Diabetic Complications Consortium

Application Title: Complex, 3D Skin-like Tissues for Preclinical Testing of Diabetic Ulcer Therapy
Principal Investigator: Jonathan Garlick, DDS, PhD

1. Project Accomplishments:

In the year of this grant we completed the following goals:

1. We have improved upon existing 3D *in vitro* tissue wound model for diabetic foot ulcer-derived (DFU) fibroblasts by testing and characterizing additional Type 2 diabetes patient-derived cells.
2. We have constructed higher throughput, "miniaturized" 3D tissue models that incorporate DFU patient-derived fibroblasts, macrophages and an endogenous, human extracellular matrix and found that is can be used to monitor wound healing responses.
3. We have determined that 8 of the 9 diabetic or control fibroblast lines that were tested can be used to make 24-well format, "miniaturized" 3D tissue models containing macrophages demonstrating the broad applicability of this model.
4. We have developed a wounding system for a 24-well format, "miniaturized" 3D tissue models and learned that there is variability in re-epithelialization between tissues made with diabetic (DFU) or control (NFF) cells.
5. We establish proof of concept that a diabetes-specific, bioengineered, 24-well format, "miniaturized" 3D tissue wound model mimics chronic wound repair in DFU using patient-derived fibroblasts and macrophages as a critical step towards optimization of this tissue models as a novel drug testing platform for DFU.
6. Our findings support the feasibility of applying this tissue engineering approach to construct such complex, human-derived 3D tissue wound healing models that can be used to study the biology of DFU in a more *in vivo*-like setting.

2. Specific Aims:

Aim 1: To optimize all-human, higher throughput, DFU-specific 3D tissue models harboring previously untested fibroblasts and macrophages from Type 2 diabetic patients (T2DM).

Result 1- Newly-tested diabetic (DFU) and control (NFF) fibroblasts demonstrate differences in markers PLOD2 and MIF by RNA analysis

We previously isolated 22 primary fibroblast cell lines, including 12 from patients with DFUs and 10 from site- and age-matched, healthy control patients (NFF). As we had previously tested only 3 DFU-derived patient fibroblast cell lines 24-well tissues, the first goal of this aim was to study tissues generated from a larger number of previously untested DFU patients and NFF cell lines to establish the reproducibility of tissue construction and wound response in these higher-throughput tissue formats. We first analyzed these previously untested fibroblasts isolated from DFU or from site-matched controls (NFF) for biomarkers that we found to be differentially expressed between NFF

and DFU fibroblasts, and wanted to confirm that the untested cells showed these differences. Specifically, we measured PLOD2 (involved in collagen maturation and crosslinking) and MIF (a pro-inflammatory cytokine involved in regulating innate immunity) and found that these previously untested patient-derived fibroblasts have a general trend of grouping by NFF and DFU, however there was variability between specific cell lines (Figure 1).

Result 2- 24-well format, "miniaturized" 3D tissue models form a well-differentiated and full thickness, self assembled skin equivalent (SASE) tissues using previously untested fibroblast cell lines.

It is known that some primary fibroblast lines do not form fully functional 3D skin equivalents. Based on this, we screened fibroblasts from previously untested DFU and NFF patient-derived cell lines to determine their ability to generate robust, 24-well format, "miniaturized" 3D tissue models (known as self-assembled skin equivalents or SASE). We used five DFU (DFU7, DFU8, DFU21, DFU25, DFU37) and four NFF control lines (NFF12, NFF14, NFF23, NFF27) that were previously untested and uncharacterized. Histology for 8 of the 9 tested cell lines showed a mature and contracted connective tissue supporting a fully differentiated epithelium (Figure 2). *This demonstrated that 8 of the 9 cell lines were able to successfully generate a full thickness skin-like tissues using the 24-well format, "miniaturized" 3D tissue models (SASE), proving the broad applicability of these tissues with DFU and control NFFs from many different patients.*

Result 3- Patient-derived macrophages persist and function in 24-well format, "miniaturized" 3D tissue models as "self assembled skin equivalent (SASE) tissues when incorporated directly into tissues as peripheral blood mononuclear cells (PBMC).

Before incorporating macrophages into tissue wounds, we first tested how to best incorporate these macrophages into 24 well 3D tissues harboring DFU and NFF fibroblasts. We first attempted to polarize peripheral blood mononuclear cells (PBMC) to different macrophage subtypes, using techniques established in our lab. We first isolated PBMC from the "blood collars" of blood draws to isolate peripheral blood mononuclear cells using our existing IRB approvals. PBMC were isolated by negative selection using magnetic bead columns and non-adherent CD14+ monocytes were collected as flow through. Macrophages were polarized to M1 and M2a, M2c states as follows: Macrophages were first generated *in vitro* by incubation in rhM-CSF (50 ng/mL) in RPMI 1640 and cells and cells were positively identified by flow cytometry. Cells were then polarized to M1 or M2 macrophage phenotype by adding recombinant rhIFN-γ and LPS or rhIL-4 and rhIL-13 to media. M2c macrophages were generated by adding rhIL-10 (50 ng/mL). We then fabricated 24-well format tissues with fibroblasts and patient-matched macrophages polarized to M0, M1, M2 phenotypes and cells were cultured for 2 weeks and evaluated tissue and cellular benchmarks to determine their functional properties when grown first in 6-well formats before moving on to 24 well formats. After multiple experiments, we found that the profile of macrophage subtypes was quite variable using the *in vitro* polarization of PBMCs. We therefore decided to

incorporate PBMCs directly into the SASE in 24 well formats at the time of tissue fabrication with DFU and NFF fibroblasts. We found that PBMCs were able to directly differentiate into stable and functional macrophage phenotypes in SASEs without previous differentiation to macrophage subtypes. This allowed us to greatly streamline the fabrication of the SASE model with functional macrophages. This was demonstrated by staining for CD163, a macrophage marker, which provided proof of concept that the SASEs were able to support macrophage differentiation and function directly from PBMCs (Figure 3).

Figure 1: No significant trends were found in when comparing expression of MIF (A) and PLOD2 (B) between the NFF, DFF, and DFU groups, or between Type I and Type II diabetics.

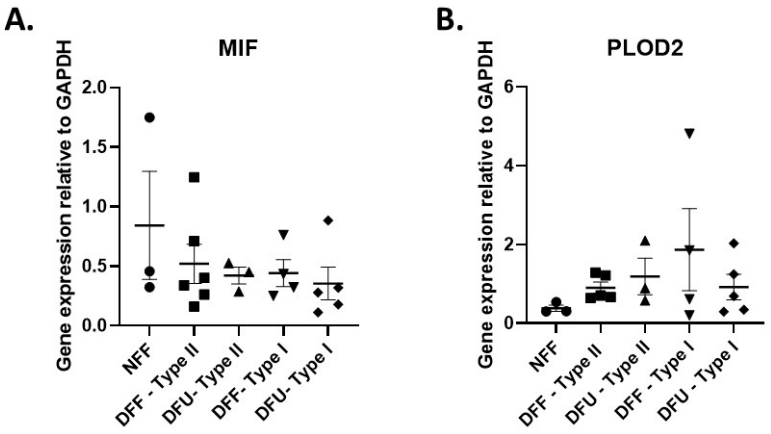


Figure 2: H&E images showing normal tissue morphology, with fully differentiated epithelium for all NFF and DFU fibroblasts except for DFU37, thus expanding the number of cell lines used in SASEs

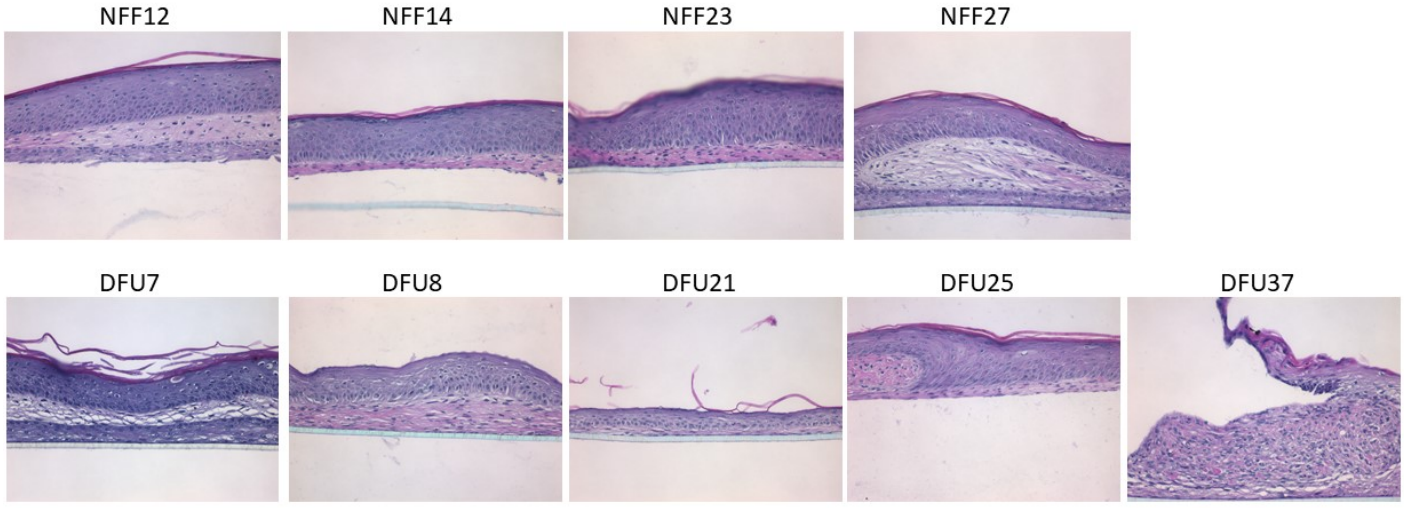
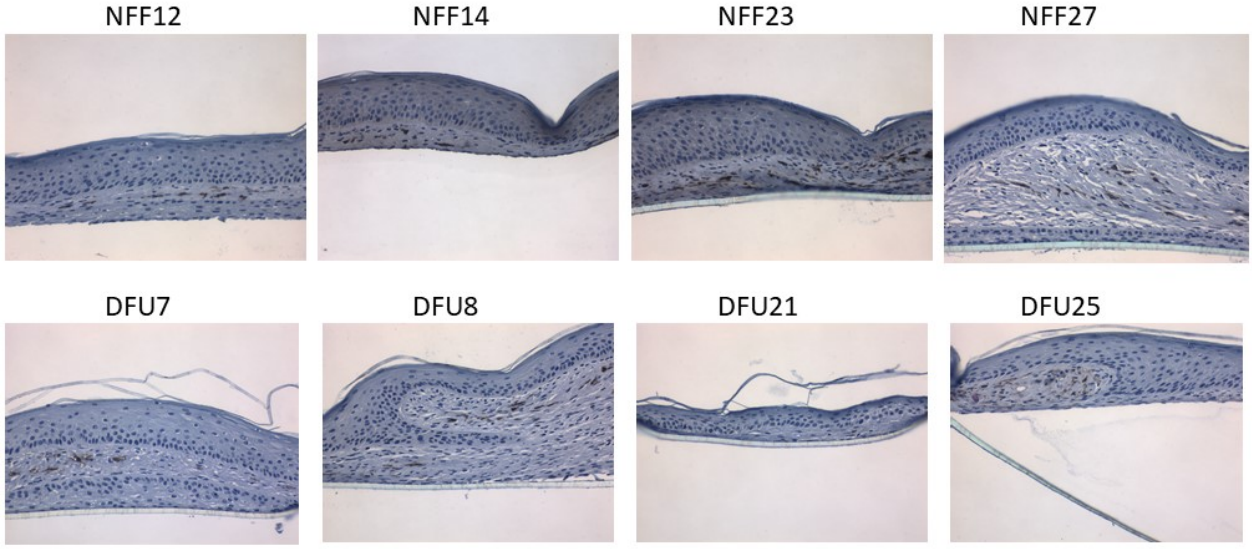


Figure 3: Immunohistochemical staining for CD163 shows the presence of macrophages in the SASE tissues.



Aim 2: To test and characterize all-human 3D tissues as screening platforms of DFU wound re-epithelialization.

Result 1- The importance of our work was to determine for the first time if it is possible to grow 24-well format, "miniaturized" 3D tissue models containing DFU-derived cells and monitor wound responses in these tissues. 24-well format tissue models were constructed as described above in AIM 1, wounded using a 3 mm punch biopsy and re-epithelialization monitored for 72 hours.

We performed several rounds of optimization to create a working wound healing model that would function in our SASE system. We focused on optimizing factors such as wound size, healing time, media formulation, and the transfer method for moving wounded tissues onto a new dermis.

Wounds were created in tissues constructed from 2 previously untested DFU cell lines and 2 previously untested NFF fibroblast cell lines which were performed in triplicate to allow us to test the reproducibility of the wound response with diabetic and control macrophages in these tissues. *These results illustrated that wounding and monitoring wounds harboring DFU-containing tissues was possible in this SASE model (Figure 4). Due to the variability seen between tissues using different DFU and NFF cell types, this*

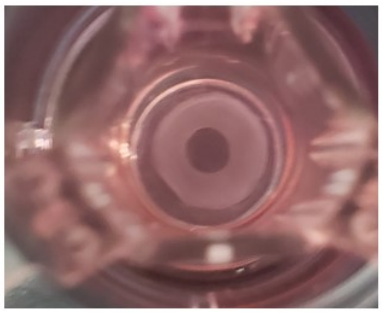
indicated the need to increase the number of biological replicates when performing these wound healing analyses.

Result 2- NFF and DFU fibroblasts demonstrate different degrees of reepithelialization in 24-well format, "miniaturized" 3D tissue models containing DFU-derived cells- Following wounding of 3D skin-like tissues, epithelialization closure measured after 72 hours and degree of closure from the wound margin was calculated to quantify re-epithelialization in multiple tissue sections. The degree of wound coverage by surface keratinocytes on DFU cell-containing stromal tissues was measured from the wound margin to the tip of the epithelial tongue migrating onto the wound and compared migration onto NFF- and foreskin fibroblast-containing stromal tissues. Wound responses were calculated and expressed as the % of wound re-epithelialization (data not shown). After optimization of wound healing conditions such as wound size, healing time and media formulation were determined we tested differences in re-epithelialization between DFU and NFF fibroblasts. Results showed that SASEs with both NFF and DFU fibroblasts were able to fully re-epithelialize, however with different levels of epithelial maturation. SASEs containing NFFs showed a fully differentiated epithelium at the 24hr time-point. In contrast, SASEs containing DFUs exhibited a more gradual healing that was complete at 48hr time-point but this tissue did not have a differentiated epithelium (Figure 4). *Thus, we established for the first time that DFU-derived cells could be used to monitor wound healing in a higher throughput format. This opens up new opportunities to use these tissues to screen new compounds that may stimulate wound healing of DFU in humans. Importantly, our data suggested that higher throughput tissues can be used as a tissue based platform and incorporated into "organs on chips" formats to screen drugs targeted to DFU at an early stage in their development.*

Result 3- Effect of macrophages on wound healing- It is known that delayed re-epithelialization and healing of DFU is linked to the polarization state of DFU macrophages. As a result, we began to test SASEs constructed with DFU or NFF fibroblasts, combined with either diabetic or control patient macrophages to determine how fibroblasts and monocyte in 24 well tissues can modify healing. Wounds were created in tissues constructed from 2 previously untested DFU cell lines and 2 previously untested NFF fibroblast cell lines which were performed to measure wound response with diabetic and control macrophages in these tissues. The results of this experiment are still being evaluated.

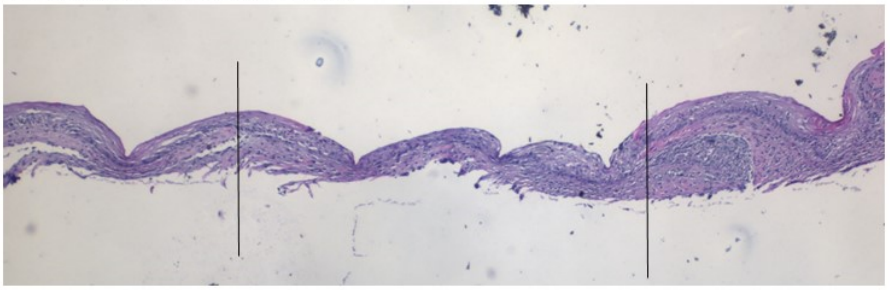
Figure 4: (A) Overhead image of wounded SASE. (B) H&E staining showed improved healing in wounds containing NFFs over those containing DFUs in preliminary data. Black lines indicate original wound edges.

A.

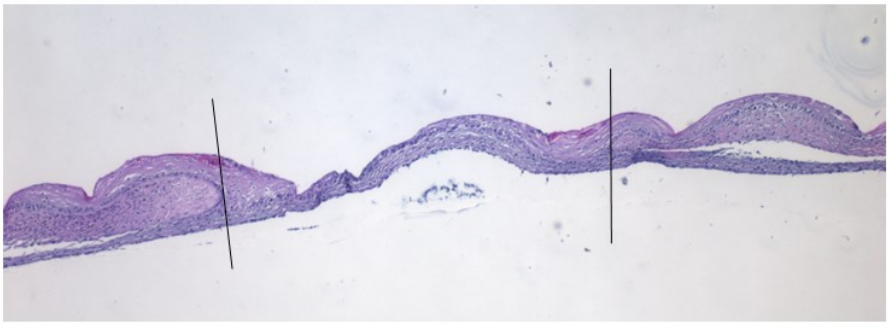


B.

NFF12 wound: after 24 hours



DFU8 wound: after 48 hours



3. Publications:

We are currently preparing two publication to present these findings.