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Coacervate delivery of HB-EGF accelerates healing of type 2 diabetic wounds

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Abstract

Chronic wounds such as diabetic ulcers pose a significant challenge as a number of underlying deficiencies prevent natural healing. In pursuit of a regenerative wound therapy, we developed a heparin-based coacervate delivery system that provides controlled release of heparin-binding epidermal growth factor (EGF)-like growth factor (HB-EGF) within the wound bed. In this study, we used a polygenic type 2 diabetic mouse model to evaluate the capacity of HB-EGF coacervate to overcome the deficiencies of diabetic wound healing. In full-thickness excisional wounds on NONcNZO10 diabetic mice, HB-EGF coacervate enhanced the proliferation and migration of epidermal keratinocytes, leading to accelerated epithelialization. Furthermore, increased collagen deposition within the wound bed led to faster wound contraction and greater wound vascularization. Additionally, in vitro assays demonstrated that HB-EGF released from the coacervate successfully increased migration of diabetic human keratinocytes. The multifunctional role of HB-EGF in the healing process and its enhanced efficacy when delivered by the coacervate make it a promising therapy for diabetic wounds.

The global prevalence of diabetes mellitus was 382 million in 2013 and is projected to rise to 592 million by 2035.¹ Fifteen percent of diabetics will develop a skin ulcer in their lifetime resulting from neuropathies, peripheral ischemia, and infection.² Healing of diabetic ulcers does not progress normally but rather is plagued by an abnormal inflammatory response, low growth factor availability, and impaired cell function and angiogenesis, among other deficiencies.³ Unsuccessful treatment of diabetic ulcers drives exorbitant healthcare costs, leads to substantial pain and morbidity, and precedes 85% of all nontraumatic foot

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amputations.⁴ Clearly, therapies to initiate and accelerate the healing of diabetic wounds are important and timely.

Growth factors (GF) orchestrate the process of tissue repair in the body and thus hold great potential for regenerative therapies if applied strategically. However, their clinical use is currently limited by the fact that if applied alone, they are rapidly degraded and removed from the site of application.⁵ Chronic wounds have particularly high protease activity and are deficient in extracellular matrix heparan sulfate proteoglycans which play a critical role in GF signaling.^{6,7} As a result, most experimental GF therapies have required frequent applications and high dosages to have positive albeit marginal effects on healing outcomes.^{5,8} We hypothesized that a delivery system releasing the GF pre-stabilized by heparin could circumvent the deficiencies of chronic wounds and promote their healing.

Heparin-binding epidermal growth factor (HB-EGF) is a pleiotropic factor influencing many stages of the wound healing process such as reepithelialization, granulation tissue deposition and remodeling, wound contraction, and angiogenesis.^{9–12} HB-EGF is also a matrikine factor which requires the presence of heparin for binding and activation of its cell receptors.^{13,14} Thus, we developed a heparin-based coacervate delivery system to provide sustained, localized release of HB-EGF in a chronic wound environment. We designed a simple polycation, poly ethylene argininyaspartate diglyceride (PEAD) that self-assembles with heparin to form a complex coacervate, a liquid–liquid separation stabilized by electrostatic interactions between oppositely charged molecules. HB-EGF is bound to heparin and thus encapsulated by the liquid coacervate droplets and separated from the wound environment. The coacervate may then be applied directly to the wound and covered with an occlusive dressing where it will slowly and steadily release HB-EGF.¹⁵ This delivery system is well-suited for HB-EGF as it offers both protection from proteolytic degradation, and potentiation of GF bioactivity by mimicking the signaling environment in normal wounds where healing proceeds unhindered. The aim of this study was to evaluate the potential of HB-EGF coacervate as a therapy for diabetic wound healing, using a genetically type 2 diabetic mouse model.

MATERIALS AND METHODS

HB-EGF coacervate preparation

PEAD was synthesized as we have described previously.¹⁶ PEAD and heparin (Scientific Protein Labs, Waunakee, WI) were each dissolved in 0.9% saline at 10 mg mL⁻¹ and filtered at 0.22 µm to sterilize. Heparin was initially combined with recombinant human HB-EGF (R&D Systems, Minneapolis, MN) and PEAD was then added. Immediately following addition of the polycation the HB-EGF coacervate self-assembled and was visible as a turbid solution.

Diabetic mouse wound healing model

Approval was received by the University of Pittsburgh's Institutional Animal Care and Use Committee prior to beginning all animal work. Male 10-week old NON-cNZO10/LtJ mice (Jackson Labs) were fed a minimum 10% fat diet to maintain their diabetic phenotype.

Blood glucose was assessed prior to wounding and at the time of sacrifice using an AlphaTRAK veterinary glucometer (Abbott). A blood glucose level of 200 mg dL⁻¹ was considered diabetic and animals with lower levels were excluded from the study. Mice were anesthetized with isoflurane and dorsal hair was removed with depilatory cream (Nair). Two identical 5 mm full-thickness wounds, including the panniculocutaneous layer were created using a round skin biopsy punch. Saline, 1 µg Free HB-EGF, or 1 µg HB-EGF coacervate (10 µg heparin and 50 µg PEAD) was applied to the wound by sterile pipet as a 10 µl solution. Both wounds on each mouse received the same treatment to avoid cross-contamination. A Polyskin II hydrocolloid dressing (Tyco Healthcare) was applied followed by Coban self-adhesive wrap (3 M) around the midsection. Animals were sacrificed on days 3, 7, 14, and 28 and wounds and surrounding tissue were collected for histological analysis.

Histological analysis

Skin tissue was immersed in Tissue-Tek O.C.T. Compound (Sakura), frozen, and 5 µm sequential sections were cut using a MICROM HM525 cryomicrotome (Thermo Fisher Scientific). Only sections from the center of the wound were used for analysis. Slides were fixed in 2% paraformaldehyde and blocked in 5% normal goat serum (Sigma) for 1 hour at 37 °C. Primary antibodies used included rabbit anti-wide spectrum cytokeratin (1: 200, Abcam), rabbit anti-Ki-67 (1: 400, Abcam), rabbit anti-von Willebrand Factor (Vwf) (1: 400, Abcam), and anti-alpha smooth muscle actin (α-SMA) (1: 500, Sigma). Primary antibodies were incubated for either 1 hour at 37 °C or overnight at 4 °C. Goat anti-rabbit IgG Alexa Fluor 488 and Alexa Fluor 594-conjugated secondary antibodies (1: 200, Invitrogen) were incubated for 45 min at 37 °C. All slides were counterstained with 4',6-diamidino-2-phenylindole (DAPI; Sigma). Slides were also stained with hematoxylin and eosin (Ricca) or with Picosirius Red Stain Kit (Polysciences Inc.) following the manufacturer's protocols. All images were acquired using an Eclipse Ti inverted microscope (Nikon) and all image analyses were performed using NIS Elements Version 3.2 software (Nikon).

Wound healing assessments

Reepithelialization was measured in day 3 and 7 wound sections stained for pan-cytokeratin. The total wound length (WL), left neo-epithelial length (LL), and right neo-epithelial length (RL) were measured using the innermost hair follicles on each side to delineate the wound margins. Percent reepithelialization was calculated as: $\text{Reepithelialization (\%)} = \frac{(\text{LL} + \text{RL})}{\text{WL}} \times 100\%$. Proliferating keratinocytes were identified in day 3 wound sections stained for pan-cytokeratin and Ki-67 as cells coexpressing these markers in the basal layer of the neo-epidermis. The number of these cells beyond the wound margin was counted and normalized to the total number of DAPI nuclei counted in the basal epidermis. Collagen content was measured in day 14 and 28 wound sections stained with Picosirius Red. Wounds were observed under polarized light which illuminates collagenous structures bright red. The wound zone perimeter was traced as the region of interest and area fraction positive for collagen was quantified using image thresholding. Wound contraction was evaluated in day 14 and day 28 sections stained with hematoxylin and eosin. The gap between wound margins, indicated by the innermost hair follicles on each side of the wound, was measured and reported as the contraction gap. Blood vessels were observed in day 28 wound sections stained for vWF and α-SMA. The number of endothelial tubes was counted as vWF-positive

cross-sectional or longitudinal tubes in the neo-dermal region. Mature vessels were counted as those endothelial tubes which also had adjacent α -SMA-positive cells. Both measurements were normalized to the total wound area (mm^2) for each wound, determined using the same boundary delineations described above.

Keratinocyte scratch migration assay

Diabetic and normal human epidermal keratinocytes (Lonza) were cultured in Keratinocyte Growth Medium-Gold (Lonza) and used at between passages 3 and 4. Cells were seeded at 2×10^6 per well in a 24-well plate and cultured to confluency. A single scratch was made through the center of each well using a 1 mL pipette tip and wells were then rinsed once with Dulbecco's Phosphate-Buffered Saline (DPBS) to remove cell debris and 0.5 mL media was added. HB-EGF dosages of 10, 100, or 250 ng mL^{-1} were added to the media in free-form or incorporated within the coacervate, formed at a 500: 100: 1 mass ratio of PEAD:heparin:HB-EGF. One group of diabetic human epidermal keratinocytes (DHEK) and the normal human epidermal keratinocytes (NHEK) received no HB-EGF as controls. Scratch width was measured in the center of 3 wells per group at 0, 6, 12, and 18 hours and reported as percent scratch closure.

Statistical analysis

Statistical analysis was performed using Minitab 16.0 software (Minitab, State College, PA). Data was tested for normality and equal variance before analysis. Statistical differences were calculated using analysis of variance (ANOVA) followed by Tukey post-hoc testing. Differences were considered significant at $p < 0.05$.

RESULTS

HB-EGF coacervate accelerates reepithelialization of diabetic wounds

To evaluate the effects of HB-EGF in diabetic wounds, we used the NONcNZO10/LtJ strain of genetically type 2 diabetic mice available from Jackson Labs. Although porcine skin is most similar to that of humans, there is no established porcine model that fully captures the pathology of type 2 diabetes. Of the rodent models currently available, the NONcNZO10/LtJ best resembles the presentation of type 2 diabetes in humans.^{17–19} We created 5 mm full-thickness excisional dorsal wounds, then applied a single dose of HB-EGF coacervate (containing 1 μg HB-EGF), the same amount of HB-EGF in free-form, or saline only, and tracked healing over four weeks. We first assessed the rate of reepithelialization, characterized by epithelial cell migration into the wound to form a new protective epidermis. We identified epidermal cells in the wounds by immunofluorescence staining for cytokeratin and reported their lengths of migration as a percent of the total wound length (Figure 1A). Three days after wounding, we observed that HB-EGF coacervate had significantly increased reepithelialization compared with the other groups (Figure 1B). Furthermore, the same 1 μg dose of HB-EGF applied in free-form without the protection of the coacervate showed no significant difference from the saline group. This trend continued to day 7, at which point reepithelialization in the HB-EGF coacervate group was more than 85% complete whereas in saline and free HB-EGF groups it was less than 55%. These data

indicate that HB-EGF coacervate can induce diabetic mouse epidermal cell migration and we next evaluated its effect on their proliferative capacity.

HB-EGF coacervate stimulates diabetic keratinocyte proliferation

In the classical paradigm of wound repair, keratinocyte proliferation occurs to contribute to the reepithelialization process, however, it is limited to the cells located at the wound margin.²⁰ We wondered if stimulation by HB-EGF throughout the wound bed might encourage epidermal cells to maintain their proliferative capacity even as they migrated into the wound. We identified proliferating keratinocytes in the basal layer of the neo-epidermis by immunofluorescence staining for Ki-67, which is exclusively expressed in the nuclei of cells in active cell cycle.²¹ As expected, in the saline and free HB-EGF treated wounds, Ki-67⁺ keratinocytes were mostly located at the wound margin on day 3 (Figure 2A and B), and only 24% and 28% of keratinocytes beyond the wound margin were proliferative, respectively (Figure 2D). Conversely, 61% of keratinocytes beyond the wound margin were positive for Ki-67 in HB-EGF coacervate treated wounds and these cells were distributed throughout the basal neo-epidermis, even out to the tip of the leading edge (Figure 2C). These results demonstrate that HB-EGF can increase keratinocyte proliferation during diabetic wound repair, provided sufficient concentrations of active GF exist in the wound bed. They also indicate that cell proliferation likely contributed to the faster reepithelialization observed in HB-EGF coacervate wounds, although the relative contributions of migration and proliferation to this end require further investigation.

HB-EGF coacervate increases collagen deposition in diabetic wounds

To further test the benefit of HB-EGF coacervate in diabetic wound healing, we examined the wound granulation and remodeling processes after two and four weeks. We evaluated the quantity and arrangement of collagen fibers in Picosirius Red stained wound sections by polarized light microscopy. The stain accentuates the birefringent properties of collagen and allows observation as bright red fibers against a black background, permitting quantification of the collagen-positive area (Figure 3A). Within the granulation region on day 14, there was no statistically significant difference in collagen-positive area between groups (Figure 3B). However, by day 28 collagen content in wounds treated with HB-EGF coacervate was more than twice that of the other groups and closest to that of the native NON-cNZO10 mouse dermis with most fibers in parallel orientation, although the fibers were shorter and thinner. These data indicate that over four weeks, HB-EGF plays a role in the activities of dermal fibroblasts in the wound bed including provisional matrix remodeling and collagen deposition.

HB-EGF coacervate enhances diabetic wound contraction

The functional readout of a healthy, collagenous wound matrix is wound contraction. During this process myofibroblasts anchor to the collagen fibers and pull the wound margins together. We evaluated wound contraction at week 2 and week 4 by measuring the gap between the wound margins, identified by the innermost hair follicle on each side of the wound (Figure 4A–C). On day 14, wounds treated with HB-EGF coacervate had a significantly smaller contraction gap compared with saline treated wounds, while free HB-EGF showed no difference compared with either group (Figure 4D). On day 28, contraction

in HB-EGF coacervate treated wounds was nearly complete and the contraction gap was significantly smaller than in both the saline and free HB-EGF groups. Clearly, the robust collagen matrix in HB-EGF coacervate wounds preceded an improved contraction response and faster wound closure.

HB-EGF coacervate encourages diabetic wound vascularization

Another sign of neo-dermal maturity is the formation of blood vessels to support the new tissue. We used immunofluorescence staining to evaluate the vessel numbers present in the granulation region on day 28, staining for vWF as an endothelial cell (EC) marker and α -SMA to identify mural cells (pericytes or smooth muscle cells) (Figure 5A). We found significantly more endothelial tubes within the neo-dermis of HB-EGF coacervate treated wounds compared with others (Figure 5B), and also more mature vessels, classified as those endothelial tubes which had mural cell stabilization (Figure 5C). These data demonstrate that sustained delivery of HB-EGF can promote wound vascularization in a diabetic wound environment. We conclude that HB-EGF coacervate treatment leads to an advanced state of healing compared with saline and free HB-EGF treatment by four weeks after wounding.

HB-EGF coacervate stimulates migration of diabetic human cells in vitro

As a test of clinical relevance, we assessed the effect of HB-EGF coacervate on diabetic human epidermal keratinocytes (DHEK) in a scratch wound migration assay. We scratched confluent monolayers of the cells to mimic epithelial injury and then measured the percent scratch closure. With no HB-EGF added, DHEK displayed significantly impaired migration at 18 hour post-wounding compared with normal human epidermal keratinocytes (NHEK) (Figure 6A). However, 10 ng mL⁻¹ of HB-EGF, both free and in the coacervate, significantly increased DHEK migration by 6 hours, and the scratches were completely healed by 12 hours (Figure 6B). Overexpression of HB-EGF has been previously shown to exert an inhibitory effect on epithelial cells.²² Consistent with those findings, we observed no significant benefit of free HB-EGF at concentrations of 100 ng mL⁻¹ at 6 hours and 250 ng mL⁻¹ at 12 and 18 hours (Figure 6C and D). Conversely, those same concentrations of HB-EGF delivered by the coacervate did significantly stimulate cell migration compared with the DHEK control group and the corresponding free HB-EGF group. These data demonstrate that HB-EGF coacervate can stimulate diabetic human cells and can circumvent the limitations of free GF administration.

DISCUSSION

Conventional treatment of diabetic ulcers includes debridement, off-loading pressure, and maintenance of a moist wound environment with frequent dressing changes.² If wounds do not show signs of healing with standard treatment, advanced biological therapies are used such as Regranex, the only recombinant GF therapy currently approved by the FDA for skin wound healing.²³ Regranex contains recombinant human platelet-derived GF (PDGF) suspended in a carboxymethyl cellulose gel which offers minimal protection of the GF, leaving it prone to rapid degradation in the protease-rich wound environment.⁶ Frequent applications and high doses are therefore necessary to produce a positive, albeit marginal effect on healing outcomes.²⁴ However, high doses also pose a risk of inducing tumor

formation²⁵ and can also be cost-prohibitive in low- and middle-income countries where diabetes is also prevalent.¹ Unfortunately, these shortcomings have significantly limited the clinical use of Regranex.^{26,27} Dosage reduction is therefore a key aim of our approach. In this study, we used a GF dosage per wound area that is approximately 1,000-fold lower than Regranex. Furthermore, the coacervate is phase-separated from wound fluid which keeps the GF localized to site of application by preventing systemic uptake. Altogether, we do not anticipate undesirable side effects of this therapy in humans.

The half-lives of GFs exogenously applied in the body are very short and are likely to be even shorter in a wound environment that has high proteolytic activity.⁶ Consequently, numerous delivery systems have been developed for protection and sustained release of EGF family proteins and tested in animal models of diabetic wounds.⁸ One focus has been employment of sulfated proteoglycans such as heparan sulfate, which play a key role in the human body of providing stabilization, localization, and potentiated bioactivity of signaling molecules.^{7,28} These benefits have motivated the incorporation of heparin or heparin-mimetic components into many different types of delivery systems for proteins.²⁹ However, few systems use heparin in its native form, unconjugated to any scaffold or gel. Covalent conjugation may affect properties such as its charge density and macromolecular structure which could reduce its binding affinity to certain proteins which rely on specific oligosaccharide sequences.^{30–32} Several benefits of heparin in its native form have been elucidated by complexing it with peptide amphiphiles for protein delivery.^{33,34} Intact heparin is particularly appealing for delivery of HB-EGF as it is required for binding and phosphorylation of EGF cell receptors.^{13,14} This motivated our development of a simple delivery system utilizing intact heparin to load HB-EGF and facilitate its biological activities.

PEAD is a synthetic polycation designed to mimic the strongly cationic domain of the fibroblast GF receptor (FGFR) which interacts with heparin through polyvalent charge attraction.^{35,36} By similar mechanism, PEAD: heparin: GF complexes self-assemble and form micron-sized coacervate droplets which are phase-separated from water and thereby protect the incorporated GF from proteolysis.¹⁶ Preservation of heparin in its native conformation enables its interaction with cell receptors and potentiates GF bioactivity.^{37–42} The coacervate incorporates 1 µg HB-EGF with greater than 92% loading efficiency and then releases approximately 15 ng per day with no initial burst release.¹⁵ PEAD was designed with a hydrolytically cleavable backbone, resulting in slow biodegradation which releases HB-EGF and heparin. Release may also occur in part by disassembly of the coacervate due to ionic interference. PEAD has been demonstrated to be noncytotoxic; it does not elicit an appreciable inflammatory response or any adverse effects when injected subcutaneously in rats.¹⁶ We have previously shown that the unloaded coacervate at these same minute concentrations has no significant effect on the healing process of excisional wounds in C57BL/6 mice.¹⁵ Furthermore, we have observed that the coacervate has no appreciable effect on other rodent tissues including heart muscle,³⁷ spinal cord,⁴³ and bone.⁴¹ For these reasons, we elected not to include the coacervate alone as a control group in this study.

NONcNZO10 mice were selected for this study from numerous diabetic mouse models available. Unlike chemical induction with streptozotocin or the db/db monogenic strain, NONcNZO10 mice display moderate obesity, whole-body insulin resistance, and an age-associated progression of metabolic abnormalities, resembling those of the human diabetic condition.¹⁹ The shortcomings of monogenic models in replicating a polygenic-disease like diabetes mellitus are underscored in studies showing that wound closure rates of db/db mice do not correlate with the severity of their diabetic condition.^{17,44} Conversely, models with polygenic etiology provide a better representation of the genetic and metabolic profiles of diabetes in humans. In a recent comparison of four common diabetic mouse strains, only NONcNZO10 mice exhibited impairment in every step of the wound healing cascade.¹⁷ It is worth noting that a second polygenic strain, TallyHo/JnJ, displays similar phenotype to NONcNZO10 and will also prove useful for studies of diabetic wound healing in the future.

We previously reported that coacervate delivery of HB-EGF accelerates scratch wound closure of NHEK and avoids the inhibitory effect of HB-EGF on keratinocyte proliferation in vitro.¹⁵ We also demonstrated in vivo that HB-EGF coacervate accelerates reepithelialization and stimulates keratinocyte proliferation in full-thickness wounds on C57BL/6 mice.¹⁵ We performed similar evaluations in diabetic wounds as reduced cell proliferation is one key complication leading to nonhealing chronic ulcers.³ We observed more than twice the number of Ki-67⁺ cells in HB-EGF coacervate treated diabetic wounds compared with saline treatment. Moreover, these cells were distributed out to the tips of epithelial leading edges, whereas proliferative cells in saline wounds were mainly limited to the margins as is typical.⁹ These results indicate that, additional to enhanced migration, keratinocyte proliferation also contributed to wound reepithelialization. We conclude that stimulation of diabetic keratinocytes by HB-EGF coacervate is sufficient for them to overcome several phenotypical impairments of their diabetic condition.

HB-EGF plays an important role in the process of remodeling the largely fibrin-based provisional matrix to a robust collagenous matrix.¹¹ The quantity and quality of collagen deposited is critical to other processes such as wound contraction and revascularization. Unfortunately, matrix remodeling is often severely impaired in chronic wounds.^{45,46} Nonetheless, we found the collagen content of HB-EGF coacervate treated wounds to be significantly higher than that of other groups after four weeks of healing. Consequently, wound contraction was also accelerated because myofibroblasts were able to securely attach to the collagen matrix and begin to pull the wound margins together. Taken together, these data demonstrate that sustained release of HB-EGF in a diabetic wound environment can stimulate dermal fibroblasts to improve the overall wound maturation process and contribute to timely closure.

Angiogenesis is a critical process in the wound healing cascade which provides oxygen, nutrients, and cells to the wound bed and contributes to waste removal.⁴⁷ HB-EGF has been previously demonstrated to promote angiogenesis through some direct and some indirect pathways acting on ECs and vascular smooth muscle cells.^{10,48–50} Our results indicate that HB-EGF coacervate increases the numbers of both endothelial tubes and stable, mature vessels in diabetic wounds after four weeks of healing. Although this is beyond the

timeframe of angiogenesis, these data suggest that HB-EGF plays a role in wound revascularization.

We and others have reported that the migratory capacity of NHEK is enhanced by HB-EGF.^{9,15} Here, we demonstrate that HB-EGF has a similar effect on diabetic cells. Low concentration (10 ng mL⁻¹) of HB-EGF, both free and in the coacervate, significantly stimulated DHEK migration compared with control groups. However, higher concentrations of free HB-EGF did not have this effect. The benefit of controlled release is apparent when the same concentrations of HB-EGF in the coacervate did successfully stimulate DHEK. These results indicate that the coacervate alters the concentration of HB-EGF available to cell receptors, avoiding the inhibitory effect of high dosages which oversaturates receptors.^{15,22} Furthermore, we proved that diabetic human cells respond well to HB-EGF released from the coacervate, which clears a major translational hurdle toward making this experimental therapy a clinical reality.

In summary, we elucidated the multifunctional role of HB-EGF in the chronic wound healing process, provided sufficient levels exist in the wound bed. HB-EGF released from the coacervate stimulated human keratinocyte migration in vitro, and in a diabetic wound model enhanced reepithelialization, wound matrix remodeling, and wound contraction and revascularization. HB-EGF coacervate represents a promising regenerative therapy for diabetic wounds.

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Figure 1.

Wound reepithelialization on day 3 and day 7. (A) Wound sections were stained for pan-cytokeratin which shows the native epidermis, dermal appendages, and the epithelial leading edges. Wound sections were also counter-stained with DAPI for cell nuclei.

Reepithelialization was measured as the percentage of the total wound length (WL) covered by both the right (RL) and left neo-epithelial lengths (LL). Bar 5 100 μ m. (B) Percent reepithelialization measured on day 3 and day 7. Data represent means $\pm$ SD of 6–8 wounds per group per timepoint, * $p < 0.05$, ** $p < 0.01$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Figure 2.

Basal keratinocyte proliferation on day 3. Wounds treated with (A) saline, (B) free HB-EGF, or (C) HB-EGF coacervate, were stained for pan-cytokeratin which shows the native epidermis, dermal appendages, and the epithelial leading edge. Wound sections were also stained for Ki-67 cell proliferation marker which brightly stains the nuclei of proliferating cells. Finally, DAPI counterstaining was performed which dimly stains the nuclei of nonproliferating cells. Indicated regions of the left column are magnified in the center and right columns. Dotted lines indicate the wound margin. Arrows indicate Ki-67⁺ keratinocytes in the basal neo-epidermis. Images show only one half of the wound. Bars = 100 μ m. (d) Ki-67⁺ cells beyond the wound margin were counted and reported as a percent of the total number of DAPI nuclei in the basal epidermis. Data represent means $\pm$ SD of 6–

8 wounds per group, $**p < 0.01$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Figure 3.

Collagen content of wounds on day 14 and day 28. (A) Representative images of day 28 wounds and normal skin stained with Picosirius Red and evaluated under polarized light to observe collagen. Bars = 100 μ m. (B) The percent of the wound region or native skin positive for collagen was quantified. Data represent means $\pm$ SD of 6–8 wounds per group per timepoint, ** $p < 0.01$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Figure 4. Wound contraction on day 14 and day 28. Wounds treated with (A) saline, (B) free HB-EGF, or (C) HB-EGF coacervate were stained with hematoxylin and eosin. Arrows indicate boundaries of the contraction gap. Bars = 500 μm. (D) Wound contraction was quantified by measuring the gap between the wound margins. Data represent means ± SD of 6–8 wounds per group per timepoint, * $p < 0.05$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Figure 5.

Wound revascularization on day 28. (A) Wounds and native skin were stained for vWF to indicate ECs (tubular cross-sections and longitudinal sections) and α-SMA for mural cells (surrounding the endothelial tubes). Bars = 100 μm. (B) EC tubes were identified as vWF⁺ vessel structures and quantified. (C) Mature vessels were identified as endothelial tubes stabilized by mural cells and quantified. Data represent means ± SD of 6–8 wounds per group, * $p < 0.05$, ** $p < 0.01$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Figure 6.

In vitro scratch wound migration assay with human keratinocytes. (A) NHEK or DHEK cultured with no HB-EGF as controls. (B) DHEK cultured with 10 ng mL⁻¹ HB-EGF free or in the coacervate, (C) DHEK cultured with 100 ng mL⁻¹ HB-EGF free or in the coacervate, and (D) DHEK cultured with 250 ng mL⁻¹ HB-EGF free or in the coacervate. Data represent means $\pm$ SD of three samples per group per timepoint, * $p < 0.05$ compared with DHEK control group at similar timepoint, # $p < 0.05$ compared with free HB-EGF group at similar timepoint and dosage.