

Novel Approaches for Understanding the Mechanisms of Wound Repair

Lillian B. Nanney^{1,2}, Robert L. Caldwell³, Alonda C. Pollins¹, Nancy L. Cardwell¹, Susan R. Opalenik⁴ and Jeffrey M. Davidson^{4,5}

Mechanisms that drive wound repair are complex and have challenged wound-healing investigators for many years. In this review, we present four examples of new tools that are being utilized to discover events that drive wound repair and regeneration. Laser capture microdissection facilitates the focused collection of tissue for purposes of genomic or proteomic analysis from specific cell populations within the wound bed. Tissue profiling and protein imaging by matrix-assisted laser desorption ionization mass spectrometry are two proteomic-based tools that permit rapid analysis with spatial orientation and relative abundance of hundreds to thousands of molecules from intact tissues. Another approach uses an *in vivo* porcine model to harness a strategy of adenoviral-driven receptor overexpression. This biological model closely mimics the human setting and permits transient stimulation along a specific cytokine pathway to tip the balance in favor of accelerated repair. The advent of new approaches that collect cell samples from within their *in vivo* circumstance while preserving discrete cellular localizations is likely to move the field of wound repair forward.

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INTRODUCTION

Cutaneous wound healing is a multi-step process that involves numerous cell types. For years, various wound-healing models have relied on biochemical and molecular techniques that were based on homogenization techniques. RNA analyses usually proceeding along the line of Northern blotting or reverse transcriptase-PCR and standard protein studies employed Western blotting, ELISA or immunoprecipitation. These techniques utilized a homogenization approach and typically were limited to a relatively small screening for 1–5 molecules of interest. Recent array-based technologies have broadened the scope of investigation. The present overview focuses on four differing technical examples and two diverse *in vivo* approaches that add significant refinement to such tried and true approaches. The new tools discussed herein are being used to address long-standing questions within wound healing. Herein we delineate several approaches that are under exploration in our laboratories and are just beginning to move us down the road of discovery.

Technique no. 1: Laser capture microdissection

Samples collected from wound beds always contain a complex mixture of cell types. These typically include

keratinocytes from the surface and more deeply positioned epidermal appendages (hair follicles, sweat ducts, and sebaceous glands), fibroblasts, endothelial cells, peripheral nerve cells, and a variable number of immunocytes ranging from neutrophils and monocytes to macrophages, T-cells, and mast cells. Cells of interest in a particular wound-healing study can represent a minor population within this larger context. Homogenization of these heterogeneous cell populations can dilute and mask the assignment of genes or proteins of interest from certain populations. These past limitations can now be overcome with the advantages offered by laser capture microdissection (LCM). This valuable new tool allows investigators to gather selected cell populations within the complex milieu of the wound bed.

LCM was introduced into medical research approximately 10 years ago (Emmert-Buck *et al.*, 1996; Simone *et al.*, 1998). In that early period, investigators began to use it to isolate tumor cells from their surrounding stroma (Emmert-Buck *et al.*, 1996; Leethanakul *et al.*, 2000). The use of LCM within the field of dermatology is still in its nascent phase. In 2003, a report compared the genomic profiling of wound keratinocytes with pathological perturbations in squamous cell carcinoma (Pederson *et al.*, 2003). In this study, LCM was

¹Department of Plastic Surgery Research Laboratory, Vanderbilt School of Medicine, Nashville, Tennessee, USA; ²Department of Cell and Developmental Biology, Vanderbilt School of Medicine, Nashville, Tennessee, USA; ³Vanderbilt Orthopaedic Institute, Vanderbilt School of Medicine, Nashville, Tennessee, USA; ⁴Department of Pathology, Vanderbilt School of Medicine, Nashville, Tennessee, USA and ⁵Department of Veterans Affairs, Nashville, Tennessee, USA

Correspondence: Dr Lillian B. Nanney, Department of Plastic Surgery Research Laboratory, S-2221 MCN Vanderbilt School of Medicine, Nashville, Tennessee 37232, USA. E-mail: lillian.nanney@vanderbilt.edu

Abbreviations: EGF, epidermal growth factor; LCM, laser capture microdissection; IMS, imaging mass spectrometry; MALDI, matrix-assisted laser desorption ionization; MS, mass spectrometry

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used as a practical tool to isolate RNA from specific populations of mouse keratinocytes. Changes in gene patterns were analyzed on a microarray and candidate genes that were either similar or different were reported. In the realm of dermatopathology, the LCM-assisted technique is also in its beginning stages and still qualifies as a tentative new approach. The plucking of selected cells populations as a prelude to DNA amplification proved useful for detecting a range of infectious agents or for clonal analysis of lymphocytic populations (Yazdi *et al.*, 2004). Herein we describe the general technique of LCM and show current examples from our ongoing research that illustrate its potential application to wound healing.

We are presently applying LCM with a goal of defining gene expression differences in keratinocytes in healing human burn wounds. Keratinocytes undergo dramatic but transient changes as a part of the healing response. It is reasonable to assume that these phenotypic changes are accompanied by dramatic changes in keratinocyte gene expression. In our laboratory, RNA samples that have been targeted for selection with the assistance of an LCM technique are either amplified for microarray analysis or are utilized for quantitative reverse transcriptase-PCR analysis to define gene expression differences for ErbB family members in wounded epidermis after burn injury.

Figure 1a is an illustration of the complexity of regions that one encounters within a typical human burn wound. Within the wound bed of a partial-thickness injury that by definition contains viable cells with the capacity to mount a healing response, one can note the upward and lateral outgrowths from surviving epithelial cells within epidermal appendages (Figure 1b-f). These epithelial cell populations often appear as isolated islands within the wound bed (Figure 1b and g). In other sections, one can observe that new growth originates either from the depths of eccrine sweat ducts (Figure 1c) or from surviving hair follicles (Figure 1d). At the edge of burn wounds, one can observe the lateral migration and proliferation of keratinocytes toward the damaged region (Figure 1e

and f). All of these keratinocytes appear to serve as the sole source of cells that will be responsible for wound resurfacing and they are being selectively captured by LCM in our current studies.

LCM works on the principle that pulsed laser light can precisely pinpoint clusters of cells or potentially single cells and isolate them from the surrounding tissue section. Quick stained frozen sections or more recently paraffin-embedded samples containing regions of interest are placed on an inverted microscope (Figure 2a). The laser pulse from a PixCell II LCM workstation (Arcturus Engineering Inc, Mountain View, CA) is used transiently to melt a sticky plastic film onto the cluster of selected cells. This process is visualized with the aid of the microscope (Figure 2b). The pulsed light and heat is transient and the plastic film retracts back upward on the cap carrier as it cools. When the cap is lifted away from the section, one can make a visual check to determine if clusters of cells have been selected as they are in Figure 2c. The LCM operator can double check by reviewing the original microscopic section to gauge whether there is an expected tissue ablation as shown in Figure 2d. The pulse-firing sequence is controlled by a moveable joystick with a button. A firing pattern can be individualized as shown in the Figure 2a-d series or by *en bloc* melting and ablation that can be accomplished as demonstrated by the Figure 2e-h series where a hair follicle was dissected away from the surrounding tissue. We envision a host of applications for this powerful new tool. For example, LCM technology should find a niche in focused selection of stem cell populations from the bulge region of hair follicles. While these cells obviously contribute to the resurfacing of wound healing (Ito *et al.*, 2005). The most recent investigators have been forced to rely on either fluorescence-activated cell sorting analysis to further their selective isolation of stem cells or have relied on elaborate suicide gene ablations in mice (Morris *et al.*, 2004).

Once tissues are successfully microdissected, a range of possibilities for analysis are available. RNA can be isolated

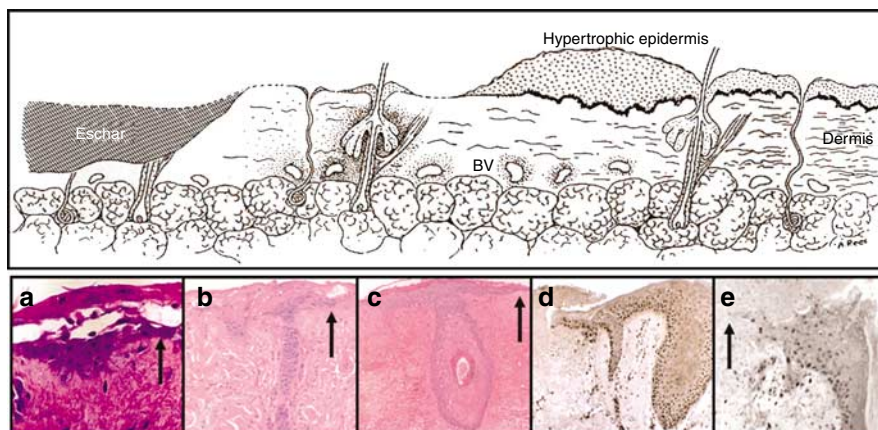


Figure 1. A diagram of a healing burn wound illustrates the diversity of cell types and spatial niches. (a) Focal regions within an actual 5-day-old burn are shown in Hematoxylin- and eosin-stained sections. (b) An isolated epithelial island. (c) Healing eccrine sweat duct. (d) Epithelium spreading outward from a healing hair follicle. (e) Hair follicles showing proliferating cell nuclear antigen-positive nuclei in the proliferating cells and arrows to indicate the migratory tip of epithelium where the cell nuclei are proliferating cell nuclear antigen negative.

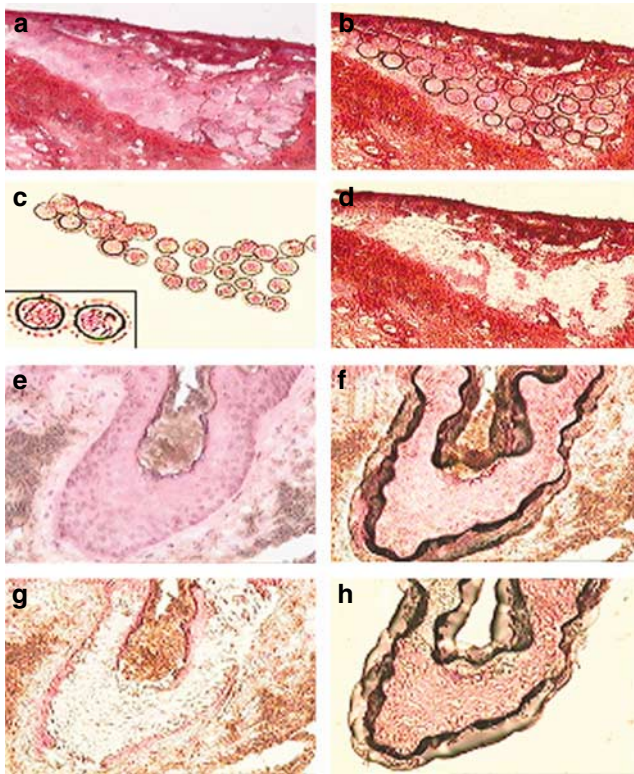


Figure 2. LCM images of a healing epithelial island within a 6-day burn injury. (a) Hematoxylin- and eosin-stained frozen section showing the cell population of interest before LCM. (b) Section showing circular outlines of laser meltings of the film prior to extraction from the section. (c) View of the circular outlines of the epithelial cells that were targeted for capture on the film on the cap. Inset confirms that cells were captured. (d) View of the deficit in the burn section where the epithelial island was once located. LCM image of a hair follicle. (e) Hair follicle prior to LCM. (f) Hair follicle targeted for capture. Note where film that is melted over this region of interest. (g) Image of the tissue deficit after successful LCM. (h) Image of the epithelial cells from the hair follicle that are successfully captured.

for subsequent quantitative reverse transcriptase-PCR assays. Validation of specific genes on LCM cells of interest can mesh nicely with immunohistochemical localization of protein distribution. Alternatively, LCM can be combined with a more global analysis using cDNA microarrays (Pederson *et al.*, 2003). Recently, investigators in fields outside of dermatology have actually collected sufficiently large numbers of cells for subsequent extraction of proteins of interest and protein analysis by matrix-assisted laser desorption ionization (MALDI) mass spectrometry (MS) (Xu *et al.*, 2002).

The application of LCM to the fields of wound repair and the greater field of dermatology is still in its infancy. Possibilities abound. For example we are currently selecting proliferative populations of keratinocytes from within healing epidermis with the goal of comparing these cells to normal keratinocytes in undisturbed basal keratinocytes in non wounded epidermis. Such extreme selectivity from among the complexity of wound cell types is possible by pre-staining of frozen burn sections using proliferating cell nuclear antigen immunostaining (Figure 1e and f). At present our

own studies have allowed us to conclude that LCM is a powerful tool in the study of simultaneous gene expression patterns in discrete cell populations in the multiple cell niches that are illustrated in the burn diagram in Figure 1a.

Tissue profiling by MALDI mass spectrometry

Tissue profiling by MALDI time-of-flight MS was first introduced in 1999 (Chaurand *et al.*, 1999). This new tool permits highly sensitive and rapid detection of hundreds of proteins from intact tissues. Among many applications, it has been increasingly used to detect protein biomarkers that predict the outcome and behavior of various tumor types (Caldwell, 2005a; Caldwell and Caprioli, 2005b; Caldwell *et al.*, 2005c). Tissue profiling has been recently introduced for dermatologic applications but these reports have been far removed from the field of tissue repair. For example, tissue profiling was used to characterize the distribution and penetration of ketoconazole delivered by shampoo to the surface of skin (Bunch *et al.*, 2004). Furthermore, Kato *et al.* (2003) documented that tissue profiling has advantages as a simple screening assay for assessing the sensitization potential of cutaneous allergens. More recently, the proteomic analysis of skin following heat or cold shock has been reported (Huang *et al.*, 2003). They were able to use this technique to show increased levels of HSP27, HSP60, and HSP70 suggesting involvement of these chaperones in the cutaneous response mechanism to temperature stress. Their data have also established numerous reference markers within the proteome map of BALB/c murine skin to provide an important framework for future efforts aimed at characterizing epidermal and subepidermal responses to environmental environment stresses which could include wound healing.

Sample preparation for tissue profiling is quick and simple. Frozen tissues are sectioned in a cryostat and are transferred to MALDI-compatible glass plates. A matrix solution containing a UV absorbent acid matrix (~250 nl) is directly deposited onto regions of interest within the tissue. The sample is then transferred to a desiccator to allow matrix crystallization. For analysis, the sample is inserted into the mass spectrometer and a series of laser shots are fired upon the matrix deposits. After desorption by the laser, ionized molecules are accelerated down the flight tube of the mass spectrometer, thus the mass to charge ratio (m/z) is calculated for each ion. MALDI-MS produces singly charged protonated molecules so the m/z ratio represents the protein molecular weight plus one proton. Each spectrum contains hundreds to thousands of protein peaks with the selected tissue area.

Recently, we have begun to harness this technology in murine wounds to discover biomarkers that distinguish the regenerative phenotype from a typical wound-healing phenotype based on its expected contraction and scarring. We selected the MRL genotype based on considerable regenerative data in the literature (Heber-Katz, 1999; Masinde *et al.*, 2005a, b). We initially created full-thickness excisional wounds on the dorsal surface and punch wounds on the ears of MRL mice and C57BL/6 mice. At various time points after injury, these tissues were submitted for routine histological analysis and immunohistochemical localization. Microscopic

examination indicated that the skin of the MRL mouse was capable of developing hair buds and subsequent hair follicle development within these full-thickness defects (Figure 3a and b). The skin defect was filled in with a cellular composition that was visibly different from the control C57BL/6 mouse that exhibited the typical wound contracture that is a characteristic feature of these loose-skinned animals (data not shown).

We hypothesized that tissue profiling would discover proteins that were differentially expressed between these two mouse models. The box and circle in Figure 4a and b illustrate how specific cell populations can be covered with a tiny volume of matrix. Figure 5 shows a typical spectrum that was obtained using tissue profiling from a healing mouse ear on day 7 of the wound-healing period. Clear differences were noted between the profile for six MRL (regenerative phenotype) mice and six C56BL/6 (control scarring) mice. We noted over 20 proteins that were differentially expressed between mouse phenotypes in these experiments. Preliminary data (Figure 5) reveal two proteins with m/z ratios that correspond to recently touted candidate proteins thymosin β 4 and β 10, which are thought to play pivotal roles in wound repair (Philp *et al.*, 2004; Goldstein *et al.*, 2005). Tissue profiling spectra provide relative quantification data. In this particular experiment, the data allowed us to determine that thymosin β 4 and β 10 were elevated in the ears of control mice compared to the regenerative ear phenotype. These data could be interpreted to mean that either the MRL mice show accelerated repair (which they do) and thymosin molecules were then downregulated by this phase of healing.

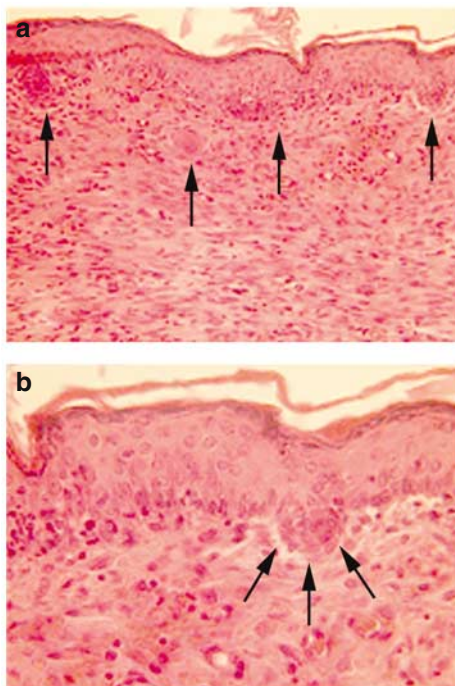


Figure 3. MRL mouse begins to regenerate hair follicles by 7 days after a full-thickness excisional wounding. Arrows indicate nascent hair buds.

Alternatively, the case could be made that the two thymosin β proteins participate in the scarring/contraction phenomenon and not to the same degree in mice with a regenerative phenotype. In summary, our preliminary characterizations of these healing mouse wounds are encouraging and suggest that molecular profiling of proteins in injured tissues may present a new avenue for distinguishing the nuances between tissue repair and cutaneous regeneration. Other protein peaks specific to the control or regenerative mouse phenotype have been detected and are currently being identified as detailed at the end of the following section.

Imaging mass spectrometry

Gene expression can be quite transient and dynamic in healing burn tissues; therefore, we wished to mine proteomic changes as well as to describe for the spatial orientation and abundance of proteins specific to burn tissues. We turned to a proteomic-based technology, imaging mass spectrometry (IMS). IMS takes advantage of MALDI tissue profiling in that a series of high-resolution laser pulses are rastered over a

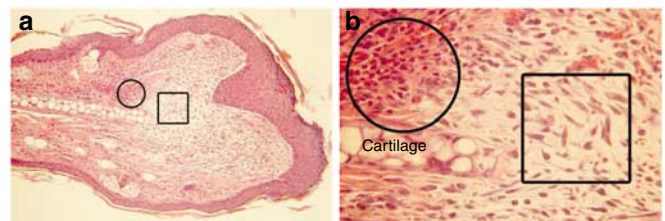


Figure 4. Wound region subjected to MALDI-TOF-MS. (a) This micrograph illustrates the extent of healing/regeneration in a full thickness ear punch in the MRL mouse after 7 days. The surface is completely resurfaced with epithelium while in the dermis distinctive cell populations can be covered with a matrix and subjected to a focal protein analysis by MALDI time of flight MS. (b) The circle illustrates a cluster of mononuclear or possible stem cells that exhibit a distinctly different phenotypic appearance than the mesenchymal type shown in the square box. Hematoxylin and eosin staining.

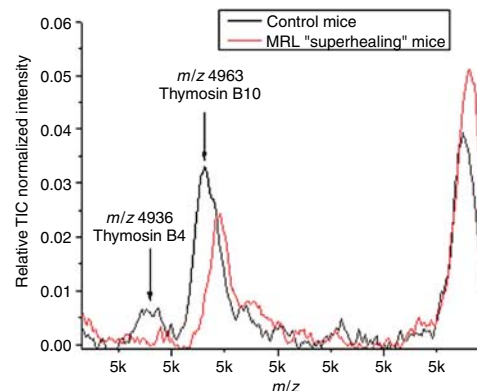


Figure 5. MALDI time of flight MS spectra. Illustrates two candidate proteins with a m/z ratio of 4936 and 4963 that are differentially expressed in the wounded ears of MRL and control mouse at 7 days after a full-thickness excisional ear punch ($N=6$). Both putative forms of thymosin β (4 and 10) are strongly expressed at 7 days in the control mice (C57BL/6) but are expressed to a lesser degree at 7 days in the regenerative MRL mouse wound.

selected region of a tissue section. To obtain IMS data, tissue sections are cut in a cryostat, mounted on a MALDI-compatible glass plate, and matrix is evenly spray-coated over tissue sections. Additionally, serial sections of the tissue are stained with hematoxylin and eosin to identify histological regions of interest (Figure 6b). Thousands of laser spots are fired over the tissue, taking approximately 3 hours to process a 1 cm² piece of tissue depending on the chosen resolution. Each laser spot represents a discrete pixel associated with a mass spectrum. Spectra are collected and plotted in raster fashion, and then compiled to render a two-dimensional representation of the analyte distribution within the tissue (Figure 6a). After all spectra within the tissue section have been acquired and processed, the investigator inspects an “averaged mass spectrum” from the imaged tissue. Each protein peak within the averaged spectrum can be individually highlighted to discover its localization and relative abundance throughout the entire imaged tissue. The results shown in Figure 6c and d represent the distribution of two proteins (or protein fragments) at m/z 3426 and m/z 3439. These images delineate two entirely different distributions during the healing process. Since the first reports nearly 9 years ago, this technique has developed increasing growing popularity and utility (Caprioli *et al.*, 1997; Stoecki *et al.*, 2001; Chaurand *et al.*, 2004a, b; Reyzer and Caprioli, 2005). IMS has been used to generate images depicting the

distribution of dermatological drugs, demonstrating that this technique is capable of producing spatially useful data in skin. In our group, IMS has allowed us to maintain a spatial degree of specificity to conduct proteomic evaluation of human burn wounds. This technology has distinct advantages over other tissue-based experimentation. For example, because proteins are imaged in intact tissues, spatial orientation of proteins is not lost as there is no need for tissue homogenization. Second, IMS permits the investigator to study hundreds to thousands of proteins very rapidly from one IMS experiment. Finally, because the technology is not antibody based, proteins can be studied without the need to purchase or create antibodies.

For both MALDI tissue profiling and IMS, proteins of interest must be identified by crude purification and sequencing. Protein identification normally involves (1) homogenizing the original tissue (~300 mg), (2) crude separation of tissue substructures by gradient centrifugation, (3) separation of proteins within a gradient by HPLC, (4) and separation of HPLC by one-dimensional SDS-PAGE. After staining the gel, (5) the band consistent with the protein molecular weight ($m/z = -1$) of the MALDI analyte is excised from the gel and digested with trypsin. Peptide sequences are then compared to sequence information in national databases and the protein identification can be made.

A number of different, but complimentary, approaches can now be taken with this model. An alternative approach is to perform two-dimensional gel electrophoresis to compare proteomes between two experimental subsets. Briefly, proteins are differentially labeled (i.e. control *versus* burn tissue) with cyanine dyes (i.e. Cy3 and Cy5), mixed, loaded onto an SDS gel and proteins are separated first by hydrophobicity (pI) and then by molecular weight (Da). Differentially-expressed protein “spots” are visualized by exciting the Cy dyes with different wavelengths. The protein spots are excised, digested with trypsin, sequenced, and matched to national databases as described above.

Adenoviral boosting strategy for a prototypic tyrosine kinase receptor

For the past 20 years, the wound-healing field has experienced an increasing number of reports where the study design was based on a simple addition of growth factor. Such studies have produced evidence that growth factors and cytokines do indeed play a role in the reparative process. However, the clinical impact of this plethora of growth factor studies has been lackluster. Recently, we and others have begun to enter into more sophisticated approaches as we pursue the strive to favorably manipulate the wound-healing process. It has become apparent that merely supplying a wound with topical growth factor is perhaps not the only way to approach the problems and questions. In 2000, we moved downstream in our efforts to impact wound repair. We first reported a successful strategy for boosting receptor numbers (Nanney *et al.*, 2000). This was based on our hypothesis that receptor numbers in the case of the ErbB1 receptor tyrosine kinase were likely the rate-limiting factor along the complex epidermal growth factor-mediated signaling

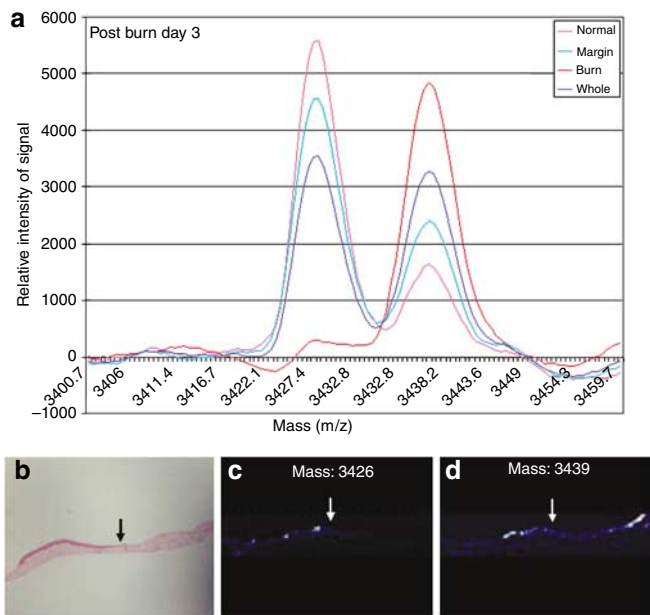


Figure 6. MALDI-IMS was used in a proteomic approach to analyze a human burn wound at day 6 after injury. (a) MALDI-IMS spectrum with the distribution and relative levels of these proteins within the three defined areas (burned area, adjacent burn margin covered with epithelium and the distal “normal” non-burned skin). (b) Hematoxylin- and eosin-stained frozen section from which the spectra were captured. (c) Tissue distributions for a protein with a mass of 3,426 expressed predominantly in the epidermal margin immediately adjacent to the burn region. (d) Tissue distributions for a protein with a mass of 3,439 which is predominantly expressed in the injured area (wound bed and adjacent hypertrophic epidermis) but not the more distal “normal” non-burned epidermis.

pathway (Nanney *et al.*, 2000). In that report, a gene gun transfection of cDNA was utilized. This mode of gene delivery proved effective but limited since this technique inflicted modest additional injury. In the intervening 5 years, the field of wound repair has experienced a resurgence in attempts to discover better therapeutic means to deliver genes and indeed to silence genes with siRNA approaches (Eming *et al.*, 2004; Eriksson and Velander, 2004).

In this review, we show evidence that *in vivo* adenoviral transfection strategies have considerable utility as an experimental system for the manipulation and examination of the biological outcomes associated with the various cytokine signaling pathways. We hypothesized that transient overexpression of various forms of the ErbB (epidermal growth factor (EGF)) receptor could differentially modulate and possibly stimulate wound repair. Herein, we use our study of the role of ErbB4 receptor and its relevant ligands as an example of newer ways to tease out differential signals that are generated when the ErbB receptor is overexpressed and manipulated with various topical ligands. Strategies for intracellular manipulation of signaling pathways downstream of growth factor signals are showing considerable promise. Other investigators have opened the door to adenoviral delivery in the wound-healing setting. Adenoviral gene transfer of an NF-kappaB super-repressor was used to successfully produce a biological impact on collagen and wound repair (Schreiber *et al.*, 2005). Two of the authors in this review (Davidson, Opalenik) manipulated angiogenesis and wound repair by the adenoviral introduction of a protein known as CARP, a transcriptional regulator (Shi *et al.*, 2005). Of course, adenoviral delivery of cytokines themselves continue to show a slightly efficacious impact (Keswani *et al.*, 2004).

Within the EGF signaling pathway, a host of questions remain unanswered. Why is there a seeming redundancy with some many highly similar ligands (EGF, transforming growth factor- α , heparin-binding-EGF, amphiregulin, epiregulin, betacellulin, and others)? Given the multitude of ErbB receptor forms with both homo and heterodimer receptor assemblies, which ones predominate in each biological setting? Can differential responses that are possibly triggered by these ligands, their receptors and the signaling pathways be detected in the *in vivo* setting? Figure 7a-d show the biological response to adenoviral overexpression of this prototypic tyrosine kinase receptor. In addition to the adenoviral transfection strategy with an ErbB receptor, we also coupled our investigation with a porcine wound model. We selected this expensive model since it provides a full complement of epidermal appendages (hair follicles, sweat ducts), and exhibits the same mosaic hair growth pattern that is found in humans. This *in vivo* model has a microvascular (thermoregulatory) architecture similar to human skin. When the ErbB4 gene was transiently transfected immediately after creation of a partial thickness injury and individual wounds were supplied with daily topically delivered ligands, we noted differential differences in the character of both the epidermis and underlying neodermis. The most dramatic effect was evident in the wounds receiving the

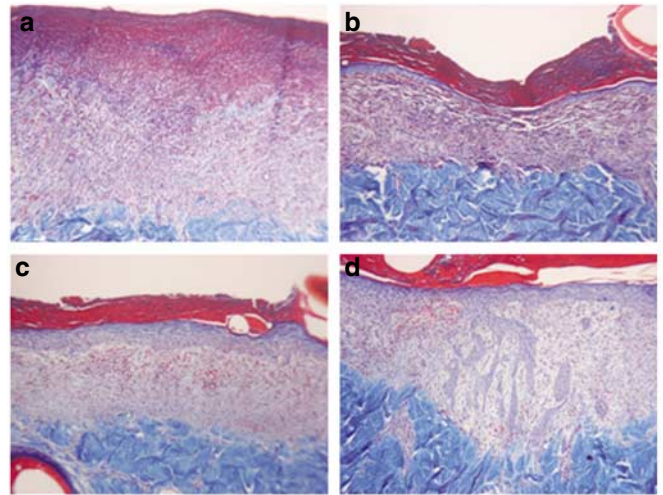


Figure 7. Histological views of porcine partial thickness wounds at 5 days after injury. Wounds were transfected by adenoviral delivery of either ErbB4 and treated topically with EGF family members or by delivery of the LacZ vector without receptor. (a) The LacZ control wound receiving the placebo gel. Granulation tissue is immature and the surface is devoid of epithelium. (b) Poorly stratified epidermis beneath the surface exudate with a minimal neodermis in a wound supplied with ErbB4 + topical EGF. (c) Epidermal resurfacing with moderate stratification over a robust neodermis in a wound supplied with ErbB4 + topical epiregulin. (d) Epidermal resurfacing with moderate stratification and remarkable downward extensions of the epidermis. The neodermis is very thick in wounds supplied with ErbB4 + heparin-binding-EGF. Gomori's Trichrome stain.

heparin-binding-EGF topical treatment on wound where ErbB4 was the predominant receptor (Figure 7d). The streaming phenotype of epithelial cells throughout the height of the dermis was remarkable. The significance of this finding remains unknown at this time but support *in vitro* studies where heparin-binding-EGF serves as a mediator along multiple pathways (Nishi and Klagsbrun, 2004; Shirakata *et al.*, 2005). This experimental overexpression strategy allowed us to provide clear evidence that EGF + ErbB4 has a modest stimulatory impact on epidermal maturity and dermal cellularity (Figure 7b).

Another ligand in the EGF family, epiregulin, appears to have an intermediate impact in these comparative studies (Figure 7c). The epidermis showed a greater degree of differentiation indicating that epiregulin + ErbB4 also has a powerful yet different biological impact on multiple wound-healing processes. The coupling of the porcine *in vivo* model with adenoviral gene delivery of supplemental receptors did confirm our hypothesis that this set of experimental circumstances would cause subtle differential responses that might enhance particular aspects of repair. Our studies support growing evidence that ErbB4 and the other receptor forms can evoke differential signaling in a variety of cells types. A recent report with another population of cells in the skin indicated that ErbB4 differentially mediates either migratory or proliferative activities (Gordon-Thomson *et al.*, 2005). In that study, tumor cells exhibited differential signaling suggesting multiple switches in ErbB signaling pathways via

EGFR/ErbB heterodimer formation. Different ligands either did or did not evoke a migratory phenotype depending on the types of receptor forms that were present in melanocytes and melanoma cells (Gordon-Thomson *et al.*, 2005). Our own studies with keratinocytes and fibroblasts *in vivo* and *in vitro* have shown that epiregulin is 10× more potent than EGF (Draper *et al.*, 2003b) in part due to its ability to signal through the extracellular signal-regulated kinase/mitogen-activated protein kinase pathway (Draper *et al.*, 2003a). The ErbB4 receptor remains rather unique within this small family of receptor tyrosine kinases family in that following binding with its ligand, dimerization, and phosphorylation, a fragment is cleaved and translocates to the nucleus where it is presumed to serve as a transcription factor (Carpenter, 2003). The present wound-healing model allowed us to derive convincing evidence for biological activity in the *in vivo* setting. These data also supply evidence that augmentation of ErbB4 receptors when coupled with certain EGF ligands holds therapeutic promise to hasten wound maturity. The data indicate that the use of the porcine model has the degree of sensitivity that makes it a suitable choice that is appropriate for confirmation of differential *in vivo* responses to similar ligands within the EGF family. We continue to believe that the field of wound healing will increasingly be advanced by creative *in vivo* manipulations of signal transduction pathways.

CONCLUSION

Regeneration of skin following serious burn or ablative injury has been a worthy but elusive goal for decades. It is our supposition that the tools and foundational information to address this intractable problem are presently at our fingertips. We suspect that investigators will continue to be captivated by their own particular molecule/pathway of interest but will increasingly employ techniques that allow them to pursue readouts with spatial information. "Location, location, location" a well-known phrase in the real-estate world, may become a frequently used phrase by cutaneous investigators. In wound repair, eventual outcomes are undoubtedly driven by multiple molecules of interest, but we are now poised to merge sophisticated tools from both camps that will allow the exploration of molecules within a discrete spatial context.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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