SPECIAL FOCUS: ANIMAL MODELS IN TISSUE ENGINEERING. PART I*

Porcine Ischemic Wound-Healing Model for Preclinical Testing of Degradable Biomaterials

Prarthana Patil, MS,^{1,**} John R. Martin, PhD,^{1,**} Samantha M. Sarett, PhD,¹ Alonda C. Pollins, MLI,² Nancy L. Cardwell, BS,² Jeffrey M. Davidson, PhD,³ Scott A. Guelcher, PhD,^{1,4,5} Lillian B. Nanney, PhD,² and Craig L. Duvall, PhD¹

Impaired wound healing that mimics chronic human skin pathologies is difficult to achieve in current animal models, hindering testing and development of new therapeutic biomaterials that promote wound healing. In this article, we describe a refinement and simplification of the porcine ischemic wound model that increases the size and number of experimental sites per animal. By comparing three flap geometries, we adopted a superior configuration (15 × 10 cm) that enabled testing of twenty 1 cm² wounds in each animal: 8 total ischemic wounds within 4 bipedicle flaps and 12 nonischemic wounds. The ischemic wounds exhibited impaired skin perfusion for ~1 week. To demonstrate the utility of the model for comparative testing of tissue regenerative biomaterials, we evaluated the healing process in wounds implanted with highly porous poly (thioketal) urethane (PTK-UR) scaffolds that were fabricated through reaction of reactive oxygen species (ROS)-cleavable PTK macrodiols with isocyanates. PTK-lysine triisocyanate (LTI) scaffolds degraded significantly *in vitro* under both oxidative and hydrolytic conditions whereas PTK-hexamethylene diisocyanate trimer (HDIt) scaffolds were resistant to hydrolytic breakdown and degraded exclusively through an ROS-dependent mechanism. Upon placement into porcine wounds, both types of PTK-UR materials fostered new tissue ingrowth over 10 days in both ischemic and nonischemic tissue. However, wound perfusion, tissue infiltration and the abundance of pro-regenerative, M2-polarized macrophages were markedly lower in ischemic wounds independent of scaffold type. The PTK-LTI implants significantly improved tissue infiltration and perfusion compared with analogous PTK-HDIt scaffolds in ischemic wounds. Both LTI and HDIt-based PTK-UR implants enhanced M2 macrophage activity, and these cells were selectively localized at the scaffold/tissue interface. In sum, this modified porcine wound-healing model decreased animal usage, simplified procedures, and permitted a more robust evaluation of tissue engineering materials in preclinical wound healing research. Deployment of the model for a relevant biomaterial comparison yielded results that support the use of the PTK-LTI over the PTK-HDIt scaffold formulation for future advanced therapeutic studies.

Keywords: animal model, ischemia, bipedicle flap, reactive oxygen species, poly (thioketal), porcine

Introduction

CHRONIC SKIN WOUNDS cause significant patient morbidity and a substantial, expanding financial burden on global healthcare systems.¹ Unlike acute cutaneous injuries, chronic wounds are often characterized by diminished vascularization.² Such ischemic conditions limit the supply of oxygen, nutrients, immune cells, and other important factors

carried by the blood into the wound site, while prolonging the inflammatory phase and increasing levels of damaging reactive oxygen species (ROS).^{3,4} Although a multitude of treatments have emerged for treating impaired and chronic cutaneous wounds,^{5–9} current clinically approved therapies are expensive, labor-intensive, and lackluster in terms of improving outcomes since they frequently fail to accelerate or complete wound closure in patients.¹⁰

¹Department of Biomedical Engineering, Vanderbilt University, Nashville, Tennessee.

²Department of Plastic Surgery, Vanderbilt University Medical Center, Nashville, Tennessee.

³Department of Pathology Microbiology and Immunology, Vanderbilt University Medical Center, Nashville, Tennessee.

⁴Department of Chemical and Biomolecular Engineering, Vanderbilt University, Nashville, Tennessee.

⁵Department of Medicine and Division of Clinical Pharmacology, Vanderbilt Center for Bone Biology, Vanderbilt University School of Medicine, Nashville, Tennessee.

**These two authors contributed equally to this work.

*This article is part of a special focus issue on Animal Models in Tissue Engineering. Part I.

Efforts to develop improved therapies for patients with chronic wounds are hampered by the lack of animal models that accurately recapitulate the clinical manifestations of human chronic skin injuries or allow for high-throughput comprehensive testing.¹¹ Porcine models are considered the most relevant preclinical approach due to the pig's anatomical and physiological similarities with humans, featuring a relatively thick epidermis, sparse hair, extensive thermoregulatory vasculature, and dermal viscoelasticity.¹² Highlighting this point, a study by Sullivan *et al.* concluded that 78% of evaluated pig wound models were concordant with human studies, compared to only 53% for rodent models,¹³ and the Wound Healing Society has designated the pig as the most relevant preclinical model for human translational value.¹⁴ Recently, Roy *et al.* created an ischemic skin model in pigs to study delayed wound healing¹⁵ and used this model to document enhanced angiogenesis in ischemic wounds after treatment with a modified collagen gel.¹⁶ However, technical challenges posed by the small wound dimensions (8 mm punch biopsy) and low numbers of ischemic wounds (four per animal) limit the practicality of the model, particularly for biomaterials testing.

The work presented in this study refines the surgical procedure and increases the size and number of ischemic test sites per animal, thereby decreasing animal usage and increasing statistical robustness. To evaluate the characteristics of the model, we implanted two cell-degradable scaffold formulations into ischemic and nonischemic wounds and assessed healing outcomes. Our previous work has demonstrated the enhanced cutaneous healing achieved with porous, biodegradable poly (ester urethane) (PEUR) scaffolds embedded into acute excisional pig wounds.¹⁷ In this study, we used two formulations of recently developed, porous poly (thioketal) urethane (PTK-UR) scaffolds that are degraded by cell-generated ROS. These designs couple polymer degradation to new tissue growth and consequently encourage more robust tissue formation than PEUR implants⁸ while undergoing more controlled *in vivo* degradation.¹⁸ The two PTK-UR scaffold formulations, made with PTK diols in combination with either hydrolysable lysine triisocyanate (LTI) or nonhydrolysable hexamethylene diisocyanate trimer (HDI_t), were compared for scaffold-mediated healing responses in excisional wounds in our optimized porcine ischemic model.

Materials and Methods

Materials

2-mercaptoethyl-ether (MEE) was purchased from Tokyo Chemical Industry Co. (Tokyo, Japan). Tertiary amine catalyst (TEGOAMIN33) was obtained from Goldschmidt (VA). HDI_t was received as a gift from Bayer Material Science. LTI was acquired from Kyowa Hakko USA. All other reagents were obtained from Sigma-Aldrich (Milwaukee, WI).

PTK-UR scaffold synthesis and *in vitro* degradation

As described previously,¹⁸ PTK diol was synthesized with a condensation polymerization in acetonitrile at 80°C using MEE, 2,2-dimethoxypropanone (DMP), and *p*-toluenesulfonic acid catalyst. The generated thiol-terminated PTK polymer was then functionalized with hydroxyl groups via reaction with 2-bromoethanol and cesium carbonate to generate polyols for suitable reaction with isocyanates. The PTK-UR scaffolds

were fabricated using liquid reactive molding of the PTK diol (1100 Da) with water (creates CO₂ bubbles for foaming), TEGOAMIN33 (foaming and gelling catalyst), and either LTI or HDI_t isocyanate. *In vitro* PTK-UR scaffold degradation was assessed at days 0, 1, 3, 5, 10, 19, and 30 by incubating 10 mg scaffold pieces in accelerated hydrolytic conditions (phosphate-buffered saline at 77°C), high oxidative conditions (20 wt% H₂O₂ in 0.1 M CoCl₂ at 37°C), or low oxidative conditions (2 wt% H₂O₂ in 0.01 M CoCl₂ at 37°C). At predetermined time points, *n* = 3 scaffold samples were removed from the incubation media, rinsed with H₂O, lyophilized, and weighed to determine mass loss.

Porcine ischemic flap and excisional wounds

All surgical procedures were reviewed and approved by the Vanderbilt University Institutional Animal Care and Use Committee. Four adolescent female Yorkshire pigs, ~12 weeks of age (32 kg) were preanesthetized with telazol, ketamine, and xylazine and maintained under anesthesia with isoflurane during surgery. Shaved dorsal skin was prepared by cleaning with 70% ethanol, chlorohexidine, and betadine wash. Four pairs of full-thickness, longitudinal incisions parallel to the spine were made with cautery under sterile conditions, and the skin was lifted from the underlying fascia to create bipedicle cutaneous flaps that were reapproximated with staples (Fig. 3A, B).

During model optimization, a sterilized, medical grade silicone sheeting (0.005", Sil-Tec; Technical Products, Inc., GA) was placed underneath the bipedicle flaps to prevent premature reperfusion and reattachment as previously described.¹⁵ The initial pig received two 15 × 7.5 cm bipedicle flaps (one cephalic, one caudal), a cephalic 15 × 10 cm flap, and a caudal 15 × 15 cm flap. Full-thickness 1 × 1 cm² excisional wounds, down to—but not extending through—the subcutaneous fat layer, were produced within the most ischemic tissue—the middle one-third within each flap. A single 1 × 1 cm full-thickness wound was placed in the 7.5 cm wide flaps while two side-by-side wounds were placed in the 10 and 15 cm wide flaps as depicted in Figure 1A.

In three additional pigs, paravertebral longitudinal incisions were created on the dorsal region, and four (15 × 10 cm) bipedicle skin flaps were raised. The two cephalic and two caudal flaps on each side of the spine were placed to create a flap in each "quadrant" of the dorsal skin as shown in Figure 3A and B. Two laterally symmetrical, full-thickness 1 × 1 cm² excisional wounds were incised in each ischemic flap, while three nonischemic wounds were created adjacent to each flap. This wounding placement and scheme resulted in a total of 8 flap (ischemic) wounds and 12 nonischemic wounds per pig (schematic in Fig. 3A).

PTK-UR scaffolds trimmed to 0.2 × 1 × 1 cm pieces were sterilized by ethylene oxide before being implanted into the wound voids after hemostasis was achieved. In each of the four test quadrants, a single LTI and HDI_t scaffold was placed in both an ischemic and a nonischemic wound site, and one nonischemic wound was devoid of a scaffold. Treatment locations were randomized between the cephalic or caudal location at the time of application to avoid any location specific bias. All wounds were cushioned and protected by a series of dressings as follows: (1) Mepilex Transfer™ (Molnlycke Healthcare, Sweden), (2) Mextra™ absorbent pad

(Molnlycke), (3) Opsite™ adhesive (Smith&Nephew, St. Petersburg, FL) before being secured with (4) MediChoice™ Tubular Net Bandage (Owens & Minor, Mechanicsville, VA), and (5) Vetwrap™ (3M, St. Paul, MN) bandaging.

Following the surgical procedure, dressings were changed every 2–3 days. Antibiotic prophylaxis (Excede™; Zoetis) was begun during the presurgical skin preparation period and was administered weekly. Buprenex was given immediately postsurgery and analgesia was maintained over the course of the experiment using a transdermal Fentanyl patch (50 mcg/h) applied at the time of surgery and changed every 2–3 days. Pigs were euthanized at 10 days postsurgery, and full-thickness wound samples (including adjacent wound margins) were collected for histology, immunohistochemistry, and subsequent quantitative morphometric analysis.

Laser Doppler perfusion imaging

A laser Doppler imager (moorLDI2-HIR; Moor Instruments, Wilmington, DE) was used to confirm the creation of ischemic skin conditions and to track the resolution of ischemia over time. Blood perfusion images of ischemic and non-ischemic skin were obtained after scaffold implantation and subsequently during dressing changes at days 1, 3, 6, 8, and 10 postsurgery. Skin perfusion in a 1.5×1.5 cm region of interest around each wound was quantified using Moor analysis software, and the total signal flux values for both ischemic and nonischemic wounds were normalized to an untreated skin region ($n=8$ wounds in two independent animals).

Histology

As previously described,¹⁷ wound tissue samples were excised from the euthanized animal, fixed in formalin, embedded in paraffin, and sectioned at 5 μ m. Tissue sections were stained with Gomori's trichrome and used to quantify tissue infiltration by calculating percent area of granulation tissue in the total scaffold wound area ($n=4$ /scaffold group). For immunohistochemical (IHC) evaluation, blood vessels were identified using rabbit anti-von Willebrand factor (VWF)¹⁹ (Cat No. A0082, 1:8000; Dako, Carpinteria, CA), M2 phenotype macrophages were identified using rabbit anti-liver Arginase-1^{20,21} (ab183333, 1:8000; Abcam, Cambridge, United Kingdom), and M1 macrophages were identified with rabbit anti-CCR7^{21–23} (Cat No. ab32527, 1:2500; Abcam).

Histology quantification

The percentage of blood vessel staining per area was quantified from VWF IHC images of the interface of the scaffold and the wound bed using ImagePro Plus 7.0 software. Before quantification of pixel-positive areas, the brown, positive immunoreactivity for diaminobenzidine (DAB) was pre-defined. A minimum of three fields of view per wound ($n=3$ wounds/scaffold group) were collected in a blinded manner and then quantified. Sections that had been immunostained with anti-Arginase-1 or anti-CCR7 were scanned via Leica SCN 400 Slide Scanner at 10 $\times$ and 20 $\times$ magnifications and analyzed using the web-based digital slide-viewing environment Digital Image Hub (Leica Technologies). The numbers of positively stained cells were counted and normalized to the total number of nuclei that were identified with hematoxylin. Quantification was performed for a minimum of three fields

of view per wound ($n=3$ wounds/scaffold group) and expressed as a ratio of cells expressing Arginase-1 (M2 macrophages) to cells expressing CCR7 (M1 macrophages).

Spatial quantification of staining intensity for Arginase-1 and CCR7 was performed by color deconvolution of DAB (brown) staining using the color deconvolution plug-in on ImageJ software. After deconvolution of the color, a 200 μ m perpendicular line was drawn from the edge of the scaffold, and a plot intensity profile (expressed as inverse gray scale; 255–0) was generated. Quantified data are expressed as a ratio of staining intensity for cells expressing Arginase-1 (M2) to cells expressing CCR7 (M1).

Statistics

One-way analysis of variance (ANOVA) was used to evaluate the statistical difference between treatment groups at day 10. Three-way ANOVA was used to elicit significance between the four groups at different time points (blood perfusion analysis).

Statistical significance was defined by p -values <0.05 . Graphical data are presented as the mean $\pm$ standard error.

Results

Double-wound bipedicle skin flap model in pigs

As an initial optimization, four raised bipedicle flaps with underlying silicone sheeting were created in an adolescent female pig (Fig. 1A). Three differing flap geometries, 15×7.5 , 15×10 , and 15×15 cm (with flap width as the variable) were tested. Two 1×1 cm wounds were created in the 10 and 15 cm width flaps, while only a single 1×1 cm wound was created in the 7.5 cm flaps to reflect the geometry of a recent porcine ischemic model.¹⁵ Ischemia was evident in these flaps based on the dark and dusky central coloration in the middle of the skin flap at the conclusion of the surgical period (Fig. 1A).

Laser Doppler perfusion imaging (LDPI) flow mapping at 24 h postsurgery quantified reduced blood perfusion in both 10 and 7.5 cm width flaps, while perfusion in the 15 cm wide flap was minimally impaired (Fig. 1B). Normal perfusion was re-established by day 10 for all flap geometries (Fig. 1B), a finding consistent with the flap delay phenomenon in the established plastic surgery literature.²⁴ Although the staples on the dependent side of the cutaneous flap were purposely not approximated closely to allow for wound fluid drainage, there was substantial fluid buildup (seroma) under the skin flap, resulting in dehiscence along the incisional line (Fig. 1C). Consequently, all subsequent studies eliminated the silicone sheeting step and used 15×10 cm raised bipedicle flaps with two ischemic wounds per flap.

In vitro scaffold degradation

Porous PTK-UR scaffolds were fabricated through reactive liquid molding using the PTK diol with either HDIt or LTI (Fig. 2A), yielding a covalent network of urethane bonds after the reaction of terminal diols and isocyanates from the respective precursors. Under accelerated hydrolytic conditions, LTI scaffolds were completely degraded by 19 days, while HDIt scaffolds were resistant to hydrolysis for at least 30 days. Both scaffold formulations degraded when incubated in oxidative solutions of 2% or 20% H_2O_2 . Both material varieties also displayed dose-dependent degradation kinetics with respect to ROS concentration, and

FIG. 1. Optimization of the bipedicle flap dimensions and implementation. (A) Variable bipedicle flap dimensions (15×10, 15×7.5, and 15×15 cm) with one or two wounds per flap. The dusky coloration of skin in the central portion of each flap indicates localized ischemia. (B) Appearance and perfusion in day 1 and 10 wounds as a function of flap width. Laser Doppler (LDPI) images at day 1 show impaired perfusion. Flow was reestablished by day 10 in ischemic flaps of all dimensions tested. (C) Image demonstrating problematic fluid buildup under wounds, in which silicone sheeting was placed as a barrier between the skin and the underlying muscle. LDPI, Laser Doppler perfusion imaging. Color images available online at www.liebertpub.com/tec

LTI samples degraded significantly faster than HDIt scaffolds in both oxidative solutions (Fig. 2B).

In vivo scaffold implantation and tissue infiltration

Three female Yorkshire pigs were used. Four bipedicle cutaneous flaps were created on each pig to yield 8 ischemic

and 12 nonischemic wounds per animal (Fig. 3A). Both ischemic and nonischemic wounds were implanted with either LTI or HDIt-based PTK-UR scaffolds, and four additional nonischemic wounds were left without scaffold (Fig. 3B). At 10 days, untreated wounds underwent extensive contraction with minimal granulation tissue formation evident from histology (Fig. 3C). By contrast, implanted

FIG. 2. PTK-UR scaffolds are tunable to exhibit selective degradation in oxidative media (HDIt) or degradation through a combination of hydrolytic and oxidative mechanisms (LTI). (A) The poly (thioketal) diol polymer was synthesized and then used to form PTK-URs through reaction with the LTI or HDIt compounds, each of which contains three isocyanate (N=C=O) functional groups that react with OH bifunctional groups of PTK. (B) *In vitro* degradation of PTK-LTI and PTK-HDIt scaffolds, expressed as % degradation versus time. The HDIt-based materials were selectively ROS degradable (H₂O₂). The LTI-based scaffolds were more susceptible to oxidative degradation and were also susceptible to hydrolytic breakdown (PBS, 77°C). HDIt, hexamethylene diisocyanate trimer; LTI, lysine triisocyanate; PBS, phosphate-buffered saline; PTK-UR, poly (thioketal) urethane; ROS, reactive oxygen species. Color images available online at www.liebertpub.com/tec

FIG. 3. Bipedicle wound model shows delayed biomaterial tissue infiltration in ischemic relative to nonischemic wounds, and ischemic wounds are more sensitive to detecting materials differences in tissue infiltration than nonischemic wounds. **(A)** Schematic of the bipedicle flap design. Red arrows point to areas of restricted blood flow in the center of each flap. Ischemic wounds, black; nonischemic wounds, white. **(B)** Image at day 0 showing the arrangement of scaffold-implanted ischemic and nonischemic wounds. **(C)** Histological illustration of untreated empty wound, trichrome stain. **(D)** Representative images of trichrome staining showing scaffold degradation and tissue infiltration in all four treatment groups. **(E)** Quantification of tissue infiltration into scaffolds at day 10 showing decreased tissue infiltration in both ischemic wound scaffold groups and improved infiltration of LTI-based scaffolds over HDIt-based scaffolds in the ischemic wounds (mean ± SEM, $n = 4$ wounds, $*p < 0.05$). Color images available online at www.liebertpub.com/tec

scaffolds were integrated into the wounds and minimized contraction through physical stenting (Fig. 3D). The scaffolds in nonischemic wounds exhibited significantly more tissue infiltration than ischemic scaffolds at the 10-day time point, while there was no significant difference in granulation tissue infiltration between the two scaffold types in nonischemic wounds (Fig. 3E). In ischemic wounds, LTI implants were significantly more infiltrated than HDIt scaffolds (Fig. 3E).

Skin perfusion and blood vessel quantification

Blood perfusion in the ischemic and nonischemic wounds was quantified following surgery by LDPI (Fig. 4A). At early time points, the ischemic wounds were significantly less perfused than nonischemic wounds (gray highlight, Fig. 4B), and ischemic wounds began to reperfuse by day 5–7 post-surgery. By day 8, ischemic LTI wounds showed significantly higher perfusion compared with HDIt wounds, and nonischemic LTI scaffolds also displayed greater perfusion than analogous HDIt implants, although differences were not statistically significant (Fig. 4B). VWF IHC staining was used to visualize neovascularization in

granulation tissue (Fig. 4C). LTI scaffold treatments in both nonischemic and ischemic regions showed slightly greater vessel density compared with HDIt, but these differences were subtle and not statistically significant (Fig. 4D).

Macrophage recruitment

The IHC markers CCR7 (M1 phenotype) and Arginase-1 (M2 phenotype) identified macrophage presence and phenotypic polarization in day 10 tissues (Fig. 5A). Nonischemic scaffolds were associated with a higher number of M2 macrophages compared with ischemic scaffolds, while M1 macrophage frequency was similar across all four treatment groups (data not shown). These trends dictated a higher ratio of M2/M1 macrophages in nonischemic scaffold-filled wounds compared with the ischemic areas (Fig. 5B), although there were no differences between the two scaffold types in either ischemic or nonischemic wounds. The M2/M1 ratio also decreased in granulation tissue as a function of distance from the edge of the scaffold (Fig. 5A, C). The Arginase-1 (M2) staining was more focal in close proximity to scaffold remnants, while the CCR7 (M1)

FIG. 4. Ischemic flaps hinder wound blood flow and implanted scaffolds trigger a robust vascular response over 10 days postinjury. **(A)** LDPI images of HDIt and LTI polymers show increased and impaired blood perfusion in nonischemic and ischemic wounds, respectively. The corresponding quantified perfusion data **(B)** show reestablishment of blood perfusion in ischemic wounds over ~ 1 week. LTI-based scaffolds also induce higher wound perfusion relative to HDIt-based scaffolds in the ischemic flap wounds (mean $\pm$ SEM, $n = 8$ [two independent animals], $*p < 0.05$ [day 8] and $\#p < 0.05$ [day 10]). **(C)** Representative images ($20\times$) showing blood vessels (stained with VWF anti-sera) at day 10 after scaffold implantation and corresponding quantification of positively stained pixels. **(D)** (mean $\pm$ SEM, $n = 3$). These data suggest robust vascular response in both materials and that LTI-based materials have a trend for higher vascular density than HDIt-based materials when placed into ischemic wounds. S, scaffold fragments; VWF, von Willebrand factor. Color images available online at www.liebertpub.com/tec

staining was relatively evenly dispersed throughout the tissue without any strong association with the scaffold (Fig. 5A).

Discussion

Due to increasing geriatric and diabetic populations, the economic burden from patients with chronic wounds is rapidly expanding. To facilitate the testing of new therapies in a highly predictive preclinical setting, there has been a renewed effort to develop impaired porcine wound models that resemble the poorly perfused and impaired healing conditions commonly present in patients.²⁵ Roy *et al.* recently developed a porcine model of chronic ischemic skin wounds using bipedicle flaps. These wounds were created on the dorsal surface of the pigs, and they included separating the skin from the underlying fascia with silicone sheeting to prevent revascularization from underlying capillary sources.^{15,26} However, we found that this model was limited by production of seroma (Fig. 1C), insufficient number of wound sites per animal, and insufficient yield of tissue for analysis per sample due to the small wound dimensions.

We directed our efforts at developing an improved porcine wound-healing model to evaluate the performance of PTK-UR scaffolds in a chronic wound environment, while increasing wound dimensions and reducing complications associated

with fluid buildup under an interposed silicone sheet. Based on previous rat cutaneous flap models^{27–29} and knowledge that the ratio of flap width to length has a predictable impact on the degree of skin ischemia that can be tolerated in bipedicle flaps,^{26,30} we made a simple increase in flap width. Our adjustment in the configuration of the dimensions of the flap allowed us to double the number of wounds per flap, maintain extended ischemia, and avoid overt and irreversible flap necrosis. These modifications increased the number of ischemic wounds that could be tested on a single pig, thus improving experimental efficiency and enhancing statistical power with additional, internal control samples.¹¹ In accordance with appropriate animal reduction principles in research,³¹ this strategy could potentially reduce the number of animals required for a study.

Although a silicone sheeting underlay has been reported to significantly decrease cutaneous blood flow in rats,²⁹ its use in the porcine model also impedes the efflux of interstitial fluid, necessitating the removal of trapped liquid underneath the skin flap as previously described.^{15,16} By eliminating the silicone sheeting in our model, we avoided these surgical limitations that complicate its implementation for testing of therapeutic biomaterials. In addition, our protocol doubled the surface area of each wound from a circular 8 mm punch biopsy (50.24 mm^2) to a $10 \times 10 \text{ mm}^2$

FIG. 5. Wound ischemia reduces M2 macrophage presence at day 10, and PTK scaffolds are associated with an M2 response at the cell-material interface. (A) Immunohistochemical identification of M1 (CCR7⁺) and M2 (Arginase-1⁺) macrophage recruitment (brown) to the infiltrated scaffolds (white). (B) Quantification of M2/M1 ratio in ischemic and nonischemic wounds demonstrates that the ischemic wounds have a delayed or ineffective transition to a more M2-dominated macrophage phenotype within the wound environment. (mean $\pm$ SEM, $n=3$, $*p<0.05$). (C) A higher M2/M1 ratio was associated with the material surface relative to within granulation tissue filling the material pores (mean $\pm$ SEM, $n=4$). Color images available online at www.liebertpub.com/tec

(100 mm²) (Fig. 3B)—increasing the volume of granulation tissue, extending the healing time, and permitting easier allocation of tissue to both histological and biomolecular analysis. Taken together, these improvements improve the data yield per pig while reducing animal usage and cost.

While evaluating the performance of this model with novel biomaterials, we were able to define multiple differences between ischemic and nonischemic wounds. The diminished tissue infiltration in ischemic compared with nonischemic implants at day 10 showed that our optimized flap model limits the infiltration and/or growth of cells at the wound site. These results were supported by noninvasive LDPI analysis of blood flow, where ischemic wounds had significantly less perfusion than nonischemic wounds for 5–7 days.

However, the duration of ischemic in the widely used flap model does not fully reflect the ischemic conditions that prevail in the more severe human pressure injuries or the diabetic state, although it is certainly useful for evaluating the angiogenic response. Despite the lack of chronic ischemic conditions in our model, acute ischemia allows us to study the early cellular response to wounds with impaired perfusion. This feature will permit the design and testing of biomaterials to augment compromised wound healing in a model with skin characteristic very similar to humans. In support of the utility of the model, our studies with PTK-based scaffolds confirmed significant differences in the healing conditions between surgically induced ischemic and nonischemic porcine skin.

Our previous reports have demonstrated enhanced tissue repair in rat wounds with PTK-UR scaffolds.^{8,18} However, the performance of LTI or HDIIt-based PTK-UR implants has not been directly compared in more clinically relevant porcine wounds. As expected from previous data,^{18,32} LTI scaffolds were sensitive to both hydrolysis and oxidation *in vitro*, while HDIIt scaffolds are only sensitive to oxidative degradation of the PTK component (Fig. 2B). In the present impaired healing model, we showed that LTI scaffolds encouraged significantly more tissue infiltration than the HDIIt counterpart in ischemic wounds; this difference in biomaterial performance was not detectable in the control, nonischemic wound sites, suggesting that testing in ischemic flap wounds provides better sensitivity for measuring differences in biomaterial performance. It is also notable that these two PTK-UR scaffolds performed similarly in rat wounds^{8,18} further suggest that cellular responses can be more effectively distinguished in the pig ischemic wound model relative to alternative approaches. Importantly, the enhanced tissue infiltration in ischemic LTI scaffolds was correlated with significantly improved perfusion relative to ischemic HDIIt scaffolds (Fig. 4B). Together, these data favor the usage of LTI-based PTK-UR scaffolds in future studies while more importantly demonstrating the utility of this impaired wound-healing model to distinguish differential responses in restorative therapeutic treatments.

The importance of macrophages in normal wound healing and the contribution of proinflammatory macrophage

persistence to formation of chronic wounds are both widely recognized.³³ In normal healing processes, macrophages are polarized toward an anti-infection, proinflammatory M1 phenotype in the few days following injury, followed by the transition toward a more restorative, M2 phenotype.³⁴ Wounds with macrophages that are stalled in the M1 phase are exposed to an excess of inflammatory cytokines, which can significantly compromise the wound-healing process and cause chronic wound persistence.³⁵ Previous reports with ischemic skin flaps in pigs have demonstrated elevated levels of M1-associated proinflammatory markers,¹⁵ while biomaterials that promote macrophage polarization toward an M2 phenotype have shown promise in enhancing wound restoration.³⁶

In our modified ischemic model, the day 10 histological samples from PTK-UR scaffold implants displayed robust signals for both CCR7 and Arginase-1, respective markers for M1 and M2 macrophages. Notably, the ratio of M2/M1 positive cells was significantly higher in nonischemic wounds compared with ischemic, although the scaffold formulation did not significantly impact macrophage presence or polarization (Fig. 5B). Coupled with the spatial organization patterns, these data suggest that M1 macrophages are broadly present in both ischemic and nonischemic tissues, while M2 macrophages are significantly reduced in the ischemic wounds. Based on the idea that M2 cells should replace M1 cells as wound healing progresses, these data confirm that the ischemic wounds are delayed or aberrant in their healing program relative to the nonischemic injuries. The M2 cell population was primarily associated directly with the scaffold surface (Fig. 5C). This distribution demonstrates that the implanted PTK-UR materials likely promote localized M2 polarization, which could enhance the scaffold-mediated healing response. The observed macrophage polarization pattern further validates this optimized porcine model as an impaired preclinical modality and also suggests that PTK-UR scaffolds support enhanced M2 macrophage activity.

Conclusions

The porcine ischemic flap model developed in this study focused on streamlining the surgical procedure by eliminating the interfacing silicone sheeting and increasing the number and dimensions of excisional wounds for testing of our degradable scaffold biomaterials. The model maintained tissue ischemia for a 1-week period with impaired wound healing in ischemic flaps in the absence of seroma. The model revealed the favorable capabilities of our novel LTI-based PTK-UR scaffolds in this challenged wound environment, promoting higher tissue infiltration, increased blood perfusion, and recruitment of proremodeling M2 macrophages. Overall, this new model is a robust, clinically relevant platform for drug and material testing.

Acknowledgments

Funding was provided by the National Institutes of Health (NIH) through R01EB019409 and the Department of Veterans Affairs. The authors acknowledge the Translational Pathology Shared Resource supported by National Cancer Institute (NCI)/NIH Cancer Center Support Grant 2P30 CA068485-14 and the Vanderbilt Mouse Metabolic Phenotyping Center Grant 5U24DK059637-13. The authors

confirm that there are no known conflicts of interest associated with this publication and that there has been no significant financial support for these efforts that could have influenced their outcome.

Disclosure Statement

No competing financial interests exist.

References

1. Sen, C.K., Gordillo, G.M., Roy, S., Kirsner, R., Lambert, L., Hunt, T.K., Gottrup, F., Gurtner, G.C., and Longaker, M.T. Human skin wounds: a major and snowballing threat to public health and the economy. *Wound Repair Regen* **17**, 763, 2009.
2. Catrina, S.-B., and Zheng, X. Disturbed hypoxic responses as a pathogenic mechanism of diabetic foot ulcers. *Diabetes Metab Res Rev* **32**, 179 2016.
3. Arya, A.K., Pokharia, D., and Tripathi, K. Relationship between oxidative stress and apoptotic markers in lymphocytes of diabetic patients with chronic non healing wound. *Diabetes Res Clin Pract* **94**, 377, 2011.
4. Dunnill, C., Patton, T., Brennan, J., Barrett, J., Dryden, M., Cooke, J., Leaper, D., and Georgopoulos, N.T. Reactive oxygen species (ROS) and wound healing: the functional role of ROS and emerging ROS-modulating technologies for augmentation of the healing process. *Int Wound J* **14**, 89, 2017.
5. Nelson, C.E., Kim, A.J., Adolph, E.J., Gupta, M.K., Yu, F., Hocking, K.M., Davidson, J.M., Guelcher, S.A., and Duvall, C.L. Tunable delivery of sirna from a biodegradable scaffold to promote angiogenesis in vivo. *Adv Mater* **26**, 607, 2014.
6. Golebiewska, E.M., and Poole, A.W. Platelet secretion: from haemostasis to wound healing and beyond. *Blood Rev* **29**, 153, 2015.
7. Castleberry, S.A., Almquist, B.D., Li, W., Reis, T., Chow, J., Mayner, S., and Hammond, P.T. Self-assembled wound dressings silence MMP-9 and improve diabetic wound healing in vivo. *Adv Mater* **28**, 1809, 2016.
8. Martin, J.R., Nelson, C.E., Gupta, M.K., Yu, F., Sarett, S.M., Hocking, K.M., Pollins, A.C., Nanney, L.B., Davidson, J.M., Guelcher, S.A., and Duvall, C.L. Local delivery of PHD2 siRNA from ROS-degradable scaffolds to promote diabetic wound healing. *Adv Healthc Mater* **5**, 2751, 2016.
9. Pop, M.A., and Almquist, B.D. Biomaterials: a potential pathway to healing chronic wounds? *Exp Dermatol* **1**, 2017.
10. Senet, P., Vicaut, E., Beneton, N., Debure, C., Lok, C., and Chosidow, O. Topical treatment of hypertensive leg ulcers With platelet-derived growth factor-BB: a randomized controlled trial. *Arch Dermatol* **147**, 926, 2011.
11. Nunan, R., Harding, K.G., and Martin, P. Clinical challenges of chronic wounds: searching for an optimal animal model to recapitulate their complexity. *Dis Model Mech* **7**, 1205, 2014.
12. Davidson, J.M. Animal models for wound repair. *Arch Dermatol Res* **290 Suppl**, S1, 1998.
13. Sullivan, T.P., Eaglstein, W.H., Davis, S.C., and Mertz, P. The pig as a model for human wound healing. *Wound Repair Regen* **9**, 66, 2001.
14. Gordillo, G.M., Bernatchez, S.F., Diegelmann, R., Di Pietro, L.A., Eriksson, E., Hinz, B., Hopf, H.W., Kirsner, R., Liu, P., Parnell, L.K.S., Sandusky, G.E., Sen, C.K., Tomic-Canic, M., Volk, S.W., Baird, A. Preclinical models of wound

- healing: is man the model? Proceedings of the Wound Healing Society Symposium. *Adv Wound Care* **2**, 1, 2013.
15. Roy, S., Biswas, S., Khanna, S., Gordillo, G.M., Bergdall, V., Green, J., Marsh, C.B., Gould, L.J., and Sen, C.K. Characterization of a preclinical model of chronic ischemic wound. *Physiol Genomics* **37**, 211, 2009.
 16. Elgharably, H., Ganesh, K., Dickerson, J., Khanna, S., Abas, M., Ghatak, P. Das, Dixit, S., Bergdall, V., Roy, S., and Sen, C.K. A modified collagen gel dressing promotes angiogenesis in a preclinical swine model of chronic ischemic wounds. *Wound Repair Regen* **22**, 720, 2014.
 17. Adolph, E.J., Guo, R., Pollins, A.C., Zienkiewicz, K.J., Cardwell, N.L., Davidson, J.M., Guelcher, S.A., and Nanney, L.B. Injected biodegradable polyurethane scaffolds support tissue infiltration and delay wound contraction in a porcine excisional model. *J Biomed Mater Res B Appl Biomater* **104**, 1679, 2016.
 18. Martin, J.R., Gupta, M.K., Page, J.M., Yu, F., Davidson, J.M., Guelcher, S.A., and Duvall, C.L. A porous tissue engineering scaffold selectively degraded by cell-generated reactive oxygen species. *Biomaterials* **35**, 3766, 2014.
 19. Adolph, E.J., Pollins, A.C., Cardwell, N.L., Davidson, J.M., Guelcher, S.A., and Nanney, L.B. Biodegradable lysine-derived polyurethane scaffolds promote healing in a porcine full-thickness excisional wound model. *J Biomater Sci Polym Ed* **25**, 1973, 2014.
 20. Pawar, A.S., Zhu, X.-Y., Eirin, A., Tang, H., Jordan, K.L., Woollard, J.R., Lerman, A., and Lerman, L.O. Adipose tissue remodeling in a novel domestic porcine model of diet-induced obesity. *Obesity* **23**, 399, 2015.
 21. Fishman, J.M., Lowdell, M.W., Urbani, L., Ansari, T., Burns, A.J., Turmaine, M., North, J., Sibbons, P., Seifalian, A.M., Wood, K.J., Birchall, M.A., and De Coppi, P. Immunomodulatory effect of a decellularized skeletal muscle scaffold in a discordant xenotransplantation model. *Proc Natl Acad Sci U S A* **110**, 14360, 2013.
 22. Brown, B.N., Valentin, J.E., Stewart-Akers, A.M., McCabe, G.P., and Badylak, S.F. Macrophage phenotype and remodeling outcomes in response to biologic scaffolds with and without a cellular component. *Biomaterials* **30**, 1482, 2009.
 23. Keane, T.J., Londono, R., Turner, N.J., and Badylak, S.F. Consequences of ineffective decellularization of biologic scaffolds on the host response. *Biomaterials* **33**, 1771, 2012.
 24. Dhar, S.C., and Taylor, G.I. The delay phenomenon: the story unfolds. *Plast Reconstr Surg* **104**, 2079, 1999.
 25. Kalani, M., Brismar, K., Fagrell, B., Ostergren, J., and Jörneskog, G. Transcutaneous oxygen tension and toe blood pressure as predictors for outcome of diabetic foot ulcers. *Diabetes Care* **22**, 147, 1999.
 26. Xu, R.X., Allen, D.W., Huang, J., Gnyawali, S., Melvin, J., Elgharably, H., Gordillo, G.M., Huang, K., Bergdall, V., Litorja, M., Rice, J.P., Hwang, J., and Sen, C.K. Developing digital tissue phantoms for hyperspectral imaging of ischemic wounds. *Biomed Opt Express* **3**, 1433, 2012.
 27. Schwarz, D.A., Lindblad, W.J., and Rees, R.S. Altered collagen metabolism and delayed healing in a novel model of ischemic wounds. *Wound Repair Regen* **3**, 204, 1995.
 28. Chen, C., Schultz, G.S., Bloch, M., Edwards, P.D., Tebes, S., and Mast, B.A. Molecular and mechanistic validation of delayed healing rat wounds as a model for human chronic wounds. *Wound Repair Regen* **7**, 486, 1999.
 29. Gould, L.J., Leong, M., Sonstein, J., and Wilson, S. Optimization and validation of an ischemic wound model. *Wound Repair Regen* **13**, 576, 2005.
 30. Gnyawali, S.C., Elgharably, H., Melvin, J., Huang, K., Bergdall, V., Allen, D.W., Hwang, J., Litorja, M., Shirley, E., Sen, C.K., and Xu, R. Hyperspectral imaging of ischemic wounds. *Measurement* **8229**, 822910, 2012.
 31. Olsson, I.A.S., Franco, N.H., Weary, D.M., and Sandoe, P. The 3Rs principle—mind the ethical gap! *ALTEX WC8*, 333, 2012.
 32. Hafeman, A.E., Zienkiewicz, K.J., Zachman, A.L., Sung, H.J., Nanney, L.B., Davidson, J.M., and Guelcher, S.A. Characterization of the degradation mechanisms of lysine-derived aliphatic poly(ester urethane) scaffolds. *Biomaterials* **32**, 419, 2011.
 33. Brown, B.N., Ratner, B.D., Goodman, S.B., Amar, S., and Badylak, S.F. Macrophage polarization: an opportunity for improved outcomes in biomaterials and regenerative medicine. *Biomaterials* **33**, 3792, 2012.
 34. Koh, T.J., DiPietro, L.A. Inflammation and wound healing: the role of the macrophage. *Expert Rev Mol Med* **13**, 23, 2011.
 35. Sindrilaru, A., Peters, T., Wieschalka, S., Baican, C., Baican, A., Peter, H., Hainzl, A., Schatz, S., Qi, Y., Schlecht, A., Weiss, J.M., Wlaschek, M., Sunderkötter, C., and Scharffetter-Kochanek, K. An unrestrained proinflammatory M1 macrophage population induced by iron impairs wound healing in humans and mice. *J Clin Invest* **121**, 985, 2011.
 36. Brown, B.N., Londono, R., Tottey, S., Zhang, L., Kukla, K.A., Wolf, M.T., Daly, K.A., Reing, J.E., and Badylak, S.F. Macrophage phenotype as a predictor of constructive remodeling following the implantation of biologically derived surgical mesh materials. *Acta Biomater* **8**, 978, 2012.

Address correspondence to:

Lillian B. Nanney, PhD

Department of Plastic Surgery

Vanderbilt University Medical Center

Nashville, TN 37232

E-mail: lillian.nanney@vanderbilt.edu

Craig L. Duvall, PhD

Department of Biomedical Engineering

Vanderbilt University

5824 Stevenson Center

Nashville, TN 37240-0002

E-mail: craig.duvall@vanderbilt.edu

Received: April 14, 2017

Accepted: July 14, 2017

Online Publication Date: September 29, 2017