Tri-Layer Amniotic Membrane Allografts promote re-epithelialization *in vitro* and *ex vivo*

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INTRODUCTION

Re-epithelialization is the process of restoring the skin barrier by forming a new epithelial layer over a wound. This process is regulated by various proteins that coordinate keratinocyte cell migration and proliferation [1]. This study investigates the ability of a tri-layer lyophilized human amnion chorion membrane (LHACM*), composed of the amnion, intermediate, and chorion layers, to enhance re-epithelialization using both an *in vitro* and *ex vivo* model.

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 1 mg/mL LHACM extract. Basal and basal media supplemented with 10 ng/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).

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 extracts were assayed at 20 mg/mL. Extracts 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 quantified using Luminex instrument (Luminex FLEXMAP 3D, R&D Systems).

***Ex vivo* wound healing model:** 11 mm-diameter human skin biopsies (Genoskin, MA) were wounded by excising a 2 mm-diameter circle of epidermis. Controls included untreated wounds, or wounds treated with culture media supplement 10 ng/mL recombinant EGF. LHACM treated wounds were treated by overlaying a 4 mm-diameter LHACM graft, with the chorion side facing down. Tissue was cultured for 7 days at 37 ˚C with 5% CO_2.

RESULTS

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

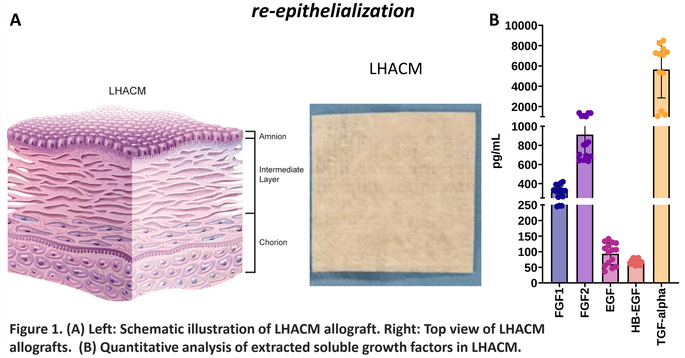

Figure 1. (A) Left: Schematic illustration of LHACM allograft. Right: Top view of LHACM allografts. (B) Quantitative analysis of extracted soluble growth factors in LHACM.

RESULTS

LHACM promotes cellular proliferation of HaCaT cells in vitro

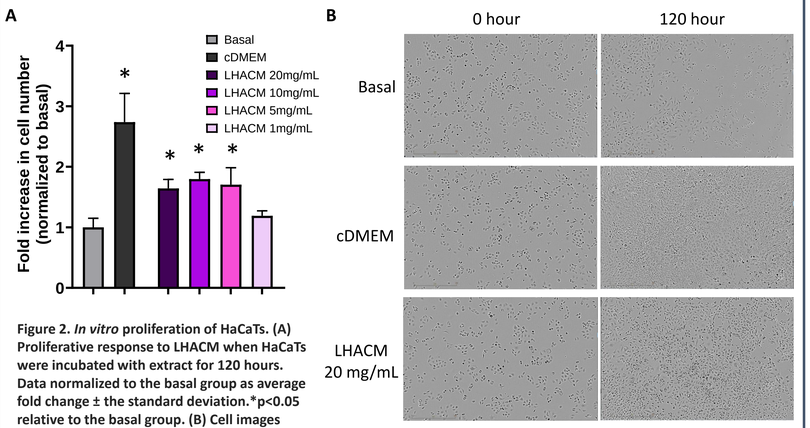

Figure 2. *In vitro* proliferation of HaCaTs. (A) Proliferative response to LHACM when HaCaTs were incubated with extract for 120 hours. Data normalized to the basal group as average fold change ± the standard deviation.*p<0.05 relative to the basal group. (B) Cell images showing confluence of cells 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

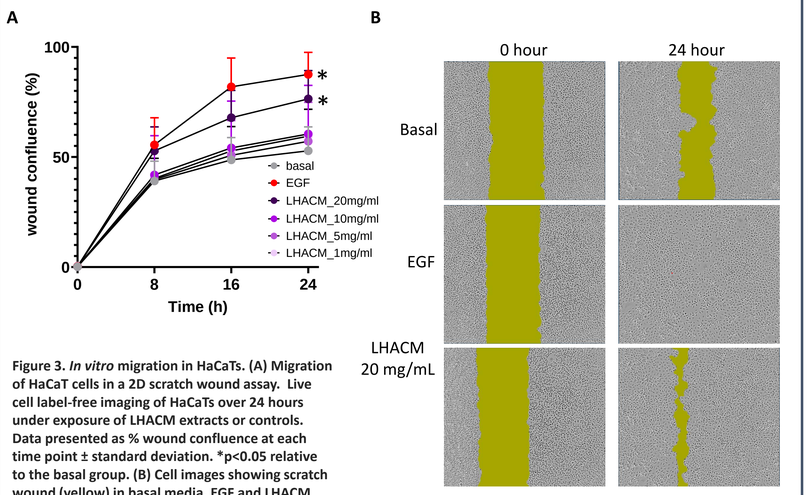

Figure 3. *In vitro* migration in HaCaTs. (A) Migration of HaCaT cells in a 2D scratch wound assay. Live cell label-free imaging of HaCaTs over 24 hours under exposure of LHACM extracts or controls. Data presented as % wound confluence at each time point ± standard deviation. *p<0.05 relative to the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

RESULTS

LHACM promotes keratinocyte migration over wound site in an ex vivo model

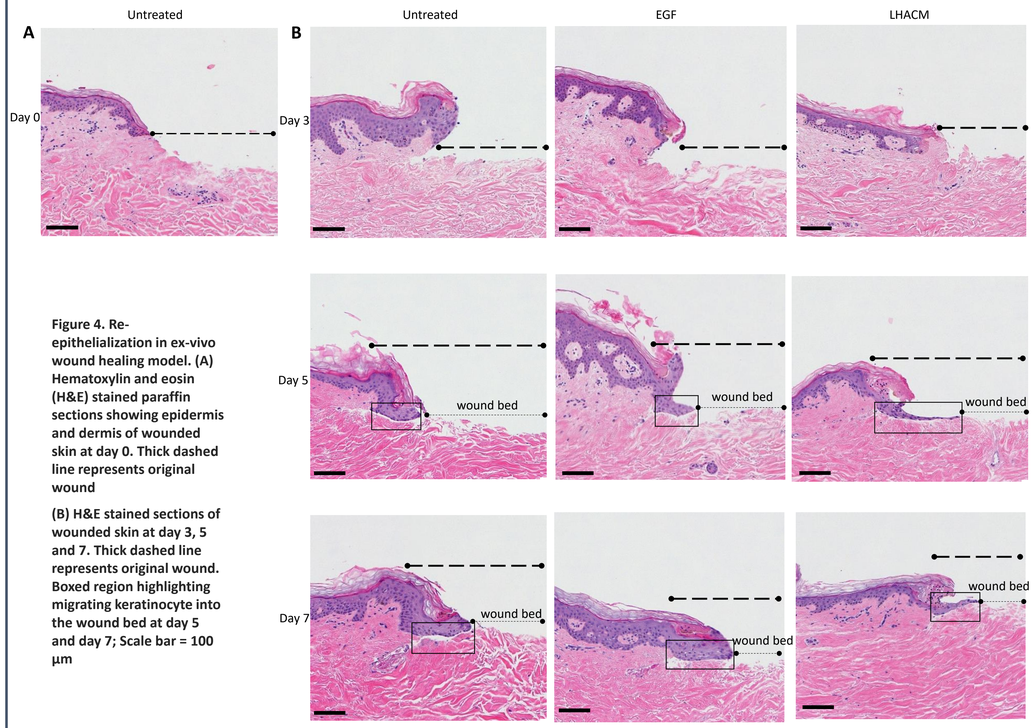

Figure 4. Re-epithelialization in ex-vivo wound healing model. (A) Hematoxylin and eosin (H&E) stained paraffin sections showing epidermis and dermis of wounded skin at day 0. Thick dashed line represents original wound

(B) H&E stained sections of wounded skin at day 3, 5 and 7. Thick dashed line represents original wound. Boxed region highlighting migrating keratinocyte into the wound bed at day 5 and day 7; Scale bar = 100 µm

CONCLUSION

These findings demonstrate that LHACM stimulates keratinocyte activity, enhancing both proliferation and migration *in vitro* and promoting keratinocyte migration in an *ex vivo* model. The results highlight the potential of LHACM to create an optimal wound healing environment by supporting key aspects of re-epithelialization. This study provides valuable insights into the therapeutic applications of LHACM for improving wound healing outcomes.

REFERENCES

1. Pastar, I., et al., Epithelialization in Wound Healing: A Comprehensive Review. Advances in Wound Care, 2014. 3(7): p. 445-464.

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