Epidermal α3β1 is required for efficient wound healing and is downregulated in aged skin

Sanjana Dhulipalla[2], Giesse Albeche Duarte[2], Lei Wu[1], Palak Patel[2], Rogger Andrade[2], John M. Lamar[2], C. Michael DiPersio[1,2] and Whitney M. Longmate[1,2]

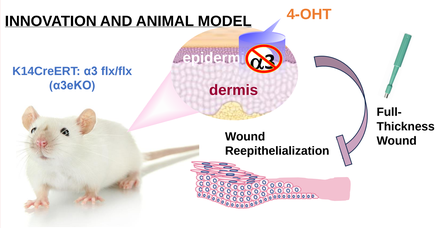
[1] Department of Surgery and [2] Department of Molecular & Cellular Physiology, Albany Medical College, Albany, NY

Background

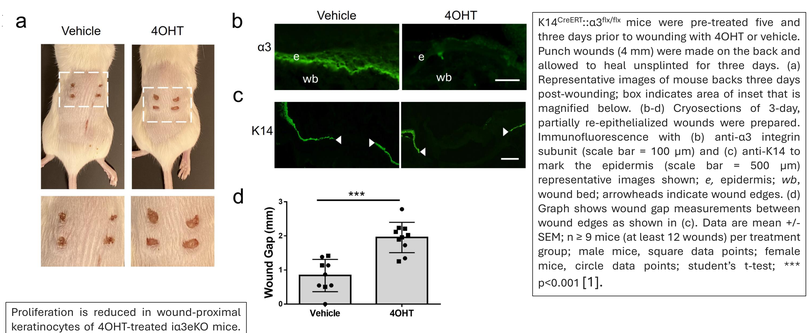
The mechanisms that contribute to wound healing capabilities remain elusive and present substantial gaps in knowledge. Despite strong efforts to explore interactions in the dermis layer of the skin, the epidermal layer is left largely understudied. Studying epidermal adhesion receptors of the extracellular matrix (ECM), such as integrins, could reveal wound healing mechanisms previously undetected.

In this context, epidermal integrin α3β1 has emerged as a promising therapeutic target in impaired wound healing as it has been shown to be highly expressed during wound healing and has been linked to keratinocyte migration *in vitro*. Although it is well-established that α3β1 binds to the ECM, its precise role in re-epithelialization *in vivo* remains unclear, as different results were observed using the global versus constitutive epidermal-specific knockout models. **This study aims to fill this knowledge gap by using a novel inducible epidermis-specific α3 knockout (iα3eKO) murine model**.

INNOVATION AND ANIMAL MODEL

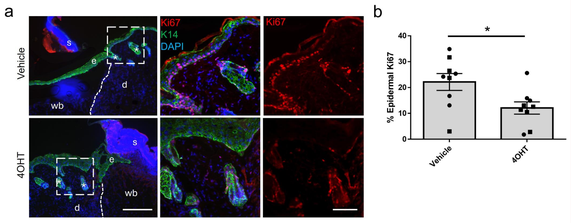


Induced deletion of integrin α3β1 in adult murine epidermis inhibits cutaneous wound reepithelialization. 8–10-week-old iα3eKO mice were treated topically with vehicle (control) or 4-OHT to locally induce deletion of α3β1 specifically in the epidermis. Following integrin deletion, cutaneous full-thickness wounds were generated with a 4 mm biopsy punch. Wounds were allowed to heal for three days, then wound gap measurements were assessed [1].



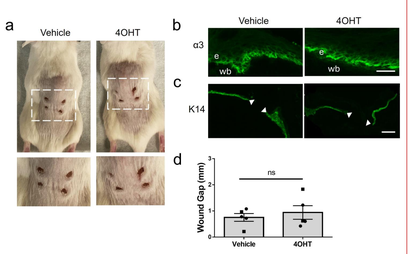
$K14^{CreERT}::\alpha3^{flx/flx}$ mice were pre-treated five and three days prior to wounding with 4OHT or vehicle. Punch wounds (4 mm) were made on the back and allowed to heal unsplinted for three days. (a) Representative images of mouse backs three days post-wounding; box indicates area of inset that is magnified below. (b-d) Cryosections of 3-day, partially re-epithelialized wounds were prepared. Immunofluorescence with (b) anti-α3 integrin subunit (scale bar = 100 μm) and (c) anti-K14 to mark the epidermis (scale bar = 500 μm) representative images shown; *e,* epidermis; *wb*, wound bed; arrowheads indicate wound edges. (d) Graph shows wound gap measurements between wound edges as shown in (c). Data are mean +/- SEM; n ≥ 9 mice (at least 12 wounds) per treatment group; male mice, square data points; female mice, circle data points; student's t-test; *** p<0.001 [1].

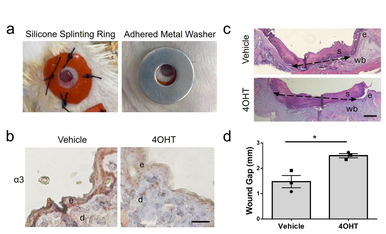
Proliferation is reduced in wound-proximal keratinocytes of 4OHT-treated iα3eKO mice. Cryosections of 3-day unsplinted wounds with wound-proximal skin from $K14^{CreERT}::\alpha3^{flx/flx}$ mice were prepared as in figure above (a) Immunofluorescence with proliferation marker anti-Ki67 (red), epidermal marker anti-K14 (green), and nuclear marker DAPI (blue). Representative images shown. Left; box indicates area of inset that is magnified on right that includes wound-proximal hair follicles; *s*, eschar; *e,* epidermis; *d*, dermis; *wb,* wound bed; *, hair follicle; scale bar, 200 μm. Right; inset panels shown with (left) and without (right) anti-k14 and DAPI; scale bar, 50 μm. (b) Graph shows percentage of epidermal cells that are Ki67-positive. Data are mean +/- SEM; n = 9 mice (14 wounds) per treatment group; male mice, square data points; female mice, circle data points; student's t-test; * p<0.05 [1].



CONTROL EXPERIMENTS

4OHT treatment has no effect on wound re-epithelialization in mice that express epidermal α3β1. Control mice with wildtype *Itga3* alleles (i.e., $K14^{CreERT}::\alpha3^{+/+}$) were pre-treated five and three days prior to wounding with 4OHT or vehicle. Punch wounds (4 mm) were made on the back and allowed to heal unsplinted for three days. (a) Representative images of mouse backs three days post-wounding; box indicates area of inset that is magnified below. (b-d) Cryosections of 3-day, partially re-epithelialized wounds were prepared. Immunofluorescence with (b) anti-α3 integrin subunit (scale bar = 100 μm) and (c) anti-K14 to mark the epidermis (scale bar = 500 μm); representative images shown; *e,* epidermis; *wb*, wound bed; arrowheads indicate wound edges. (d) Graph shows wound gap measurements between wound edges as shown in (c). Data are mean +/- SEM; n = 5 mice (5wounds) per treatment group; male mice, square data points; female mice, circle data points; student's t-test; ns, not significant [1].



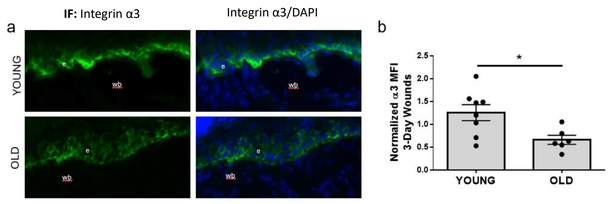


Induced deletion of epidermal α3β1 results in reduced re-epithelialization of splinted wounds that cannot contract. $K14^{CreERT}::\alpha3^{flx/flx}$ mice were pre-treated five and three days prior to wounding with 4OHT or vehicle as in Figure 1. Punch wounds (4 mm) were made on the back then splinted to prevent wound contraction by suturing on a silicone splinting ring (a; left), then adhering a metal washer on top (a; right); representative images shown. Wounds were allowed three days to heal, then paraffin wound sections were prepared. (b) Immunohistochemistry for anti-α3 integrin subunit confirms deletion of α3β1 in 4OHT-treated skin; representative images shown; *e*, epidermis; *d*, dermis; scale bar, 100 μm. (c) H&E staining shows the regenerating wound epidermis; arrowheads indicate wound edges and dotted line indicates the wound gap; *s*, eschar; *e*, epidermis; *wb*, wound bed; scale bar, 500 μm. (d) Graph shows wound gap measurements between wound edges as shown in (c). Data are mean +/- SEM; n = 3 mice (3 wounds) per treatment group; male mice, square data points; female mice, circle data points; student's t-test; * p<0.05 [1].

Hypothesis

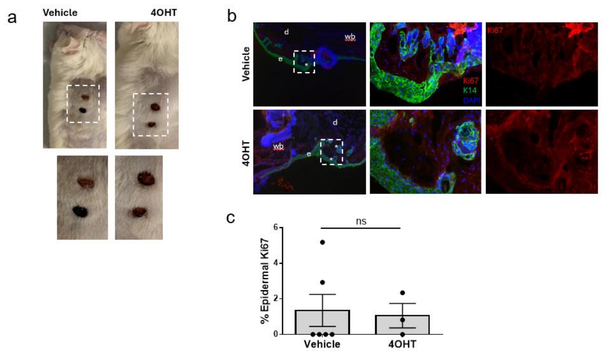
Expression of epidermal integrin α3β1 will reduce with increased age and contributes to decreased wound healing capabilities in old mice and elderly humans.

Figure 1. Integrin α3β1 expression declines with increased murine age.



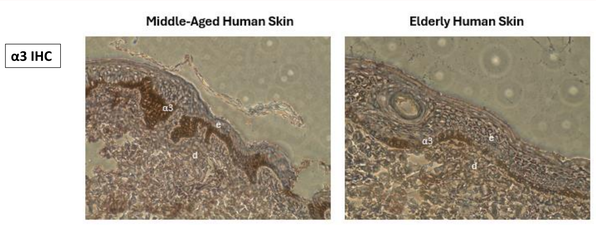
Vehicle-treated (α3-expressing) young (8-week-old) and old (22-month-old) $K14^{CreERT}::\alpha3^{flx/flx}$ mice were wounded with a punch biopsy (4 mm) on the back and allowed to heal unsplinted for three days. (a) Cryosections of 3-day, partially re-epithelialized wounds were prepared. Immunofluorescence with anti-α3 integrin subunit (green) and DAPI (blue) to assess integrin α3 expression; representative images shown.(b) Graph shows quantitation of data shown in (a), whereby the mean fluorescence intensity (MFI) of α3 IF was determined using Image J software. Data are mean +/-SEM; n ≥ 6 mice (at least 9 wounds) per treatment group; Young: 4 male mice and 4 female mice; Old: 4 male mice and 2 female mice; student's t-test; * p<0.05.

Figure 2. Markedly reduced keratinocyte proliferation is observed in both vehicle and 4OHT-treated aged iα3eKO mice, consistent with impaired wound healing with age and low integrin α3β1 level.



Proliferation is reduced in wound-proximal keratinocytes of both aged vehicle and 4OHT-treated iα3eKO mice. Cryosections of 3-day unsplinted wounds with wound-proximal skin from $K14^{CreERT}::\alpha3^{flx/flx}$ mice were prepared as in Figure 1. (a) Representative images of mouse backs three days post-wounding; box indicates area of inset that is magnified below. (b) Immunofluorescence with proliferation marker anti-Ki67 (red), epidermal marker anti-K14 (green), and nuclear marker DAPI (blue). Representative images shown. Left; box indicates area of inset that is magnified on right that includes wound-proximal hair follicles; *e,* epidermis; *d*, dermis; *wb,* wound bed; *, hair follicle. Right; inset panels shown with (left) and without (right) anti-k14 and DAPI. (b) Graph shows percentage of epidermal cells that are Ki67-positive. Data are mean +/- SEM; n ≥ 3 mice (wounds) per treatment group; student's t-test; ns, not significant.

Figure 3. Expression of integrin α3β1 appears to decrease in human skin with increased age.



Initial studies with human skin samples have elucidated preliminary results suggesting an age-dependent decline of integrin α3 expression with increased human age. Left image depicts α3 expression in shoulder skin from a 46-year-old male. Right image depicts α3 expression in forehead skin from an 87-year-old female. Formaldehyde fixed, paraffin embedded (FFPE) samples of disease-free skin were purchased from Precision for Medicine and were stained using α3 Immunohistochemistry and counterstained with hematoxylin. Future studies will focus on obtaining samples from more age groups and staining skin from more areas of the human body.

Future Directions

Future studies on our murine model will focus on quantifying wound gaps in aged $K14^{CreERT}::\alpha3^{flx/flx}$ vehicle-treated and 4OHT-treated mice.

Additionally, studies in human skin will focus on acquiring more samples that span a larger age range (i.e. infant, adolescent, young adult, middle-aged, elderly) to determine an age-dependent relation of α3 expression, with the ultimate goal of developing therapeutic strategies to promote activation of integrin a3b1 in order to aid in cutaneous healing for the treatment of wounds in elderly patients or in hard-to-heal wounds in general.

Methods

Pretreatment and Wounding: The mice (8-10 weeks old) are topically treated with the active form of tamoxifen (4OHT; 1 mg dissolved in 200 μL acetone per mouse) or vehicle-treated (200 μL acetone per mouse) 5 and 3 days prior to wounding. The backs of the mice are shaved, disinfected, then wounded with 4mm full-thickness biopsy punches.
Histology: Wounds were collected 3 days after wounding. Frozen sections were prepared and immunostained with anti-integrin α3, anti-K14 and anti-Ki67, as well as DAPI (marker for nuclei). Fixed tissue was embedded in paraffin and stained with Hematoxylin/Eosin (H&E) or immunostained with anti-α3 integrin subunit.
Imaging and Quantification: Images were acquired with a Nikon Eclipse 80i microscope with a Photometrics Cool Snap ES camera. Wound gaps were quantified histologically by measuring the distance between each K14-positive wound edge using NIS Elements AR 3.2 software. Keratinocyte proliferation was quantified using ImageJ software; the number of Ki67 labeled cells in epidermis were divided by the total number of DAPI-positive nuclei within epidermis, then multiplied by 100 to determine the percentage of proliferating cells in the epidermis.
Statistical Analyses: Data will be collected for at least 10 wounds per subgroup (i.e. 2 wounds per mouse, at least 5 mice per test group: equal males and females). All data is expressed as mean +/- S.E.M. P<0.05 will be considered significant.

Conclusions

- This study confirms an essential role for integrin α3β1 in promoting timely wound closure. Induced deletion of α3β1 results in delayed wound reepithelialization, likely through reducing keratinocyte proliferation and migration **(Fig. 1, 2 and 4).**
- Additionally, preliminary results suggest α3β1 expression decreases with increased murine age (Fig.1)
- Results displaying decreased proliferating wound-proximal hair follicles in aged mice suggest that could be a reason for decreased wound healing capabilities in mice, a potential contributor in human skin, as well. (Fig 2)
- Preliminary results suggest a similar age-dependent relationship of integrin α3 in human skin (Fig.3)

Acknowledgments

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Reference

[1] Dhulipalla S, Duarte GA, Wu L, DiPersio MR, Lamar JM, DiPersio CM, Longmate WM, Keratinocyte Integrin α3β1 Promotes Efficient Healing of Wound Epidermis, *JID Innovations* (2024), doi: https://doi.org/10.1016/j.xjidi.2024.100310.