

ORIGINAL ARTICLE

Bone marrow-derived cells in palatal wound healing

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OBJECTIVE: Myofibroblasts are responsible for contraction and scarring after cleft palate repair. This leads to growth disturbances in the upper jaw. We hypothesized that cells from the bone marrow are recruited to palatal wounds and differentiate into myofibroblasts.

METHODS: We transplanted bone marrow from green fluorescent protein (GFP)-transgenic rats into lethally irradiated wild-type rats. After recovery, experimental wounds were made in the palatal mucoperiosteum, and harvested 2 weeks later. GFP-expressing cells were identified using immunostaining. Myofibroblasts, activated fibroblasts, endothelial cells, and myeloid cells were quantified with specific markers.

RESULTS: After transplantation, $89 \pm 8.9\%$ of mononuclear cells in the blood expressed the GFP and about 50% of adherent cells in the bone marrow. Tissue obtained during initial wounding contained only minor numbers of GFP-positive cells, like adjacent control tissue. Following wound healing, $8.1 \pm 5.1\%$ of all cells in the wound area were positive, and $5.0 \pm 4.0\%$ of the myofibroblasts, which was significantly higher than in adjacent tissue. Similar percentages were found for activated fibroblasts and endothelial cells, but for myeloid cells it was considerably higher ($22 \pm 9\%$).

CONCLUSIONS: Bone marrow-derived cells contribute to palatal wound healing, but are not the main source of myofibroblasts. In small wounds, the local precursor cells are probably sufficient to replenish the defect.

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Introduction

Wound healing consists of three partly overlapping phases; inflammation, tissue formation, and tissue

remodeling (Singer and Clark, 1999). During tissue formation and remodeling, fibroblasts differentiate into myofibroblasts in response to mechanical tension, transforming growth factor-beta 1, and the extra domain-A splice variant of fibronectin (Gabbiani, 2003). Myofibroblasts are responsible for wound contraction and the production of abundant extracellular matrix leading to a scar (Powell *et al*, 1999; van Beurden *et al*, 2005). They typically express alpha-smooth muscle actin (α SMA), which enables them to generate the contractile forces. The highest myofibroblast density in a wound is generally found around 2 weeks postwounding (Singer and Clark, 1999). After wound closure, myofibroblasts and other cells disappear by apoptosis (Desmoulière *et al*, 1997). Ultimately, a rigid scar with a low cell density remains, which may lead to functional and esthetic problems. Scar formation after cleft palate repair impairs maxillary growth (Berkowitz, 1977; Ross, 1987).

Upon injury, local stem cells are activated and start to proliferate to regenerate the lost or damaged tissue (Ghazizadeh and Taichman, 2001; Li *et al*, 2004). In addition, circulating bone marrow-derived cells (BMDCs) may be recruited (Wang *et al*, 2006). These cells include hematopoietic stem cells (HSC), mesenchymal stem cells (MSC), endothelial progenitor cells (Wu *et al*, 2007), and circulating fibrocytes. The latter are a distinct mononuclear subpopulation of fibroblast and myofibroblast precursors with both hematopoietic and mesenchymal markers (Bucala *et al*, 1994; Abe *et al*, 2001; Mori *et al*, 2005; Bucala, 2007). HSCs are known to give rise to all blood cell lineages but possibly also to non-blood cells such as hepatocytes, endothelial cells, smooth muscle cells, and cardiac myocytes (Wu *et al*, 2007). MSCs are far less abundant precursors that can differentiate *in vitro* into non-hematopoietic cells like osteoblasts, chondrocytes, fibroblasts, astrocytes and others (Wu *et al*, 2007). BMDCs are also able to differentiate into several cell types in normal and regenerating tissues *in vivo* such as macrophages, satellite cells, epithelial cells and endothelial cells, but also myofibroblasts (Krause *et al*, 2001; Badiavas *et al*, 2003; Direkze *et al*, 2003; Dreyfus *et al*, 2004; Taylor, 2004). BMDCs also have the capacity to differentiate into oral and craniofacial tissues but, as in other adult

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tissues, local populations of progenitor cells also seem to be present in the lamina propria of the oral mucosa (Maria *et al*, 2007; Stephens and Genever, 2007). However, no studies are available on the contribution of BMDCs to oral mucosa wounds.

We hypothesized that circulating BMDCs are recruited to palatal wounds and differentiate into myofibroblasts. If so, this might offer new opportunities for anti-scarring therapy in cleft palate surgery and other fibrotic conditions, by preventing the recruitment of specific myofibroblast precursors. To investigate the recruitment of myofibroblast precursors originating from the bone marrow, we transplanted bone marrow from green fluorescent protein (GFP)-transgenic rats into wild-type rats. Then, we investigated the contribution of BMDCs to the myofibroblast population after the inflammation phase in experimental wounds in the palate. In addition, we estimated the contribution of BMDCs to other cell populations.

Materials and methods

Animals

Five GFP-transgenic Sprague-Dawley rats were used as donors for the bone marrow transplantation (BMT) (rats provided by Dr M. Okabe and Dr T. Suzuki, Japan SLC, Inc., Shizuoka, Japan). Ten wild-type Sprague-Dawley rats (Janvier, Le Genest, France) were used as recipients. The rats were 6 weeks old at the start of the experiment and kept under sterile housing conditions with free access to food and water. The Board for Animal Experiments of the Radboud University Nijmegen Medical Centre has approved these experiments (RU-DEC 2005-104).

Bone marrow transplantation

The recipient rats received two doses of 5 Gy total body irradiation with an interval of 18 h. Bone marrow from the femurs of the GFP-transgenic donor rats was harvested by flushing with phosphate-buffered saline (PBS) (Invitrogen/GIBCO, San Diego, CA, USA), supplemented with 2% penicillin/streptomycin (Invitrogen/GIBCO, San Diego, CA, USA). After washing, mononuclear cells were isolated by density centrifuga-

tion over Lymphoprep ($\rho = 1.077 \text{ g ml}^{-1}$) (Axis-Shield POC AS, Oslo, Norway), and washed twice in 0.9% NaCl. Subsequently, 2.3×10^8 mononuclear cells per kg rat (0.4 ml) were injected in the tail vein, 3 h after the second irradiation. The weight of the rats was monitored, and only a temporary weight reduction was observed. Five weeks after the BMT, blood was drawn and mononuclear cells were analyzed for GFP by flow cytometry on a FACScan (Becton and Dickinson, Franklin Lake, NJ, USA). Blood from 15 GFP-transgenic rats and seven wild-type rats was analyzed for comparison. The wild-type blood contained no GFP-positive cells. Bone marrow from four transplanted rats was put in culture to analyze GFP expression of mesenchymal precursors using flow cytometry.

Experimental wounding

Seven weeks after the BMT, 4-mm palatal wounds were made (Figure 1) in the mucoperiosteum between the third molars under anesthesia of a mix of fentanyl and fluanisone (Hypnorm, Vetapharma Ltd, Leeds, UK) and midazolam (Dornicum, Deltaselect, Dreieich, Germany). Buprenorphinehydrochloride (Temgesic®, Schering-Plough, Brussels, Belgium) was used postoperatively as an analgesic. Two weeks after wounding, when the inflammation reaction has faded, the rats were killed by CO_2/O_2 inhalation. Wound and adjacent control tissue was harvested with a 5-mm biopsy punch, and cut into two parts (Figure 1). The tissue samples were fixed in 4% paraformaldehyde for 24 h, and embedded in paraffin.

Bone marrow cultures

The femurs from four recipient rats were dissected, washed in 80% ethanol and subsequently in PBS with 4% penicillin/streptomycin and 2% fungizone (all from GIBCO, Paisley, UK). The femurs were flushed with 2% penicillin/streptomycin in PBS and the marrow was resuspended through an injection needle. The cell suspension was then centrifuged at 400 g for 5 min, the pellet was resuspended in culture medium. Cells were plated in non-coated culture flasks in culture medium (LG-DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with penicillin/streptomycin and 10% FBS (Hyclone,

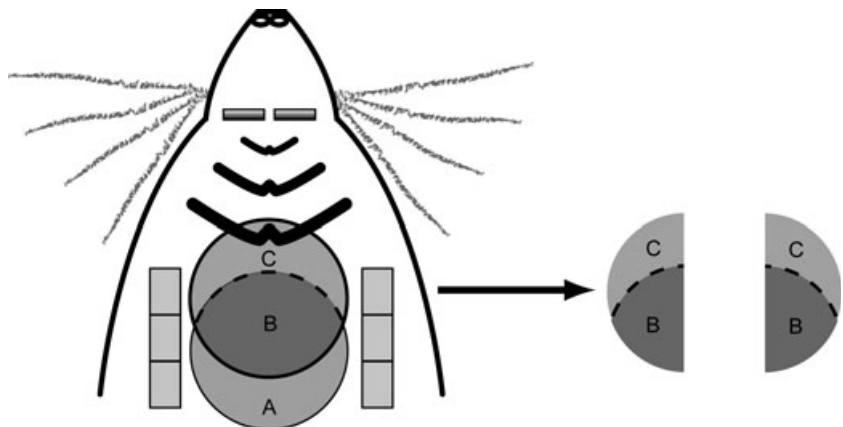


Figure 1 The wound model. Left panel: Initially, a 4-mm ϕ palatal wound was created between the third molars and behind the third ruga (a) Two weeks later, wound (b) and control (c) tissue was harvested. Right panel: The biopsy was cut into two parts and embedded in paraffin

South Logan, UT, USA) and cultured for 9 days. We used FBS batches preselected for their potential to support MSC expansion. The MSC were harvested by trypsinization, and analyzed by flow cytometry.

Histology and immunohistochemistry

Five-micrometer sections were cut and stained with hematoxylin and eosin (H&E) for general tissue survey. The initial biopsy tissue from five rats was also processed. For immunohistochemical staining, three sections per sample (125 μm apart) containing both wound and control tissue were mounted on Superfrost Plus slides (Menzel-Gläser, Braunschweig, Germany). The sections were deparaffinated, rehydrated, and incubated with 3% H_2O_2 . The sections were postfixed with 4% formalin and washed in 0.75 $\mu\text{g ml}^{-1}$ glycine in PBS (PBS-G). Antigens were retrieved with citrate buffer (pH 6.01) at 70°C, followed by incubation in 0.075 g ml^{-1} trypsin (Difco Laboratories, Detroit, MI, USA) in PBS at 37°C. Then, the sections were preincubated with 10% normal donkey serum (NDS) (Chemicon, Temecula, CA, USA) in PBS-G.

All antibodies and the Vectastain ABC Standard alkaline phosphatase mix (ABC-AP) (Vector Laboratories, Burlingame, CA, USA) were diluted in 2% NDS. To detect GFP, the sections were incubated overnight at 4°C with a polyclonal rabbit-anti-GFP antibody (1:300) (Invitrogen/Molecular Probes, Eugene, OR, USA). Subsequently, biotinylated donkey-anti-rabbit (1:500) (Jackson Labs, West Grove, PA, USA) was added. Next, the sections were treated with ABC-AP, and washed with TRIS-HCl (pH 8.2). The Fast Blue substrate (Sigma Chemical Co., St Louis, MO, USA) was freshly prepared, and applied to the sections. The reaction was stopped in demineralized water (Milli-Q pore system, Millipore SA, Molsheim, France), and the sections were washed in PBS and preincubated again for double-staining with the following primary mouse monoclonal antibodies:

- A) Anti αSMA (Sigma Chemical Co.) 1:1600, 1 h at room temperature to detect myofibroblasts
- B) Anti CD-68 (Serotec, DPC, Breda, the Netherlands), 1:100, overnight at 4°C to detect macrophages
- C) Anti heat-shock protein (HSP)-47 (Stressgen, Ann Arbor, MI, USA), 1:24 000, overnight at 4°C to detect activated fibroblasts.
- D) Anti collagen type IV (The Developmental Studies Hybridoma Bank/University of Iowa, Iowa City, IA, USA), 1:300, overnight at 4°C to detect blood vessels.

The primary antibodies were omitted for negative controls, which were always blank. Next goat-anti-mouse-AlexaFluor-594 (1:200, 1 h at room temperature) (Invitrogen/Molecular Probes, Eugene, OR, USA) was added. Finally, the sections were washed and the nuclei were stained with 4',6-diamidino-2-phenylindole (Roche Diagnostics Nederland BV, Almere, the Netherlands). A 1,4-Diazabicyclo[2.2.2]octane solution (DABCO; Sigma Chemical Co.) solution in TRIS buffered glycerin was

used as anti-fading agent. Slides were stored in the dark at 4°C. Photographs were taken on a Carl Zeiss Imager Z.1 system (Carl Zeiss Microimaging GmbH, Jena, Germany). GFP photos were acquired under bright field conditions. The other stainings were photographed with fluorescent settings. The GFP images were inverted and merged with the fluorescent images to reveal co-localization using ImageJ (National Institutes of Health, Bethesda, MD, USA).

Cell counting and statistics

The fraction of GFP-positive mononuclear cells was determined by the FACScan in wild-type blood, GFP-transgenic blood, and recipient blood. In three sections of each tissue sample, αSMA -positive cells and nuclei were counted in the wound and control area within a frame with a width of 50 μm and a depth of 300 μm . GFP-positive and GFP/ αSMA double-positive cells were counted in a larger area of 200 μm wide. The epithelium was excluded from counting. The results were considered statistically significant when a paired one-tailed Student's *t*-test returned a *P*-value < 0.05.

To estimate the fraction of the other bone marrow-derived cell types, three rats with a high number of GFP-positive cells in the wound area were selected. Three tissue sections were used to determine the number of double-positive cells. To estimate the number of GFP-positive endothelial cells, only the cells in the inner layer of the blood vessel walls were counted. No statistics was applied to these results.

Results

Nearly all mononuclear cells in the blood of the GFP-donors and the recipients were GFP-positive ($89.0 \pm 8.9\%$ and $89.2 \pm 4.5\%$, respectively), indicating a successful bone marrow transplantation (Figure 2a). The fraction of GFP-positive adherent cells from the cultured recipient bone marrow was $49 \pm 6.6\%$ (Figure 2b).

All wounds were closed at 2 weeks postwounding. General histology of the samples is shown in Figure 3a. The tissue consists of a multilayered keratinized epithelium overlying the lamina propria. The wound area shows a much higher cell density than the adjacent control tissue. The presence of a ruga further marks the control area.

Figure 3b shows the immunohistochemical stainings for each marker. In the wound area of the samples, much more GFP-positive cells were present than in the control area (first panel). Abundant αSMA -positive were present in the wound area (second panel), but only few of them were GFP-positive. The adjacent control tissue only showed αSMA -positive cells in the blood vessels. Relatively more GFP-positive cells were also CD68-positive in the wound area compared with the control (third panel). Only few double-positive cells were found with the HSP-47 and collagen IV staining (fourth and fifth panel).

The percentage of GFP-positive cells (Figure 4a) was significantly higher in the wound area ($8.1 \pm 5.1\%$)

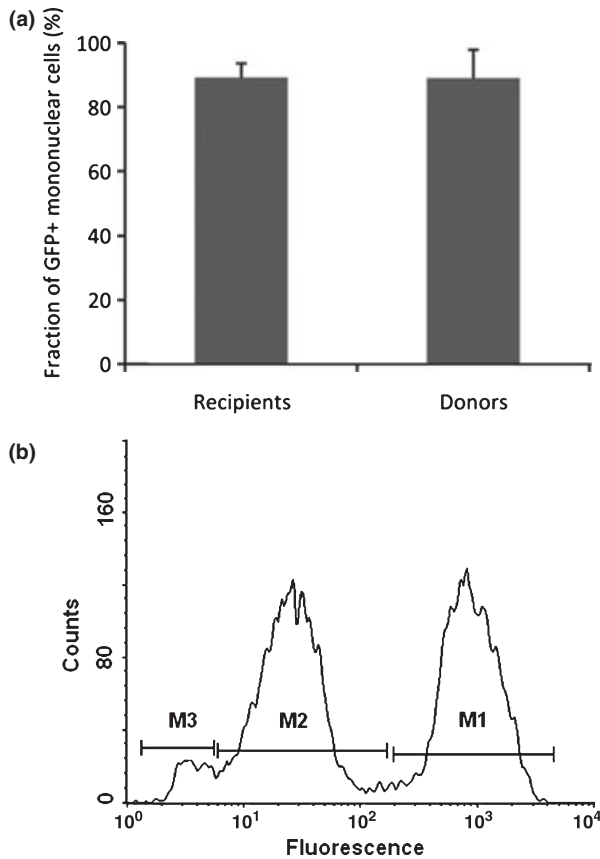


Figure 2 (a) Flow cytometry of blood mononuclear cells 5 weeks after BMT. Recipients and transgenic donors respectively. 89% of the mononuclear blood cells of the recipients and donors was GFP-positive. (b) Flow cytometry graph of a representative bone marrow culture from a recipient rat. On average $49 \pm 6.6\%$ of the cultured cells were GFP-positive ($n = 4$). M1 represents the GFP-positive cells, M2 the GFP-negative cells, and M3 the cell culture debris

than in the control area ($0.7 \pm 0.8\%$, $P = 0.0025$) and the initial biopsy ($1.0 \pm 0.4\%$). In the wound area $46 \pm 24\%$ of all cells were myofibroblasts, but only $4.6 \pm 3.0\%$ of them were GFP-positive (Figure 4b). In the control area, nearly no myofibroblasts were detected ($0.3 \pm 0.8\%$) and none of them were GFP-positive. The fraction of GFP-positive myofibroblasts was significantly higher in the wound area ($P = 0.007$).

Figure 4c shows the percentages of the other cell types. In the wound area, $14 \pm 5.6\%$ of all cells were CD68-positive and $21 \pm 8.2\%$ of these were GFP-positive. The control area contained $7.5 \pm 5.7\%$ CD68-positive cells, of which $9.1 \pm 6.9\%$ were GFP-positive. In the wound area, $57 \pm 13\%$ of all cells were HSP47-positive cells, and $7.3 \pm 3.8\%$ of them were also GFP-positive. In the control area, $35 \pm 12\%$ of all cells were HSP47-positive, and $1.9 \pm 2.0\%$ of them were also GFP-positive. Only $2 \pm 2\%$ of the endothelial cells in the wound area were GFP-positive. In the control area, no GFP-positive endothelial cells were detected. Finally, very few GFP-positive cells were detected in the epithelium of both control and wound areas (Figure 3b).

Discussion

The aim of our study was to investigate whether circulating BMDCs contribute to palatal wound healing. The bone marrow transplantation was very efficient as about 90% of the mononuclear cells in the blood were GFP-positive, which was similar to the percentage in the donor animals. In the remaining cells the transgene is apparently silenced (Torensma and Figdor, 2004). By contrast, only about 50% of the cultured adherent cells from the bone marrow were GFP-positive, which shows that the engraftment of mesenchymal precursors was less efficient. This is also a common observation in human bone marrow transplantation (Rieger *et al*, 2005; Bartsch *et al*, 2009).

We showed that a small percentage (8.1%) of all cells in the wounds was bone marrow derived. Studies on wounded skin show that up to 37% of the cells originates from the bone marrow (Fathke *et al*, 2004; Rea *et al*, 2009). Both studies on skin used larger wound sizes, and it has been suggested that this could be an important factor for the recruitment of BMDCs (Brittan *et al*, 2005; Verstappen *et al*, 2009). In patients with large burn wounds, the number of circulating BMDCs is up-regulated and correlates with wound size (Yang *et al*, 2002; Mansilla *et al*, 2006). The number of bone marrow-derived cells recruited to burn wounds also increases with wound size (Rea *et al*, 2009). This suggests that the limited wound size in our model restricts the contribution of BMDCs to palatal wounds. In small wounds, the available local stem cells might be sufficient to replenish the wound. As mesenchymal precursors are engrafted less efficiently, the actual contribution of precursors from this population might be higher in this type of studies.

We also found a small percentage (4.6%) of bone marrow-derived myofibroblasts in the wound tissue. This population largely overlaps with the HSP47-positive population, which contains both activated fibroblasts and myofibroblasts (Kuroda and Tajima, 2004). In other wounded or chemically damaged tissues, the number of bone marrow-derived myofibroblasts is generally higher (4–67%) (Direkze *et al*, 2003; Brittan *et al*, 2005; Mori *et al*, 2005). (Myo)fibroblasts originate from mesenchymal precursors but they can also differentiate from circulating fibrocytes, which have both mesenchymal and hematopoietic properties (Bucala *et al*, 1994; Mori *et al*, 2005). In burn wounds, a positive correlation was also found between wound size and the number of circulating fibrocytes (Mansilla *et al*, 2006), and their subsequent recruitment to the wound (Yang *et al*, 2005). In small and relatively fast healing wounds (Von den Hoff *et al*, 2006), only a low fraction of bone marrow-derived myofibroblasts might therefore be found.

By contrast, a higher fraction of macrophages and other myeloid cells was derived from the bone marrow. Macrophages differentiate from circulating monocytes (Dipietro *et al*, 2001). A high percentage of the blood mononuclear cells in the recipient rats was derived from donor bone marrow. In addition, macrophage

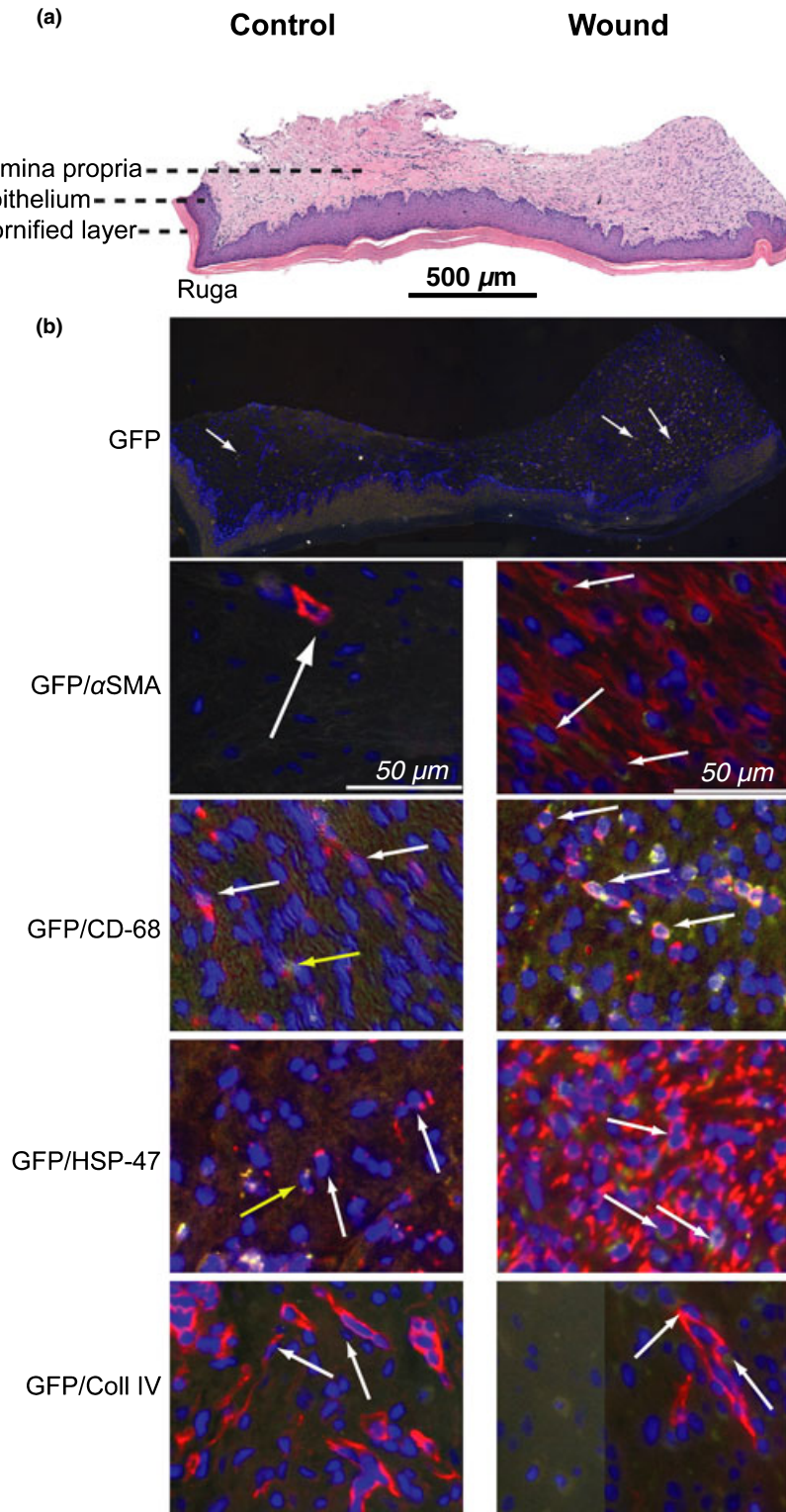


Figure 3 Histology and immunohistochemistry of palatal wounds. (a) H&E staining. The wound area shows a higher cell density than the control area. (b) Staining with 4',6-diamidino-2-phenylindole (blue, nuclei), and antibodies against GFP (green), and cell type markers (red). The first merged picture shows the GFP distribution in the wound and control area. The next merged pictures show GFP co-localization with: α SMA, CD-68, HSP-47, and with GFP-positive cells at the inner side of blood vessels stained by collagen type IV. The latter cells were considered as endothelial cells. Arrows indicate the GFP-positive cells with the co-localized cell type marker in the wound area. The yellow arrows in the control area indicate double-positive cells

recruitment may be promoted by other BMDCs in the wound (Chen *et al*, 2008). However, the majority of myeloid cells in our study were not donor derived and thus originates from local tissue precursors.

Only few epithelial cells were bone marrow-derived, both in the wound area and the adjacent tissue. In

studies on skin, the percentage of bone marrow-derived epithelial cells ranges from 0.0001% to 11% (Borue *et al*, 2004; Brittan *et al*, 2005; Fan *et al*, 2006). However, these studies do not show a positive correlation between wound size and the number of bone marrow-derived epithelial cells. These and our data therefore

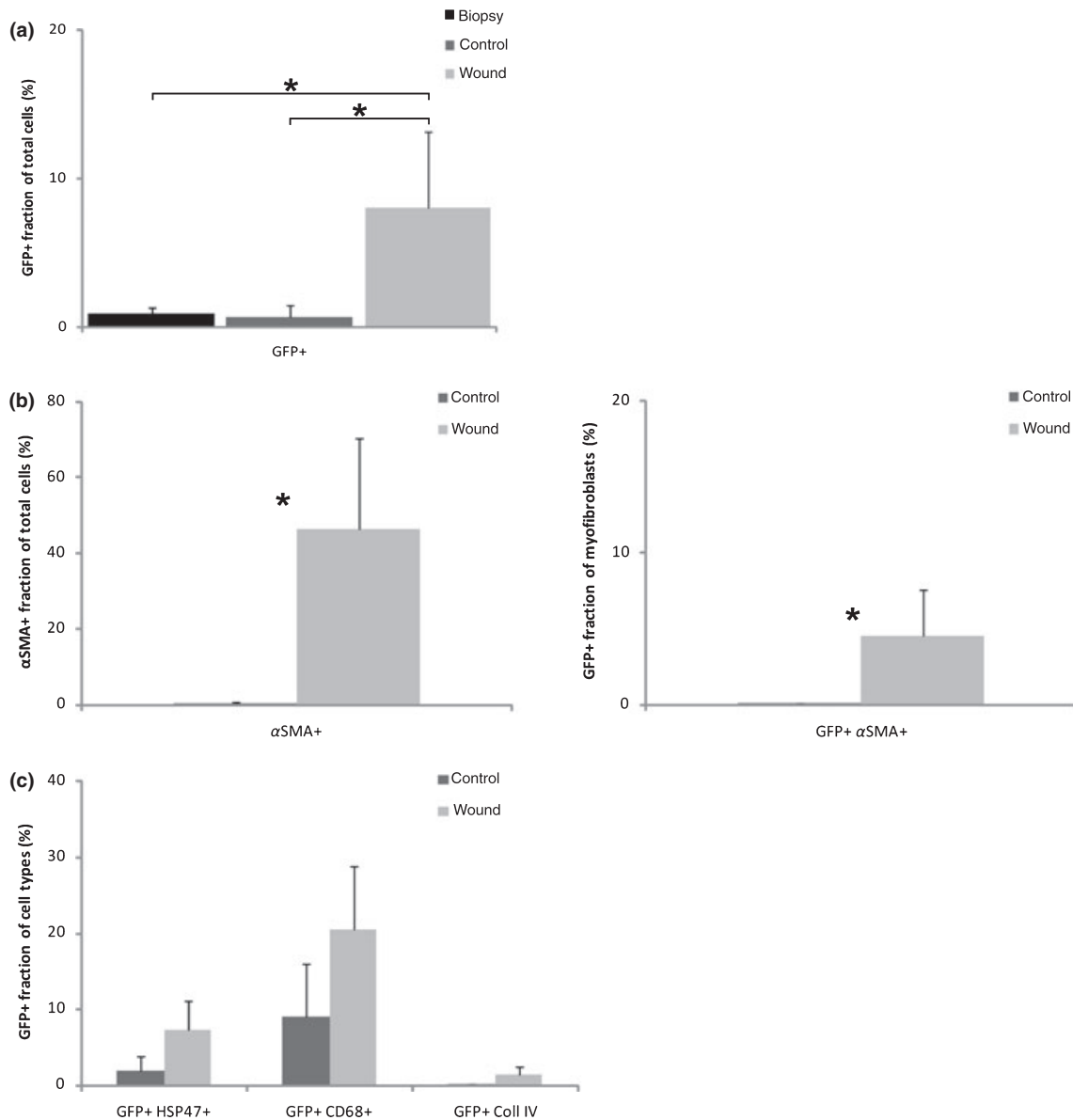


Figure 4 The fractions of stained cells in the tissue obtained from experimental wounding, control tissue and the wounds. (a) GFP-positive cells. (b) Myofibroblasts (left) and GFP-positive myofibroblasts (right). (c) GFP-positive macrophages (CD-68), activated fibroblasts (HSP-47), and endothelial-like cells (Coll IV). The asterisk indicates a statistically significant difference ($P < 0.05$)

strongly suggest that the palatal wounds re-epithelialize mainly from the surrounding local stem cells.

In conclusion, only a small fraction of the myofibroblast population in palatal wound healing is derived from circulating BMDCs. The recruitment of BMDCs is probably limited by the small wound size. The vast majority of wound myofibroblasts in this model may be derived from local fibroblasts invading from the adjacent tissue.

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