

Stem cells from human exfoliated deciduous teeth (SHED) enhance wound healing and the possibility of novel cell therapy

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Abstract

Background aims. In recent years, stem cells from human exfoliated deciduous teeth (SHED) have received attention as a novel stem cell source with multipotent potential. We examined the effect on wound-healing promotion with unique stem cells from deciduous teeth as a medical waste. **Methods.** An excisional wound-splinting mouse model was used and the effect of wound healing among SHED, human mesenchymal stromal cells (hMSCs), human fibroblasts (hFibro) and a control (phosphate-buffered saline; PBS) was evaluated by macroscopy, histology and enzyme-linked immunosorbent assay (ELISA), and the expression of hyaluronan (HA), which is related to wound healing, investigated. **Results.** SHED and hMSCs accelerated wound healing compared with hFibro and the control. There was a statistically significant difference in wound healing area among hFibro, hMSCs and SHED compared with the control after day 5. At days 7 and 14 after cell transplantation, the histologic observation showed that transplanted PKH26-positive cells were surrounded by human HA binding protein, especially in hMSCs and SHED. HA expression volume values were 1558.41 ± 60.33 (control), 2092.75 ± 42.56 (hFibro), 2342.07 ± 188.10 (hMSCs) and 2314.85 ± 164.91 (SHED) ng/mg, respectively, and significantly higher in hMSCs and SHED compared with hFibro and control at days 7 and 14 ($P < 0.05$). **Conclusions.** Our results show that SHED hMSCs have similar effects of wound-healing promotion as hFibro and controls. This implies that SHED might offer a unique stem cell resource and the possibility of novel cell therapies for wound healing in the future.

Key Words: cell therapy, human mesenchymal stromal cells, hyaluronic acid, stem cells from human exfoliated deciduous teeth, wound healing

Introduction

An intractable wound such as a chronic wound causes a patient great stress and treatment is very difficult (1,2). Until now, such a wound has been treated by using surgical operations and medical treatments. But there are various problems, such as imperfect wound healing and scarring. They have also not resulted in dramatic changes in wound closure or outcome (3). Accordingly, establishment of a treatment is essential. Now various examinations as the treatment method of the wound such as the development of the ointment treatment, artificial skin, and skin substitute, are done. Therefore cell therapy toward promotion of wound healing and scar-less treatment has been paid to attention. Several lines of evidence have reported that fibroblasts and mesenchymal stromal cells (MSCs), applied to accelerate wound healing, have differentiation and paracrine

effects (4–6). Fibroblast injections are based on the hypothesis that autologous fibroblasts are capable of producing collagens for ongoing improvement without immune or allergic reactions. But clinical reports have recommended repeated injections in order to continue longer rhytide correction, and the results are less than optimal (7). Fibroblast injections can reportedly increase inflammation and scar formation (7–10). On the other hand, MSCs are referred to as stromal progenitor, self-renewing and expandable stem cells and are able to differentiate into osteoblasts, adipocytes, chondrocytes, etc. (11). However, bone marrow aspiration is an invasive procedure for the donor. In addition, the number, proliferation and differentiation potential of MSCs declines with increasing age (12). In a recent study, dental pulp contained a population of multipotent stem cells with the capacity to differentiate into several different cells

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lineages *in vitro* and *in vivo* (13,14). These studies have facilitated experiments addressing an important property of stem cells. Human dental pulp cell also shows properties that resemble MSCs (15). Therefore it has been used as a new source of stem cells and been applied to treat many of the same diseases as MSCs. Dental pulp cells have already been applied to cardiac infarction, Alzheimer's and Parkinson's diseases and muscular dystrophy (16,17). However, there are few reports regarding the application of dental pulp stem cells for wound healing.

Wound healing consists of inflammation, granulation tissue formation, re-epithelization and remodeling. The process of wound healing depends upon a variety of interactions between cells and the extracellular matrix (ECM). One of the extracellular matrices is hyaluronic acid (HA), which is well known to not

only support tissue architecture as a passive structural component of the matrix in various connective tissues but also be involved in dynamic cellular processes during wound healing. Thus, it is important to observe HA in the process of wound healing.

We examined the effect of stem cells from human exfoliated deciduous teeth (SHED) in wound healing by using a wound-healing skin defect model as described previously (18) and comparing SHED with human (h)MSCs, human fibroblasts (hFibro) and a control (phosphate buffered saline; PBS). SHED was also evaluated in terms of HA expression, which is thought to have important biologic roles in skin wound healing. Taken together, the results suggest that SHED may offer a unique stem cell resource with the potential for novel cell therapies for wound healing.

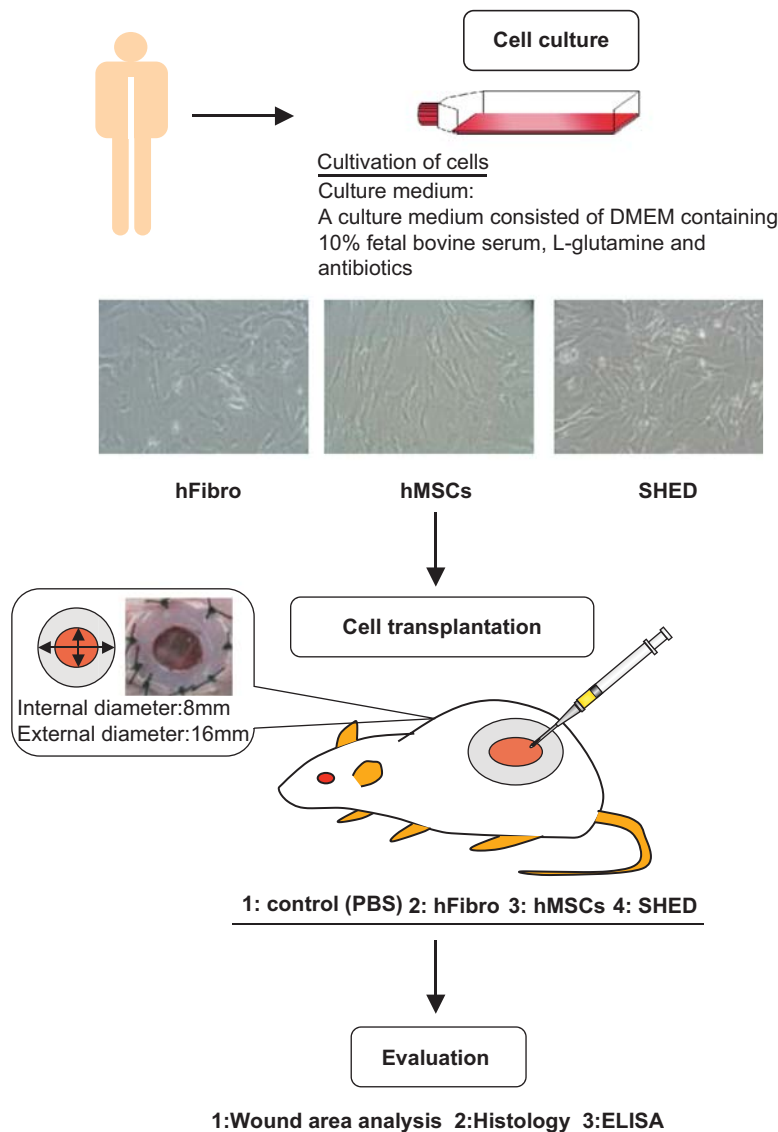


Figure 1. Schema of the experimental protocol.

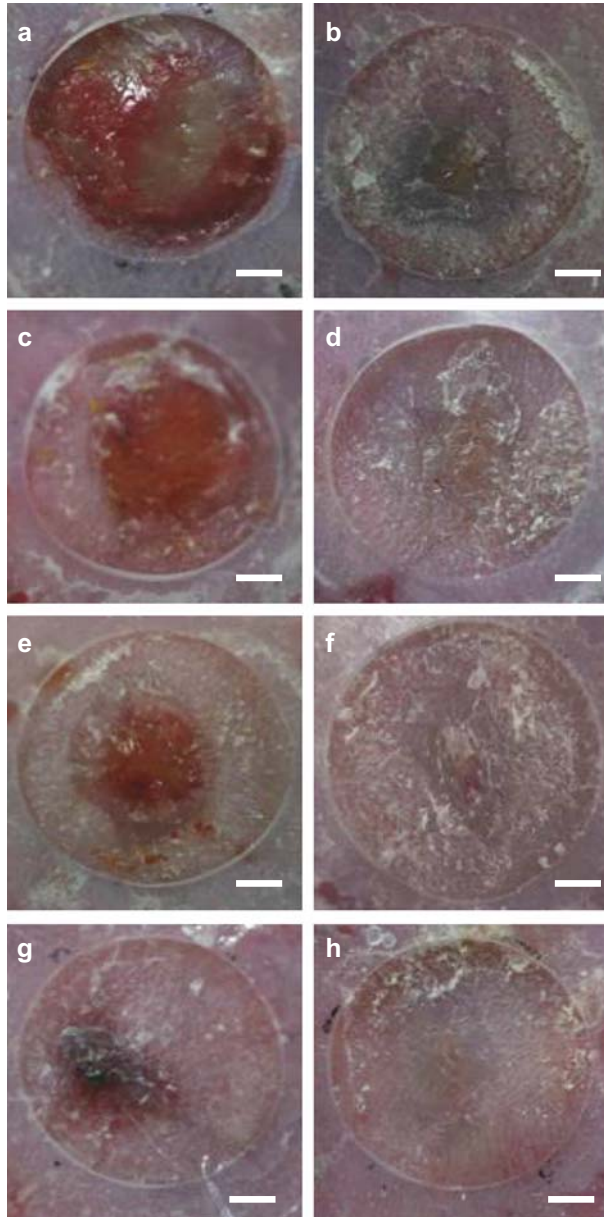


Figure 2. Effects of SHED on wound closure. Representative photographs of the wounds at days 7 and 14 after cell transplantation in a murine excisional wound-splinting model, in which wounds received implantation of PBS (control), hFibro, hMSCs or SHED. Left panel (a, c, e, g) is at day 7 after cell transplantation. Right panel (b, d, f, h) is at day 14 after cell transplantation. (a, b) Control, (c, d) hFibro, (e, f) hMSCs, (g, h) SHED. Bars = 2 mm.

Methods

Animals

Seven-week-old KSN/Slc nude mice, obtained from the Chubu Kagaku Shizai Corporation (Nagoya, Japan), were used. The animal experiments were performed in accordance with the *Guide for Animal Experimentation of Nagoya University School of Medicine* (Nagoya, Japan).

Cell culture of hFibro, hMSCs and SHED

SHED were obtained from clinically extracted deciduous teeth at the Regenerative Medicine Clinic and Department of Oral and Maxillofacial Surgery, Nagoya University Hospital (Nagoya, Japan), after obtaining informed consent from each patient. The research protocol was approved by the Nagoya University Ethics Committee (permission number 548-3). They were isolated and cultured as described previously (13,14). hMSCs and hFibro were purchased from LONZA® Inc. (Walkersville, MD, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM), containing low glucose supplemented with 10% fetal bovine serum (FBS), 100 U penicillin G, 100 µg streptomycin and 0.25 µg amphotericin B (Sigma, St Louis, MO, USA), at 37°C in 95% humidified air and 5% CO₂.

Wound-healing model and cell transplantation

The experimental protocol is summarized in Figure 1. Before cell transplantation, cells were labeled with a fluorescent cell linker PKH26 (Sigma-Aldrich, St Louis, MO, USA), according to the manufacturer's protocol. The animals were divided randomly into four groups: a control group, with PBS, and three experimental groups, with hFibro, hMSCs and SHED, respectively. The excisional wound-splinting model was generated as described previously (18). Briefly, two 8-mm full-thickness excisional skin wounds were created on each side of the midline. A donut-shaped silicone splint was placed so that the wound was centered within the splint. An immediate-bonding adhesive (Krazy Glue Columbus) was used to fix the splint to the skin, followed by interrupted sutures to stabilize its position. Each wound received 4×10^6 cells in PBS injected intradermally around the wound at four injection sites, and 1×10^6 cells in PBS applied onto the wound bed. Tegaderm (3M, London, Canada) was placed over the wounds.

Assessment of wound area

Digital photographs of the wounds were taken on days 0–14 after cell transplantation. The wound area was measured using an image analysis program (Scion Corporation, Frederick, MD, USA). The percentage of the wound area was calculated as: area of actual wound/area of original wound \times 100. Some mice were killed on days 7 and 14 after cell transplantation and evaluated using an image analysis program.

Histologic examination and immunofluorescent staining

Extraction tissue were fixed in 4% paraformaldehyde and embedded in OCT compound (Tissue-Tek;

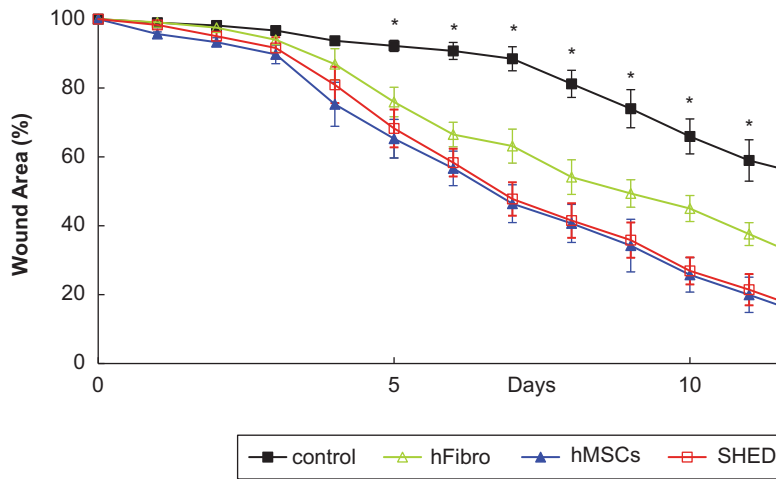


Figure 3. Wound measurement of control, hFibro, hMSCs and SHED groups. Each point represents the mean percentage area of the original wound size. ANOVA versus cell transplantation groups or control, * $P < 0.05$.

Miles Inc., Elkhart, IN, USA). Sections were taken at 5- μm intervals and performed with hematoxylin and eosin staining. Immunofluorescent staining was used to confirm the presence of human HA generated by the injected cells (Rockland Immunochemicals Inc., Gilbertsville, PA, USA). HA was identified with a biotinylated HA-binding protein (Seikagaku bio, Tokyo, Japan) diluted 1:100 in PBS for 2 h at room temperature. After incubation, the slides were rinsed in PBS and visualized with fluorescein isothiocyanate (FITC)-conjugated streptavidin (Beckman Coulter Inc., Brea, CA, USA) diluted 1:200 in PBS for 15 min at room temperature. The slides were mounted with mounting medium and DAPI (Vector Laboratories Inc., Burlingame, CA, USA). Before the reaction of HA-binding protein, tissue sections were pre-incubated with 1% bovine serum albumin (BSA) for 1 h to reduce autofluorescence. Endogenous biotin was blocked with a streptavidin biotin blocking kit (Vector Laboratories Inc.) for 20 min at room temperature. Histologic examination and immune fluorescent staining were observed under a fluorescence microscope (Microscope BX51; Olympus, Tokyo, Japan).

Enzyme-linked immunosorbent assay for HA

To determine the level of HA in each sample at 7 and 14 days, an HA quantitative test kit was used (Seikagaku bio) according to the manufacturer's protocol. The HA test kit is an enzyme-linked binding protein assay that uses a capture molecule known as HA-binding protein (HABP). First, the biotinylated HABP solution was reacted. Next, horseradish peroxidase (HRP)-conjugated streptavidin solution was added as a secondary reaction. Stop solution added with them and a

colored product was formed at a level proportional to the amount of HA in each sample measured at 450 nm. HA levels in all samples were determined against a reference curve prepared from the reagent blank and HA reference solutions provided with the kit.

Statistical analysis

Statistical differences among the volumes in different cells were evaluated with a Tukey-Kramer test following one-way analysis of variance (ANOVA). A value of $P < 0.05$ was considered to be statistically significant.

Results

Macroscopic observation of wound-healing area by effect of transplanted cells

For each wound, samples were collected from the dorsum of mice on days 7 and 14 *in vivo*. At day 7, healing was still incomplete in all groups; particularly in the control group, the surface was still raw under macroscopic observation. Exudate was observed in control, hFibro and hMSCs groups. At day 14, wound contraction in hFibro, hMSCs and SHED groups was almost complete, in contrast with the control group, which was not sufficiently closed (Figure 2). Digital image analysis showed that the percentage of the wound area was $88.48 \pm 3.49\%$ and $42.64 \pm 5.36\%$ (control), $63.15 \pm 4.93\%$ and $19.33 \pm 3.77\%$ (hFibro), $46.41 \pm 5.49\%$ and $4.90 \pm 2.36\%$ (hMSCs), and $47.77 \pm 4.86\%$ and 5.74 ± 2.85 (SHED) at 7 and 14 days, respectively.

Cell-transplantation groups hFibro, hMSCs, and SHED demonstrated accelerated wound healing

compared with the control group. There was a statistically significant difference in wound area observed between the hFibro, hMSCs and SHED groups and control group at all time-points after day 5, and between hMSCs and SHED groups and the hFibro group at all time-points after day 6 ($P < 0.05$; Figure 3). However, there was no statistically significant difference between the hMSCs and SHED groups (Figure 3).

Histologic observation and detection of human HA produced by transplanted hFibro, hMSCs and SHED

At day 7 after cell transplantation, an infiltrate of inflammatory cells was admitted in all groups. The wound epidermis was particularly lacking in the control group. Cell transplantation groups, especially hMSCs and SHED groups, showed thick granulation tissue. HABP detection was performed to confirm the presence of HA derived from transplanted

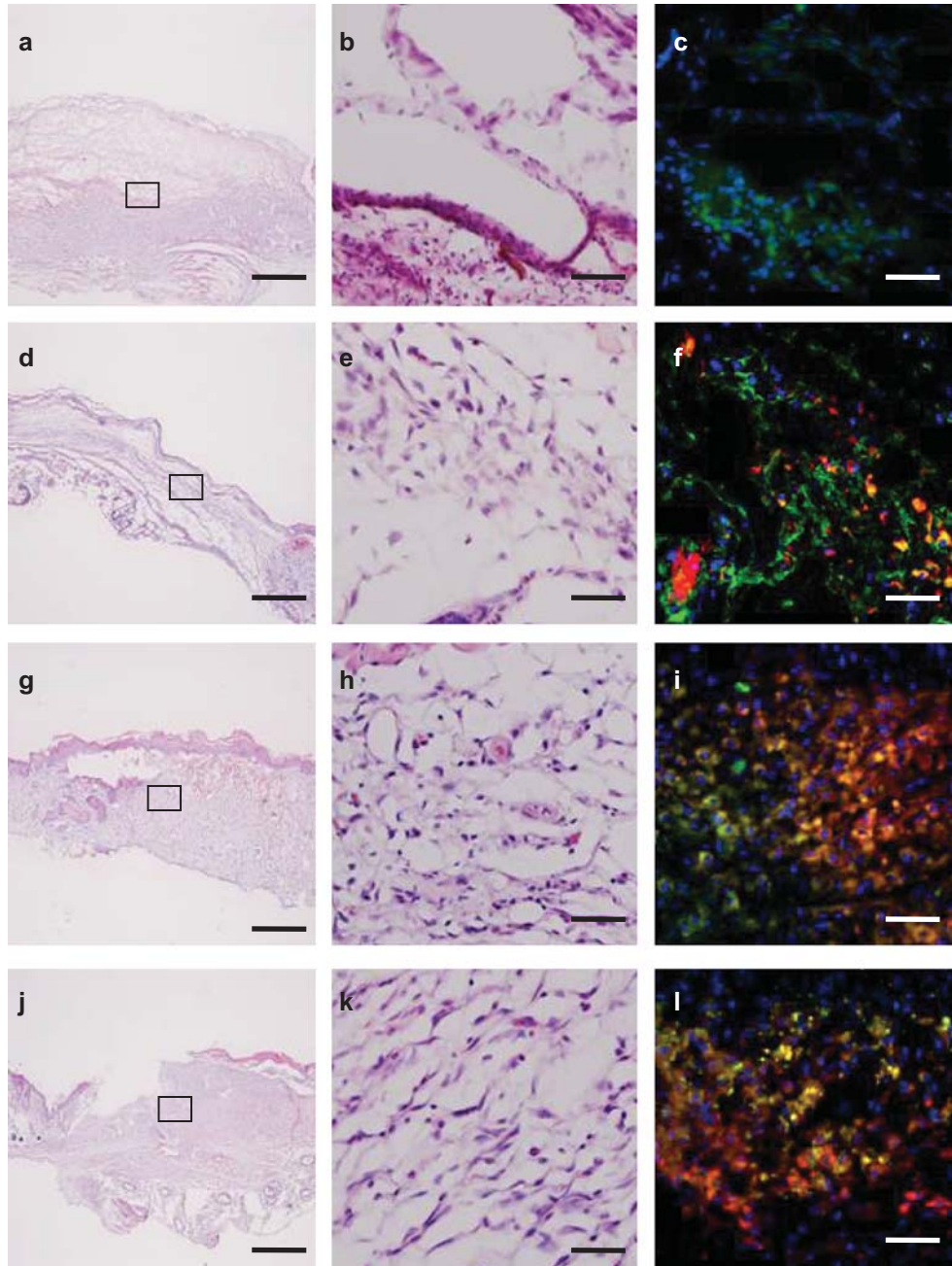


Figure 4. Histologic evaluation of the effect of transplanted cells at day 7. Left and middle panels show hematoxylin and eosin staining, the right panel shows PKH26-labeled cells (red fluorescence) and human HA staining (green fluorescence); images were obtained by fluorescence microscopy. DAPI was used to visualize the nuclei (blue fluorescence). Control (a, b, c), hFibro (d, e, f), hMSCs (g, h, i) and SHED (j, k, l). Bars = 30 mm (a, d, g, j) and 25 μ m (b, c, e, f, h, i, k, l).

cells at the wound sides. Evaluation by fluorescence microscope showed that PKH26-positive cells were surrounded by human HA in hFibro, hMSCs and SHED groups (Figure 4). These results showed that the hFibro, hMSCs and SHED groups, particularly the hMSCs and SHED groups, produced human HA derived from the injected cells.

At day 14 after cell transplantation, the number of inflammatory cells in the wound area was reduced compared with day 7. Cell transplantation groups

were re-epithelialized with epithelium (Figure 5). Evaluation by fluorescence microscope showed that PKH26-positive cells (hFibro, hMSCs and SHED) and HA production were also reduced (Figure 5).

HA production measurement by enzyme-linked immunosorbent assay

The HA volume was determined by measuring with an enzyme-linked immunosorbent assay (ELISA)

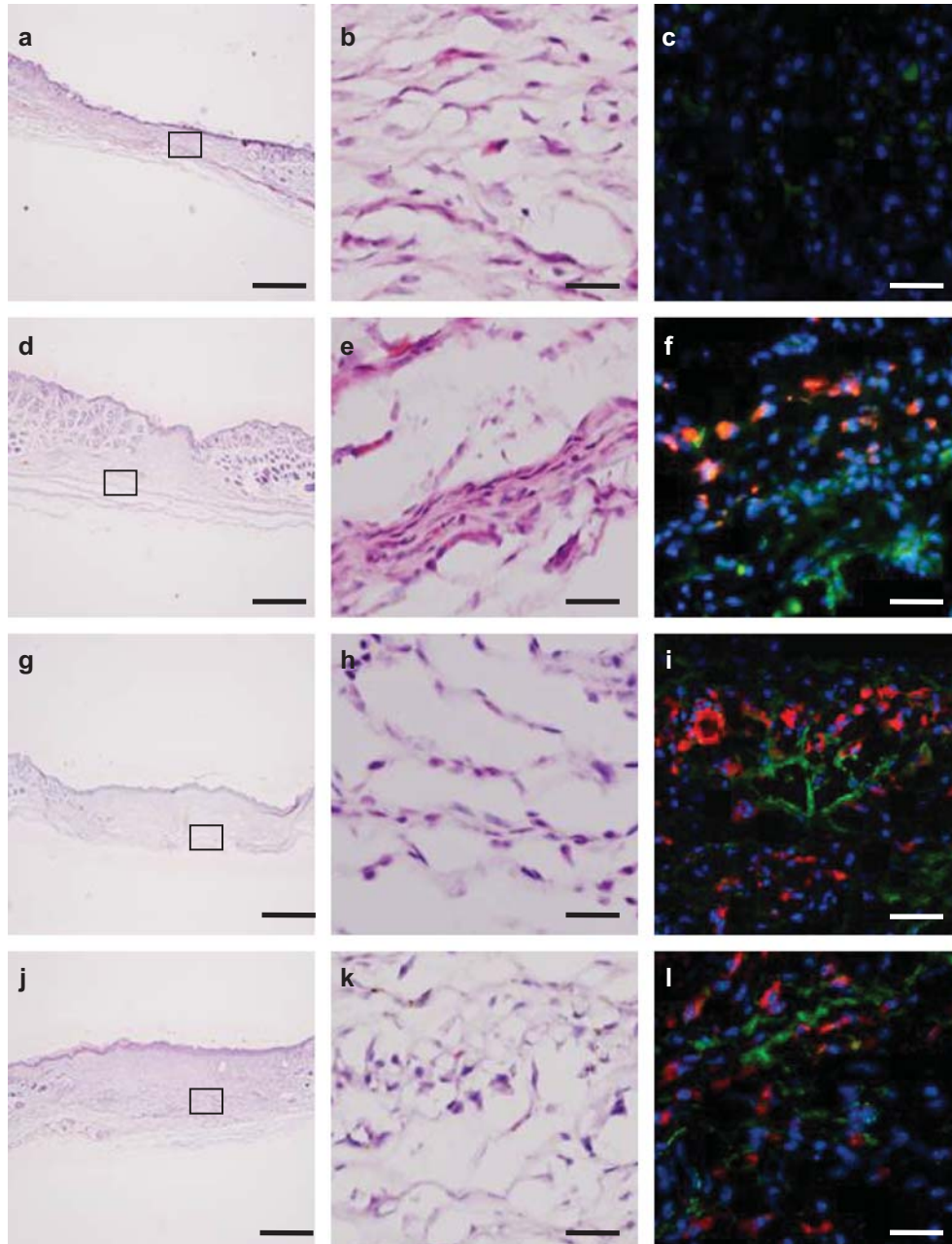


Figure 5. Histologic evaluation of the effect of transplanted cells at day 14. Left and middle panels show hematoxylin and eosin staining, the right panel shows PKH26-labeled cells (red fluorescence) and human HA staining (green fluorescence); images were obtained fluorescence microscopy. DAPI was used to visualize the nuclei (blue fluorescence). Control (a, b, c), hFibro (d, e, f), hMSCs (g, h, i) and SHED (j, k, l). Bars = 30 mm (a, d, g, j) and 25 μ m (b, c, e, f, h, i, k, l).

(Table I). The HA volumes at day 7 and 14 were 1558.41 ± 60.33 ng/mg and 704.17 ± 40.26 ng/mg (control), 2092.75 ± 42.56 ng/mg and 1425.79 ± 56.31 ng/mg (hFibro), 2342.07 ± 188.10 ng/mg and 1653.10 ± 120.84 ng/mg (hMSCs), and 2314.85 ± 164.91 ng/mg and 1644.98 ± 120.70 ng/mg (SHED), respectively. Significantly increased amounts of HA in wounded tissues were observed in hMSCs, SHED and hFibro groups compared with the control group at days 7 and 14 ($P < 0.05$). Moreover the hMSCs and SHED groups showed significantly higher HA levels compared with the hFibro group at days 7 and 14 ($P < 0.05$). There was no statistically significant differences between hMSCs and SHED groups at days 7 and 14 ($P < 0.05$; Table I).

Discussion

Chronic wounds are difficult to heal, and little improvement has been made in preventing the associated morbidity and disability over the past few decades (19). The best available treatment for chronic wounds achieves only a 50% healing rate. The wound healing area in this study was also about 50% in the control group (Figure 3). Therefore innovative treatments to enhance wound healing and regeneration are needed. The major goal of wound-healing biology is to discover how skin can be induced to reconstruct damaged parts more perfectly (20). We have shown that SHED and hMSCs enhance wound healing by promoting re-epithelialization and the relationship with the extracellular matrix, especially HA.

An excisional wound-splinting mouse model was used, which prevents skin contraction and allows wounds to heal through granulation and re-epithelialization (18), to study the effect of therapeutic reagents on wound healing. Our results indicated that wound closure in the control group was significantly delayed compared with hFibro, hMSCs and SHED groups. The implantation of the hMSCs and SHED groups significantly promoted the wound-healing process compared with hFibro and control groups after day 6 (Figures 2 and 3). Consistent with our findings, previous reports have not shown enhancement of

wound healing in allogeneic fibroblast-treated mice and shown that MSCs promotes wound healing (21). In short, the benefit of fibroblasts in wound healing remains controversial (22); fibroblasts have been found to cause increased inflammation and scar formation (10). On the other hand, MSCs have been found to repair epithelium *in vitro* through differentiation (23), and MSCs engrafted in the wound may contribute to cells in the skin epidermis and appendages, thus mediating dermal regeneration (21). A previous study has reported a substantial engraftment of MSCs in day-7 wounds and a rapid reduction of cells in day-14 wounds (24). Our findings are consistent with these results: transplanted MSCs and SHED reduced from 7 to 14 days (Figures 4 and 5). The mechanisms involved with the decline of transplanted cells are not fully understood. With progression of the wound-healing process, cytokines and ECM molecules favorable to SHED and MSCs survival and engraftment decrease (21). The injected cells might come loose by a turnover of the skin, and the quantity of HA in the ECM decrease. A recent study showed that HA levels peaked on day 7 post-wound (25). This study also confirmed HA expression. HA has an important role in keratinocyte proliferation and migration, which implies importance in the re-epithelialization process (26–28), and also reduces collagen deposition, therefore leading to reduced scarring (29).

Treatments using MSCs would be effective, but the number, proliferation and differentiation potential of MSCs decline with increasing age (12). On the other hand, SHED can be obtained without any invasion and could be a substitute for MSCs (14). There has been no prior report regarding the influence on wound healing by local application of SHED. This study has demonstrated that hMSCs and SHED accelerated wound healing compared with hFibro and control groups. As a result, SHED might be a useful cell origin as well as hMSCs.

In conclusion, our results show that SHED significantly promotes wound healing compared with hFibro and control groups. Deciduous teeth, which are considered to be medical waste, could provide novel therapeutic approaches for the treatment of wounds and novel stem-cell sources for wound healing.

Table I. HA volume at days 7 and 14.

	Day 7 (ng/mg)	Day 14 (ng/mg)
control	1558.4 ± 60.33	704.17 ± 40.26
hFibro	2092.75 ± 42.56	1425.79 ± 56.31
hMSCs	2342.07 ± 188.10	1653.10 ± 120.84
SHED	2314.85 ± 164.91	1644.98 ± 120.70

* $P < 0.05$.

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