A Pulsed Current Electric Field Alters Protein Expression Creating a Wound Healing Phenotype in Human Skin Cells

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A Pulsed Current Electric Field Alters Protein Expression Creating a Wound Healing Phenotype in Human Skin Cells

Running title: Electric Fields Promote Skin Cell Regeneration

Abstract

Aims

Pulsed current (PC) electric field (EF) devices promote healing in chronic wounds but the underpinning mechanisms are largely unknown. The gap between clinical evidence and mechanistic understanding limits device uptake in clinics.

Materials & Methods

Migration, proliferation and gene/protein expression profiles were investigated in the presence/absence of PCEF, in skin: keratinocytes (NHK); dermal fibroblasts (HDF); dermal microvascular endothelial cells (HDMEC) and macrophages (THP-1).

Results

While PCEF had little effect on migration or proliferation, it significantly altered the expression of 31 genes and the secretion of 7 pro-angiogenic and pro-regenerative growth factors using ELISAs.

Conclusions

PCEF significantly altered skin cell genomes/proteomes which provides some evidence of how PCEF devices promote healing of chronic wounds.

Introduction

Skin regeneration is one of the most complex biological processes that occurs in the human body. Skin wound repair requires a number of physiological processes to occur in overlapping time frames including: inflammation; reepithelialisation; angiogenesis and neodermal synthesis and remodelling. However, underlying pathophysiology (e.g. diabetes, obesity, vascular disorders) or external factors (e.g. radiation exposure) can disrupt wound repair, resulting in a non-healing chronic wound [1-3]. A chronic wound is defined as a wound that does not heal in an orderly set of stages and in a predictable amount of time the way most wounds do; wounds that do not heal within three months are often considered chronic.

Chronic wounds are a major therapeutic challenge and the disease burden of failing skin repair and non-healing ulcers is extensive [4-7]. Their lack of healing in a "normal" time frame of three months can lead to a number of morbidities and challenges for the patients which vary widely depending on underlying pathology (e.g. diabetic foot ulcers), amount of blood supply (e.g. arterial or venous ulcers), or tissue damage due to pressure (e.g. pressure ulcers) [8-12].

All wound healing processes are impaired in chronic wounds. Inflammation is excessive, with particularly high numbers of neutrophils and both neutrophils and macrophages are phenotypically different from their equivalents in normal, healing wounds with reduced phagocytic capacity leading to a build up of necrotic debris in the chronic wound bed. Keratinocytes are hyper-proliferative at the wound edge with little or no migration into/over the wound bed. They appear to be in a state of partial activation but with suppression of checkpoint regulators. The wound bed is filled with exudate, loaded with proteases and necrotic debris, degrading any growth factors secreted into the environment.

There is little angiogenesis with vessels surrounded by fibrin cuffs and little to no vessel sprouting. Finally, fibroblasts in the chronic wound bed become senescent, due to the toxic environment, and no longer proliferate or differentiate into myofibroblasts resulting in no

wound contraction and hardly any granulation tissue formation [3].

Overall, underlying pathology, infection and the resultant cell changes create an environment which facilitates the persistence of the wound in opposition to restoration of tissue and healing [13]. The mainstream options for healing chronic wounds include debridement, moisture-retentive dressings, negative pressure therapy and various topical treatments [14-16]. When a full-thickness skin wound occurs, an electric field (EF) is generated immediately, with the cathode of the EF located at the wound centre [17]. This physiological EF can be measured in acute skin wounds using a Dermacorder® (Bioelectromed, California, USA) and is generally between 100-150mV/mm [18, 19]. *In vitro* studies demonstrate that the majority of skin cell types undergo EF-mediated directional migration or galvanotaxis and can be activated by applied EFs [20] leading to increases in growth factor (GF) secretion [8] and alterations in gene and protein expression [21, 22].

Due to cell damage and senescence, it is highly likely that the physiological EF is absent in chronic wounds, although there is no published data to support this theory. This is likely due to the difficulties in access to consenting patients and the very small numbers of Dermacorders® available worldwide. It is no surprise, therefore, that applied PCEFs are very effective at healing chronic wounds. Clinical studies show that the WoundEL™ device (Mölnlycke Health Care, Göteborg, Sweden), which generates a monophasic rectangular waveform of low frequency pulses or pulsed current (PC) EF, promoted healing in wounds that had not healed during four months of hospital treatment. Following WoundEL™ treatment, the wounds started to granulate and heal within two months [23]. Applied EFs are a mainstream healthcare option for persistent, non-closing wounds in the USA, and have an A grade rating for improving healing in chronic wounds in Europe [24-27]. Although applied EFs are proven to heal chronic wounds in the clinic, the underpinning mechanisms by which applied EFs alter

wound cell behaviour to switch a chronic non-healing wound environment to a pro-healing environment are unclear and this lack of knowledge of the underlying mechanisms hinders the uptake of these devices into mainstream healthcare systems worldwide. To start to bridge the gap and help to explain why pulsed current EF devices work clinically, we investigate how PCEF alters wound healing physiological processes (migration, proliferation) and assess gene and protein expression in the presence and absence of PCEF in four of the major cell types involved in wound repair (keratinocytes, endothelial cells, fibroblasts and macrophages) to further understand and optimize this technology for the use in wound care clinics worldwide. Here, we investigate how PCEF alters wound healing physiological processes (migration, proliferation) and assess gene and protein expression at various time points in the presence and absence of PCEF (with some variance in duty cycle and frequency of pulses) in all the major cell types involved in wound repair to further understand and optimize this technology for the use in wound care clinics worldwide.

Materials and Methods

Cell culture

Primary human: keratinocyte (NHK); dermal fibroblast (HDF) and dermal microvascular endothelial cell (HDMEC) strains used in this study were derived from neonatal donors' foreskin, purchased from Invitrogen (Carlsbad, California, USA). The macrophages were derived from the human monocyte cell line THP-1 (ATCC), differentiated with 30ng/ml phorbol 12-myristate 13- acetate (PMA, Sigma-Aldrich Ltd, Gillingham, UK). The cells were cultivated and grown in media as follows: NHK - Keratinocyte growth medium containing supplement mix, 0.06mM $CaCl_2$ (PromoCell, Heidelberg, Germany) and 25U/ml penicillin and 25μg/ml streptomycin (Invitrogen, Carlsbad, California, USA), (NHK were used up to P5 and

passaged at 80%); HDF - Dulbecco's modified Eagle's medium with Glutamax, w/o pyruvate (DMEM) (Invitrogen, Carlsbad, California, USA) supplemented with 10% foetal bovine serum and 25U/ml Penicillin and 25μg/ml Streptomycin (Invitrogen), (HDF were used up to P9 and passaged at 80-90%); HDMEC - Endothelial cell growth media containing endothelial cell growth supplement (PromoCell) and 25U/ml penicillin and 25μg/ml streptomycin (Invitrogen), (HDMEC were used up to P5 and passaged at 80%); THP-1 - RPMI 1640 (Invitrogen) supplemented with 10% FBS, 2mM Glutamine, 25U/ml penicillin and 25μg/ml streptomycin (Invitrogen), THP-1 were used at P2 and passaged at 100%). For each experiment, cells were removed from liquid nitrogen and grown for 3-4 days in the humidified incubator (37ºC, 5% CO_2) before removing them from the culture plate by trypsinization and culturing on PMP collagen I- coated plates (30μg/ml, Gibco, Thermofisher, Waltham, Massachusetts, USA).

PCEF device

In order to generate the rectangular pulsed monophasic PCEF waveforms, a digital Series Arbitrary Function Generator AFG-2005 (GW-Instek, Farnell, Newark, UK) was used. For single cell migration, proliferation and the majority of gene and protein expression experiments monophasic pulses at frequencies of 128Hz (1.8% duty cycle; time 7812 μs; pulse duration 140 μs; signal off 7672 μs, amplitude of 50mA) were used. We chose this particular amplitude as it has been tested preclinical in an animal study and showed the greatest percentage of survival in rat and the lowest rate of necrosis compared with the other amplitudes [28].

Single cell migration

Single cell migration experiments were performed using the galvanotaxis method previously used and described [29]. Briefly, the method uses a customised galvanotaxis chamber that allows application of direct current (DC) or PCEF through 6cm agar bridges (2% Agar in PBS, Sigma-Aldrich Ltd). Cells were seeded at a density of 4 x 10^4/ml in the central channel of the galvanotaxis chamber, coated with collagen I for 24 hours and allowed to settle in the

humidified incubator for 2-3 hours prior to placement on a heated microscope stage. DC (100 mV/mm) or PCEF (128 Hz), generated in the lateral reservoirs by Ag/AgCl electrodes, was carried into the central channel via agar bridges. The middle reservoir was sealed with a coverslip on the top (using vacuum grease) to maintain current and prevent heat loss.

Proliferation

Cells (7 x 10^5) were seeded into autoclavable polymethylpentene (PMP) 100mm dishes (WolfLabs, York, UK), coated with collagen I for 24h prior to PCEF stimulation (128 Hz). A custom device was designed and manufactured (Figure 1) to apply PCEF to cells for up to 48 hours within a humidified incubator (37ºC, 5% CO_2). Before PCEF application, the media was changed and the central section of the device was inserted (Figure 1). PCEF was applied using agar, cured inside plastic tubes, to form a bridge between the Ag/AgCl electrode wires (1.5mm diameter) in the reservoirs, connected to the pulsed current generator box, and the media surrounding the cells. The plastic tubes were inserted through the holes in the insert. The electrical signal was measured, both before and after the experiment, using an oscilloscope (TekScope® THS730A, Berkshire, UK) (Figure 1). Cells were incubated in the presence or absence of PCEF, trypsinised to remove them from the device post-treatment and incubation and counted at different time points (24 and 48h) using a haemocytometer.

Quantitative real-time polymerase chain reaction

Cells (7 x 10^5) were seeded into 100 mm autoclavable PMP dishes coated with collagen I, 24 hours before PCEF stimulation. The custom chamber described in the proliferation method was used. All samples were incubated for either 7 or 23 hours after the 1h PCEF treatment (128 Hz) as previous studies have revealed that EF application can induce changes of gene expression after a minimum of 7h incubation time [30, 31]. Subsequently, cells were washed twice with 10 mL sterile PBS, and lysis buffer was added to the cells. The cell layer was detached using a cell scraper and frozen at -80ºC. RNA was isolated according to the Manufacturer's instructions

(Qiagen, Hilden, Germany) and cDNA synthesis was generated using the RT² First Strand Kit (Qiagen). Subsequently, RT²-PCR was performed with the RT² Profiler PCR Array (Human Wound Healing PCR Array, Qiagen) using the LightCycler 480 (Roche Diagnostics GmbH, Mannheim, Germany). Starting with raw C_T values the data was uploaded, normalized, and the biologically relevant fold-change data was exported. The C_T cut-off was 35 (C_T value is the cycle where a statistically significant increase in fluorescence above the background signal detection). The method of analysing was the relative quantification using the $2^{-\Delta\Delta C_T}$ using the Qiagen Gene globe Data Analysis on-line facility. The experiments all passed the data quality control (QC), the RT Efficiency and Genomic DNA contamination. Five reference genes were available for normalising the data (ACTB - Actin, beta; B2M - Beta-2-microglobulin; HPRT1 - Hypoxanthine phosphoribosyltransferase 1; RPLP0 - Ribosomal protein, large, P0; GAPDH - Glyceraldehyde-3-phosphate dehydrogenase). Data was assessed by fold-regulation, which represents fold-change results in a biologically meaningful way. Fold-change values greater than one indicate a positive- or an up-regulation and the fold-regulation is equal to the fold-change. Fold-change values less than one indicate a negative or down-regulation and the fold-regulation is the negative inverse of the fold-change. The experiments were repeated at least three times and a significant ($p \leq 0.05$) fold change of ≥ 1.3 compared to the untreated control is presented.

Enzyme-linked Immunosorbent assay (ELISA)

Culture medium was collected post PCEF application and subsequent incubation and used to measure protein secretion by means of ELISA assay. The ELISA assays were conducted according to the Manufacturer's instructions (Qiagen; R&D Systems, McKinley, Minneapolis, USA; Peprotech, London, UK). ELISA's were employed to detect CXCL1, IL1β, IL6, TNFα, CTGF, CCL2, FGF2, IL10, VEGF and CSF2.

Statistical analysis

All quantitative data are presented as mean ± SEM. To compare the statistical difference between any pair of data, the unpaired t-test assuming equal variance was used to calculate a p-value that is considered significant when it is less than or equal to 0.05. For measures that contained multiple groups, an ANOVA with a Tukey's multiple comparisons post-test was carried out to determine significance.

Results

Single cell migration

Keratinocyte migration is essential for reepithelialisation [1]. The average speed of NHK in the absence (NF) and presence of DCEF (100 mV/mm) and PCEF (128Hz) was 0.93±0.02 μm/min (NF and DCEF) and 0.89±0.02 μm/min (PCEF), respectively (Figure 2a). The directionality factor for NHK in the absence of DCEF was close to zero (0.081±0.02 μm/min), while in the presence of DCEF it increased by 6.8-fold to 0.55±0.03 μm/min. In contrast, the directionality factors for NHK in the absence (0.081±0.02 μm/min) or presence of PCEF (0.116±0.02 μm/min) were very similar and close to zero (Figure 2b).

Proliferation

Using our custom device (Figure 1), a PCEF of 128 Hz was applied for 1 hour and then the cells were incubated for either 23 or 47 hours, as described. PCEF had no effect on NHK number after 24 hours but decreased NHK number by 31.6% after 48 hours. In contrast, the application of PCEF had no effect on either HDF or HDMEC proliferation (Figure 3).

Gene and protein expression results

The RT² Profiler™ PCR Array Human Wound Healing was used to assess wound healing gene expression in control and PCEF-treated NHK, HDF, HDMEC and differentiated THP-1 cells. The array contains a focused panel of 84 genes that play a role in different stages of wound

healing (Supplementary Table S1 online). Cells were subjected to PCEF for 1 hour, followed

by either 7 or 23 hours of culture prior to lysis and RNA extraction, as described in the methods.

All genes where expression was significantly ($p \leq 0.05$) altered by $\geq$ 1.3-fold upon PCEF (128

Hz) treatment for 1 hour followed by incubation for either 7 or 23 hours are listed in Table 1.

In NHK, the expression of 11 genes were significantly altered. NHK gene expression was down

regulated by PCEF apart from COL5A2 expression, which was upregulated after 1 hour of

PCEF stimulation and 23 hours of incubation (Table 1). ELISA was performed to determine

the effect of PCEF application on CXCL1, IL1β, IL6 and TNFα secretion from NHK. No

significant changes to the secretion of IL1β or 1L6 were observed upon PCEF treatment in

NHK (results not shown). CXCL1 secretion from NHK was decreased by 48% (P = 0.05) after

1 hours of PCEF treatment (128 Hz) followed by 11 hours of incubation. TNFα secretion from

NHK was increased by 5.5-fold (P < 0.05) after 1 hours of PC EF treatment (128 Hz) followed

by 11 hours of incubation (Figure 4).

In HDF, the expression of 8 genes was significantly altered. Gene expression was down

regulated by PCEF apart from CTGF expression after 1 hour of PCEF stimulation and 23 hours

of incubation (Table 1). ELISA was performed to determine the effect of PCEF application on

CTGF secretion. Although significant changes to the expression of CTGF was only observed

in HDFs, ELISAs were performed on supernatants from all 4 cell types in the presence and

absence of PCEF treatment (128 Hz; I hour), followed by varying times of incubation post-

treatment. No significant changes to the secretion of CTGF were observed upon PCEF

treatment in HDFs (results not shown). However, significant changes to CTGF secretion were

seen after 1 hour of PCEF treatment in all other cell types. In NHKs, CTGF secretion was

decreased by 47.6% (p < 0.001) after 7 hours incubation post treatment, while no significant

difference was observed after 23 hours incubation post treatment (Figure 4). In HDMEC a

20.7% (p < 0.001) decrease in CTGF secretion were seen after 7 hours incubation post-

treatment. In THP-1 cells, no effect to the level of CTGF secretion was observed after 7 and 23 hours incubation post treatment, while there was an increase in CTGF secretion of 3-fold ($p < 0.001$) after 27 hours incubation post-treatment, respectively (Figure 4).

In HDMEC, the expression of 9 genes were significantly down regulated by PCEF (Table 1). None of the gene expression changes were explored with ELISA.

Finally, in differentiated THP-1 cells, the expression of 4 genes was significantly up regulated by PCEF (Table 1). ELISA was performed to determine the effect of PCEF application on CCL2 and FGF2 secretion from THP-1 cells. No significant changes to the secretion of CCL2 were observed upon PCEF treatment in THP-1 cells (results not shown). In contrast, FGF2 secretion was increased by 2.4-fold ($p < 0.01$) after 1 hour of PCEF treatment followed by incubation for 23 hours (Figure 4).

In addition to the significant gene expression changes described above, the expression of 3 genes was altered by more than 50-fold, IL10 in THP-1 cells (53 fold), Coagulation factor XIII A1 polypeptide (F13A1) in HDMEC (149 fold) and Cathepsin G (CTSG) in HDF (-271 fold) (Table 1).

ELISA was performed to determine the effect of PCEF application on IL10 in all cell types (Figure 5). All cell types were treated with PCEF (128 Hz) for 1 hour. After 11 hours of post treatment incubation, IL10 secretion was increased 2-fold ($p < 0.05$) from NHKs and 16.2-fold ($p < 0.01$) from HDMEC while, after 27 hours of post treatment incubation, IL10 was increased 2.1-fold ($p < 0.05$) from THP-1 and 3.2-fold ($p < 0.01$) from HDF (Figure 5).

Finally, as VEGF secretion and CSF2 gene expression were increased upon application of DCEF (200 mV/mm (VEGF); 100 mV/mm (CSF2)), VEGF and CSF2 ELISAs were performed on supernatants from all cell types (VEGF) and NHK (CSF2) after 1 hour of PCEF (128 Hz) (Figure 6). There was no detectable difference in VEGF secretion from HDF or HDMEC upon PCEF treatment (results not shown). There was a 29.6% ($p < 0.001$) increase and a 14.9% ($p <$

0.01) decrease in VEGF secreted from PCEF-treated NHK (128 Hz) with a post-treatment incubation of 23 and 27 hours, respectively, compared to control. In differentiated THP-1 cells, there was a 411-fold (p < 0.001) increase in VEGF secreted from PCEF (128 Hz)-treated cells incubated for 11 hours post treatment, compared to untreated controls. Upon extending the incubation period to either 23 or 27 hours post treatment, levels of VEGF secretion dropped dramatically from both PCEF treated and untreated THP-1 cells and there was no difference in secretion between treated and untreated cells (Figure 6).

In PCEF treated NHKs, CSF2 secretion was increased by 2.7-fold (p < 0.05) after 11 hours of incubation post treatment, compared to untreated controls (Figure 6).

Discussion

The ability of PCEF (128 Hz) to alter the physiological behaviour of 4 major cell types in wound healing was tested. The PCEF signal was modelled on a device used to treat chronic wounds, WoundEL™. No effect on migration speed or directionality was observed in any cell type (Figure 2). However, there was a 32% decrease in NHK proliferation upon PCEF treatment, compared to untreated NHK after 48 hours, while no changes to proliferation were observed in either HDF or HDMEC (Figure 3). The ability of an applied EF to alter the speed of migration varies widely, but previous studies on human keratinocyte strains have shown that they migrate at the same rate in the presence or absence of an applied DCEF [18], therefore it is not surprising that there was no effect on human keratinocyte migration rate in the presence of absence of an applied PCEF (Figure 2a).

Gene expression studies were performed with the Profiler RT^{2}PCR array, which contains 84 wound-related genes. Within the 4 cell types tested, there were 32 significant gene changes overall, plus 3 genes altered more than 50-fold, post PCEF treatment for 1 hour (128 Hz),

followed by 7 or 23 hours of incubation. While in NHKs and HDFs, 10/11 and 7/8 genes were down regulated, respectively, in sharp contrast, in HDMEC and differentiated THP-1 cells, 8/9 and 4/4 genes were up regulated, respectively (Table 1). ELISA confirmed that PCEF treatment significantly altered the secretion of 7 gene products: CXCL1; IL 10; FGF2; VEGF; GM CSF2; TNFα and CTGF (Figures 4-6).

PCEF-mediated alteration to cell migration and proliferation

Previously, almost all cell types measured have displayed directional migration when placed in a DCEF for a minimum of one hour *in vitro* [18]. In a DCEF, cells receive a consistent directional signal which initiates cell turning by either lamellipodial re-arrangement or physical turning followed by directional migration towards either the cathode (majority of cells [17]) or the anode (dermal fibroblasts [31] macrophages [32]). The mechanisms underpinning the directional sensing of DCEFs have recently been elucidated [33]. However, PCEF is a pulsed cathodal signal, rather than the continuous cathodal DC signal. The constant on/off pulsing of the PC signal likely underpins the lack of effect on directionality as the cells' polarity signalling scaffolds would require a continuous cathodal signal in order for the cells to turn/reform lamellipodia towards the cathode (Figure 2).

In addition, PCEF significantly altered the expression of 7 integrin genes (NHK-2; HDMEC-3; THP-1-2) which could aid the initiation of cell migration into the chronic wound bed [34, 35].

PCEFs also had little effect on cell proliferation, with only a 32% PCEF-mediated decrease in keratinocyte proliferation after 48 hours which could reflect the creation of a "migration zone" close to the wound edge, where the endogenous EF is strongest [4], as keratinocytes stop migrating when undergoing mitosis.

PCEF-mediated alterations to genes that play a role in wound Inflammation

Many of the PCEF-mediated inflammatory gene changes would benefit the healing of chronic wounds including: the reduction in secretion of CXCL1 (GROα) from NHKs, which both recruits and activates neutrophils [36]; the decrease in both pro-inflammatory IL1β and IL6 gene expression in NHKs [37], the decrease in PTGS2 (COX2) expression in both NHKs and HDFs, strongly elevated in diabetic wounds [38, 39] and a target for non-steroidal anti-inflammatory drugs; the increase in MIF gene expression in HDMEC, an anti-inflammatory factors which limits/terminates the inflammatory response [40, 41] and finally the increase in secretion of IL 10 from all 4 cell types. IL 10 is a cytokine which limits and terminates the inflammatory response, inhibiting neutrophil and macrophages infiltration [42]. Over-expression of IL10 is observed in fetal, scar-less wound healing and is a potential therapy for promoting wound healing and reducing skin scarring [43] (Figure 5).

Finally, uncontrolled and excessive protease activity is a major contributor to wound healing impairments [2]. Cathepsins contribute to the chronicity of chronic wounds and inhibitors of cathepsins (K and S) are in development for treatment of various immune disorders [44]. Two cathepsin genes were downregulated in HDF upon PCEF treatment, cathepsin L2 (CTSV) and cathepsin G (CTSG).

These PCEF-mediated changes to the pro-inflammatory signature of a chronic wound could significantly shift conditions towards a pro-healing environment. In addition, the PCEF-mediated increase in IL10 secretion and decreases in CXCL1, IL1β and IL6 gene expression could also contribute to a reduction in wound scarring and fibrosis [45].

What was quite striking was that while a 3.2-fold increase in IL10 secretion was observed in HDFs treated with PCEF (128 Hz) for 1 hour followed by 27 hours of incubation, but all other conditions remained the same, there was no increase in IL10 secretion at all (Figure 5). This underpins the importance of the particular WoundEL™ electrical signal in promoting gene and protein expression changes and, therefore, wound healing.

PCEF mediated alteration to genes that play a role in angiogenesis

Angiogenesis is essential for wound repair and in chronic wounds it can be severely disrupted, depending on the type of chronic wound [3].

PCEF treatment increased the gene expression of F13A1 (FXIII) (149-fold; HDMEC) and CCL2 (1.88-fold THP-1) (Table 1). FXIII plays an essential role in wound healing, promoting angiogenesis and stabilising granulation tissue [46]. Both congenital or acquired FXIII deficiencies are associated with impaired wound healing [47]. In addition, CCL2 plays a major role in neovascularisation in wound repair [48-50].

PCEF treatment also increased protein secretion of a number of pro-angiogenic factors including CSF2 (2.7-fold NHK); VEGF [51] (411-fold THP-1) and FGF2 [52] (37.3%- 2.4-fold THP-1) (Figures 4 and 6). CSF2 is a pleotropic GF which promotes angiogenesis by increasing VEGF expression. Indeed, topical recombinant CSF2 enhances healing and prevents recurrence of chronic venous ulcers [53, 54]. Previously a 2.5-fold increase in CSF2 gene expression in NHKs was observed upon DCEF treatment for 1 hour (100 mV/mm) [30].

PCEF-mediated changes to genes that influence/are central to fibroblast function

Fibroblasts in chronic wounds are senescent and granulation tissue is sorely absent in chronic wounds. 5 ECM genes were significantly upregulated by PCEF: COL1A1; COL4A1; COL5A3 and vitronectin (HDMECs) and COL5A2 (NHK) (Table 1). The upregulation of all 5 ECM genes could aid the healing of chronic wounds as they are all reduced or impaired in chronic wounds or associated with impaired healing [55, 56].

PCEF increased the secretion of both TNFα (5.5 fold NHK) and CTGF (THP-1). TNFα is classically thought of as a pro-inflammatory factor but it plays an important role in increasing the expression of alpha smooth muscle actin in fibroblasts and promoting fibroblast differentiation [57]. CTGF is a pleiotropic GF which promotes angiogenesis, dermal fibroblast differentiation and wound healing [58]. Indeed, endogenous wound fluid CTGF levels from

human diabetic foot ulcers correlated positively with foot ulcer healing rates [59]. The extensive and sustained PCEF mediated increases in secretion of both TNFα (5.5 fold; NHK) and CTGF ($\leq$ 3-fold; THP-1) would be very beneficial in activating the resident fibroblasts in the periphery of the chronic wound to stimulate wound repair [60, 61].

Conclusion

Chronic wounds are a major therapeutic global challenge set to rise sharply as our elderly population grows exacerbated by the increasing incidence of underlying pathophysiologies. They cause significant morbidity, mortality and lead to extensive Healthcare costs.

Applied EFs have an A-grade rating for improving chronic wound healing in Europe yet they are not a mainstream UK healthcare option due to a lack in understanding as to how these electrical stimuli actually alter cellular signalling and cell behaviour in the wound. Here, we attempt to start to bridge the gap between the laboratory and the clinic by exploring how a specific PCEF alters the behaviour of human keratinocytes, microvascular endothelial cells, macrophages and dermal fibroblasts. The results demonstrate that the applied PCEF alters gene and protein expression in the 4-major skin wound cell types. These PCEF-mediated alterations could potentially generate a less inflammatory wound environment with improved angiogenesis and fibroblast function, which could underpin the observed improvements with PCEF device treatment in the Clinic.

Future work

The PCEF signal was modelled according to a particular device, WoundEL^{TM}, that has been used successfully to treat chronic wounds since the 1980's. The current ex vivo study tested the potential mechanisms underpinning the improvement to chronic wounds observed in vivo. In our future work we aim to test the PCEF signal on tissue samples from a patient's chronic

wound edge and on biopsies over time to capture proteome changes. While 32% decrease in keratinocyte proliferation in vitro could be relevant clinically, to explore underpinning mechanisms, experiments must be performed initially in vitro and then further experiments can be designed in more complex organisms with specific gene or protein markers to determine effects in more complex organisms.

The present study demonstrates that PCEF treatment initiates significant changes that could underpin a switch in the wound environment in vivo to promote angiogenesis. We do indeed know that this signal has beneficial effects in the clinic, so we already know that PCEF application must alter cell behaviour in vivo to switch a wound from a chronic into a healing phenotype. We did not see any significant increases in antiangiogenic factors or any significant decreases in proangiogenic factors supporting the theory that the PCEF signal alters gene expression to promote angiogenesis. We are aware that porcine chronic wound models are in development and when available, it will be possible to directly test the PCEF signal in vivo.

Translational Perspectives

Bridging the gap between laboratory and clinic requires a step wise progression from the *in vitro* results presented here to clinical studies including the measurements of growth factors in chronic wound fluid before and after treatment with PCEF. In order to progress this work further, therefore, the next steps would require the design of a clinical trial to recruit patients with perhaps diabetic foot ulcers who were willing to allow tissue/fluid to be collected pre and post PCEF device application over time for gene and protein expression analysis, specifically including the proteins identified here: CXCL1; IL1β; IL6; TNFα; CTGF; CCL2; FGF2; IL10; VEGF and CSF2. The timings of observed significant changes *in vitro* could form a starting point for analysis *in vivo*. When the link between the observed *in vitro* cellular responses and

the tissue responses in chronic wounds *in vivo* is established, then PCEF devices should start to be more widely available in wound clinics worldwide.

Summary Points

- A form of PCEF significantly altered skin cell genomes/proteomes
- The genes that were differentially expressed upon PCEF treatment are involved in multiple stages of wound healing
- PCEF-dependent expression changes at the gene level translated into altered protein expression for 7 proteins tested
- WoundEl could significantly alter the wound microenvironment, promoting wound healing and reducing scarring.

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Table 1.

Gene name (for each cell type) showing significant or >50-fold PCEF mediated changes in gene expression	Gene Symbol	Fold Change in Gene Expression 8h	p Value	Fold Change in Gene Expression 24h	p Value
Keratinocyte (NHK)					
Collagen, type V, alpha 2	COL5A2	-1.80	0.135	**1.91**	**0.039**
Chemokine (C-X-C motif) ligand 1	CXCL1	**-2.59**	**0.046**	-1.43	0.412
Heparin-binding EGF-like growth factor	HBEGF	**-2.74**	**0.041**	-2.26	0.379
Interleukin 1, beta	IL1B	**-2.58**	**0.034**	-2.41	0.305
Interleukin 6 (interferon, beta 2)	IL6	**-3.88**	**0.002**	1.06	0.838
Integrin, alpha 6	ITGA6	**-3.52**	**0.016**	-1.17	0.478
Integrin, beta 3	ITGB3	**-3.37**	**0.025**	1.24	0.707
Plasminogen activator, urokinase receptor	PLAUR	**-2.29**	**0.018**	-2.35	0.415
Signal transducer & activator of transcription 3	STAT3	1.03	0.568	**-1.9**	**0.016**
Tumour necrosis factor α	TNF	**-2.72**	**0.018**	-2.97	0.402
Prostaglandin-endoperoxide synthase 2, COX2	PTGS2	**-2.74**	**0.05**	-1.15	0.954
Dermal Fibroblast (HDF)					
Cathepsin L2	CTSV	1.15	0.539	**-1.52**	**0.031**
Fibroblast growth factor 10	FGF10	1.03	0.981	**-3.24**	**0.012**
Plasminogen activator, tissue	PLAT	-1.84	0.361	**-1.49**	**0.023**
Prostaglandin-endoperoxide synthase 2, COX2	PTGS2	23.19	0.374	**-1.5**	**0.04**
TIMP metallopeptidase inhibitor 1	TIMP1	-1.19	0.3	**-1.45**	**0.02**
Collagen, type III, alpha 1	COL3A1	**-1.31**	**0.03**	**-1.31**	**0.033**
Fibroblast growth factor 7	FGF7	**-1.39**	**0.041**	-1.46	0.203
Connective tissue growth factor	CTGF	1.24	0.442	**1.33**	**0.05**
Cathepsin G	CTSG	1.01	0.877	**-271.35**	0.374
Endothelial Cell (HDMEC)					
Collagen, type I, alpha 1	COL1A1	1.44	0.335	**1.53**	**0.035**
Collagen, type IV, alpha 1	COL4A1	1.39	0.46	**2.31**	**0.001**
Collagen, type V, alpha 3	COL5A3	1.25	0.727	**2.34**	**0.001**

Integrin, alpha 2	ITGA2	1.31	0.431	**1.62**	**0.021**
Integrin, alpha 5	ITGA5	**8.67**	**0.03**	**1.26**	**0.019**
Integrin, beta 5	ITGB5	**1.6**	**0.035**	1.16	0.517
Macrophage migration inhibitory factor	MIF	**2.07**	**0.006**	1.06	0.593
Vitronectin	VTN	1.76	0.223	**2.08**	**0.019**
Ras homolog gene family, member A	RHOA	1.45	0.161	**-1.71**	**0.05**
Coagulation factor XIII, A1 polypeptide	F13A1	1.78	0.292	**148.67**	0.374
Macrophage (THP-1)					
Chemokine (C-C motif) ligand 2	CCL2	-1.59	0.131	**1.88**	**0.016**
Fibroblast growth factor 2 (basic)	FGF2	1.42	0.29	**1.59**	**0.02**
Integrin, alpha 4	ITGA4	1.08	0.98	**2.4**	**0.03**
Integrin, alpha 6	ITGA6	1.72	0.3	**2.22**	**0.04**
Interleukin 10	IL10	-1.15	0.718	**52.75**	0.373

Figure Legends

Figure 1. Experimental design for proliferation, gene and protein expression studies. Customised device designed and manufactured to apply pulsed current (PC) electric field (EF) to cells for up to 48 hours within a humidified incubator (37°C, 5% CO2).

Figure 2. Direct current (**DC) electric field (EF) (100mV/mm) and pulsed current (PC) EF (128 Hz) had no effect on NHK migration rate while only DCEF induced a directional response**. Human keratinocytes (NHK) were plated, incubated and visualised on a Nikon T200 microscope at 10x magnification in the absence (no field (NF)) or presence of DCEF (100 mV/mm) or PCEF (128 Hz). Images from multiple fields were captured at time zero and every 10 minutes for one hour using Volocity software. (**a**) Migration rate was calculated as the average distance that the cells travelled per minute (average speed μm/min) (**b**) The directionality factor was calculated as the cosine of the angle (Ө) of each cells migration path (straight line from its position at time 0 to its position at 60 mins). Data presented are means ± SEM (N = 3; ***p<0.001).

Figure 3. Pulsed current (PC) EF had no effect on human dermal fibroblast (HDF) or human dermal microvascular endothelial cell (HDMEC) proliferation but decreased human keratinocyte (NHK) number after 48 hours. 7 x 10^5 cells (NHK, HDF or HDMEC) were seeded into polymethylpentene (PMP) 100mm dishes, pre-coated with collagen I for 24h. Cells were treated with either no electric field (NF) or using our custom device, a PCEF of 128 Hz was applied for 1 hour (+PCEF). The cells were then incubated for a further 23 or 47 hours and counted using a haemocytometer. Data presented are means ± SEM (N = 3; *p<0.05).

Figure 4. Pulsed current (PC) electric field (EF) significantly altered the secretion of growth factors known to promote wound healing and regeneration. Human keratinocytes (NHK), human dermal microvascular endothelial cells (HDMEC) and human macrophages (THP-1) were treated for 1 hour with and without PCEF (128 Hz). Supernatants were collected following varying times of incubation post treatment and protein levels were determined using enzyme-linked immunosorbent assay (ELISA) (a) connective tissue growth factor (CTGF) (b) transforming growth factor (TNF)α (c) c-x-c-motif chemokine ligand 1 (CXCL1) (d) fibroblast growth factor 2 (FGF2). Corrected Absorbance Values (CAV) have been used where indicated. Data presented are means ± SEM (N = 3-6; *p<0.05, ** p<0.01, *** p<0.001)

Figure 5. Pulsed current (PC) electric field (EF) (128Hz) significantly increased the secretion of the pro-regeneration growth factor interleukin (IL) 10. (a) Human keratinocytes (NHKs), human dermal fibroblasts (HDF), human dermal microvascular endothelial cells (HDMEC) and human macrophages (THP-1) were treated for 1 hour with and without PCEF (128 Hz). Supernatants were collected following varying times of incubation post treatment and IL10 levels were determined using an enzyme-linked immunosorbent assay (ELISA) (b) HDFs were treated for 1 hour with and without PCEF at 128Hz and IL10 secretion was measured. Corrected Absorbance Values (CAV) have been used where indicated. Data presented are means ± SEM (N = 3-6; *p<0.05, ** p<0.01)

Figure 6. Pulsed current (PC) electric field (EF) significantly increased the secretion of pro-angiogenic growth factors. Human keratinocytes (NHK) and macrophages (THP-1) were treated for 1 hour with and without PCEF (128 Hz). Supernatants were collected following varying times of incubation post treatment and protein levels were determined using enzyme-linked immunosorbent assay (ELISA) (a) Vascular endothelial growth factor (VEGF) (b)

colony stimulating factor 2 (CFS2). Corrected Absorbance Values (CAV) have been used where indicated. Data presented are means ± SEM (N = 3-6; *p<0.05, ** p<0.01, *** p<0.001).

Table 1.

This table displays the significant (p ≥ 0.05) or >50-fold changes in gene expression at 8 and 24 hours evaluated by a Human Wound Healing RT² Profiler™ PCR Array.

The significant (p ≥ 0.05) or >50-fold changes are highlighted in bold and the gene names/symbols selected for further investigation in an ELISA are underlined (N =3).

Supplementary Table S1

This table displays the 84 genes in the Human Wound Healing RT² Profiler™ PCR Array

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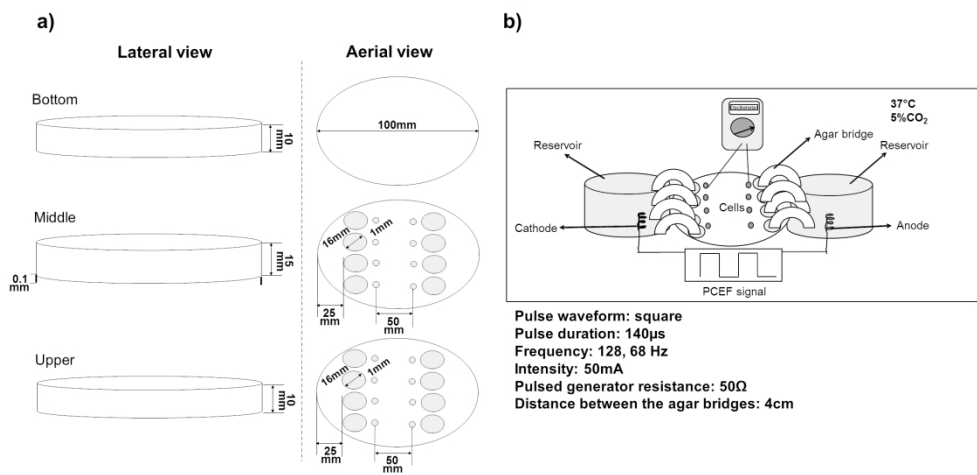
Figure 1. Experimental design for proliferation, gene and protein expression studies. Customised device designed and manufactured to apply pulsed current (PC) electric field (EF) to cells for up to 48 hours within a humidified incubator (37ºC, 5% CO2).

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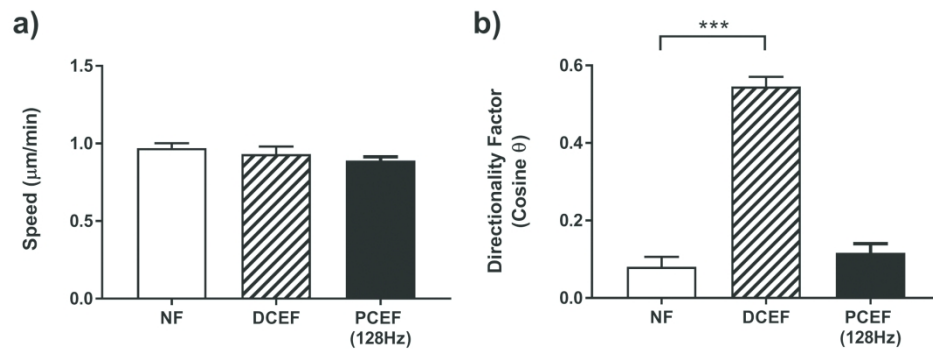
Figure 2. Direct current (DC) electric field (EF) (100mV/mm) and pulsed current (PC) EF (64 or 128 Hz) had no effect on NHK migration rate while only DCEF induced a directional response. Human keratinocytes (NHK) were plated, incubated and visualised on a Nikon T200 microscope at 10x magnification in the absence (no field (NF)) or presence of DCEF (100 mV/mm) or PCEF (128 Hz). Images from multiple fields were captured at time zero and every 10 minutes for one hour using Volocity software. (a) Migration rate was calculated as the average distance that the cells travelled per minute (average speed µm/min) (b) The directionality factor was calculated as the cosine of the angle (Ө) of each cells migration path (straight line from its position at time 0 to its position at 60 mins). Data presented are means ± SEM (N = 3; ***$p<0.001$).

Figure 3. Pulsed current (PC) EF had no effect on human dermal fibroblast (HDF) or human dermal microvascular endothelial cell (HDMEC) proliferation but decreased human keratinocyte (NHK) number after 48 hours. 7 x 105 cells (NHK, HDF or HDMEC) were seeded into polymethylpentene (PMP) 100mm dishes, pre-coated with collagen I for 24h. Cells were treated with either no electric field (NF) or using our custom device, a PCEF of 128 Hz was applied for 1 hour (+PCEF). The cells were then incubated for a further 23 or 47 hours and counted using a haemocytometer. Data presented are means ± SEM (N = 3; *p<0.05).

Figure 4. Pulsed current (PC) electric field (EF) significantly altered the secretion of growth factors known to promote wound healing and regeneration. Human keratinocytes (NHK), human dermal microvascular endothelial cells (HDMEC) and human macrophages (THP-1) were treated for 1 hour with and without PCEF (128 Hz). Supernatants were collected following varying times of incubation post treatment and protein levels were determined using enzyme-linked immunosorbent assay (ELISA) (a) connective tissue growth factor (CTGF) (b) transforming growth factor (TNF)α (c) c-x-c-motif chemokine ligand 1 (CXCL1) (d) fibroblast growth factor 2 (FGF2). Corrected Absorbance Values (CAV) have been used where indicated. Data presented are means ± SEM (N = 3-6; *p<0.05, ** p<0.01, *** p<0.001)

Figure 5. Pulsed current (PC) electric field (EF) (128Hz) significantly increased the secretion of the pro-regeneration growth factor interleukin (IL) 10. (a) Human keratinocytes (NHKs), human dermal fibroblasts (HDF), human dermal microvascular endothelial cells (HDMEC) and human macrophages (THP-1) were treated for 1 hour with and without PCEF (128 Hz). Supernatants were collected following varying times of incubation post treatment and IL10 levels were determined using an enzyme-linked immunosorbent assay (ELISA) (b) HDFs were treated for 1 hour with and without PCEF at 128Hz and IL10 secretion was measured. Corrected Absorbance Values (CAV) have been used where indicated. Data presented are means ± SEM (N = 3-6; *p<0.05, ** p<0.01)

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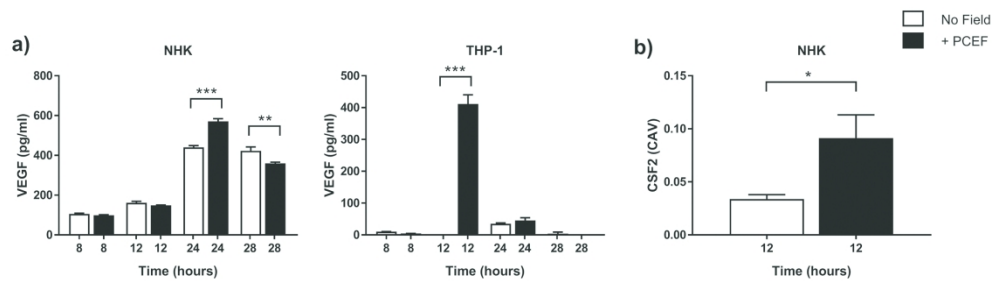
Figure 6. Pulsed current (PC) electric field (EF) significantly increased the secretion of pro-angiogenic growth factors. Human keratinocytes (NHK) and macrophages (THP-1) were treated for 1 hour with and without PCEF (128 Hz). Supernatants were collected following varying times of incubation post treatment and protein levels were determined using enzyme-linked immunosorbent assay (ELISA) (a) Vascular endothelial growth factor (VEGF) (b) colony stimulating factor 2 (CFS2). Corrected Absorbance Values (CAV) have been used where indicated. Data presented are means ± SEM (N = 3-6; *p<0.05, ** p<0.01, *** p<0.001).

Table 1. Significant and >50-fold changes in gene expression at 8 and 24 hours evaluated by a Human Wound Healing RT² Profiler™ PCR Array.

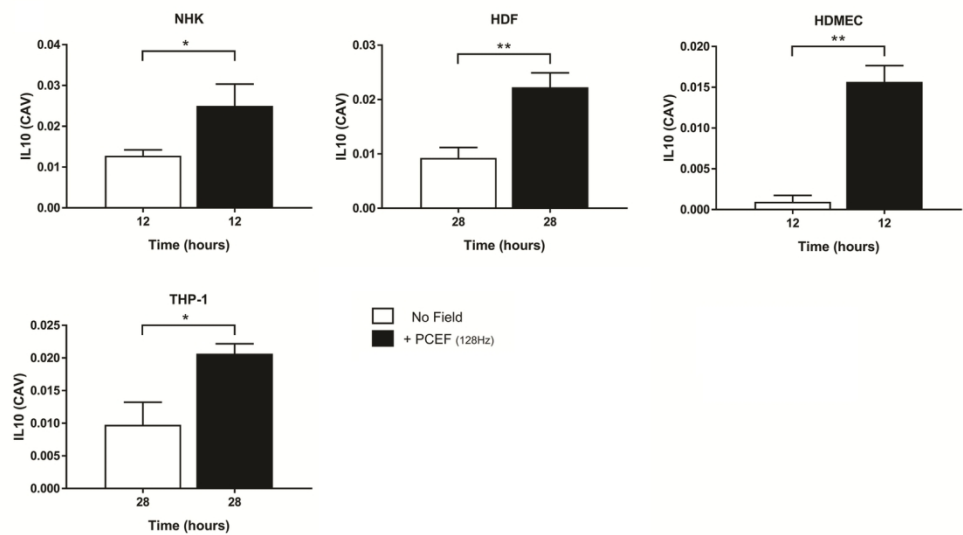|	Symbol	Fold Regulation 8h	p Value	Fold Regulation 24h	p Value
NHK					
Collagen, type V, alpha 2	COL5A2	-1.80	0.135	**1.91**	**0.039**
Chemokine (C-X-C motif) ligand 1	**CXCL1**	**-2.59**	**0.046**	-1.43	0.412
Heparin-binding EGF-like growth factor	HBEGF	**-2.74**	**0.041**	-2.26	0.379
Interleukin 1, beta	**IL1B**	**-2.58**	**0.034**	-2.41	0.305
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Integrin, beta 3	ITGB3	**-3.37**	**0.025**	1.24	0.707
Plasminogen activator, urokinase receptor	PLAUR	**-2.29**	**0.018**	-2.35	0.415
Signal transducer & activator of transcription 3	STAT3	1.03	0.568	**-1.9**	**0.016**
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HDF					
Cathepsin L2	CTSV	1.15	0.539	**-1.52**	**0.031**
Fibroblast growth factor 10	FGF10	1.03	0.981	**-3.24**	**0.012**
Plasminogen activator, tissue	PLAT	-1.84	0.361	**-1.49**	**0.023**
Prostaglandin-endoperoxide synthase 2, COX2	PTGS2	23.19	0.374	**-1.5**	**0.04**
TIMP metallopeptidase inhibitor 1	TIMP1	-1.19	0.3	**-1.45**	**0.02**
Collagen, type III, alpha 1	COL3A1	**-1.31**	**0.03**	**-1.31**	**0.033**
Fibroblast growth factor 7	FGF7	**-1.39**	**0.041**	-1.46	0.203
Connective tissue growth factor	**CTGF**	1.24	0.442	**1.33**	**0.05**
Cathepsin G	CTSG	1.01	0.877	**-271.35**	0.374
HDMEC					
Collagen, type I, alpha 1	COL1A1	1.44	0.335	**1.53**	**0.035**
Collagen, type IV, alpha 1	COL4A1	1.39	0.46	**2.31**	**0.001**
Collagen, type V, alpha 3	COL5A3	1.25	0.727	**2.34**	**0.001**

Integrin, alpha 2	ITGA2	1.31	0.431	**1.62**	**0.021**
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Integrin, beta 5	ITGB5	**1.6**	**0.035**	1.16	0.517
Macrophage migration inhibitory factor	MIF	**2.07**	**0.006**	1.06	0.593
Vitronectin	VTN	1.76	0.223	**2.08**	**0.019**
Ras homolog gene family, member A	RHOA	1.45	0.161	**-1.71**	**0.05**
Coagulation factor XIII, A1 polypeptide	F13A1	1.78	0.292	**148.67**	0.374
THP-1 (macrophage)					
Chemokine (C-C motif) ligand 2	CCL2	-1.59	0.131	**1.88**	**0.016**
Fibroblast growth factor 2 (basic)	**FGF2**	1.42	0.29	**1.59**	**0.02**
Integrin, alpha 4	ITGA4	1.08	0.98	**2.4**	**0.03**
Integrin, alpha 6	ITGA6	1.72	0.3	**2.22**	**0.04**
Interleukin 10	**IL10**	-1.15	0.718	**52.75**	0.373

Significant ($p \geq 0.05$) and >50-fold changes are highlighted in bold as well as the proteins selected for ELISA (N =3).

Supplementary Table S1 - 84 genes in the Human Wound Healing RT² Profiler™ PCR Array

Description	Symbol
1. Actin, alpha 2, smooth muscle, aorta	ACTA2
2. Actin, alpha, cardiac muscle 1	ACTC1
3. Angiopoietin 1	ANGPT1
4. Chemokine (C-C motif) ligand 2	CCL2
5. Chemokine (C-C motif) ligand 7	CCL7
6. CD40 ligand	CD40LG
7. Cadherin 1, type 1, E-cadherin (epithelial)	CDH1
8. Collagen, type XIV, alpha 1	COL14A1
9. Collagen, type I, alpha 1	COL1A1
10. Collagen, type I, alpha 2	COL1A2
11. Collagen, type III, alpha 1	COL3A1
12. Collagen, type IV, alpha 1	COL4A1
13. Collagen, type IV, alpha 3 (Goodpasture antigen)	COL4A3
14. Collagen, type V, alpha 1	COL5A1
15. Collagen, type V, alpha 2	COL5A2
16. Collagen, type V, alpha 3	COL5A3
17. Colony stimulating factor 2	CSF2
18. Colony stimulating factor 3 (granulocyte)	CSF3
19. Connective tissue growth factor	CTGF
20. Catenin (cadherin-associated protein), β1, 88kDa	CTNNB1
21. Cathepsin G	CTSG
22. Cathepsin K	CTSK
23. Cathepsin L2	CTSV
24. Chemokine (C-X-C motif) ligand 1	CXCL1
25. Chemokine (C-X-C motif) ligand 11	CXCL11
26. Chemokine (C-X-C motif) ligand 2	CXCL2
27. Chemokine (C-X-C motif) ligand 5	CXCL5

1
2
3   **28.** Epidermal growth factor   EGF
4
5   **29.** Epidermal growth factor receptor   EGFR
6
7   **30.** Coagulation factor XIII, A1 polypeptide   F13A1
8
9   **31.** Coagulation factor III   F3
10
11   **32.** Fibrinogen alpha chain   FGA
12
13   **33.** Fibroblast growth factor 10   FGF10
14
15   **34.** Fibroblast growth factor 2 (basic)   FGF2
16
17   **35.** Fibroblast growth factor 7   FGF7
18
19   **36.** Heparin-binding EGF-like growth factor   HBEGF
20
21   **37.** Hepatocyte growth factor   HGF
22
23   **38.** Interferon, gamma   IFNG
24
25   **39.** Insulin-like growth factor 1 (somatomedin C)   IGF1
26
27   **40.** Interleukin 10   IL10
28
29   **41.** Interleukin 1, beta   IL1B
30
31   **42.** Interleukin 2   IL2
32
33   **43.** Interleukin 4   IL4
34
35   **44.** Interleukin 6 (interferon, beta 2)   IL6
36
37   **45.** Interleukin 6 signal transducer (gp130, oncostatin MR)   IL6ST
38
39   **46.** Integrin, alpha 1   ITGA1
40
41   **47.** Integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 R)   ITGA2
42
43   **48.** Integrin, alpha 3 (antigen CD49C, α 3 subunit of VLA-3R)   ITGA3
44
45   **49.** Integrin, alpha 4 (antigen CD49D, α 4 subunit of VLA-4R)   ITGA4
46
47   **50.** Integrin, alpha 5 (fibronectin receptor, alpha polypeptide)   ITGA5
48
49   **51.** Integrin, alpha 6   ITGA6
50
51   **52.** Integrin, alpha V   ITGAV
52
53   **53.** Integrin, beta 1   ITGB1
54
55   **54.** Integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)   ITGB3
56
57   **55.** Integrin, beta 5   ITGB5
58
59   **56.** Integrin, beta 6   ITGB6
60
           57. Mitogen-activated protein kinase 1   MAPK1

58. Mitogen-activated protein kinase 3   MAPK3

59. Macrophage migration inhibitory factor   MIF

60. Matrix metallopeptidase 1 (interstitial collagenase)   MMP1

61. Matrix metallopeptidase 2   MMP2

62. Matrix metallopeptidase 7 (matrilysin, uterine)   MMP7

63. Matrix metallopeptidase 9   MMP9

64. Platelet-derived growth factor alpha polypeptide   PDGFA

65. Plasminogen activator, tissue   PLAT

66. Plasminogen activator, urokinase   PLAU

67. Plasminogen activator, urokinase receptor   PLAUR

68. Plasminogen   PLG

69. Phosphatase and tensin homolog   PTEN

70. Prostaglandin-endoperoxide synthase 2   PTGS2

71. Ras-related C3 botulinum toxin substrate 1   RAC1

72. Ras homolog gene family, member A   RHOA

73. Serpin peptidase inhibitor, clade E, member 1   SERPINE1

74. Signal transducer and activator of transcription 3   STAT3

75. Transgelin   TAGLN

76. Transforming growth factor, alpha   TGFA

77. Transforming growth factor, beta 1   TGFB1

78. Transforming growth factor, beta receptor III   TGFBR3

79. TIMP metallopeptidase inhibitor 1   TIMP1

80. Tumor necrosis factor   TNF

81. Vascular endothelial growth factor A   VEGFA

82. Vitronectin   VTN

83. WNT1 inducible signaling pathway protein 1   WISP1

84. Wingless-type MMTV integration site family, member 5A   WNT5A