Enhanced Neurogenic Biomarker Expression and Reinnervation in Human Acute Skin Wounds Treated by Electrical Stimulation

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Electrical stimulation (ES) is known to promote cutaneous healing; however, its ability to regulate reinnervation remains unclear. First, we show that ES treatment of human acute cutaneous wounds (n = 40) increased reinnervation. Next, to define neurophysiologic mechanisms through which ES affects repair, microarray analysis of wound biopsy samples was performed on days 3, 7, 10, and 14 after wounding. This identified neural differentiation biomarkers TUBB3 (melanocyte development and neuronal marker) and its upstream molecule FIG4 (phosphatidylinositol (3,5)-bisphosphate 5-phosphatase) as significantly up-regulated after ES treatment. To demonstrate a functional ES-TUBB3 axis in cutaneous healing, we showed increased TUBB3+ melanocytes and melanogenesis plus FIG4 and nerve growth factor expression, suggesting higher cellular differentiation. In support of this role of ES to regulate neural crest-derived cell fate and differentiation in vivo, knockdown of FIG4 in neuroblastoma cells resulted in vacuolization and cell degeneration, whereas ES treatment after FIG4-small interfering RNA transfection enhanced neural differentiation, survival, and integrity. Further characterization showed increased TUBB3+ and protein gene product 9.5+ Merkel cells during in vivo repair, after ES. We demonstrate that ES contributes to increased expression of neural differentiation biomarkers, reinnervation, and expansion of melanocyte and Merkel cell pool during repair. Targeted ES-assisted acceleration of healing has significant clinical implications.


INTRODUCTION

Reinnervation of cutaneous wounds is temporally regulated by molecular and biochemical cues during the processes of neurogenesis, synaptogenesis, and subsequent organization of the nervous tissue (Pomeroy and Kim, 2000; Singer et al., 1994). Furthermore, spatial regulation of reinnervation of the neodermis and epidermis involves neural differentiation and synthesis of functional neuropeptides (Hsu et al., 2014). During reinnervation, the central trunk of individual nerve fibers terminates in the dermis. Subsequent branches end in the dermis (Karanth et al., 1991) or extend into the overlying epidermis (Reilly et al., 1997). Mechanisms regulating epidermal reinnervation, particularly the spatiotemporal regulation of proteins that guide neural differentiation, remain ill defined.

Neural differentiation is characterized by early neurite outgrowth (Chang et al., 2013), which is required for reinnervation. Several biomarkers for neural differentiation have been identified, including mitotic spindle components (Jouhilathi et al., 2008) and lipid membrane domains (Pinvesti et al., 2001). TUBB3 has been classified as a neural-specific differentiation marker (Geisert and Frankfurter, 1989) of reinnervation in tissue regeneration (Knox et al., 2013), although at least one report suggests expression by nonneural cell types (Luduena, 1998). FIG4, a key plasma membrane protein with critical roles in phosphatidylinositol (3,4,5)-triphosphate synthesis and neural differentiation (Winters et al., 2011), has also been established as a biomarker for reinnervation. Of particular relevance for reinnervation after cutaneous injury, nerve growth factor (NGF), a key molecule in cell migration (Chen et al., 2014), has also been shown to modulate gene expression associated with neural differentiation in neural crest-derived cells such as epidermal melanocytes (Yaar et al., 1991) and Merkel cells (Vos et al., 1991), suggesting its role in promoting epidermal reinnervation after wounding.

Therapeutic strategies that can improve physiologic reinnervation of the dermis and overlying epidermis after
cutaneous injury are critical to restoring nociceptive integrity and limiting postinjury neuropathic pain. Several studies support the ability of electrical stimulation to induce reinnervation of human tissues, particularly muscle (Gordon et al., 2010, Guzman-Venegas et al., 2016); however, assessments have been largely based on functional outcomes, with much less attention focused on mechanisms by which these more favorable functional outcomes are achieved. Electrical stimulation (ES) induction of neuronal tubulin components, neurofilament 200, and c-fos gene expression have been implicated as mechanisms mediating neurite outgrowth and differentiation in chick embryos (Wood and Willits, 2006) and rats (Udina et al., 2008); however, similar mechanistic studies examining reinnervation of human tissues in response to ES are lacking. Specifically, a gap in knowledge remains regarding its effect on reinnervation of human skin after injury, despite the fact that ES has been shown to promote cutaneous wound repair in both animal models (Kloth, 2005; Ojingwa and Isseroff, 2003) and humans (Sebastian et al., 2011b).

In our initial study examining the effects of ES on human cutaneous wound healing, we identified enhanced angiogenesis and re-epithelialization in human cutaneous wounds (Sebastian et al., 2015). In addition, expression of substance P (neurotransmitter) and protein gene product 9.5 (PGP9.5) (panneuronal marker) were found to be significantly up-regulated on day 14 after injury (50- and 10-fold, respectively) in nontreated wounds compared with uninjured skin. ES treatment further increased expression of these markers (60- and 30-fold, respectively) compared with unwounded skin at that time point. This provoked the hypothesis that ES could up-regulate neural differentiation processes in wound healing. To explicitly understand the role of ES in reinnervation during repair, temporal examination of neuronal marker expression and quantification of nerve fibers in healing human cutaneous wounds was performed (days 3, 7, 10, and 14 after injury). We demonstrate that ES increases neural differentiation and reinnervation in healing human cutaneous wounds and elucidate key neural differentiation biomarkers differentially expressed in response to ES treatment.

RESULTS

Reinnervation and neuropeptide synthesis were up-regulated in ES-treated wounds

Temporal punch biopsy samples from ES-treated and non–ES-treated wounds undergoing spontaneous healing were obtained from the upper inner arm of 40 healthy volunteers on days 3, 7, 10, and 14, following the previously published experimental design (Sebastian et al., 2015) (see Supplementary Materials online). Initial expression analyses for potential biomarkers associated with ES-induced reinnervation identified a significant increase in PGP9.5 transcripts in ES-treated wounds compared with control wounds (non–ES-treated wounds) at day 14 after injury (see Supplementary Figure S1a online). Corresponding PGP9.5 protein expression in day 14 ES-treated wounds was also significantly up-regulated compared with control wounds, reaching levels seen in uninjured skin (Figures 1a and b). A trend for earlier induction of PGP9.5 (day 7) by ES was noted but was not significant. A progressive increase in PGP9.5+ nerve fibers within healing wounds was noted over time, with significant increases seen in ES-treated wounds by day 14 after injury compared with control wounds (Figure 1c and d, and see Supplementary Figure S1b). In support of ES-mediated wound reinnervation, we also noted an increase in substance P expression (Figure 1e and f) and number of substance P+ cells in wounded human skin (Figure 1g and h, and see Supplementary Figure S2 online), with significant increases noted in ES-treated wounds compared with control wounds (P < 0.05) by day 14 after injury.

TUBB3 was up-regulated in ES-treated wounds

To identify targets of ES in reinnervation, we performed whole-genome transcriptional profiling using microarrays of (i) uninjured skin, (ii) control wounds, and (iii) ES-treated wounds. (Wounds were assessed on days 3, 7, 10, and 14 after injury). TUBB3 (Akasaka et al., 2009; Shibazaki et al., 2012), a neural differentiation and melanocyte-associated gene, was identified as one of the most highly up-regulated genes (highest fold change between ES-treated and uninjured skin) in electrically stimulated wounds on healing day 14 (ES14) compared with uninjured skin and control wounds on day 14 after injury (C14) (Figure 2a, and see Supplementary Dataset S1 from microarray online). Subsequent quantitative real-time reverse transcriptase–PCR analysis confirmed up-regulation of intracutaneous TUBB3 transcription (P < 0.05) in ES and corresponding control wounds on days 10 and 14 after wounding (see Supplementary Figure S3a online) and a significant induction of TUBB3 associated with ES treatment at days 10 and 14 (~70% ES10 vs. C10 and 28% ES14 vs. C14, respectively). Western blot analysis and immunolocalization of TUBB3 in wounds also showed corresponding significant increases in the percentage of TUBB3+ cells in day-10 and -14 ES-treated wounds compared with controls (Figure 2b and c, and see Supplementary Figure S3b–f).

In addition to the quantitative differences noted in TUBB3 expression between uninjured skin, control, and ES-treated wounds, spatial differences in TUBB3 protein distribution in wounds were also noted. In normal healthy skin, TUBB3–like immunoreactivity was seen in isolated cells scattered along stratum basale, in addition to few intradermal cells (Figure 2c). TUBB3 was observed in differentiating epidermal layers in the center of healing wounds (see Supplementary Figure S3d). However, in the wound edge and the flanking neodermis, TUBB3 was scattered haphazardly in stratum basale cells, with TUBB3+ dendritic-like structures branching out to the upper layers of the epidermis (Figure 2c). Protein results confirmed significant up-regulation (P < 0.05) of TUBB3 expression in ES-treated wounds compared with nontreated healing wounds (~67% and 34% more cells were TUBB3+ in ES10 and ES14, respectively, compared with the corresponding control wounds) (Figure 2b and c, and see Supplementary Figure S3e). In wound granulation tissue, TUBB3 expression was mostly restricted to stellate and spindle-shaped cells (presumptive fibroblasts/myofibroblasts) and occasional cells lining neocapillaries (see Supplementary Figure S3f).
ES expands the pool of melanocytes, up-regulates TUBB3 expression in melanocytes, and promotes melanogenesis in cutaneous healing.

To further characterize the role of TUBB3 in wound healing, we examined melanocyte TUBB3 expression in human cutaneous wounds. Although TUBB3 has been shown to regulate melanocyte developmental lineage (Locher et al., 2013), the effect of ES on melanocyte-associated genes and gene products has not been previously reported to our knowledge. Here, we show that ES treatment significantly...

**Figure 1. Increased reinnervation and neuropeptide synthesis in ES-treated wounds.**

(a) Representative Western blot of PGP9.5 expression. (b) Quantification of PGP9.5 expression in uninjured skin and cutaneous wounds after injury. (c) Quantification of immunoreactivity to PGP9.5 (%PGP9.5 area) in uninjured skin (US), control, and ES-treated wounds. (d) IHC images of PGP9.5 expression. (e) Representative Western blot of SP expression. ES14 is ES day 14, C14 is control day 14. (f) Quantification of percentage of SP⁺ cells in uninjured skin and cutaneous wounds at 7 and 14 days after injury. (g) Quantification of IHC analysis of SP expression. (h) Representative IHC of SP expression in skin (indicated with arrows). Dotted lines indicate epidermal-dermal junction. Scale bar = 100 μm. Star indicates statistical significance of P < 0.05. C, control; D, day; DER, dermis; EP, epidermis; ES, electrical stimulation; IHC, immunohistochemistry; PGP9.5, protein gene product 9.5; SP, substance P; US, uninjured skin.
increased expression of the melanocyte-lineage specific antigen (gp100) over control wounds (ES14 vs. C14, $P < 0.05$) (Figure 2d). Although gp100 expression was found to be present in both the dermis and epidermis of healing wounds, the predominant population of gp100+ cells was localized to the epidermis of ES-treated wounds, compared with relatively fewer gp100+ cells in control wounds (see Supplementary Figure S4a online). Co-immunostaining of TUBB3 with gp100 showed that ES treatment up-regulated the number of intraepidermal TUBB3/gp100 double-positive cells ($P < 0.05$) (Figure 2e and f, and see Supplementary Figure S4a). Given the fact that TUBB3 is an active component of microtubule tracks, which transport melanin-containing melanosomes packed in mature melanocytes (Locher et al., 2013), we next investigated the role of ES in stimulating intraepidermal
melanogenesis using quantitative Masson-Fontana histochemistry (see Supplementary Figure S4b and c). The results indicate that ES promotes TUBB3 expression outside the developing nervous system and up-regulates melanogenesis, suggesting that ES may determine the extent of repigmentation in healing human skin.

Absence of proliferating melanocytes in healing wounds and up-regulated NGF expression in ES-treated wounds

To elucidate mechanisms by which ES increased numbers of melanocytes during cutaneous wound repair, we examined proliferation in gp100⁺ cells in human cutaneous wounds. The results failed to show any evidence of proliferation in gp100⁺ cells (as evidenced by Ki67⁺ co-localization in gp100⁺ melanocytes) in the wound healing niche (see Supplementary Figure S5 online), suggesting that the increased number of melanocytes after ES was due to enhanced intracutaneous differentiation and migration of resident precursor cells such as melanoblasts, diminished apoptosis, a decrease in senescence that may account for differences in cell numbers, or a combination of these mechanisms. Therefore, to further define the effect of ES on melanocyte differentiation, we examined neurotrophic protein epidermal NGF expression in control and ES-treated wounds. NGF has previously been correlated to higher chemotaxis and dendriticity of melanocytes in vitro and to higher innervation density in various tissues (Korschning and Thoenen, 1983). We found that ES-treated wounds showed significant up-regulation of NGF mRNA (Fig. S6a) and protein expression (immunohistochemistry [IHC] and Western blot analyses) (Figure 3a–d, and see Supplementary Figure S6b online) compared with control wounds. This result suggests that ES enhances melanocyte differentiation in human skin after injury partly through its ability to up-regulate NGF expression.

ES up-regulates FIG4 expression and enhances differentiation in a human neuroblastoma cell line

Having observed enhanced expression of neural differentiation genes after cutaneous injury in vivo, we investigated whether ES can promote neural differentiation in vitro. Further analysis of microarray data from our human cutaneous wounds showed up-regulation of another neural differentiation biomarker, FIG4 (phosphatidylinositol [3,5]-bisphosphate 5-phosphatase) (Winters et al., 2011), in ES14 compared with C14 wounds (see Supplementary Dataset S1 from microarray). Moreover, ingenuity pathway analysis after microarray suggested that FIG4 is an upstream signaling molecule to TUBB3. The up-regulation of FIG4 expression in ES wounds was confirmed by transcript analysis (see Supplementary Figure S7a online), IHC (P < 0.05, ES10 vs. C10 and ES14 vs. C14) (Figure 3e and f, and see Supplementary Figure S7b), and Western blot analyses (P < 0.05, ES7 vs. C7 and ES14 vs. C14) (Figure 3g and h). In uninjured skin, FIG4 expression was found to be restricted to differentiating keratinocytes (cytoplasmic) and occasional scattered cells in the dermis. In contrast, during the repair process, FIG4⁺ cells were found more widely distributed throughout the granulation tissue and the neoeipidermis (nuclear localization in cells) (see Supplementary Figure S7b).

To assess whether ES could directly induce FIG4 expression during neural differentiation, we analyzed its expression in SHSY5Y human neuroblastoma cells cultured under differentiation conditions using retinoic acid (RA) and brain-derived neurotrophic factor (BDNF) as previously described (Mastroeni et al., 2009). As anticipated, differentiation using RA+BDNF promoted up-regulation of both NeuN, a predominant postmitotic neural nuclei marker (see Supplementary Figure S8a online) and FIG4 expression (see Supplementary Figure S8b) in TUBB3⁺ cells. To determine whether ES could accentuate induction of neural differentiation biomarkers, we also subjected the cells to ES (1 hour/day for 10 days) while they were treated with RA+BDNF. The applied electric field (100 mV/mm) corresponds to the endogenous “current of injury” prevailing in cutaneous wound healing during initiation of granulation tissue, fibrosis, wound contraction, and neovascularization (Nuccitelli et al., 2008, 2011). Quantitative real-time reverse transcriptase–PCR indicated that FIG4 mRNA expression was increased on the 10th day of RA+BDNF+ES treatment compared with RA+BDNF treatment alone (P < 0.05) (see Supplementary Figure S8c). Moreover, Western blot analysis of neuroblastoma cells showed significant up-regulation of class II phosphatidylinositol-3-kinase and FIG4 after RA+BDNF+ES treatment (P < 0.05, increase of ~77% and ~43%, respectively) (Figure 4a and b).

Next, to understand the functional role of the FIG4 gene in the phosphatidylinositol (3,4,5)-triphosphate pathway (see Supplementary Figure S8d), FIG4 was knocked down in neuroblastoma cells by small interfering RNA (siRNA) transfection (see Supplementary Figure S8e and f). Intracellular vacuologenesis was observed 3 days after transfection (see Supplementary Figure S8g). Positive immunostaining results of these vacuoles for lysosomal-associated membrane protein 2 (LAMP2) suggest that these represent late-stage endosomes (Huynh et al., 2007) (see Supplementary Figure S8h). Next, we investigated whether FIG4 siRNA-transfected cells were capable of neural differentiation and whether ES could affect the process. For this, after FIG4 siRNA transfection, we subjected cells to (i) RA+BDNF and (ii) RA+BDNF+ES treatments. ES treatment of FIG4 siRNA-transfected cells grown under differentiation conditions increased nuclear NeuN, FIG4, and TUBB3 expressions (see Supplementary Figure S8i and j) and decreased LAMP2 expression (see Supplementary Figure S8k) compared with non–ES-treated cells. Quantitative real-time reverse transcriptase–PCR analysis showed a 2-fold increase in FIG4 mRNA expression after ES treatment (see Supplementary Figure S8l), which corroborated with Western blot analysis results (Figure 4c and d). FIG4 was the only protein that was up-regulated by ES treatment in both mRNA and protein analyses. Next, to assess cell integrity of differentiating cells by RA+BDNF-alone treatment after FIG4 siRNA treatment, cells were subjected to a single course of ES on the final day of RA+BDNF addition. Here, all cells were lysed within 35 minutes of ES (see Supplementary Video S1 online). In contrast, cells exposed to ES throughout differentiation (RA+BDNF+ES) after FIG4 siRNA treatment survived (see Supplementary Video S2 online). This result indicated the potential of ES to restore the integrity of neural cells after vacuologenesis and the subsequent differentiation process.
ES enhances TUBB3 expression in Merkel cells during reinnervation of cutaneous wounds in vivo

Given the induction of innervation by ES and the spatial distribution of TUBB3⁺ cells during healing, we pursued the hypothesis that ES could promote TUBB3 expression in Merkel cells. These neural crest-derived cells attached to sensory nerve fibers can be identified by the Merkel cell-selective cytokeratin 20 (CK20). TUBB3 is expressed on

Figure 3. NGF and FIG4 expression is up-regulated in human cutaneous wounds treated with ES. (a) Representative IHC and (b) quantification of NGF expression in uninjured skin (US), control (non-ES-treated), and ES-treated skin wounds at day 14. (c) Representative Western blot and (d) quantitation of NGF expression in uninjured skin, control, and ES-treated skin wounds at day 14; P < 0.05. (e) Representative IHC and (f) quantification of FIG4 expression in uninjured skin, control, and ES-treated skin wounds at day 14. (g) Representative WB and (h) quantitation of FIG4 expression in uninjured skin, control, and ES-treated skin wounds at day 14. Scale bar = 100 μm. Star indicates statistical significance of P < 0.05. C, control; ES, electrical stimulation; IHC, immunohistochemistry; NGF, nerve growth factor; US, uninjured skin.
both motor and sensory neurons, and the skin is composed of both sensory and vasomotor nerves. Therefore, the TUBB3 antibody would not differentiate between motor and sensory neurons in the skin and as such is considered a panneuronal marker in the skin.

From the co-expression results, few CK20⁺ cells in the epidermis and dermis expressed TUBB3 (see Supplementary Figure S9a online). However, TUBB3⁺/CK20⁺ cell numbers increased in the epidermis with subsequent wound healing days (Figure 5a and b). ES significantly increased TUBB3⁺/CK20⁺ cells on healing days 10 and 14 compared with respective non–ES-treated wounds (P < 0.05; fold change ES10/C10 and ES14/C14 = 1.44 and 1.50, respectively; P > 0.05 on all other healing days). Next, to determine whether ES could induce Merkel cell reinnervation, we co-stained Merkel cells with PGP9.5. A significant increase in the percentage of PGP9.5⁺/CK20⁺ cells was noted in ES-treated wounds at day 14 compared with control wounds (P < 0.05, fold change ES14/C14 = 1.48) (Figure 5c and d, and see Supplementary Figure S9b). Most PGP9.5⁺/CK20⁺ cells were localized to the dermis of wound healing tissues compared with the epidermis in uninjured skin. Taken together, the data suggest enhancement of distinct subpopulations of Merkel cells (TUBB3⁺/CK20⁺ and PGP9.5⁺/CK20⁺) after ES. Collectively, our studies suggest that ES promotes neural differentiation and reinnervation by targeting specific proteins such as TUBB3 and FIG4 during human cutaneous repair.

DISCUSSION

Reinnervation has been shown to be essential for healing without formation of chronic ulcers or pathologic scars (Johnson and Doll, 1984). Several studies have provided insights into mechanisms promoting accelerated neural differentiation and tissue regeneration (Akimoto, 2016; Migliorini et al., 2011). In complimentary clinical and experimental studies, documented imbalances of neuromediators found in denervated tissue are associated with conditions in which delayed wound healing is common. Indeed, diabetes is a good example of neural involvement (neuropathy) preceding...
the onset of wound healing problems in the form of ulcers (Ashrafi et al., 2016). However, during repair, the challenge for regenerating neurons to reach targets is counteracted by its slow rate of regeneration of 1 mm/day, together with progressive loss of regenerative capacity over time and distance (Fu and Gordon, 1995). Therefore, strategies that can accelerate neural differentiation and reinnervation hold particular promise for these problematic wounds. Our report quantitatively investigates reinnervation and the associated spatiotemporal induction of neural differentiation biomarkers in response to ES treatment in human cutaneous wounds, which to our knowledge has not been reported. ES significantly up-regulated (i) TUBB3 in human wounds, melanocyte count, TUBB3$^+$ melanocytes, and melanogenesis; (2) NGF in wound granulation tissue; (iii) FIG4 in human wounds and in vitro neural differentiation; and finally, (iv) a fraction of TUBB3$^+$ Merkel cells and reinnervation in Merkel cells. Collectively, we have evidenced an increase in reinnervation in response to ES in human cutaneous wounds and provided detailed analysis of biomarker induction by ES.

Up-regulation of TUBB3 in ES-treated wounds was associated with an increase in dendritic processes of peripheral neurons in dermis and epidermis. Shibazaki et al., noted that TUBB3 expression was cell-cycle dependent and is mediated by the binding of RE-1—silencing transcription factor to RE-1 element, which is present in the first intron of the TUBB3 gene (Shibazaki et al., 2012). Therefore, alterations in the RE-1 silencing transcription factor—TUBB3 axis could potentially cause differential expression of TUBB3. The presence of TUBB3 has been noted in the microtubules of keratinocytes (Jouhilahti et al., 2008) and fibroblasts (Drabova et al., 1998) previously, although the physiologic significance of TUBB3 expression has been ill defined in these cell types or in epidermal nerve fiber network formation during repair (Lauria et al., 2004).

As neural crest-derived cells, melanocytes share many similarities with peripheral neurons, including the presence of multiple dendritic processes, expression of similar cell surface receptors (e.g. p75NTR and c-Kit), and response to individual neurotrophins (Yaar and Park, 2012). Therefore, various pharmacological treatments have been shown to induce neurite outgrowth by overexpressing neurotrophins (Blesch et al., 2000; Hefti, 1994). NGF, in association with ES, has been reported to induce neurite outgrowth in vitro (Chang et al., 2013), which in part is correlated to enhanced cellular differentiation process. NGF binds to neural/neural cell-related surface receptor tropomyosin receptor kinase A and initiates various intracellular signaling pathways (Chang et al., 2013), leading to cell differentiation. ES enhanced NGF expression in wounds, which may explain higher chemotaxis of melanocytes from neighboring healthy interfollicular epidermis (Chou et al., 2013). Subsequent enhancement in melanogenesis is secondary to either increasing of melanocyte numbers or promoting melanogenesis in individual cells, supplying the regenerating epidermis with sufficient melanin (Paus, 2013). In addition, up-regulated TUBB3 expression in melanocytes is also an indication of higher differentiation, characterized with increased dendrite formation (Yaar et al., 1991) as observed in our in vivo results.

![Figure 5. ES expands the pool of TUBB3$^+$ Merkel cells and increases Merkel cell reinnervation during human cutaneous wound healing.](image-url)
We also observed up-regulation of TUBB3 expression after ES in Merkel cells, which are in association with nerve terminals in this case. This increased expression of TUBB3 could promote a differentiated melanocyte lineage (Uchigasaki et al., 2004). Therefore, these data provide further insight into the possible application of ES to augment innervation in undifferentiated/immature Merkel cells in the bulge area of hair follicles. Most CK20+ cells in contact with PGP9.5+ cells were found only in the dermis. However, TUBB3+/CK20+ cells were observed in both epidermis and dermis, with higher numbers in the epidermis during the final healing stages. This raises the question of whether Merkel cells migrate to the epidermis after initiation of differentiation during wound healing or, paradoxically, whether it is necessary for Merkel cells to have contact guidance with nerve elements while undergoing differentiation.

Formation of vacuoles that express late endosome markers in response to FIG4 knockdown in SHSY5Y human neuroblastoma cells in our studies is consistent with the previous work of Chow et al. (2007), who showed that inactivation of FIG4 and PIKfyve with an abrogation of phosphatidylinositol 3,5-bisphosphate synthesis resulted in late endosome accumulation (Chow et al., 2007; Zhang et al., 2007). The synthesis of phosphatidylinositol 3,5-bisphosphate from phosphatidylinositol 3-phosphate requires PIKfyve for plasma membrane lipid turnover during cell differentiation (Gary et al., 1998). The reverse reaction requires phosphatidylinositol 3,5-bisphosphate 5-phosphatase FIG4 (Duex et al., 2006), and both the proteins for the forward and backward reactions coexist in the same protein complex (Dove et al., 2009). However, our results show that the unique specificity of ES in regulating FIG4 in this multicomplex needs further investigation. In addition, the mechanism by which FIG4 may regulate keratinocyte activity, intracellular localization, and cell fate remains unknown and requires further study to precisely define the role of FIG4 in re-epithelialization during repair.

Finally, the apparent up-regulation of neuropeptides in the granulation tissue and the temporal and spatial advancement of nerve fibers in the epidermis of ES-treated wounds suggest the effect of ES to be more proximal to sensory nerves, although studies continue to examine the primary target of ES. In this context, the noninvasive methodology of ES to promote neural differentiation, in addition to our data showing increased cell survival after ES of in vitro differentiated cells, suggests new perspectives and molecular targets for ES in tissue repair. In conclusion, our findings provide evidence that ES application promotes reinnervation of human skin after acute injury and up-regulates neural differentiation biomarkers while having similar effects on melanocytes and Merkel cells in vivo. The finding of this study has potential clinical implications for targeted device-assisted acceleration of reinnervation of cutaneous wounds after injury. ES therapy may be of direct benefit in the prevention of chronic wound formation or as an adjuvant therapy to conventional treatment (debridement and skin substitute replacement) of chronic nonhealing wounds (diabetic ulcers, venous ulcers, pressure sores).

MATERIALS AND METHODS

Patient selection and recruitment
This study was conducted in accordance with the ethical principles of good clinical practice and the Declaration of Helsinki. This study received ethical approval from the local research committee (Manchester, UK), and all subjects (n = 40; see Supplementary Table S1 online) gave full written informed consent.

SHSY5Y cell culture and differentiation
Human neuroblastoma SHSY5Y cells were purchased from ATCC (CRL-2266; Manassas, VA), and cell culture is explained in Supplementary Materials.

In vitro electrical stimulation
SHSY5Y cells were subjected to degenerate waves ES waveform (Sebastian et al., 2011a) at 100 mV/mm (60 Hz).

Electrical stimulation device used on human volunteers
The electrical stimulation device used for in vivo ES-treated wounds was the Fenzian electrobiofeedback system (Fenzian Ltd., Hungerford, UK) (Sebastian et al., 2015).

Human punch biopsy sample collection for wound healing studies
The protocol that we adopted for in vivo ES studies was mentioned in our previous study (Sebastian et al., 2015). The detailed procedure is explained in Supplementary Materials.

Tissue preparation for wax embedding, section cutting, histological staining, and semiquantitation
Tissues for wax embedding were fixed in 10% neutral-buffered formalin (catalogue number F5304; Sigma-Aldrich, Dorset, UK) at 4 °C and processed as described previously (Sebastian et al., 2011b).

Microarray analysis
In microarray (n = 4), normal wound healing samples were compared with ES samples on days 3, 7, 10, and 14, explained in detail in the Supplementary Materials.

RNA isolation, cDNA synthesis, and quantitative real-time reverse transcriptase—PCR
For in vivo biopsy samples obtained on different days of healing, RNA isolation, cDNA synthesis, and quantitative real-time reverse transcriptase—PCR were performed as explained previously (Sebastian et al., 2011b). RPL32 was used as the internal control and the ΔΔCt method was used to calculate fold change of gene expression. The primers used are detailed in Supplementary Table S2 online.

IHC and immunocytochemistry
IHC staining was performed as detailed in our previous reports (Sebastian et al., 2011b). Tissue immunofluorescence and immunocytochemistry experiments are detailed in the Supplementary Materials. All antibodies and incubation parameters are detailed in Supplementary Table S3 online.

IHC image analysis
Definiens tissue studio, version 3.51 (Definiens AK, Munchen, Germany) was used to quantitate the IHC results (Sebastian et al., 2015), described in detail in the Supplementary Materials.

Western blotting
For cells. Western blotting was performed according to our previous protocol (Sebastian et al., 2015). One-step nitro blue tetrazolium/S-bromo-4-chloroindoxyl phosphate (NBT/BCIP, cat.

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Western blot band analysis
Percentage of band intensity was calculated by Image J (Fiji, 1.47; National Institutes of Health, Bethesda, MD) analysis (Sebastian et al., 2015). This is further explained in Supplementary Materials.

Masson-Fontana histochemistry
Quantitative melanin histochemistry by Masson—Fontana stain was performed as described in the Supplementary Materials.

FIG4 siRNA transfection
SHSY5Y cells (4 × 10^5 cells/well) were cultured in 22-mm collagen—coated coverslips in 6-well plates, and transfection was carried out using siRNA complexes prepared with siPORT NeoFX Transfection Reagent (Ambion, Waltham, MA) according to manufacturer's protocol.

Microscopy and nerve fiber thickness
Microscopy and nerve fiber thickness measurement were performed in NIH Image J software as described in the Supplementary Materials.

Statistical analysis
Data are presented as mean ± standard deviation from three independent experiments performed in triplicate (n = 3). Statistical analysis was calculated using one-way analysis of variance for comparison between three groups with Turkey post-hoc test, and student’s t test for comparison between two groups. Confidence intervals of 95% with corresponding P-values of 0.05 were chosen throughout analysis.

CONFLICT OF INTEREST
James Colthurst is an employee of Oxford Bioelectronics, Ltd.

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AUTHOR CONTRIBUTIONS
AB, AS, and JC conceived and designed the study, RP designed melanogenesis experiments, and AS and PH performed the experiments. AS, AB, SWV, JC, and RP analyzed and discussed the results. AS, AB, SWV, and RP wrote the manuscript.

SUPPLEMENTARY MATERIAL
Supplementary material is linked to the online version of the paper at www.jidonline.org, and at http://dx.doi.org/10.1016/j.jid.2016.09.036.

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