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The effect of fresh bone marrow cells on reconstruction of mouse calvarial defect combined with calvarial osteoprogenitor cells and collagen/apatite scaffold

Xiaohua Yu¹, Liping Wang², Fei Peng¹, Xi Jiang², Zengmin Xia¹, Jianping Huang², David Rowe^{2,*}, and Mei Wei^{1,*}

¹Department of Chemical, Materials, and Biomolecular Engineering, University of Connecticut, Storrs, CT 06269

²Department of Reconstructive Sciences, School of Dental Medicine, University of Connecticut Health Center, Farmington, CT 06030, USA

Abstract

Fresh bone marrow cells have already exhibited its advantages as osteogenic donor cells, but the combination between fresh bone marrow cells and other donor cells utilized for bone healing has not been fully explored. To highlight the impact of fresh bone marrow cells on scaffold-based bone regeneration, single or a combination of calvarial osteoprogenitor cells (OPC) and bone marrow cells (BMC) were used as donor cells combined with collagen/apatite scaffold for calvarial defect healing. The host and donor contributions to bone formation were assessed using histological and GFP imaging analysis. Although the amount of new bone formed by different cell sources did not show significant differences, the origin of the bone formation in the defects mainly depended on the types of donor cells employed: when only calvarial OPC were used as donor cells, a donor-derived bone healing instead of host-derived bone ingrowth was observed; when only fresh BMC were loaded, the host bone could grow into the defect along the lamellar structure of the scaffolds, but the amount of new bone formed was significantly lower than the defect loaded with calvarial OPC only. The combination of calvarial OPC and fresh BMC had similar amount of new bone formation as the group loaded with calvarial osteoprogenitors alone, but did not induce any host-derived bone formation. These results provide compelling evidence of the importance of fresh BMC to induce host-implant integration in bone tissue engineering.

Keywords

Bone tissue engineering; Host integration; Osteoblasts; Fresh bone marrow cells; Green fluorescence protein

*Corresponding authors: Mei Wei, 97 North Eagleville Road, U-3136, Storrs, CT, 06269, Tel: 1-860-486-9253, Fax: 1-860-486-4745, m.wei@ims.uconn.edu; David W Rowe, 263 Farmington Avenue, Farmington, CT 06030, Phone: 860-679-2324, Fax: 860-679-2910, rowe@neuron.uchc.edu.

1. Introduction

There are more than 1 million cases of skeletal defects a year in the US that requires bone-grafting operation to avoid developing into delayed unions or nonunions (Meinel et al. 2004). Unfortunately, large skeletal deficiency repair still remains a challenge in the clinic due to the limited supply of autograft tissues and potential pathogen transfer and immune rejection from allograft (Rezwan et al. 2006). None of the approaches proposed so far has proven to be ideal. Recently, tissue engineering approaches combining cells with appropriate scaffolds and osteogenic agents to regenerate bone has demonstrated to be a promising method to address this clinical problem (Langer and Vacanti 1993). The regeneration of functional bone using tissue engineering strategy requires an adequate source of healthy expandable cells, a desirable scaffold, tissue-stimulating compounds, and an efficient method to deliver these molecules to targeted tissues (Tu et al. 2009).

As one of the three key components for bone tissue engineering, cell source plays a critical role in achieving successful bone regeneration. Qualified cell source should be easily expandable and non-immunogenic, and have a protein pattern similar to the tissue to be regenerated (Heath 2000). Mesenchymal stem cell (MSC) has been proven to be the most promising cell line to regenerate new bone among the cells that have been explored (Bruder et al. 1998; Meinel et al. 2004; Kim et al. 2007; Na et al. 2007; Augst et al. 2008; Arthur et al. 2009; Tu et al. 2009). Besides, the capacity for extensive replication without differentiation makes MSC an ideal cell resource for bone repair (Bruder and Fox 1999). Calvarial osteoprogenitor cell (OPC) is one of the cell populations that can be derived from MSCs (Kalajzic et al. 2008). It has been shown that implantation of OPCs could result in new bone formation in segmental mandibular defects (Schliephake et al. 2001).

Fresh bone marrow cell (BMC) is another practical cell source for bone regeneration (Ohgushi et al. 1989; Den Boer et al. 2003). Bone marrow provides niches for both hematopoietic and mesenchymal stem cells. The MSCs in bone marrow have the potential to induce effective bone regeneration. Meanwhile, hematopoietic cells have been reported to contribute to inflammation and bone resorbing cells such as osteoclasts at the early stage of bone healing (Colnot et al. 2006). Fresh BMC is of our particular interest because bone marrow aspirate can be obtained rapidly under emergent situations without further expansion *in vitro* (Block 2005). In comparison, most other cell sources, such as MSC, adipose-derived stem cell and embryonic stem cell, require a long expansion time before transplantation. Taking into account of this consideration, BMC appears to be a superior cell source for tissue engineering due to its immediate availability. As such, the effect of BMC combined with other cell sources on bone repairing still needs to be further explored in order to take full advantage of fresh BMC.

The importance of green fluorescent protein (GFP) reporter construct for evaluating implanted cell behavior in tissue engineering has been demonstrated by various investigators (Wang et al. 2008; Aline Dumas et al. 2009). The advantages of using GFP markers in tissue engineering studies can be summarized as: easily traced, no additional detection techniques required except for fluorescence microscopy, and long-lasting fluorescence signals (Aline Dumas et al. 2009). A series of transgenic mice harboring GFP reporters that mark different

levels of osteoprogenitor lineage differentiation have been developed successfully in Dr. Rowe's lab (Kalajzic et al. 2002; Kalajzic et al. 2005). The pOBCol3.6GFP transgene is activated at an early stage of preosteoblast differentiation and continues being expressed strongly in osteoblasts lining on new bone surfaces (Wang et al. 2005a). Additionally, a frozen sectioning technique has been developed to well maintain the GFP signals in histological sections (Jiang et al. 2005). Therefore, the development of transgenic mice harboring type I collagen GFP reporters and the ability to reserve the fluorescent signal during histological processing make it possible to use transgenic mice to evaluate host/donor cell behavior during cell-based bone healing.

In this study, we evaluated new bone formation in collagen/apatite scaffold loaded with different donor cell combinations. The aim of the study is two-fold. First, the effect of fresh BMCs on bone defect reconstruction in collagen/apatite scaffolds with/without the presence of OPCs was evaluated. Second, the host/donor cell contribution during new bone formation was differentiated using different GFP reporters.

2. Materials and methods

2.1 Collagen-apatite scaffold preparation and characterization

Collagen was extracted from rat tail tendon, as described elsewhere (Rajan et al. 2006). Briefly, collagen fibers in rat tail tendon were cut after the skin was exposed and soaked in a 0.02 M acetic acid solution. The collagen solution was then kept stirring at 40°C for at least 48 h until collagen fibers were fully dissolved. The viscous solution obtained was centrifuged at 10⁴ rpm at 40°C for 15 min. After centrifugation, the supernatant was collected and stocked for scaffold preparation. A collagen solution containing modified simulated body fluid (m-SBF) (Qu and Wei 2008; Yu et al. 2009), namely, Collagen/m-SBF, was initiated by adding 33 mL distilled water to 67 mL collagen solution before adding reagents for m-SBF. Then the following chemicals at reagent grade were added to the collagen solution at the following order and concentrations: 6.0 mM NaCl, 3.0 mM K₂HPO₄·3H₂O, 3.0 mM MgCl₂·6H₂O, 50 mM HEPES, 8.0 mM CaCl₂ and 18 mM NaHCO₃. The pH of the collagen/m-SBF solution was adjusted to 7.0 by 5 M NaOH solution. The resulting solution was then maintained in a water bath at 42°C for 24 h. During the soaking, nano collagen fibers and nano HA particles were co-precipitated, which was subsequently centrifuged at 10⁴ rpm for 15 min at 4°C. The supernatant was then removed and the precipitates were rinsed with distilled water. The precipitates obtained were freeze-dried for 48-72 h to form a collagen-apatite scaffold. The scaffold was further cross-linked using 2% EDC at 40°C for 48 h and then rinsed with distilled water for 3 times. The 3-D structure of the collagen/apatite scaffold was analyzed using cone beam X-ray computed microtomography (µCT40, Scanco Medical AG, Switzerland). Three-dimensional images were reconstructed using standard convolution back-projection algorithms with Shepp and Logan filtering. To examine the morphology of the collagen/apatite scaffold, the scaffold was then sputter-coated with gold palladium and observed using field emission scanning electron microscopy (FESEM, LEO/Zeiss DSM 982). All scaffolds were cut with a dermal punch to a disc with a diameter of 3.5 mm and a thickness of 1 mm. Prior to animal test, the scaffolds were sterilized in 70% high purity ethanol for 10 min.

2.2 Transgenic mice

The pOBCol3.6GFPtpz and pOBCol3.6GFPcyan transgenic mice described above were maintained as homozygous breeders on a CD1 background. The transgenes remained stable and consistent in their expression for multiple generations.

2.3 Cell isolation and expansion

Calvarial OPCs were derived from pOBCol3.6GFPcyan transgenic mice. Neonatal calvarial cells were isolated from 4-6 day-old mice using a modified sequential digestion method described by Wang et al. (Wang et al. 2005b). Briefly, after removal of sutures and adherent mesenchymal tissues, transgenic mouse calvariae was subjected to four sequential 15 min enzyme digestion at 37°C in a solution containing 0.05% trypsin-EDTA and 0.1% collagenase P (Roche Diagnostics). Cells released from the second to fourth digestions were collected, centrifuged, re-suspended and plated at a density of 1.0×10^6 cells per 100-mm cell culture dish (Falcon, Fisher Scientific) in DMEM medium containing 10% FCS for 5-6 days. Medium was changed every other day for the entire duration of culture. Cells at a pre-confluent stage of osteoblast differentiation were harvested using 0.25% trypsin/EDTA, collected by centrifugation, and re-suspended in DMEM on ice.

2.4 Recipient mice

Wild type recipient mice were used as the recipients for the calvarial model in this study. The animals were randomly divided into four experimental groups that received the following implants as listed in Table I. All animals were irradiated with a lethal dose of 900 cGy total body gamma irradiation using a ¹³⁷Cs source (Nordion Gammacell 40 Irradiator; Canada), with a dose rate of 66.5 cGy/min (Wang et al. 2005a). Rescue of the hematopoietic system of irradiated mice was performed by systemic injection of 5.0×10^6 total bone marrow cells derived from either pOBCol3.6tpz or wild type mice based on the experimental design in Table I into retro-orbital sinus of anesthetized mice.

2.5 Mouse calvarial defect model

All surgeries were performed under a protocol approved by the University of Connecticut Health Center Animal Care Committee. The mice were anesthetized with a ketamine/xylazine mixture (135 mg/kg and 15 mg/kg). Circular calvarial defects (3.5 mm in diameter with full thickness) were created in 8 to 12 week-old CD1 non-transgenic mice. Briefly, an 8-10 mm incision was made along the sagittal suture by scalpel blade. Then, a skin flap was raised to expose the underlying bone and central 3.5 mm diameter holes were drilled through each of the two parietal bones using a trephine without dura perforation. The defect areas were filled with a scaffold loaded with different cells based on the experimental design in Table 1. The mice were sacrificed at 4 weeks post surgery. To detect new bone formation in relation to the specific host/donor cell population, mice were given an intraperitoneal injection of freshly prepared xylenol orange (XO; 30 mg/mL, 90mg/kg) 24 h prior to the sacrifice.

2.6 Radiological examination

Bone defect repair was assessed using radiological examination. The mice were sacrificed at 4 weeks, and their skulls were harvested. To evaluate the new bone formation at defect areas, all the skulls were examined by a radiography facility (Faxitron X-ray LX 60). The X-ray photographs were taken at 24 kV for 8 seconds. Quantitative new bone formation was analyzed by measuring the radiopacity of host bone and new bone with ImageJ software (National Institutes of Health, Bethesda, MD) (Abramoff et al. 2004). Briefly, the radiopacity of the defect area and host was measured by ImageJ, the relative radiopacity was calculated by dividing the defect area radiopacity with host bone radiopacity.

2.7 Fluorescent imaging analysis

All mouse skulls were carefully excised, cleaned, and fixed immediately in 10% formaldehyde/PBS (pH=7.4) at 40C for 3-4 days. They were then transferred into 30% sucrose/PBS solution overnight. The samples were subsequently embedded with a frozen specimen embedding media (Cryomatrix, Thermo-Shandon, USA). The cryostat section was prepared using a Leica CM1900 Cryostat (Leica D-69226, Germany) outfitted with a Cryojane (Instrumedics, NJ) tape transfer system. The skull tissue was cryosectioned into 7 μ m thin sections. These bone sections were examined using a Zeiss Axio Imager Z1 equipped for fluorescence imaging and a computer-controlled mechanical stage as described previously (Jiang et al. 2005). A set of filter combinations were used to distinguish the GFP signal and XO label from the autofluorescent background of bone and bone marrow. GFPtopaz/Texas Red dual filter cube was used for visualization of green fluorescent protein. Cyan/Texas red dual cube was used to identify blue fluorescent protein as described previously. All images were taken in air and recorded with a Zeiss Axio digital camera. Image processing was performed using AxioVision 4.7 (Zeiss, Germany), and Adobe Photoshop (Adobe Systems, San Jose, CA).

To evaluate the mineralized area, GFP expression and calcium deposition, original images from each channel were analyzed using ImageJ (National Institutes of Health, Bethesda, MD). For mineralized area, the region of interest (ROI) was manually defined and the total area was measured. For GFP expression and calcium deposition, the intensity of Col3.6Cyan and XO was measured by subtracting the background with "threshold" to define the ROI.

2.8 Histology

Following the analysis and imaging of GFP expressions, all the slides were soaked in PBS for 1 h to detach the sections from glass slides. Sections of all four groups were stained with hematoxylin and eosin (H&E). Briefly, the slides were first stained in hematoxylin for 1 min and then washed thoroughly using tap water. Following the washing, the slides were stained with eosin for 10 seconds and rinsed with tap water again. They were then mounted with 50% glycerin in PBS and covered by a coverslip. Images of HE staining were recorded by Leica DMR microscope equipped with AxioCam HRC camera. Besides, tartrate-resistant acid phosphatase (TRAP) staining for osteoclasts was also conducted on all the sections. Briefly, sections were rinsed by PBS thrice for 5 min at each time. The enzyme-labeled fluorescence 97 (ELF 97) substrate (Molecular Probe, E-6601) was diluted by 20-fold in a commercialized buffer (112 mM sodium acetate, 76 mM tartrate, 11 mM sodium nitrite,

pH=4.1). ELF 97 substrate was applied onto the slides and incubated at room temperature for 5 min. The reaction was then stopped by submerging the slides into the wash buffer (25 mM EDTA, 5 mM levamisole in PBS, pH=8.0) for 3 times with gentle agitation. The slides were then mounted to glass slides with 50% glycerin in PBS and covered with a cover slip. TRAP staining sections were observed using Zeiss Axio Imager Z1 equipped for fluorescence imaging and a computer-controlled mechanical stage as described above (Jiang et al. 2005).

2.9 Statistical analysis

All data were presented as mean $\pm$ standard deviation. The numerical results obtained in this study were subjected to one-way analysis of variance (ANOVA). The significance of the results was evaluated at a significance level of $p < 0.05$ based on the p value of comparison groups.

3. Results

3.1 Scaffold characterization

The three-dimensional structure of the collagen/apatite scaffold was examined using micro-CT. The porosity of the scaffold measured by micro-CT is $94.84 \pm 0.16\%$ which indicates the scaffold is highly porous. A typical lamellar structure was also illustrated by micro-CT shown in Fig 1-A. The average inter-lamella distance between two adjacent lamellae is $30 \pm 9 \mu\text{m}$, which is sufficient for cell penetration and tissue ingrowth. Although a dense skin layer was found on the surface of the scaffold, the internal structure of the scaffold was homogeneous. The scaffold used for implantation was cut from the center of a large bulk scaffold, so the skin layers on the surfaces were avoided. The lamellar structure of the scaffolds was further confirmed by the cross-section view (Fig 1-B). The morphology of the scaffold was also revealed by SEM. A layer-by-layer structure was clearly observed under SEM. It was found that besides the lamellar structure, the scaffold illustrated a porous structure allowing cells to penetrate and ingrowth.

3.2 Radiological examination

X-ray radiographs of the mice skull were obtained to determine new bone formation at the calvarial defect sites. The results showed that different orthotopic bone formation for the four experimental groups (Fig. 2). At day 3 after the implantation, no significant difference was found between the four groups as shown in Figs. 2A and 2C. Although there was slight radiopacity across the scaffold for all groups, the radiopacity was mainly contributed by apatite particles in the collagen/apatite scaffolds. At day 28 post implantation, all groups exhibited complete radiopacity across the defect. The new bone formation was also measured in term of radiopacity. It was found that all groups seeded with cells formed more bone than the control group with no cells. There was no significant difference between the groups loaded with different cell combinations (Fig. 2-E). Moreover, X-ray images suggest that the new bone formed in Groups A and B (without BMCs) appeared to be morphologically different from those in Groups C and D (with BMCs). In Groups A and B, although the calvarial defects were filled by mineralized tissue after 4 weeks of implantation, a gap was clearly seen between the newly formed bone and the host bone. In

comparison, a better integration between the new bone and the host was observed in Groups C and D than Groups A and B after 28 days of implantation (Figs. 2B and 2D) when BMCs were employed as donor cells. The difference between the two sets of studies indicates that the scaffolds loaded with BMCs have better host-implant integration regardless of new bone formation capacity.

3.3 Fluorescent imaging analysis

All four test groups were evaluated for the effect of different cell populations on new bone formation. The mice were implanted with scaffolds loaded with different cell combinations, and then rescued by fresh bone marrow from a second mouse as shown in experimental design (Table I). At day 3 after transplantation, Col3.6cyan OPCs were clearly seen in the defects of Groups B and D. Cell distribution in these groups was concentrated at the edge of the defects. No fluorescence of Col3.6tpz was detected from the defects loaded with BMCs in Groups C and D (Fig. 3, right column). The scaffolds in all the defects maintained complete lamellar structure and no degradation was observed at day 3 from DIC channel (Fig. 3, middle column). A small amount of Col3.6cyan cells were also found in Group A which was implanted with scaffold alone without cells, but cell migration from the test side to the control occurred.

After 28 days of implantation, active osteoblasts *in vivo* were identified by their strong fluorescent intensity, relative to low intensity in fibroblastic cells. New bone formation was again confirmed with the co-localization of osteoblast fluorescent reporters and the newly deposited mineralized matrix (red) which was stained by xylenol orange (XO). It was found that new bone formation in these four groups varied significantly with cell type seeded into the scaffolds. Group B, where OPC was the only donor cell, had the most new bone formation among the four testing groups (Fig. 4-A), but no significant differences were observed in term of osteoblast activities (Col3.6Cyan intensities) among Groups A, B and D (Fig. 4-B). The labeling level of calcium deposition was found to be significantly higher in groups loaded with cells than the group without cells (Group A, Fig. 4-C). This result is in agreement with the results obtained from radiological examination and mineralized area measurement. Morphologically, the entire defect was filled by well organized new bone as shown in Fig. 4 OPC panel. More importantly, it was found that the new bone formation was closely associated with blue Col3.6cyan osteoblasts, which suggests new bone formation was mainly contributed by the donor cells. Besides, no scaffold residue was visible at the defect sites in this group. The absence of green Col3.6tpz osteoblasts at the defect sites indicated that the transplanted bone marrow did not contribute to bone repair (Fig. 4, OPC panel). Local new bone formation at the edge of the defects was also found in Group A, but most of the scaffolds still remain intact at the defect site (Fig. 4, No cells panel). In Group C, new bone was mainly formed at the bottom part of the scaffold that faces the dura. It was noticed that the newly formed bone was concentrated at the edges close to the host bone, and it well bridged the defect with the adjacent host bone (Fig. 4, BMC panel). In addition, pOBCol3.6tpz positive cells (green) were easily found in this group, however, these cells did not overlay with the XO label, indicating they were probably osteoclastic cells instead of osteoblasts (Fig. 4, tpz column). Only a small portion of the scaffold was degraded, and the lamellar structure of the scaffold was clearly seen in the upper part of the defect area (Fig. 4,

BMC panel). In Group D which was loaded with a combination of OPCs and BMCs as donor cells, the defect was also filled with large amount of new bone (Fig. 4, OPC+BMC panel). However, compared to Group B, the new bone formation in this group was less organized. The collagen/apatite scaffold was still observable in some locations, indicating incomplete degradation of the scaffold.

3.4 Histology

H&E staining showed that collagen/apatite scaffolds loaded with different cell populations demonstrated significantly different bone forming capabilities. After 28 days of implantation, the major structure of the scaffold without cells (Group A) still remained except that the edge of the scaffold was slightly degraded and invaded by fibroblasts (Figs. 5A and E). New bone was found at the edge of the scaffold due to the migration of OPCs from the test scaffold to the control. Group B, which was loaded with OPCs only, had the most new bone formation and bone remodeling among the four groups. No scaffold residue was found in the defect after 4 weeks of implantation, which suggested that the scaffold was completely degraded and replaced by new bone. In addition, blood vessels were reconstructed and large bone marrow area was formed within the defects (Figs. 5B and F). In comparison, scaffold was hardly degraded in Group C. New bone was only formed at the lower edge of the scaffold, but it was well integrated with the host bone. For the rest of the scaffolds, fibrous tissue was filled in the space between the lamellae of the scaffold (Figs. 5C and G). By contrast, only a thin layer of the scaffold was remained in Group D after 28 days of implantation. New bone almost filled the entire defect. The lamellar structure of the scaffold could barely be seen and the space between the new bone and remaining scaffold was fully invaded by fibrous tissues. It was also found that new blood vessels had already started to form in Group D. All these findings demonstrated that an active bone remodeling process had begun in the defects of Group D (Figs. 5D and H).

TRAP stain was used to identify osteoclastic cell activity on both the scaffold and the bone surfaces so as to investigate the effect of osteoclast on bone remodeling. TRAP positive cells were scarcely observed in the defect site of group A (Figs. 6-A, E, and I). In contrast, the osteoclastic cell activity of Group B is significantly different from Group A. It is noteworthy that TRAP positive cells were widely distributed on bone surfaces, but they did not overlay with any blue Col3.6cyan osteoblasts (Figs. 6-B, F, and J). Different from Groups A and B, the TRAP positive cells in Group C were concentrated at the lower edge of the scaffold which was associated with the newly formed bone (Figs. 6-C, G, and K). This indicated that the bone remodeling in this region is extremely active. The TRAP stain pattern in Group D was similar to that of Group B but was not that strong. Similarly, the TRAP positive cells in Group D also did not overlay with pOBCol3.6cyan GFP positive cells (Figs. 6-D, H and L).

4. Discussion

In this study, multiple types of cells from different tissue sources, including both OPCs and BMCs, were used in combination with scaffolds for bone regeneration. New bone formation was observed in all experimental groups either with single cell type or multiple cell types. However, the amount of new bone formed, bone distribution, new bone-host bone

integration, and scaffold degradation appeared to be distinctly different among these groups. The presence of GFP markers in each cell type and rescue bone marrow makes it possible to explore the mechanisms of new bone formation in each test group. The pOBCol3.6 GFP transgenic mice utilized in this study appeared to be a powerful tool to appreciate the host/donor cell contributions to bone regeneration.

According to the results from fluorescence imaging analysis and H&E histology, new bone formation was confirmed because GFP reporters matched perfectly with our histological observations. It is known that pOBCol3.6 transgene is not specific to bone. It can also be found in other cells producing type I collagen (Wang et al. 2005a). However, when the blue fluorescence is associated with the red XO label on new bone surface, the overlay of blue and red colors on new tissue surface provides a strong evidence of new bone formation. Ample new bone has been observed and its formation is closely guided by the scaffold microstructure and affected by the type of cells seeded (Fig. 4). These results illustrated the excellent osteo-permissive and osteogenic properties of the apatite/collagen scaffolds. It was found that OPCs aligned along the lamellar structure of the scaffold and subsequently deposited mineralized matrix on these substrates. These results clearly demonstrate that bone regeneration observed in this study is a scaffold-guided process. Therefore, new bone ingrowth, orientation and structure might be closely related to the structure of the scaffold used for bone repair.

Although significant new bone formation was found in groups with or without BMCs, fresh BMCs play an important role in bone repairing, especially new bone-host bone integration. GFP scanning images suggested that new bone formation was directly contributed by the donor cells instead of the host cells when OPCs were seeded in the defects. The new bone amounts formed were similar in both Groups B and D. However, the absence of pOBCol3.6GFPtpz positive cells in defect area of Group D indicates that BMCs did not contribute directly to new bone formation. The fact that the pOBCol3.6tpz positive cells in Group C appeared to be osteoclastic cells also suggested the implanted fresh BMCs did not directly participate in bone formation (Fig. 4). Although many reports have demonstrated the importance of bone marrow to bone regeneration (Bruder et al. 1998; Tshamala and Van Bree 2006; Jegoux et al. 2009), our results demonstrated that fresh BMCs did not participate in new bone formation directly. Nevertheless, fresh bone marrow at defect sites might support new bone formation in some other ways, such as providing various growth factors (Cancedda et al. 2003; Yoshii et al. 2009). This is well supported by new bone formation observed in Group C where the scaffold was only loaded with pOBCol3.6GFPtpz BMCs, an ingrowth of host-derived bone into the scaffold was observed. The presence of Col3.6tpz positive cells, together with the lack of co-localization of Col3.6tpz and XO label indicates clearly that fresh BMCs donor cells are involved in new bone formation, but do not directly make new bone.

The degradation behavior of scaffold *in vivo* is another critical parameter for the success of new bone regeneration (Rezwan et al. 2006). The degradation rate of an ideal scaffold should match the rate of new bony matrix deposition (Mastrogiacomo et al. 2007). The degradation of the collagen/apatite scaffold appeared to be a cell mediated degradation process, where the new bone deposition was closely associated with scaffold degradation.

When the scaffold was implanted alone, most of the scaffold remained intact after 4 weeks post surgery. In comparison, the scaffold was completely resorbed when it was loaded with OPCs (Fig. 5). Two mechanisms are proposed here to explain the degradation of the scaffold in this study. First, OPC in the scaffold is an early osteoblastic progenitor lineage, which secretes biological factors that attract and recruit cellular components, such as macrophages and osteoclasts from the host. The scaffold degradation may therefore occur under the function of osteoclastic cells through phagocytosis and other cell-mediated processes. TRAP staining clearly showed that osteoclastic cells were active in all groups loaded with OPCs (Fig. 6). Second, mature osteoblasts can induce osteoclast differentiation by stimulating receptor activator of nuclear factor- κ B ligand (RANKL) and other receptors such as osteoclast-associated receptors in osteoclast precursor cells (Marie et al. 2000). The scaffold degradation is enhanced when osteoblast-osteoclast communication is well established (Boyce et al. 2009; Matsuo 2009). The successful bone regeneration with OPCs suggested that the degradation rate of the apatite/collagen scaffold matched perfectly with the bone ingrowth rate. When the scaffold was loaded with a mixture of OPCs and BMCs, only part of the scaffold was resorbed after 4 weeks of implantation. The loaded BMCs may inhibit the migration of cells from the host to the defect as BMCs occupied substantial spaces in the scaffold. However, since bone resorbing cells like macrophages and osteoclasts can also be derived from bone marrow (Colnot et al. 2006), the loaded BMCs can also help to resorb the scaffold associated with new mineral matrix deposition. This might also explain partially why scaffold degradation was observed in Group C. The overlay of TRAP staining with pOBCol3.6GFPtpz positive cells from BMC further confirmed this explanation (Fig. 6).

One problem raised in this study is the lack or little host contribution to the repair process. It was found that there was always a gap between the newly generated bone and surrounding host calvarial bone. From a tissue engineering perspective, this outcome is not an ideal defect repair. When donor OPCs are present, there is little host osteogenic activity at the edge of the defects and no cell ingrowth from host was observed. However, when BMCs were used as donor cells, ingrowth of host derived bone into the scaffold along the lamellar structure was observed. Although bone marrow has been reported to contribute to bone repair (Ohgushi et al. 1989; Colnot et al. 2006; Tshamala and Van Bree 2006), our results suggest that bone marrow cells themselves do not differentiate into osteoblasts according to the rare appearance of pOBCol3.6tpz positive cells in Group C. Instead, it is believed that bone marrow cells induce efficient host bone migration and engraftment, which is also very important to bone repair. Therefore, the application of bone marrow cells provides us with a promising approach to improve the poor implant-host bone integration.

5. Conclusion

This study reports a novel double-hole mouse calvarial model for evaluating new bone formation in a collagen/apatite scaffold. Donor dominated bone regeneration was achieved when OPCs were loaded into the scaffold. When BMCs were loaded into the scaffold, less new bone were formed than the scaffolds loaded with OPCs. However, host derived bone formation was observed, which generates better host-new bone integration. When BMCs were mixed with OPCs, similar to the group loaded with OPCs alone, ample new bone was formed but the host did not contribute to the new bone formation. It has been demonstrated

that transgenic mice harboring GFP reporters for osteoblasts is a powerful tool for appreciation of the host/donor contributions during bone repair.

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Fig. 1.
3D images and microstructure of collagen/apatite scaffold. (A) micro-CT 3D image of the scaffold; (B) cross section view of the scaffold from micro-CT; (C) FESEM micrograph of the lamellar structure of the scaffold

Fig. 2.

X-ray micrographs of the defect area of mice calvarial model at different time points. (A) Right side: scaffold loaded without any cells for 3 days; Left side: scaffold loaded with OPC for 3 days; (B) Right side: scaffold loaded without any cells for 28 days; Left side: scaffold loaded with OPC for 28 days; (C) Right side: scaffold loaded BMC for 3 days; Left side: scaffold loaded with OPC plus BMC for 3 days; (D) Left side: scaffold loaded with BMC for 28 days; Right side: scaffold loaded with OPC plus BMC for 28 days; (E) Quantification of new bone formation in term of relative radiopacity. * Indicates the test is significantly different from the control (No cells) ($p < 0.05$) at day 28.

Fig. 3.

Fluorescent imaging analysis of the cross section of the calvarial defects at day 3 after implantation. (Overlay) Scanning images of DIC channel combined with Cyan channels. (DIC) differential interference contrast channel under dark field. (Cyan) Cyan channel for blue fluorescence signal from Col3.6cyan cells.

Fig. 4.

Fluorescent imaging analysis of the cross section of the calvarial defects at day 28 after implantation. (A) Mineralized area was determined manually by defining the new bone area in DIC channel with the assistance of ImageJ. (B) Total intensity of Col3.6Cyan was quantified by measuring the average intensity of the Cyan channel, and then multiplied by total area after subtracting the background. (C) Total intensity of calcium labeling was quantified by measuring the average intensity of XO channel, and then multiplied by total area after subtracting the background. (Overlay) Scanning images of DIC channel combined with three fluorescence channels. (DIC) differential interference contrast channel under dark field. (Cyan+XO+Tpz) Cyan channel for blue fluorescence signal from Col3.6cyan cells; XO channel for red fluorescence signal from xylene orange; Tpz channel for green fluorescence signal from Col3.6tpz cells. * Indicates the test is significantly different from the control (No cells) ($p < 0.05$) at day 28.

Fig. 5.
H&E staining of the cross section of the calvarial defects at day 28 post implantation. (A) & (E): defect filled with scaffold loaded without any cells; (B) & (F): defect filled with scaffold loaded with OPC; (C) & (G): defect filled with scaffold loaded with BMC; (D) & (H): defect filled with scaffold loaded with OPC plus BMC

Fig. 6.

Osteoclastic activity revealed by TRAP staining combined with GFP fluorescent imaging at day 28 post implantation. (A), (E) & (I): defect filled with scaffold loaded without any cells; (B), (F) & (J): defect filled with scaffold loaded with OPC; (C), (G) & (K): defect filled with scaffold loaded with BMC; (D), (H) & (L): defect filled with scaffold loaded with OPC plus BMC

Table. 1

Experimental design of donor cells, transplanted total bone marrow cells, and collagen/apatite scaffold

<i>Host mice</i>	<i>Transplanted total bone marrow cells (5 Million)</i>	<i>Implants and cells</i>	
		Left side defect	Right side defect
CD-1 (n=8)	Col3.6tpz mice	Group A: collagen/apatite scaffold	Group B: collagen/apatite scaffold +1 million Col3.6Cyan OPCs
CD-1 (n=8)	CD-1	Group C: collagen/apatite scaffold +20 million Col3.6Tpz BMCs	Group D: collagen/apatite scaffold +1 million Col3.6Cyan OPCs + 20 million Col3.6Tpz BMCs