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The role of bone marrow-derived cells in bone fracture repair in a green fluorescent protein chimeric mouse model

Kazuhiro Taguchi [a,b,*], Rei Ogawa [a,c], Makoto Migita [a,d], Hideki Hanawa [c], Hiromoto Ito [b], Hideo Orimo [a]

[a] *Department of Biochemistry and Molecular Biology, Nippon Medical School, Tokyo, Japan*
[b] *Department of Orthopedic Surgery, Nippon Medical School, Tokyo, Japan*
[c] *Department of Plastic and Reconstructive Surgery, Nippon Medical School, Tokyo, Japan*
[d] *Department of Pediatrics, Nippon Medical School, Tokyo, Japan*

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Abstract

We investigated the role of bone marrow cells in bone fracture repair using green fluorescent protein (GFP) chimeric model mice. First, the chimeric model mice were created: bone marrow cells from GFP-transgenic C57BL/6 mice were injected into the tail veins of recipient wild-type C57BL/6 mice that had been irradiated with a lethal dose of 10 Gy from a cesium source. Next, bone fracture models were created from these mice: closed transverse fractures of the left femur were produced using a specially designed device. One, three, and five weeks later, fracture lesions were extirpated for histological and immunohistochemical analyses. In the specimens collected 3 and 5 weeks after operation, we confirmed calluses showing intramembranous ossification peripheral to the fracture site. The calluses consisted of GFP- and osteocalcin-positive cells at the same site, although the femur consisted of only osteocalcin-positive cells. We suggest that bone marrow cells migrated outside of the bone marrow and differentiated into osteoblasts to make up the calluses.

Keywords: Fracture; Green fluorescence protein; Bone marrow; Osteoblast

Fracture healing is a complex physiologic process that involves the coordinated participation of several cell types. In a reproducible model of experimental fracture healing in the rat, Thomas and Einhorn [1] demonstrated that remodeling of the woven bone formed by intramembranous and endochondral ossification continues for several weeks. The bone formed by intramembranous ossification develops far from the site of the fracture, and the process results in the formation of hard calluses. The bone forms directly without the prior formation of cartilage. A layer of osteoblasts appears on the surface of the developing bone, and their activity increases the thickness of the bone. Successive layers of matrix are added by apposition, and the osteoblasts, which initially lie on the surface of the bone, subsequently become included within it as osteocytes. The bone formed by endochondral ossification is formed adjacent to the fracture site, involves the development of a cartilage anlage which becomes calcified, and is replaced by bone. To understand the different processes that contribute to the healing of a fracture, it is helpful to view these healing events in terms of four distinct responses. These four responses include those that take place in the bone marrow, cortex, periosteum, and external soft tissue. However, few investigators have studied the response and differentiation of bone marrow to a fracture in vivo. We have focused in particular on the pluripotency of mesenchymal stem cells and have

* Corresponding author. Fax: +81 3 5685 1796.
E-mail address: s3061@nms.ac.jp (K. Taguchi).

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established that these cells can differentiate into osteoblasts both in vitro and in vivo [2,3]. In this study, we investigated the relationship between bone marrow cells and bone fracture repair.

A chimeric mouse model stably reconstituted with green fluorescent protein (GFP)-marked bone marrow cells was used [4]. In a previous study using GFP chimeric models, we succeeded in observing the migration of GFP-marked bone marrow cells into ischemic heart muscle lesions, cerebral infarct lesions, and nephritis lesions [5–7]. In this study, we extended our approach to investigate bone fracture lesions.

Materials and methods

Creation of GFP chimeric mouse model. Twenty-five 8-week-old male C57BL/6 mice and 25 GFP transgenic C57BL/6 mice were used for this study. These were divided into three groups to investigate conditions 1, 3, and 5 weeks after fracture. The GFP transgenic mice, in which EGFP expression was under the control of a cytomegalovirus enhancer and a chicken β-actin promoter, were generously provided by Dr. M. Okabe (Osaka University, Osaka, Japan) [4]. From these transgenic mice, we created GFP chimeric mice. In brief, bone marrow transplantation was carried out with 8-week-old C57BL/6 GFP mice as the donors and 8-week-old C57BL/6 mice as the recipients. Donor bone marrow cells (4×10^6) were obtained from the femurs and tibias of the GFP mice and injected into the tail veins of the irradiated ($5\ \mathrm{Gy} \times 2$) non-transgenic recipient mice. All the experiments were conducted in accordance with the rules and regulations of the Animal Ethics Committee of Nippon Medical School.

Fluorescence-activated cell scanning (FACS) analysis. To determine the reconstitution rate of bone marrow cells in the irradiated mice, we collected bone marrow cells 6 weeks after bone marrow transplantation and analyzed them with a fluorescence-activated cell sorter (FACS Calibur; Becton–Dickinson, Franklin Lakes, NJ, USA).

Creation of bone fracture mouse model. The GFP chimeric mice were anesthetized with pentobarbital sodium at 0.1 mg/100 g, and closed transverse fractures of the left femur were produced with a specially designed device (Tact, Tokyo, Japan) based on the fracture apparatus of Bonnarens and Einhorn [8–13]. A midline anterior knee incision was made, and a 23G-gauge needle (0.63 mm in diameter) was inserted into the left femur. After closing the knee joint, the middiaphysis of the pinned femur was fractured by applying a force perpendicular to the long axis of the bone. The force was generated by dropping a 300-g weight from a height of 5 cm to produce a standardized closed diaphyseal fracture of the left femur. Radiographs were obtained after surgery. After awakening from the anesthesia, the mice were permitted unrestricted, full weight-bearing activity.

Evaluation of fracture lesions. After 1, 3, and 5 weeks of normal activity, X-ray examinations of the mice were performed and the femurs were extirpated under anesthetization with pentobarbital sodium at 0.1 mg/100 g for evaluation. The femurs were fixed and decalcified with 4%(v/w) paraformaldehyde (Sigma, St. Louis, MO, USA) and 7%(v/w) formic acid for 4 days, after which paraffin sections were made [14]. Paraffin-embedded samples were sectioned at 4 μm thickness with a microtome. They were floated in water at 46 °C and placed on microscope slides. They were then incubated at 37 °C for 24 h. To evaluate the specimens histopathologically and immunohistopathologically, hematoxylin–eosin staining and anti-GFP (dilution of 1:500) (Medical and Biological Laboratories, Nagoya, Japan) and anti-osteocalcin (1:1000) (Takara, Kyoto, Japan) immunostainings were performed. In each staining, the sections were dewaxed in xylenes and dehydrated in an ethanol bath. For hematoxylin–eosin (HE) staining, a 20-min hematoxylin stain and 5-min eosin stain were performed. Immunohistopathological staining was done with a VECTASTAIN ABC kit (Funakoshi, Tokyo, Japan), and counterstaining was carried out with hematoxylin. All sections were then covered with permount and cover-slipped.

Results

Bone marrow cells from the GFP chimeric mice were analyzed 6 weeks after bone marrow transplantation with a fluorescence-activated cell sorter (FACS caliber). In all of the 15 mice, 99% of the bone marrow mononuclear cells were GFP-positive, and almost all of the blood cells appeared to have been reconstituted (Fig. 1). Bone fracture mouse models were created from them, and their bone marrow cells were confirmed to be GFP-positive by anti-GFP immunostaining (Fig. 2). The bone, muscle, and subcutaneous tissue cells were GFP negative. Differences between the GFP transgenic mice and the GFP chimeric mice were clearly observed in the specimens. GFP expression in the bones of the GFP transgenic mice was strong at the edges of the bones and weak in the center.

After 1, 3, and 5 weeks of normal activity, X-ray examinations of the bone fracture model mice were performed (Fig. 3). Callus formation was observed after 3 and 5 weeks, with much larger calluses at 5 weeks than at 3.

In HE stained paraffin sections made 1 week after fracture of the femur, neither synosteosis nor callus formation was observed (Fig. 4A). In HE stained paraffin sections made 3 weeks after fracture, woven bone showing early intramembranous ossification was clearly observed peripheral to the fracture site (Fig. 4C). In the anti-GFP immunostaining sections, differential expressions between the femurs and regenerated calluses were clear (Fig. 4D). In addition, GFP expression on the femur side of the calluses was much stronger than on the other side. The stained pattern of the calluses strongly resembled that of the bone in the GFP transgenic mice. In HE stained paraffin sections made 5 weeks after fracture, the calluses were composed predominantly of woven bone (Fig. 4E). Mature bone was formed by intramembranous ossification. In the specimens taken 5 weeks after fracture, GFP expression was higher than in those taken at 3 weeks (Fig. 4F). In the anti-GFP immunostaining sections, the differential expressions between the femurs and regenerated calluses were clearer than in those taken at 3 weeks. The stained pattern of the calluses also strongly resembled that of the bone in the GFP transgenic mice. GFP expression on the femur side of the calluses was much stronger than on the other side. In addition, many inflammatory cells were collected around the fracture lesion at 3 weeks and

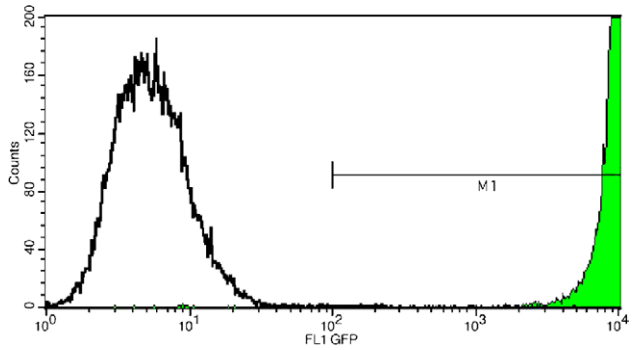

Fig. 1. FACS analysis of GFP chimeric mice. Bone marrow cells from the GFP chimeric mice were analyzed with a fluorescence-activated cell sorter (FACS caliber) 6 weeks after bone marrow transplantation. In all mice, 99% of bone marrow mononuclear cells were GFP-positive, and it appeared that almost all of the blood cells had been reconstituted.

new bone marrow was formed at 5 weeks. In the anti-GFP immunostaining sections, these cells were also GFP-positive.

Woven bone was made up of GFP- and osteocalcin-positive cells at the same site (Fig. 5). In the anti-osteocalcin immunostaining sections, osteocalcin-positive cells were observed in the calluses and femurs, but not in the bone marrow. Moreover, in the anti-GFP immunostaining sections, GFP-positive cells were observed in the calluses, but not in the femurs.

Discussion

It has been pointed out that even if osteogenic cells at the site of a fracture are working at full capacity, they will not heal the defect if too few cells are present locally nor will any drugs directed at enhancing bone formation be effective since maximal osteogenesis per cell is already occurring [11,12]. In considering the clinical therapies for bone fractures, those using pluripotent cells may be the best for minimally invasive yet efficacious treatment. Accordingly, our studies of tissue engineering and gene therapy have focused on

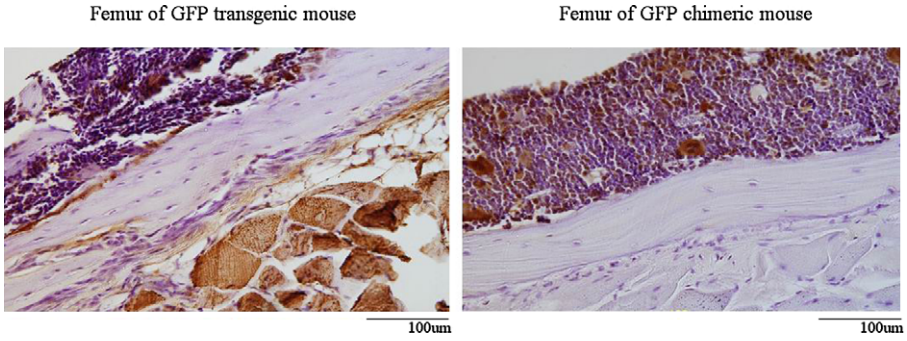

Fig. 2. Anti-GFP immunostaining: comparison of GFP transgenic and chimeric mice. Anti-GFP immunostainings of specimens from GFP transgenic and chimeric mice. In the specimens from GFP chimeric mice, the bone marrow cells were confirmed to be GFP-positive; the bone, muscle, and subcutaneous tissue cells were GFP-negative. GFP expression in the bones of the GFP transgenic mice was strong at the edges of the bones and weak in the center.

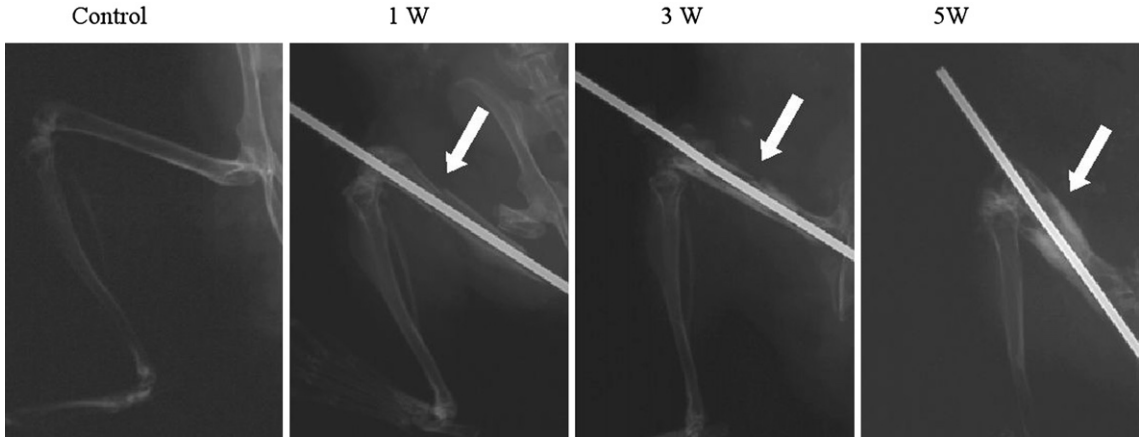

Fig. 3. Time course of X-ray study. X-ray examinations were performed 1, 3, and 5 weeks after fracture. Callus formation was observed after 3 and 5 weeks, with much larger calluses at 5 weeks than at 3. Arrows indicate the fracture site.

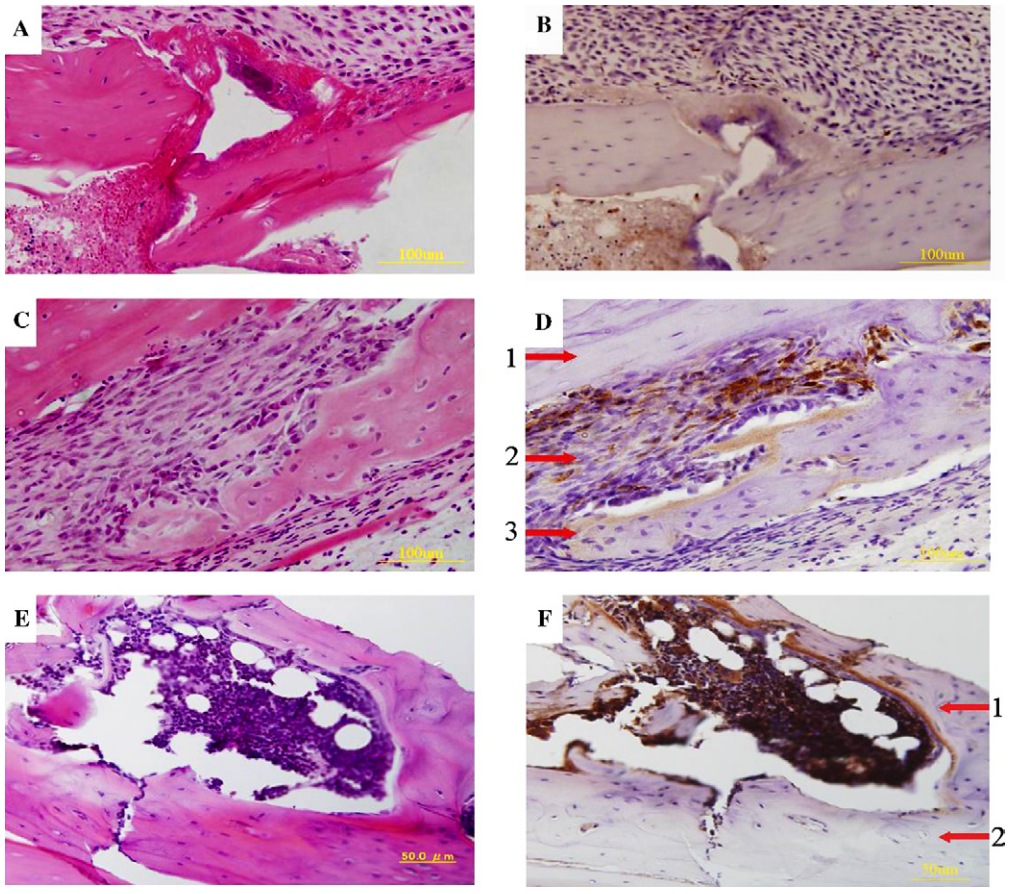

Fig. 4. HE staining and anti-GFP immunostaining of a mouse fracture callus at 1 week (A,B), 3 weeks (C,D), and 5 weeks (E,F). Arrow D-1 indicates the femur; D-2: inflammatory cells; and D-3: a callus. Arrow F-1 indicates a callus; F-2 the femur. In the HE stained paraffin sections 1 week after femur fracture, neither synosteosis nor callus formation was observed. In the HE stained paraffin sections 3 weeks after femur fracture, a callus showing early intramembranous ossification was clearly observed. Moreover, many inflammatory cells had collected around the fracture lesion. In the anti-GFP immunostaining sections, these cells were GFP-positive (arrow D-2), and the cells that made up the calluses were stained positively (arrow D-3). In the HE stained paraffin sections 5 weeks after femur fracture, a callus was clearly observed. Mature bone was formed by intramembranous ossification. Bone marrow was formed between the callus and femur. In the anti-GFP immunostaining sections, the cells that made up the callus were stained positively (arrow F-1), and GFP expression was higher than at 3 weeks.

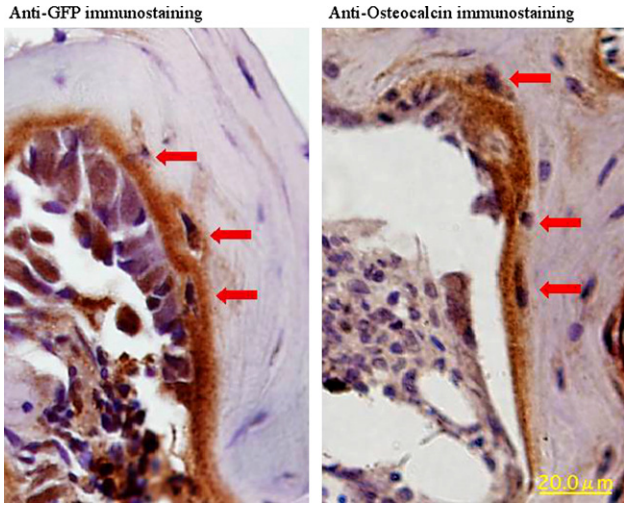

Fig. 5. Anti-GFP and anti-osteocalcin immunostaining of a mouse femur at 5 weeks. The osteoblasts that made up the calluses (osteocalcin-positive cells) were GFP-positive at the same site, and these cells were considered to have originated from bone.

the development of cell therapies for bone fracture repair [2,3]. We have already confirmed that mesenchymal stem cells harvested from bone marrow or adipose tissue can be differentiated into osteoblasts through the use of various kinds of osteogenic media, scaffolds for cell seeding, and gene transduction techniques both in vitro and in vivo [2,3]. In this study, we focused on bone fracture repair by the migration of bone marrow cells in vivo. If bone marrow cells are closely related to bone fracture repair, we will be able to develop cell therapies by reinforcing the migration and differentiation capacities of bone marrow cells.

This study was the first to use a GFP chimeric mouse model for bone fracture repair. The model mice were stably reconstituted with GFP-marked bone marrow cells. In a past study using GFP chimeric models, we succeeded in observing the migration of GFP-marked bone marrow cells into ischemic heart muscle

lesions, cerebral infarct lesions, and nephritis lesions [5–7]. In this study, we extended our approach to investigate bone fracture lesions. In all of the GFP chimeric mice, 99% of both the peripheral and bone marrow mononuclear cells were GFP-positive. Bone fracture models were created using the GFP chimeric model mice. It has to be noted, however, that this model does not perfectly simulate remodeling of fracture calluses in humans, because mouse bone lacks the haversian systems characteristic of most other mammals [11].

GFP expression in the bones was strong at the edges and weak in the center (Fig. 2). The stained pattern of the calluses strongly resembled that of the bone in the GFP transgenic mice (Fig. 4). Through the activity of the osteoblasts the bone increases in thickness. Successive layers of matrix are added by apposition, and the osteoblasts, which initially lie on the surface of bone, subsequently become included within it as osteocytes [15]. We suggest that the existence of osteocytes and increased matrix in the center of the bone makes GFP expression lower in the center and higher at the edge.

In HE stained paraffin sections, no callus formation was observed after 1 week, but immature bone was observed after 3 weeks and mature bone after 5 weeks. GFP expression was strong on the femur side of the calluses.

We performed anti-GFP and anti-osteocalcin immunostaining with paraffin sections. Because bone is rigid, it is difficult to make frozen tissue sections and perform double staining. The osteoblasts that made up the calluses (osteocalcin-positive cells) were GFP-positive at the same site (Fig. 5). In the anti-osteocalcin immunostaining sections, osteocalcin-positive cells were observed in the calluses and femurs, but not in the bone marrow. In addition, in the anti-GFP immunostaining sections, GFP-positive cells were observed in the calluses, but not in the femurs. Moreover, GFP/osteocalcin expression on the femur side of the calluses was much stronger than on the other side. We concluded that activated osteoblasts existed on the femur side of the calluses and mature osteocytes on the other side [16,17]. However, because no osteoblasts were observed in the bone marrow, we speculated that bone marrow cells such as mesenchymal stem cells or osteoblast progenitor cells migrated to the fracture lesion and formed calluses to repair the fracture from the femur side to the other side, concluding that the osteoblasts (osteocalcin-positive cells) making up the calluses were derived from bone marrow.

In other studies using various mouse models of disease, we suggested the following two roles for bone marrow cells [5–7]:

(A) Bone marrow cells are directly related to tissue regeneration (in studies using ischemic heart muscles from mice, 'bone marrow cells directly differentiated into heart muscle cells).
(B) Bone marrow cells are indirectly related to tissue regeneration (in studies using cerebral infarct models and nephritis models, bone marrow cells infiltrated the disease lesions, and these cells were considered to secrete various cytokines related to tissue repair).

In the present study, bone marrow cells may have directly differentiated into the osteoblasts that made up the calluses, so we suggest that bone marrow cells play a role in bone fracture repair as in (A) above.

Bone marrow cell homing has been studied for over 30 years [11]. It has been defined as the ability of stem cells "to seek marrow stroma selectively, to interest with it and subsequently to lodge within it to initiate hematopoiesis." Homing is central to the process of bone marrow transplantation [18]. Bone marrow cells are now considered to include not only hematopoietic stem cells but also mesenchymal stem cells; accordingly, the role of each should be considered separately [19]. We suggest from our study that it is the mesenchymal cells in the bone marrow that are closely related to bone fracture repair.

In conclusion, we have provided direct evidence of the activation and distribution of bone marrow cells in bone fracture lesions. Our findings may allow the design of an appropriate cell therapy and gene-delivery system whereby exogenous mesenchymal pluripotent cells are delivered to bone fracture lesions.

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