Targeting Cx43 and N-Cadherin, Which are Abnormally Upregulated in Venous Leg Ulcers, Influences Migration, Adhesion and Activation of Rho GTPases

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INTRODUCTION

Chronic venous leg ulcers (CVLU) have few effective therapeutic options, and represent a significant medical need.

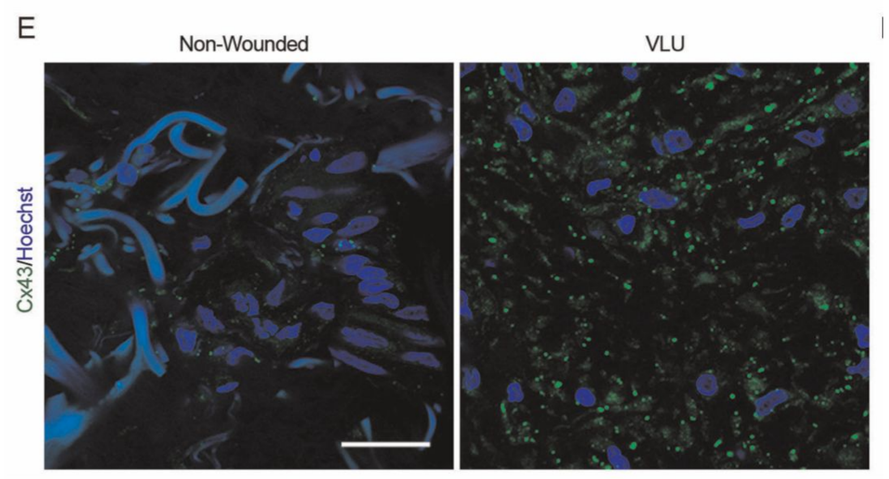

FIgure 1. Dermal Cx43 levels (in green) were significantly elevated in human VLU as opposed to matched non-wounded samples from the same patient.

- Cx43, the most ubiquitous connexin in the skin, which is normally downregulated in normal healing, is pathologically upregulated in CVLU[1].
- Topical application of Cx43 antisense gel to rodent wound models, which downregulated Cx43, accelerated healing while reducing inflammation and scar size[2].

This paper explored the effect of **knockdown** of **Cx43** and associated proteins, namely **N-cadherin** and **ZO-1** in human CVLU, which provided insight into potential therapeutic solutions to CVLU.

AIM

To investigate whether **knockdown of Cx43, N-cadherin and/or ZO-1** will change cell adhesion, migration, proliferation and cytoskeletal dynamics.

METHODS

1. Wound edge punch biopsies of human CVLU and matched intact skin samples were obtained
2. Cx43, N-Cadherin, ZO-1 were immunostained on biopsy samples
3. Knockdown and controls of Cx43, N-Cadherin, ZO-1 in 3T3 fibroblasts were compared via two modalities:
   - **Sense** (sODN) and **antisense oligodeoxynucleotide** (asODN) **treatment** (Cx43asODN, Cx43sODN, ZO-1asODN, ZO-1sODN)
   - Transfection and transduction of **pSuper vector** (Cx43shRNA, p.Sup, N-cadshRNA, Mock, Cx43-DN, Cx43-WT)
4. Control and knockdown fibroblasts were immunostained
5. Golgi reorientation measurements, fibroblast proliferation rates, wound edge cell protrusion lengths were obtained
6. Western blot was used to visualise actin, a-tubulin, b-catenin
7. Measurements were made for **leading edge cell migration** in serum starved and serum-present conditions
8. **Rho GTPase levels** were detected using GST-Rhotekin and Western blot
9. Forster resonance energy transfer was used to detect **augmented activation of Rac-1 and RhoA GTPases**
10. Hanging drop assays were used to visualise cell-cell adhesion

Statistical analysis was performed via the Wilcoxon Matched-Pairs Signed-Ranks, ANOVA and Chi-squared tests.

RESULTS

- Cx43, N-cadherin and ZO-1 were **dramatically upregulated** in human VLU dermis
- Cx43 expression is linked to ZO-1 and N-cadherin → knockdown of Cx43 via Cx43asODN or Cx43shRNA **induced significant downregulation** of ZO-1 and N-cadherin.
- Cx43 and N-cadherin knockdown **accelerated the rate of fibroblast migration**, but ZO-1 did not.
  - In serum-starved conditions, reducing Cx43 levels slowed migration; in normal serum media, reducing Cx43 levels significantly accelerated migration.
- Cx43 and N-cadherin knockdown **reduced fibroblast cell-cell adhesion**, and **increased fibroblast polarisation and migration**.
- Cx43 and N-cadherin knockdown **reduced fibroblast proliferation**.
- Cx43 knockdown fibroblasts expressed **richer lamellipodial protrusions** oriented to direction of movement, and **did not express the F-actin belt** expressed by control fibroblasts; TyrTub were predominantly oriented perpendicular to the wound edge.
  - Cx43 overexpressing fibroblasts expressed TyrTub, AcetTub and lamellipodia that were **less extensive** as compared to control fibroblasts.
- Cx43 and N-cadherin knockdown **enhanced Rac1 and RhoA GTPase** activities, but Cdc42 activity was **unchanged**.

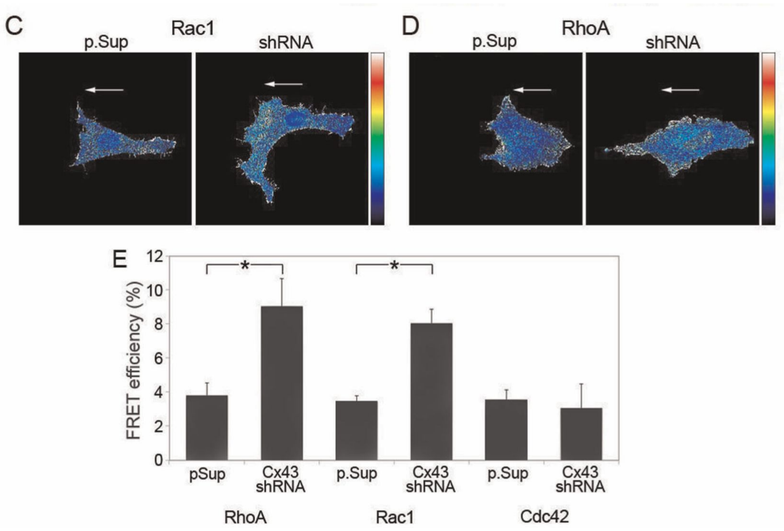

Figure 2. C and D: Leading edge cell activities of p. Sup and shRNA transduced cells, indicating elevated Rac1 and RhoA GTPase activities; E: FRET efficiency comparison of p. Sup and Cx43shRNA transduced cells, indicating elevated FRET efficiency of RhoA and Rac1, but unchanged FRET efficiency of Cdc42 when transduced with Cx43shRNA.

DISCUSSION

- Impaired healing found in human CVLU may be due to reduced fibroblast migration rate due to elevated Cx43 levels. **Therapeutically controlling Cx43**, and hence its associated proteins, may have significant effects on wound healing.
- Addition of mimetic peptide **ACT-1** to Cx43 has been reported to stabilise Cx43 in cell membranes, hence **accelerating wound healing** while reducing scar formation. Since it has similar effects to those of Cx43 antisense, ACT-1 peptide's effect on fibroblast migration has further research potential[3].
- While there are conflicting reports regarding GJ communication and cell migration, this divergence may be related to **serum starved cell conditions** in some reports[4]. As serum starvation is **not the case** in acute wounds in vivo, Cx43 downregulation should speed up fibroblast and keratinocyte migration in our target population.
- Cx43 downregulation may prevent maturation of stable cell-cell adhesions by reducing N-cadherin expression and/or docking of connexons.
- Reduction of Cx43 or N-cadherin in 3T3 with shRNA caused **reduced cell proliferation**, which is contradictory to previous reports. It was reported that GAP27 could enhance cell proliferation at wound leading edges without apparently reducing Cx43[5]. Thus, any differences observed when Cx43 was reduced significantly may be due to the direct effect of Cx43 on cell proliferation rather than effect of Cx43-based GJ communication.
- Our findings on Cx43 interactions with actin and microtubules corroborate with other reports.

CONCLUSION

Overall, this study provides insight into why CVLUs overexpressing Cx43 are slow to heal, and into the mechanisms by which reducing Cx43 and N-cadherin expression accelerate fibroblast migration, pointing to a potential therapeutic solution for CVLUs.

Our findings support the concept that the role of Cx43 is not simply to form GJ channels, but also to stabilise a nexus comprising N-cadherin and others required for cell adhesion and adhesion-dependent dynamics, and **must be broken dow**n to facilitate efficient fibroblast migration.

A large **Phase 2 clinical trial** is now underway using the Cx43asODN to reduce Cx43 expression in subjects with VLUs after an initial clinical trial showed significant improvement in complete healing.

Currently, **Phase 2 clinical trials** are taking place by downregulating Cx43 using Cx43asODN in the healing of corneal ulcers.

ACKNOWLEDGEMENTS

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REFERENCES

1. Wang C, Lincoln J, Cook J, Becker D. Abnormal Connexin Expression Underlies Delayed Wound Healing in Diabetic Skin. Diabetes. 2007;56(11):2809-2817.
2. Qiu C, Coutinho P, Frank S, Franke S, Law L, Martin P et al. Targeting Connexin43 Expression Accelerates the Rate of Wound Repair. Current Biology. 2003;13(19):1697-1703.
3. Hunter A, Barker R, Zhu C, Gourdie R. Zonula Occludens-1 Alters Connexin43 Gap Junction Size and Organization by Influencing Channel Accretion. Molecular Biology of the Cell. 2005;16(12):5686-5698.
4. Francis R, Xu X, Park H, Wei C, Chang S, Chatterjee B et al. Connexin43 Modulates Cell Polarity and Directional Cell Migration by Regulating Microtubule Dynamics. PLoS ONE. 2011;6(10):e26379.
5. Pollok S, Pfeiffer A, Lobmann R, Wright C, Moll I, Martin P et al. Connexin 43 mimetic peptide Gap27 reveals potential differences in the role of Cx43 in wound repair between diabetic and non-diabetic cells. Journal of Cellular and Molecular Medicine. 2011;15(4):861-873.

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