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ARTICLE

Dual therapeutic functions of F-5 fragment in burn wounds: preventing wound progression and promoting wound healing in pigs

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Burn injuries are a leading cause of morbidity including prolonged hospitalization, disfigurement, and disability. Currently there is no Food and Drug Administration-approved burn therapeutics. A clinical distinction of burn injuries from other acute wounds is the event of the so-called secondary burn wound progression within the first week of the injury, in which a burn expands horizontally and vertically from its initial boundary to a larger area. Therefore, an effective therapeutics for burns should show dual abilities to prevent the burn wound progression and thereafter promote burn wound healing. Herein we report that topically applied F-5 fragment of heat shock protein-90α is a dual functional agent to promote burn wound healing in pigs. First, F-5 prevents burn wound progression by protecting the surrounding cells from undergoing heat-induced caspase 3 activation and apoptosis with increased Akt activation. Accordingly, F-5–treated burn and excision wounds show a marked decline in inflammation. Thereafter, F-5 accelerates burn wound healing by stimulating the keratinocyte migration-led reepithelialization, leading to wound closure. This study addresses a topical agent that is capable of preventing burn wound progression and accelerating burn wound healing.

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INTRODUCTION

Burn injuries are a leading cause of morbidity including prolonged hospitalization, disfigurement, and disability. Burn injuries to the skin are primarily caused by heat but also by exposure to radioactivity, X-irradiation, electricity, chemicals, and friction. The World Health Organization estimated that burn injuries cause annually 265,000 deaths worldwide and 4,500 deaths in the United States. In addition to burn-induced deaths, additional 11 million patients worldwide and 500,000 patients in the United States seek medical care for skin burn wounds each year. The total annual costs of burn injuries in the Unites States exceed US$1 billion per year if direct medical costs plus hospital days and loss of productivity for care of children with burns are included.[1,2]

Burn wounds are different from acute traumatic and surgical wounds. A second-degree burn can expand to a third-degree burn within the initial 4 days.[3] During this period of time, burn skin wounds expand horizontally and vertically from the initial site of trauma and create an overall larger wound, the so-called "secondary burn progression." This burn wound-specific phenomenon is a major pathophysiological factor that involves the death of cells surrounding the direct burned site. In 1953, Jackson first described the three concentric zones of burn wounds: the central zone of coagulation, the transitional zone of stasis, and the outer zone of hyperemia.[4] The tissue in the zone of coagulation is directly destroyed by the thermal injury resulting in irreversible tissue necrosis. The phenomenon of burn wound progression refers to the zones of stasis and the hyperemia in which cells initially remain viable following the injury[5,6] but, if left untreated, soon die of necrosis, apoptosis or both, due to ischemia, infection, and accumulation of toxic metabolites.[7–9] Moreover, burn wound progression is associated with slower healing rates, worse scarring, and greater contracture.[10] Although burn injury managements and patient outcomes have improved over time, the current therapies for burn wounds are limited to metabolic and fluid support, infection control, surgical intervention, and skin grafting. These therapies are supportive but specifically directed at altering the burn wound itself. There have been no Food and Drug Administration (FDA)-approved therapeutics that target the key issue of the secondary burn wound progression and thereafter promotes burn wound healing in humans.[8,26]

While searching for critical factors that play a role in acute wound healing, we focused on the secreted molecules from reepithelializing keratinocytes because their behavior under the stressful conditions of wound healing is known to be highly different than keratinocytes in nonwounded skin. Further, keratinocyte reepithelialization and wound closure are relatively early events in wound healing. Protein purification from conditioned medium of migrating human keratinocytes allowed us to identify the secreted form of heat shock protein-90α (Hsp90α) as a critical overarching

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keratinocyte-derived molecule that orchestrates reepithelialization, fibroplasia and neoangiogenesis via the stimulation of cell migration of keratinocytes, fibroblasts, and endothelial cells, respectively.[11,12] We have since demonstrated that the topical application of recombinant Hsp90α protein dramatically shortened the time of full thickness wound closure in multiple rodent and pig models.[13–16] Several factors may contribute to the effectiveness of Hsp90α. First, the secreted form of Hsp90α is a common promotility factor for all the cell types involved in wound healing. Second, the promotility activity of Hsp90α can override transforming growth factor β inhibition in the wound environment. Third, continued replenishment of secreted Hsp90α does not need normal blood circulation, the latter is interrupted by damaged blood vessels in the wound. Based on these findings, we proposed that the keratinocyte-secreted Hsp90α plays a critical role in the early phase of wound healing, in particular completion of the reepithelialization process and wound closure.[17]

A recent finding from our laboratory prompted us to speculate that topical Hsp90α treatment might have unique effects on burn wound healing. Tsen *et al*. found that Hsp90α promotes wound healing by activating, via binding to the low-density lipoprotein receptor-related protein-1 receptor, the Akt1 and Akt2 kinases. More importantly, we showed that both Akt1- and Akt2-knockout mice show defects in wound healing.[14] Activated Akt kinases are known to phosphorylate and inhibit the pro-apoptotic Bcl-2 family members Bad, Bax, caspase 9, GSK-3, and FoxO1. Therefore, it is conceivable that topical treatment with Hsp90α may prevent secondary burn wound progression by inhibiting apoptosis of the cells surrounding the burn wound. To test this hypothesis, we first established a modified pig burn wound model that recapitulates the burn wound characteristics reported in humans and standardizes a protocol for testing topical drug candidates. Using this burn wound model, we found that topical application of the Hsp90α fragment, F-5, prevented the secondary burn wound progression by inhibiting cell apoptosis and accelerated burn wound healing by stimulating reepithelialization. We believe that the dual functional F-5 fragment represents a potentially promising therapeutic agent for topical treatment of burn wounds in humans.

RESULTS

A modified pig burn wound model that recapitulates the characteristics of human burn wounds and creates a platform for therapeutic evaluations

Of all species, the skin of the pig is the most similar to human skin,[18] and previous studies reported establishments of various burn injury models in pigs.[19–24] Having analyzed these models and, more importantly, our previous establishment of a diabetic pig wound model,[15] we needed a pig burn model that recapitulates the characteristics of human burn wounds and, more importantly, is ideal for evaluating new therapeutic candidates. As shown in Figure 1a, triplicate burn wounds created on both sides (middle rows) of the pig underwent a clear expansion especially from 48 to 96 hours following the injury under our experimental conditions (1.5 cm × 1.5 cm 115 °C brass block for 30 seconds) and the expansion slowed down afterward (day 5). Quantitation of the burn wound progression is shown in Figure 1b. These observations are consistent with the previous reports.[9,10,19] Since our goal was to test a new topical drug candidate, the burns were debrided after 48 hours to remove the top dead skin, similar to what is done in the hospital burn unit to prepare burn wounds for topical treatment. Again, as shown in Figure 2, the secondary burn wound progression was clearly detected from day 2 to day 4 following burn wound debridement (panels g to l versus panels m to r), as indicated in the selected two rows of marked wounds on both sides of the animal. This observation supports the finding of a previous study that debridement or excision could not stop the burn wound progression.[23,25] Our measurements of multiple wounds from independent experiments showed that the average horizontal expansion is from 1.5 ± 0.04 to 1.88 ± 0.14 cm (n = 3) from day 0 to day 4 (see quantitation of the data in Supplementary Figure S1a). We noticed that a direct measurement of the vertical progression of the burns were hard with the naked eye and, instead, measured by immunohistochemistry analyses, see later sections).

In our hands, however, we noticed that skin wounds, including burn wounds, created on the same side of a pig heal at different rates.[15] The likely reasons include the dramatic difference in skin thickness and elasticity, especially from the top to the bottom along the same side of the pig's torso (Supplementary Figure S2). Recognition of this difference is particularly important for studies that evaluate new therapeutic agents versus their placebo controls, where the two sets of wound must share similar healing rates to begin with. Therefore, rather than using standardized burn wounds on the same side of the torso, we used a pair of standardized skin burn wounds that were made on the opposite sides of the pig's torso at two corresponding spots for drug testing. Using this placement of the skin wounds, we found that the two corresponding burn wounds healed at nearly identical rates. As also illustrated in Figure 2, in the experiments in later sections, where we compare the healing of wounds treated with different topical agents, we always used comparisons between the wound A and the wound A′, the wound B and the wound B′, etc. (middle row). Each pair of the wounds locates at the corresponding, but the opposite, two sides of the animal.

In addition to horizontal expansion of the burn wounds, we confirmed the occurrence of the secondary burn wound progression using various histochemistry and immunohistochemistry analyses. As shown in Figure 3a, hematoxylin–eosin staining revealed an expansion of the burn-damaged area both horizontally (red arrows) and vertically (black arrows; panels a to d). Consistently, the Masson's trichrome blue staining revealed similarly expanded damage in dermal collagens (Figure 3b, as indicated by dotted red boxes). Specifically, the blue staining of the collagens disappeared over time in association with increased red staining of cell cytoplasm and purple/black staining of the nuclei (panels c′ and d′ versus panels a′ and b′). Quantitation of these data is shown (see Supplementary Figure S1b in Supplementary Data). To confirm burn wound progression-associated cell death in the areas surrounding the original burn wounds, we stained the burn wound sections with antibodies against high mobility group box 1 protein (HMGB1) for signs of necrosis and antibodies against activated caspase 3 as an indication of apoptosis, as previously reported.[20,22] As shown in Figure 4a, in comparison with normal skin (panel a), massive necrosis (brown stain) was not only detected after 48 hours in the area that was in direct contact with the heat block (panel c, indicated by arrows) but also in the areas surrounding the burn wound in a time-dependent manner (panels d and e). Also as expected, as shown Figure 4b, in comparison with normal skin, a strong apoptotic signal (brown staining), activated caspase 3, was detected on day 4 in both epidermis and dermis surrounding the burned skin (panel d′), and it continued to progress to day 7 (panel e′). Quantitation of these data is shown (see Supplementary Figure S1b). In summary, this modified pig burn wound model exhibits the characteristic features of burn wounds in humans and provides a platform for our therapeutic studies.

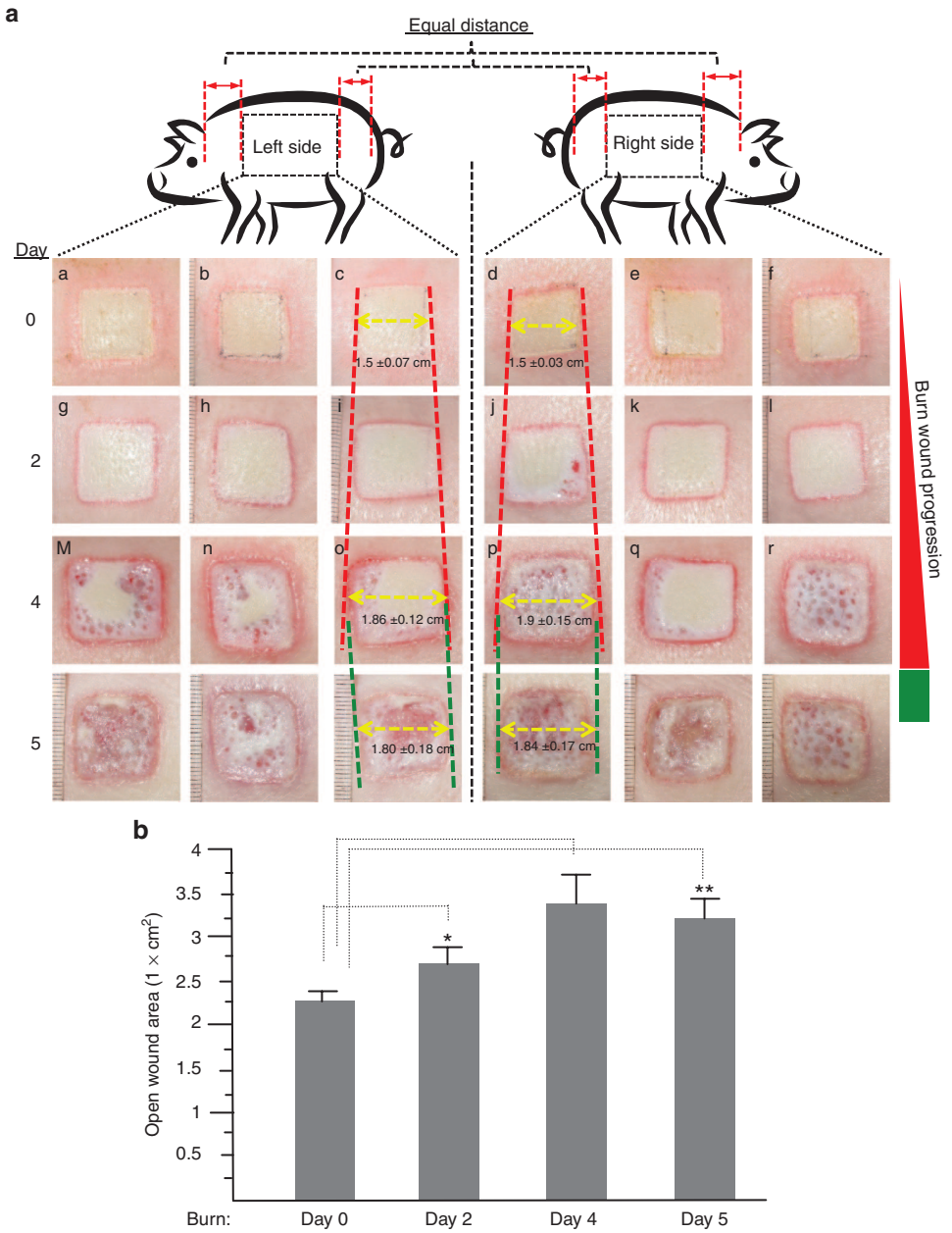

Figure 1 Skin burn wound progression in pigs. Nine 1.5 × 1.5 cm^2 burn wounds were created on each side of the pig ($n = 4$) with 4.0 cm apart between the wounds. The wounds were monitored every 24–48 hours, photographed, and measured between the two horizontal (cm) and vertical (cm) edges, to give rise to area (cm^2). (**a**) The images of the mid-row wounds from one of representative pigs ($n = 3$) are shown. Burn wound (horizontal) progression is indicated in two rows of wounds by red lines and slowed down on day 5 (green lines). (**b**) Quantitation of the wound size in triplicates is represented as mean ± SD. *$P < 0.05$; ** $P < 0.005$.

Topical F-5 treatment prevents cell apoptosis and secondary burn wound progression

Using the newly modified pig burn wound model outlined above, we tested our hypothesis that topical treatment with recombinant Hsp90α protein prevents burn wound progression. Following debridement of the burns on day 2 as previously shown in Figure 2, either carboxy methyl cellulose (CMC) vehicle control or CMC containing human recombinant F-5 fragment of Hsp90α (see purified F-5 in Supplementary Figure S3a) was topically applied to burn wounds in triplicates. The FDA-approved becaplermin gel (PDGF-BB, Regranex) was included as a comparison. Considering the fact that we only applied the tested agents once on day 2, we focused our measurements of the burn wounds only up to 9 days following wounding. In a previous study, a single application showed its strongest effect on full-thickness pigskin wound healing 4–10 days postwounding.[15] First, as shown in Figure 5a, the F-5-treated burn wounds accumulated less pus than the vehicle-treated wounds (middle row versus upper row). Most encouragingly, the F-5-treated burn wounds showed much reduced progression between day 2 and day 4 (middle row), in comparison with the CMC vehicle control or the Regranex-treated burn wounds (top and bottom rows), as indicated by the measurements. The statistic quantitation of these data together with wound closure measurements is shown in Figure 7a (see later sections).

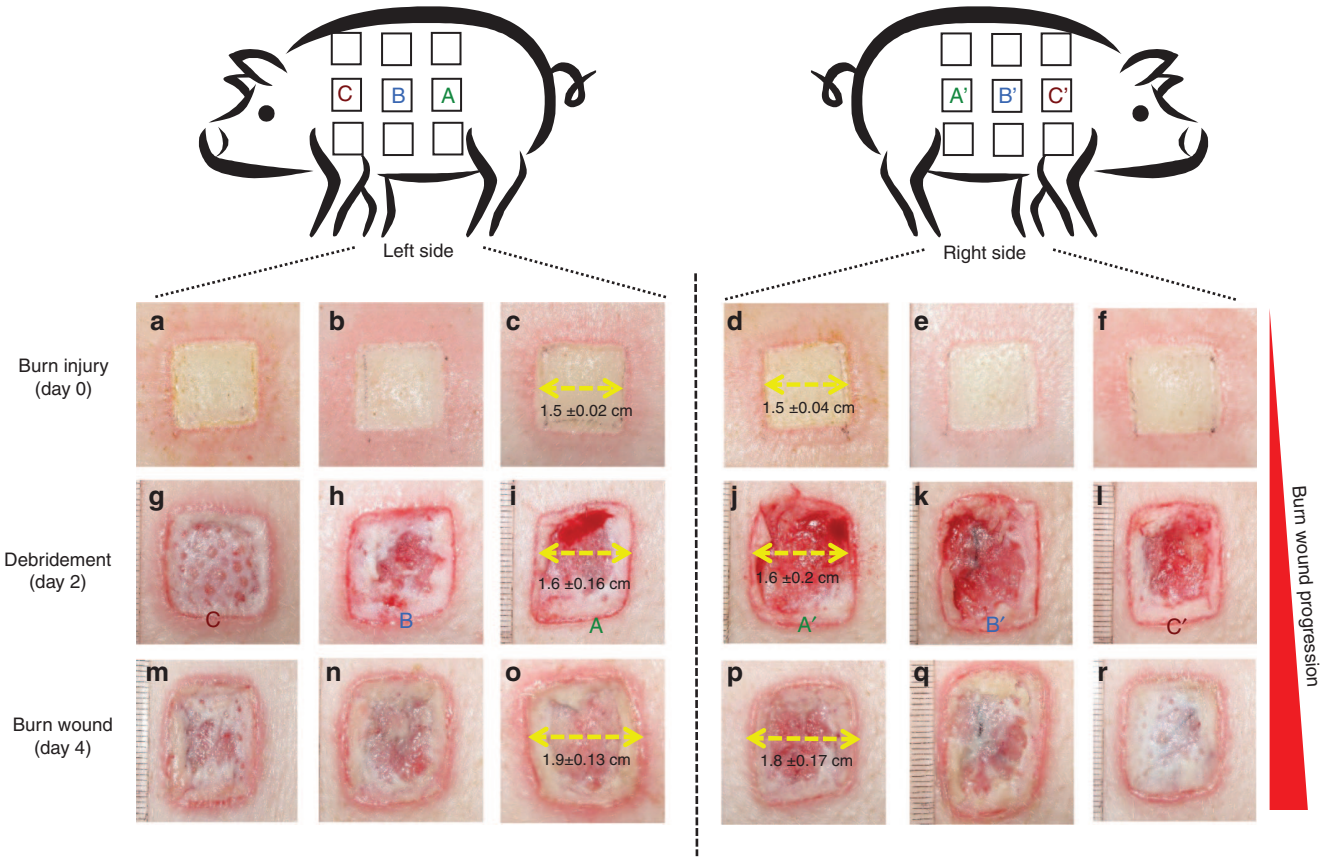

Figure 2 Debridement and wounds matching for testing topically applied agents. Total of 18 burns were created as previously mentioned (see Figure 1). Herein, however, the burns were debrided at the 48-hour time point following the burn injury to remove the (removable) dead tissue and to prepare it for topical treatments. Only the matching pairs of wounds (A to A′, B to B, and C to C′) were used for a specific treatment versus its control, since the wounds on the same side (A to C or A′ to B′) heal with different rates.[15]

To confirm this new finding, we performed immunohistochemistry on biopsy specimens of the burn wounds. As shown in Figure 5b, the specimens of CMC vehicle-treated burn wounds at day 9 exhibited massive cell apoptosis outside the original burn area (panel b, arrows) when compared with unburned skin (panel a). Interestingly, the heat-induced cell apoptosis, stained with an antibody to activated caspase 3, was almost completely prevented in the F-5 treated burn wounds (panel c). In comparison, when the same burn wound biopsies were stained with an antibody against HMGB1 to detect necrosis, as shown in Figure 5c, the F-5 treatment did not show any significant effect on heat-induced cell necrosis (panel c′ versus panel b′). Therefore, F-5 treatment selectively prevents cell apoptosis, but not cell necrosis, in burns and their surrounding tissues. Quantitation of these data is as shown (see Supplementary Figure S1c,d).

Since topical treatment with F-5 prevents apoptosis, *i.e.*, less dead cells in the wound, we speculated that the F-5–treated burn wounds might also show less host immune response and lower levels of inflammation. As shown in Figure 6a, the naphthol AS-D chloracetate esterase staining showed that the CMC vehicle-treated burn wounds exhibited a massive infiltration of neutrophils and other granulocytes (red stained; panel a and the enlarged insert, panel a′). F-5–treated burn wounds showed less accumulated pus on day 4 (as previously shown in Figure 5a) and, more intriguingly, largely the absence of neutrophils under the burn (panel b and enlarged insert panel b′). To confirm this unexpected finding using a different type of acute skin wound, we obtained biopsies of full-thickness excision wounds over time in pigs and subjected them to naphthol AS-D chloracetate esterase staining. As shown (see Supplementary Figure S4), we found that there were much less penetrated neutrophils and granulocytes in F-5–treated wounds, especially on day 2 to day 4 (panels f and h), than the CMC vehicle control-treated wounds (panels b and c). Enlarged representative images are shown (panels b′ and c′ versus panels f′ and g′; red arrows point to red-stained neutrophils). Furthermore, we stained monocytes and macrophages with an anti-CD163 antibody and found similar results. As shown in Figure 6b, F-5 treatment significantly reduced the macrophage staining (panel d versus panel c and panel d′ versus panel c′). Quantitation of the above data is shown in Figure 6c. While these appear to a correlation between the decreased apoptosis and a decrease in the level of inflammation, understanding their relationships would require additional studies in the future.

Topical F-5 treatment accelerates burn wound reepithelialization

In addition to preventing burn wound progression, F-5 greatly promoted burn wound closure. As previously shown in Figure 5a, the F-5–treated burn wounds exhibited much greater acceleration of wound closure (middle row) than either the vehicle alone-treated (top row) or the Regranex-treated (bottom row) burn wounds within the period of 9 days. This visual observation is confirmed by quantitation of the burn wound closure over time. As shown in Figure 7a, the vehicle alone-treated burn wounds expanded in size from day 2 to day 4, started to come back to the initial wound size on day 7 and, then, showed a trend of wound closure (bars 1, 4, 7,

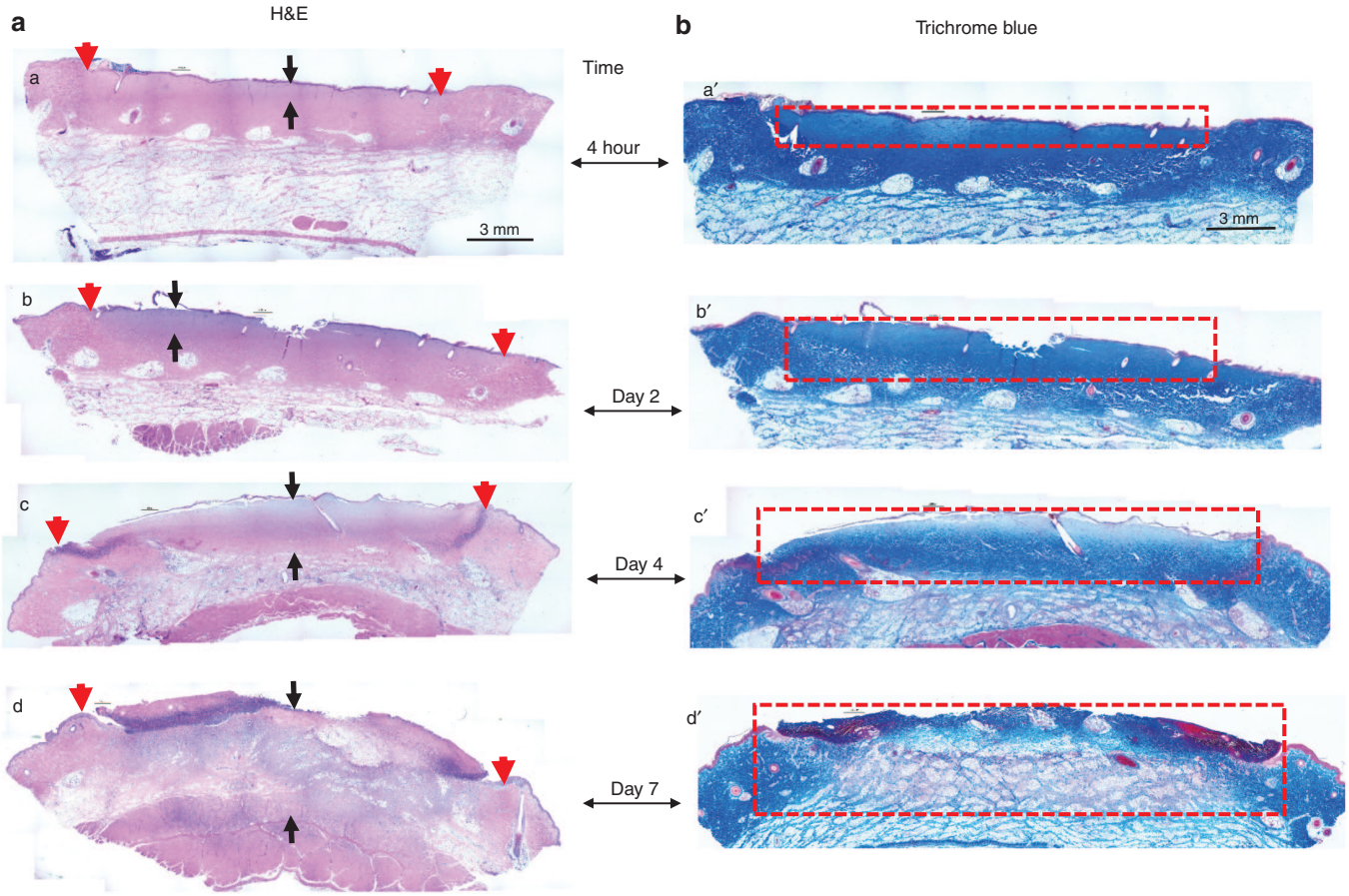

Figure 3 Histological analyses of healing burn wounds in pigs. Skin biopsies of burn wounds from day 0 to day 7 were subjected to the indicated histochemistry analyses. (**a**) hematoxylin–eosin staining showed damages of the skin structure with increasing width and depths over time by burn, as pointed out by red (horizontal) and black (vertical) arrows. (**b**) Trichrome blue staining revealed the increasing damage of the collagens over time by burn, as indicated by the increasing sizes of red boxes. Fifteen independent images under each time point of burns were analyzed and quantitated (see Supplementary Figure S1a).

and 10). Similar results were obtained from the Regranex-treated burn wounds, which showed a modest acceleration of wound closure over the vehicle control (bars 3, 6, 9, and 12; Regranex is not recommended by FDA for the treatment of acute wounds).[7,27] However, unlike the vehicle control and Regranex treatment, the F-5–treated burn wounds did not go through the phase of wound expansion phase (bar 5) and exhibited much greater acceleration of wound closure on day 7 and day 9 (bars 8 and 11). The results of these findings were reproduced by burn wound healing studies over a period of 2 weeks (see Supplementary Figure S5).

To investigate how F-5 treatment has such a robust effect on burn wound closure, we stained the day 9 (day 7 post-treatment) burn wounds with Masson's trichrome blue to (i) reveal the boundary between burned and unburned skin (due to damaged and undamaged collagens, blue stained) and (ii) examine the newly migrated epidermis (pink stained) as defined by the reepithelialization tongue. As clearly shown in Figure 7b, the vehicle alone-treated and the Regranex-treated burn wounds showed a small degree of reepithelialization (panels a and c) when compared with burn wounds treated with F-5 fragment (panel b). A close-up look at the boundary between the burned and unburned junction confirmed the observation (panels a′ and c′). In contrast, the F-5–treated burn wounds exhibited a dramatically elongated reepithelialization tongue toward the wound bed (panels b and b′), consistent with our previous report that extracellular Hsp90α serves primarily as a potent motility factor for keratinocytes.[11,12] Quantitation of the reepithelialization tongue is shown in Table 1. Taken together, the above results demonstrate that topically applied F-5 fragment of Hsp90α has two functions in wound healing: (i) to prevent burn wound progression by protecting the surrounding cells from entering apoptosis and (ii) to accelerate burn wound closure by stimulating reepithelialization.

DISCUSSION

The critical and unique pathological manifestation of skin burn wounds is the secondary wound progression within the initial week of injury, which is thought to be due to cell necrosis and apoptosis. There is no FDA-approved topical therapy for skin burn wounds. Based on the science, an effective burn wound therapy should be dual effect: preventing the burn wound progression and thereafter accelerating burn wound closure. When skin is wounded, the wound area experiences acute hypoxia that stimulates the lateral migration of keratinocytes across the wound bed and begins the process of reepithelialization within a matter of several hours.[28] Our laboratory reported that, under an acute hypoxia, the migrating keratinocytes secrete Hsp90α into the wound bed where the secreted Hsp90α acts to increase cellular motility both as an autocrine factor on the keratinocytes and as a paracrine factor for peri-wound fibroblasts and microvascular endothelial cells. Through its ability to promote cell migration, the keratinocyte-secreted Hsp90α advances wound reepithelialization, fibroplasia, and neovascularization, the three

essential processes in early wound healing.[17] Exogenous addition of excess Hsp90α to acute, full-thickness, skin wounds dramatically accelerated wound closure in normal and diabetic preclinical models including mice and pigs.[11–13] In the study herein, we show that the therapeutic entity, F-5, of the secreted Hsp90α added in excess to pigskin burn wounds greatly accelerates wound closure compared with vehicle controls or the Regranex gel. More encouragingly, we found that F-5 shows an additional useful property in burn wounds to nullify the process of burn wound progression. In our porcine burn wound model, topical application of the F-5 fragment to acute burn wounds prevented cell apoptosis, reduced heat-induced inflammation and destruction of dermal collagen, and promoted reepithelialization for accelerating wound healing. Therefore, the F-5 fragment of Hsp90α has a strong clinical implication for burns in humans.

Burns are generally described as first-degree (superficial burn), second-degree (partial-thickness), or third-degree (full-thickness) burns.[26,29] First-degree burns exhibit redness, swelling, and mild blistering and usually heal in a few days with minimal treatment. Second-degree burns affect both the epidermis and a variable portion of the dermis and are still capable of spontaneous healing with variable healing time. Third-degree burns are incapable of healing spontaneously, due to its destruction of the entire depth of the epidermis and dermis, and almost always merit excision and grafting. The potential therapeutic effectiveness of topical F-5 would likely be the most beneficial for second-degree burns, in which the surrounding cells remain viable for variable periods of time following the initial injury. Topical F-5 applied to second-degree burns could prevent burn wound progression by preventing the cells from undergoing apoptosis and salvaging the zone of stasis. The idea that topically applied Hsp90α may protect cells from environmental stress-induced apoptosis came from several of our recent studies. Using gene knockout mouse models, Tsen *et al*. demonstrated that Hsp90α accelerates wound closure through the Akt signaling pathway, the most recognized survival pathway in all cells.[14] Consistently, we found that F-5–treated burn wounds show higher levels of Akt activation (See Supplementary Figure S5). Likewise, Dong *et al*. recently showed that Hsp90α uses the low-density lipoprotein receptor-related protein-1 receptor on breast tumor cells to protect themselves from hypoxia-induced cell death.[30] Guo *et al*. observed that secreted Hsp90α blocks the intrinsic (including heat) apoptotic pathway (J. Guo, P. Jayaprakash and W. Li, unpublished data). These observations prompted us to investigate whether secreted Hsp90α could effectively lessen the degree of burn wound progression. The data generated herein support the initial speculation.

The degree of temperature and duration of contact for creating burns in animal models were often debated for their relevance to the burns in humans.[31] In reality, it is not possible to exactly relate animal burns for research to human burns seen at the clinic. For example, human burn wounds can be caused by a variety of environmental insults, including boiled water or steam (~100 °C), heated iron (sole plate, ~200 °C), boiled cooking oil (soybean oil, ~300 °C), gas fire (815 °C), to name a few. The duration of contact varies and the temperature can hardly maintain the same throughout the incidents. In laboratory research, however, due to animal safety regulations, often the least harsh conditions are allowed. For instance, one may not be able to justify using 815 °C to create burn wounds in animals, whereas these burns are mechanistically similar to burn wounds caused by 100 °C burn. Most previous burn wound studies used heat blocks with 50 °C to 120 °C and reported that only >100 °C caused "deep partial thickness wounds," required to see "burn

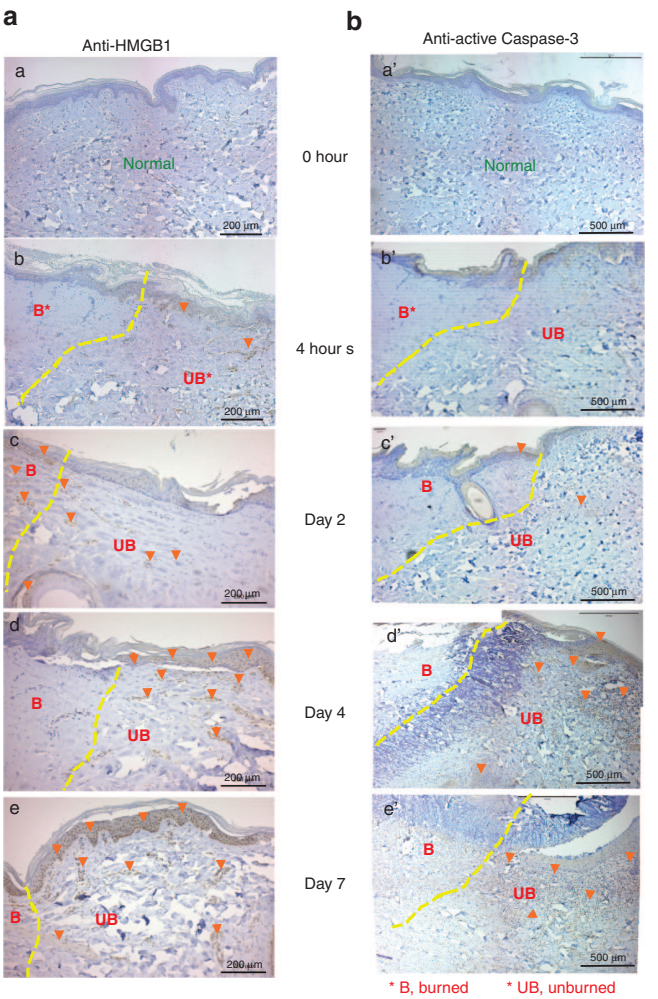

Figure 4 Burn causes necrosis and apoptosis in unburned areas in pigs. (**a**) Anti-HMGB1 antibody staining (brown) showed tissue necrosis in the areas surrounding burn wound over time, as indicated by orange arrows. (**b**) Anti-activated caspase 3 staining (dark brown) showed burn-caused tissue apoptosis in burn wound-surrounding areas over time. B (in red), burned area; UB (in red), unburned area. Fifteen independent images under each time point of burns were analyzed. The images shown represent a consensus from multiple and noncontinuous sections of a given skin specimen. Quantitation of the data is shown in Supplementary Figure S1a.

wound progression."[31] During our study, choosing the quick period of 30 seconds to avoid any drastic drop of the heat block temperature when it is applied to the animal skin, we tested the temperature range from 80 to 120 °C and found that "burn wound progression" occured most evidently and reproducibly after 120 °C heat shock. In all, creating multiple wounds with consistent pathological parameters for evaluation of therapeutics is a constant challenge and continues to be improved.[32]

There is a list of other agents tested for preventing burn wound progression in various preclinical models.[8] The therapies used to date to lessen wound progression in burn wounds are based on regulating perfusion of the tissue, inhibiting coagulation, inhibiting inflammation, and preventing apoptosis. Tobalem *et al*. used cold and warm water treatments to delay secondary burn wound progression and reported mixed results.[33] The same group investigated the effect of i.p. injection of erythropoietin on secondary burn wound progression in rats and found that erythropoietin reduced

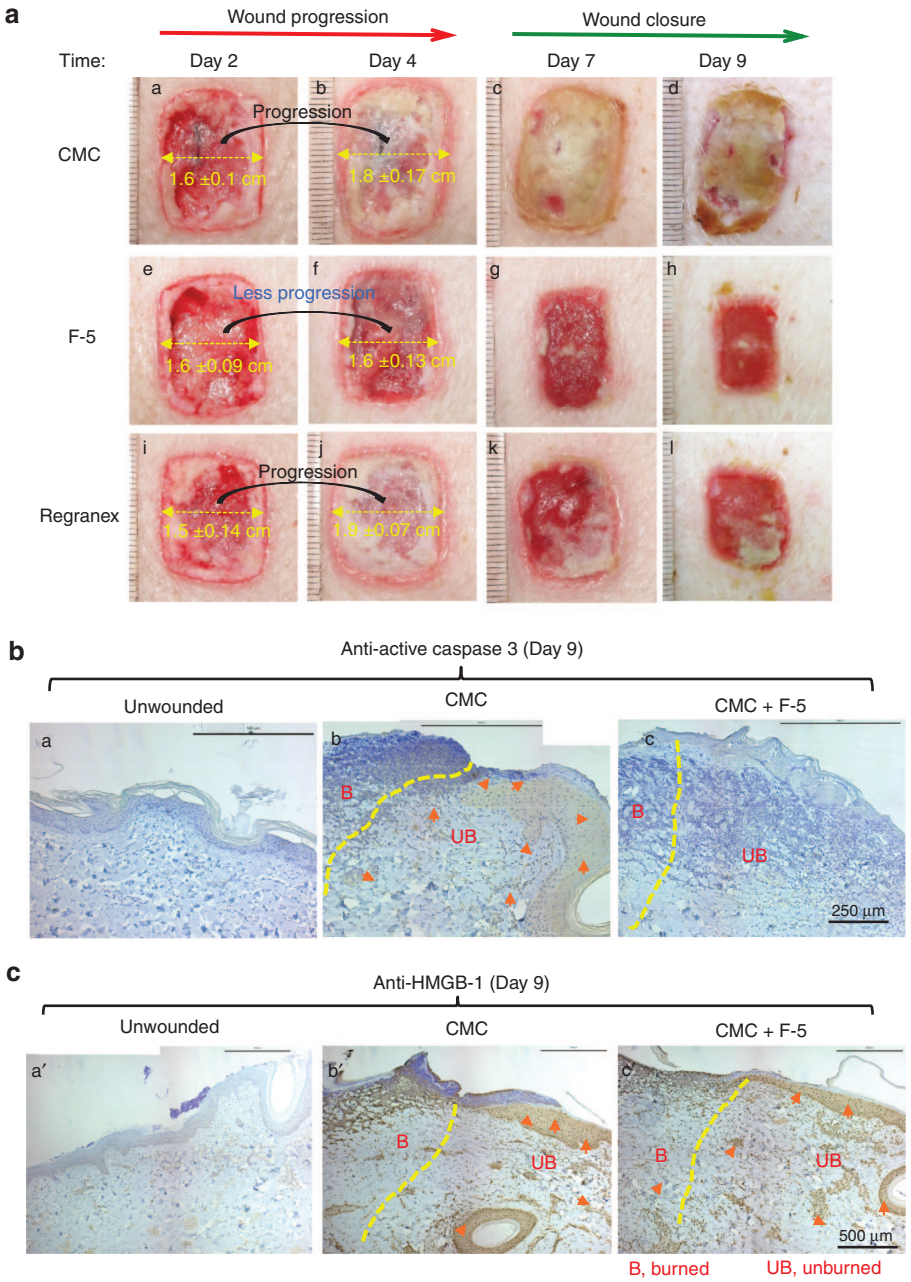

Figure 5 F-5 treatment prevents burn wound progression and accelerates burn wound closure. (**a**) Debrided burn wounds on day 2 were topically treated with carboxy methyl cellulose (CMC) vehicle, CMC plus F-5 peptide (46 µmol/l) and the commercial Regranex gel. The wound progression and wound closure were monitored on day 4, 7, and 9 following the initial injury (see Figure 7a for quantitation). (**b**) F-5 treatment prevents burn-induced apoptosis in burn wound-surrounding area (pane c versus panel b). (C) F-5 treatment shows little rescue effect on burn-caused necrosis (panel c′ versus panel b′). Regranex-treated burn wounds showed similar results as CMC-treated burn wounds. Quantitation is shown in Supplementary Figure S5a,b. B (in red), burned; UB (in red), unburned.

surface necrosis and increased baseline perfusion, which supported erythropoietin's positive effect on burn wound progression.[34] Singer *et al*. reported that inhibition of transforming growth factor β1 signaling accelerates reepithelialization and rescues scar formation in pig burn wounds, suggesting that transforming growth factor β1 plays a negative role in burn wound healing.[35] Several groups reported that topical application of inhibitors of tumor necrosis factor α production or anti- tumor necrosis factor α antibodies reduced the inflammatory response and lessened burn wound progression in pigs.[36,37] Taira *et al*. reported that Rosiglitazone, an insulin sensitizer that enhances the responses of the target cells to insulin, reduces burn progression in rats.[38] Zhang *et al*. showed that topical Hsp90α protein promotes burn wound closure in mice.[39] Eski *et al*. reported that topical treatment of burn wounds with cerium nitrate, an antiseptic agent, prevents tissue necrosis in burn wounds in rats, independently of its anti-infection effect.[40] Singer *et al*. reported that the antioxidant, curcumin, was able to reduce significantly burn wound progression in rats.[41]

As previously mentioned, burn wound progression was thought to result from cell necrosis and apoptosis. However, the processes of apoptosis and necrosis are unrelated and use completely distinct mechanisms. Necrosis is an uncontrolled and passive process

that usually affects large field of cells. Necrosis is mediated by two mechanisms, interference with the energy supply of the cell and direct damage to cell membranes. On the contrary, apoptosis is programmed controlled cell death and can affect only individual or cluster of cells. Apoptosis is regulated by three signaling pathways: extrinsic (death receptor ligand pathway), intrinsic (mitochondrial pathway), and perforin/granzyme pathway.[42,43] A previous study showed that necrosis plays a larger role in burn wound progression than apoptosis between 1 and 24 hours post burn.[10] In our experiments, we were only able to visualize a significant wound size expansion between 48 and 96 hours post burn, during which the topically applied F-5 peptide showed an inhibitory effect on apoptosis and burn wound progression. These two sets of results are not necessarily mutually exclusive due to fact that they differ spatiotemporally. Then, the finding of this study implicates that apoptosis is a major contributor of the burn wound progression in the later phase of burn progression and could be treated as effective biomarker and drug target.

In summary, it is clear that an effective treatment of burn wounds would require an agent that both inhibits the process of wound progression and thereafter accelerates the event of reepithelialization, leading to the wound closure. Second, considering that secondary burns could occur to a large area of a human body, such an agent must also be safe and show limited side effects. As far as our current understanding goes, the secreted form of Hsp90α plays little role in development and a critical role in repair of damaged tissues as a stress-responding molecule. The 115 amino acid F-5 fragment (of the 732 amino acid full-length human Hsp90α), which has eliminated the N-terminal and C-terminal domains of Hsp90α that might cause off-target effects, appears to fit nicely into these criteria. It remains to be seen if its dual effect on pig burn wounds can be translated to humans.

MATERIALS AND METHODS

Animals and housing

Female Yorkshire pigs (S&S Farms, Ramona, CA) 2 to 3 months in age and weighing 20–25 kg at arrival were acclimated for approximately 1 week prior to the experimental procedures. All pigs were housed at the University of Southern California Keck School of Medicine Animal Facility in the basement level of the Hoffman Building. These animals were fed and housed individually at atmospheric pressure at 20–23 °C with ~60% humidity and a 12-hour light/12-hour dark cycle. The University of Southern California veterinary staff under the guidelines of the University of Southern California Animal Research Committee monitored the physical well-being of the animals and all of the experimental procedures at the facility. All procedures, maintenance, and treatment of the animals were in accordance with the principles of humane treatment published by the National Academy of Sciences (US Department of Health, 1996) and The Institutional Animal Care and Use Committee of the University of Southern California protocol.

Creating burn and excision wounds

To create burn wounds, the pigs were sedated with telazol and xylazine combination 1.1–2.2 mg/kg dosage i.m. for induction and then intubated and maintained with sevoflurane or isoflurane 1–4% continuous inhalation. The pigs were shaved to remove hair from both sides of their torsos. The skin was disinfected with three repeated alternating scrubs of ethanol and a germicidal scrub (chlorohexidine or betadine). The surgical skin site on the torso was scrubbed with a betadine scrub and solution three times using the sterile prep kit provided in the operating room at the facility. Once the burn wounds were created on the right side, the animal was turned over onto its left side and the procedure was repeated. Nine burn wounds were created on each side for a total of 18 burn wounds on the animal. This results in approximately 10–15% of the body surface area being wounded. The site of the wound were designated with a black marking pen on the skin over the paravertebral area of the pigs (torso), with a template marking nine squares 1.5 × 1.5 cm^2. Wounds were separated from one another by 4 cm of unwounded skin. Contact burn injuries were created on the skin of the pigs' sides using brass thermal blocks (3 cm long, 1.5 cm wide, and weighing 140 g), which are preheated on a temperature-controlled metal heat block (Dry Heat Block, VWR, , Randor, PA) with the temperature set at 115 °C. The contact time of the brass block on the skin was 30 seconds with the blocks' gravity, allowing the creation of burn wounds with a consistent depth. The burns were either un-debrided or debrided at 48 hours after being created to remove the dead tissue.

To create full-thickness excision wounds in pigs, the animal is initially sedated with a telazol and xylazine combination 1.1–2.2 mg/kg dosage i.m. for induction and then intubated and maintained with sevoflurane or isoflurane 1–4% inhalational continuous. The pig is shaved along its torso on both sides. The pig is placed on its right side (left side up). The surgical

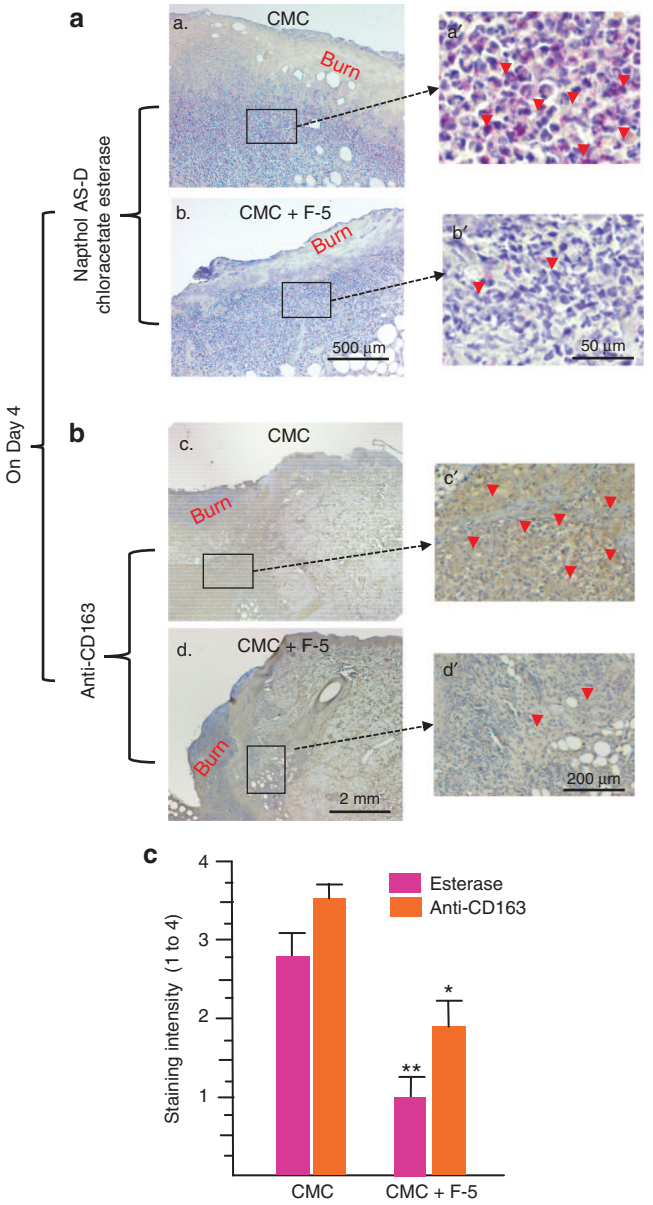

Figure 6 F-5 treatment reduces inflammation in acute skin wounds. (**a**) Napthol AS-D chloracetate esterase staining shows penetration of neutrophils and other granulocytes (panel a) in an enlarged image (panel a′). F-5 treatment almost completely prevents the neutrophil penetration (panels b and b′) in burn wounds. (**b**) Anti-CD163 antibody staining shows penetration of monocytes and macrophages (panel c) in an enlarged image (panel c′). F-5 treatment reduces the amount of macrophage penetration (panels d and d′) in burn wounds. (**c**) Quantitation of the data above, based on 15 randomly selected images (see Materials and Methods) $^{*}P < 0.05$; $^{**}P < 0.005$.

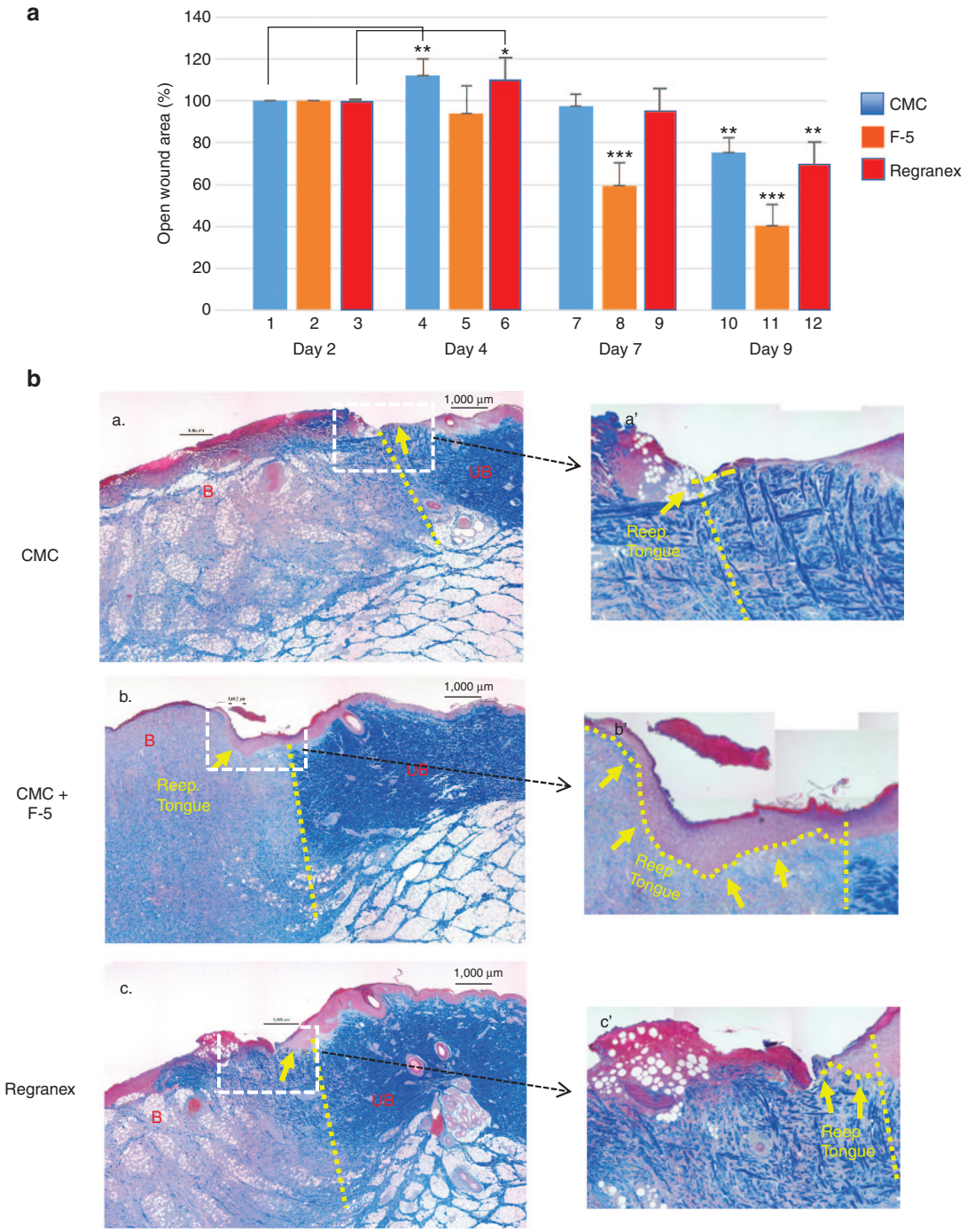

Figure 7 F-5 promotes burn wound closure by enhancing reepithelialization in pigs. (**a**) Quantitation of the burn wound healing data previously shown in Figure 5a. Wounds in triplicates were photographed on the indicated days and analyzed for wound closure rates (% of open wound area). The experiment was repeated three times (in three pigs) and data quantitated as mean ± SD. *$P \leq 0.05$; ** $P \leq 0.01$; ***$P \leq 0.001$, compared with the placebo. (**b**) Wedge biopsies were made on day 9 burn wounds and stained with trichrome blue. The reepithelialization front (pink) was enlarged and shown (a′, b′, and c′). Quantitation of the data is shown in Table 1. Reep. Tongue: Reepithelialized tongue; B, burned; and UB, unburned.

skin site on the torso is scrubbed with betadine scrub and solution three times using the sterile prep kit provided in the operating room. The outline of the wounds is created with a precut paper template and a permanent marker. Wounds will be 1.5 × 1.5 cm^2 with 4 cm of unwounded skin between two wounds. The maximum number of excision wounds on each side is 12, making a possible maximum of 24 wounds for each experimental investigation, as previously described.[15]

FDA-approved becaplermin gel (Regranex® from Smith and Nephew, Andover, MA, or recombinant human PDGF-BB) was used as a positive control. Recombinant full-length or F-5 fragment of Hsp90α was mixed in 0.1 or 0.3 g of 15% sterile CMC in ~250 µl volume. These drug or tested Hsp90α proteins were topically applied on wounds in triplicates in a pig. Wounds were covered with Opsite clear bandages (Smith and Nephew, Hull, UK), overlaid with a cotton gauze cloth and finally taped to cover the entire wound area. Finally, the entire area that included all the wounds on two sides of the pig is wrapped (360°) in elastic bandages, followed by a final wrap in an elastic tape (Elastikon®, Johnson & Johnson, New Brunswick, NJ).[15]

Table 1 Measurement of wound reepithelialization

Treatment	*Length of reepithelialization tongue (μm)*
CMC	1,375 ± 176.78
F-5	2,175 ± 106.06
Regranex	1,350 ± 212.13

Five randomly selected images under each indicated treatment, as shown in Figure 7b, were used to measure the length of newly generated reepithelialization tongue by three laboratory members who are not involved in this project. Values are represented as means ± SD. $P < 0.05$ to 0.005.

Measurements of wound healing

After surgery and treatments, digital photographs were taken individually of each wound on the indicated days from a fixed distance. Wound healing was analyzed based on measurements of wound progression, wound closure, and histology/immunohistochemistry analyses. To measure (horizontal) wound progression, the area of an open wound was measured by *H* (cm) × *W* (cm) to give rise to square centimeter of the wound. Means of the middle triplicate wounds on both side of the animal were used for the presentations. The wounds at the top and bottom rows of the animal showed similar results, while the actual numbers of the measurements varied. The results were reproducible in three pigs. The vertical wound progression was evaluated by immunohistochemistry analyses (see below). To measure wound closure, the area of an open wound on that day was measured and compared with the area of the wound on day 0 following surgery, using the software AlphaEase FC version 4.1.0 (Alpha Innotech Corporation, Miami, FL), as previously described.[13,15]

Immunohistochemistry

The histological and immunohistological analyses were carried out for skin wounds, in which Wedge biopsies measuring $3 \times 3\,cm^2$ were taken on specified day for skin wounds. All tissue samples were fixed in 10% formalin (VWR, Randor, PA) and placed in paraffin blocks for sectioning. Histological analyses included hematoxylin and eosin and Masson's trichrome blue staining. Immunohistochemistry studies on skin wounds were conducted with anti-HMGB1 antibody for necrosis (1:100, GTX101277, GeneTex, Irvine, CA), anti-activated caspase 3a antibody for apoptosis (1:200, GTX61121, GeneTex). Neutrophils and granulocytes were stained by naphthol AS-D chloracetate esterase (Sigma, St. Louis, MO). Monocytes and macrophages were stained with antibodies against the CD163 receptor (1: 2000 dilution, Novus Biologicals, Littleton, CO). Fifteen randomly selected images under each condition were visualized under microscope by three experts in immunohistochemistry analysis who were not involved in this project and pre-agreed on intensity of the staining by looking at standardized slides, to provide scores: 1 = none, 2 = weak, 3 = clear, and 4 = strong. The consensus of their readings guided us to conclusions.[14,15]

Protein purification

Complementary DNA cloning, production, and purification of recombinant Hsp90α proteins have been carried out as previously described.[14,15] Tag-free and GMP-purified F-5 (>97% purity) was from Codex Therapeutics (Gaithersburg, MD) .

Statistic analyses

Data on animal wound healing were based on three or more independent experiments. Data are presented as mean ± SD. Statistical significance for comparisons was determined by the Student's two-tailed *t*-test. The values of $P \leq 0.05$ were considered statistically significant.[14,15]

CONFLICT OF INTEREST

The authors declared no conflict of interest.

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