

# Stromal-derived factor-1 delivered via hydrogel drug-delivery vehicle accelerates wound healing in vivo

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Presented in part at the 19th Wound Healing Society Annual Meeting in Dallas, Texas, April 26, 2009.

Manuscript received: February 16, 2010  
Accepted in final form: January 25, 2011

DOI:10.1111/j.1524-475X.2011.00687.x

## ABSTRACT

Topical treatment of superficial wounds has many advantages including decreased cost and increased ease of application compared with systemic treatments. Many of the advantages, however, are lost when it is necessary for repeated doses of topical medications to be given over an extended period of time. Therefore, a drug-delivery vehicle that delivers biologically appropriate doses in a sustained fashion would prove valuable. In this study, an alginate hydrogel scaffold impregnated with the angiogenic chemokine stromal-derived factor-1 was used to provide targeted, though short-term, delivery directly into the wound bed. Wounds were created on the dorsum of mice, and either a stromal-derived factor-1-impregnated or a saline-impregnated scaffold was applied. Wounds were explanted after 1, 3, 7 days, wound area was measured, and histology and immunohistochemistry for endothelial markers were performed. The remaining wound area in stromal-derived factor-1-treated wounds vs. controls was not significant 1 day after wounding ( $96.7 \pm 0.1$  vs.  $97.5 \pm 1.1\%$ ,  $p=0.317$ ), but was significant after 3 days postwounding ( $46.7 \pm 0.1$  vs.  $82.3 \pm 2.4\%$ ,  $p=0.046$ ) and 7 days postwounding ( $2.3 \pm 1.3$  vs.  $32.0 \pm 4.0\%$ ,  $p=0.049$ ). Immunohistochemistry revealed a greater degree of endothelial cell invasion into the wound bed infiltration compared with controls. The results of this study suggest significant clinical promise for our hydrogel-delivery vehicle in the treatment of wounds.

Wounds, both acute and chronic, are a significant burden affecting hundreds of thousands of patients and resulting in billions of dollars of outlays. With improved understanding of the mechanisms by which wounds heal,<sup>1–3</sup> increasingly effective treatment regimens have been developed that involve variations in type, duration, and timing of pharmacologic intervention. Such strategies may be used not only to augment “normal” wound healing, but may also have particular value in their potential to promote healing in more challenging scenarios such as chronic, diabetic, and radiated wounds.

It is increasingly recognized that in the appropriate setting, extended or repeated delivery of an agent to promote healing, even over a relatively brief interval, can be more effective than a single dose alone. Several studies have shown that faster rates of wound closure were achieved when an agent was serially delivered over a prolonged period.<sup>4,5</sup> This extended delivery method is more biologically accurate compared with a single dose which, even if supra-physiologic, is quickly metabolized either locally (by the harsh environment of the ischemic, often bacteria-colonized wound) or systemically, resulting in significantly decreased bioavailability.

While potentially effective, treatment regimens that involve repeated dosing schedules are costly and resource-intensive. A drug-delivery vehicle that delivers agents in a

time-release fashion and therefore harnesses the advantages of continuous dosing while still being delivered in a manner which is simple and cost-effective, would be highly beneficial to patients and healthcare providers alike, and would result in significant cost savings.

Toward this end, we have developed a porous alginate scaffold that functions as a drug-delivery vehicle that can be applied directly to the wound bed.<sup>6</sup> The structure of the scaffold allows for slow, consistent release of the impregnated solution over a period of 18–24 hours. Although the scaffold can be loaded with any solution, in this study the scaffolds were saturated with the chemokine stromal-derived factor-1 (SDF-1), a molecule that is increasingly recognized to play a central role in normal wound healing. While previous work has evaluated the effect of SDF-1-impregnated scaffolds after 9 days, the purpose of this study was to evaluate for the first time the effects of this treatment in a murine cutaneous wound model at the earlier time points of 1, 3, and 7 days.

## METHODS

### Fabrication of alginate scaffolds

The sodium alginate (molecular weight: 110,000; Wako Pure Chemical Industry, Osaka, Japan) solution was

prepared according to the previously described ionotropic gelation method.<sup>7</sup> The aqueous sodium alginate was poured into an alginate-coated beaker and sprayed with calcium chloride in order to form a thin alginate gel layer. Next, an additional layer of calcium chloride was added on top of the thin gel layer to complete gelation, and the gels were washed with deionized water in order to remove free calcium ions. The process of lyophilization allows the gels to form the necessary “honeycomb”-like structure, and was performed by freezing the resultant gels at  $-80^{\circ}\text{C}$  and then placing under vacuum at room temperature for 2 days.

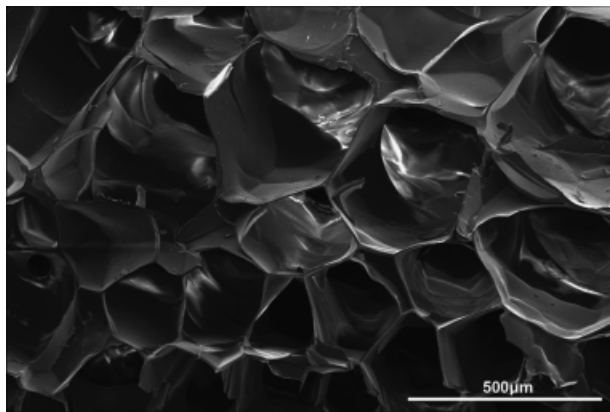
The lyophilized alginate gels were then cut into discs (5 mm in diameter by 2 mm in thickness) and covalently cross-linked with ethylenediamine (5.17 mM) by activating the carboxyl group with *N*-hydroxysuccinimide (NHS, 114 mM) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC, 305 mM) in 2-(*N*-morpholino)-ethane sulfonic acid (MES) buffer (0.2 M, pH 6.0) (all purchased from Sigma, St. Louis, MO). After 24 hours of cross-linking, the resulting scaffolds were washed with phosphate-buffered saline (PBS) and immersed in 0.015 M spermine chloride/MES buffer solution containing NHS (114 mM) and EDC (305 mM) overnight at room temperature.

### SDF-1 incorporation

The spermine-immobilized scaffolds were sterilized with 70% ethanol and allowed to air-dry in an aseptic tissue culture hood. Twenty microliters of aqueous SDF-1 protein (1.0 ng/ $\mu\text{L}$ ; Sigma) containing sodium heparin (1 U/ $\mu\text{L}$ ; Sigma) was dropped onto the sterilized scaffolds and incubated at  $+4^{\circ}\text{C}$  overnight in order to complete protein incorporation (Figure 1). Control scaffolds were prepared in exactly the same fashion, with the exception that the SDF-1 protein was omitted.

### Mice

Nine 8-week-old male C57BL/6 mice (Jackson Laboratory, Ben Harbor, ME) were used for this study. All animal care and experimental procedures were approved by the Weill



**Figure 1.** Scanning electron microscopy image of the alginate scaffold. Note the latticework-like appearance with the presence of intervening pores approximately 250  $\mu\text{m}$  in diameter.

Cornell Medical College Institutional Animal Care and Use Committee and were in compliance with the Guide for the Care and Use of Laboratory Animals.<sup>8</sup>

### Splinted excisional wound model

Mice were anesthetized via intraperitoneal injection of ketamine (80 mg/kg) and xylazine (60 mg/kg), and two circular full-thickness skin wounds (6 mm in diameter) were created on the dorsum of each mouse.<sup>9</sup> One 5 mm calcium alginate gel scaffold was placed into each wound, one of which had been saturated with SDF-1, and the other with saline. Silicone splints (6 mm in diameter) were sutured in place to secure the calcium alginate scaffolds, and the wounds were covered by an occlusive dressing (Tegaderm, 3M, Minneapolis, MN).

### Tissue harvest

After 1, 3, or 7 days, the animals were sacrificed and the wounds were explanted. Tissue was fixed for 24 hours in 10% buffered formalin, dehydrated, and embedded in paraffin. Ten-micrometer sections were prepared, and stained with hematoxylin and eosin (H&E).

### Wound measurement

At each time point, the remaining full-thickness wound within the explanted tissue specimen (defined as the area of the epithelial gap between encroaching epidermal elements) was measured based on photomicrographs taken with an upright bright field microscope (Nikon eclipse 80I, Tokyo, Japan). The wound closure speed was then calculated using the formula:

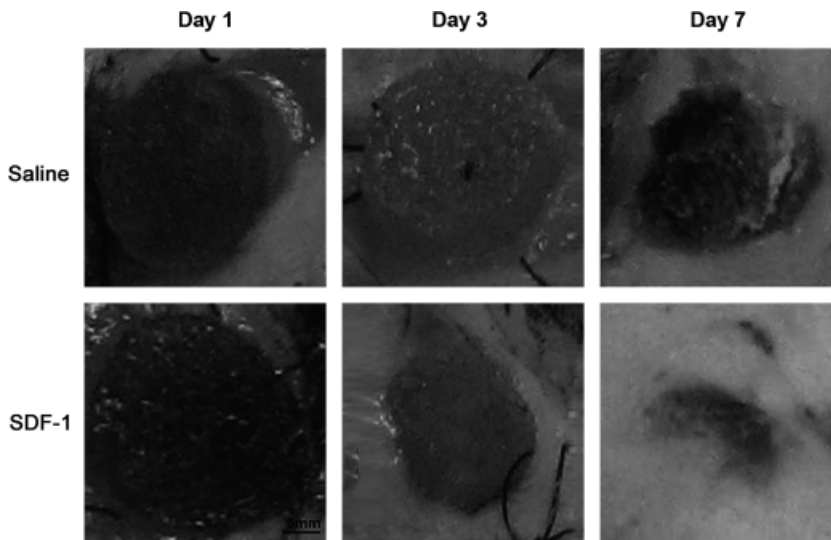
$$\text{wound closure speed (mm}^2/\text{day)} = \frac{(\text{remaining wound area at time A} - \text{remaining wound area at time B})}{\text{number of days between time A and B}}$$

### VE-cadherin and von Willebrand Factor (vWF) immunohistochemistry

For immunohistochemical (IHC) analysis, the sections were deparaffinized, rehydrated, and then incubated in 3%  $\text{H}_2\text{O}_2$  to eliminate endogenous peroxidase activity. Next, the sections were washed with PBS and incubated with goat serum for 20 minutes. Then, sections were incubated for 30 minutes with antibody to either VE-cadherin or vWF, washed with PBS, and incubated for 20 minutes with biotinylated anti-goat secondary antibody. Next, the specimens were incubated for 30 minutes with avidin-biotin complex, and then for 30 minutes with a biotinylated horseradish H reagent. Finally, the slides were treated with diaminobenzidine (all from Sigma) and hematoxylin counterstain.

### Statistical analysis

Values are presented as mean  $\pm$  standard error of the mean. Statistical significance was determined with the use



**Figure 2.** Gross images of SDF-1- and saline-impregnated scaffolds after 1, 3, and 7 days. Note the decreased remaining wound area in animals treated with SDF-1-impregnated scaffolds compared with saline-impregnated scaffolds. SDF-1, stromal-derived factor-1.

of a Mann–Whitney U test for comparison of two groups. The significance level was set at  $p < 0.05$ .

**RESULTS**

Time-released delivery of SDF-1 to the wound bed decreased wound-healing time compared with blank controls on gross analysis (Figure 2). Twenty-four hours postwounding, the area of remaining wound when treated with scaffold-containing SDF-1 was reduced to  $96.7 \pm 0.1\%$  of the initial wound area, compared with  $97.5 \pm 1.1\%$  seen with the blank scaffold ( $p=0.317$ ). Three days postwounding, the remaining wound area in the mice treated with SDF-1 was  $46.7 \pm 0.1\%$ , and in the blank group was  $82.3 \pm 2.4\%$  ( $p=0.046$ ). Seven days postwounding, the SDF-1 group wounds were nearly closed, with a remaining wound area of  $2.3 \pm 1.3\%$ , compared with the blank group, which had a remaining wound area of  $32.0 \pm 4.0\%$  ( $p=0.049$ ) (Figure 3).

The wound closure rate for the period between days 0 and 1 postwounding in the SDF-1 group was  $0.94 \text{ mm}^2/\text{day}$  and in the saline group it was  $0.70 \text{ mm}^2/\text{day}$  (Figure 4). For the period between 1 and 3 days postwounding, the rate of wound closure in the SDF-1 group was  $7.07 \text{ mm}^2/\text{day}$  and in the saline group was  $2.15 \text{ mm}^2/\text{day}$ . Finally, between days 3 and 7 postwounding, the rate in the SDF-1 group was  $3.14 \text{ mm}^2/\text{day}$  and in the saline group was  $3.55 \text{ mm}^2/\text{day}$ .

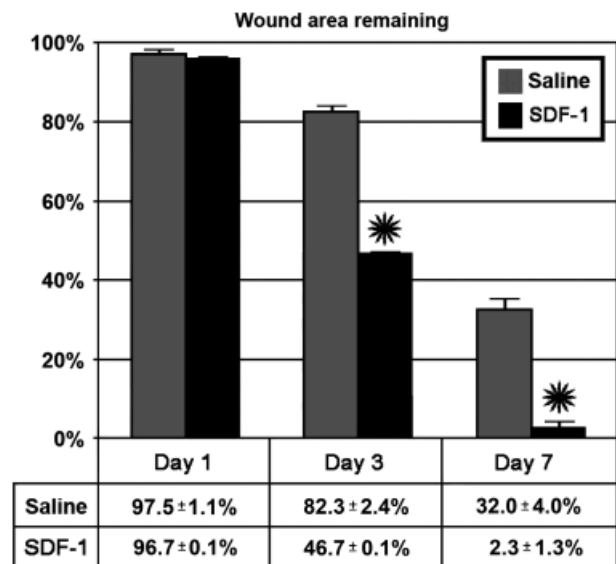
Histological analysis showed that 3 days after wounding, significantly more cells had invaded the wounds treated with SDF-1-impregnated scaffolds (Figure 5A) compared with those that had been treated with the saline control (Figure 5D). Seven days postwounding, the wounds treated with the SDF-1-impregnated scaffold were almost completely closed (Figure 6D). This was in contrast to the wounds treated with the saline-impregnated scaffolds, which were still clearly present (Figure 6A).

There was greater VE-cadherin expression in the wounds treated with the SDF-1-impregnated scaffold at both 3 and 7 days postwounding (Figures 5E and 6E),

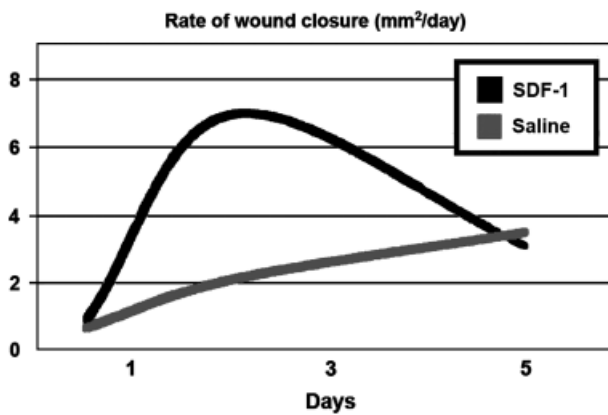
compared with saline control (Figures 5B and 6B). Furthermore, vWF expression was more intense in the SDF-1-treated-impregnated scaffold wounds at both 3 and 7 days postwounding (Figures 5F and 6F), compared with the saline control (Figures 5C and 6C).

**DISCUSSION**

Wounds, particularly those that are chronic or occur in the setting of diabetes and/or radiation, are a particularly challenging clinical entity to treat, because the elements



**Figure 3.** Remaining wound area for measured for day 1, 3, and 7. Shown as a percentage of the initially created wound area at day 0. The stars represent statistically significant values with  $p < 0.05$ . Error bars signify mean  $\pm$  standard error of the mean.



**Figure 4.** Wound closure rate (mm<sup>2</sup>/day) calculated for the time intervals 0–1 day, 1–3 days, and 3–7 days.

that normally comprise effective healing, including angiogenesis, reepithelialization, and tissue remodeling, are significantly impaired.<sup>2,10,11</sup> Of these, angiogenesis is particularly important, as the growth and development of new blood vessels allow for the transportation of necessary cellular and humoral elements to the site of injury.<sup>12</sup> Furthermore, in specific pathologic conditions such as diabetes mellitus, the process of wound healing is further delayed as the formation of new blood vessels is impaired due to suboptimal mobilization of endothelial progenitor cells (EPCs).<sup>13</sup>

Previous research has shown that delivery of wound-healing agents over an extended period of time can allow for enhanced wound healing, and it has been shown that bioengineered scaffolds have the potential to do so in a cost-effective way. Kim et al.<sup>5</sup> have shown that a biodegradable scaffold seeded with EPCs can result in improved wound healing, as a significantly higher distribution of EPCs and higher rates of vascular regeneration were noted in the wounds of the EPC-seeded scaffold group compared with the wounds of the empty scaffold group or the empty scaffold group that was also given a single dose of EPCs. One disadvantage of their technique is that the scaffold was composed of poly-L-lactic acid (PLLA), which has a highly hydrophobic nature that leads to the nonspecific binding of SDF-1 and eventually impairs the bioactivity of

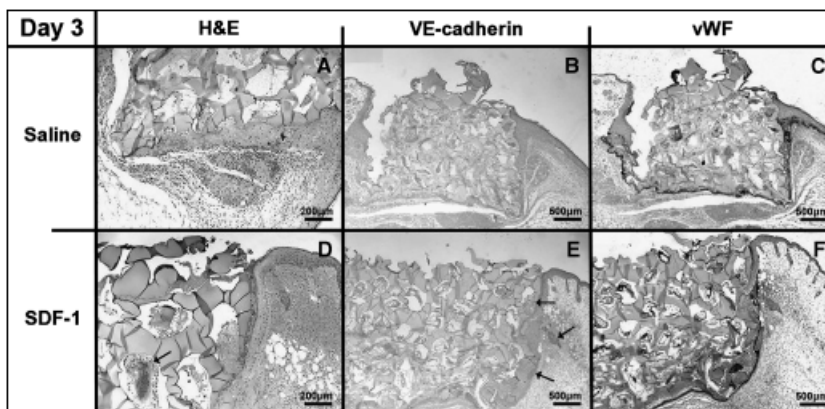
SDF-1. The degradation product of PLLA is lactic acid, which leads to local inflammation due to the acidic pH. Furthermore, because PLLA is soluble only in organic solvents (e.g., chloroform, methylenechloride), it is very difficult to fabricate cytokine-containing PLLA devices, such as sheets and microspheres, without losing bioactivity.

An increasing body of evidence indicates that cytokines such as SDF-1 can trigger the recruitment of EPCs and accelerate wound-healing processes.<sup>13,14</sup> Recent evidence suggests that an increase in SDF-1 expression may lead to mobilization and recruitment of stem cells or EPCs for tissue repair which affects reepithelialization and neovascularization, ultimately leading to functional healing of the wounded area.<sup>15–17</sup> SDF-1 elicits a chemotactic response by binding to CXCR4, regulating the trafficking of proinflammatory cells as well as other marrow-derived stem or progenitor cells (such as endothelial, hematopoietic, and mesenchymal lineages) to facilitate wound healing. Also, SDF-1 has been shown to have a beneficial effect when delivered directly to the wound site.<sup>18,19</sup>

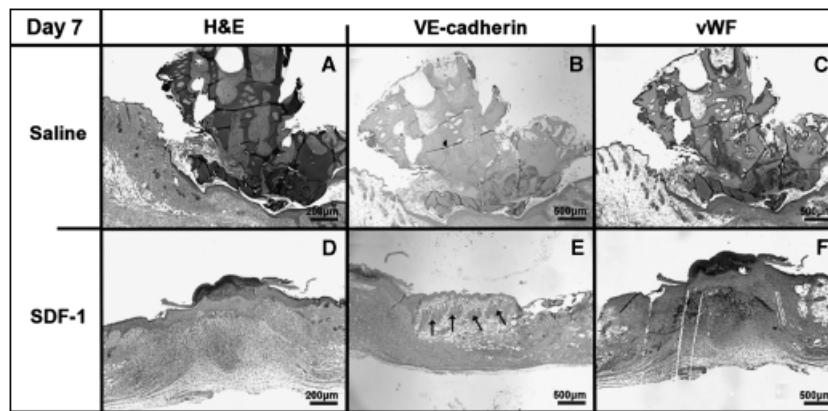
Furthermore, there are reasons to believe that SDF-1 (and by extension, other inflammatory cytokines) may be more effective when delivered over a prolonged period. Because the intravascular half-life of SDF-1 is known to be 25.6 minutes,<sup>12</sup> the physiologic effect is short-lived when delivered as a single dose. Schantz et al.<sup>4</sup> showed that when SDF-1 was delivered via an explantable scaffold and micro-delivery apparatus over a period of 15 days, the degree of angiogenesis and cellular migration into the scaffold was greater than in control mice. A significant limitation of their technique was the requirement for explantation of their apparatus, thereby making it an invasive and costly procedure for clinical usage.

The advantages of our scaffold are that it has a high biocompatibility without generating any toxic by-products, it can easily incorporate SDF-1, and it can function as a reservoir without losing the bioactivity of SDF-1 due to its binding to heparin for incorporation. Previous in vitro studies have showed that the time until complete dissociation for different materials that were loaded onto this formulation of the alginate scaffold varied between 15.8 and 24.5 hours.<sup>6</sup>

The results of our study reveal that sustained delivery of SDF-1 resulted in improved wound healing. On gross examination, wounds treated with the SDF-1-impregnated



**Figure 5.** Hematoxylin & eosin (H&E), VE-cadherin, and vWF staining at day 3. (A) H&E-stained saline scaffold with ×100 magnification, (B) VE-cadherin stained saline scaffold with ×40 magnification, (C) vWF-stained saline scaffold with ×40 magnification, and (D) H&E-stained SDF-1 scaffold with ×100 magnification. The arrow points to a vessel. (E) VE-cadherin-stained SDF-1 scaffold with ×40 magnification. The arrows point to vessels. (F) vWF-stained SDF-1 scaffold with ×40 magnification; note the greater degree of staining compared with the saline control. SDF-1, stromal-derived factor-1; vWF, von Willebrand Factor.



**Figure 6.** Hematoxylin & eosin (H&E), VE-cadherin, and vWF staining at day 7. (A) H&E-stained saline scaffold with  $\times 40$  magnification, (B) VE-cadherin-stained saline scaffold with  $\times 40$  magnification, (C) vWF-stained saline scaffold with  $\times 40$  magnification, and (D) H&E-stained SDF-1 scaffold with  $\times 40$  magnification. (E) VE-cadherin-stained SDF-1 scaffold with  $\times 40$  magnification. The arrows point to vessels. (F) vWF-stained SDF-1 scaffold with  $\times 40$  magnification; again, note the greater degree of staining compared with the saline control. SDF-1, stromal-derived factor-1; vWF, von Willebrand Factor.

scaffolds healed more rapidly than those treated with saline control. The SDF-1-treated wounds closed at a significantly more rapid rate during the period between 1 and 3 days postwounding. This is in contrast to the period between days 3 and 7 postwounding, when the speed of wound closure was the same in the SDF-1-treated and saline-treated control groups, presumably because the SDF-1 reservoir had been depleted from the scaffold.

The assessment of healing wounds by IHC markers for various cellular components revealed changes at multiple time points. The markers used for endothelial cells were VE-cadherin and vWF. VE-cadherin (also known as Cadherin-5) is a member of cadherin superfamily, expressed by vascular endothelial cells. It is the major adhesion junction protein, which, by modulating cell–cell adhesion, regulates angiogenesis and vascular permeability and therefore is used to assess neovascularization. Other endothelial markers, such as vWF, have also been used to assess wound healing. In the present study, IHC analysis showed a significantly higher number of endothelial cells in SDF-1-treated wounds as compared with saline-treated controls. This suggests that time-release SDF-1 therapy triggers endothelial cell migration, and therefore neovascularization and angiogenesis.

While only a single cytokine was delivered in the current study, our scaffold can provide a platform for the delivery of multiple agents (alone or in combination). In addition to other cytokines, antibiotics could be delivered locally into the wound at very high levels, without fears of systemic toxicity. We believe that the relatively brief period of “sustained” delivery (18–24 hours) is advantageous, as prolonged delivery of potent cytokines may have unpredictable and untoward consequences.<sup>20–23</sup>

Although treatment with a single scaffold may be sufficient for acute/normal wounds, current scaffold kinetics may require reapplication in the setting of more challenging wounds. Further studies are currently underway to determine the efficacy of our cytokine-loaded scaffolds in the treatment of such difficult wounds, and future studies will aim to evaluate the potential risk of carcinogenic interactions related to increased angiogenesis.

In conclusion, this study has shown that a drug-delivery vehicle that allowed for time-release delivery of SDF-1 resulted in increased recruitment of EPCs to the site of injury and an overall improved wound-healing process compared

with saline control. Furthermore, our scaffold, which can be placed directly onto the wound bed, obviates the need to remove the implant and allows a desired dosage to be delivered to the area of interest over a sustained time interval without the need for repeated application.

## ACKNOWLEDGMENTS

This project was funded in part by a grant from the New York State Empire Clinical Research Investigator Program (ECRIP) to Peter W. Henderson, and a grant from the Empire State Stem Cell Fund to Sina Y. Rabbany. Masaya Yamamoto was supported by a grant from the Kyoto University Foundation for Promotion of Education and Research for a young faculty member. The authors would like to thank Ms. Alice Harper for lending her expertise in animal handling.

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