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Bi-layered living cellular construct resulted in greater healing in an alloxan-induced diabetic porcine model

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Abstract

Tissue-engineered skin constructs, including bi-layered living cellular constructs (BLCC) used in the treatment of chronic wounds, are structurally/functionally complex. While some work has been performed to understand their mechanisms, the totality of how BLCC may function in wound healing remains unknown. To this end, we have developed a delayed wound healing model to test BLCC cellular and molecular mechanisms of action. Diabetes was chemically-induced using alloxan in Yucatan miniature pigs, and full-thickness wounds were generated on their dorsum. These wounds were either allowed to heal by secondary intention alone (control) or treated with a single or multiple treatments of a porcine autologous BLCC. Results indicated a single treatment with porcine BLCC resulted in statistically significant wound healing at day 17, while four treatments resulted in statistically significant healing on days 10, 13, and 17 compared to control. Statistically accelerated wound closure was driven by re-epithelialisation rather than contraction or granulation. This porcine diabetic model and the use of a porcine BLCC allowed evaluation of healing responses *in vivo* without the complications typically seen with either xenogenic responses of human/animal systems or the use of immune compromised animals, expanding the knowledge base around how BLCC may impact chronic wounds.

KEYWORDS

allogenic, BLCC, diabetes, re-epithelialisation, wound care

Key Messages

- successfully generated a diabetic pig model and porcine BLCC (pBLCC) to investigate the mechanism by which BLCCs promote healing in full-thickness wounds
- alloxan-induced diabetic pigs were given full-thickness wounds and treated with pBLCC (1 or 4 times) or left untreated. Wound healing was monitored

Justin T. Avery and Jizeng Qiao contributed equally to the manuscript.

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over the course of 33 days for contraction, closure, and granulation with planimetry and histology.
- both 1x and 4x BLCC treatments resulted in significant improvements in wound healing; however, 4x BLCC resulted in significant differences earlier and at more time points, suggesting a greater impact from multiple treatments
- while BLCC treatment did not significantly impact granulation or contraction, increased wound closure was primarily driven by increased rate of re-epithelialisation in response to BLCC treatment

1 | INTRODUCTION

Chronic wounds are a major clinical challenge, with hallmarks including persistent elevated levels of inflammation, infection/biofilm generation, increased MMP levels, and stalled re-epithelialisation.[1] Individuals acutely impacted by chronic wounds are those with diabetes, since their disease makes them prone to infection, have poor epidermal barrier function, and decreased or imbalanced growth factors, ECM components, and MMPs.[2] 537 million adults between the ages of 20 to 79 years of age are living with diabetes as of 2021, meaning 1 in 10 adults have impaired wound healing as a result of their disease.[3] As such, the need to modulate the chronic wound healing process is at an all time high. There is widespread use of a number of dressings and skin substitutes to support and cover these complex wounds. Of particular interest, treatment with a bi-layered living cellular construct ("BLCC" Apligraf, Organogenesis, Canton, MA) has resulted in statistically improved wound healing compared to controls in both venous leg ulcers (VLUs)[4] and diabetic foot ulcers (DFUs).[5] BLCC has a robust clinical data set and has been demonstrated as an effective therapeutic product for both venous leg and diabetic foot ulcers.[4,6-9]

BLCC is a living cellular product engineered with cultured human foreskin fibroblasts, keratinocytes, and a bovine collagen matrix.[10-12] Studies by Stone et al evaluating potential mechanisms of healing with BLCC in chronic VLUs focused on changes in the wound edge and wound bed of non-healing VLUs.[13,14] Transcriptomics of wound edge biopsies found that a single BLCC application provoked an acute wound healing phenotype, reversing some of the dysregulation observed in chronic VLUs.[13] Assessment of biopsies from the wound bed found a shift from an inflamed/fibrotic gene profile to an acute, pro-healing phenotype through the downregulation of TGF-β and upregulation of ECM remodelling proteins like matrix metalloproteinase-8 (MMP-8).[14] Outside of these studies of VLUs performed by Stone *et al*, the mechanistic data surrounding wound care treatments is lacking overall. *In vivo* studies for human-derived products are often limited by the xenogeneic responses elicited using a human-derived product in an animal model. Alternatively, immune-compromised species may be used, but this approach negates the important impact that the immune microenvironment plays in wound healing.[15]

Porcine models have been widely considered as one of the best wound healing models due to their anatomical, physiological, and metabolic similarities to human skin.[16,17] Various wound types, including full-thickness excision, partial thickness excision, and thermal wounds, have been used in wound healing studies with porcine models[18-21]. In order to more appropriately mimic the delayed healing responses seen in chronic wounds, chemically-induced diabetes[22] with alloxan[23] or streptozotocin[24] is the gold standard and has been successfully used in previous studies.[17,25,26] To remove the constraints of using a human product in a porcine model, an allogeneic porcine replicate of BLCC was developed. Using modified protocols developed for human cells, porcine BLCCs (pBLCCs) were successfully produced with porcine fibroblasts and keratinocytes.[27] In this study, we evaluated the impact of BLCC treatment in a diabetic delayed full-thickness porcine model using a porcine-derived BLCC.

2 | MATERIALS AND METHODS

2.1 | Cell culture and pBLCC fabrication

Detailed methods for fabricating porcine BLCC (pBLCC) are described elsewhere.[27] Briefly, dorsal skin of a neonatal (<24 hours old) male Hanford miniature swine was incubated with trypsin and collagenase to release cells into suspension. These cells were serially passaged in growth media (Fibroblasts: Dulbecco's Modified Eagle's Medium [DMEM] + 15% Fetal Bovine Serum; Keratinocytes: Minimally Supplemented Basal Medium [MSBM] (Organogenesis, Canton, MA)). Keratinocyte cultures were supplemented with Triiodothyronine, Bovine pituitary extract, and Cholera toxin, as described in Johnson et al,[26] to prevent differentiation during expansion in monolayer cultures.

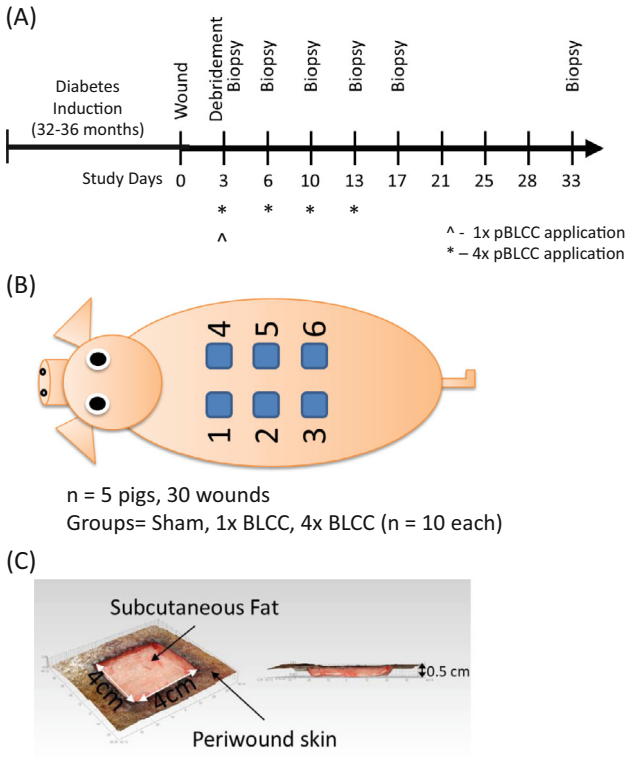

FIGURE 1 Study design. (A) 4 cm × 4 cm full-thickness excision wounds were created on the back of each pig where a 4 cm space was maintained between wounds on Day 0. Wounds were allowed to granulate for 3 days, and then sharply debrided and treated with application of pBLCC(s). Biopsies were taken from one set of wounds while the other set of wounds were used for photographic analysis. (B) Image depicting wound layout – 2 wounds for each treatment type were generated and location was randomised. (C) 3D rendering of the full-thickness wounds extending 0.5 cm to the subcutaneous fat layer

2.2 | Animals

The animal study protocol was approved by the IACUC of Sinclair Research Center (IACUC Protocol #: SRC11325). Five castrated male Yucatan miniature pigs (Sinclair Research Centre, Auxvasse, Missouri) were chemically-induced to become diabetic with alloxan 32 to 36 months prior to study initiation to faithfully model diabetes rather than just hyperglycemia.[28] The age of animals at the time of study initiation ranged from 2.75 to 3.05 years, at which time the animals weighed between 54.4 and 68.2 kg. Successful induction of diabetes was confirmed by blood glucose levels of 255 to 600 mg/dL maintained with insulin for the duration of the study.

2.3 | Surgical wounding procedure

Pigs were tattooed and full-thickness wounds (4 cm × 4 cm), extending to the subcutaneous fat layer, were created on the backs of each pig on study day 0 (Figure 1). Wounds were allowed to granulate for 3 days, sharply debrided, and then treated with either 1x pBLCC (day 3), sequentially 4x with pBLCC (day 3, 6, 10, 13), or left open (sham) (Figure 1). For wounds treated with 4x sequential pBLCC, if any residual pBLCC was present at the next application, it was removed from the wound prior to reapplication. All wounds were covered with petrolatum gauze, moist gauze, and Tegaderm (3 M, St. Paul, Minnesota). Wound sites were randomised among six wounds (n = 2 of each group) on each animal. Per animal, n = 1 of each group was used for wound photography, and n = 1 of each group was used for histological biopsies.

2.4 | Photography and analysis

Wounds were imaged on post-operative days 0, 6, 10, 13, 17, 21, 25, 28, and 33 with both 2D (D90, Nikon, Melville, New York) and 3D (LifeViz, QuantifiCare, Valbonne, France) digital cameras.

For 2D planimetric analysis, NIH ImageJ software was used to quantify wound healing. Briefly, the scale was synchronised with a 5 cm ruler in each picture in order to determine the pixel to cm ratio. To calculate wound closure, the total wound area and unhealed wound area were contoured and calculated, and the percentage of wound closure was calculated using Equation (1). Additionally, exponential plateau fits were performed using GraphPad Prism 9.2 with constraints $Y_0 = 0$ and $Y_{Max} = 100$ to interpolate wound healing rate. Equation (2) was then used from the Exponential Plateau fits values to determine theoretical wound healing based on individual animal fit values. Percent wound contraction at each time point was calculated from the total area within the tattooed at baseline and at each timepoint, using Equation (3).

$$\left(1 - \frac{\text{Unhealed Wound Area}}{\text{Total Wound Area}}\right) * 100, \tag{1}$$

$$Y_{\text{Max}} - (Y_{\text{Max}} - Y_O) * e^{-k * \text{Days after Closure Initiation}}, \tag{2}$$

$$\left(1 - \frac{\text{Wound Area inside tattoo at Time point}}{\text{Initial Wound Area inside tattoo at Day 0}}\right) * 100. \tag{3}$$

For 3D volumetric analysis, DermaPix software was used. A square contour was drawn around the wound to construct 3D images and mesh. A second contour along the wound edges of 3D image was drawn for the

volumetric analysis. Percentage of volume decrease was calculated using Equation (4).

$$\left(1-\frac{\text{Wound Volume at Time point}}{\text{Wound Volume at Day 0}}\right)*100. \quad (4)$$

2.5 | Histology and analysis

Rectangular biopsies of 0.5 mm × 5 mm were taken from each wound (n = 1 per group, per animal) on Days 3, 6, 10, 13, 17, and 33. Biopsy tissues were processed using standard histological techniques. Briefly, biopsies were fixed in 10% formalin and embedded in paraffin, 5 μm thick sections were cut, and sections were stained with haematoxylin and eosin (H&E) and Masson-Golder's Trichrome (MGT). Qualitiative assessments of healing were assessed by evaluating images.

2.6 | Statistical analysis and graph generation

Statistical analysis and graph generation were performed using GraphPad Prism v9.3. Two-way ANOVA with Uncorrected Fisher's LSD comparisons was performed for comparing multiple groups to the sham group. In cases where repeated measures were assessed (wound closure, contraction, and volume), Repeated Measures Two-way ANOVA with Uncorrected Fisher's LSD comparisons was performed. For comparing fit curves on the exponential plateau data set, an extra sum-of-squares *F* test was performed.

3 | RESULTS

3.1 | pBLCCs were successfully fabricated and showed same characteristics as human analogs

pBLCCs were fabricated from porcine fibroblasts and keratinocytes using comparable methodologies to human BLCC (BLCC).[27] BLCC and pBLCCs were analysed by H&E to determine whether pBLCCs closely represent BLCCs. The pBLCCs (Figure 2A) consisted of a lower layer of fibroblasts within a bovine type I collagen lattice termed "dermal equivalent" and an upper layer containing keratinocytes that differentiated and stratified to form an epidermis, having comparable morphologic characteristics to human BLCC (Figure 2B). The morphology of pBLCCs suggested a live cellular construct with the characteristics of cell proliferation and differentiation during the production process *in vitro*.

3.2 | Wound granulation and contraction are not primary drivers of wound closure from pBLCC

In this diabetic swine model, full-thickness excisional wounds were either left open (sham), treated 1x with pBLCC, or treated 4x with pBLCCs (Figure 1). Throughout the course of the study, the majority of pBLCCs were

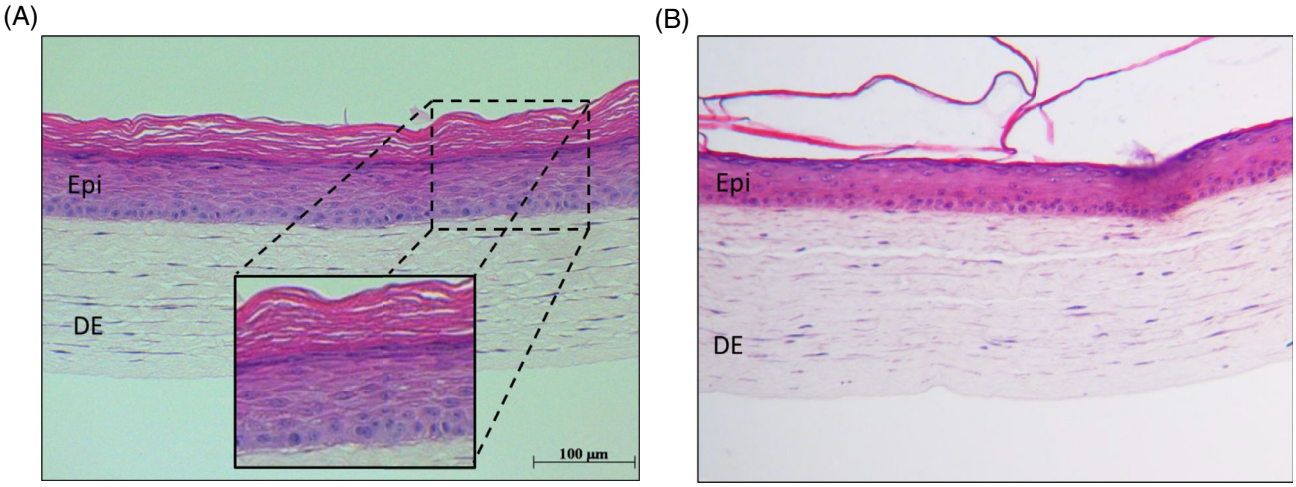

FIGURE 2 Porcine BLCC showed comparable morphology as human analogs using H&E staining. (A) Porcine BLCC is composed with intact epidermis (Epi) and dermal equivalent (DE). Stratified epidermal layers are clearly visible (insert). (B) Human BLCC is composed with similar epidermis (Epi) and dermal equivalent (DE)

degraded between reapplications, modelling the clinical experience seen with human BLCCs.[6,29,30] All treatment groups initially produced a robust granulation bed, irrespective of their treatment as shown in representative images (Figure 3A). Using wound volume analysis over time, results indicated a decrease in wound volume which was biphasic and most rapid between Days 3 and 10, while granulation continued to form Days 10 to 33, albeit at a slower rate (Figure 3B). No statistical differences in granulation deposition were measured by wound volume between the three treatment groups.

In order to evaluate the possibility of wound contraction driving the wound closure, wound contraction was quantified by measuring the surface area within the tattoo at baseline and at each timepoint (representative images, Figure 4A). Rapid contraction was observed between day 3 and 13 at comparable levels across all treatment groups, with a small but statistically elevated contraction percentage in the 1x pBLCC group compared to sham on day 13 only ($P = 0.0189$) (Figure 4B); however, the impact of this finding on the closure results is expected to be minimal, as there were no differences in closure observed for the 1x pBLCC group compared to sham at this time point (Figure 4C). Average contraction percentage of 54% was seen in all groups by the end of study with no statistical differences in contraction between treatment groups.

3.3 | pBLCC stimulated wound epithialisation in diabetic porcine model

Wound closure started between days 6 and 10 and wounds were > 90% epithelialised by the end of study (Figure 4A). Wound closure progressed from day 6 to 25 irrespective of treatment group (Figure 4C). At days 10 and 13, 4x pBLCC treated wounds had statistically greater wound closure compared to the sham group ($P = 0.0459$ and $P = 0.0117$, respectively). By day 17, both 1x and 4x pBLCC treated wounds had statistically improved wound closure compared to sham ($P = 0.0403$ and $P = 0.0005$, respectively). By day 21, both 1x (86.2%) and 4x (93.2%) pBLCC trended with higher percent closure than sham (73.8%), but statistical significance between groups was lost.

Since contraction and granulation were not the mechanistic drivers of wound closure, we assessed whether epithelialisation was the primary healing factor promoted by BLCC treatment using histological assessment with Masson-Golder's Trichrome (MGT) staining (Figure 5). With pBLCC treatment, tissue granulation was observed on Day 6 and nearly filled the entire wound bed by Day 13, matching the observations made with 3D planimetry analysis. An epithelial tongue, originating from the periwound epidermis, was observed as early as day 6 (red arrow) and coincided with initial contact between the periwound keratinocytes and newly-formed granulation tissue. At days 10 and 13, both extension and increasing maturity of the epithelial layer advancing from the periwound area can be seen. Together, these qualitiative observations suggest that pBLCC treatment promoted wound healing through epithelialisation.

3.4 | Interpolation modelling indicate multiple pBLCC treatments has statistically greater rate of wound closure

To determine whether the overall rate of wound closure was statistically different between treatment groups, an

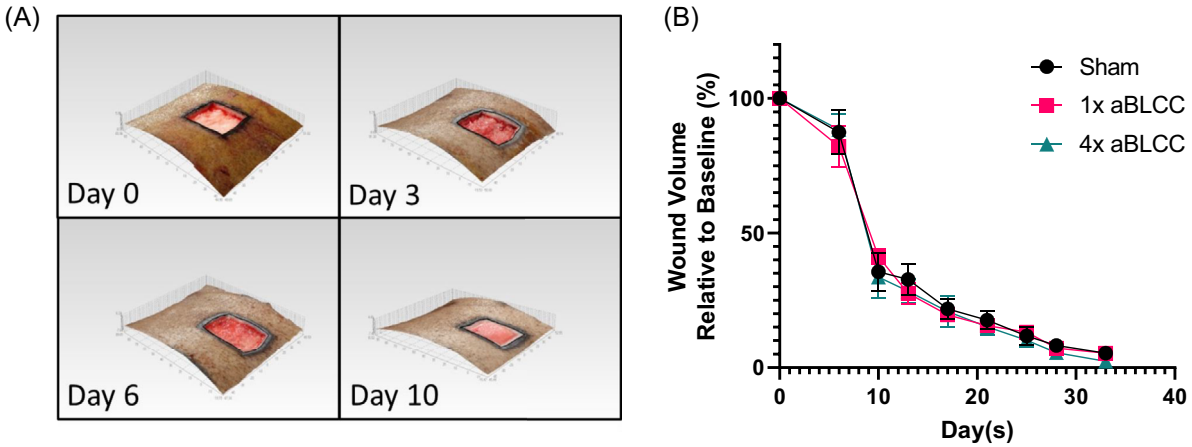

FIGURE 3 pBLCCs do not influence the rate of wound granulation. (A) Representative 3D wound photographs from Days 0, 3, 6, and 10. (B) pBLCCs do not influence granulation/wound volume reduction (N = 5 pigs per group). Data presented as means ± standard error of the mean

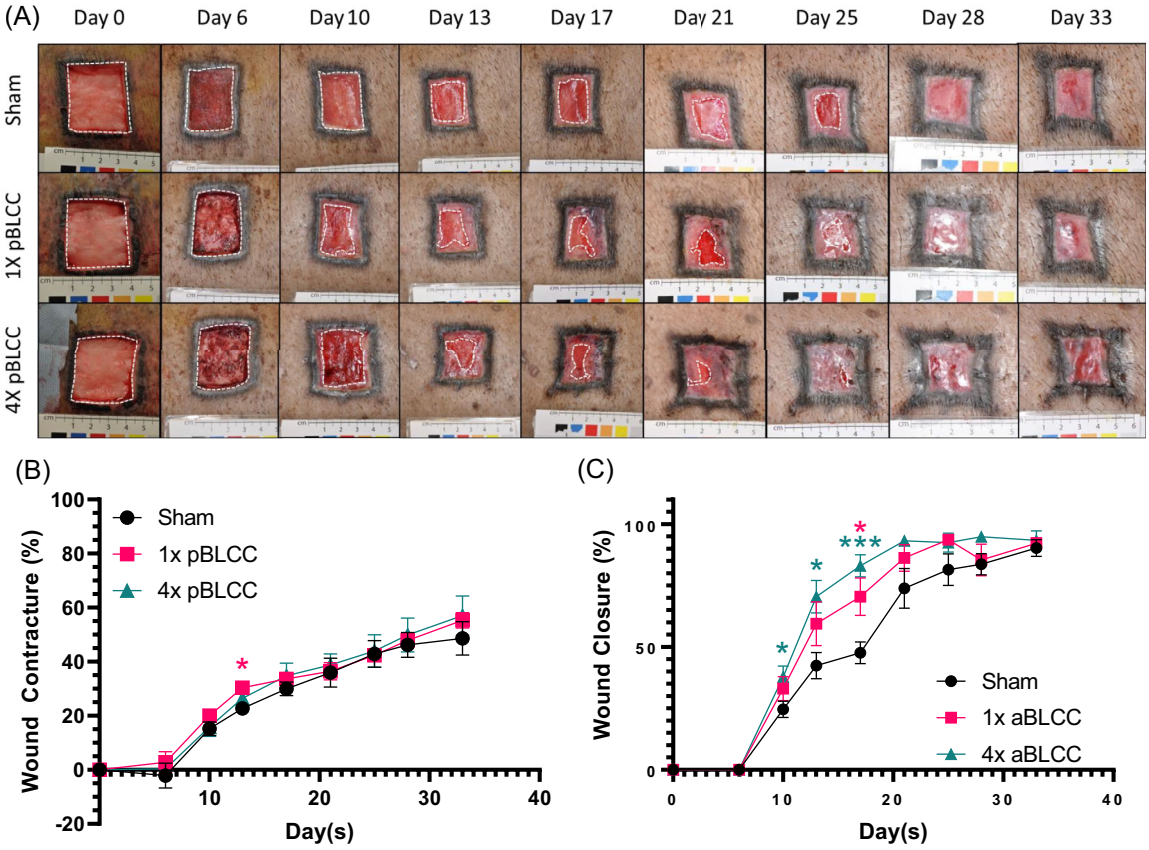

FIGURE 4 Analysis of wounds indicate pBLCC treated wounds close at a quicker rate than sham. (A) Representative photographic analysis of wounds across a single animal for each group. (B) Wound contraction analysis. A mathematical, but not biological, statistical difference in contraction was observed for 1x pBLCC vs sham on Day 13. (C) Wound closure analysis. Statistical significance was achieved at Day 10 for both 4x pBLCC ($P = 0.0459$) and Day 17 for 1x pBLCC ($P = 0.0403$) vs sham. Statistical significance lost at Day 21 for both pBLCC treatments vs sham. Repeated Measures Two-way ANOVA with Greisser-Greenhouse correction and Uncorrected Fisher's LSD comparisons. *$P \leq 0.05$, ***$P \leq 0.001$. Data presented as means ± standard error of the mean

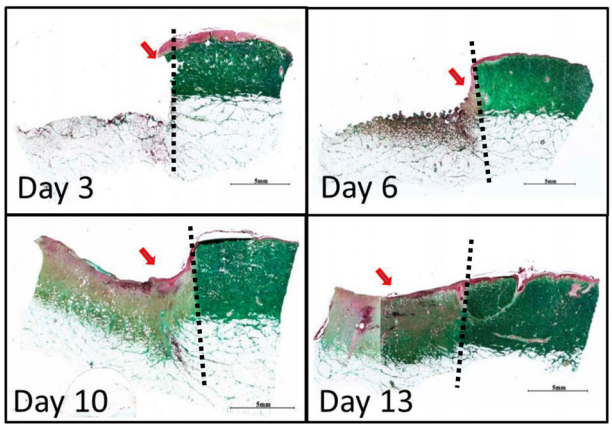

FIGURE 5 Wound closure is achieved through re-epithelialisation. Mason's Trichrome-Goldner staining of sections from a 4x pBLCC application of wound at Days 3, 6, 10, and 13. Red arrow indicates an epithelial tongue, black dashed line represents the approximate border of the original wound

exponential plateau fit was performed (Figure 6A). The k value of each fit was statistically different from one another based on extra sum-of-squares F test ($P < 0.0001$), supporting the observed statistical differences observed in wound closure. Using this interpolation model, it was determined that 4x pBLCC treatment would have a 50% closure rate at a statistically earlier timepoint compared to sham (10.60 days vs 15.06 days, respectively), and 1x pBLCC would achieve statistically earlier closure compared to sham at 60% healing (13.87 days vs 17.98 days, respectively). Additionally, it was determined that 4x pBLCC (21.27 days) and 1x pBLCC (25.79 days) would have a 90% closure rate statistically earlier than sham (36.09 days) with p values of ($P < 0.0001$ for both) (Figure 6B). Kaplan–Meier plots using the *in vivo* closure results determined that the 4x pBLCC was statistically different from sham both at early (25% closed, $P = 0.0495$) and late (75% closure, $P = 0.0112$) time points (Figure 6C,D).

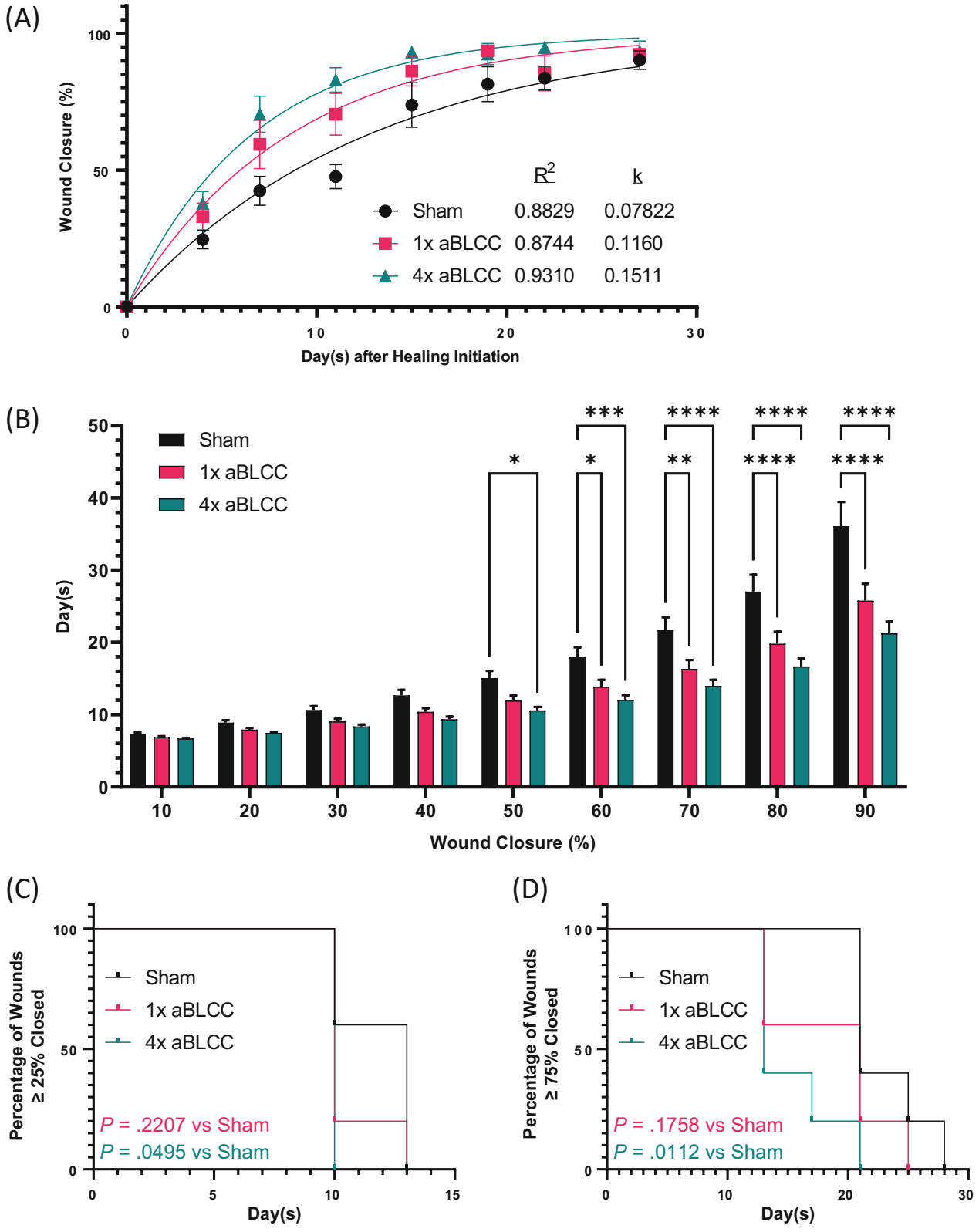

FIGURE 6 Analysis of wound closure indicates additive effect of multiple BLCC treatments. (A) Exponential plateau fit was used to model the rate of healing for wounds (see methods for equation). *k* value was statistically different for all equations (Extra sum-of-squares *F* test). (B) Exponential plateau interpolation of wound closure based on *in vivo* data acquisition. 4x pBLCC treated animals are predicted to achieve 50% wound closure quicker than sham. 1x pBLCC treated animals are predicted to achieve 60% wound closure more rapidly than sham. Two-way ANOVA with Uncorrected Fisher's LSD comparisons was performed. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, ****$P \leq 0.0001$ (C and D) Kaplan–Meier plots for wound closure at 25% and 75%, respectively, where 4x pBLCC achieved statistical significance at both times (Log-rank [Mantel-Cox] test). Data presented as means ± standard error of the mean

4 | DISCUSSION

In this study, we demonstrated that porcine and human BLCCs had comparable structure despite the animal origin of the keratinocytes and fibroblasts making up the pBLCC. pBLCC treatment increased the rate of wound closure significantly between Days 10 and 21 after initial application, which coincides with the most rapid phase of wound epithelialisation observed in other diabetic pig models.[17] In general, while one treatment with BLCC achieves statistically greater wound closure compared to control by day 17 of treatment, multiple applications of pBLCC resulted in more robust healing in this model. The similarity of observed wound contraction and granulation rates across all three treatments and histological analysis strongly supported that wound closure promoted by pBLCCs was primarily driven by more robust epithelialisation.

Pig and human skin are anatomically close in both hair follicle density and thickness of epidermis/dermis, making pigs a better preclinical model compared to other species such as rabbits and mice.[31] For regeneration of full-thickness wounds, the wound bed must first be filled with granulation tissue consisting of various cell types, collagen, fibronectin, and hyaluronic acid.[32,33] We measured granulation using 3-D volumentric analysis and found across the treatment groups, there were no statistical difference in tissue granulation regardless of treatment. With the primary outcome of wound healing measured by wound closure, we needed to determine whether wound contraction, which is driven by the presence of myofibroblasts in granulation tissue in pigs,[34] played a significant role in the results seen and overall healing process. The minor difference in contraction did not align with statistical differences in closure and resolved at the next time point, indicating contraction was not responsible for the differences observed in closure.

Epithelialisation of full-thickness wounds starts from the wound edges, which is in contrast from that of partial thickness wounds, where it starts from both wound edges and hair follicles.[35,36] Starting 6 days after wounding, an epithelial tongue was observed, which migrated along the deposited granulation tissue between days 6 and 13. The tip of the epithelial tongue expressed hyper-proliferated keratinocytes; histology confirmed that pBLCC application(s) promoted faster migration of newly formed epidermis. Wound healing of BLCC have also been tested *in vitro* and Falanga et al demonstrated that wounded (fenestrated) BLCCs healed themselves by re-epithelialisation, similarly to the process observed in this study, with an epithelial tongue extending over the edge of the wound bed and full healing by 5 days post injury.[37] As such, we determined that statistical increase in wound closure was the result of increased rate of epithelialisation.

BLCCs are considered a biological skin substitute since they promote wound healing by secondary intention rather than engrafting to the patient.[7,29,30,38-40] BLCCs are thought to supply matrix materials, secrete cytokines and other soluble factors, which promote wound healing.[13,14,41] Use of BLCCs to treat deep-dermal excisional wounds found that BLCCs promoted marginal epithelium to spread from the edge of the wound, resulting initially in an immature epithelial layer and potential scarring.[39] Furthermore, mechanistic studies of BLCC in VLUs suggested that changes in gene expression in the wound bed are predicted to enhance epithelial proliferation after BLCC treatment,[14] which is consistent with increased re-epithelialisation observed in our model.

While there are some limitations to this study (use of a pig model, porcine BLCC rather than human BLCC, lack of scar formation assessment), findings mimic expectations from previous studies and allows for mechanistic assessment without concern over xenograft rejection. We have demonstrated that pBLCCs increase the rate of re-epithelialisation in a diabetic porcine full-thickness wound model. A single treatment with BLCC resulted in earlier statistically significant wound closure compared to control treated wounds by day 17 of treatment. Treatment with multiple applications of pBLCC resulted in rapid wound closure, achieving 90% wound closure 4 days faster than a single application and approximately 2 weeks faster than sham treated controls with both interpolation and experimental data. As such, this study builds upon the important literature focused on elucidating how these advanced products stimulate wound healing responses.

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CONFLICT OF INTEREST

Justin T. Avery, Erika Medeiros, Kelly A. Kimmerling, and Katie C. Mowry are employed by Organogenesis. Jizeng Qiao and Thomas J. Bollenbach were employees of Organogenesis when this study was originally carried out.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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