stromal keratinocytes in the wounded area of recovery day 1 cornea. In contrast, Hsp90 expression was significantly increased in the corneal epithelium, stroma, and keratinocytes of recovery day 3 cornea, where the injured area was covered completely by the regenerated corneal epithelium. These results suggested that Hsp90α is induced during the recovery of injured epithelial cells (see Fig. 1D, right panel). Quantitative PCR results showed that Hsp90α mRNA was induced in recovery day 1 and day 3 cornea (see Fig. 1E). HSF1 is a major upstream transcription factor that regulates the expression of heat shock proteins under stress conditions. In line with that, the phosphorylation of HSF1 at Serine 326, which activates HSF1, was upregulated in recovery day 3 cornea after injury (see Figs. 1F, 1G). In addition, Hsp70, Hsp27, and Hsp40 expression were similarly induced during recovery of the injured corneal epithelium (see Supplementary Fig. S1), which is consistent with previous reports.[35] Taken together, these results suggested that the HSF1-Hsp90α pathway was activated during corneal epithelial cell proliferation and migration after injury.

Stress-induced secretion of Hsp90α is reported in different tumors and during wound healing in skin.[19] We found that Hsp90α was detectable in the corneal stroma during the healing of the injured epithelium (see Fig. 1D), which implied that Hsp90α was secreted extracellularly during cornea wound healing. To prove this, debridement wounds were created in the right corneas of the mice in the same way as that in Figure 1A. Similarly, the injured areas were covered almost completely by epithelium by recovery day 3 (data not shown). To determine whether Hsp90 was secreted into the tear film, we rinsed the left control cornea and right injured corneal surface during wound healing with PBS twice a day, and the solutions were collected for immunoblotting. Hsp90α expression was detected in the tear film from recovery day 2 to day 7 cornea, but not in the tear film of the uninjured cornea or recovery day 1 cornea (Fig. 2A, top panel, compared lanes 1–4 to lanes 5–14). Coomassie blue staining was used to control for protein loading (see Fig. 2A, lower panel). The intensity of the Hsp90α protein bands in Figure 2A were quantified using image-J. The results showed that secretion of Hsp90α increased from recovery day 2 to day 5 after injury, but this increase was attenuated with prolong recovery time (see Figs. 2A, 2B). The induction of secreted Hsp90α in the tear film was also confirmed with the ELISA assay (see Fig. 2C). Taken together, these results indicated

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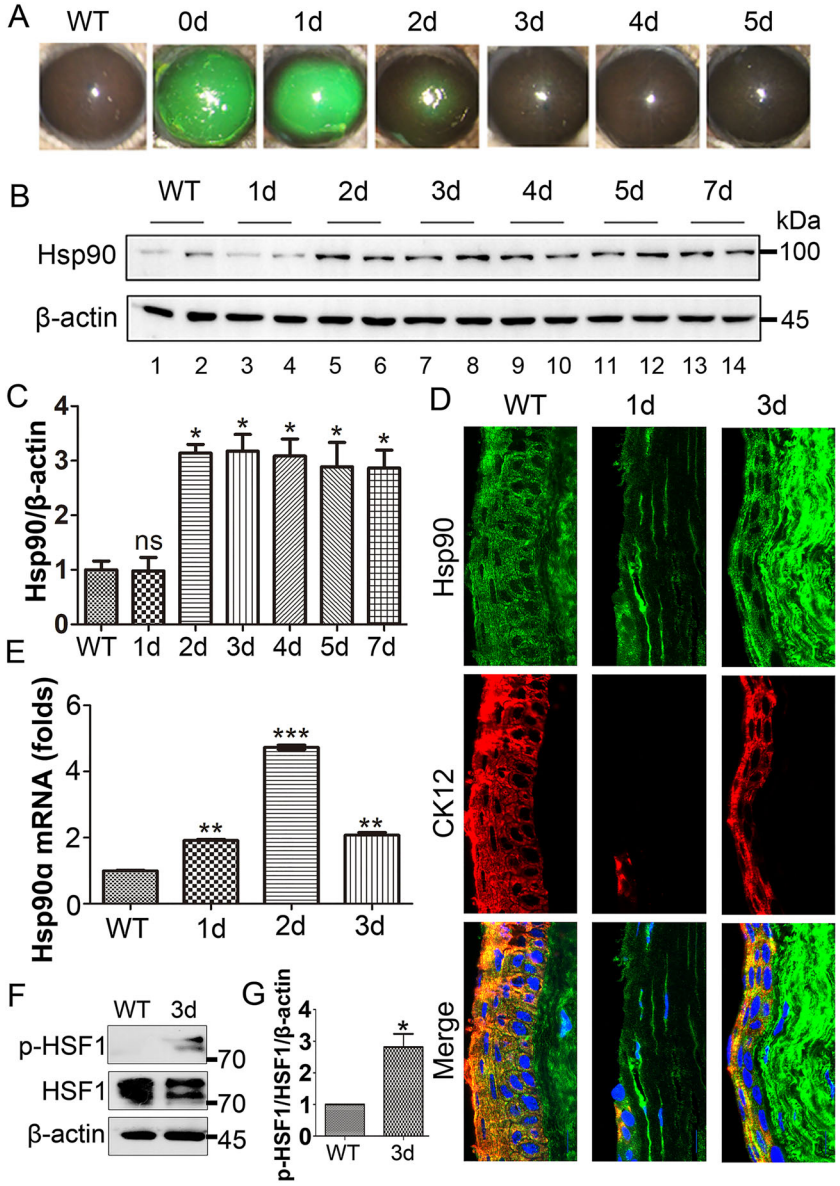

Figure 1. Upregulation of Hsp90 expression in wounded corneal epithelium in mice. (**A**) Sodium fluorescein stain of the injured corneal epithelium under a slit lamp. Scale bar: times 25. (**B**) Immunoblot of the expression of Hsp90α and β-actin in uninjured and injured cornea at the indicated recovery days post injury. Each lane represents tissue from one cornea. (**C**) Densitometry quantitation of Hsp90α versus β-actin in **B**. The results are reported as mean ± SD ($n = 4$). $^{*}P < 0.05$; $^{**}P < 0.01$. (**D**) Immunofluorescence assay of the expression of Hsp90α (green) and CK12 (red) in normal (wt) and injured cornea at recovery day 1 and 3. The cell nuclei were stained with DAPI. The scale bar represents 10 µm. (**E**) Quantitative PCR to measure the expression of Hsp90α mRNA expression in normal and injured corneas from days 1 to 3 of recovery. The data shown in the bar graph represent mean ± SD ($n = 3$). $^{**}P < 0.01$; $^{***}P < 0.001$. (**F**) Immunoblot of the expression of phosphorylated HSF1 (at Serine 326), HSF1, and β-actin proteins in normal and recovery day 3 cornea. (**G**) Densitometry quantification of phosphorylated-HSF1 in **F** using image J. The results represent mean ± SD ($n = 3$). Unpaired 2-tailed t-test was used for statistical analysis, $^{*}P < 0.05$.

that the expression and secretion of Hsp90α were upregulated during the recovery of wounded corneal epithelium.

Recombinant Hsp90α Accelerates Repair of Wounded Corneal Epithelium

The administration of recombinant Hsp90α promotes skin wound healing.[16] The data in Figures 1 and 2 suggested that Hsp90 was secreted extracellularly during corneal epithelium wound healing. We therefore proposed that eHsp90α may act as a drug for corneal injury therapy. To test this, we generated LPS-free bacterial GST and GST-Hsp90α proteins (Supplementary Fig. S2). The injured right corneas were treated with PBS (sham), 15 µM of GST, or GST-Hsp90α in PBS. The recovery area of the injured cornea was measured using Image-J. GST-Hsp90α shortened the healing time when compared to PBS or GST protein alone (Figs. 3A, 3B).

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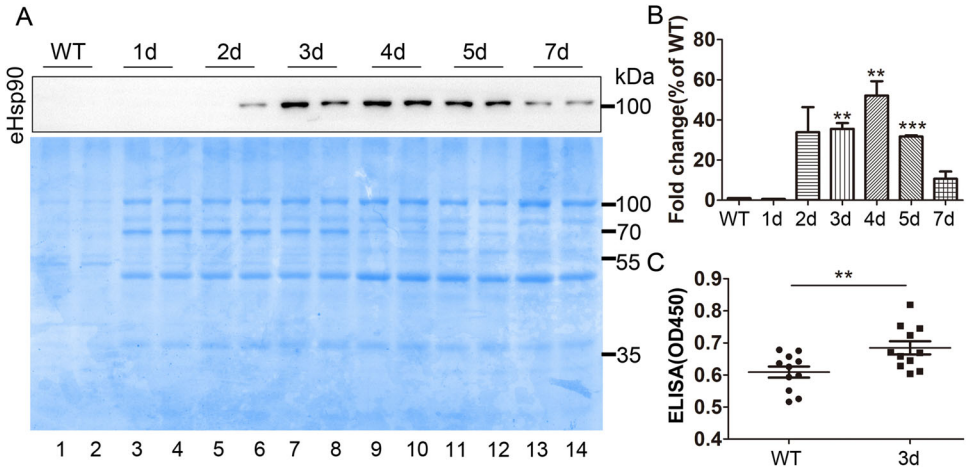

Figure 2. The eHsp90α is secreted extracellularly into the tear film on the surface of the cornea during epithelium wound healing. (**A**) Top panel: Immunoblot of eHsp90α in tear film of normal and injured corneas up to 7 days after recovery. Each lane represents one cornea sample. Bottom panel: Coomassie billiant blue (CBB) staining of proteins in the tear film used for **A**. (**B**) Densitometry quantitation of eHsp90α in **A** using image J. The fold induction was calculated by dividing the densitometry of eHsp90α in tear film of injured corneas by that in normal corneas. The results are reported as mean ± SD ($n = 6$) $^{**}P < 0.01$; $^{***}P < 0.001$). (**C**) ELISA assay to detect eHsp90 in tear film of normal (wt) and recovery day 3 corneas. The data represent mean ± SD ($n = 11$). Unpaired 2-tailed *t*-test was used for statistical analysis, $^{**}P < 0.01$.

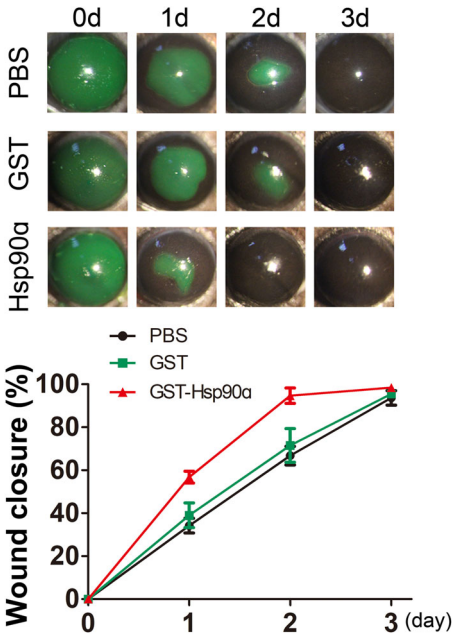

Figure 3. Recombinant Hsp90α accelerates the repair of wounded corneal epithelium. (**A**) Sodium fluorescein stain to visualize changes in the wounded cornea that were treated with PBS, GST, or GST-Hsp90α for 0, 1, 2, and 3 days. (**B**) Quantitation of the wound area in **A** using Image J. The data are presented as mean ± SD ($n = 12$). Unpaired 2-tailed *t*-test was used for statistical analysis. PBS versus GST-Hsp90α, $P < 0.01$; GST versus GST-Hsp90α, $P < 0.05$.

We further compared the histological differences between recovery day 2 and day 7 that were treated with either GST alone or GST-Hsp90α to normal cornea. The central and peripheral regions of the cornea were stained with H&E. The results showed that the epithelium of the recovered cornea, whether treated with GST or GST-Hsp90α, is thinner than that of the normal cornea (Figs. 4A, 4B). However, the recovered epithelium treated with GST-Hsp90α is significantly thicker than that treated with GST (see Figs. 4A, 4B). Cytokeratin-12(CK12), a marker of corneal epithelial differentiation, was detectable in the re-epithelialized cells treated with both GST and GST-Hsp90α, suggesting that these recovered epithelial cells underwent differentiation (see Fig. 4C). We extended the treatment time of GST and GST-Hsp90α to 14 days on the injured cornea. The thickness of recovered corneal epithelium increases in both GST- and GST-Hsp90α-treatment without statistical significance, but the thickness is still less than normal corneal epithelium (Supplementary Fig. S3). Furthermore, using whole mount immunofluorescence staining assay, we assessed cell proliferation by staining for Ki-67. Recovery day 2 corneal epithelium treated with GST-Hsp90α demonstrated significantly increased Ki-67 staining when compared to that treated with GST alone (see Figs. 4D, 4E). These results suggested that GST-Hsp90α promoted both proliferation and differentiation of corneal epithelial cells during corneal epithelial wound-healing in mice. GST-Hsp90α exhibits its therapeutic role on early stage of corneal wound healing.

The Recombinant Hsp90α Protein Increases the Proliferation and Migration of Human Corneal Epithelial Cells In Vitro

Next, we studied the regulation of eHsp90α on the proliferation and migration of corneal epithelial cells in vitro. The HCEC line was cultured in serum-free media containing either GST or GST-Hsp90α at different concentration for 48 hours. Cell proliferation was measured with the MTS assay (Fig. 5A). The results showed that GST-Hsp90α was able to increase HCEC proliferation in a dose dependent manner from 0.078 to 0.625 µg/mL, and the growth plateaued with increasing GST-Hsp90α concentration. This increased proliferation was not observed with GST treatment alone (see Fig. 5A). GST-Hsp90α-induced cell proliferation was also observed using an EdU-incorporation assay.

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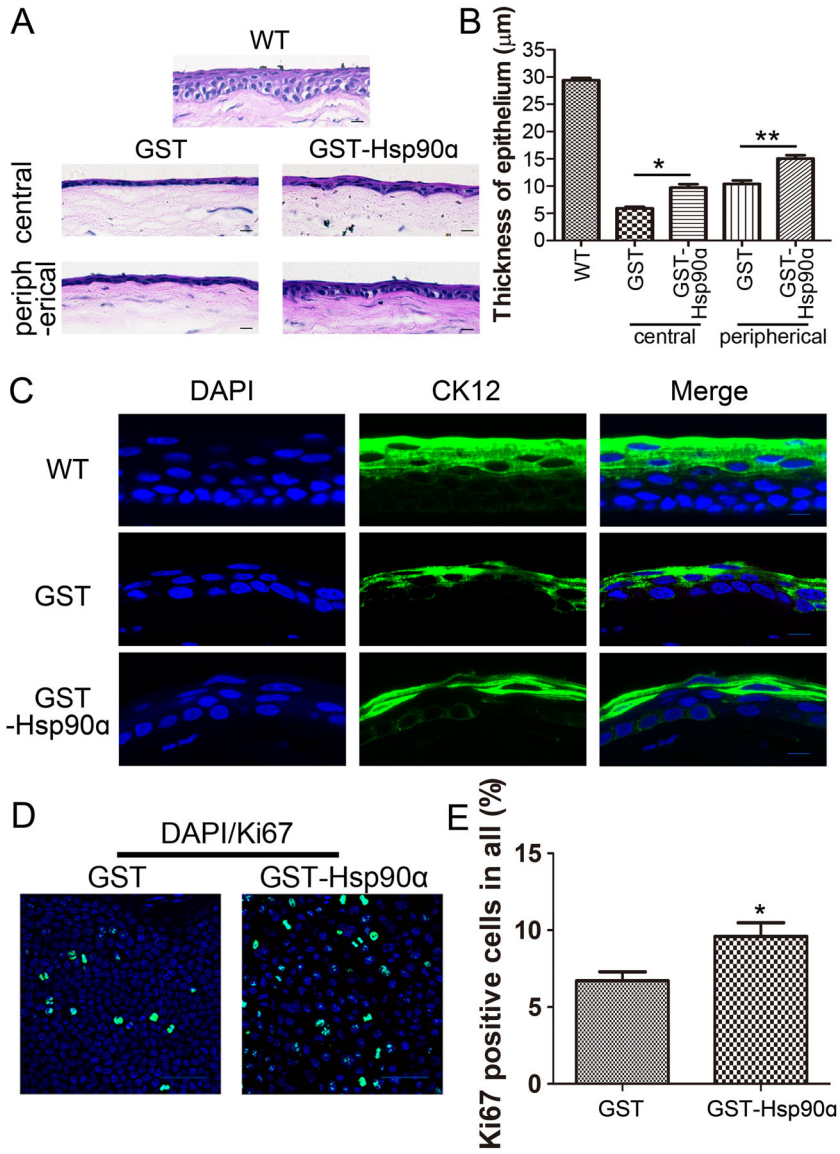

Figure 4. Recombinant eHsp90α upregulates corneal epithelial cell proliferation and migration during cornea wound healing in mice. (**A**) H&E stain of central and peripheral corneal tissues of normal cornea (wt, 2 months old) and recovery day 7 cornea that were treated with GST or GST-Hsp90α after injury. Scale bar represents 10 μm. (**B**) Measuring the thickness of corneal epithelium in **A** using image J. The bar graph represents mean ± SD ($n = 6$). Unpaired 2-tailed *t*-test was used for statistical analysis, $^{*}P < 0.05$; $^{**}P < 0.01$. (**C**) Immunofluorescence staining of CK-12 expression in uninjured or injured corneas that were treated with GST or GST-Hsp90α for 3 days. The cell nuclei were stained with DAPI. The scale bar represents 10 μm. (**D**) Immunofluorescence staining with antibody against Ki-67 in whole mount corneas that were treated GST or GST-Hsp90α for 2 days. The scale bar represents 50 μm. (**E**) Quantitation of Ki67-positive cells versus total cell numbers in **D**. The data represent mean ± SD ($n = 6$). $^{*}P < 0.05$.

The HCECs were treated with GST or GST-Hsp90α for 24 hours in serum-free media. After that, EdU was added to the media for 1 hour before terminating cell culture. The EdU-positive cells were photographed (see Fig. 5B) and quantified (see Fig. 5C). The number of EdU-positive cells increased significantly in the GST-Hsp90α-treated HCECs compared to GST-treated cells (see Fig. 5C). To determine the regulatory effect of GST-Hsp90α on HCEC migration, we scratched a fully confluent HCEC plate and then treated the cells with serum-free media containing GST or GST-Hsp90α for up to 16 hours. Scratch-injured cells demonstrated increased the intracellular Hsp90α expression compared to the unwounded cells (see Figs. 5D, 5E). The administration of GST-Hsp90α increased HCEC migration compared to GST protein alone (see Figs. 5F, 5G). These results suggested that GST-Hsp90α induced HCEC proliferation and migration in vitro.

Recombinant eHsp90α Promotes Corneal Epithelial Cell Proliferation and Migration Through the LRP-1/AKT Signaling Pathway

The LDL receptor related protein 1, which is a receptor for low-density lipoprotein, is also a receptor for eHsp90.[16,22] By binding to LRP-1, eHsp90 activates multiple downstream pathways, such as PI3K-AKT and ERK1/2 to regulate cell migration and proliferation.[22] To determine whether

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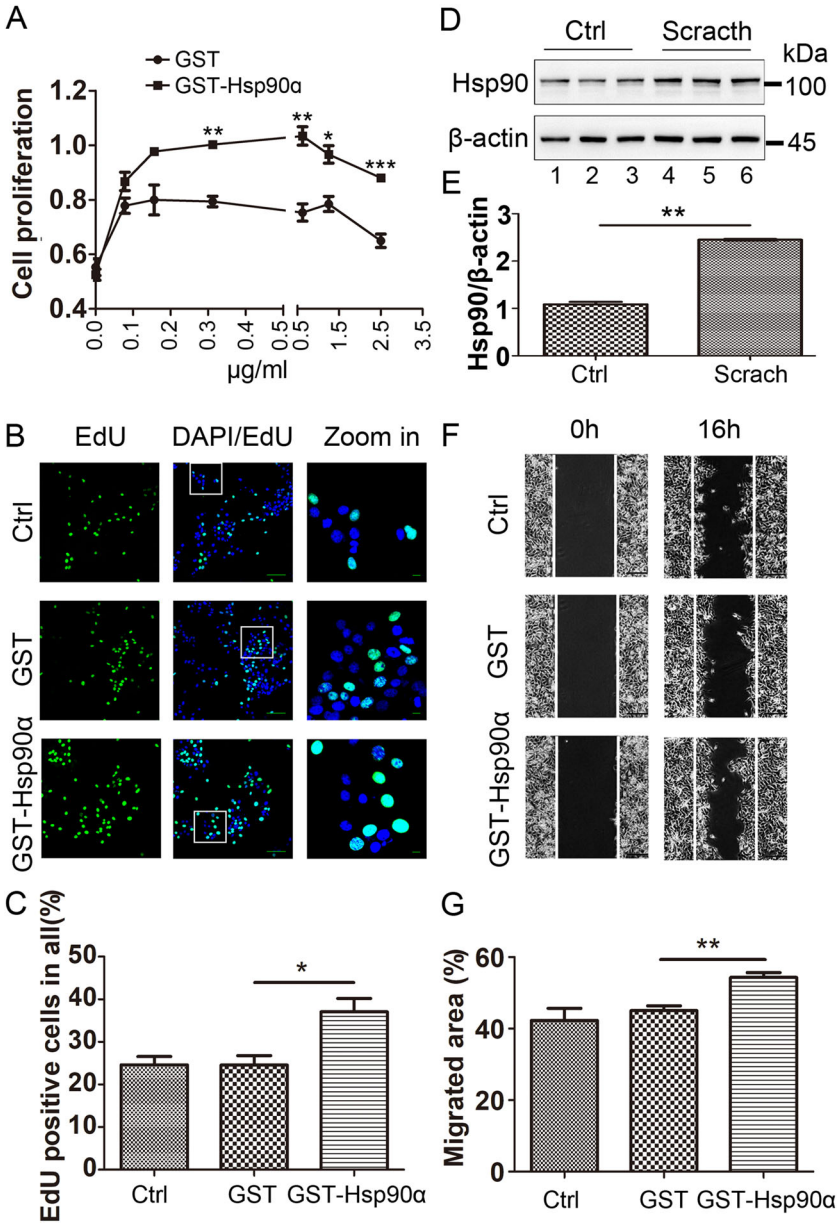

Figure 5. Recombinant Hsp90α promotes HCEC cell proliferation and migration in vitro. (**A**) MTS assay to measure HCEC proliferation after treatment with GST or GST-Hsp90α at the indicated concentrations for 48 hours. The results represent mean ± SD ($n = 3$). Unpaired 2-tailed *t*-test was used for statistical analysis, $^{*}P < 0.05$; $^{**}P < 0.01$. (**B**) EdU incorporation assay to assess cell proliferation. The HCECs were treated with media containing GST (0.625 μg/mL) or GST-Hsp90α (0.625 μg/mL) for 24 hours. EdU was added to cells for 1 hour before terminating cell culture. The EdU positive cells were photographed under confocal microscope. The scale bar represents 100 μm. (**C**) Quantitation of EdU positive cells in **B**. The percentage of EdU positive cells out of total number of cells was calculated. The data represent mean ± SD ($n = 3$). (**D**) Immunoblot of the expression of Hsp90α and β-actin in normal (lanes 1–3) and scratched HCECs (lanes 4–6). (**E**) Densitometry of protein bands in **D** using Image J. Fold induction was calculated by dividing β-actin-normalized Hsp90α in GST-Hsp90α-treated cells by that in GST-treated cells. (**F**) Wound healing assay: confluent HCEC tissue culture plates were scratched and incubated in media containing GST (0.625 μg/mL) or GST-Hsp90α (0.625 μg/mL) for 16 hours. The healing area was photographed. The scale bar represents 50 μm. (**G**) Quantitation of the healing area in **F** using Image J. The data are reported as mean ± SD ($n = 4$). Unpaired 2-tailed *t*-test was used for data statistical analysis, $^{**}P < 0.01$.

LRP-1 is involved in GST-Hsp90's regulatory effect on corneal epithelial cells, we tested the expression of LRP-1 in HCECs that were treated with media containing PBS, GST (0.625 μg/mL,) or GST-Hsp90α (0.625 μg/mL) for 24 hours. The results demonstrated that LRP-1 was constitutively expressed in HCEC cells (Fig. 6A, top panel). Treatment with GST-Hsp90α increased LRP-1 expression more than PBS or GST treatment alone (see Fig. 6A, compared lane 4 to lanes 1, 2, and 3, and 6B). GST did not increase LRP-1 expression compared to PBS (see Fig. 6A, lanes 2 and 3). Furthermore, GST-Hsp90α upregulated the phosphorylation of AKT at S473 (a phosphorylation site for PI3K) compared to PBS or GST treatment alone (see Fig. 6A, comparing lane 4 to lanes 1, 2, 3, and C). The immunofluorescence assay

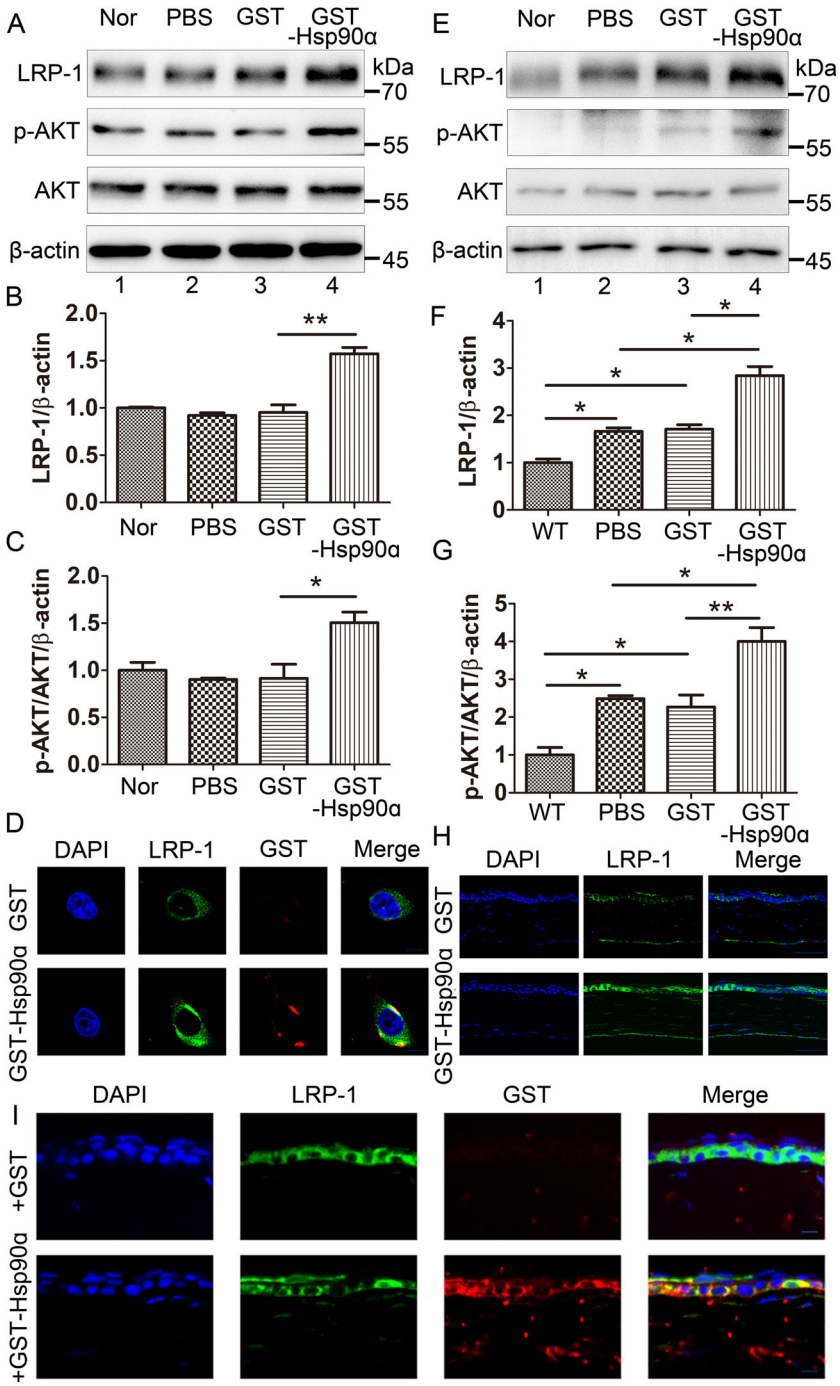

Figure 6. Recombinant Hsp90α associates with LRP-1 and activates AKT pathways in corneal epithelial cells. (**A**) Immunoblot of LRP-1, phospho-AKT, AKT, and β-actin expression in HCECs treated with media (lane 1), or media containing PBS (lane 2), GST (lane 3), or GST-Hsp90α (0.625 µg/mL, lane 4) for 24 hours. (**B, C**) Densitometry quantitation the ration of LRP-1/ β-actin, p-AKT versus AKT in **A** using Image J. The data represent mean ± SD ($n = 3$). *$P < 0.05$; **$P < 0.01$. (**D**) Immunofluorescence staining to assess colocalization of GST-Hsp90α and LRP-1 on HCEC cell surface in vitro. GST was used as control. The scale bar represents 10 µm. (**E**) Immunoblot of LRP-1, phospho-AKT, AKT, and β-actin expression in normal corneas without treatment (lane 1) or injured corneas that were treated with PBS (lane 2), GST (lane 3), or GST-Hsp90α (lane 4) for 3 days. (**F, G**) Densitometry quantitation of the ratio of LRP-1/β-actin, p-AKT versus AKT, in **E** using Image J. The data represent mean ± SD ($n = 3$). Unpaired 2-tailed *t*-test was used for statistical analysis, *$P < 0.05$; **$P < 0.01$. (**H**) Immunofluorescence staining to detect the expression of LRP-1 in the injured cornea tissue treated with GST or GST-Hsp90α for 3 days. The scale bar represents 50 µm. (**I**) Immunofluorescence staining to detect the colocalization of GST-Hsp90α and LRP-1 on the surface of recovery day 3 corneal epithelial cells after injury. The scale bar represents 10 µm.

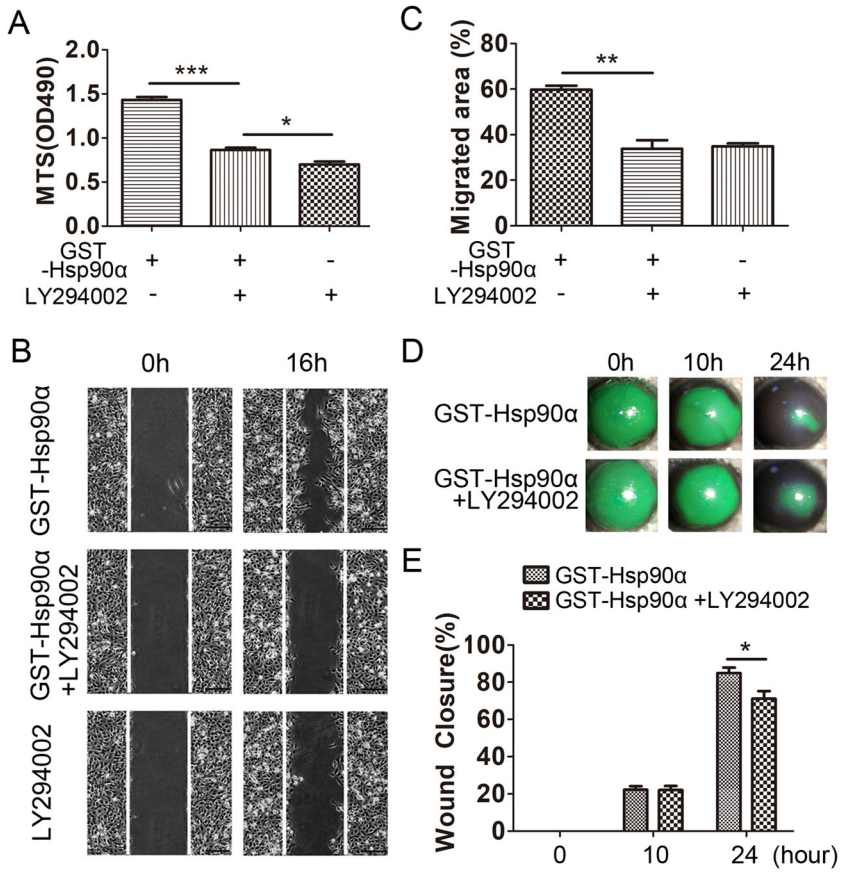

Figure 7. AKT inhibitor LY294002 attenuates GST-Hsp90α-induced corneal epithelial cell proliferation and migration. (**A**) MTS cell proliferation assay: HCEC cells were treated in serum free media containing GST-Hsp90α (0.625 μg/mL), GST-Hsp90α (0.625 μg/mL) + LY294002 (10 μM), or LY294002 (10 μM) alone for 48 hours. The data shown in bar graph represent means ± SD ($n = 4$), $^{*}P < 0.05$. (**B**) Wound healing assay: wound healing was assessed in HCECs that were treated with GST-Hsp90α (0.625 μg/mL), GST-Hsp90α + 10 μM LY294002 or 10 μM LY294002 alone for 0 and 16 hours. The scale bar represents 50 μm. (**C**) Quantitation of the wound closure in **B** using Image J. The data shown represent mean ± SD ($n = 4$). $^{**}P < 0.01$. (**D**) Sodium fluorescein stain to assess wound-healing of injured corneas that were treated with GST-Hsp90α or GST-Hsp90α plus LY294002 for 0, 10, and 24 hours. (**E**) Quantitation of the wound area in **D** using Image J. The data represent mean ± SD. Unpaired 2-tailed *t*-test was used for statistical analysis ($n = 8$), $^{*}P < 0.05$.

demonstrated co-localization of GST-Hsp90α and LRP-1 on the HCEC cell surface, and this was not observed with GST protein alone (see Fig. 6D). These results suggested that GST-Hsp90α could bind LRP-1 and activate AKT pathway. Furthermore, we analyzed the expression of LRP-1 in normal corneas or the injured corneas that were treated with PBS, GST, or GST-Hsp90α for 3 days. The immunoblot results showed that the expression of LRP-1 and phosphorylated AKT was upregulated in injured corneas treated with PBS, GST, and GST-Hsp90α compared to that in normal corneas without treatment (see Fig. 6E). However, the induction of LRP-1 and p-AKT by GST-Hsp90α is more than that by PBS or GST protein alone (see Figs. 6F, 6G). There was no difference of LRP-1 and phosphor-AKT expression between PBS and GST treatment (see Fig. 6E). In addition, the induction of LRP-1 by GST-Hsp90α was also observed in the immunofluorescence assay (see Fig. 6H). GST-Hsp90α but not GST colocalized with LRP-1 on recovery day 3 corneal epithelial cells (see Fig. 6I). These results suggested that the LRP-1-AKT pathway was involved in the GST-Hsp90α induced the wound-healing process in the cornea. To confirm this, we performed the wound-healing assay and cell proliferation assay (MTS) using HCECs that were treated with GST-Hsp90α or GST-Hsp90α plus LY294002 (PI3K inhibitor) or LY294002 alone (Figs. 7A, 7B, 7C). The results showed that inhibition of AKT activation by LY294002 reduced GST-Hsp90α mediated cell proliferation (see Fig. 7A) and migration (see Figs. 7B, 7C). These results indicated that AKT acts downstream of GST-Hsp90α to promote HCEC proliferation and migration. In the mouse model, we administrated eye drop solution containing 15 μM GST-Hsp90α or GST- Hsp90α plus LY294002 to the injured corneas, and the results showed that inhibition of AKT by LY294002 efficiently inhibited the recovery promoting effect of GST-Hsp90α on the injured cornea (see Figs. 7D, 7E). These results suggested that GST-Hsp90α promotes the recovery of injured corneal epithelium by activating the LRP-1-AKT pathway.

Discussion

Epithelial cell wound healing is fundamental to corneal repair after injury. This process is regulated by multiple factors, such as TGF-β,[36] FGF,[17] and heat shock proteins (e.g. Hsp70 and Hsp27).[32] In this paper, we found that Hsp90α is upregulated and secreted extracellularly during the recovery of injured corneal epithelium (see Fig. 2). The administration of bacterially purified recombinant GST-Hsp90α can

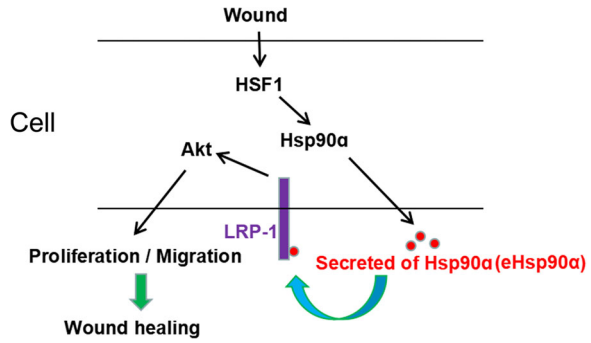

Figure 8. Schematic map of eHsp90α in the treatment of corneal injury. When cornea epithelium is wounded, the acute stresses activate HSF1, which in turn upregulates the expression and secretion of Hsp90α. The secreted Hsp90α (eHsp90α) binds to and activates LRP1-AKT pathways leading to the proliferation and migration of corneal epithelial cells and accelerating corneal epithelium wound healing.

increase the speed of healing of injured corneal epithelium (see Fig. 3). GST-Hsp90α can upregulate the proliferation and migration of corneal epithelial cells both in vivo and in vitro (see Figs. 4, 5), and this regulation is accomplished through activation of the LRP1-AKT pathway (see Fig. 6). The inhibition of AKT activation by its inhibitor ly294002 abolishes GST-Hsp90α's regulatory effect (see Fig. 7). These results demonstrate for the first time that eHsp90α is involved in regulating the corneal wound-healing process and may be considered a novel drug candidate for cornea injury therapy.

Hsp90α is a stress-induced chaperone, and it regulates cellular proteostasis intracellularly and extracellularly.[1] Hsp90α is secreted extracellularly into the tumor microenvironment by many types of tumor cells.[19,20] It interacts with diverse clients, such as metalloproteinase MMP2 and MMP9, FGF2, TGF-β, and VEGF, to regulate tumor cell proliferation, angiogenesis and metastasis.[3,12] Hsp90α is secreted extracellularly by squamous epithelial cells and keratinocytes in wounded skin.[8] Administrating eHsp90α facilitates the wound-healing process of injured skin in mouse and pig models,[15] and this regulation is inhibited by anti-Hsp90 antibody.[15] In addition, administration of human recombinant Hsp90α proteins promotes wound healing in burn injuries. The diverse effects of eHsp90α are attributable to its role in regulating various cellular processes, such as cell proliferation, migration, anti-inflammation, and anti-apoptosis.[8]

Like skin, corneal epithelium is composed of seven layers of squamous epithelial cells.[17] After injury, the corneal stem cells at the corneo-limbal area proliferate and migrate to cover the injured area.[37] This ability to self-heal is important for the biological function of the cornea. It is reported that Hsp70, a direct downstream target of HSF1, is upregulated during the wound-healing process of the cornea.[28] This suggests that the HSF1-mediated heat shock response is activated in the corneal epithelium after injury. In this paper, we show that eHsp90α participates in corneal wound healing. The eHsp90α is induced at both the mRNA and protein level during recovery of the wounded cornea, and this induction is associated with activation of its upstream regulator HSF1 (see Fig. 1). In addition, we find that Hsp90α is secreted into the corneal stroma and tear film during recovery of the injured epithelium (see Figs. 1, 2). It is difficult to detect eHsp90α protein in the tear film of uninjured cornea (see Fig. 2A, top panel, lanes 1–2), but eHsp90α is easily detectable in the tear film during reepithelization (see Fig. 1, Fig. 2). We proposed that the Hsp90α in tear film facilitates the recovery process of injured epithelial cells, and tested this proposal by administering recombinant Hsp90α to the injured cornea in mice. We find that administration of GST-Hsp90α to the injured cornea accelerates the healing speed when compared to GST protein alone (see Fig. 3). This regulation is suppressed by AKT inhibitor (see Figs. 7D, 7E), but not by Hsp90 inhibitor 17-AAG (data not shown), implying that the regulation of GST-Hsp90α on corneal epithelial cell wound healing does not rely on Hsp90α's chaperone activity. Hsp90α regulates skin wound-healing by binding to and activating LRP-1 pathways (e.g. p-ERK1 and p-AKT).[15] Consistently, the data in Figure 6 shows that GST-Hsp90α colocalizes with LRP-1 in the injured mouse corneal epithelial cells and HCEC cell line in vitro. This activates AKT, which is involved in regulating cell proliferation and migration. The data in Figure 7 suggests that inhibition of AKT reduces GST-Hsp90α-induced migration and proliferation of corneal epithelial cells. Together, these results suggest that GST-Hsp90α promotes corneal wound healing by activating the LRP-1-AKT pathway (see Figs. 4, 7).

The cornea injury model used for this study was generated by removing the epithelial layer of the cornea mechanically with a blade. This is a common model for studying corneal wound healing. Clinically, the cornea can be injured by divergent factors, such as alkali, acid, and mechanical trauma. Administration of recombinant Hsp90α protein can accelerate the healing process of burn injuries in mice.[34,38] We show here that the administration of recombinant Hsp90α can promote wound healing after mechanical injury of the cornea. However, it is unclear whether eHsp90α utilizes the same pathways to facilitate recovery after different mechanisms of injury. These experiments are still currently under investigation in our laboratory.

Conclusion

Hsp90α is induced and secreted extracellularly during recovery of injured corneal epithelial cells (Fig. 8). The administration of recombinant Hsp90α protein helps the wound-healing process of mechanically injured corneal epithelium. Hsp90α is a promising therapeutic candidate for corneal injury.

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