Myo/Nog cell presence is negatively correlated with neovascularization during wound healing.

Eric Gdovin[1], Apeksha Ghumatkar[1], David Broytman[1], Jessica L. Kim[1], Bryanna Guthridge[1], Monica A. Villegas[1], E-Jine Tsai[1], Mark Martin[1], Rushil Brahmbhatt[1], Jacquelyn Gerhart[1], Mindy George-Weinstein[1], Zeus A. Antonello[2] and Arturo Bravo-Nuevo[1]

[1]Philadelphia College of Osteopathic Medicine, Philadelphia, PA
[2]Cooper University Hospital, Camden, NJ

Introduction

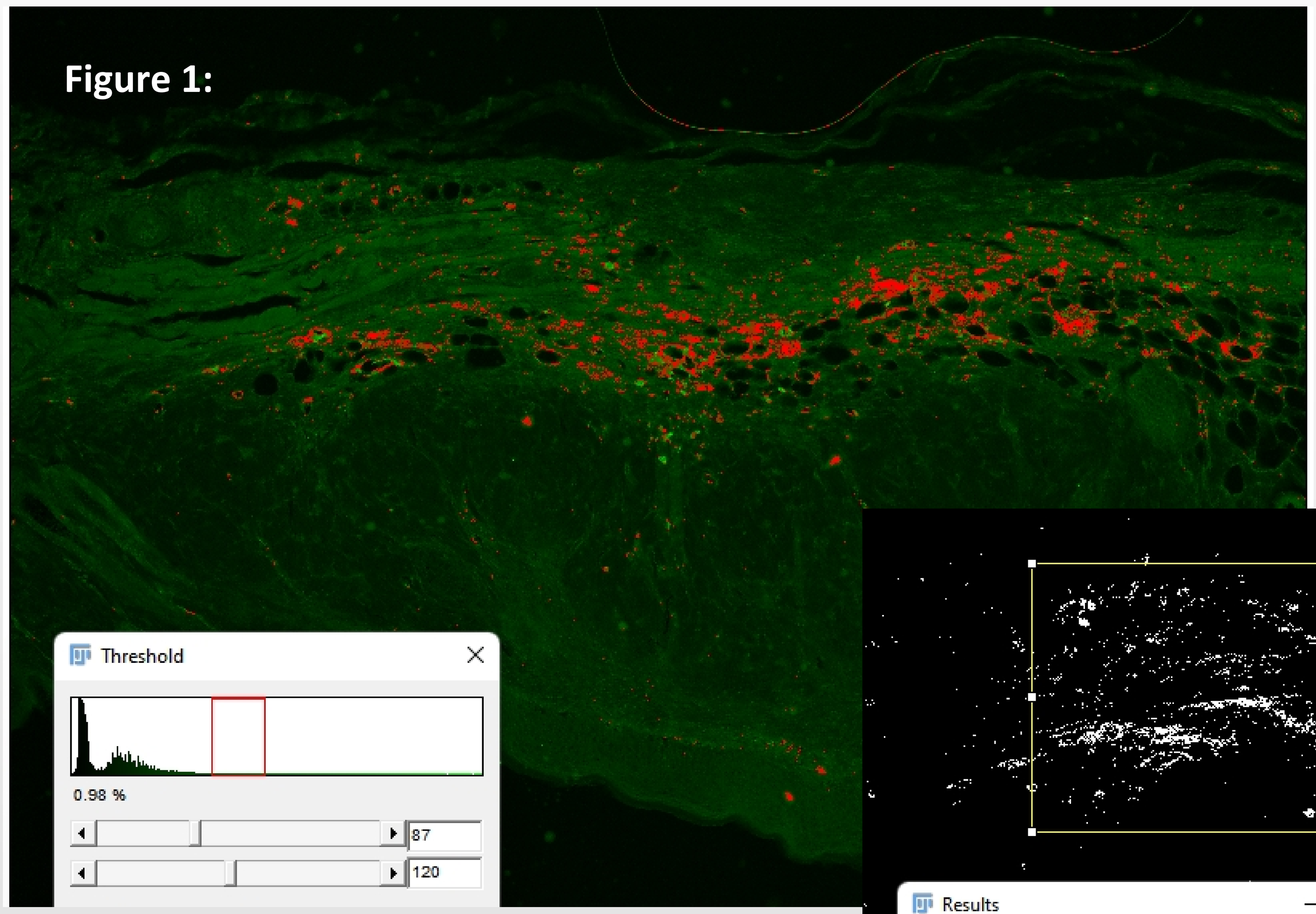
Both Type 1 and Type 2 diabetes mellitus (T1DM, T2DM) contribute to delayed wound healing and systemic vasculopathies. It has been previously established that, along with macrophages and fibroblasts, there are increased numbers of Myo/Nog cells present in the dermis of mice shortly after wounds are induced. Myo/Nog cells are so named because they express the skeletal muscle transcription factor MyoD, along with Noggin, a bone morphogenic protein inhibitor. In addition to these established traits, Myo/Nog cells also express brain-specific angiogenesis inhibitor protein 1 (BAI-1), which is an inhibitor of angiogenesis and a target of the G8 monoclonal antibody (mAb). Initially we assess the relationship between the glycemic state of mice, and the number of Myo/Nog cells preset in the dermis. We then expand on this knowledge to assess the relationship between the presence of Myo/Nog cells and the level of neovascularization in both wounded and unwounded mice, under hyperglycemic and normoglycemic conditions. These comparisons are repeated under both control and Myo/Nog depletion mediated by their specific immunotoxic targeting.

Methodology

To assess the effects of hyperglycemia on neovascularization, T1DM was induced in C57BL/6J male mice, 5-8 per treatment group, via administration of streptozotocin (STZ) at a dose of 50mg/kg per day, from postnatal days 21 through 25, causing destruction of pancreatic beta cells. Two wounds per mouse were made under anesthesia using a 6mm punch biopsy. To further investigate the effect of Myo/Nog cell presence on neovascularization, Myo/Nog depletion subgroup was formed via topical treatment of wounds 3 times per week for 3 weeks with a G8 mAb conjugated to a complex consisting of cytotoxic doxorubicin (DOX) in a DNA dendrimer (3DNA). The wounded area was measured daily, and healing was allowed to progress until samples were taken and embedded for cryosectioning. After sectioning, samples were processed for immunofluorescence imaging. One subset of samples was stained with a mAb targeting BAI1[A] to obtain Myo/Nog cell counts, and a second subset of samples was stained with a mAb targeting α -SMA[B] to highlight vasculature. These slides were then imaged within NIH Elements on a Nikon Eclipse 80i at their respective fourescence wavelengths, with the BAI1 mAb subset being utilized for cell counts within NIH Elements, and the α -SMA mAb subset being imaged for analysis in Fiji.

A: (1 º: G8, Genisphere; 2 º: AlexaFlour-488, ThermoFisher)
B: (1º: α-SMA, Sigma-Aldrich; 2 º: AlexaFlour-488, ThermoFisher)

Fiji Process



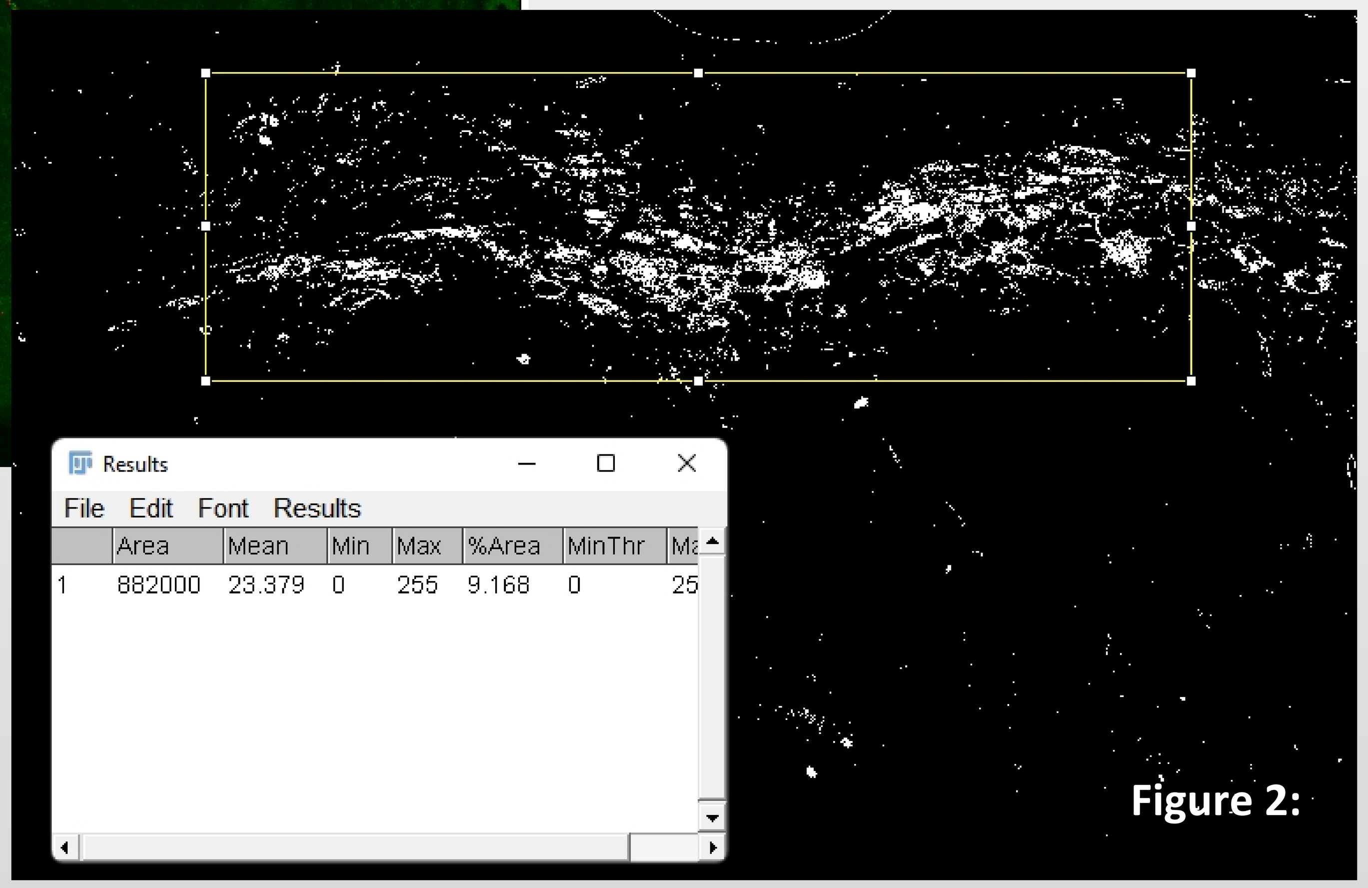
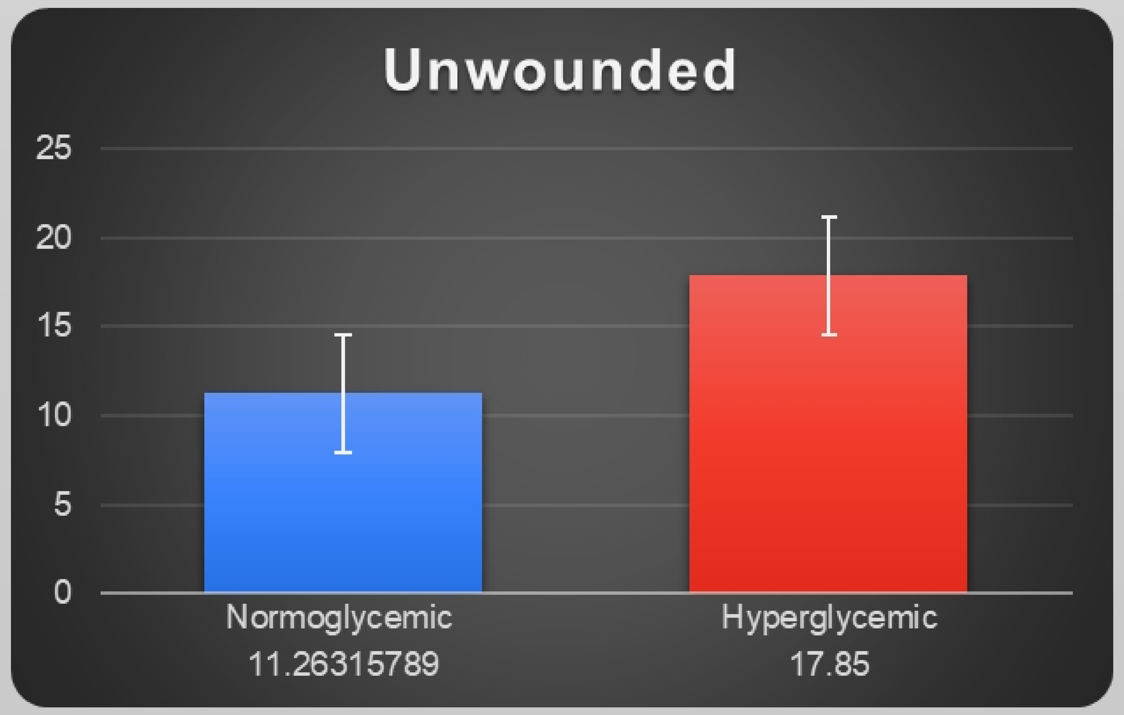


Fig 1: In Fiji, the color channel corresponding to α -SMA fluorescence was isolated, a high/low-pass intensity filter (red) was utilized to both eliminate background noise and isolate the fluorescence corresponding to alpha-SMA.

Fig 2: The image was then converted to binary, and a percentage area was taken in software consisting of only the narrow bandpass defined earlier, that percentage represents the amount of neovascularization.

Results



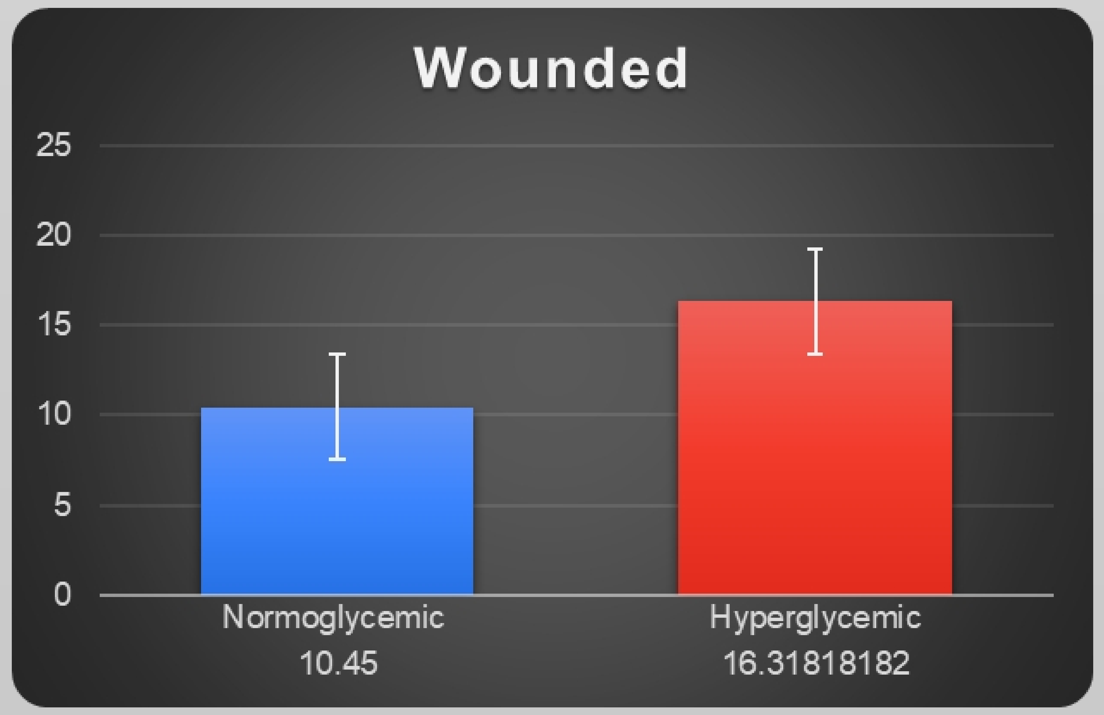


Figure 1: Increased counts of Myo/Nog cells are found in diabetic as compared to normoglycemic mice. Here we show the results of the initial project phase. We see increased numbers of Myo/Nog cells in diabetic mice, in both unwounded (left) and wounded (right) conditions. Statistical significance was seen between diabetic and normoglycemic mice in both wounded (P<.001) and unwounded (P=.004) mice.

Conclusion

There is a statistically significant, direct correlation between heightened glycemic state and increased numbers of Myo/Nog cells present in dermal tissue. Hyperglycemic tissue sections showed a significant increase of 6.57 cells/section, on average, as compared to normoglycemic tissue (17.85 vs. 11.26 cells/section P=.004) in the unwounded condition. A smaller, yet more statistically significant increase of 5.87 cells/section on average was seen in hyperglycemic tissue as compared to control (16.32 vs. 10.45 P<.001) when looking at the wounded group.

We expanded on this knowledge to explore the role Myo/Nog cells play in the angiogenesis necessary for proper wound healing. Here, we developed a novel workflow using immunofluorescence microscopic imaging and Fiji, an open-source image analysis software, to determine the correlation between Myo/Nog cell presence and the percentage of area per image containing neovascularization. While we have refined the process to the point that we are confident in the data being output through this workflow, a bottleneck has been discovered, that is the time spent in acquisition of images for the purpose of analysis. We have determined our next step is to transition to confocal microscopic imaging. We will increase throughput by way of resolution-stacking in software, a methodology typically utilized in landscape and astrophotography, to produce larger images for processing with fewer fields of view necessary, reducing the time directly required for obtaining images by a significant margin.

Acknowledgements

1. Baltzis, D., Eleftheriadou, I. & Veves, A. Pathogenesis and Treatment of Impaired Wound Healing in Diabetes Mellitus: New Insights. *Adv Ther* 31, 817–836 (2014). https://doi.org/10.1007/s12325-014-0140-x
2. Gerhart, J., Hayes, C., Scheinfeld, V., Chernick, M., George-Weinstein, M., & Gilmour, S. (2012). Myo/Nog cells in normal, wounded and tumor-bearing skin. *Experimental Dermatology*, *21*(6), 466–468. doi: 10.1111/j.1600-0625.2012.01503.x
3. Gerhart, J., Werner, L., Mamalis, N., Infanti, J., Withers, C., Abdalla, F., … George-Weinstein, M. (2019). Depletion of Myo/Nog Cells in the Lens Mitigates Posterior Capsule Opacification in Rabbits. *Investigative Opthalmology & Visual Science*, *60*(6), 1813. doi: 10.1167/iovs.19-26713
4. Guo S, Dipietro LA. Factors affecting wound healing. *J Dent Res*. 2010;89(3):219-229. doi:10.1177/0022034509359125
5. Center for Disease Control and Prevention. National Diabetes Statistics Report, 2020.
6. Okonkwo, U., & Dipietro, L. (2017). Diabetes and Wound Angiogenesis. *International Journal of Molecular Sciences*, *18*(7), 1419. doi: 10.3390/ijms18071419
7. Okonkwo, U. A., Chen, L., Ma, D., Haywood, V. A., Barakat, M., Urao, N., & Dipietro, L. A. (2020). Compromised angiogenesis and vascular Integrity in impaired diabetic wound healing. *Plos One*, *15*(4). doi: 10.1371/journal.pone.0231962