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Porcine-derived collagen peptides promote re-epithelialisation through activation of integrin signalling

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Abstract

Chronic non-healing cutaneous wounds represent a major burden to patients and healthcare providers worldwide, emphasising the continued unmet need for credible and efficacious therapeutic approaches for wound healing. We have recently shown the potential for collagen peptides to promote proliferation and migration during cutaneous wound healing. In the present study, we demonstrate that the application of porcine-derived collagen peptides significantly increases keratinocyte and dermal fibroblast expression of integrin α2β1 and activation of an extracellular signal-related kinase (ERK)-focal adhesion kinase (FAK) signalling cascade during wound closure in vitro. SiRNA-mediated knockdown of integrin β1 impaired porcine-derived collagen peptide-induced wound closure and activation of ERK-FAK signalling in keratinocytes but did not impair ERK or FAK signalling in dermal fibroblasts, implying the activation of differing downstream signalling pathways. Studies in ex vivo human 3D skin equivalents subjected to punch biopsy-induced wounding confirmed the ability of porcine-derived collagen peptides to promote wound closure by enhancing re-epithelialisation. Collectively, these data highlight the translational and clinical potential for porcine-derived collagen peptides as a viable therapeutic approach to promote re-epithelialisation of superficial cutaneous wounds.

KEYWORDS
collagen peptides, keratinocytes, re-epithelialisation, wound healing

1 | INTRODUCTION

Cutaneous wound healing is a complex biological process involving multiple overlapping phases, the impairment of which contributes to the development of chronic non-healing wounds. The emergence of chronic non-healing wounds thereby inflicts a heavy burden onto worldwide healthcare providers, emphasising the need for more efficacious treatment options to treat chronic non-healing wounds.[1,2]

Bioactive peptides generated from the hydrolysis of collagen, an extracellular matrix (ECM) protein found within animal skin and bones have shown potential for promoting cutaneous wound healing, with preliminary studies demonstrating enhanced wound closure through the promotion of cellular proliferation.[3–6] However, the mechanisms by which collagen peptides promote cutaneous wound healing remain undefined, thus limiting their translational potential.

Abbreviations: DDR, discoidin domain receptor; EGF, epidermal growth factor; ECM, extracellular matrix; ERK, extracellular-related kinase; FAK, focal adhesion kinase; FGF, fibroblast growth factor; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; PI3K, phosphoinositide 3-kinase; PCP, porcine collagen peptides; TGF-β, transforming growth factor-β.

Previous studies have shown collagen peptides promote osteoblast proliferation in vitro through the activation of the phosphoinositide 3-kinase (PI3K)/Akt pathway, while inhibition using an PI3K inhibitor abolished collagen peptide-induced activation of Akt, leading to impaired osteoblast proliferation.[7] This corroborates with previous observations of collagen peptide-induced proliferation and the potential involvement of PI3K/Akt activation during cutaneous wound healing.[5] However, studies using scratch-wounded NIH-3 T3 mouse fibroblasts revealed collagen peptide-induced proliferation is mediated through activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signalling pathway and increased expression of wound healing-associated growth factors; epidermal growth factor, fibroblast growth factor and transforming growth factor-β (TGF-β).[8] Collectively, these data suggest that collagen peptides may promote cutaneous wound healing through multiple mechanisms rather than the activation of just one signalling pathway.

Cell-ECM interactions play a crucial role in cutaneous wound healing by promoting cellular proliferation, adhesion and migration, with integrin receptors constituting the most abundant receptors mediating these interactions between cells and their surrounding ECM.[9] In particular, integrin α2β1, a collagen receptor expressed by both keratinocytes and dermal fibroblasts recognises specific motifs present within collagen such as GFOGER, leading to the autophosphorylation of focal adhesion kinase (FAK) and downstream activation of signalling pathways such as Akt and extracellular signal-related kinase (ERK) to promote cellular proliferation and migration.[10,11] Given collagen peptides are derived from native collagen suggests the presence of similar motifs that allow for similar interactions with integrins to activate downstream signalling pathways during cutaneous wound healing.

Despite attempts to elucidate the mechanistic action of collagen peptides in various cell and animal models, the mechanism by which they promote cutaneous wound healing still remains undefined. Therefore, the present study aimed to delineate the mechanisms mediating porcine-derived collagen peptide (PCP)-induced wound closure of keratinocytes and dermal fibroblasts in vitro and in ex vivo wounded 3D skin equivalents to determine their potential as a therapeutic approach for cutaneous wounds.

2 | MATERIALS AND METHODS

2.1 | Cell culture

Primary human keratinocytes and dermal fibroblasts were isolated from surplus human skin following informed consent (REC reference 19/NE/004_Lovat) and maintained as previously described.[12] Primary keratinocytes were cultured in either EpiLife media (ThermoFisher Scientific, Waltham, MA, USA) supplemented with 1% human keratinocyte growth factor supplement (HKGS) (ThermoFisher Scientific, USA) and 1% penicillin–streptomycin-amphotericin (PSA) (Lonza, Verviers, Belgium), or Keratinocyte Growth Medium 2 (Promocell, Heidelberg, Germany) supplemented with SupplementMix (Promocell, Germany) and 1% PSA (Lonza, Belgium). Primary dermal fibroblasts were cultured in Dulbecco Modified Eagle Medium (DMEM) supplemented with 10% foetal bovine serum (FBS) (Sigma-Aldrich, Gillingham, UK) and 1% PSA. For all experiments, both primary keratinocytes and dermal fibroblasts were only used up to passage 5.

2.2 | Preparation of porcine-derived collagen peptides (PCP)

Hydrolysed collagen peptides from porcine skin (PCP) were provided by Rousselot. PCP was generated through the enzymatic hydrolysation of type I collagen, isolated from porcine skin to obtain a mixture of single chain amino acid peptide sequences with a molecular weight between 2 and 5 kDa. For in vitro 2D experiments, PCP was dissolved in ultra-pure water and filter-sterilised to give a final concentration of 1 mg/mL, with 100 μL used to coat the wells of a 96 well ImageLock plate (Essen Biosciences, Royston, Hertfordshire, UK) or 1 mL used to coat the wells of a 6 well plate (Greiner, Stonehouse, UK). After 24 h, plates were aspirated to remove excess solution before leaving at room temperature to air dry. For ex vivo 3D experiments, PCP was dissolved in 1:1 EpiLife/DMEM to give a final concentration of 1 mg/mL before application to wounded 3D skin equivalent models.

2.3 | Small interfering RNA (siRNA) knockdown of integrin β1

For transient knockdown of integrin β1, ON-TARGETplus SMARTpool *ITGB1* siRNA (Dharmacon, Horizon Discovery, Lafayette, CO, USA) or ON-TARGETplus Non-targeting Control Pool siRNA (siCtrl) (Dharmacon, Horizon Discovery, USA) were transfected into cells as previously described.[13] After 6 h, cells were scratched using a 200 μL pipette tip and supplemented with fresh culture medium. Cells were harvested at 24, 48, and 72 h post-wounding.

2.4 | Western blot

Western blotting in wounded keratinocytes and dermal fibroblasts treated in the presence/absence of 1 mg/mL PCP and harvested at 24, 48, and 72 h post-wounding was performed as previously described.[14] Briefly, protein was separated by electrophoresis using 4%–20% SDS-PAGE gels (Bio-Rad, California, USA) and transferred onto PVDF membranes (Bio-Rad, California, USA) before incubating with primary antibodies overnight at 4°C (Supplementary Table 1). For detection, anti-mouse or anti-rabbit secondary antibodies (Vector Laboratories, California, USA) were diluted 1:2500 and protein expression was visualised using a LI-COR Odyssey Fc Imager and quantified using Image Studio Software (LI-COR Biosciences, Nebraska, USA).

2.5 | Two-dimensional scratch assays

Scratch assays were performed as previously described.[5] Briefly, 30,000 primary keratinocytes or 15,000 primary dermal fibroblasts were seeded onto uncoated or wells pre-coated with 1 mg/mL PCP of a 96-well ImageLock Plate (Essen Biosciences, UK). Cells were transfected with either siCtrl or *ITGB1* siRNA as previously described.[13] After 6 h, cells were scratched using a 96-well WoundMaker (Essen Biosciences, UK), with continued incubation at 37°C inside an Incucyte ZOOM system (Essen Biosciences, UK) and monitoring of wound closure over 72 h with images taken every 2 h.

2.6 | Flow cytometry

1×10^6 primary keratinocytes or dermal fibroblasts were treated in the presence/absence of 1 mg/mL PCP and added to cell culture media for 72 h. Cells were detached using 5 mL Accutase (Biolegend, San Diego, CA, USA), centrifuged at 600 g for 5 min in 5 mL fluorescent-activated cell sorting (FACS) tubes (ThermoFisher Scientific, USA) and re-suspended in 500 μL FACS buffer (5% FBS in PBS). Cells were blocked with 5 μL of Human TruStain FcX (Biolegend, USA) in 100 μL FACS buffer for 10 min at room temperature, prior to the addition of 5 μL of primary antibody (see Supplementary Table 2) and continued incubation on ice for 30 min in the dark. Cells were then washed three times in FACS buffers before re-suspending in 300 μL FACS buffer and analysis using a Fortessa X20 system (BD Biosciences, New Jersey, USA). FCS Express 7 software (De Novo Software, Pasadena, CA, USA) was used to identify CD49b+, CD49c+, CD49e+, CD49f + and CD29+ cell populations following gating (Supplementary Figure 1) and calculate median CD49b+, CD49c+, CD49e+, CD49f + and CD29+ expression in control and PCP-treated cells relative to the control.

2.7 | Cell adhesion assays

For experiments looking at integrin receptor blocking on keratinocyte adhesion to collagen peptide-coated surfaces, 50,000 keratinocytes were incubated in serum-free Keratinocyte growth medium 2 (Promocell, Germany) in the presence or absence of either 10 μg/mL integrin α2β1 or 10 μg/mL integrin α5β1 blocking antibodies for 45 min at 37°C. The cells were then seeded into the wells of a 96-well ImageLock Plate (Essen Biosciences, UK), coated with either 5 $\mu g/cm^2$ rat-tail collagen I (ThermoFisher Scientific, USA), 50 μg/mL. fibronectin (Sigma-Aldrich, USA) or 1 mg/mL PCP and incubated at 37°C for 2 h. After 2 hs, cells were washed twice with PBS to remove any unbound cells before adding Keratinocyte growth medium 2 (Promocell, Germany) and capturing images of adhered cells using an Incucyte ZOOM system (Essen Biosciences, UK). Image J software was used to calculate the number of cells adhered. Cell counts were normalised against the positive controls of either collagen I for integrin α2β1 blocking experiments or fibronectin for integrin α5β1 blocking experiments.

2.8 | Three-dimensional wounded skin equivalents

For the generation of wounded skin equivalents, full-thickness skin equivalents were generated using primary keratinocytes and dermal fibroblasts as previously described.[12] To provide additional support for the wounded skin equivalents, an extra dermal equivalent for each full-thickness skin equivalent was also generated.[1] Briefly, full-thickness skin equivalents were harvested from inserts before using a 1 mm punch biopsy to generate a full-thickness wound. Wounded skin equivalents were placed on top of the extra dermal equivalent to provide support prior to incubation at 37°C and supplementation with 5 mL 1:1 EpiLife/DMEM (ThermoFisher Scientific, USA) containing 100 μg/mL Vitamin C (Sigma-Aldrich, UK). Wounded skin equivalents were treated in the presence/absence of 1 mg/mL PCP applied directly to the wound. Full-thickness wounded skin equivalents were harvested 1, 2, 3, and 4 weeks post-wounding, washed in sterile PBS, and fixed in 10% formalin prior to paraffin embedding.

2.9 | Haematoxylin and eosin (H&E) staining of formalin-fixed paraffin-embedded (FFPE) skin equivalent sections

H&E staining was performed on 4 μm sections of FFPE skin equivalents as previously described.[12] Images of H&E stained sections of skin equivalents were acquired by scanning slides at 40× magnification using an Aperio AT2 Slide Scanner (Leica Biosystems, Wetzlar, Germany).

2.10 | Analysis of wound closure in 3D wounded skin equivalents

Aperio ImageScope software (Leica Biosystems, Germany) was used to measure both epidermal and dermal width and thickness of 3D wounded skin equivalents at 1–4 weeks post-wounding, whilst mathematical equations (see Supplementary Table 3) were used to calculate 3D wound healing.

2.11 | Statistics

All data were analysed, tested for normal distribution and presented as mean ± SD using statistical software, GraphPad Prism Version 8 (GraphPad, San Diego, CA, USA). p <0.05 was considered significant. Unpaired t-tests were used to determine relative integrin α2, α3, α5, α6, and β1 subunit expression. Two-way ANOVA with Tukey's multiple comparisons test was used to compare the effect of PCP on integrin α2, integrin β1, p-FAK/FAK, p-Akt/Akt and p-ERK/ERK expression in wounded keratinocytes and dermal fibroblasts treated in the

[1]Al-Shaibani M., Deciphering the Role of Mesenchymal Stem Cells (MSCs) in Cutaneous Wound Healing. [PhD Thesis]. Newcastle University, 2018.

presence/absence of *ITGB1* siRNA and compare healing of 3D wounded skin equivalents treated in the presence/absence of PCP.

3 | RESULTS

3.1 | PCP promotes a trend-wise increase in FAK and ERK activation in wounded keratinocytes

Previous studies have shown that PCP promotes cutaneous wound healing in vitro by enhancing cellular proliferation.[5] To evaluate the potential interaction of PCP with the integrin α2β1 receptor and subsequent activation of downstream ERK and Akt signalling pathways to enhance cellular proliferation (Figure 1A), both primary keratinocytes and dermal fibroblasts were subjected to scratch wound induction prior to Western blot analysis of integrin α2, integrin β1 ERK, FAK, and Akt expression at 24, 48, and 72 h post-wounding. Results demonstrated no significant difference in integrin α2 and integrin β1 expression between untreated and PCP-treated wounded keratinocytes (Figure 1B–D); however, PCP promoted a trend-wise increase in both FAK and ERK activation from 48 h post-wounding (Figure 1B, E, F), with no significant effect on Akt activation observed (Figure 1B, G). Moreover, similar results were observed in wounded dermal fibroblasts, whereby PCP promoted a significant increase in ERK and FAK activation in wounded fibroblasts at 48 and 72 h post-wounding (Supplementary Figure 2, $*p$ <0.05). Taken together, these data suggest that PCP activates ERK and FAK signalling in order to promote both keratinocyte and dermal fibroblast wound closure in vitro.

3.2 | Knockdown of integrin β1 significantly impairs PCP-induced activation of ERK and FAK during keratinocyte wound closure

In order to confirm the interaction of PCP with the integrin α2β1 receptor and subsequent activation of ERK and FAK to promote keratinocyte wound closure, primary keratinocytes were subjected to transient knockdown of the integrin β1 subunit using *ITGB1* siRNA prior to scratch-wound induction and Western blot analysis of integrin α2, integrin β1, FAK, and ERK expression at 24, 48, and 72 h post-wounding. Results confirmed significant *ITGB1* siRNA-induced reduction in integrin β1 expression in both untreated and PCP-treated wounded keratinocytes (Figure 2A, B, $*p$ <0.05, $**p$ <0.01, $***p$ <0.001, $****p$ <0.0001), which also decreased integrin α2 expression (Fiigure 2C, *p <0.05, $**p$ <0.01, $***p$ <0.001). Furthermore, this decrease in integrin α2β1 expression resulted in a significant decrease in PCP-induced activation of ERK at 24 h post-wounding and FAK at 48 and 72 h post-wounding (Figure 2D, E, $*p$ <0.05, $**p$ <0.01, $***p$ <0.001), collectively suggesting that PCP interacts with the integrin α2β1 receptor to activate ERK signalling during the early phases of keratinocyte wound closure with subsequent activation of FAK occurring during later phases of keratinocyte wound closure (Figure 2F). Additionally, knockdown of integrin β1 significantly impaired PCP-induced keratinocyte wound closure (Figure 2G, $***p$ <0.001, $****p$ <0.0001), supporting PCP interaction with integrin receptors as a key event required to enhance keratinocyte wound closure. However, studies in dermal fibroblasts demonstrated that while *ITGB1* siRNA significantly decreased integrin α2β1 expression and impaired PCP-induced wound closure (Supplementary Figure 3A–C, F, $*p$ <0.01, $***p$ <0.001, $****p$ <0.0001), it did not affect ERK or FAK activation, suggesting that PCP may activate other downstream signalling pathways to promote dermal fibroblast wound closure (Supplementary Figure 3D, E, ns). Collectively, these data suggest PCP promotes keratinocyte wound closure through its interaction with integrin α2β1 and downstream activation of an ERK-FAK signalling cascade.

3.3 | PCP increases expression of integrin α2, integrin α5 and integrin β1 in keratinocytes

It has been suggested that the hydrolysis of collagen to collagen peptides exposes motifs that would typically be inaccessible to cells, with previous studies suggesting that some exposed motifs not only bind to collagen receptors but also bind to other ECM receptors expressed by cells.[15,16] To explore this potential in cutaneous cells, unwounded keratinocytes were treated in the presence/absence of 1 mg/mL PCP before analysis of cell surface expression of different integrin subunits by flow cytometry. Results demonstrated that PCP significantly increased integrin α2, integrin α5 and integrin β1 expression (Figure 3A–C, $*p$ <0.05, $**p$ <0.01) but was unable to increase integrin α3 and integrin α6 expression (Figure 3D, E). Studies in unwounded dermal fibroblasts also demonstrated PCP increased integrin α2 and integrin α5 expression but not integrin α3 or integrin α6 (Supplementary Figure 4, $*p$ <0.05, $**p$ <0.01). Taken together, these data demonstrate the ability of PCP to upregulate integrin α2β1 and integrin α5β1 expression by both unwounded keratinocytes and dermal fibroblasts.

3.3.1 | Blocking of integrin receptor α2β1 inhibits keratinocyte interaction with PCP

To further elucidate the potential interactions of PCP with integrin receptors α2β1 and α5β1 present on keratinocytes, cell adhesion assays were performed to determine a direct or indirect interaction of PCP with these integrin receptors. Results demonstrated that keratinocytes were able to adhere to collagen I, fibronectin and PCP-coated surfaces, with significantly more keratinocytes adhering to collagen I and fibronectin compared to PCP after 2 h (Figure 4, $*p$ ≤0.05), suggesting lower binding affinity between keratinocytes and PCP compared to collagen I or fibronectin. However, blocking of the α2β1 integrin receptor on keratinocytes resulted in the significant inhibition of their adherence on both collagen I and PCP-coated surfaces (Figure 4A, $**p$ ≤0.01, $***p$ ≤0.001). Moreover, results revealed blocking of the α5β1 integrin receptor had no significant effect on keratinocytes adherence to either fibronectin or PCP-coated surfaces (Figure 4B).

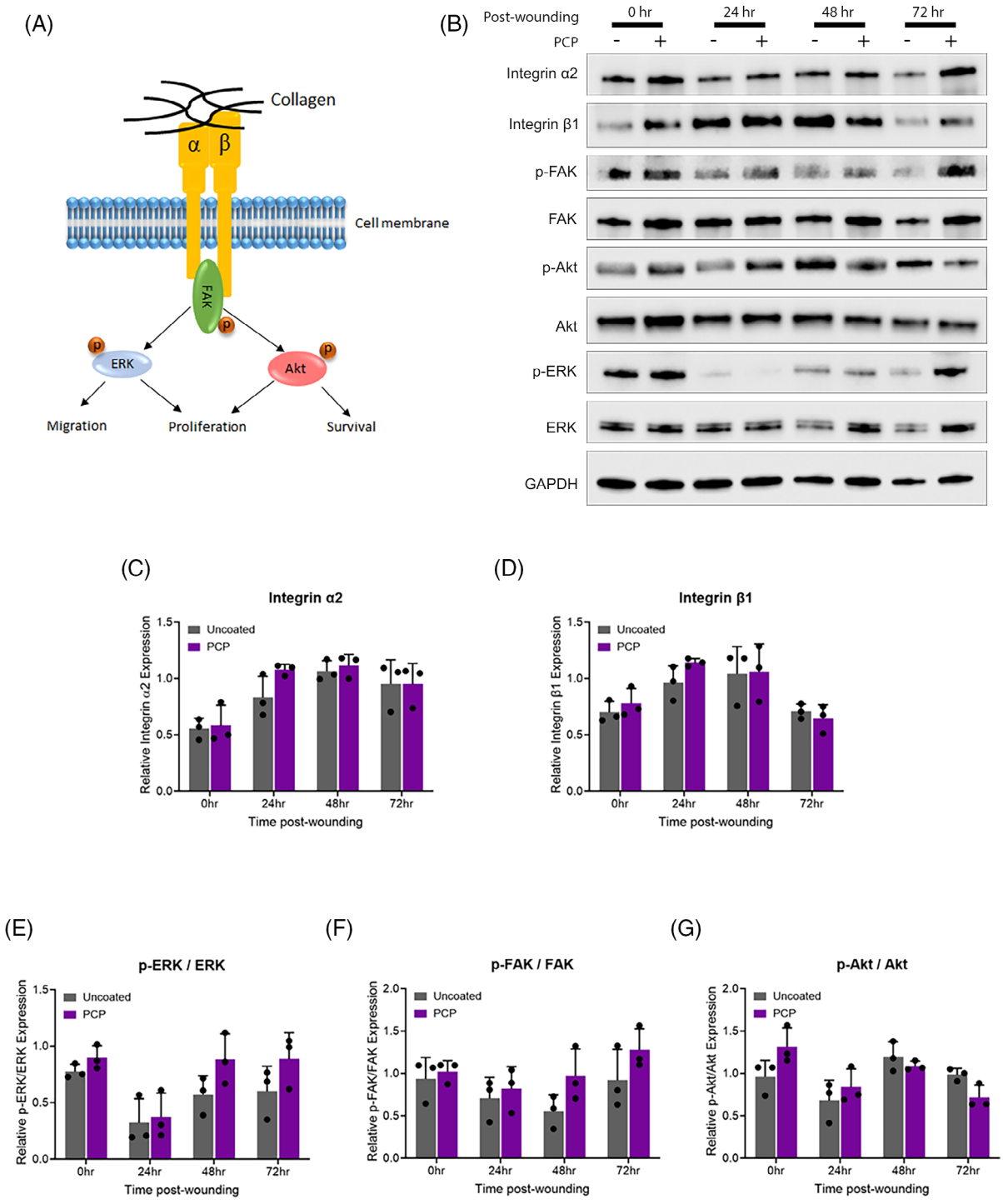

FIGURE 1 Porcine-derived collagen peptides promotes a trend wise increase in FAK and ERK signalling but not Akt in wounded keratinocytes. (A) Schematic diagram illustrating collagen interaction with cell surface integrin receptors leads to downstream activation of signalling pathways associated with cellular proliferation. (B) Representative western blot for integrin α2 (130 kDa), integrin β1 (130 kDa), p-FAK (110 kDa), FAK (110 kDa), p-Akt (60 kDa), Akt (60 kDa), p-ERK (42–44 kDa), ERK (42–44 kDa) and GAPDH (37 kDa) expression in wounded keratinocytes taken at 0, 24, 48, and 72 h post-wounding in the presence or absence of 1 mg/mL porcine-derived collagen peptides (PCP). Densitometric expression of (C) integrin α2, (D) integrin β1, (E) p-ERK/ERK, (F) p-FAK/FAK or (G) p-Akt/Akt expression relative to GAPDH expression in wounded keratinocytes taken at 0, 24, 48, and 72 h post-wounding the presence or absence of 1 mg/mL PCP (Mean ± SD, $N = 3$ independent experiments using 3 different biological samples, Two-way ANOVA with Tukey's multiple comparisons test, ns).

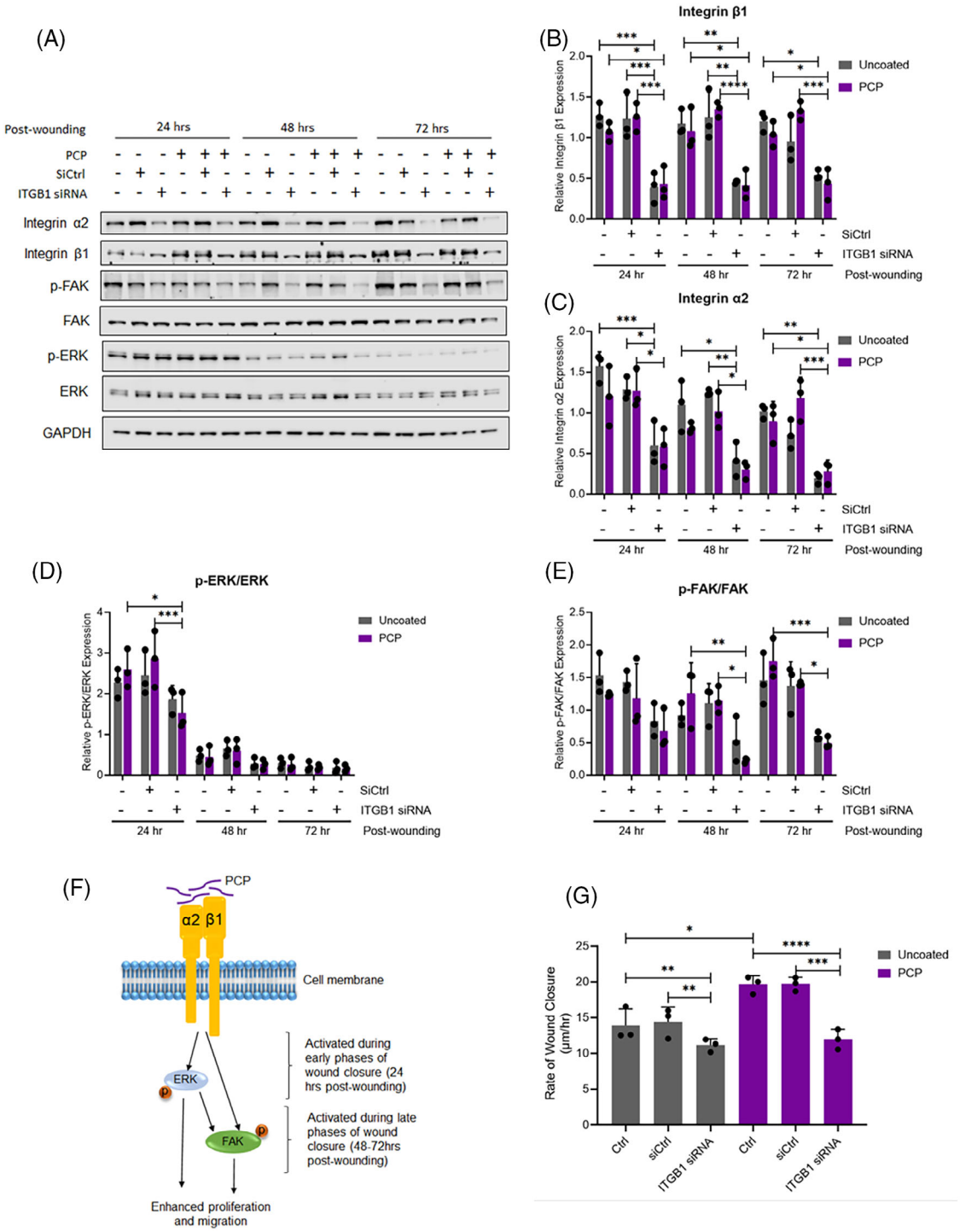

FIGURE 2 Knockdown of integrin β1 significantly impairs porcine-derived collagen peptide-induced activation of ERK and FAK and wound closure of keratinocytes. (A) Representative western blot for integrin α2 (130 kDa), integrin β1 (130 kDa), p-FAK (110 kDa), FAK (110 kDa), p-Akt (60 kDa), Akt (60 kDa), p-ERK (42–44 kDa), ERK (42–44 kDa), and GAPDH (37 kDa) expression in wounded keratinocytes at 24, 48, and 72 h post-wounding in the presence or absence of 1 mg/mL porcine-derived collagen peptides (PCP) following treatment with either SiCtrl or ITGB1 siRNA. Densitometric expression of (B) integrin β1, (C) integrin α2, (D) p-ERK/ERK, (E) p-FAK/FAK expression relative to GAPDH expression in wounded keratinocytes at 24, 48, and 72 h post-wounding in the presence or absence of 1 mg/mL PCP following treatment with either SiCtrl or ITGB1 siRNA (Mean ± SD, $N = 3$ independent experiments using 3 different biological samples, Two-way ANOVA with Tukey's multiple comparisons test, $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, $^{****}p \leq 0.0001$). (F) Schematic diagram illustrating potential downstream pathway activation following PCP binding to the integrin α2β1 receptor in order to promote keratinocyte wound closure. (G) Primary keratinocytes were seeded onto either uncoated wells or wells pre-coated with 1 mg/mL PCP before being treated with ITGB1 siRNA or siCtrl, scratch wound induction and monitoring of wound closure over 72 h (Mean ± SD, $N = 3$ independent experiments using three different biological samples, One-way ANOVA with Tukey's multiple comparisons test, $^{*}p<0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, $^{****}p \leq 0.0001$).

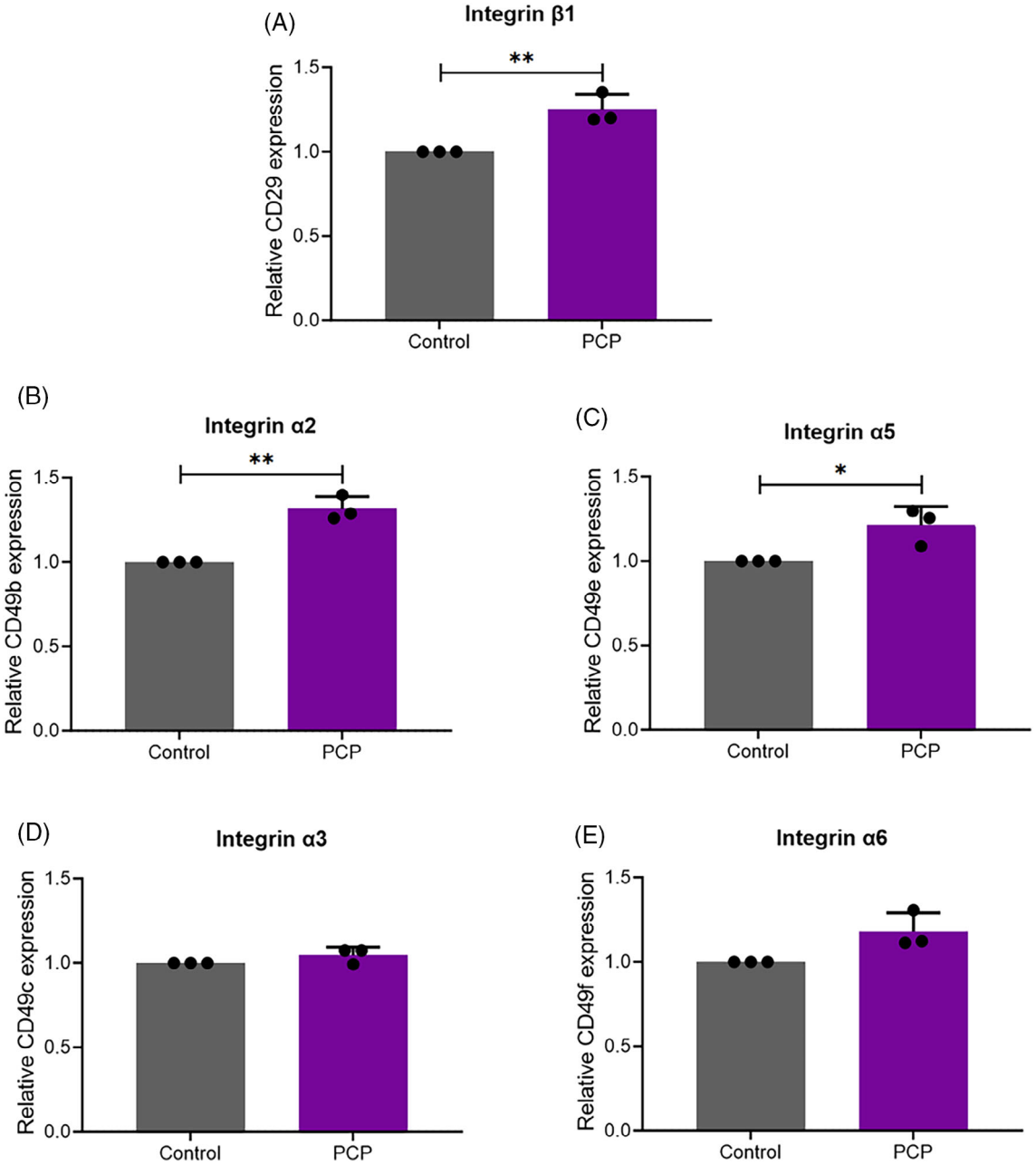

FIGURE 3 Porcine-derived collagen peptides increase expression of integrin α2, integrin α5 and integrin β1 in keratinocytes. Relative cell surface expression of (A) integrin β1, (B) integrin α2, (C) integrin α5, (E) integrin α3, and (E) integrin α6 compared to the control in unwounded primary keratinocytes treated in the presence or absence of 1 mg/mL porcine-derived collagen peptides (PCP) for 72 h before analysis using flow cytometry (Mean ± SD, $N = 3$ independent experiments using three different biological samples, Unpaired t-test, $^{*}p \leq 0.05$, $^{**}p \leq 0.01$).

Overall, these results suggest that PCP interacts with integrin α2β1 receptors to promote keratinocyte adhesion.

3.4 | PCP promotes re-epithelialisation of 3D wounded skin equivalents ex vivo

To evaluate the potential for PCP to promote cutaneous wound healing in a more representative cutaneous environment, 3D human skin equivalents were constructed on an additional dermal equivalent layer prior to the induction of a 1 mm punch biopsy wound and subsequent treatment in the presence/absence of 1 mg/mL PCP and analysis of wound closure at 1–4 weeks post-wounding (Figure 5A). Results demonstrated PCP significantly promoted wound closure by 3 weeks post-wounding by enhancing re-epithelialisation (Figure 5B, C, $^{*}p$ <0.05, $^{**}p$ <0.01). However, PCP had no significant effect on dermal regeneration (Figure 5D). Theoretically, the induction of a 1 mm punch biopsy creates a cylindrical void within the skin equivalents. Assuming equal healing occurs across the entire void of the punch wound, additional calculations were performed to determine the volume of epidermal and

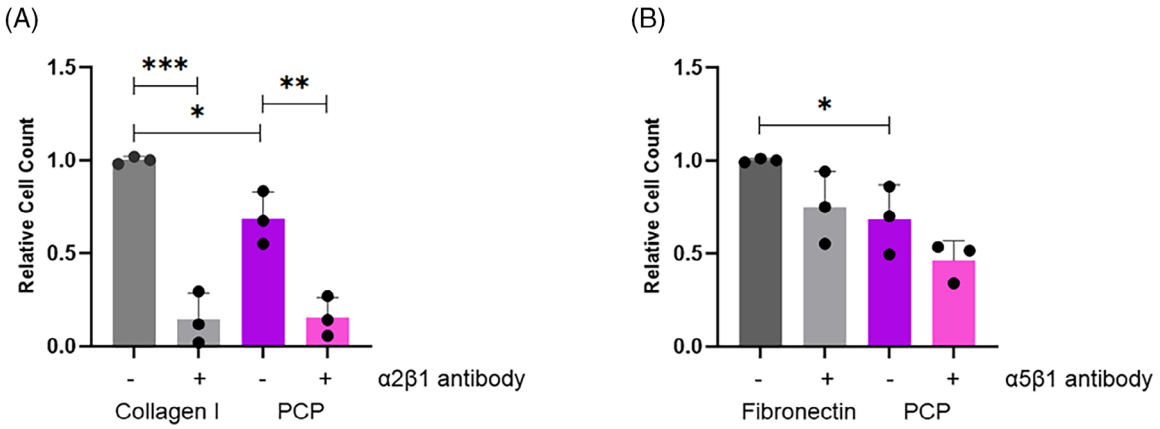

FIGURE 4 Inhibition of integrin α2β1 impairs keratinocyte adhesion to PCP. (A) Relative cell count of keratinocytes adhered to either collagen I or PCP-coated wells in the presence or absence of 10 μg/mL integrin α2β1 blocking antibody after 2 h (Mean ± SD, $N = 3$ independent experiments using three different biological samples, one-way ANOVA with Tukey's multiple comparisons test, $^{*}p$ <0.05, $^{**}p$ <0.01, $^{***}p$ <0.001). (B) Relative cell count of keratinocytes adhered to either fibronectin or PCP-coated wells in the presence or absence of 10 μg/mL integrin α5β1blocking antibody after 2 h (Mean ± SD, $N = 3$ independent experiments using three different biological samples, one-way ANOVA with Tukey's multiple comparisons test, $^{*}p$ <0.05).

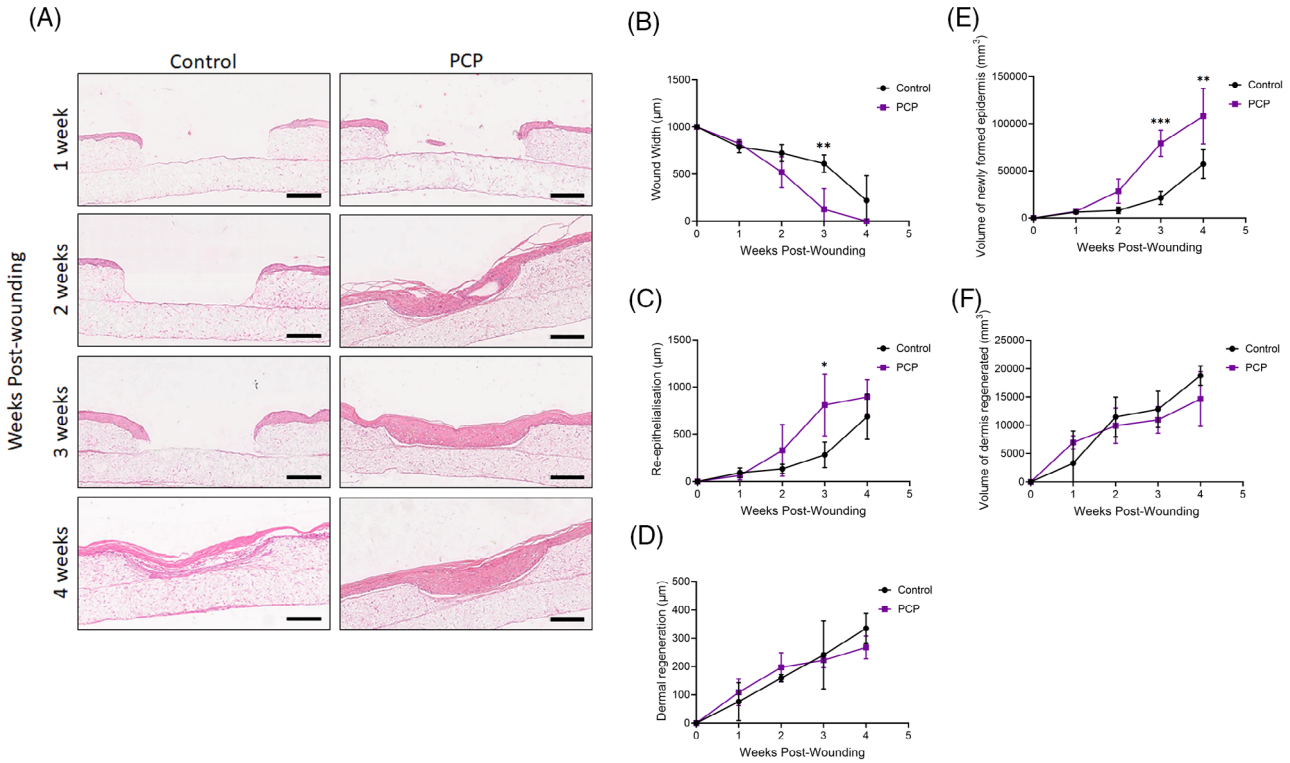

FIGURE 5 Porcine-derived collagen peptides promote re-epithelialisation of 3D wounded skin equivalents ex vivo. (A) Representative photomicrographs of H&E stained patient-matched 3D full thickness wounded human skin equivalents that were treated in the presence of absence of 1 mg/mL porcine-derived collagen peptides (PCP) added topically at 1–4 weeks post-wounding. Scale bar = 200 μm. Measurement of (B) Wound width (μm), (C) re-epithelialisation (μm) and (D) dermal recovery (μm) of 3D wounded human skin equivalents treated in the presence or absence of 1 mg/mL PCP added topically and harvested at 1–4 weeks post-wounding. (Mean ± SD, $N = 3$ independent experiments, two-way ANOVA with Tukey's multiple comparisons test, $^{*}p \leq 0.05$, $^{**}p \leq 0.01$). Volumetric analysis of newly regenerated (E) epidermis and (F) dermis in 3D wounded skin equivalents treated in the presence or absence of 1 mg/mL PCP added topically at 1–4 weeks post-wounding. (Mean ± SD, $N = 3$ independent experiments, two-way ANOVA with Tukey's multiple comparisons test, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$).

dermal regeneration. Results again confirmed significant PCP-induced re-epithelialisation by increasing the volume of the newly regenerated epidermis at 3 and 4 weeks post-wounding (Figure 5E, $^{**}p$ <0.01, $^{***}p$<0.001), whilst having no significant effect on dermal regeneration (Figure 5F). Collectively, these data suggest that PCP promotes cutaneous wound healing by enhancing re-epithelialisation.

4 | DISCUSSION

Biomaterials developed from ECM proteins such as collagen have been used in a variety of therapeutics for the treatment of both acute and chronic wounds due to their biodegradability and low toxicity; however, they are not always effective in treating every type of wound.[17] Over the years, focus has shifted towards the use of bioactive collagen peptides as a potential therapeutic strategy for treating cutaneous wounds. The hydrolysis of native collagen generates various peptides that differ in both molecular weight and bioactivity, with previous studies highlighting their beneficial biological effects such as antioxidant activity and enhanced cellular proliferation.[5,18,19]

The ECM plays a critical role in promoting cellular proliferation, migration and adhesion through various cell-ECM interactions mediated through interactions between specific motifs present within ECM proteins and cell surface receptors such as integrin receptors. Motifs present within collagen such as GFOGER interact with collagen receptors such as integrin α2β1 leading to autophosphorylation of FAK and downstream activation of MAPK and PI3K/Akt signalling pathways, promoting cellular proliferation (Figure 1A). Results demonstrated PCP-induced a trend-wise increase in both FAK and ERK activation in both keratinocytes and dermal fibroblasts, with no observed effect on Akt activation (Figure 1B, E–G, Supplementary Figure 2), suggesting PCP activates FAK and ERK signalling in order to promote cutaneous wound healing. However, whilst PCP was able to increase cell surface expression of integrin α2β1 on unwounded keratinocytes and dermal fibroblasts, PCP did not significantly increase integrin α2 or integrin β1 expression in either wounded keratinocytes or dermal fibroblasts, implying that the activation of a FAK and ERK signalling cascade by PCP may in fact occur independently of integrin binding during cutaneous wound healing (Figure 1B–D, Supplementary Figure 2A–C, Figure 3A, B, Supplementary Figure 4A, B).

Investigations into the potential interactions between PCP and keratinocytes revealed that keratinocytes were able to adhere to PCP-coated surfaces; however, relatively fewer cells adhered to PCP compared to the positive control of collagen I after 2 h (Figure 4A), thereby suggesting keratinocytes may have a lower binding affinity to PCP compared to collagen I. The fact that keratinocytes adhered more readily to collagen I compared to PCP could be due to the structural differences between native triple-helical collagen I and collagen peptides, with previous studies demonstrating that while integrin receptors present on cells can interact with collagen motifs such as GFOGER, other cell surface receptors such as discoidin domain receptors (DDRs) are only activated when binding to triple-helical collagen and thereby may not be able to interact with collagen peptides.[20–22] Furthermore, investigations into the interactions between PCP and integrin α2β1 revealed that blocking of the integrin α2β1 receptor significantly impaired keratinocyte adhesion to PCP, indicating that integrin α2β1 is integral to PCP interactions with keratinocytes (Figure 4A). Supporting the ability for collagen peptides to interact and bind to integrin α2β1, previous studies using HT1080 cells demonstrated the ability for these cells to attach to collagen peptides that contained the motif GFOGER, suggesting that the collagen peptides used in the present study may also contain the GFOGER motifs.[23] However, further studies are required to determine whether keratinocyte adhesion to PCP is due to interactions with GFOGER motifs or due to interactions with other novel motifs. Further investigations into the interaction between PCP and integrin receptors using transient knockdown of integrin β1 revealed significant impairment in expression of integrin α2β1 as well as impaired PCP-induced keratinocyte wound closure and ERK activation at 24 h post-wounding and FAK activation at 48 and 72 h post-wounding (Figure 2B–E, G), thereby confirming a potential interaction between PCP and integrin β1 receptor to activate ERK and FAK signalling during cutaneous wound healing.

Previous studies have implicated an ERK-FAK-Paxillin signalling cascade in corneal epithelial cell wound healing, demonstrating the inhibition of ERK with a MEK1 inhibitor impaired wound closure and also inhibited FAK phosphorylation and the formation of focal adhesions.[24] It is, therefore, possible that this signalling cascade may also play a role in PCP-induced keratinocyte wound closure, with early activation of ERK required to promote PCP-induced FAK activation at later stages of keratinocyte wound closure following integrin ligation (Figure 2A, D–F). Furthermore, phosphorylated ERK has been shown to be localised to cells at the wound margin, regulating the formation of lamellipodia and focal adhesions through interactions with FAK and Paxillin, further supporting the potential for PCP-induced activation of ERK to promote FAK activation and stimulate additional signalling pathways to promote keratinocyte wound closure following integrin ligation.[24] However, whilst knockdown of integrin β1 impaired PCP-induced wound closure and diminished integrin α2 expression in wounded dermal fibroblasts, there was no significant effect on FAK or ERK activation (Supplementary Figure 3B–E, G). The continued activation of ERK regardless of PCP treatment or *ITGB1* siRNA suggests PCP may not play a role in ERK activation to promote dermal fibroblast wound closure. Despite the fact that FAK activation was not significantly impaired by knockdown of integrin β1, a trend-wise decline in PCP-induced FAK activation was observed (Supplementary Figure 3E), indicating FAK may still play a role in PCP-induced wound closure of dermal fibroblasts and may be involved in the activation of other downstream signalling pathways that promote dermal fibroblast wound closure (Supplementary Figure 3E, F). Further studies are hence warranted to explore additional signalling pathways potentially activated by PCP during cutaneous wound healing.

Most understandings of cell-ECM interactions are derived from population or whole cell-based assays such as Western blots and immunostaining, where cells are viewed in a uniform state. Whilst these analyses provide averaged metrics, they do not reveal important relationships at the cell-ECM interface.[25] The use of these assays within the present study does not allow for the observation of interactions between leading wound edge and trailing cells at various time points making it difficult to draw definitive conclusions as to how PCP activates various signalling pathways to promote cutaneous wound healing. The heterogeneity and plasticity of cells during wound healing means that cells at the wound edge and trailing cells will not express the same proteins or genes at the same time, as indicated by previous

studies demonstrating ERK activation occurs in waves during collective cell migration, with ERK activation first occurring at the leading wound edge before being activated by trailing cells.[26] Additional studies need to be conducted integrating the use of reporter genes and live-cell imaging techniques to better understand PCP-induced activation of ERK-FAK signalling and determine whether this increase in activation is observed by all cells within the wound or whether its only observed within a subpopulation such as the cells at the wound edge. Despite the fact that the use of Western blotting within the present study did not clearly define which cells within the wound (leading edge vs trailing edge) are being stimulated by PCP to promote FAK and ERK activation, the results clearly demonstrate PCP mediates its effects via interactions with the integrin β1 receptor, with loss of receptor β1 expression impairing PCP-induced wound closure.

The hydrolysis of collagen to peptide fragments exposes cryptic motifs that enable collagen peptides to interact with other ECM receptors, unlike native collagen which can only interact with collagen receptors in order to promote wound healing. Previous studies demonstrated that some exposed cryptic motifs interact with integrin α5β1, a known fibronectin receptor found on the cell surface of various cells.[16] Supporting this potential, PCP increased cell surface expression of integrin α2β1 and integrin α5β1 on both unwounded keratinocytes and dermal fibroblasts, implying that whilst PCP interacts with integrin α2β1, PCP may also contain specific motifs that are capable of interacting with fibronectin receptors such as integrin α5β1 (Figure 3A–C, Supplementary Figure 4A–C). Fibronectin plays an important role during cutaneous wound healing, promoting the adhesion and migration of dermal fibroblasts, endothelial cells and keratinocytes,[27,28] and suggesting that PCP may behave in a similar manner by providing additional anchorage points within the cutaneous wound that allows keratinocytes to form adhesion complexes via integrin α5β1 and integrin α2β1 in order to build up traction, thereby promoting cellular migration and enhancing re-epithelialisation. Results from the present study revealed that the blocking of integrin α5β1 did not affect the ability of keratinocytes to adhere to PCP-coated surfaces suggesting that integrin α5β1 is not required for keratinocyte binding and adhesion to PCP (Figure 4B), thereby confirming that keratinocyte interaction with PCP via integrin α2β1 and further suggesting that the ability for PCP to upregulate integrin α5β1 expression by keratinocytes occurs through other unexplored mechanisms (Figures 3C and 4). Interestingly, integrin α5β1 recognises RGD motifs present within fibronectin, thus allowing integrin α5β1-expressing cells to adhere to fibronectin.[29] However, our data revealed that blocking of the integrin α5β1 receptor on keratinocytes did not significantly inhibit their ability to adhere to fibronectin-coated surfaces (Figure 4B). Previous studies have demonstrated that other integrin receptors that recognise fibronectin such as integrin αvβ6 are expressed by keratinocytes and play key roles in keratinocyte adhesion and migration on fibronectin during wound healing.[10,30–32] This suggests that while the blocking of integrin α5β1 prevented integrin α5β1-mediated keratinocyte adhesion, keratinocytes were potentially still able to adhere to fibronectin via integrin αvβ6. In the present study, potential interactions between various β1-containing integrins and PCP were explored; however, further studies are required in order to determine whether PCP may interact with other integrin receptors such as integrin αvβ6 to activate various downstream signalling pathways that promote keratinocyte adhesion and migration during cutaneous wound healing.

Confirming the potential for PCP to promote re-epithelialisation during cutaneous wound healing, studies in wounded ex vivo 3D skin equivalents demonstrated PCP promoted complete epidermal restoration 3 weeks post-wounding (Figure 5A–C, E). Importantly, these data support the potential use of PCP in the treatment of superficial cutaneous wounds, with the restoration of an effective epidermal barrier offering an effective means of preventing associated complications such as wound infections and dehydration.[33] Further studies are required using non-invasive fluorescent tracer dyes to accurately monitor re-epithelialisation in the wounded 3D skin equivalents to further assess the effects of PCP on wound healing and observe any cell–cell or cell-ECM interactions in real-time to help elucidate any further additional mechanisms activated by PCP during cutaneous wound healing.[34,35] Interestingly however, studies in wounded 3D skin equivalents revealed no significant effect of PCP on dermal regeneration, contradicting previous studies performed using 2D scratch assays (Figure 5A, D, F).[5] These results likely reflect the lack of environmental factors in the 2D environment such as the ECM that affect the physiology, morphology and spatial orientation of dermal fibroblasts and thereby the potential response of dermal fibroblasts to PCP treatment.[36–38]

Overall, results from the present study highlight the translational and clinical potential for PCP as a viable therapeutic approach to promote re-epithelialisation of superficial cutaneous wounds.

AUTHOR CONTRIBUTIONS

Conceptualisation: E.G. (equal), P.E.L. (equal). *Data Curation*: K.M. (lead), G.R. (supporting). *Formal Analysis*: K.M. (lead), G.R. (equal), S.V. (equal), E.G. (equal), P.E.L. (equal). *Funding Acquisition*: E.G. (equal), P.E.L. (equal). *Investigation*: K.M. (equal), G.R. (equal), S.V. (equal), R.S. (equal), E.G. (equal), P.E.L. (equal). *Methodology*: K.M. (equal), G.R. (equal), S.V. (equal), E.G. (equal), P.E.L. (equal). *Project Administration*: E.G. (equal), P.E.L. (equal); Resources: E.G. (supporting), P.E.L. (lead). *Supervision*: E.G. (supporting), P.E.L. (lead). *Validation*: K.M. (equal), G.R. (equal). *Visualisation*: K.M. (equal), G.R. (equal), S.V. (equal), E.G. (equal), P.E.L. (equal). *Writing-Original Draft Preparation*: K.M. (lead), E.G. (equal), P.E.L. (equal). *Writing-Review and Editing*: K.M. (equal), G.R. (equal), R.S. (equal), S.V. (equal), E.G. (equal), P.E.L. (equal).

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CONFLICT OF INTEREST STATEMENT

K.M. declares no conflicts of interest. P.E.L. is the CSO of AMLo Biosciences. G.R. and R.S. are employees of AMLo Biosciences. S.V. and E.G. are employees of Rousselot.

DATA AVAILABILITY STATEMENT

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

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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