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RNA Interference-based Investigation of the Function of Heat Shock Protein 27 during Corneal Epithelial Wound Healing --Manuscript Draft--

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Abstract:	Small interfering RNA (siRNA) is among the most widely used RNA interference methods for the short-term silencing of protein-coding genes. siRNA is a synthetic RNA duplex created to specifically target a mRNA transcript to induce its degradation and it has been used to identify novel pathways in various cellular processes. Few reports exist regarding the role of phosphorylated heat shock protein 27 (HSP27) in corneal epithelial wound healing. Herein, cultured human corneal epithelial cells were divided into a scrambled control-siRNA transfected group and a HSP27-specific siRNA-transfected group. Scratch-induced directional wounding assays, and western blotting, and flow cytometry were then performed. We conclude that HSP27 has roles in corneal epithelial wound healing that may involve epithelial cell apoptosis and migration. In the accompanying video, step-by-step descriptions of sample preparation and the study protocol are provided.
Author Comments:	Feb 23th, 2016 Dear Editor in Chief, We appreciate again all your comments to make our manuscript better. We have done our best to answer your comments and revise our manuscript as your recommendations. Please do not hesitate if there is any query.

	<p>Thank you in advance for considering our manuscript.</p> <p>With best regards,</p> <p>Jae Yong Kim, MD, PhD, Associate Professor, Department of Ophthalmology, University of Ulsan College of Medicine, Asan Medical Center #388-1 Pungnap-2-dong, Songpa-gu, Seoul, Korea 138-736 Tel: +82-2-3010-3680. Fax: +82-2-470-6440, E-mail: jykim2311@amc.seoul.kr</p>
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TITLE:

RNA Interference-based Investigation of the Function of Heat Shock Protein 27 during Corneal Epithelial Wound Healing

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KEYWORDS:

Heat shock protein 27, corneal epithelium, corneal epithelial wound healing, epithelial cell apoptosis, epithelial migration

SHORT ABSTRACT:

Herein, we present a protocol to use heat shock protein 27 (HSP27)-specific small interfering RNA to assess the function of HSP27 during corneal epithelial wound healing. RNA interference is the best method for effectively knocking-down gene expression to investigate protein function in various cell types.

LONG ABSTRACT:

Small interfering RNA (siRNA) is among the most widely used RNA interference methods for the short-term silencing of protein-coding genes. siRNA is a synthetic RNA duplex created to specifically target a mRNA transcript to induce its degradation and it has been used to identify novel pathways in various cellular processes. Few reports exist regarding the role of phosphorylated heat shock protein 27 (HSP27) in corneal epithelial wound healing. Herein, cultured human corneal epithelial cells were divided into a scrambled control-siRNA transfected group and a HSP27-specific siRNA-transfected group. Scratch-induced directional wounding assays, and western blotting, and flow cytometry were then performed. We conclude that HSP27 has roles in corneal epithelial wound healing that may involve epithelial cell apoptosis and migration. In the accompanying video, step-by-step descriptions of sample preparation and the study protocol are provided.

INTRODUCTION:

Corneal epithelial cells (CECs) are continuously shed into tear film, while they are simultaneously replaced by cells from the limbus and corneal epithelial basal layers.¹ Various extrinsic stressors can induce the apoptosis and desquamation of CECs.² The heat shock proteins (HSPs) are highly conserved and can be divided into two families according to molecular size.³ The largest HSP family includes HSP90, HSP70, and HSP60, and the smaller family includes HSP27.⁴ The phosphorylation of HSP27 is known to play an important role in cell survival and is required for cell migration because of the role of this protein in actin remodeling.⁵⁻⁷ Therefore, we attempted to test the potential role of HSP27 phosphorylation in CEC migration and apoptosis in an *in vitro* model of epithelial wound healing.

RNA interference (RNAi) using either small or short interfering RNAs (siRNA) has generated interest in both basic and applied biology, as it potentially allows the expression of any gene of interest to be knocked-down.⁸ Herein, we used HSP27-specific siRNA to assess the contribution of HSP27 to CEC wound healing and apoptosis. Traditional RNAi methods for gene knock-down in cells use synthetic RNA

duplexes, including two unmodified 21-mer oligonucleotides that can be assembled to create siRNAs. The RNAi siRNA that we used in this present study is a simple and highly efficient method to transfect cells, and this reagent works with various immortalized cell lines. In this present study and the accompanying video, we demonstrate the methods used for this analysis, including a scratch-induced directional wound assay, western blotting, siRNA transfection assay, immunofluorescence assay, and flow cytometry.

PROTOCOL:

1. Cell line

1.1) Culture 10^6 telomerase-immortalized human corneal epithelial cells (HCECs) in a 6-well plate (density: 1039.9 cell/mm²) in a 37 °C incubator with a 5% CO₂ atmosphere using bronchial epithelium growth medium (BEGM) until they reach 95% confluence.

2. Western blot analysis after creating epithelial scratch wounds

2.1) Streak a sterile 200 µL pipette tip across the surface of a well of confluent cultured HCECs four times per dish in a biological safety cabinet (Class II, Type A2) and incubate HCECs separately in a 37 °C incubator with a 5% CO₂ atmosphere for 1, 5, 10, 30, 60, and 120 min.

2.2) Use one 6-well plate for six different samples according to the incubation time after corneal epithelial wounding.

2.3) Wash wounded HCEC monolayers three times with 1× PBS and then add 2.0 mL BEGM to each well.

2.4) Detach HCECs using 2 mL 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) per well for 5 min, centrifuge at $900 \times g$ for 5 min in a 15 mL tube, and aspirate trypsin-EDTA using a 1 mL pipette.

2.5) Suspend HCECs with 1 mL 1X PBS and transfer them to a 1.5 mL tube.

2.6) Centrifuge HCECs at $10,000 \times g$ for 15 sec and aspirate 1X PBS using a 1 mL pipette.

2.7) Resuspend HCECs in 100 µL ice-cold lysis buffer (10 mM Tris, 10 mM NaCl, 2 mM EDTA, 25 mM NaF, 2 mM Na₃VO₄, 1 mM phenylmethanesulfonyl fluoride [PMSF], protease inhibitors [1 µM pepstatin A, 1 µM leupeptin, and 0.1 µM aprotin] and 0.5% Triton X-100 [pH 7]) and mix them well.

2.8) Incubate cells for 30 min on ice to induce cell lysis.

2.9) Centrifuge lysates at 4 °C at $10,000 \times g$ for 15 min, and then transfer supernatants to fresh 1.5 mL tubes (90 µL aliquots) and store them at –80 °C.

2.10) Determine total protein concentrations of cell lysates using the Bradford protein assay.⁹

2.11) Load 30 µg total cell proteins into a 10% or 12% acrylamide gel for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and then electrophoretically transfer separated protein bands to nitrocellulose filters with a 200 mA current for 1 hr at 4 °C to use in western blot assays.

2.12) Block nitrocellulose filter membranes with 5% skimmed milk in Tris-buffered saline with Tween 20 (TBST) for 1 hr, add primary rabbit polyclonal antibodies against non-phosphorylated HSP27 (1:1000 dilution) or primary rabbit polyclonal antibody against phosphorylated HSP27 (1:1000 dilution) in 5% bovine serum albumin (BSA), and incubate the membranes overnight at 4 °C on a shaker.

2.13) Detect immunoreactive bands using horseradish peroxidase-conjugated goat anti-rabbit antibodies (1:10000 dilution) in 5% BSA after washing for 10 min 3 times with TBST.

2.14) Incubate the membrane in the western blotting luminol reagents (6–7 mL per 10 cm × 5 cm membrane) for 1 min at room temperature.

2.15) Remove the membrane from reagent solution, remove excess liquid with an absorbent towel, and place in a plastic sheet protector.

2.16) Working in a dark room with a safe light, place covered membrane in a film cassette with protein side facing up.

2.17) Place X-ray film on top of membrane and expose for 1 min.

3. siRNA transfection assay¹⁰

3.1) Culture HCECs at 5×10^5 cells/well in a 6-well plate in a 37 °C incubator with a 5% CO₂ atmosphere using BEGM until they reach 95% confluence.

3.2) Dilute the transfection reagent (2.5 or 7.5 µL) with 100 µL reduced serum medium for transfection (the dilution factor was 41 or 14.3) and dissolve the HSP27-specific and scrambled control siRNA in 100 µL reduced serum medium to create 10 or 50 nM of HSP27-specific and scrambled control siRNA.

3.3) Mix 100 µL siRNA solution with 100 µL diluted transfection reagent (1:1 ratio) and incubate the mixture for 15 min at room temperature.

3.4) Add the siRNA–lipid complexes to cells. Then after 4 hr change media to complete BEGM and incubate the cells for 2 days at 37 °C.

3.5) Analyze transfected cells by western blotting, as described from sections 4.1 to 4.7.

4. Western blot assay for siRNA-transfected cells¹¹

4.1) Extract HSP27-specific and scrambled control siRNA-transfected HCECs in a biological safety cabinet using 100 μ L ice-cold lysis buffer (10 mM Tris, 10 mM NaCl, 2 mM ethylenediaminetetra-acetic acid (EDTA), 25 mM NaF, 2 mM Na_3VO_4 , 1 mM phenylmethanesulfonyl fluoride (PMSF), protease inhibitors, and 0.5% Triton X-100, pH 7).

4.2) Incubate cells for 30 min on ice to induce cell lysis.

4.3) Pellet lysates at 10,000 \times g for 15 min and then transfer supernatants to fresh 1.5 mL tubes (90 μ L aliquots) and store them at -80°C .

4.4) Determine protein concentrations of the cell lysates using the Bradford protein assay.⁹

4.5) Load samples with equal amounts of total cell proteins on a 10% or 12% acrylamide gel, subject the gel to SDS–PAGE, and electrophoretically transfer the separated protein bands to nitrocellulose filters with a 200 mA current for 1 hr at 4°C to use in western blot assays.

4.6) Block nitrocellulose filter membranes with 5% skimmed milk in Tris-buffered saline with Tween 20 (TBST) for 1 hr, add primary antibodies against phosphorylated and non-phosphorylated HSP27 (1:1000 dilution), phosphorylated Akt (1:1000 dilution), non-phosphorylated Akt (1:1000 dilution, used as a cell survival marker), Bcl-2-associated X protein (1:1000 dilution, used as a pro-apoptotic protein), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:200 dilution, used as a loading control) in 5% bovine serum albumin (BSA), and incubate the membranes overnight at 4°C on a shaker.

4.7) Detect immunoreactive bands using horseradish peroxidase-conjugated goat anti-rabbit antibodies (1:10000 dilution) in 5% BSA after washing 3 times with TBST, 10 min each wash.

4.8) Incubate the membrane in the western blotting luminol reagents (6–7 mL per 10 cm \times 5 cm membrane) for 1 min at room temperature.

4.9) Remove the membrane from reagent solution, remove excess liquid with an absorbent towel, and place in a plastic sheet protector.

4.10) Working in a dark room with a safe light, place the covered membrane in a film cassette with the protein side facing up.

4.11) Place X-ray film on top of the membrane and expose for 1 min.

5. Scratch-induced directional wounding assay evaluation of cell migration¹²

5.1) In a biological safety cabinet, make a wound by dragging a sterile pipette tip across the surface of a well of confluent cultures of HSP27-specific siRNA-transfected or scrambled control siRNA-transfected HCECs.

5.2) Immediately after wounding, wash the cells twice with 1× phosphate buffered saline (PBS) and maintain them in BEGM cultures in a 37 °C incubator with a 5% CO₂ atmosphere for 24 hr after wounding.

5.3) Photograph HCEC images using an upright microscope at x100 magnification 24 hr after wounding and perform background flattening using the Filter command in image analysis software.

5.4) Using the Select Measurements command, define the Area of Interest (AOI) with the same sized polygonal shape which can cover perpendicularly from end to end of initial wound, and determine three different AOI in the wounded area of each sample.

5.5) Automatically count the cell number in each field using the Count/Size Measure menu options.

6. Flow cytometry analysis of apoptosis

6.1) Culture HSP27-specific siRNA-transfected and control siRNA-transfected HCECs containing each 10 nM siRNA at a concentration of 5×10^5 cells/well in 6-well plates until the cells reach 95% confluence in a 37 °C incubator with a 5% CO₂ atmosphere in BEGM.

6.2) Detach HCECs using 2 mL 0.25% trypsin-EDTA per well for 5 min, centrifuge at $900 \times g$ for 5 min in a 15 mL tube, and aspirate trypsin-EDTA using a 1 mL pipette.

6.3) Wash cells twice with cold 1× PBS and then resuspend the cells in 1× binding buffer (0.1 M HEPES/NaOH [pH 7.4], 1.4 M NaCl, and 25 mM CaCl₂) at a concentration of 10^6 cells/mL.

6.4) Transfer 100 µL cell suspension (1×10^5 cells) into a 5 mL culture tube.

6.5) Add 5 µL fluorescein isothiocyanate-conjugated Annexin V and 5 µL propidium iodide.

6.6) Gently vortex the cells and incubate them for 15 min at room temperature in the dark.

6.7) Add 200 µL of 1×-binding buffer to each tube and analyze the cells by a flow cytometer within 1 hr.

REPRESENTATIVE RESULTS:¹³

The expression of phosphorylated HSP27 significantly increased at 5, 10, and 30 min after scratch wounding compared with unwounded HCECs. Western blot analysis revealed that the expression of phosphorylated HSP27 and phosphorylated Akt were both significantly reduced, whereas the expression of Bax was significantly increased in HSP27-specific siRNA-transfected HCECs (Figure 1A-E). The phosphorylated HSP27 expression was reduced by 30% and 40% in 10 nM and 50 nM of HSP27-specific siRNA-transfected cells, respectively, compared with control siRNA-transfected cells, but the non-phosphorylated HSP27 expression was not reduced (Figure 1A-B). Moreover, the non-phosphorylated HSP27 expression was reduced by 20% and 30% in 10 nM and 50 nM of HSP27-specific siRNA transfected cells, respectively, but the non-phosphorylated HSP27 expression was not reduced (Figures 1A and C).

The scratch-induced directional wounding assay indicated that at 24 hr after wounding, HSP27-specific siRNA-transfected cells at 10 and 50 nM exhibited reduced migration (Figure 2). Moreover, HSP27-specific siRNA-transfected HCECs underwent more apoptotic and necrotic cell death compared with scrambled control siRNA-transfected cells by flow cytometry (Figure 3).

Figure 1. Western blot analysis using antibodies against phosphorylated HSP27 (p-HSP27), non-phosphorylated HSP27 (non-p-HSP27), phosphorylated Akt (p-Akt) as a cell-survival marker, non-phosphorylated Akt (non-p-Akt), Bcl-2-associated X protein (Bax) as a pro-apoptotic protein, and GAPDH (A). The expression of phosphorylated and nonphosphorylated HSP27 and phosphorylated Akt significantly decreased (BeD), however, the expression of Bax significantly increased in the HSP27-specific siRNA-transfected HCECs (E), compared with that observed in the control siRNA-transfected cells (all $p < 0.05$). The phosphorylated HSP27 expression was reduced by 30% and 40% in 10 nM and 50 nM of HSP27-specific siRNA-transfected cells compared with mock control, respectively, but the phosphorylated HSP27 expression was not reduced in 10 nM and 50 nM of control siRNA-transfected cells (B). **, *, †, ††; ‡, ‡‡; §, §§: a statistically significant difference among groups ($p < 0.05$).

Figure 2. Scratch-induced directional wounding assay to evaluate cell migration after wounding in siRNA-transfected HCECs. A scratch wound was created in control and HSP27-specific siRNA-transfected cells (A). Cells were removed from the 'dragged' areas. At 24 hr after wounding, 10 and 50 nM of HSP27-specific siRNA-transfected cells exhibited lower numbers of migrating cells compared with the 10 and 50 nM control siRNA-transfected cells (B). ** and * indicate a statistically significant difference among groups ($p < 0.05$). The data are shown as means \pm standard deviations.

Figure 3. Flow cytometry of 50 nM of scrambled control siRNA and HSP27-specific siRNA-transfected human corneal epithelial cells (HCECs) labeled with annexin V and PI (A and B). The percentage of the total cells in quadrants corresponded to early apoptotic cells (annexin V-positive and PI-negative cells, Q4, lower right), late apoptotic cells (annexin V-positive and PI-positive cells, Q2, upper right), and necrotic cells (annexin V-negative and PI-positive cells, Q1, upper left). HSP27-specific siRNA-transfect HCECs had more apoptotic and necrotic cell death than control siRNA-transfected cells.

DISCUSSION:

In this present study, we evaluated the potential role of HSP27 in corneal epithelial wounding using *in vitro* approaches. The critical steps involved siRNA transfection for HSP27 knock-down to observe the function of HSP27 in cells subjected to stress. Notably, a role for HSP27 was revealed by these experiments in epithelial cell migration and apoptosis during corneal epithelial wound healing. Unlike previous studies¹⁰ that used rat HSP27-specific siRNA to transfect vascular smooth muscle cells, we used a siRNA transfection technique to modify gene expression in human CECs to effectively knock-down HSP27-specific gene expression and study HSP27 function. Although there were differences in the target sequence that we used as well as in the cell density, final siRNA concentration, and incubation time, the protocol recommended by the manufacturer was explicitly followed. In terms of alternative methods, HSP27 knock-out mouse may be used to show if HSP27 phosphorylation involves epithelial migration and cell apoptosis. However, it is difficult to monitor the change of HSP27 phosphorylation in mouse model, because its phosphorylation occurs in very short period during epithelial wound healing.

There were several limitations to the present study. First, the *in vitro* environment in which we cultured human CECs certainly differed from the *in vivo* environment for human CECs, especially regarding cell survival. Second, the siRNA used in this study was not specific to the phosphorylated form of HSP27 as it affected the overall expression levels of HSP27, including both phosphorylated and non-phosphorylated forms.

In the future, a clinical application of these procedures would be to apply HSP27 to live human wounded corneas. We hope that the current findings will help to advance treatments of corneal epithelial tissue damage.

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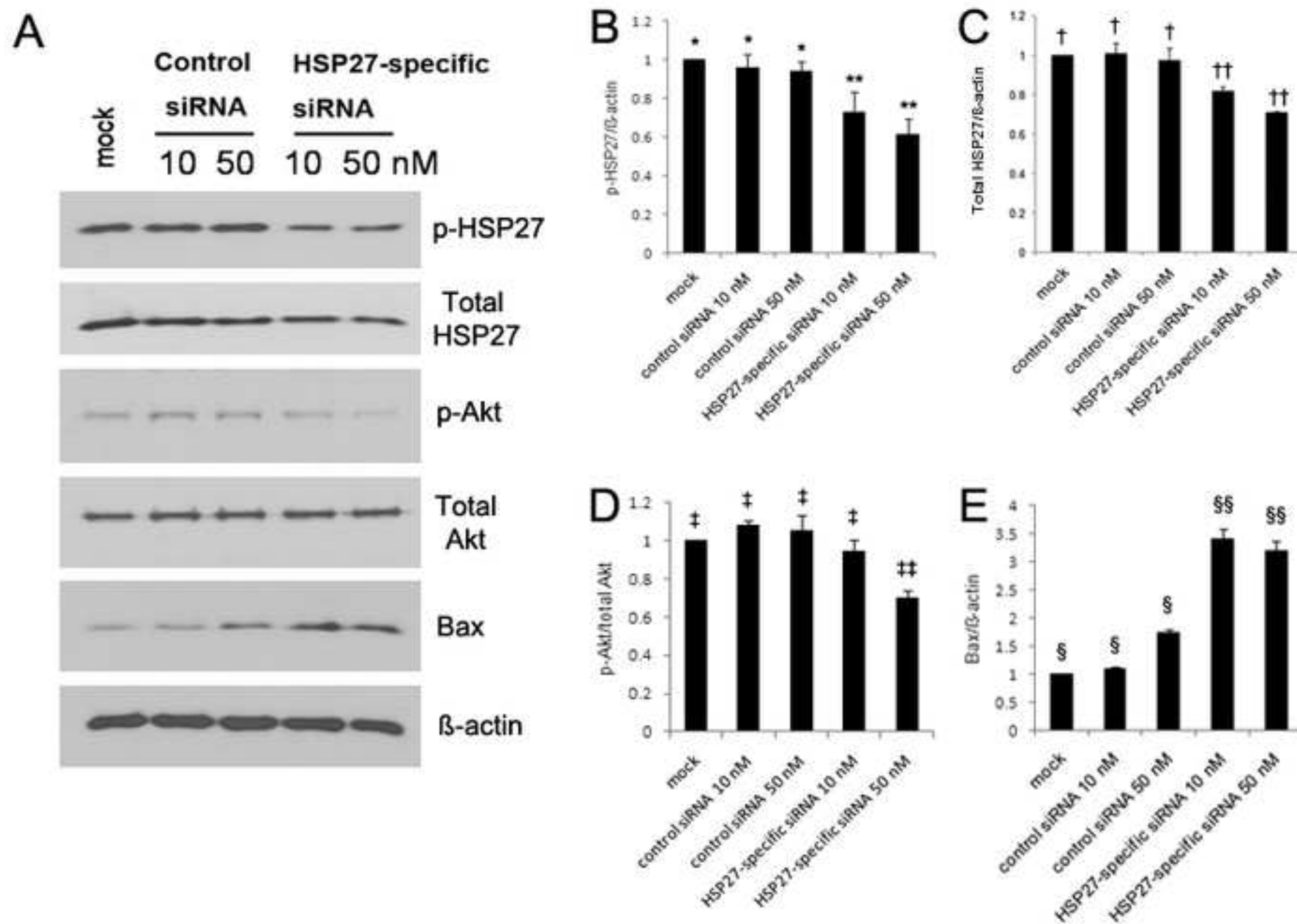
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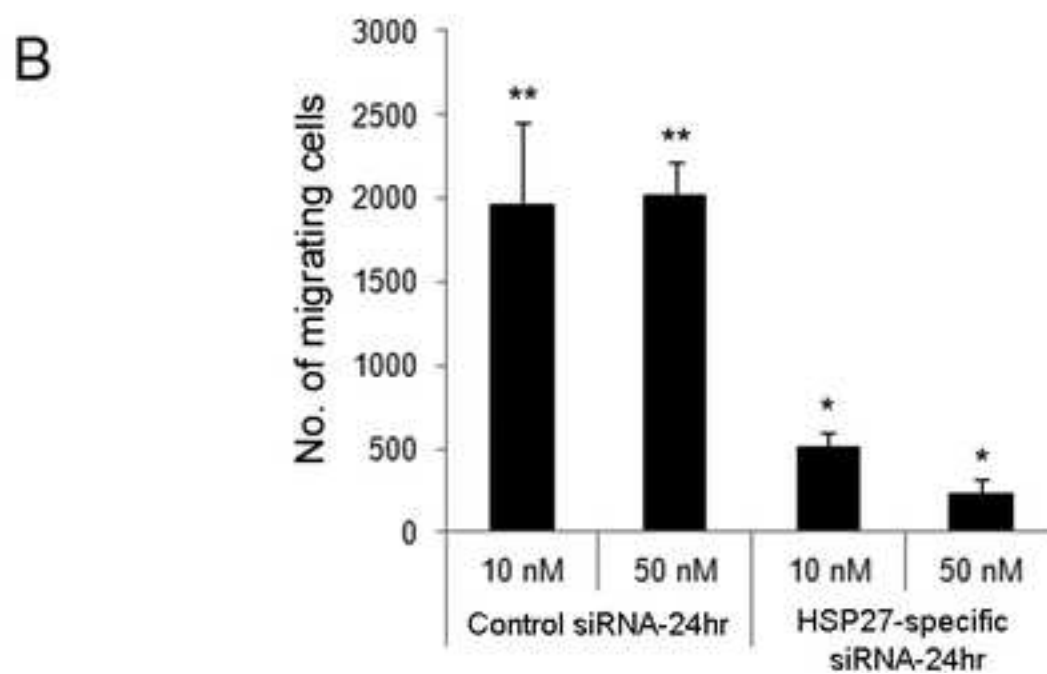
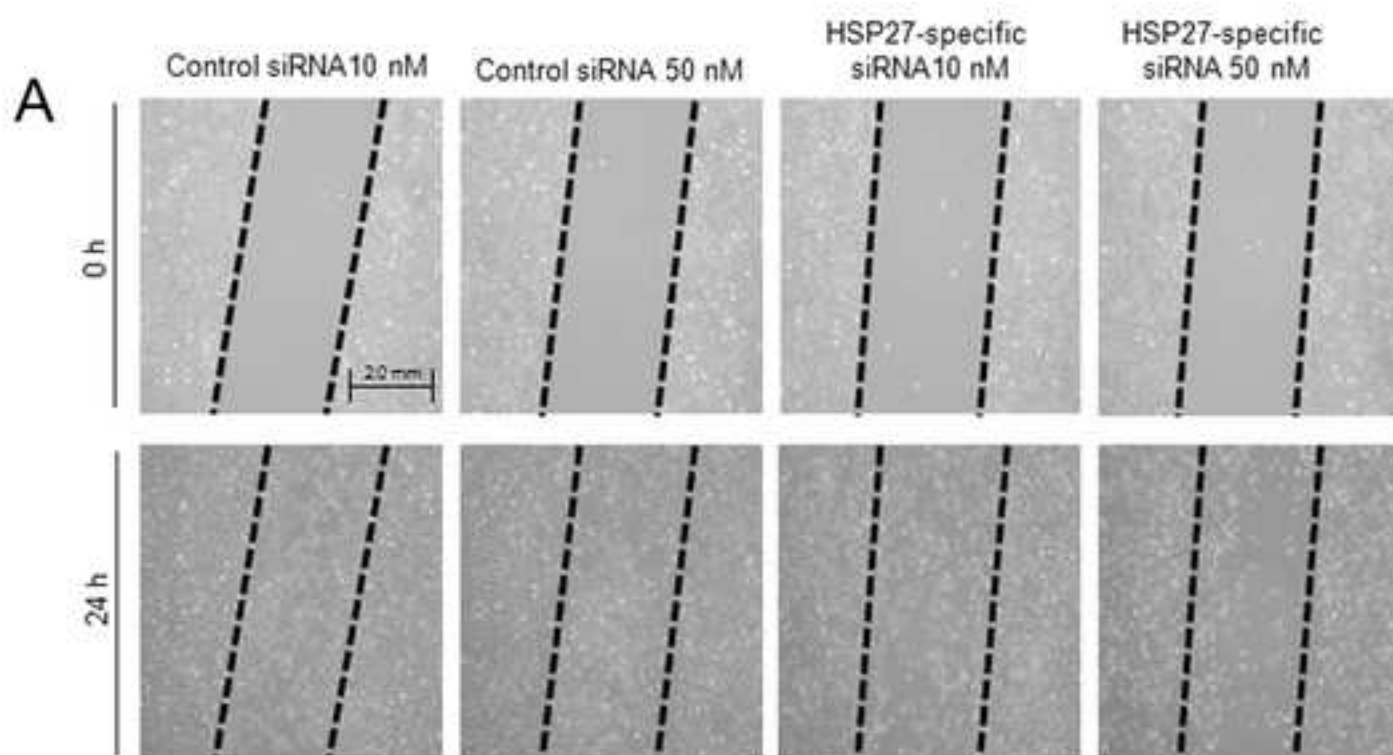
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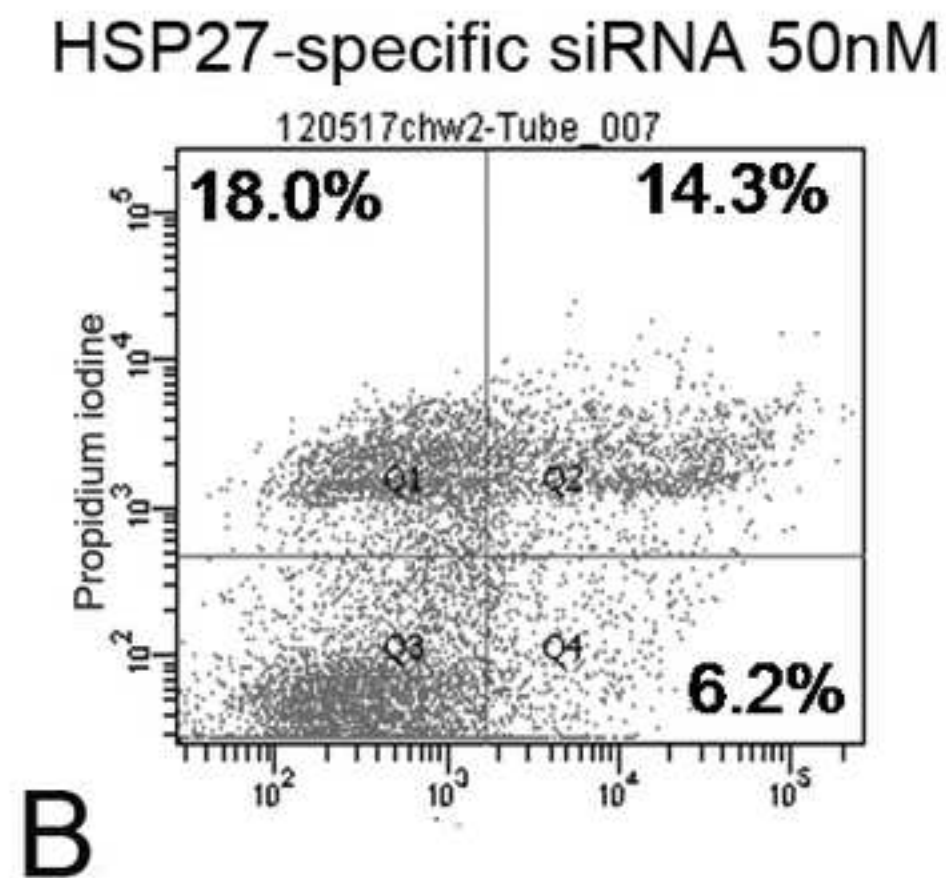
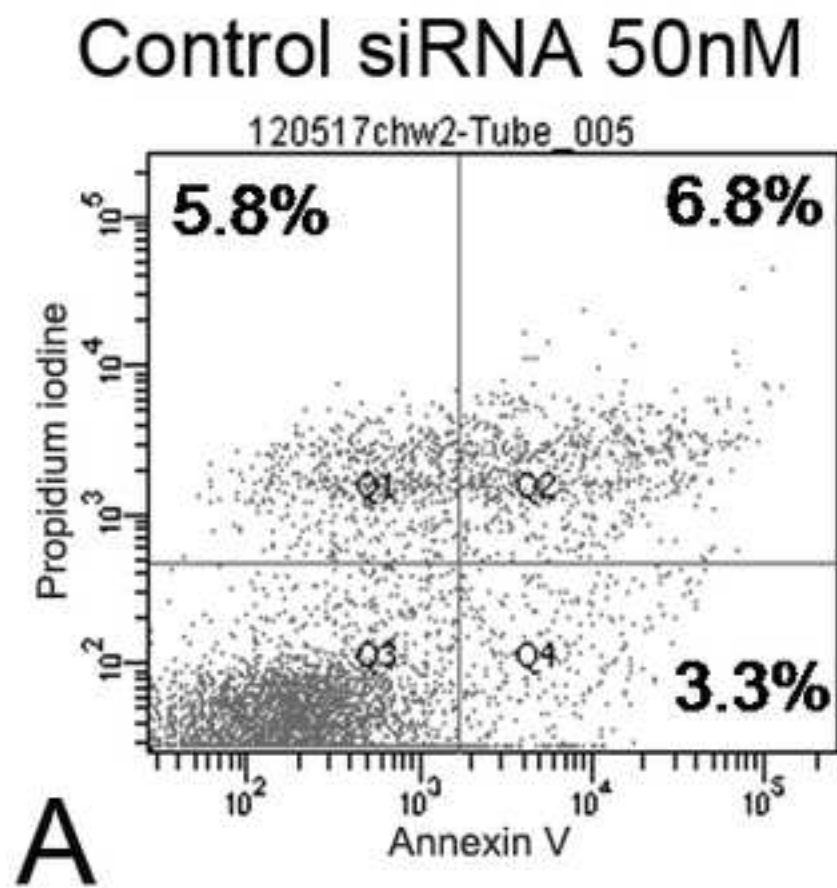
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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Biological safety cabinet	CHC LAB Co.Ltd, Daejeon, Republic of Korea	CHC-777A2-06	Class II, Type A2
Stealth RNAi™ siRNA	Thermo Fisher Scientific, Inc., Waltham, MA		RNAi siRNA; scrambled control-siRNA and HSP27-specific siRNA
BEGM™	Lonza, Inc., Walkersville, MD	CC-3171, CC4175	Bronchial epithelium growth medium
Protease inhibitor	Sigma-Aldrich, Inc., St. Louis, MO	P8340 ,P7626	1 uM Pepstatin A, 1 uM Leupeptin, 0.1 uM Aprotin
Bradford protein assay	Bio-Rad Laboratories, Hercules, CA	#500-0001	Bradford protein assay
Nitrocellulose filters	Amersham, Little Chalfont, UK	RPN3032D	Western blotting membrane
Non-phosphorylated HSP27	Abcam Inc., Cambridge, MA	ab12351	1:1000 dilution (Total HSP27)
Phosphorylated HSP27 (Ser85)	Abcam Inc., Cambridge, MA	ab5594	1: 1000 dilution HSP27 was phosphorylated at Ser85
Lipofectamine® RNAiMAX reagent	Invitrogen, Carlsbad, CA	13-778-075	Transfection reagent
Phosphorylated Akt (Ser473)	Cell Signaling Technology, Danvers, MA	No. 4060	1: 1000 dilution Akt was phosphorylated at Ser473 (cell survival marker)
Non-phosphorylated Akt	Cell Signaling Technology, Danvers, MA	No. 4061	1:1000 dilution (Total Akt)
Bcl-2-associated X protein	Cell Signaling Technology, Danvers, MA	No. 4062	1: 1000 (anti-apoptotic protein marker)
GAPDH	Santa Cruz Biotechnology, Santa Cruz, CA	No. 4063	1:1000 loading control marker (house keeping gene)
Horseradish peroxidase-conjugated goat anti-rabbit antibodies	Thermo Fisher Scientific, Inc., Waltham, MA	NCI1460KR	1:10000 dilution
OPTI-MEM	Invitrogen, Carlsbad, CA	31985	reduced serum medium for transfection
Image analysis software	Olympus, Inc., Tokyo, Japan	Image-Pro Plus 5.0	

Skimmed milk powder	Carl Roth GmbH + Co. KG, Karlstruhe, Germany	T145.2	
Tris	Amresco LCC, Inc. Solon, OH	No-0497	
Sodium Chloride	Amresco LCC, Inc. Solon, OH	No-0241	
Six well culture plate	Thermo Fisher Scientific, Inc., Waltham, MA	140675	35.00 mm diameter / well
24-well culuture dish	Thermo Fisher Scientific, Inc., Waltham, MA	142475	
Orbital shaker	N-Bioteck, Inc., Seoul, South Korea	NB1015	
Bovine serum albumin	Santa Cruz Biotechnology, Santa Cruz, CA	sc-2323	
BDFACSCanto™ II	BD Biosciences, Franklin Lakes, NJ		Flow cytometry
X-Ray Film	Kodak, Rochester, NY		Medical X-Ray Cassette with Green 400 Screen
western blotting luminol reagent	Santa Cruz Biotechnology, Santa Cruz, CA	sc-2048	
FITC Annexin V Apoptosis Detection Kit I	BD Biosciences, Franklin Lakes, NJ	556547	



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Scratch-induced directional wounding assay to investigate the role of HSP21

Author(s):

Aen Yoo, Soon-Suk Kang, Eun-Soon Kim, Hungwan Tchah, Jae Song Kim during corneal epithelial wound healing

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We appreciate all your comments to make our manuscript better. We have done our best to answer your comments and revise our manuscript as your recommendations. Please do not hesitate if there is any query.

1. Formatting:

-Please use “hr” as the abbreviation for hours.

We have used “hr” in the whole manuscript.

-Please remove all references to the video in the manuscript.

We will remove all references to the video in the manuscript.

2. Grammar:

- Long abstract – “process” should be “processes”

We have changed “process” to “processes” in long abstract.

-2.7 – There’s a typo involving parentheses near Triton X-100.

We have corrected the typo involving parentheses near Triton X-100.

-2.8, 4.2 – Should be “Incubate cells” since they have not lysed yet.

We have changed “incubate lysates” to “incubate cells” as you recommend.

-5.2 – “immediately” should only be used once in the sentence

We have removed one of them in this sentence.

-5.3 – “microscopy” should be “microscope”

We have changed “microscopy” to “microscope”.

-6.1 – “on the coverslip”

We have removed these protocols as the reviewer #2 recommends.

-7.7 – Should be “flow cytometer”

We have changed the “flow cytometry” to “flow cytometer”.

3. Additional detail is required:

-3.5 – Shouldn't the referenced section be section 4 rather than steps in section 2?

We have changed the referenced section from section 2 to section 4.

-4.5 – What voltage is used? How long are gels run? What is the voltage/temperature/run time of the transfer?

We have added the voltage/temperature/run time of the transfer in the protocols 2.11 and 4.5.

-4.6 – Aren't blots blocked first? What are the antibodies diluted in? How long are the blots incubated?

We have added all the information in protocol 4.6 as follows.

“4.6. Block nitrocellulose filter membranes with 5% skimmed milk in Tris-buffered saline with Tween 20 (TBST) for 1 hr, add primary antibodies against phosphorylated and non-phosphorylated HSP27 (1:1000 dilution), phosphorylated Akt (1:1000 dilution), non-phosphorylated Akt (1:1000 dilution, used as a cell survival marker), Bcl-2-associated X protein (1:1000 dilution, used as a pro-apoptotic protein), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:200 dilution, used as a loading control) in 5% bovine serum albumin (BSA), and incubate the membranes overnight at 4 °C on a shaker.”

-4.7 – What are the antibodies diluted in? How long are the washes? How are blots developed? Is film used?

We have added all the information in protocols 2.13-17 and 4.7-11 as follows.

4.7) Detect immunoreactive bands using horseradish peroxidase-conjugated goat anti-rabbit antibodies (1:10000 dilution) in 5% BSA after washing 3 times with TBST, 10 min each wash.

4.8) Incubate the membrane in the western blotting luminol reagents (6–7ml per 10cm × 5cm membrane) for 1 min at room temperature.

4.9) Remove the membrane from reagent solution, remove excess liquid with an absorbent towel, and place in a plastic sheet protector.

4.10) Working in a dark room with a safe light, place covered membrane in a film cassette with protein side facing up.

4.11) Place X-ray film on top of membrane and expose for 1 min.

-6.7 – What volume is placed on each coverslip?

We have removed these protocols as the reviewer #2 recommends.

-7.3 – What is the composition of the binding buffer? The materials used for FACS should be included in the materials table (propidium iodide, etc.) unless in a kit, in which case the kit should appear in the materials table.

We have added the composition of binding buffer in the protocol 7.3 and the FITC Annexin V Apoptosis Detection Kit I for FACS in the materials table.

4. Results:

-Please provide a control western blot to show that siRNA transfection specifically reduced HSP27 production.

We have provided the control western blot to show that siRNA transfection specifically reduced HSP27 production as figure 1 and described our results in the representative results section.

-Please define the error bars in 1B (SEM, SD, etc.)

We have added “The data are shown as means \pm standard deviations” in the legend of figure 2.

5. Discussion: Please discuss the potential modifications/troubleshooting that can be performed. Also, please expand on the significance with respect to alternative methods, starting with what the alternative methods are.

We have already added the following comment regarding the potential modifications.

“Unlike previous studies that used rat HSP27-specific siRNA to transfect vascular smooth muscle cells, we used a siRNA transfection technique to modify gene expression in human CECs to effectively knock-down HSP27-specific gene expression and study HSP27 function. Although there were differences in the target sequence that we used as well as in the cell density, final siRNA concentration, and incubation time, the protocol recommended by the manufacturer was explicitly followed.”

In terms of alternative methods, we have added following comment in the discussion.

“In terms of alternative methods, HSP27 knock-out mouse may be used to show if HSP27 phosphorylation involves epithelial migration and cell apoptosis, however, it is difficult to monitor the change of HSP27 phosphorylation in mouse model, because its phosphorylation occurs in very short period during epithelial wound healing.”

Reviewer #1:

Manuscript Summary:

The authors attempted to present a protocol to use heat shock protein 27 (HSP27)-specific small interfering RNA to assess the function of HSP27 during corneal epithelial wound healing.

First of all, in that many revisions were attached in their account, I could guess that repetitive editorial reviews should be carried out before peer review, which made this manuscript close to the perfect. I think that this manuscript was well-written and quite helpful for the other research to perform the research with siRNA for RNA Interference.

Their main findings were also described in their previous work,¹ but they did not cite it in their reference. If they cite the original article, it would be more helpful for reader to follow the methodology.

References:

1. Song IS, Kang SS, Kim ES, et al. Heat shock protein 27 phosphorylation is involved in epithelial cell apoptosis as well as epithelial migration during corneal

epithelial wound healing. Experimental eye research 2014;118:36-41.

We have added our previous work in the reference list as reference #13.

Reviewer #2:

Manuscript Summary:

Prior to this submission, the authors have published an article in Experimental Eye Research titled " Heat shock protein 27 phosphorylation is involved in epithelial cell apoptosis as well as epithelial migration during corneal epithelial wound healing" (Exp Eye Res 118 (2014) 36-41). This manuscript is principally the extended methodology for RNAi-based investigation of the function of HSP 27 during corneal epithelial wound healing using an in vitro scratching model, which has been described in that paper. Since the complete study has been published in a prestigious ophthalmological journal, the significance of the study is justified.

Major Concerns:

1. Have the authors obtained a permission from Exp Eye Res to publish this paper?

The permission from Exp Eye Res to reuse figures and excerpt data was obtained to publish this manuscript.

2. Along with the siRNA transfection assay, they also described in the Protocol Western blot analysis, actin cytoskeleton by immunofluorescence, and flow cytometry analysis of apoptosis. However, they did not show the result of these

experiments.

The protocol and data description for immunofluorescence to show the actin cytoskeleton were removed in the manuscript. The results of flow cytometry were added as figure 3.

Minor Concerns:

In this article the authors didn't mention the source of siRNA they used, nor did they mention the sequence of the siRNA. The information did appear in their paper in Exp Eye Res.

Because we add our previous work in the reference list, the information regarding the source and sequence of siRNA can be found.