

## Original Article

# Autologous fibroblasts, peripheral blood mononuclear cells, and fibrin glue accelerate healing of refractory cutaneous ulcers in diabetic mice

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**Abstract:** Background/Aims: We have developed a method to transplant sheets of autologous fibroblasts and peripheral blood mononuclear cells, which are highly angiogenic and regenerative, as treatment against refractory cutaneous ulcers in mice and rabbits. The cell sheets are also sealed with fibrin to further enhance effectiveness. Methods: Secretion of growth factors from cells incubated with or without fibrin in vitro was assessed by enzyme-linked immunosorbent assays, while angiogenesis and fibroblast migration were assessed by tube formation and scratch assays. Healing of cutaneous ulcers following transplantation of cells was evaluated in mice with diabetes mellitus. Results: Secretion of vascular endothelial growth factor, hepatocyte growth factor, and transforming growth factor was much higher from cell sheets supplemented with fibrin than from cell sheets only. Accordingly, supernatants from the former enhanced angiogenesis and fibroblast migration in vitro. Cutaneous ulcers treated with fibrin-glued cell sheets also healed more quickly than untreated ulcers or ulcers treated with cell sheets only. Conclusion: Fibrin-sealed cells accelerate wound healing and microvascularization by supplying growth factors, and thus are promising as treatment against refractory cutaneous ulcers.

**Keywords:** Fibrin glue, fibroblast, peripheral blood mononuclear cell

## Introduction

Ischemic disorders such as peripheral arterial disease, diabetes mellitus, and pressure and congestive disorders can cause refractory skin ulcers, for which corresponding treatments are available [1-3]. Of note, more than 200 million people worldwide are affected by peripheral arterial disease, e.g., arteriosclerosis obliterans, Buerger's disease, and blue toe syndrome, of whom many undergo revascularization by surgery to improve blood flow. However, limb amputation is unavoidable in some patients because of progressing infection, insufficient response to pharmacotherapy, and lack of healing [4]. For such patients, we previously reported a method to promote neovascularization by transplanting hypoxically preconditioned bone marrow cells [5, 6]. In an effort to avoid invasive collection of bone marrow for transplantation, we also found that transplantation of hypoxically preconditioned peripheral blood

mononuclear cells also enhances microvessel density and limb blood flow in ischemic hindlimbs in comparison to normoxically preconditioned cells [7, 8].

As cell sheets are more efficiently engrafted [9], we also tested mixed sheets consisting of autologous fibroblasts and peripheral blood mononuclear cells. These sheets secrete growth factors needed throughout the wound healing process, and were found to be effective against ulcers in mice and rabbits [10, 11]. Moreover, we constructed multilayered mixed sheets that were similarly effective in mice, but were easier to transplant [12]. However, cell sheets also do not mold perfectly into wounds typically seen in the clinic, which are of complex shape. Therefore, we tested fibrin glue, which is already used in the clinic to promote wound healing [13-15], to mold cell sheets to the shape of ulcers and perhaps enhance efficacy.

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Notably, we found that, *in vitro*, mixed cell sheets supplemented with fibrin secrete vascular endothelial growth factor more abundantly than cell sheets only, suggesting that fibrin glue might even enhance angiogenic activity. In addition, fibrin-sealed cells were similarly or more effective than pure cells against cutaneous ulcers in diabetic mice. Taken together, the data suggest that transplanting mixed cells and sealing with fibrin is a potentially useful and novel treatment for irregularly shaped cutaneous ulcers.

### Materials and methods

#### Animals

Male C57BL/6 mice were purchased from Japan SLC (Shizuoka, Japan). All animal procedures were approved by the Institutional Animal Care and Use Committee at Yamaguchi University (#31-002 and 31-093), and were compliant with relevant guidelines.

#### Isolation of peripheral blood mononuclear cells and fibroblasts

Mononuclear cells were isolated from mouse peripheral blood using Lympholyte<sup>®</sup>-M (Cedar-Lane Laboratories, Hornsby, Ontario, Canada), and cultured in CTS<sup>™</sup> AIM V<sup>®</sup> medium (Thermo Fisher Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific). Fibroblasts were isolated from mouse tails using collagenase (Wako, Osaka, Japan), and cultured in CTS<sup>™</sup> AIM V<sup>®</sup> medium supplemented with 10% fetal bovine serum.

#### Preparation and transplantation of cell sheets

As described previously [12], peripheral blood mononuclear cells ( $2.0 \times 10^6$ /well) were incubated under normoxic conditions (37°C, atmospheric O<sub>2</sub> concentration) for 2 days followed by hypoxic conditions (33°C, 2% O<sub>2</sub>) for 1 day, using 2 mL medium consisting of CTS<sup>™</sup> AIM V<sup>®</sup>, HFDM-1 (+) (Cell Science & Technology Institute, Sendai, Japan), and 5% fetal bovine serum. Cells were collected by centrifugation, mixed with  $5.0 \times 10^5$  fibroblast cells and thrombin, and applied to wounds directly by pipetting (**Figure 1A**). Cells were then sealed with Beriplast<sup>®</sup> (CSL Behring K.K.). Sealing is achieved within 1 minute. For *in vitro* experiments, cell mixtures in thrombin were seeded in a

24-well dish and supplemented with or without fibrinogen.

#### Histology

Cells supplemented with or without fibrin were incubated *in vitro* for 3 days, fixed overnight at room temperature with neutrally buffered 10% formalin, embedded in paraffin, and stained with hematoxylin and eosin.

#### Cell viability

Fibroblast ( $5.0 \times 10^5$ ) and peripheral blood mononuclear cells ( $2.0 \times 10^6$ ) were seeded in a 24-well plate, supplemented with and without fibrin glue, incubated for 3 days, and reacted for 2 h at 37°C in a CO<sub>2</sub> incubator with 400 µL of a 1:5 mixture of MTS reagent (CellTiter 96 Aqueous One Solution Cell Proliferation Assay, Promega, Madison, WI, USA) and media. After incubation, 200 µL of supernatant from each well was transferred to a 96-well plate and assayed at 490 nm on a 2030 ARVO X4 microplate reader (PerkinElmer, Boston, MA, USA). Fibrin glue without cells was incubated and assayed in the same manner as negative control.

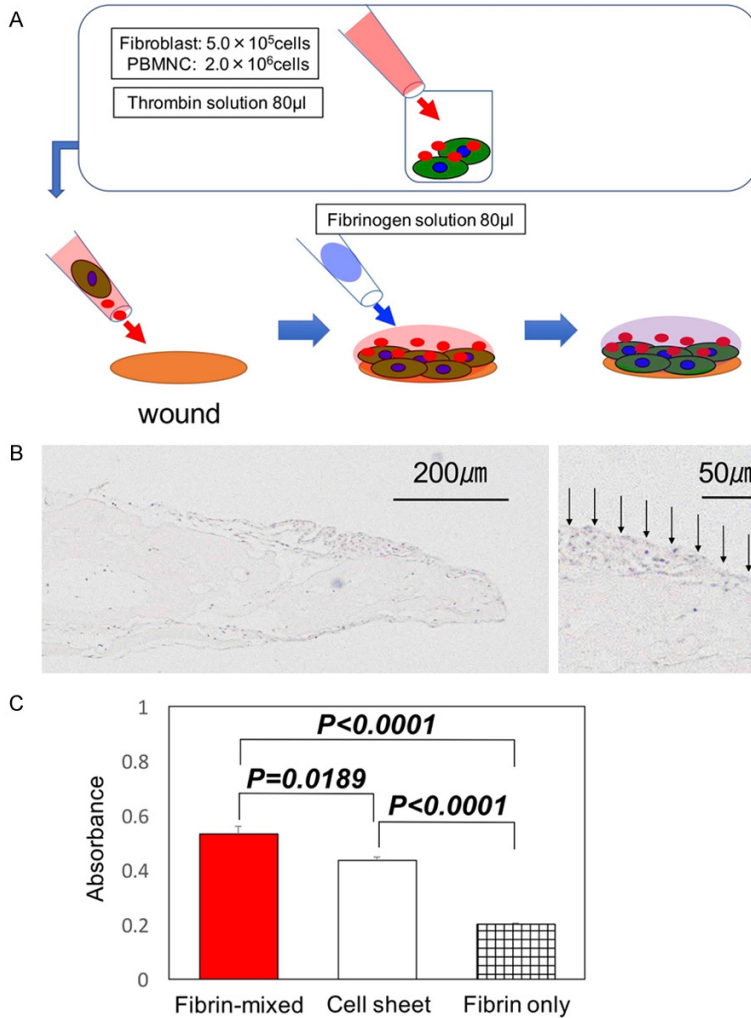
#### Enzyme-linked immunosorbent assay (ELISA)

Fibroblast ( $5.0 \times 10^5$ ) and peripheral blood mononuclear cells ( $2.0 \times 10^6$ ) were seeded in a 24-well plate with and without fibrin glue, incubated under normoxic conditions (37°C, atmospheric O<sub>2</sub> concentration) for 2 days and then under hypoxic conditions (33°C, 2% O<sub>2</sub>) for 1 day. Supernatants were collected, and assayed for vascular endothelial growth factor, hepatocyte growth factor, and transforming growth factor-β1 using Quantikine Immunoassay Kits (R&D Systems, Minneapolis, MN, USA). To assess secretion of vascular endothelial growth factor over time in the presence or absence of fibrin glue,  $5.0 \times 10^5$  fibroblasts and  $2.0 \times 10^6$  peripheral blood mononuclear cells were seeded in a 24-well plate and incubated under normoxic conditions (37°C, atmospheric O<sub>2</sub> concentration) for 7 days. Supernatants were collected every day, and assayed for vascular endothelial growth factor as described.

#### Tube formation assay

Human umbilical cord vascular endothelial cells were cultured in EGMTM-2 medium (Lonza,

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**Figure 1.** Construction and characteristics of cell sheets with fibrin glue. **A.** Transplantation of mixed fibroblasts and peripheral blood mononuclear cells to injured sites and sealing with fibrin glue. **B.** Hematoxylin and eosin staining of fibroblasts and peripheral blood mononuclear cells incubated with fibrin glue for 3 days in a 24-well plate in low (left) and high (right) power fields. Arrows indicate fibroblast cells and peripheral blood mononuclear cells on fibrin glue. **C.** Cell viability was assessed by MTS assay after  $5.0 \times 10^5$  fibroblasts and  $2.0 \times 10^6$  peripheral blood mononuclear cells were seeded in a 24-well plate with and without fibrin glue and incubated for 3 days. Fibrin glue incubated under the same conditions but without cells was used as negative control. Error bars indicate standard error. Fibrin-mixed, cells with fibrin glue; Cell sheet, cells without fibrin glue; Fibrin only, fibrin glue without cells ( $n = 4$  per group).

Basel, Switzerland) supplemented with EGM-TM BulletKit™ (Lonza), trypsinized, and resuspended in 10% fetal bovine serum. To assess tube formation, cells were seeded at  $2.0 \times 10^4$ /well (in  $100 \mu\text{L}$ ) in 96-well plates coated with Matrigel® (Corning, Corning, NY). Subsequently, wells were incubated with  $100 \mu\text{L}$  fresh or conditioned medium from mixed cells with or with-

out fibrin, and imaged after 6 h. Tube formation was assessed using Angiogenesis Analyzer for ImageJ (National Institutes of Health, Bethesda, MD) [16, 17].

### Migration assay

Fibroblasts were seeded in 24-well cell culture dishes to high confluence, scratched with  $200 \mu\text{L}$  tips, and cultured for another 16 h in conditioned medium from cells with or without fibrin. Phase-contrast images were obtained at 0 and 18 h thereafter using a BZ-X710 All-in-One fluorescence microscope (Keyence, Osaka, Japan). Wound area was measured using the associated software (Keyence).

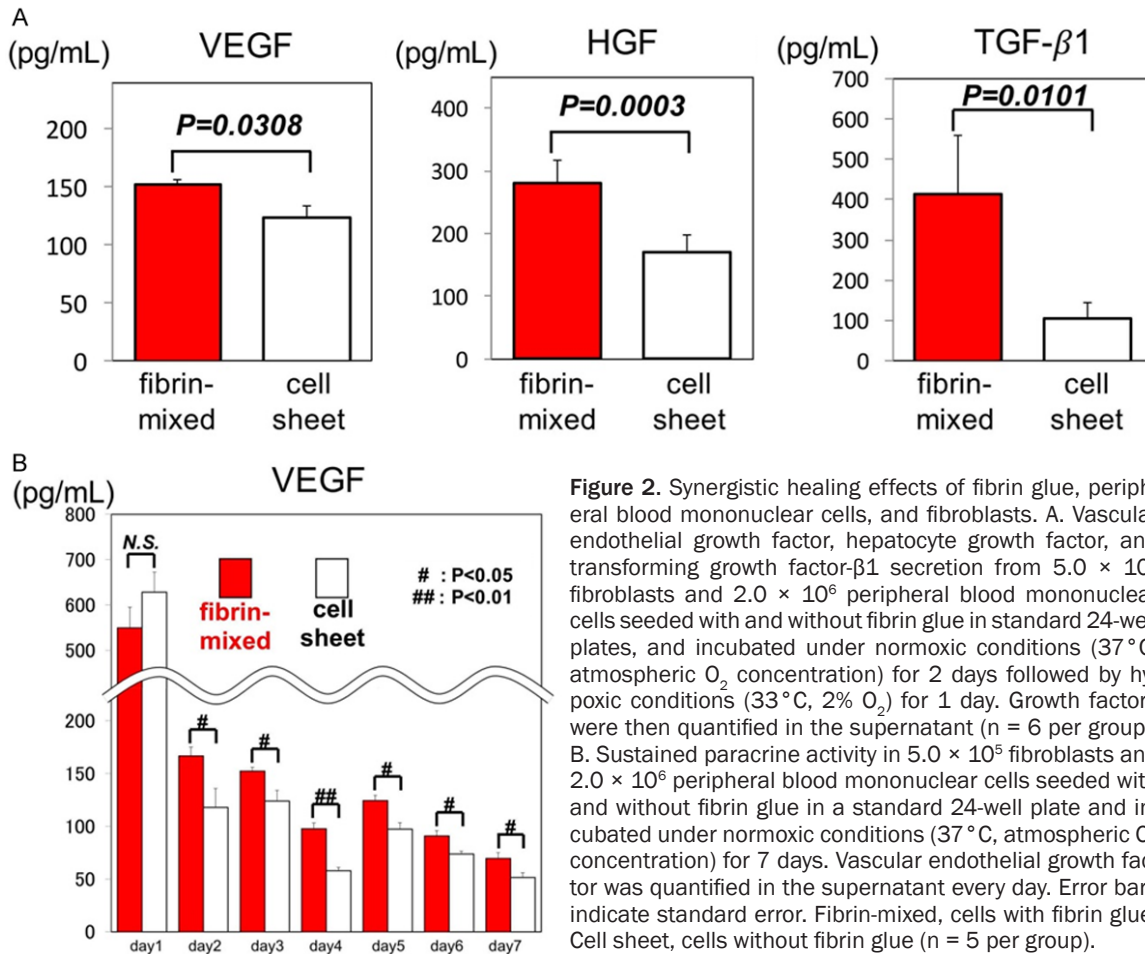
### Model of cutaneous ulcer

Mice were injected with streptozotocin (Sigma-Aldrich, St. Louis, MO) to induce diabetes mellitus. Animals with blood glucose more than  $300 \text{ mg/dL}$  were anaesthetized with 1.5% isoflurane via inhalation, and full-thickness skin injuries 8 mm in diameter were surgically created on the back.

### Wound healing

Surgically created defects were left untreated (controls,  $n = 6$ ), or were transplanted with mixed cell sheets cultured under hypoxic conditions ( $n = 6$ ), sealed with fibrin ( $n = 6$ ), or transplanted with mixed cells and sealed with fibrin ( $n = 6$ ) (Figure 1A). Cell sheets were collected from plates and delivered to wounds using a  $1,000 \mu\text{L}$  tip (FUKAE-KASEI, Kobe, Japan) shortened to widen the mouth and preserve cell structure. All ulcers were covered with Urgotul® (Laboratoires Urgo, Chenove, France), wrapped with Derma-aid® (ALCARE,

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**Figure 2.** Synergistic healing effects of fibrin glue, peripheral blood mononuclear cells, and fibroblasts. **A.** Vascular endothelial growth factor, hepatocyte growth factor, and transforming growth factor-β1 secretion from  $5.0 \times 10^5$  fibroblasts and  $2.0 \times 10^6$  peripheral blood mononuclear cells seeded with and without fibrin glue in standard 24-well plates, and incubated under normoxic conditions ( $37^\circ\text{C}$ , atmospheric  $\text{O}_2$  concentration) for 2 days followed by hypoxic conditions ( $33^\circ\text{C}$ , 2%  $\text{O}_2$ ) for 1 day. Growth factors were then quantified in the supernatant ( $n = 6$  per group). **B.** Sustained paracrine activity in  $5.0 \times 10^5$  fibroblasts and  $2.0 \times 10^6$  peripheral blood mononuclear cells seeded with and without fibrin glue in a standard 24-well plate and incubated under normoxic conditions ( $37^\circ\text{C}$ , atmospheric  $\text{O}_2$  concentration) for 7 days. Vascular endothelial growth factor was quantified in the supernatant every day. Error bars indicate standard error. Fibrin-mixed, cells with fibrin glue; Cell sheet, cells without fibrin glue ( $n = 5$  per group).

Tokyo, Japan), bandaged (ALCARE), and the injured area was measured at days 0, 9, and 14, under anesthesia and using Image J.

### Statistical analysis

Data were analyzed in Stata/SE 12.1 (StataCorp, College Station, TX). Statistical differences between two groups were assessed by two-tailed unpaired t-test, while statistical differences among multiple groups were assessed by one-way analysis of variance followed by a Bonferroni *post hoc* test.  $P < 0.05$  was considered statistically significant.

### Results

#### Production of mixed cells

We previously reported that multi-layered, mixed cell sheets consisting of autologous fibroblasts ( $5.0 \times 10^5$  cells) and peripheral blood mononuclear cells ( $2.0 \times 10^6$  cells) can

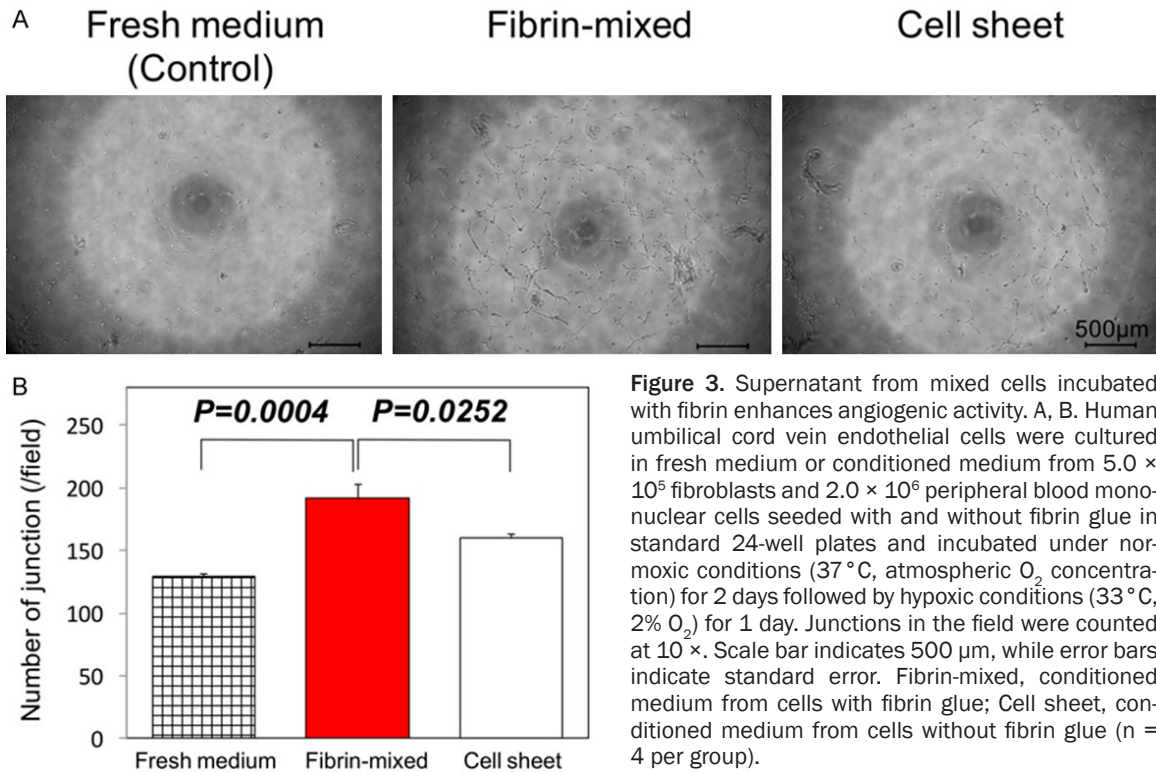
be harvested from 24-well plates using 10 PU/mL dispase [12]. This method has now enabled transplantation of mixed cells and molding into irregularly shaped wounds with fibrin glue. Of note, hematoxylin and eosin staining indicated that cells proliferated around, but not inside, fibrin glue when present (**Figure 1B**), suggesting that fibrin glue provides a good scaffold for cells.

#### Viability of mixed cells with fibrin

**Figure 1C** shows that viability after 3 days in culture, as measured by MTS proliferation assay, was higher in cells supplemented with fibrin, highlighting fibrin glue as a favorable scaffold for cells.

#### Secretion of growth factors and cytokines from fibrin-supplemented cells

Secretion of vascular endothelial growth factor, hepatocyte growth factor, and transforming



**Figure 3.** Supernatant from mixed cells incubated with fibrin enhances angiogenic activity. A, B. Human umbilical cord vein endothelial cells were cultured in fresh medium or conditioned medium from  $5.0 \times 10^5$  fibroblasts and  $2.0 \times 10^6$  peripheral blood mononuclear cells seeded with and without fibrin glue in standard 24-well plates and incubated under normoxic conditions ( $37^\circ\text{C}$ , atmospheric  $\text{O}_2$  concentration) for 2 days followed by hypoxic conditions ( $33^\circ\text{C}$ , 2%  $\text{O}_2$ ) for 1 day. Junctions in the field were counted at  $10\times$ . Scale bar indicates  $500\ \mu\text{m}$ , while error bars indicate standard error. Fibrin-mixed, conditioned medium from cells with fibrin glue; Cell sheet, conditioned medium from cells without fibrin glue ( $n = 4$  per group).

growth factor- $\beta 1$ , as measured by ELISA of culture supernatants, was significantly higher from mixed cells supplemented with fibrin than from cell sheets only (**Figure 2A**). Moreover, fibrin-supplemented cells abundantly secreted vascular endothelial growth factor for up to 7 days, in comparison to cell sheets only (**Figure 2B**).

#### *Angiogenic potency of factors secreted by fibrin-supplemented cells*

Tube formation was significantly stimulated in human umbilical cord vein endothelial cells cultured for 6 h in conditioned medium from fibrin-supplemented cells than in cells cultured in fresh medium or in conditioned medium from cell sheets without fibrin (**Figure 3A** and **3B**). This result suggests that fibrin glue augments the angiogenic activity of mixed cells.

#### *Fibroblast migration potency of factors secreted by fibrin-supplemented cells*

Migration of primary mouse fibroblasts over 16 h across scratches made in confluent cells was enhanced by conditioned medium from fibrin-supplemented cells (**Figure 4A** and **4B**). This result highlights the potential of fibrin-sup-

plemented cells to induce fibroblast migration at injured sites.

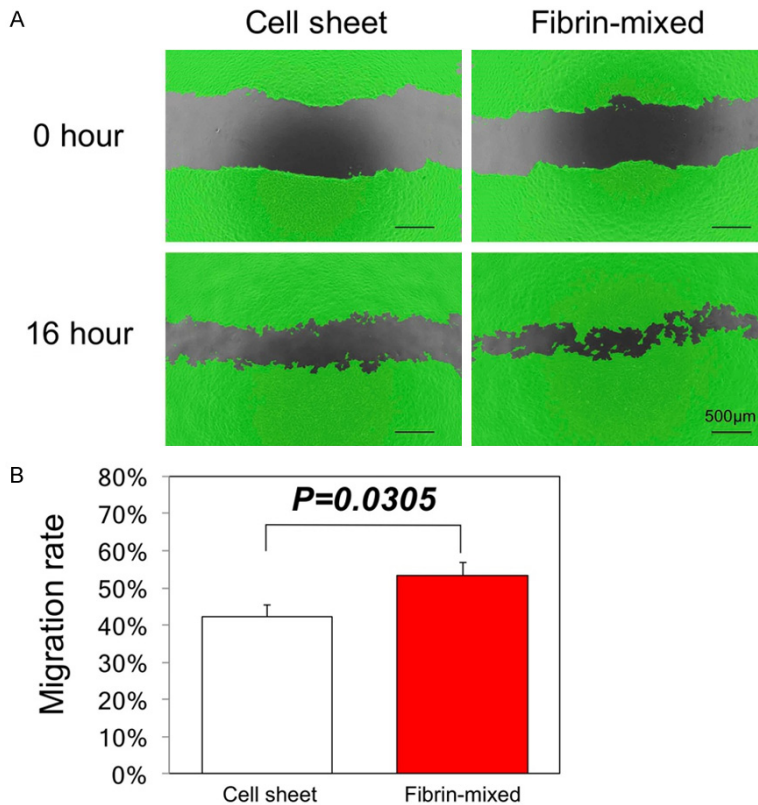
#### *Healing of cutaneous ulcers in mice using mixed cells sealed with fibrin*

To evaluate as therapy for cutaneous ulcers, mixed cells were transplanted to full-thickness skin injuries on the backs of mice with diabetes mellitus (**Figure 5A**), and sealed with or without fibrin. Untreated sites (Control) and sites treated with fibrin glue (Fibrin only) were used as control. After 9 days, wound healing was significantly more advanced at sites treated with mixed cells and sealed with fibrin than at control sites (**Figure 4B**). At 14 days, wound healing was significantly more advanced in the same sites relative to sites treated with mixed cells but not sealed with fibrin (**Figure 4B**). In any case, injuries were nearly closed in all animals by 21 days.

#### **Discussion**

Ulcer healing requires a well-orchestrated interplay of cell-matrix and cell-cell signaling. For example, fibroblasts facilitate the interaction of keratinocytes and endothelial cells with the extracellular matrix to promote healing [18]. In

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**Figure 4.** Supernatant from mixed cells incubated with fibrin enhances fibroblast migration. A, B. A scratch approximately 1.0 mm wide was made in confluent mouse fibroblasts, which were then cultured for another 18 h in conditioned media from  $5.0 \times 10^5$  fibroblasts and  $2.0 \times 10^6$  peripheral blood mononuclear cells seeded with and without fibrin glue in standard 24-well plates and incubated under normoxic conditions ( $37^\circ\text{C}$ , atmospheric  $\text{O}_2$  concentration) for 2 days followed by hypoxic conditions ( $33^\circ\text{C}$ , 2%  $\text{O}_2$ ) for 1 day. Wound closure was measured after 16 h, and cells are rendered green for clarity. Scale bar indicates 500  $\mu\text{m}$ , while error bars indicate standard error. Fibrin-mixed, cells with fibrin glue; Cell sheet, cells without fibrin glue (n = 7 per group).

addition, these cells secrete vascularization-associated growth factors, including vascular endothelial growth factor, hepatocyte growth factor, and transforming growth factor- $\beta 1$  [19], that are essential for normal wound-healing [23, 24]. Accordingly, we have now developed mixed cell sheets of fibroblasts and peripheral blood mononuclear cells as potential therapy when transplanted to skin ulcers and sealed with fibrin. Sealing with fibrin appears to accelerate wound healing more efficiently than transplantation of cells alone, especially in mice with streptozotocin-induced diabetes, which typically delays healing. Importantly, fibroblasts are conveniently isolated and cultured from various tissues, while peripheral blood mononuclear cells are harvested easily and indeed are now transplanted to ischemic tis-

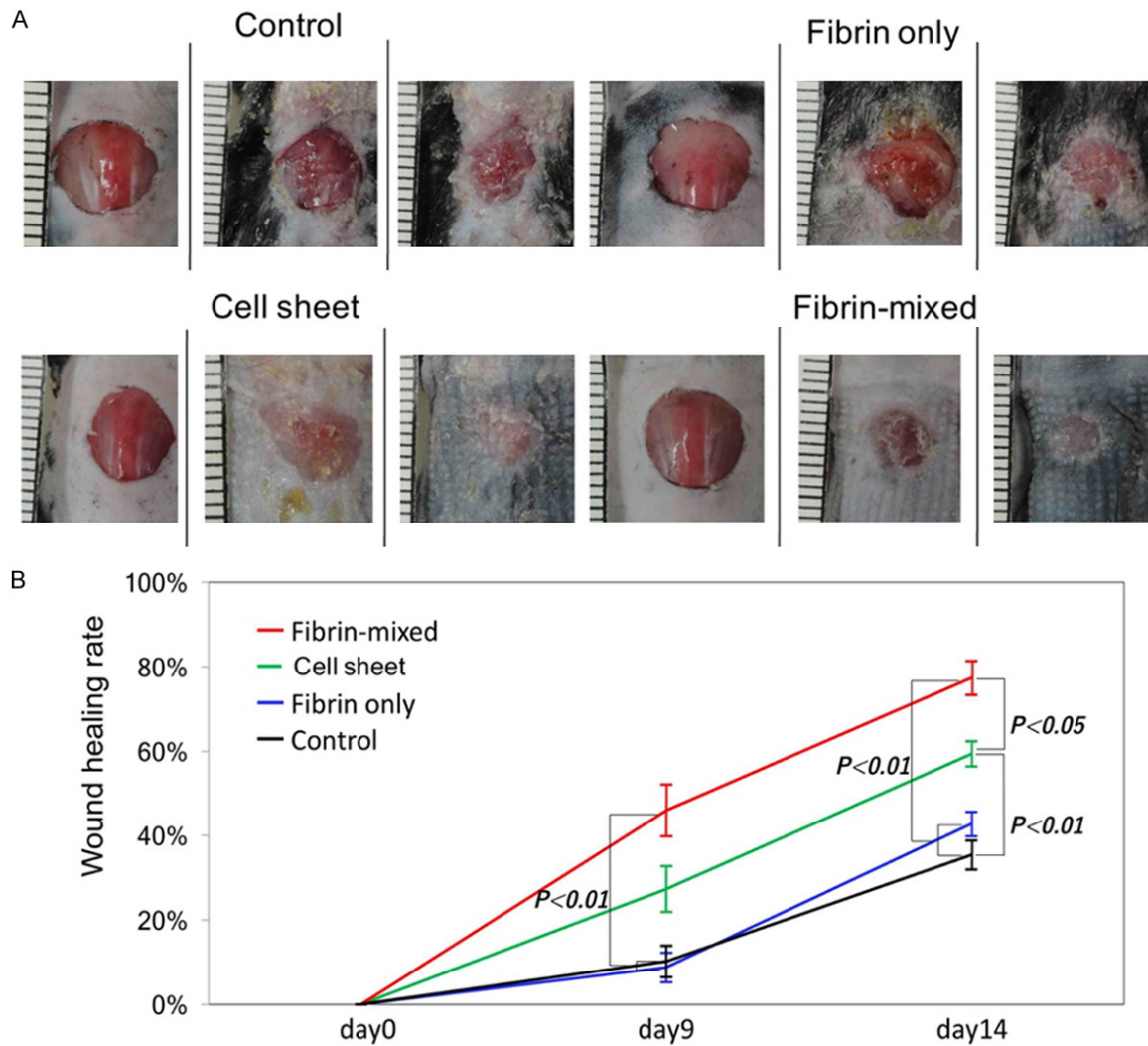
sues to induce angiogenesis [20-22].

Normal healing is regulated by a complex signaling network involving growth factors, cytokines, and chemokines and can be divided into overlapping inflammatory, proliferative, and tissue remodeling phases [23]. Of note, vascular endothelial growth factor, transforming growth factor- $\beta 1$ , and platelet-derived growth factor BB are required throughout the process [27, 28], of which the last two are believed to be supplied by peripheral mononuclear cells [26-29]. Transforming growth factor- $\beta 1$  and platelet-derived growth factor BB also induce expression of vascular endothelial growth factor and collagen in fibroblasts [10]. Accordingly, abnormal expression of growth factors in refractory cutaneous ulcers such as venous and diabetic leg ulcers induces chronic inflammation and delays healing [25, 26].

Cell sheet technology, which was only recently developed, depends on detaching cells from a temperature-sensitive

culture dish or with a small amount of dispase while keeping the extracellular matrix intact [30]. This technology is now widely used and was reported to enhance the effectiveness of cell transplants in regenerative medicine [12, 31]. However, these cell sheets are difficult to mold to cutaneous ulcers with complex shapes, which are common in the clinic, and is a specific issue for dermatological injuries. Therefore, we attempted to use fibrin glue to mold transplanted cells to the injured site. We note that fibrin glue is safe, keeps cells in place, and promotes healing by enhancing cell survival, providing a good scaffold, and shielding against oxygen stress [13-15]. Indeed, we found that fibrin glue enhances the ability of mixed cells to promote angiogenesis and fibroblast migration.

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**Figure 5.** Therapeutic effect of cells with fibrin. A. Healing of full-thickness skin wounds in diabetic mice after transplantation of mixed cells with and without fibrin glue, as imaged on days 1, 9, and 14 (left to right). B. Time course of wound healing as measured in ImageJ. Error bars indicate standard error. Fibrin-mixed, mixed cells with fibrin glue; Cell sheet, cells without fibrin glue; Fibrin only, fibrin glue without cells; Control, no treatment (n = 6 for each group).

In addition, we developed a novel and feasible protocol to hypoxically precondition cells to enhance expression heme oxygenase-1 and hexokinase-2 [32] and growth factors, and to improve retention of transplanted cells in mice through CXCR4 and integrin  $\alpha$ M [33, 34]. Similarly, we found previously that hypoxically preconditioned peripheral blood mononuclear cells enhance cell adhesion and angiogenesis and improve blood flow into muscle at ischemic hindlimbs in rabbits [8]. Hypoxic preconditioning was also reported to increase secretion of vascular endothelial growth factor in single-layered sheets [10, 11].

In summary, the data clearly show that mixed cells sealed with fibrin are effective against

refractory skin ulcers, with fibrin glue acting as a functional scaffold.

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### Disclosure of conflict of interest

None.

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### References

- [1] Singh N, Armstrong DG, Lipsky BA. Preventing foot ulcers in patients with diabetes. *JAMA* 2005; 293: 217-228.
- [2] Wu SC, Driver VR, Wrobel JS, Armstrong DG. Foot ulcers in the diabetic patient, prevention and treatment. *Vasc Health Risk Manag* 2007; 3: 65-76.
- [3] Vuorisalo S, Venermo M, Lepäntalo M. Treatment of diabetic foot ulcers. *J Cardiovasc Surg* 2009; 50: 275-291.
- [4] Vartanian SM, Conte MS. Surgical intervention for peripheral arterial disease. *Circ Res* 2015; 116: 1614-1628.
- [5] Li TS, Hamano K, Suzuki K, Ito H, Zempo N, Matsuzaki M. Improved angiogenic potency by implantation of ex vivo hypoxia prestimulated bone marrow cells in rats. *Am J Physiol Heart Circ Physiol* 2002; 283: H468-H473.
- [6] Kubo M, Li TS, Kurazumi H, Takemoto Y, Ohshima M, Murata T, Katsura S, Morikage N, Furutani A, Hamano K. Hypoxic preconditioning enhances angiogenic potential of bone marrow cells with aging-related functional impairment. *Circ J* 2012; 76: 986-994.
- [7] Kubo M, Li TS, Suzuki R, Shirasawa B, Morikage N, Ohshima M, Qin SL, Hamano K. Hypoxic preconditioning increases survival and angiogenic potency of peripheral blood mononuclear cells via oxidative stress resistance. *Am J Physiol Heart Circ Physiol* 2008; 294: H590-H595.
- [8] Kudo T, Hosoyama T, Samura M, Katsura S, Nishimoto A, Kugimiya N, Fujii Y, Li TS, Hamano K. Hypoxic preconditioning reinforces cellular functions of autologous peripheral blood-derived cells in rabbit hindlimb ischemia model. *Biochem Biophys Res Commun* 2014; 444: 370-375.
- [9] Matsuura K, Utoh R, Nagase K, Okano T. Cell sheet approach for tissue engineering and regenerative medicine. *J Control Release* 2014; 190: 228-239.
- [10] Ueno K, Takeuchi Y, Samura M, Tanaka Y, Nakamura T, Nishimoto A, Murata T, Hosoyama T, Hamano K. Treatment of refractory cutaneous ulcers with mixed sheets consisting of peripheral blood mononuclear cells and fibroblasts. *Sci Rep* 2016; 6: 28538.
- [11] Takeuchi Y, Ueno K, Mizoguchi T, Samura M, Harada T, Oga A, Murata T, Hosoyama T, Morikage N, Hamano K. Development of novel mouse model of ulcers induced by implantation of magnets. *Sci Rep* 2017; 7: 4843.
- [12] Mizoguchi T, Ueno K, Takeuchi Y, Samura M, Suzuki R, Murata T, Hosoyama T, Morikage N, Hamano K. Treatment of cutaneous ulcers with multilayered autologous mixed sheets consisting of fibroblasts and peripheral blood mononuclear cells. *Cell Physiol Biochem* 2018; 47: 201-211.
- [13] Kim I, Lee SK, Yoon JI, Kim DE, Kim M, Ha H. Fibrin glue improves the therapeutic effect of MSCs by sustaining survival and paracrine function. *Tissue Eng Part A* 2013; 19: 2373-2381.
- [14] Marcelo D, Beatriz PM, Jussara R, Fabiana B. Tissue therapy with autologous dermal and epidermal culture cells for diabetic foot ulcers. *Cell Tissue Bank* 2012; 13: 241-249.
- [15] Litvinov RI, Weisel JW. What is the biological and clinical relevance of fibrin? *Semin Thromb Hemost* 2016; 42: 333-343.
- [16] Arnaoutova I, George J, Kleinman HK, Benton G. The endothelial cell tube formation assay on basement membrane turns 20: state of the science and the art. *Angiogenesis* 2009; 12: 267-274.
- [17] Tanaka Y, Shirasawa B, Takeuchi Y, Kawamura D, Nakamura T, Samura M, Nishimoto A, Ueno K, Morikage N, Hosoyama T, Hamano K. Autologous preconditioned mesenchymal stem cell sheets improve left ventricular function in a rabbit old myocardial infarction model. *Am J Transl Res* 2016; 8: 2222-2233.
- [18] Cerqueira MT, Pirraco RP, Martins AR, Santos TC, Reis RL, Marques AP. Cell sheet technology-driven re-epithelialization and neovascularization of skin wounds. *Acta Biomater* 2014; 10: 3145-3155.
- [19] Liu Y, Luo H, Wang X, Takemura A, Fang YR, Jin Y, Suwa F. In vitro construction of scaffold-free bilayered tissue-engineered skin containing capillary networks. *Biomed Res Int* 2013; 2013: 561410.
- [20] Horie T, Onodera R, Akamastu M, Ichikawa Y, Hoshino J, Kaneko E, Iwashita C, Ishida A, Tsukamoto T, Teramukai S, Fukushima M, Kawamura A; Japan Study Group of Peripheral Vascular Regeneration Cell Therapy. Long-term clinical outcomes for patients with lower limb ischemia implanted with G-CSF-mobilized autologous peripheral blood mononuclear cells. *Atherosclerosis* 2010; 208: 461-466.
- [21] Moriya J, Minamino T, Tateno K, Shimizu N, Kuwabara Y, Sato Y, Saito Y, Komuro I. Long-term outcome of therapeutic neovascularization using peripheral blood mononuclear cells for limb ischemia. *Circ Cardiovasc Interv* 2009; 2: 245-254.



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- [22] Tateishi-Yuyama E, Matsubara H, Murohara T, Ikeda U, Shintani S, Masaki H, Amano K, Kishimoto Y, Yoshimoto K, Akashi H, Shimada K, Iwasaka T, Imaizumi T; Therapeutic Angiogenesis Using Cell Transplantation Study Investigators. Therapeutic angiogenesis for patients with limb ischaemia by autologous transplantation of bone-marrow cells: a pilot study and a randomized controlled trial. *Lancet* 2002; 360: 427-435.
- [23] Behm B, Babilas P, Landthaler M, Schreml S. Cytokines, chemokines and growth factors in wound healing. *J Eur Acad Dermatol Venereol* 2012; 26: 812-820.
- [24] Zaja-Milatovic S, Richmond A. CXC chemokines and their receptors: a case for a significant biological role in cutaneous healing. *Histol Histopathol* 2008; 23: 1399-1407.
- [25] Demidova-Rice TN, Hamblin MR, Herman IM. Acute and impaired wound healing: pathophysiology and current methods for drug delivery, part 1: normal and chronic wounds: biology, causes, and approaches to care. *Adv Skin Wound Care* 2012; 25: 304-314.
- [26] Vellayappan MV, Jaganathan SK, Manikandan A. Nanomaterials as a game changer in the management and treatment of diabetic foot ulcers. *RSC Adv* 2016; 6: 114859-114878.
- [27] Eming SA, Krieg T, Davidson JM. Inflammation in wound repair: molecular and cellular mechanisms. *J Invest Dermatol* 2007; 127: 514-525.
- [28] Röhl J, Zaharia A, Rudolph M, Murray RZ. The role of inflammation in cutaneous repair. *Wound Pract Res* 2015; 23: 8-15.
- [29] Samura M, Morikage N, Suehiro K, Tanaka Y, Nakamura T, Nishimoto A, Ueno K, Hosoyama T, Hamano K. Combinatorial treatment with Apelin-13 enhances the therapeutic efficacy of a preconditioned cell-based therapy for peripheral ischemia. *Sci Rep* 2016; 6: 19379.
- [30] Murakami D, Yamato M, Nishida K, Ohki T, Takagi R, Yang J, Namiki H, Okano T. The effect of micropores in the surface of temperature-responsive culture inserts on the fabrication of transplantable canine oral mucosal epithelial cell sheets. *Biomaterials* 2006; 27: 5518-5523.
- [31] Sawa Y, Miyagawa S. Cell sheet technology for heart failure. *Curr Pharm Biotechnol* 2013; 14: 61-66.
- [32] Kubo M, Li TS, Suzuki R, Ohshima M, Qin SL, Hamano K. Short-term pretreatment with low-dose hydrogen peroxide enhances the efficacy of bone marrow cells for therapeutic angiogenesis. *Am J Physiol Heart Circ Physiol* 2007; 292: H2582-H2588.
- [33] Liu H, Xue W, Ge G, Luo X, Li Y, Xiang H, Ding X, Tian P, Tian X. Hypoxic preconditioning advances CXCR4 and CXCR7 expression by activating HIF-1 $\alpha$  in MSCs. *Biochem Biophys Res Commun* 2010; 401: 509-515.
- [34] Kubo M, Li TS, Kamota T, Ohshima M, Qin SL, Hamano K. Increased expression of CXCR4 and integrin  $\alpha$ M in hypoxia-preconditioned cells contributes to improved cell retention and angiogenic potency. *J Cell Physiol* 2009; 220: 508-514.