Self-assembling elastin-like peptides growth factor chimeric nanoparticles for the treatment of chronic wounds

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Chronic wounds are associated with poor epidermal and dermal remodeling. Previous work has shown the efficacy of keratinocyte growth factor (KGF) in reepithelialization and elastin in dermal wound healing. Here we demonstrate the fabrication of a fusion protein comprising of elastin-like peptides and KGF. This fusion protein retains the performance characteristics of KGF and elastin as evidenced by its enhancement of keratinocyte and fibroblast proliferation. It also preserved the characteristic elastin-like peptides inverse phase transitioning allowing the recombinant protein to be expressed in bacterial hosts (such as *Escherichia coli*) and purified rapidly and easily using inverse temperature cycling. The fusion protein self-assembled into nanoparticles at physiological temperatures. When applied to full thickness, wounds in Lepr^{db} diabetic mice these particles enhanced reepithelialization and granulation, by 2- and 3-fold respectively, when compared to the controls. The data strongly suggests that these self-assembled nanoparticles may be beneficial in the treatment of chronic wounds resulting from diabetes or other underlying circulatory conditions.

skin | fusion peptides | regeneration

Chronic wounds such as venous stasis ulcerations, pressure sores, and diabetic foot ulcers are challenging clinical problems with limited treatment options. Often drastic operative interventions such as amputations or free flaps with clear loss of function are necessary to heal tissue defects. Because these wounds are associated with poor reepithelialization, angiogenesis, and granulation (1) most treatments are based on enhancing either one or a combination of these processes. These include the use of growth factors like keratinocyte growth factor (KGF), EGF, PDGF (2), collagen sponges containing growth factors (3) or other wound healing compounds (4), glucosamine nanofibers (5), or dermal substitutes (6).

KGF also known as FGF-7 is a monomeric peptide belonging to the fibroblast growth factor family and plays a prominent role in epidermal morphogenesis and wound healing (7). It is mainly expressed by cells of mesenchymal origin such as fibroblasts, microvascular endothelial, and smooth muscle cells but affects epithelial cells (8, 9). This paracrine mode of action of KGF on epithelial cells is mediated through the KGF receptor (KGFR or FGFRIIIb), a splice variant of the FGF-2 receptor encoded by the gene *fgfr-2* (10, 11). KGF is present at very low levels in skin under normal conditions but is highly up-regulated after injury (12). Exogenous KGF significantly enhances reepithelialization of full and partial thickness wounds in porcine and rabbit ear wound models (13). Interestingly, the expression of KGF is both reduced and delayed during diabetic wound healing (14) and the injection of KGF DNA accelerated wound closure and reduced inflammation in the genetically diabetic mouse (15). These studies suggest that KGF is beneficial for wound healing. However, in most cases the growth factor is delivered topically to the wound limiting its bioavailability. Hence, repeated applications are necessary to see a clinically beneficial effect. This requires the use of large quantities of the growth factor making these wound healing therapies expensive. Moreover, KGF only affects keratinocytes that need the underlying dermis or granulation tissue to migrate and heal the wound. So in instances of deep wounds requiring dermal repair or chronic wounds where there is limited granulation tissue formation, the benefits of KGF become limited. Hence for KGF to be successfully used as a wound healing therapeutic, it has to be used in combination with factors that enhance dermal healing.

Elastin is a major constituent of skin elastic fibers and may be beneficial for dermal regeneration (16). Several studies have explored the application of elastin containing materials for healing of chronic wounds. This includes scaffolds of collagen and solubilized elastin (17), dermal substitutes coated with 3% α-elastin (6, 18), and alginate wound dressings linked with hybrid peptides of elastin (19). Tropoelastin is the soluble precursor form of elastin. ELPs are composed of tandemly repeated blocks of $(\text{Val-Pro-Gly-X-Gly})_N$, a sequence motif derived from the hydrophobic domain of tropoelastin (20). An interesting property of ELPs is their ability to undergo phase transition at physiological temperatures. At temperatures below their inverse transition temperature, also known as a lower critical solution temperature, they are soluble in aqueous solutions. However as the temperature is raised above the transition temperature, they undergo an entropically driven, temperature induced contraction and self assembly, rendering them insoluble. This property enables recombinant ELPs to be expressed in bacteria and rapidly purified to high homogeneity using inverse temperature cycling (ITC) (21). ELPs also exhibit a variety of biological effects on fibroblasts that play an important role in dermal remodeling. These include enhanced chemotactic activity (22), increased fibroblast proliferation (23), and up-regulation of collagenase in cultured fibroblasts (24). However, ELPs have also been shown to induce terminal differentiation of cultured keratinocytes (25). This might make their use in skin wound healing limited as they may interfere with reepithelialization while enhancing dermal remodeling.

Here, we have fabricated a fusion protein comprising of recombinant human KGF and ELPs that may address the above mentioned issues. The fusion protein maintained the performance characteristics of KGF and ELPs as evidenced by its enhancement of keratinocyte and fibroblast proliferation. More-

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The authors declare no conflict of interest.

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over, the recombinant protein also preserved the characteristic phase transition behavior of ELPs allowing it to be expressed in bacteria and purified rapidly using ITC. Furthermore, the inverse transitioning behavior of ELPs allowed the protein to be self-assembled into nanoparticles. These particles when applied to full-thickness wound in genetically diabetic mice improved wound healing by enhancing reepithlialization (2-fold) and granulation (3-fold) when compared to controls. Our data strongly suggests that these self-assembled nanoparticles may be beneficial in the treatment of chronic wounds resulting from diabetes or other underlying circulatory conditions.

Results

Synthesis of KGF–ELP Fusion Protein. Human recombinant KGF was cloned at the N-terminus of the elastin-like peptide (ELP, V40C2) encoding cassette (Fig. 1*A*) within the pUC19 vector. The DNA fragment encoding the fusion protein was then excised from the PUC19 vector and cloned into the modified pET25b+ vector through the SfiI site (Fig. S1). We chose an elastin cassette containing 50 elastin pentapeptides because this cassette has a transition temperature lower than physiological temperature (26). This fusion protein (KGF–ELP) was expressed in a bacterial host (*Escherichia coli*), and purified using inverse temperature cycling. Three cycles of inverse temperature cycling yielded a highly purified protein (Fig. 1*B*), and this protein was reactive to KGF antibody, as demonstrated by the corresponding Western blots (Fig. 1*C*).

Formation of KGF–ELP Particles. ELPs undergo an entropically driven contraction and self-assembly rendering them insoluble above a transition temperature T_t. This property of ELPs was maintained in the fusion protein because we were able to purify the protein from bacterial lysates using ITC (Fig. 1*B*). Due to the inverse transitioning property of ELPs in the fusion protein, when the KGF–ELP or ELP solution was heated to its transition temperature spherical particles with a diameter of 500 nm were formed (Fig. 2 *A* and *C*). These particles exhibited a single peak (Fig. 2 *B*, 510 nm, and *D*, 530 nm) and the polydispersity defined

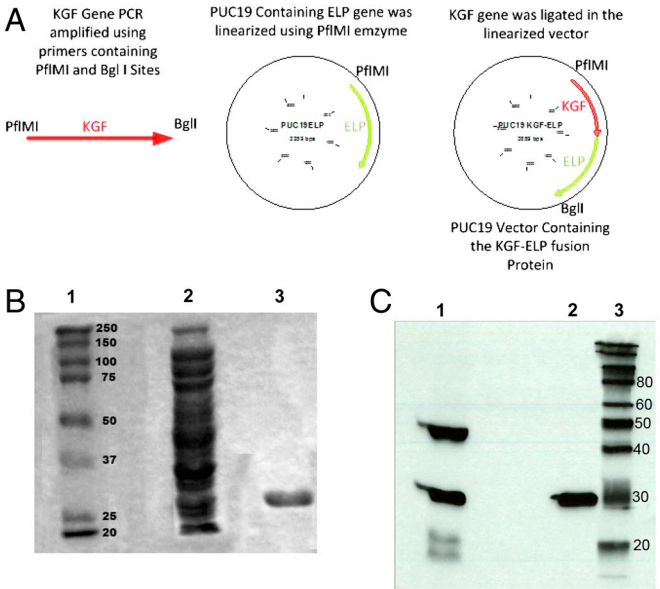

Fig. 1. Preparation of the fusion peptide. (*A*) Recombinant KGF was cloned in front of the elastin cassette V40C2 in the cloning vector PUC19 using PflMI and BglI sites. (*B*) The bacterial lysate and purified KGF fusion protein was run on a SDS-PAGE gel and stained with simply safe blue stain for total protein. Lane 1, ladder; lane 2, bacterial lysate; and lane 3, purified KGF–ELP fusion. (*C*) The corresponding gel was then subjected to Western blot with a monoclonal KGF antibody. Lane 1, bacterial lysate; lane 2, purified KGF–ELP fusion protein; and lane 3, ladder.

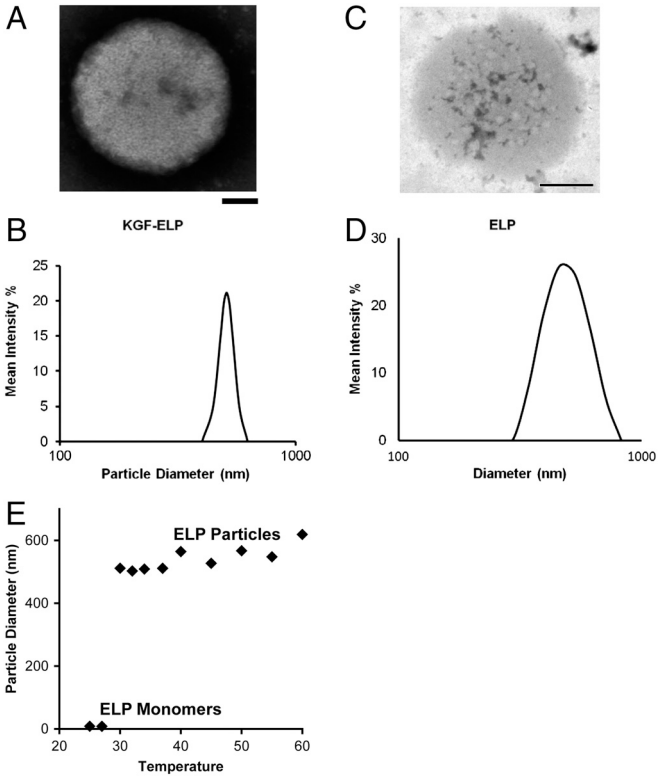

Fig. 2. Formation of particles. (*A*, *C*) TEM imaging of the formed particles. (*A*) KGF–ELP or (*C*) ELP particles. Either KGF–ELP or ELP was dissolved in deionized water and incubated at 37 °C to initiate particle formation. These particles were stained and then imaged using TEM. Bar = 100 nm. (*B*) Particle size distribution of the formed (*B*) KGF–ELP or (*D*) ELP particles. Either KGF–ELP or ELP was dissolved in PBS and particle size was determined as described in *Materials and Methods*. (*E*) Particle diameter as a function of temperature. The fusion peptide was dissolved in PBS and then determination of particle size was done by incubating the solution at the indicated temperatures. At temperatures lower than the transition temperature the fusion peptide exists as monomers and as the temperature is increased the elastin chains come together and result in the formation of submicron sized particles.

as the standard deviation of the distribution were 20 and 78 nm for KGF–ELP and ELP particles respectively. Moreover, these particles were stable over a wide range of temperatures (Fig. 2*E*).

KGF–ELP Fusion Retains the Performance Characteristics of KGF and ELP. Originally identified as a potent mitogen of keratinocytes (27), KGF is a powerful enhancer of epithelial regeneration (7). To assess the biological activity of recombinant KGF in the KGF–ELP fusion we carried out a proliferation assay using a KGF responsive epithelial cell line A431 (28). We serum-starved the cells overnight and the next day added ELP, KGF–ELP, KGF alone, or KGF with ELP particles. Indeed KGF–ELP-induced proliferation in the cells after 2 d (Fig. 3*A*, 2.31-fold, $p < 0.05$) similar to free KGF (Fig. 3*A*, 2.0-fold, $p < 0.05$). ELP by itself had no effect on keratinocyte proliferation nor did it affect the enhancement of proliferation by KGF (Fig. 3*A*). Furthermore, KGF–ELP phosphorylated the downstream targets of KGF, ERK1, and ERK2 (28) similar to exogenous KGF in the presence of ELP (Fig. 3 *B*–*D*). Although, free KGF in the presence of ELP led to higher phosphorylation of ERK compared to the KGF fusion protein. This suggests that whereas the performance characteristic of KGF in the fusion was maintained, the activity of KGF was diminished due to the fusion. Additionally, we also investigated whether the fusion protein was active by itself or became active by proteolysis (Fig. S2). As can be seen in the figure the

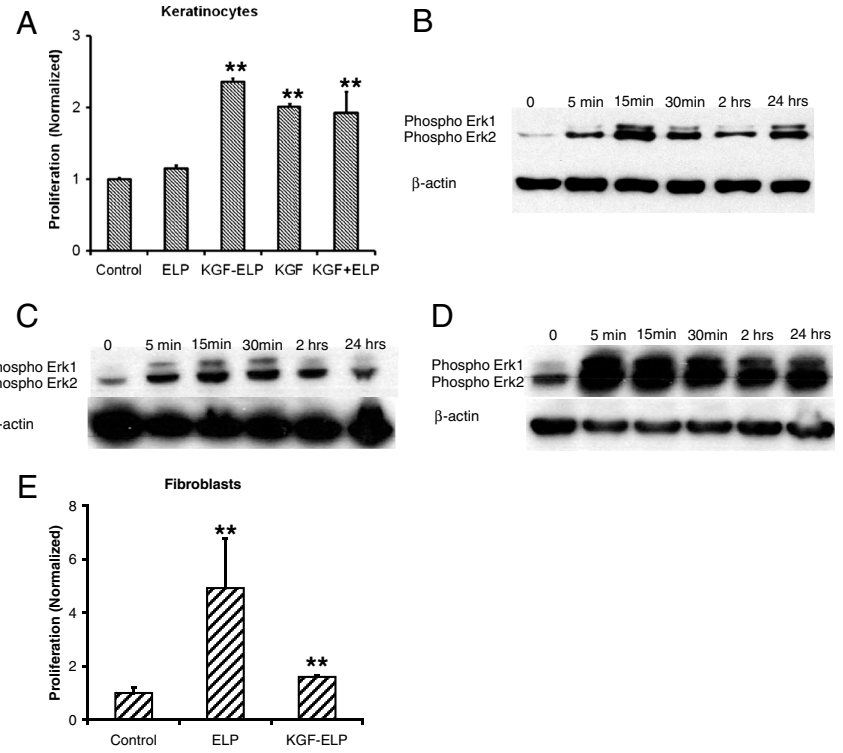

Fig. 3. KGF–ELP fusion retains the performance characteristics of KGF and ELP. (*A*) Keratinocytes were serum-starved overnight and next day were treated with either Serum free DMEM (Control), ELPs (10 μg/mL), KGF–ELP fusion protein (10 μg/mL), KGF (50 ng/mL), or KGF (50 ng/mL) + ELP (10 μg/mL). After 2 d cell number was assessed by Hoechst assay, and was normalized to the control cell number (Y axis). This experiment was repeated two times with triplicates and a representative experiment is shown. ** indicates $p < 0.05$ when compared to control. (*B–D*) Phosphorylation of ERK 1 and ERK 2 in cultured keratinocytes. Keratinocytes were serum-starved overnight and then treated with (*B*) KGF–ELP (10 μg/mL), (*C*) ELP (10 μg/mL) or (*D*) Free KGF (25 ng/mL) + ELP (10 μg/mL) for the indicated times. The cells were then lysed and the lysates were subjected to Western blot using an antibody specific to phosphorylated ERK1 and ERK2. For loading control, the blots were stripped and reprobed for β-actin. (*E*) Fibroblasts were serum-starved overnight and next day were treated with either Serum free DMEM (control), KGF–ELP fusion protein (10 μg/mL) or ELPs (10 μg/mL). After 2 d cell number was assessed by Hoechst assay, and was normalized to the control cell number (Y axis). This experiment was repeated two times with triplicates and a representative experiment is shown. ** indicates $p < 0.05$ when compared to control.

fusion protein remains intact throughout the duration of the experiment.

Similarly, previous studies have indicated that elastin induces proliferation of fibroblasts (23). To test the biological activity of ELPs we added either ELPs or KGF–ELP to serum-starved fibroblasts. Indeed after 2 d, ELPs induced a dramatic 4.9-fold increase in fibroblast proliferation (Fig. 3*E*, $p < 0.05$). On the other hand, the fusion protein KGF–ELP showed a 1.6-fold increase in fibroblast proliferation (Fig. 3*E*, $p < 0.05$) suggesting that the fusion peptide had retained some biological activity of ELPs, though it was significantly less than ELPs alone. Because, fibroblasts lack the receptor of KGF it is unlikely that KGF inhibits fibroblast proliferation or has any effect on fibroblasts.

KGF–ELP Particles Improve Healing of a Full-Thickness Wound in Genetically Diabetic Mice. Finally, we examined the efficacy of KGF–ELP particles in the healing of full-thickness wounds in B6.BKS(D) – *Lepr^{db}*/J mice. This naturally occurring mutation causes insulin resistance and significantly retards wounds healing. Full-thickness wounds were created on the dorsal area of the mouse. The particles suspended in saline cannot be used because it leads to loss of the solution when the animal moves resulting in the drying of the wound. To avoid this difficulty, all the treatments were done in fibrin hydrogel as a vehicle. Treatment included onetime administration of fibrin gel, KGF in fibrin gel, ELP particles, KGF and ELP particles, or KGF–ELP fusion particles suspended in fibrin gel. Fourteen days after the treatment, the mice were euthanized and the wounded tissues were harvested and processed for histology. Histological examination of the wound revealed that animals treated with ELP (Fig. 4*C*), KGF–ELP fusion particles (Fig. 4*D*) or KGF and ELP particles (Fig. 4*E*) exhibited thicker granulation tissue as compared to other treatments (KGF and Control, Fig. 4 *A* and *B*). When we quantified the thickness we found that wounds treated with KGF–ELP fusion particles exhibited significantly less granulation (Fig. 4*F*, 0.232 mm, * $= p < 0.01$) than either ELP particles or Free KGF with ELP particles treatments (Fig. 4*F*, ELP = 0.403 mm, and free KGF with ELP = 1.185 mm). Interestingly, mice treated with free KGF with ELP had thicker granulation tissue than ELP by themselves, however there was considerable variability (Fig. 4*F*, # denotes, $p = 0.043$).

On the other hand, only wounds treated with KGF–ELP nanoparticles and KGF with ELP particles showed significant reepithelialization when compared to other treatments (Fig. 5 *A–E*). We quantified the extent of reepithelialization and found that whereas the fusion protein resulted in 36% coverage and free KGF in presence of ELP particles resulted in 31% coverage (Fig. 5*F*).

Discussion

In this work we demonstrated the engineering of a fusion protein that affects two cell types, namely keratinocytes and fibroblasts. Keratinocytes and fibroblasts play an important role in chronic wound healing and their morphology and activity is significantly altered in diabetic wounds (29). Various growth factors that enhance the functionality of these cells have been suggested as possible treatments; but failed to show beneficial effects clinically. This might be due to failure to deliver the growth factor where it is needed or its rapid loss in the circulation. On the other hand, dermal substitutes rely on successful integration with the host through vascularization and cellular in growth. This integration is limited when the cells are impaired as in the case of diabetic wounds. Treatments involving cell suspensions of autologous suspensions of keratinocytes and fibroblasts have yielded some promising results (29). However, this approach involves significant time and resources as cells need to be grown in significant quantities for successful grafting.

Our fusion protein significantly enhanced the healing of full-thickness wounds in *Lepr^{db}* diabetic mice by enhancing dermal and epidermal regeneration. We found that ELPs in agreement with previous studies induced fibroblast proliferation in vitro (23). However, the most interesting finding was that ELPs and KGF–ELP fusion protein induced significant granulation in vivo. This is significant as it eliminates the use of dermal scaffolds that is necessary if KGF is to be used successfully for wound healing. Furthermore, mice treated with ELP or a mixture of exogenous KGF and ELP exhibited much higher granulation than the KGF–ELP fusion protein. Whereas granulation is necessary, excessive granulation leads to fibrosis and scarring that are not desired out-

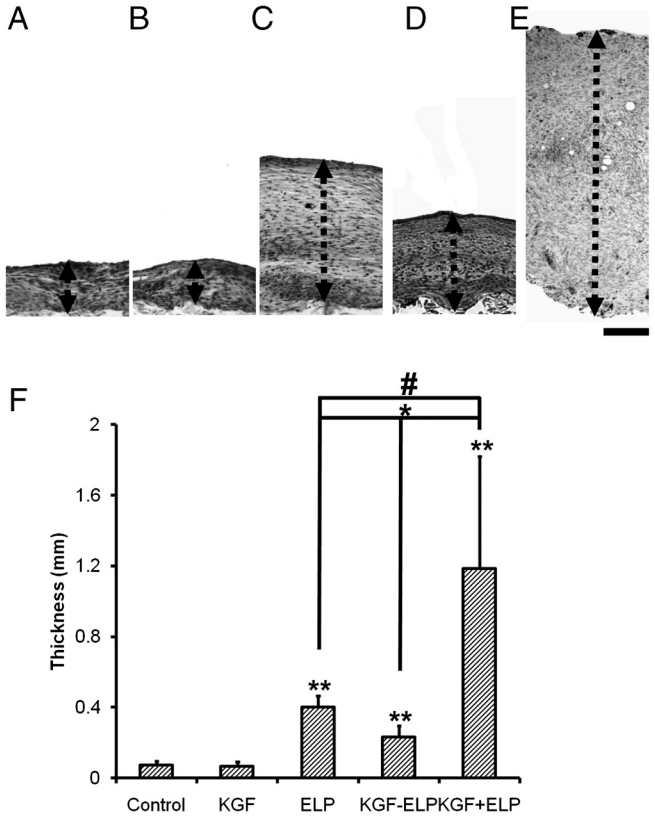

Fig. 4. Elastin enhances granulation tissue of full-thickness wounds in genetically diabetic mice. Full-thickness wounds were created on the back of genetically diabetic mice. The mice were then treated with either (*A*) Fibrin gel, (*B*) Fibrin gel containing KGF, (*C*) Fibrin gel containing elastin particles, (*D*) Fibrin gel containing KGF–ELP particles, or (*E*) Fibrin gel containing free KGF + ELP particles. The figure shows the middle of the wound after 14 d. The dotted arrow in the middle of the wounds was quantified. Bar = 500 μm. (*F*) Quantification of the thickness of granulation tissue. Each value represents the mean thickness from 7 mice ($n = 7$). ** denotes $p < 0.05$ when compared to control or KGF. # denotes $p = 0.043$ when compared to ELP particles. * denotes $p < 0.01$ when KGF–ELP particles are compared with either ELP particles treatment or free KGF + ELP particles treatment.

comes of wound healing (30). Interestingly, the effect of the KGF fusion protein on granulation was less dramatic than ELPs, but it was enough to induce the migration of keratinocytes in the wounds. This is a significant benefit of using KGF–ELP fusion protein over just blending KGF with ELP particles.

Interestingly, ELPs alone did not have any effect on reepithelialization. This might be due to excessive granulation preventing cutaneous wound healing (31). However, it is more likely that ELPs simply affected keratinocyte proliferation, possibly due to induction of keratinocyte differentiation (25). On the other hand, our fusion protein contains KGF, a known mitogen that delays differentiation and induces proliferation of keratinocytes (32). Hence we saw significant increase in reepithelialization induced by either KGF mixed with ELPs or KGF fusion protein in full-thickness wounds. This data suggests that whereas enhancing keratinocyte function is necessary by using a growth factor like KGF; enhancing fibroblast function is equally if not more necessary for developing successful wound healing therapies.

Interestingly, the fusion protein not only retained the biological activity of elastin but also its physical phase transition property. This unique property allowed the expression and purification of large quantities of the protein rapidly through ITC as previously described (21). This technique enables the production of large quantities of the growth factor fusion protein through a series of hot and cold spins eliminating the need of costly chromatographic or antibody-based protein purification steps. Moreover, the inverse transitioning behavior of ELPs allowed the fusion protein to self-assemble into nanoparticles at the transition temperature. These particles may serve as "drug depots" at the wound site as described for other elastin-based fusion peptides (33). This might explain how a single treatment of KGF–ELP showed dramatic effects on keratinocytes in vivo whereas KGF alone did not. The formation of elastin-based particles using block copolymers of different elastin chains having different hydrophobicities has been described before (34) (35). However our work suggests that a fusion of a growth factor with an elastin chain also yields nanoparticles, without the introduction of multiple block copolymers. These particles may be spherical micelles arising from the fusion of hydrophilic KGF and hydrophobic ELPs because the molecular weights of both are comparable, or they could be simple aggregates resulting from the coacervation of the elastin chains at the transition temperature. Further studies are underway in our laboratory to determine the exact nature of these particles. The particle diameter (500 nm) was a result of the length of the elastin chain length that we chose to fine tune the transition temperature. Different sizes of particles may also have different effect on wound healing outcomes. Further studies are underway in our laboratory to investigate the effect of particle sizes on wound healing.

The concept of using elastin-based fusion proteins as delivery vehicles has been previously described. Shamji and coworkers have reported the expression of a fusion protein consisting of ELP and IL2R or ELP and TNFα antagonist (33, 36), for treatment of inflammation. These fusion proteins maintained the binding property of the antagonists as well as the inverse phase transitioning property of the elastin. The proteins worked by competing with the binding of IL-2 or TNF-α to the receptor; both well known players of inflammation. Both of these cases exploited the phase transitioning property ELPs to make a temperature responsive drug delivery system. However, in our case ELPs by themselves led to granulation, which is beneficial and important for wound healing. So the developed fusion protein not only exploits the phase transitioning property of ELPs but also their intrinsic biological activity that enhanced granulation in the wounds.

In summary, we developed a fusion protein that enhances granulation and reepithelialization, two crucial processes in wound healing. Due to the coacervation property of elastin, large quantities of the fusion protein can be manufactured with relative ease and low costs. The fusion protein can also self-assemble into particles that are easier to administer to the wound and ensure bioavailability of the growth factor. Moreover, it also doesn't lead to excessive granulation that is detrimental to the outcome of wound healing. Therefore, it is likely that these fusion proteins will be advantageous in the treatment of injuries in which growth factor therapies have been shown to be beneficial but impractical due to high costs associated with continuous growth factor delivery. Whereas we have shown this strategy for a single growth factor, KGF, it could be readily extended to include other monomeric growth factors or therapeutic proteins. Furthermore, due to the aggregating property of the elastin chains a heterogeneous particle consisting of multiple ELP chains functionalized with different growth factors or proteins can be constructed. These heterogeneous particles will be useful for the delivery of combination therapy, where these growth factors can act synergistically to improve wound healing or other conditions.

Materials and Methods

Materials. PBS, FBS, DMEM, and serum free medium were all purchased from Invitrogen. The restriction enzymes and other enzymes used for cloning were purchased from New England Biolabs unless mentioned otherwise. A431 and human dermal fibroblasts were purchased from ATCC and they were cultured in DMEM supplemented with 10% FBS. Primary keratinocytes were isolated from neonatal foreskins and cultured as described previously (32).

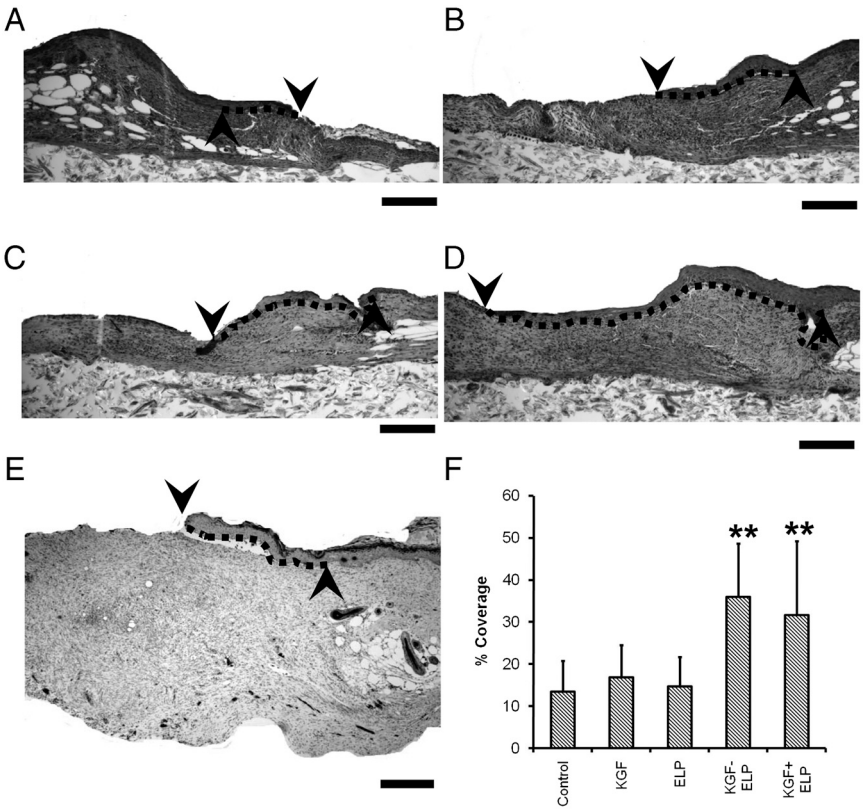

Fig. 5. KGF–ELP enhances reepithelialization in full-thickness wounds in diabetic mice. Full-thickness wounds were created on the back of genetically diabetic mice. The mice were then treated with either (*A*) Fibrin gel, (*B*) Fibrin gel containing KGF, (*C*) Fibrin gel containing elastin particles, (*D*) Fibrin gel containing KGF–ELP particles, or (*E*) Fibrin gel containing free KGF + ELP particles. Fourteen days after wounding the animals were killed; the tissue was harvested and stained with Hematoxylin and Eosin. The up arrow indicates the edge of the created wound and the down arrow indicates the tip of the migrating tongue of the wound. Dotted line represents the extent of reepithlialization. Bar = 400 μm. (*F*) Quantification of reepithelialization. Each bar represents the length of reepithelialization normalized to the initial wound gap (% Coverage). The value is representative of a mean of 7 animals for each group ($n = 7$). ** = $p < 0.05$ when compared to control, KGF, or ELP.

Generation of the Fusion Protein Expression Plasmid. The plasmids containing the elastin cassette V40C2 and recombinant KGF have been described previously (32, 37). We used PflMI and BglI enzymes for generating of the fusion protein as has been described previously (26). We PCR-amplified the KGF encoding sequence using primers having PflMI and BglI sites at the 5′ and 3′ end respectively using PfuUltra high fidelity polymerase (Stratagene) as per manufacturer's recommendations. The PCR-amplified fragment was then cloned using a zero blunt TOPO PCR cloning kit (Invitrogen). After TOPO cloning, the KGF fragment was excised using PflMI and BglI enzymes. The pUC19 vector containing the V40C2 sequence was linearized by PflMI and the excised KGF fragment was then cloned in frame with the elastin cassette (Fig. 1*A*). This yielded PUC19 vector containing the KGF–ELP fusion protein flanked by PflMI and BglI sites (Fig. 1*A*). This sequence encoding the fusion protein was then cut out from PUC19 vector using PflMI and BglI enzymes and cloned in a modified pET25b+ expression vector through SfiI site (Novagen, Fig. S1). We modified this vector to incorporate an additional SfiI site in the multiple cloning sites for cloning of the fusion protein (Fig. S1). For generating control ELP particles, the V40C2 sequence was cloned from the PUC19 vector to the PET25b+ expression vector in a similar fashion as the fusion protein.

Expression and Purification of the Fusion Peptide. The pET25b+ vector containing the KGF–ELP or ELP sequence cassette was transformed into *E. coli* cells through heat shock at 42 °C. A starter culture of 75 mL was then inoculated overnight and added to a 1 L culture the next day. The 1 L culture was then propagated overnight. The next day, bacterial cells were harvested by centrifugation at 4 °C. The bacterial pellet was resuspended in 1× phosphate buffered saline (PBS), and the cells were disrupted by sonication on ice. The lysate was cleared by centrifugation followed by a polyethyleneimine treatment (final concentration: 0.5% w/v). After another round of centrifugation, the cleared supernatant containing KGF–ELP or ELP was transferred to a clean centrifuge tube. The tube was heated to 40 °C in the presence of 1 M NaCl and a warm centrifugation at 40 °C was carried out to pellet KGF–ELP. The supernatant was then discarded, and the pellet was resolubilized in PBS in the presence of 10 mM DTT on ice followed by another cold spin at 4 °C. This cycle was repeated twice. For the final resuspension step, the KGF–ELP or ELP was resuspended in purified water and then dialyzed overnight against deionized water at 4 °C using a Spectra/Por 14 kDa cutoff membrane (Spectrum Laboratories). The protein was then lyophilized using a Virtis Advantage lyophilizer (Virtis).

Animals. Genetically diabetic male B6.BKS(D) – *Lepr^{db}*/J mice were obtained from The Jackson Laboratory. A total of seven mice were used for each treatment group. All mice were used experimentally when 9 weeks old at the time of wounding. Each mouse was housed individually. The animals were cared for in accordance with the guidelines set forth by the Committee on Laboratory Resources, National Institutes of Health, and Subcommittee on Research Animal Care and Laboratory Animal Resources of Massachusetts General Hospital. All animals had free access to food and water, both before and after the operation.

Surgical Procedures for Wounding. The diabetic mice were anesthetized using 2–2.5% vaporized inhaled isoflurane (Iso Flo, Abbott Laboratories). Under sterile conditions, the dorsal area was totally depilated and a single full-thickness excisional square wound (1×1 cm^2) was created on the upper back of each mouse using a pair of sharp scissors and a scalpel. 100 μL of Fibrin gel containing the desired treatment was administered into the wounds of the mice and dressed with a 2.5 cm × 2.5 cm piece of Tegaderm™ (3M Health Care). After 14 d of wounding, the mice were euthanized using pentobarbital anesthesia. The skin around the wound was then excised and processed further for histology.

Preparation of Fibrin Gels. Fibrin gel was prepared on the wound of the mice by mixing two fractions: one fraction containing Fibrinogen (6.25 mg/mL, Sigma), KGF–ELP (0.45 nM), KGF (0.023 nM), or Elastin (0.45 nM) and the other containing thrombin (12.5 U/mL, Sigma), $CaCl_2$ (12.5 mmol/L, Sigma) in 1× Tris buffered saline. Fibrinogen fraction containing the particles was incubated at 37 °C to initiate particle formation. After wounding the mice, 80 μL of the warm fibrinogen fraction containing the particles was mixed with 20 μL of the thrombin fraction in a tube and immediately applied to the wound topically. After the mixture was gelled (in < 2 minutes) the wounds were covered with tegaderm.

Histological Evaluation of Tissues. After 14 d the mice were killed. The tissue surrounding the wound area was excised and the excised tissue was embedded in paraffin. Six-micrometer tissue sections were cut and mounted on slides. The sections were then stained for hematoxylin and eosin. Pictures of the wound were then taken using a Nikon Microscope.

Statistics and Data Analysis. For statistical significance, the p value was calculated for the indicated groups by using ANOVA. The analysis of in vivo data

was done using Image J software (National Institutes of Health) and histology data. Histological pictures were taken from multiple sections (at least 4 sections per animal) and the wound edges were determined using the position of the first hair follicle. The reepithelializatinon tongue was traced and quantified using Image J. For granulation tissue thickness, pictures at the middle of the wound were taken and quantified using Image J.

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