Tri-Layer Amniotic Membrane Allografts Modulate Keratinocyte Activity *In Vitro*

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INTRODUCTION

Re-epithelialization is the process of restoring the barrier between the wound and the environment with a new epithelial layer. The cellular mechanisms by which this occurs are coordinated by a variety of regulatory proteins to promote the migration and proliferation of keratinocytes[1]. In this in vitro study, we evaluate the potential of a tri-layer lyophilized human amnion chorion membrane (LHACM*), which includes the amnion, intermediate, and chorion layers, to promote re-epithelialization by activating keratinocytes.

MATERIALS AND METHODS

Extract Preparation: Human amniotic tissue (amnion, intermediate, and chorion layers) was processed using a proprietary and patent-pending cleansing process followed by lyophilization and terminal sterilization. Soluble factors from LHACM were extracted in assay-appropriate basal media at 4°C for 16 hours.

Proliferation: HaCaT cells were treated with basal media supplemented with LHACM extract at final concentrations of 20, 10, 5 and 1mg/mL. Basal (DMEM (with 0% FBS) and complete media (DMEM with 10% FBS) media served as controls. Following a 120 hour incubation at 37˚C, cellular proliferation was determined by CyQuant Assay.

Migration: HaCaT cells were plated at confluence on ImageLock plates (Sartorius) and incubated for 3 hours at 37°C. Monolayers were scratched using the WoundMaker (Sartorius) and treatments applied at final concentrations of 20, 10, 5 and 1mg/mL LHACM extract. Basal and basal media supplemented with 10ng/mL recombinant human epidermal growth factor (EGF) media served as controls. Cellular migration was determined by live cell imaging for 24 hours with automated image processing to determine % Wound Confluence at each time point (S3 IncuCyte, Sartorius).

Western blotting: HaCaT cells were treated with basal media supplemented with LHACM extract at final concentration of 20 mg/mL for 3, 6 or 24 hours. Protein extracts from both control and treatment groups were resolved by SDS-PAGE and subsequently transferred onto a nitrocellulose membrane. Following membrane blocking to prevent nonspecific binding, primary antibodies specific to the target proteins were incubated, allowing for the formation of antigen-antibody complexes. After thorough washing, membranes were probed with fluorescently labelled secondary antibodies. Membranes were imaged and signal was quantified (Odyssey M, Li-cor Biosciences)

Luminex Assay: Soluble growth factors in LHACM extract were assessed using bead-based Luminex multiplex assay (R&D systems). Elution was done as described above and extractswere assayed at 20mg/mL. Eluates were incubated with beads coated in antibodies to specific growth factors of interest. Samples were then incubated with biotinylated detection antibodies followed by a streptavidin-phycoerythrin (PE) reporter. Analytes of interest were quantifies using Luminex instrument (Luminex FLEXMAP 3D, R&D Systems).

Immunofluorescence: Immunofluorescence was performed on HaCaT cells treated with basal media supplemented with LHACM extract at final concentration of 20mg/mL for 3, 6 or 24 hours. Basal (DMEM (with 0% FBS) and basal media supplemented with 10 ng/mL recombinant human epidermal growth factor (EGF) served as controls. Briefly, cells were fixed with 4% paraformaldehyde at room temperature for 30 minutes. Incubation with primary antibodies against cJUN and phospho-cJUN was carried out overnight at 4°C. For visualization, cells were incubated with Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 488 (Thermo Fisher). Images were acquired on a Leica microscope fitted with 10x objective, using Leica Application Suite Advance Fluorescence software and the THUNDER Imager (Leica Microsystems).

RESULTS

Soluble fraction of LHACM contains growth factors that promote re-epithelialization

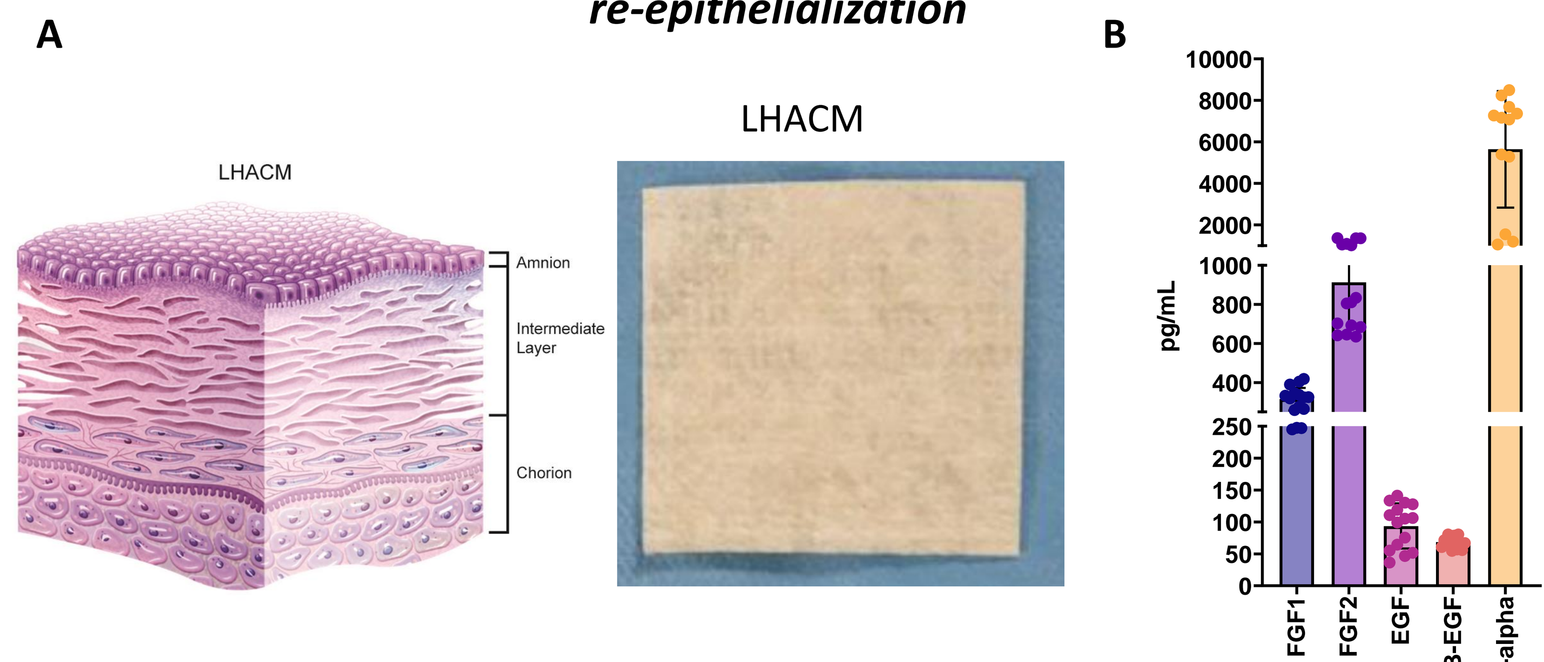

Figure 1. (A) Left: Schematic illustration of LHACM allograft. Right: Top view of LHACM allografts. (B) Quantitative analysis of extracted soluble growth factors in LHACM.

RESULTS

LHACM promotes cellular proliferation of HaCaT cells in vitro

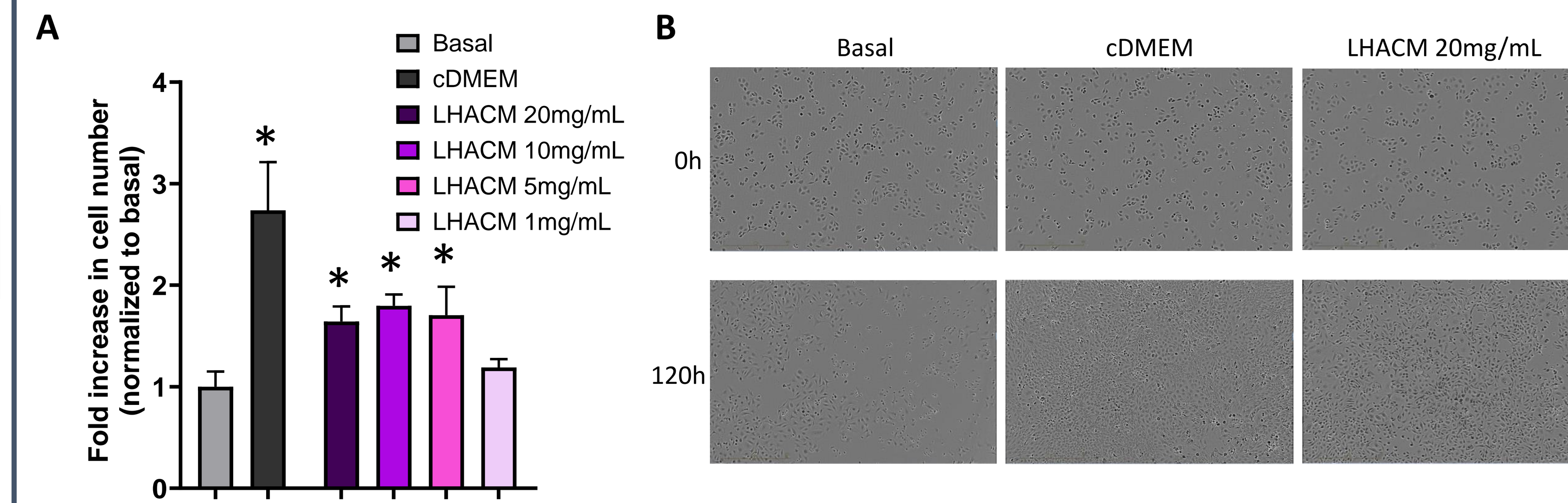

Figure 2. *In vitro* proliferation in HaCaTs. (A) Proliferative response to LHACM when HaCaTs were incubated with extract for 120 hours. Data normalized to the basal group as average fold change ± the standard deviation.*p<0.05 relative to the basal group. (B) Cell images showing confluence of cells in basal media, complete media (cDMEM) and LHACM (20 mg/mL) at 0 hour and 120 hour.

LHACM promotes cellular migration of HaCaT cells in vitro

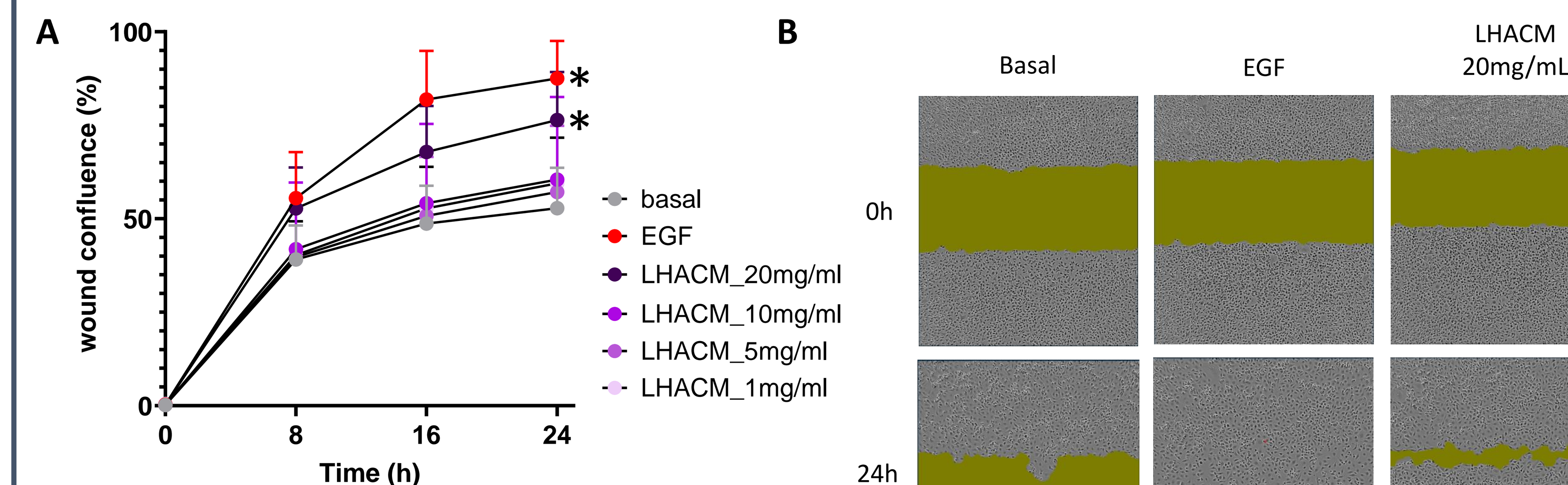

Figure 3. *In vitro* migration in HaCaTs. (A) Migration of HaCaT cells in a 2D scratch assay. Live cell label-free imaging of HaCaTs over 24 hours under exposure of LHACM extracts or controls. Data presented as % coverage of the initial scratch area at each time point ± standard deviation. *p<0.05 relative to the basal group. (B) Cell images showing scratch wound (yellow) in basal media, EGF and LHACM (20mg/mL) at 0 hour and 120 hour.

LHACM treatment upregulates activated cJUN expression in vitro

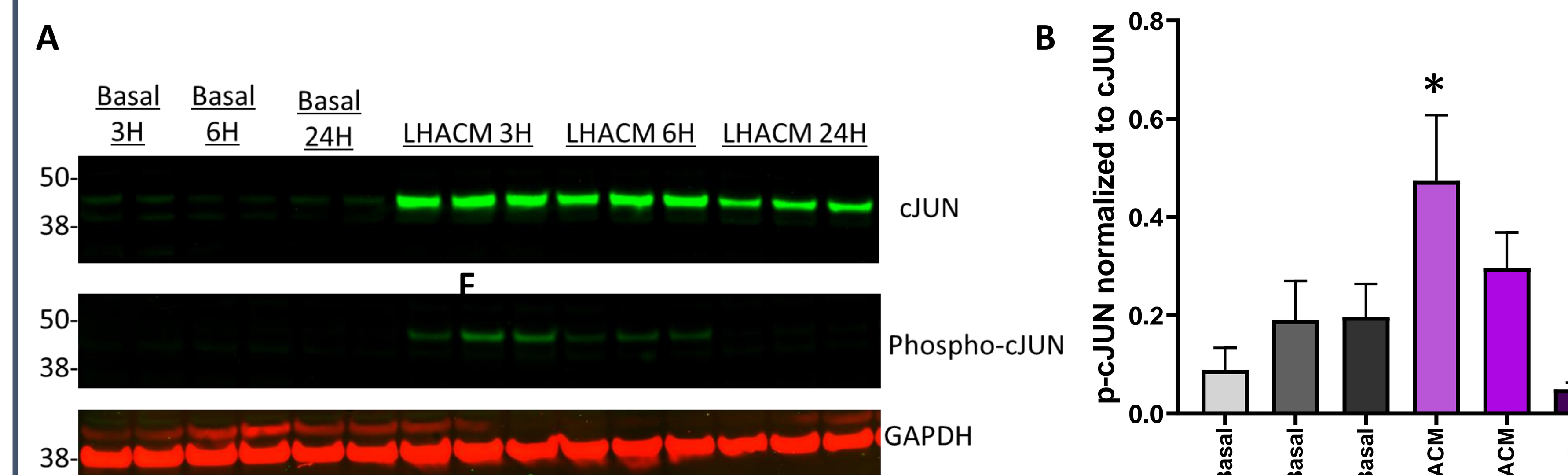

Figure 4. cJUN expression with LHACM treatment (A) Transcription factor, cJUN, protein expression in response to LHACM when HaCaTs were incubated with extract for 3, 6 or 24 hours. (B) Fluorescent signal of phospho-cJUN normalized to the total cJUN signal plotted as normalized signal ± the standard deviation. *p<0.05 relative to the basal group at each time point.

RESULTS

LHACM treatment upregulates activated cJUN expression at the wound edge in vitro

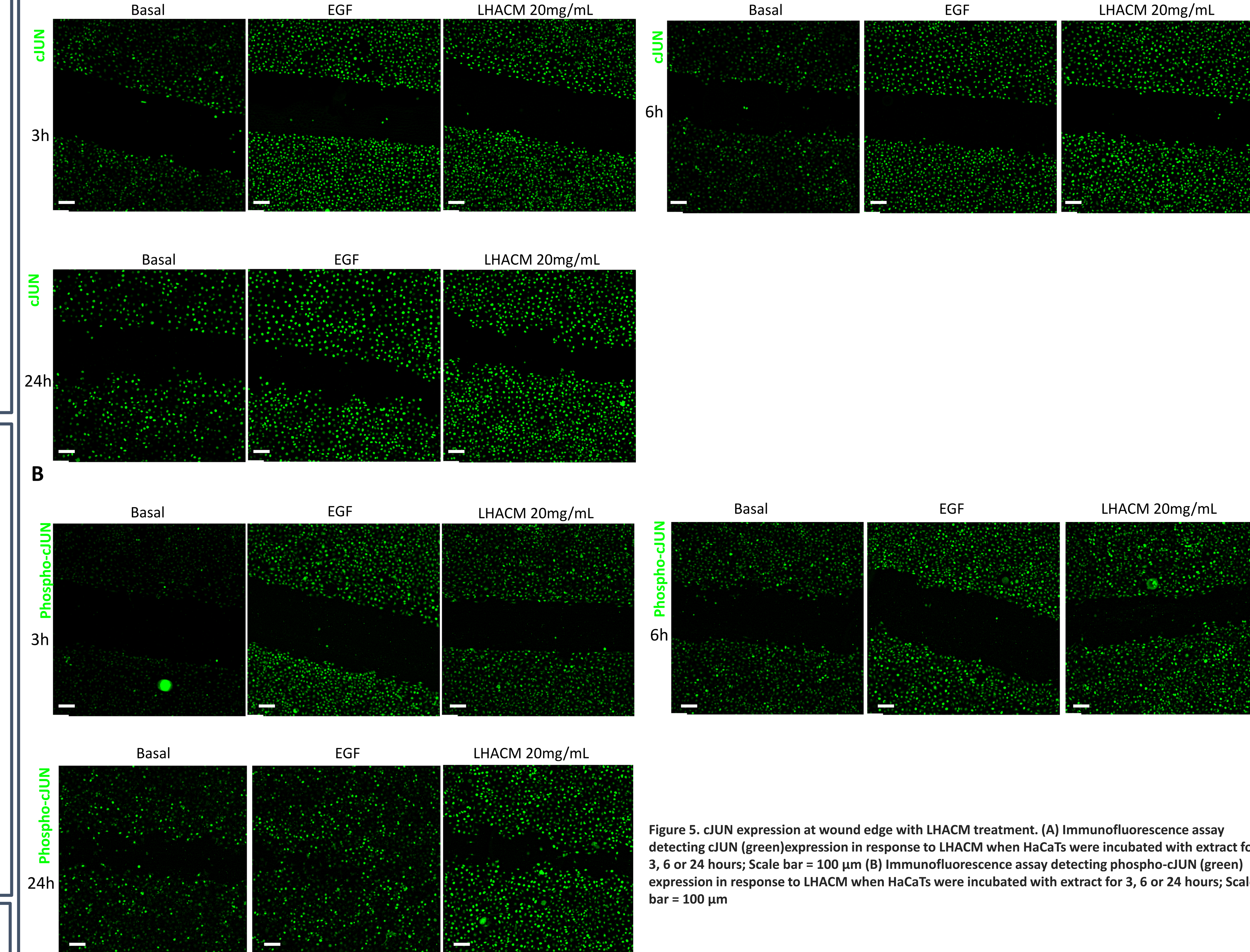

Figure 5. cJUN expression at wound edge with LHACM treatment. (A) Immunofluorescence assay detecting cJUN (green)expression in response to LHACM when HaCaTs were incubated with extract for 3, 6 or 24 hours; Scale bar = 100 µm (B) Immunofluorescence assay detecting phospho-cJUN (green) expression in response to LHACM when HaCaTs were incubated with extract for 3, 6 or 24 hours; Scale bar = 100 µm

CONCLUSION

The findings from this study suggest that LHACM activates keratinocytes, promoting both proliferation and migration in vitro. This research contributes valuable insights into the potential uses of LHACM to create an optimal wound healing environment, emphasizing its role in fostering keratinocyte function during the critical process of re-epithelialization.

REFERENCES

1. Pastar, I., et al., Epithelialization in Wound Healing: A Comprehensive Review. Advances in Wound Care, 2014. 3(7): p. 445-464.
2. Li, G., et al., c-Jun Is Essential for Organization of the Epidermal Leading Edge. Developmental Cell, 2003. 4(6): p. 865-877.

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