Delivery of mesenchymal stem cells in biomimetic engineered scaffolds promotes healing of diabetic ulcers

Aim: We hypothesized that delivery of mesenchymal stem cells (MSCs) in a biomimetic collagen scaffold improves wound healing in a diabetic mouse model. **Materials & methods:** Rolled collagen scaffolds containing MSCs were implanted or applied topically to diabetic C57BL/6 mice with excisional wounds. **Results:** Rolled scaffolds were hypoxic, inducing MSC synthesis and secretion of VEGF. Diabetic mice with wounds treated with rolled scaffolds containing MSCs showed increased healing compared with controls. Histologic examination showed increased cellular proliferation, increased VEGF expression and capillary density, and increased numbers of macrophages, fibroblasts and smooth muscle cells. Addition of laminin to the collagen scaffold enhanced these effects. **Conclusion:** Activated MSCs delivered in a biomimetic-collagen scaffold enhanced wound healing in a translationally relevant diabetic mouse model.

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One of the most important complications of diabetes is the development of lower extremity ulcers. Approximately 2–3% of diabetics develop foot ulcers each year, affecting approximately 15–25% of diabetics during their lifetime [1–5]. The etiology of delayed and poor wound healing in diabetic ulcers is complex and includes poor vascular perfusion, neurological injury and trauma, as well as limited cellular angiogenic potential, a diminished population of mesenchymal stem cells (MSCs) and reduction in the deposition of appropriate extracellular matrix [2,3].

Wound healing requires multiple cell processes, including proliferation, migration, differentiation, matrix deposition and remodeling, angiogenesis, resulting in eventual re-epithelialization of the wound [6]. Current tissue engineering approaches to advanced wound care therapy have been to enhance these processes via delivery of either matrix-

based or cell-based scaffolds. Matrix-based scaffolds include Alloderm®, dCell®, Integra® and GraftJacket® that use matrix without cells; cell-based scaffolds include Derga-graft® and MySkin® that do not have significant matrix, and Apligraf® and Epifix that have both cells and matrix [7–9]. Although these products promote wound healing, no treatment paradigm has shown superior long-term efficacy [8].

MSCs are a promising source of cells for multiple regenerative processes, including patches used for myocardial infarction and skin regeneration [10–14]. MSCs give rise to mesodermal lineages including muscle, fat and pericytes and can transdifferentiate into epidermal cells [15,16]; MSCs also enhance angiogenesis via multiple cytokines that ameliorate ischemic environments, including VEGF, MCP-1 and MIP-1α [17,18]. Culturing MSCs in hypoxic conditions enhances their angio-

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Figure 1. *In vitro* validation of rolled collagen scaffold model (see facing page). (A) FACS analysis confirms identity of murine mesenchymal stem cells with positive expression of CD34, CD44, Sca-1 and lack of expression of CD117. (B) The collagen scaffold is created by compressing the collagen into a flat configuration ('F') and then rolling it into a rolled configuration ('R'). (C) Graph shows serial measurements of oxygen tension at the core of a rolled collagen scaffold. (D) Bar graph shows relative values of growth factor release into the medium after 72-h incubation from a flat or rolled collagen scaffold. VEGF: * $p = 0.0006$ (*t*-test); $n = 8-9$. TGF- β : $p = 0.9$; $n = 3$. FGF: $p = 0.5$; $n = 3$. (E) Left panel shows representative immunohistochemical analysis for VEGF in the core (top row) and periphery (bottom row) of the rolled collagen scaffold at 0 h (left column) and 72 h (right column); arrowheads show positive cells. Scale bar, 50 μ m. Right panel shows change in percentage of VEGF-positive cells over time; $p < 0.0001$, ANOVA; *, core is significant at all time points versus time 0 (*post hoc*); **, periphery is significant at all time points versus time 0 (*post hoc*).

genic potential [19], which is not surprising since MSCs reside in the bone marrow niche *in vivo* that has lower oxygen tension compared with typical atmospheric conditions that are used for tissue culture [20].

The extracellular matrix plays a significant role in controlling cell behavior, and the stem cell niche directly and indirectly modulates stem cell functions including self-renewal and differentiation [21]. The bone marrow MSC niche is made up of multiple matrix proteins, including collagen I, collagen IV, laminin and fibronectin. By culturing cells within a 3D scaffold, we can impart some physiological cues that cells would normally be exposed to in tissues. The 3D geometry of a biomimetic scaffold is an important factor that enhances its regenerative capacity, suggesting the importance of mimicking the physical geometry of the native cellular niche [22–24].

We hypothesized that use of a biomimetic scaffold to deliver MSCs will enhance their therapeutic potential. We previously showed that plastic compression of a collagen scaffold to a density approaching that of tissue also creates a hypoxic central core that would be of particular use as a biomimetic scaffold for MSCs [24–26]. We used these compressed collagen scaffolds to deliver MSCs into a mouse model of diabetic wounds to determine their therapeutic potential in this translationally relevant model.

Materials & methods

Cells

Murine bone marrow-derived MSCs were obtained from Cyagen (CA, USA). MSCs were cultured in EGM-2 media (Lonza, Basel, Switzerland). MSC identity was confirmed with FACS analysis of passage 2 MSC using anti-CD34 (BioLegend, CA, USA), anti-CD44 (BD Pharmingen, CA, USA), anti-Sca-1 (BioLegend), and anti-CD117 (BioLegend) using an LSRII flow cytometer (BD Biosciences, CA, USA). Cell passages 2–6 were used for this study.

Collagen scaffolds

Collagen scaffolds were created by adding type 1 rat tail collagen (3 ml of 5% solution; Enzo Life Sciences, NY, USA), phosphate-buffered saline (PBS; 1 ml) and

10 $\times$ DMEM (0.5 ml; Sigma-Aldrich, MO, USA) and then pH adjusted to 7.0 using dropwise addition of 1 M NaOH according to the supplier directions. For cellular constructs, 2×10^6 MSCs in 0.5 ml solution were added to the collagen gel; for acellular constructs, an equal volume PBS was added (final volume 5 ml). MSC number was determined from previous work showing high cell viability at this seeding density, and the subsequent hypoxia gradient formed by cell consumption of oxygen resulted in an upregulation of the angiogenic growth factor cascade [27]. For laminin containing scaffolds, 25 μ g laminin (Corning, MA, USA) was added. The collagen solution was allowed to solidify within a rectangular mold (room temperature, 30 min) and then removed from the mold and compressed between glass plates (5 min). The compressed collagen sheet was then rolled tightly and placed in media (37°C, 72 h).

Core oxygen tension

Fiberoptic fluorescent probes (Oxford Optronix Ltd, Oxford, UK) were used to measure oxygen tension in 3D rolled scaffolds in real time [25]. A probe was embedded into the core of a rolled collagen scaffold, with care taken to achieve 360 transillumination. The scaffold was then placed into sterile media (originally 160 mmHg), and covered with parafilm. Oxygen tension was measured at single-minute intervals.

Growth factor release

Collagen scaffolds were incubated at 37°C for 72 h and then the medium was collected and growth factor concentrations were determined using mouse Quantikine ELISA kits for VEGF, FGF or TGF- β according to the manufacturer's instructions (R&D Systems, MN, USA).

Immunohistochemistry

Sections were heated in citric acid buffer (pH 6.0) at 100°C (10 min) for antigen retrieval. The sections were treated with hydrogen peroxide (0.3%) in methanol for 30 min at room temperature to block endogenous peroxidase activity and incubated with bovine serum albumin (5%) in PBS containing Triton X-100

Figure 2. Delivery of mesenchymal stem cells in a rolled collagen scaffold enhances wound healing in a diabetic mouse leg wound model (see facing page). (A) Line graph shows wound size over time. * $p < 0.0001$ (two-way ANOVA). Control: $n = 6$; MSC injection: $n = 2$; acellular collagen: $n = 4$; MSC collagen: $n = 3$. (B) Photographs show representative images of wounds, days 0 and 3. Scale bar: 2 mm. (C) Bar graph shows mean wound area at day 1 in a replicate experiment with additional control group to include acellular + MSC. $p = 0.0041$ (ANOVA). * $p = 0.0037$ versus control (*post hoc*) and $p = 0.0347$ versus acellular + MSC injection (*post hoc*). Control: $n = 4$; acellular + MSC injection: $n = 12$; MSC: $n = 6$. (D) Representative images and bar graph showing percentage of cells positive for Ki-67 adjacent to acellular and MSC scaffolds. * $p = 0.0001$ (*t*-test); $n = 4$. (E) Representative images and bar graph showing percentage of cells positive for cleaved caspase-3 adjacent to acellular and MSC scaffolds. $p = 0.6$ (*t*-test); $n = 4$. (F) Representative images and bar graph showing VEGF-positive cells adjacent to acellular and MSC scaffolds. * $p = 0.04$ (*t*-test); $n = 3-4$. (G) Representative images and bar graph showing relative number of capillaries adjacent to acellular and MSC scaffolds. * $p < 0.0001$ (*t*-test); $n = 4$. (D–G) Scale bars: 10 μm . (H) Bar graph showing F4/80-positive cells adjacent to acellular and MSC scaffolds. * $p = 0.04$ (*t*-test); $n = 3-4$. (I) Bar graph showing smooth muscle actin-positive cells adjacent to acellular and MSC scaffolds. $p = 0.2$ (*t*-test); $n = 3$. (J) Bar graph showing vimentin-positive cells adjacent to acellular and MSC scaffolds. * $p = 0.003$ (*t*-test); $n = 3$. Red arrows show positive cells. MSC: Mesenchymal stem cell.

(0.05%; T-PBS) for 1 h at room temperature to block nonspecific protein-binding sites. Sections were then incubated in T-PBS at 4°C with the primary antibodies: anti-VEGF (rabbit; 1:100; Santa Cruz Biotechnology, TX, USA), Ki67 (rabbit; 1:200; Abcam, MA, USA), anti-caspase 3 (rabbit; 1:1000; Cell signaling, MA, USA), F4/80 (rat; 1:100; Abcam), α -SMA (rabbit; 1:50; Abcam), vimentin (rabbit; 1:500; Abcam). After overnight incubation, the sections were incubated with either secondary anti-rabbit antibody Dako EnVision™ + Dual Link System-HRP (Dako, CA, USA) or secondary anti-rat antibody (Dako) for 1 h at room temperature and treated with Dako Liquid DAB+ Substrate Chromogen System (Dako) to visualize the reaction products. Finally, the sections were counterstained with Dako Mayer's Hematoxylin (Lillie's Modification) Histological Staining Reagent (Dako). Photomicrographs were then obtained of the slides and positive staining cells were manually counted and reported as a percentage of positive cells.

Diabetic hind limb wound model

All animal studies were performed in strict compliance with Federal guidelines and approved by Yale University's Institutional Animal Care and Use Committee. Male C57BL/6 mice (8–12 weeks; 20–30 g; Jackson Laboratory, ME, USA) were used for induction of diabetes with streptozotocin [28]. In brief, mice were injected daily for 7 days with streptozotocin (50 mg/kg IP; Tocris Biosciences, Bristol, UK). After at least 1 week, hyperglycemia was confirmed with a glucometer; only mice with blood glucose greater than 300 mg/dl were used.

Mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg). One of the lower extremities was removed of hair using a chemical depilatory (Nair, NJ, USA). A full-thickness skin wound (6 mm diameter) was then made over the anterior thigh of the lower extremity. A subcutaneous pocket was cre-

ated with blunt dissection immediately adjacent to and proximal to the wound, and a collagen scaffold (3 mm length, containing 2×10^5 cells) was inserted into the subcutaneous pocket. Control mice received either no collagen scaffold, an acellular collagen scaffold, and/or an injection of a matched number of MSCs (2×10^5) without a scaffold. For mice treated with an MSC injection, the injection was made through the wound into the subcutaneous space; a subcutaneous pocket was not dissected to prevent creating additional surgical trauma as well as avoiding leakage of MSCs out of the injection site. Photographs of the wounds were taken daily using a dissecting microscope and the wound area analyzed (Image J software, NIH). At the completion of study, mice were euthanized and the wounds and surrounding tissues were harvested and processed. Paraffin embedded sections were prepared and stained using immunohistochemistry for markers of proliferation, apoptosis, VEGF, actin and markers of macrophages and fibroblasts. The percentage of positive cells was determined using manual counting in four high power fields. Capillaries were identified on H&E stained slides by their typical tubular morphology and the presence of intraluminal red blood cells; quantification was performed by averaging the capillary density in four manually counted high power fields.

MSC labeling & detection *in vivo*

MSCs were labeled with Molday ION Rhodamine B (12.5 ng/ml; Biopal, MA, USA) for 18 h prior to incorporation into the collagen scaffold. Immediately after collagen incorporation, the mice ($n = 3$ for non-labeled control and labeled groups, respectively) were artificially ventilated (70% N_2O and 30% O_2) and kept under 2% isoflurane anesthesia. The mice were then positioned in a custom-built MRI compatible holder. The spin-echo MR datasets were obtained using 11.7 T horizontal-bore spectrometer (Agilent,

Figure 3. Retention of mesenchymal stem cells within the collagen scaffold *in vivo*. (A) Representative images showing detection of nanoparticle-labeled mesenchymal stem cells *in vivo*; first two columns, MRI; middle two columns, histology; right two columns, fluorescence. Top row, day 0; middle row, day 3; bottom row, day 7. Red arrows identify signal attenuation from labeled mesenchymal stem cells, yellow arrows show corresponding region in control mice. Histology scale bar: 25 μm. (B) Left panel, representative image showing presence of labeled cells in the collagen scaffold, and no labeled cells in adjacent tissue; red arrow shows unlabeled cells at periphery of the collagen scaffold. Right panel, bar graph showing cell density in the core and periphery of the collagen scaffold. * $p = 0.002$ (t -test). $n = 4$. HPF: High power field.

CA, USA) with a ^1H RF surface coil of 1.5 cm diameter positioned on top of the wound of the thigh. Axial slices of 128×128 image matrix and 1-mm thickness were acquired using a field of view of 25.6 mm^2 (in plane resolution of $200 \times 200 \text{ μm}$), a recycle time of 1500 ms and echo time of 9 ms (gradient echo). The resulting MR images were processed in Matlab (MathWorks Inc., MA, USA). Longitudinal MR scanings were performed on the same slice of the wounds for the same animal at day 0, 3 and 7 for both nonlabeled

control and labeled mice. Rhodamine fluorescence was detected using fluorescence microscopy. Iron oxide was detected using Prussian blue staining.

Splinted back wound model

The splinted excisional wound model was created as previously described [29]. In brief, diabetic mice were anesthetized and removed of hair over their back using a chemical depilatory. A full-thickness skin wound (5 mm diameter) was created on the back. The colla-

gen scaffold was divided into four pieces (7.5 mm each containing 5×10^5 cells), and each piece implanted into a subcutaneous pocket adjacent to the wound in four equally spaced directions (total of 2×10^6 cells implanted per mouse). Control mice received equivalent-sized acellular collagen scaffolds. A silicone ring (6 mm inner diameter, 0.5 mm thick) was sutured into place surrounding the wound and a Tegaderm dressing (3M, St Paul, MN, USA) was placed over the splinted wound. Photographs of the wounds were taken daily using a dissecting microscope and the wound area analyzed (Image J software, NIH). At the completion of study, mice were euthanized and the wounds and surrounding tissues were harvested and processed as described above.

Topical application of collagen scaffolds

Acellular and MSC-containing collagen scaffolds were created as described. Some scaffolds were then immediately unrolled back into a flat configuration (nonhypoxic) or kept in rolled configuration (physiological hypoxia) for the 72-h incubation *in vitro* (37°C). At the end of 72 h, all scaffolds were unrolled into flat configuration. The flat scaffolds were then secured on top of splinted back wounds of diabetic mice using eight evenly spaced nylon sutures at the wound edge; no scaffolds were implanted in mice receiving topical scaffolds. Photographs of the wounds were taken daily using a dissecting microscope and the wound area analyzed (Image J software). At the completion of study, mice were euthanized and the wounds and surrounding tissues were harvested and processed as described above.

Statistical analysis

Results are presented as mean value $\pm$ SEM. A two-tailed *t*-test or analysis of variance was used for comparison between groups. Results were considered significant when $p < 0.05$. The Tukey's *post hoc* test was used for correction of results that were significant by ANOVA. GraphPad Prism 6 software (La Jolla, CA, USA) was used for statistical analysis and creation of graphs.

Results

A collagen scaffold increases VEGF secretion from MSC *in vitro*

In order to determine whether a biomimetic scaffold enables MSC survival and function *in vitro*, MSCs were embedded into rolled collagen scaffolds as previously described [30]. FACS analysis confirmed that the murine MSC expressed CD34, CD44 and Sca-1, but not CD117, prior to implantation (Figure 1A), as expected from the supplier documentation [31]. MSCs were embedded in compressed collagen gels, which were either left as flat sheets or rolled, to provide a com-

plex 3D geometry (Figure 1B) with a central hypoxic core (Figure 1C). The hypoxia generated through cell consumption of O_2 in rolled scaffolds resulted in a higher level of VEGF secretion into the medium, but this hypoxic environment did not promote the secretion of TGF- β or FGF (Figure 1D), suggesting that the geometrically complex scaffolds provided MSCs with a microenvironment promoting secretion of the angiogenic factor VEGF. VEGF-positive MSCs were detectable at baseline; within 12 h MSCs in the core of the rolled scaffold were nearly uniformly VEGF-positive, whereas MSCs at the periphery required approximately 72 h to develop near uniform VEGF positivity (Figure 1E). These results suggest that the hypoxic core environment of the rolled scaffold stimulates MSC production of VEGF that likely stimulates the MSCs in the periphery of the scaffold to increase VEGF synthesis and secretion.

Activated MSC in collagen scaffolds increase wound healing *in vivo*

Since MSCs have increased VEGF upregulation and release in rolled scaffolds, compared with flat scaffolds, *in vitro*, we determined whether delivery of MSCs in a rolled scaffold enhances wound healing in a diabetic mouse leg wound model. Preoperative age, weight and the rate of diabetes achieved after streptozotocin therapy were similar between all groups (data not shown). The basal rate of wound healing was significantly reduced in diabetic mice compared with nondiabetic mice ($p < 0.0001$, ANOVA; $n = 4$; data not shown). In diabetic mice, rolled scaffolds without or with MSCs were implanted subcutaneously just proximal to the wound at the time of wound creation. Control diabetic mice healed the leg wounds within approximately 7 days. Wounds to which MSCs were delivered in a rolled scaffold showed significant improvement in wound healing by day 1 compared with mice receiving either collagen scaffolds without MSCs, or MSC injected directly into the wound (Figures 2A & B), suggesting that the combination of a relevant cell type delivered in a biomimetic scaffold was critical to enhance wound healing. Mice receiving rolled scaffolds with MSCs not only healed more quickly compared with mice receiving both acellular collagen scaffolds and MSCs directly injected into the wound, but closed the wound area by a significant margin (64% closure vs 49% with MSC injection vs 37% with acellular scaffold on day 1, $p = 0.004$; Figure 2C). Examination of the tissue immediately adjacent to the collagen scaffolds showed that there was increased numbers of proliferating cells (Figure 2D) with no change in apoptotic cells (Figure 2E) in scaffolds containing MSC compared with acellular scaffolds. Since MSC-containing scaffolds

Figure 4. Delivery of mesenchymal stem cells in a rolled collagen scaffold enhances wound healing in a diabetic mouse splinted back wound model (see facing page). (A) Line graph shows wound size over time. * $p < 0.0001$ (two-way ANOVA); $n = 6$. (B) Photographs show representative images of wounds, days 0 and 5. Scale bar 2 mm. (C) Representative images and bar graph showing percentage of cells positive for Ki-67 adjacent to acellular and MSC scaffolds. * $p = 0.003$ (t -test); $n = 3$. (D) Representative images and bar graph showing percentage of cells positive for cleaved caspase-3 adjacent to acellular and MSC scaffolds. $p = 0.8$ (t -test); $n = 3$. (E) Representative images and bar graph showing VEGF-positive cells adjacent to acellular and MSC scaffolds. * $p < 0.0001$ (t -test); $n = 4$. Scale bars (C–F), 10 μm . (F) Representative images and bar graph showing relative number of capillaries adjacent to acellular and MSC scaffolds. * $p = 0.02$ (t -test); $n = 5$ –6. (G) Bar graph showing F4/80-positive cells adjacent to acellular and MSC scaffolds. * $p = 0.02$ (t -test); $n = 3$. (H) Bar graph showing smooth muscle actin-positive cells adjacent to acellular and MSC scaffolds. * $p = 0.01$ (t -test); $n = 3$. (I) Bar graph showing vimentin-positive cells adjacent to acellular and MSC scaffolds. * $p = 0.003$ (t -test); $n = 3$. Red arrows show positive cells. MSC: Mesenchymal stem cell.

secrete more VEGF *in vitro* in the rolled configuration compared with flat scaffolds (Figure 1D), we examined VEGF secretion *in vivo*; there were increased numbers of VEGF-positive cells in the tissue adjacent to MSC-containing scaffolds compared with acellular scaffolds (Figure 2F). Similarly there were more capillaries adjacent to MSC-containing scaffolds compared with acellular scaffolds (Figure 2G). There were more cells that were F4/80-positive, consistent with macrophages as well as vimentin-positive, consistent with fibroblasts, and a trend toward increased numbers of smooth muscle actin (SMA)-positive cells, consistent with myofibroblasts, near MSC-containing scaffolds compared with acellular scaffolds (Figures 2H–J). These data show that MSCs in a rolled scaffold enhance wound healing *in vivo*, in multiple ways, of which enhancing wound vascularization is one such mechanism. These findings are consistent with our original hypothesis that by creating a biomimetic physiological environment for the MSCs, we enhance their wound healing capability.

Since there was increased cell proliferation and enhanced wound healing in diabetic mice with wounds treated with MSC-containing scaffolds, we determined whether MSCs persist in the scaffold *in vivo* after implantation. MSCs were labeled with nanoparticles containing both iron and rhodamine prior to implantation. MRI was used to detect the labeled cells *in vivo*. Iron-labeled cells were detectable in the collagen scaffold immediately after implantation as well as on postoperative days 3 and 7 (Figure 3A; first two columns). The MRI data from iron labeled mice showed a specific darkening (red arrow in Figure 3A) that was absent in the control mice (yellow arrow in Figure 3A). Similarly, iron-labeled cells were also detectable with histology through day 7 (Figure 3A; middle two columns). In addition, the rhodamine-labeled cells were directly detectable through day 7 (Figure 3A; last two columns). Since labeled MSCs were detectable in the collagen scaffold *in vivo* postoperatively, and proliferating cells were present adjacent to the scaffold (Figure 2D), we examined scaffolds to determine if any unlabeled cells were also present. Unlabeled cells were present in the

scaffold, with more unlabeled cells in the periphery of the scaffold compared with the core of the scaffold (Figure 3B). These results suggest that cells migrate into the scaffold after implantation, for example, biomimetic scaffolds promote cell survival *in vivo*.

Activated MSCs increase wound healing in a splinted back wound model

Since the diabetic mouse wound healing model is thought to measure both wound healing as well as wound contraction, and human wounds generally heal with little contraction, we determined whether MSC-containing scaffolds promote wound healing in the splinted back wound model, as this model prevents wound contraction [32]. Rolled collagen scaffolds without and with MSCs were implanted subcutaneously adjacent to splinted back wounds in diabetic mice. Wounds treated with MSC-containing scaffolds had increased wound healing compared with acellular rolled scaffolds as early as postoperative day 4 (36 vs 31%, day 4; 51 vs 35%, day 5; 68 vs 55%, day 6; $p < 0.0001$; Figures 4A & B). Similar to the diabetic leg wounds treated with MSC-containing scaffolds, splinted diabetic back wounds treated with MSC-containing scaffolds had adjacent increased proliferation without increased apoptosis, increased VEGF-positive cells and capillaries and increased numbers of F4/80-, SMA- and vimentin-positive cells (Figures 4C–I). These results suggest that MSC-containing scaffolds promote wound healing in splinted diabetic back wounds, for example, they promote wound healing in a clinically relevant model.

Laminin improves wound healing efficiency

Since laminin promotes MSC function in tissue-engineered constructs [33], and we have previously shown that laminin promotes vascular network formation in 3D collagen scaffolds [34], we determined the effects of laminin in the splinted diabetic mouse back wound model. Relative to mice treated with acellular laminin-containing collagen scaffolds, mice treated with MSC in laminin-containing collagen scaffolds

showed an increased rate of wound healing (64 vs 31%, day 4; 52 vs 22%, day 5; $p < 0.0001$; Figures 5A & B). Wounds treated with MSC + laminin-containing collagen scaffolds showed increased proliferation without increased apoptosis, increased VEGF-positive cells and capillaries and increased numbers of F4/80-, SMA- and vimentin-positive cells in comparison to wounds treated with acellular laminin-containing collagen scaffolds (Figures 5C–I).

Interestingly, wounds that were treated with MSCs in laminin-containing collagen scaffolds (Figure 5A) had an even further increased rate of wound healing in comparison to treatment with MSCs in collagen scaffolds without laminin (Figure 4A; $p < 0.0001$, ANOVA). Furthermore, wounds that were treated with MSCs in laminin-containing collagen scaffolds had increased VEGF-positive cells (83 vs 65%; $p = 0.002$, *t*-test), increased numbers of F4/80-positive cells (76 vs 60%; $p = 0.0007$, *t*-test) and a dramatically increased number of vimentin-positive cells (70 vs 11%; $p < 0.0001$, *t*-test) compared with treatment with MSCs in collagen scaffolds without laminin. These results show that addition of laminin to the collagen scaffold augments the rate of diabetic wound healing, suggesting that the biomimetic scaffold promotes MSC survival and/or function *in vivo*.

MSC delivered topically increase wound healing

Since biomimetic scaffolds containing MSCs heal diabetic leg wounds as well as diabetic splinted back wounds, we determined if scaffolds that were unrolled prior to topical application would be similarly successful, for example, the improved MSCs function would retain its efficacy in a clinically useful model. Topical application of collagen scaffolds containing MSCs that were cultured in rolled configuration for 72 h *in vitro* and then unrolled prior to implantation *in vivo* ('rolled *in vitro*') showed improved wound healing compared with acellular scaffolds (Figure 6A, red line vs black line; $p < 0.0001$, ANOVA). Wounds treated with topical scaffolds containing MSCs had increased proliferation without increased apoptosis, increased VEGF-positive cells and capillaries, and increased numbers of F4/80-, SMA- and vimentin-positive cells in comparison to wounds treated with acellular collagen scaffolds (Figures 6C–I). These results show that rolled biomimetic scaffolds contain MSCs with enhanced survival and/or function that retain the ability to enhance wound healing even when applied topically to the wound, for example, without the continued hypoxic or biomimetic environment.

Since these results suggest the importance of the cells being in the rolled scaffold configuration during

the *in vitro* culture period to promote MSCs survival and/or function, we hypothesized that MSCs cultured in flat configuration would lose the enhancement of MSCs survival and/or function. Therefore, MSCs were seeded in scaffolds and rolled, but immediately unrolled and cultured *in vitro* for 72 h in flat configuration prior to topical application to the wound. These scaffolds ('flat *in vitro*') showed reduced rates of wound healing compared with scaffolds that were cultured in rolled configuration *in vitro* (Figure 6A, blue line vs red line; $p < 0.0001$, ANOVA). Furthermore, unrolling the scaffolds immediately after creation and culturing in a flat configuration resulted in reduced VEGF-positive cells (63 vs 82%; $p < 0.05$, ANOVA; Figure 6E), reduced capillary formation (1.7 vs 2.5%; $p < 0.05$, ANOVA; Figure 6F) and reduced numbers of vimentin-positive cells (16 vs 37%; $p < 0.05$, ANOVA; Figure 6I) compared with wounds treated with scaffolds cultured in the rolled configuration. These results confirm that culture of MSCs in a biomimetic scaffold in the rolled configuration promotes MSC survival and/or function to enhance wound healing and that is retained with topical application to the wound.

Discussion

We show that MSC-seeded collagen scaffolds rapidly develop a hypoxic core that increases VEGF secretion (Figure 1), and that delivery of MSCs in a biomimetic collagen scaffold increases wound healing in the diabetic mouse leg model (Figure 2). Interestingly, MSCs can be found up to 1 week after implantation *in vivo*, suggesting that the collagen scaffold promotes MSC survival (Figure 3). Use of the splinted back wound model also shows improved wound healing with MSCs (Figure 4), suggesting the translatability of this strategy to human diabetic patients. Addition of laminin to the scaffold improves wound healing, confirming the importance of the scaffold in reproducing the MSCs niche environment (Figure 5). Finally we show that MSCs conditioned in collagen scaffolds can be delivered topically to enhance diabetic wound healing, and that the rolled configuration of the biomimetic scaffold is important to promoting MSC survival and/or function (Figure 6). These results show that biomimetic scaffolds can promote stem cell survival and function, enabling a new therapeutic modality for diabetic wounds.

There has been a recent appreciation for the use of 3D scaffolds to aid regeneration and healing of diseased tissues. Recent work includes the use of acellular patches made of extracellular matrix, mainly collagen type I, to aid the regeneration process in infarcted cardiac tissue; these patches provided superior healing compared with polymeric patches [35]. Similarly

Figure 5. Addition of laminin to the rolled collagen scaffold further enhances wound healing in a diabetic mouse splinted back wound model. (A) Line graph shows wound size over time. * $p < 0.0001$ (two-way ANOVA); $n = 3$. (B) Photographs show representative images of wounds, days 0 and 4. Scale bar: 2 mm. (C) Representative images and bar graph showing percentage of cells positive for Ki-67 adjacent to acellular + laminin and MSC + laminin collagen scaffolds. * $p = 0.02$ (t -test); $n = 3$. (D) Representative images and bar graph showing percentage of cells positive for cleaved caspase-3 adjacent to acellular + laminin and MSC + laminin collagen scaffolds; $p = 0.8$ (t -test); $n = 3$. (E) Representative images and bar graph showing VEGF-positive cells adjacent to acellular + laminin and MSC + laminin collagen scaffolds; * $p < 0.0001$ (t -test); $n = 3$. (F) Representative images and bar graph showing relative number of capillaries adjacent to acellular + laminin and MSC + laminin collagen scaffolds. * $p = 0.02$ (t -test); $n = 3$. Scale bars (C–F), 10 μ m. (G) Bar graph showing F4/80-positive cells adjacent to acellular + laminin and MSC + laminin collagen scaffolds. * $p < 0.0001$ (t -test); $n = 3$. (H) Bar graph showing smooth muscle actin-positive cells adjacent to acellular + laminin and MSC + laminin collagen scaffolds. * $p = 0.01$ (t -test); $n = 3$. (I) Bar graph showing vimentin-positive cells adjacent to acellular + laminin and MSC + laminin collagen scaffolds. * $p = 0.0005$ (t -test); $n = 3$. Red arrows show positive cells. MSC: Mesenchymal stem cell.

Figure 6. Topical application of collagen scaffolds containing mesenchymal stem cells enhances wound healing in a diabetic mouse splinted back wound model. (A) Line graph shows wound size over time. * $p < 0.0001$ (two-way ANOVA); $n = 3$. **(B)** Photographs show representative images of wounds, days 0 and 5. Scale bar: 2 mm. **(C)** Bar graph showing percentage of cells positive for Ki-67 adjacent to acellular and MSC collagen scaffolds applied topically; $p < 0.0001$ (one-way ANOVA); $n = 3$. * $p < 0.05$ (*post hoc*) MSC flat versus acellular rolled and acellular flat. ** $p < 0.05$ (*post hoc*) MSC rolled versus acellular rolled and acellular flat. **(D)** Bar graph showing percentage of cells positive for cleaved caspase-3 adjacent to acellular and MSC collagen scaffolds applied topically. $p = 0.7$ (one-way ANOVA); $n = 3$. **(E)** Bar graph showing percentage of VEGF-positive cells adjacent to acellular and MSC collagen scaffolds applied topically. $p < 0.0001$ (one-way ANOVA); $n = 3$. * $p < 0.05$ (*post hoc*) MSC flat versus acellular rolled and acellular flat. ** $p < 0.05$ (*post hoc*) MSC rolled versus MSC flat, acellular rolled and acellular flat. **(F)** Bar graph showing relative number of capillaries adjacent to acellular and MSC collagen scaffolds applied topically. $p = 0.0003$ (one-way ANOVA); $n = 3$. * $p < 0.05$ (*post hoc*) MSC flat versus acellular rolled and acellular flat. ** $p < 0.05$ (*post hoc*) MSC rolled versus MSC flat, acellular rolled and acellular flat. **(G)** Bar graph showing F4/80-positive cells adjacent to acellular and MSC collagen scaffolds applied topically. $p = 0.01$ (one-way ANOVA); $n = 3$. * $p < 0.05$ (*post hoc*) MSC flat versus acellular flat. ** $p < 0.05$ (*post hoc*) MSC rolled versus acellular flat. **(H)** Bar graph showing smooth muscle actin-positive cells adjacent to acellular and MSC collagen scaffolds applied topically. $p = 0.009$ (one-way ANOVA); $n = 3$. * $p < 0.05$ (*post hoc*) MSC flat versus acellular flat. ** $p < 0.05$ (*post hoc*) MSC rolled versus acellular flat and acellular rolled. **(I)** Bar graph showing vimentin-positive cells adjacent to acellular and MSC collagen scaffolds applied topically. $p < 0.0001$ (one-way ANOVA); $n = 3$. * $p < 0.05$ (*post hoc*) MSC rolled versus MSC flat, acellular flat and acellular rolled. MSC: Mesenchymal stem cell.

a 3D vicryl graft was used to deliver adipose-derived stromal vascular fraction cells to improve cardiac function after infarct [36]. We used a biomimetic scaffold to optimize MSC angiogenic potential, enhancing wound healing in diabetic mice. Our approach was to control three physiological parameters to optimize the biomimetic nature of the environment based on the MSC niche environment *in vivo*. These parameters included biomimetic matrix composition, tissue-like matrix density and physiological oxygen environment. The matrix material tested was either collagen I only or collagen I with laminin. Matrix density was controlled and increased to approximately 10%, which is approaching tissue density, by the process of plastic compression [24]. Cell-embedded scaffolds were rolled in a spiral configuration to create a hypoxic gradient maximally at the scaffold core; we have previously shown that this scaffold configuration stimulates multiple cell types to increase VEGF synthesis and secretion [25]. Our data suggest that use of the proper matrix components, at physiological density and configuration, promotes MSC survival and function to promote wound healing. Interestingly, diabetes is associated with diminished subpopulations of MSCs, suggesting that diabetic ulcers may be amenable to therapies that restore MSC numbers and/or function [37,38].

The stem cell niche provides a microenvironment conducive for stem cell survival and maintenance of multipotency. Within the bone marrow, the physiological site of the MSCs niche environment, reported levels of physiological pO_2 range between 0 and 7% [20,39]. MSCs within rolled collagen scaffolds generated physiological hypoxia due to the formation of O_2 consumption gradients [25]. The level of O_2 within the core of our constructs dipped below 1% for the first 12 h of culture, which is nearing pathological hypoxia

(Figure 1C). We hypothesize that changes in MSC O_2 metabolism then result in a lower consumption of O_2 , allowing stabilization of O_2 levels at 50 mmHg (6.6%) from 24 h onward (Figure 1C), since this low O_2 environment does not result in loss of cell viability of implanted scaffolds (Figure 3). Indeed, we were able to detect persistent MSCs *in vivo* even after 1 week (Figure 3). Interestingly, after 72 h of hypoxia, subsequent exposure of the MSCs to the ambient environment with topical application retains efficacy of wound healing (Figure 6), suggesting that the hypoxic cellular activation is not transient.

Addition of laminin to the collagen scaffold promotes wound healing as well as increases the number of VEGF-positive cells, macrophages and fibroblasts near the implanted scaffold (Figure 5), suggesting that the biomimetic scaffold promotes MSC survival and/or function *in vivo*. We have also shown that inclusion of laminin increases cell expression of integrin $\alpha 6$ and VEGFR2 as well as VEGF uptake [34]. These data suggest that the matrix composition of the scaffold to which the cells are attached is critical for optimal cell function and wound healing. Other groups have shown that MSCs cultured in defined matrix scaffolds can direct lineage commitment, particularly true of laminins [40]. The importance of the topographical and mechanical properties of scaffolds in which cells are cultured is a driver of multiple cell functions [41]. However, the optimal niche composition and physical organization for MSCs remains to be defined [42]. For example, the role of hydroxyapatite in promoting MSC survival and function is yet to be determined.

We also show that topical application of the unrolled flat MSC-containing scaffold improves wound healing in the splinted back wound model (Figure 6). These results suggest that delivering MSCs with a biomimetic

scaffold may be translatable to human patients with diabetic wounds, either via subcutaneous implantation near the wound (Figures 2, 4 & 5) or topically on the wound (Figure 6). Since the subcutaneous implants were in the rolled configuration and the topical application was in the flat configuration, these results suggest that the hypoxia-activated MSCs are the key therapeutic agent, rather than the scaffold, for example, the critical aspect of the biomimetic scaffold is its ability to activate MSCs to promote cellular survival and/or function. One limitation of this study is the relatively small *n* used in each of the individual *in vivo* experiments; however, the reproducibility of the results using multiple delivery parameters suggests, in toto, that the biomimetic scaffold improves MSC function sufficiently to promote wound healing *in vivo*. Furthermore, the consistently improved rate of early wound healing shown in all the experiments may be translatable to clinically meaningful results for patients.

Conclusion

In summary, we show that delivery of MSCs in biomimetic scaffolds through implantation or by topical application promotes wound healing in a translationally relevant diabetic mouse model. These results suggest that a tissue engineering approach is a viable approach to this difficult and complex clinical prob-

lem. These results also suggest that a biomimetic scaffold can mimic a stem cell niche environment, promoting stem cell survival and/or function *in vivo*, potentially increasing their therapeutic application.

Financial & competing interests disclosure

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

Tissue-engineered collagen scaffolds activate mesenchymal stem cells

- Collagen scaffolds, with or without laminin, can be used to both activate stem cells and to deliver them for therapeutic applications.
- Stem cells cultured in collagen scaffolds release detectable levels of angiogenic mediators including VEGF.
- Collagen scaffolds may mimic the natural stem cell niche environment promoting stem cell survival and/or function.

Activated mesenchymal stem cells in collagen scaffolds increase wound healing *in vivo*

- Lower extremity wounds remain an important clinical problem for patients with diabetes.
- Activated mesenchymal stem cells (MSCs) implanted adjacent to a wound promote healing in diabetic mouse hindlimb wound and splinted back wound models.

Topical application of activated MSCs promotes wound healing

- Activated MSCs placed topically on a wound promote healing in a diabetic mouse splinted back wound model.
- Topical application of activated stem cells with a collagen scaffold is a viable and translationally relevant model for stem cell treatment of wounds.
- Tissue-engineering approaches may yield solutions to management of diabetic wounds.

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