thogens. Sfakiuanakis et al. (2001a, b) demonstrated that *Actinobacillus actinomycetemcomitans* extracts induced dose- and time- dependent expression of IL-1α, IL-1β, and IL-8 in cultured gingival epithelial cells and that IL-1 inhibitors inhibited IL-8 induction by IL-1α and IL-1β. TNF-α and IL-1α also induced the dose-dependent expression of IL-8 in cultured gingival epithelial cells (Bickel et al. 1996). Moreover, using in situ hybridization methods, Tonneti et al. (1994) demonstrated that IL-8 mRNA positive cells were selectively located in the JE or pocket epithelium in close spatial relationship with subgingival plaque microorganisms. In our previous studies with the present animal model, we reported that JE cells revealed transient expression of IL-1α, IL-1β, and TNF-α peaking at 3 h after LPS application (Miyauchi et al. 2001). The results support that JE cells recruit PMNs by their CXC chemokine production induced via their own proinflammatory cytokine secretion and indicate that JE cells may be responsible for initiation of periodontal inflammation and the acute transformation of periodontal disease.

Another possible role of CXC chemokines produced from JE cells may be the upregulation of the proliferative activity of the JE cells themselves. It is reported that the recombinant rat MIP-2 stimulated proliferation of alveolar epithelial cells (Driscoll et al. 1995) and that cultured gastric epithelial cells treated with GRO/CINC-1 showed a significant increase in cell number and BrdU incorporation in a dose-dependent manner (Suzuki et al. 2000). In addition, Jarnbring et al. (2000) immunohistochemically demonstrated that IL-8 was expressed in PCNA-positive proliferating keratinocytes in a periodontal patient group and suggested that IL-8 may have a role in keratinocyte proliferation. In this animal model, we previously reported that number of PCNA-positive JE cells was significantly increased by LPS application on day 2 (Takata et al. 1997). Although in this animal model the lateral proliferation or downgrowth of JE cells along the root surface was not observed, MIP-2 and CINC-2 produced in JE cells may also play an important role in upregulation of the proliferative activity of the JE cells.

Interestingly, in both the untreated animals and the LPS-treated animals, epithelial remnants of Malassez showed constitutive expression of CINC-2. We also demonstrated constitutive expression of IL-1β in the epithelial remnants (Miyauchi et al. 2001). Therefore, IL-1β constitutively produced by the epithelial remnant may cause the following CINC-2 production and the inductive CINC-2 may exert some critical effects in the functions of this epithelium such as their survival in periodontal ligament.

In summary, in gingival tissue after topical application of LPS, JE cells are a major source of CXC chemokines including MIP-2 and CINC-2. In the period associated with the marked enhancement of CXC chemokine production, a prominent increase of PMN infiltration in the JE and sub-JE area was also detected. These findings suggest that MIP-2 and CINC-2 may be responsible for PMN migration toward the periodontal pathogen and may play an important role in the initiation of inflammation and subsequent periodontal tissue destruction. Further studies will be required to clarify the critical role of JE cells in the pathogenesis of periodontitis and the possibility of new cytokine therapy of periodontitis targeting CXC chemokines.

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Enhancement of Periodontal Tissue Regeneration by Transplantation of Bone Marrow Mesenchymal Stem Cells

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Background: The use of suitable cells transplanted into periodontal osseous defects appears to be a powerful strategy to promote periodontal tissue regeneration. Mesenchymal stem cells (MSCs) isolated from bone marrow have the potential for multilineage differentiation. The purpose of this study was to examine whether auto-transplantation of MSCs into periodontal osseous defects would be useful for periodontal tissue regeneration.

Methods: Bone marrow MSCs were isolated from beagle dogs and expanded in vitro. The expanded MSCs were mixed with atelocollagen (2% type I collagen) at final concentrations of 2×10^6, 5×10^6, 1×10^7, or 2×10^7 cells/ml, and auto-transplanted into experimental Class III defects. Atelocollagen alone was implanted into the defects as a control. Periodontal tissue healing was evaluated by histological and morphometric analyses 1 month after transplantation.

Results: The defects were regenerated with cementum, periodontal ligament, and alveolar bone in the MSC-atelocollagen groups. Less periodontal tissue regeneration was observed in the control group compared to the MSC-atelocollagen groups. Morphometric analysis revealed that the percentage of new cementum length in the 5×10^6 and 2×10^7 cells/ml groups and the percentage of new bone area in the 2×10^7 cells/ml group were significantly higher than in the control group ($P < 0.01$).

Conclusion: These findings suggest that auto-transplantation of bone marrow mesenchymal stem cells is a novel option for periodontal tissue regeneration. *J Periodontol 2004;75:1281-1287.*

KEY WORDS

Atelocollagen; bone marrow cells; periodontal regeneration; stem cells, mesenchymal; tissue engineering.

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Previous studies have shown that conventional regenerative therapies such as guided tissue regeneration (GTR), topical application of enamel matrix derivative (EMD), or various polypeptide growth factors can successfully regenerate periodontal tissue.[1-7] Since the strategy of these treatments is to stimulate regeneration from endogenous somatic stem cells, the presence of resident tissue is the most important factor for periodontal tissue regeneration. Therefore, indications for these treatments are limited to cases of 2- or 3-wall bony defects or to Class II furcation lesions.

It is generally accepted that tissue regeneration can be accomplished by combining three key elements: cells, scaffolds, and signaling molecules.[8] Scaffolds and signaling molecules are already being used clinically in regenerative therapies; however, the other key cells have not yet been identified. In this context, the use of suitable cells seeded into periodontal defects would appear to be a powerful strategy to promote regeneration of periodontal tissue.[4] The cells should be non-immunogenic, highly proliferative, and easy to harvest, with the ability to differentiate into various types of cells comprising periodontal tissue.

In our research, we have focused on bone marrow-derived mesenchymal stem cells (MSCs). Bone marrow MSCs can easily be obtained repeatedly and differentiate into osteoblasts, chondrocytes, tenocytes, adipocytes, muscle cells, or nerve cells in vitro and in vivo.[9-15] Thus, transplantation of bone marrow MSCs

might provide a new method for treatment of osteoporosis, arthritis, cardiac diseases, and degenerative nerve diseases. Considering these findings,[9-15] it is conceivable that MSCs might be useful for periodontal tissue regeneration. We previously developed a new culture system with fibroblast growth factor-2 (FGF-2) to expand MSCs with their multilineage differentiation potential.[15] The purpose of the present study was to evaluate the potential of bone marrow mesenchymal stem cells, expanded by the culture system, on periodontal tissue regeneration in vivo.

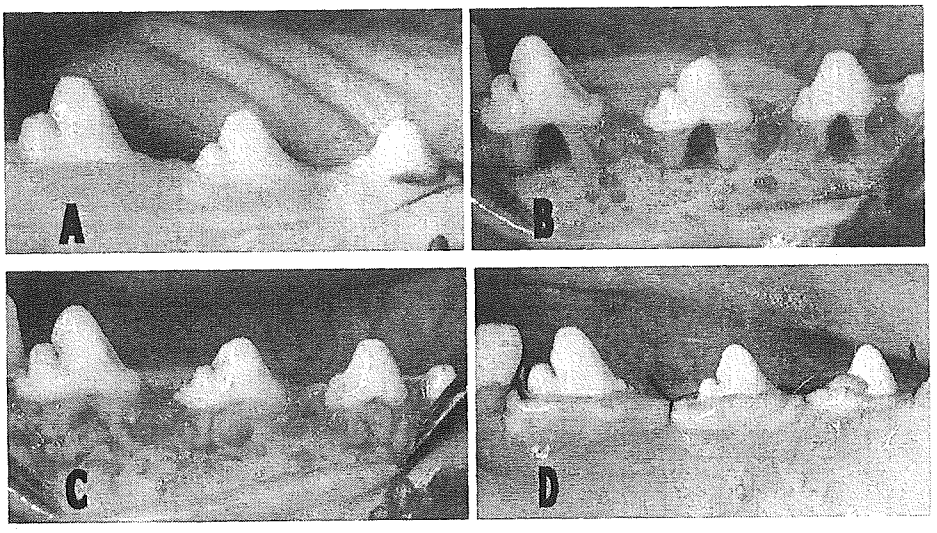

Figure 1.
Presurgical view of the mandibular premolar area ***(A)****; after defect preparation* ***(B)****; after transplantation of various concentrations of MSCs in atelocollagen* ***(C)****; after repositioning of gingival flaps and suturing* ***(D)****.*

MATERIALS AND METHODS

After receiving approval from the Committee of Research Facilities for Laboratory Animal Science, Hiroshima University School of Medicine, 12 female beagle dogs weighing 10 to 14 kg and aged 12 to 20 months were used in this study. Good oral health was established by scaling and mechanical toothbrushing.

Isolation and Development of Bone Marrow MSCs

Bone marrow aspirates of 1 ml were taken from the iliac crest of each animal under sodium pentobarbital (40 mg/kg) anesthesia. Cell culture was performed in accordance with the technique described by Tsutsumi et al.[15] The cells, including erythrocytes, were seeded at 2×10^8 cells/100 mm in tissue culture dishes and maintained in 10 ml of Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum,§ 0.05 unit/ml penicillin,‖ and 0.05 mg/ml streptomycin.‖ Three days after seeding, floating cells were removed and the medium was replaced with fresh medium. Thereafter, attached cells were fed with fresh medium every 3 days. Passages were performed when cells were approaching confluence. Cells were seeded at 5×10^3 cells/cm^2 in 100 mm dishes and maintained in the medium containing 3 ng/ml FGF-2¶ for 2 weeks. The cells were harvested with trypsin plus EDTA, washed with phosphate buffered saline, and stored in liquid nitrogen until use. In this study, cell passage 3 was used for transplantation.

Creation of Class III Furcation Defects and Transplantation of MSCs

All surgical procedures were performed under general anesthesia with sodium pentobarbital (40 mg/kg), and local infiltrated anesthesia with 2% lidocaine with 1:80,000 noradrenaline. Experimental Class III furcation defects prepared in this study were based on the model described by Lindhe et al.[16] The second, third, and fourth premolars (P2, P3, and P4) in each dog were selected for experimentation (Fig. 1). Following sulcular incisions, mucoperiosteal flaps were raised, and Class III furcation defects were created surgically at P2, P3, and P4. The Class III defect height from the cemento-enamel junction to the reduced alveolar crest was 4 mm. Denuded root surfaces were prepared to remove all periodontal ligament and cementum. The roots were denuded only in the area within the furcation and extending to the mesial line angle for the mesial root and to the distal line angle for the distal root. Reference notches were placed around the circumference of the mesial and distal roots at the bottom of the bone level.

The MSCs cultured for 2 weeks were suspended, and four groups of MSC-collagen gel material were prepared. That is, 2×10^6, 5×10^6, 1×10^7, and 2×10^7 MSCs were mixed with 1 ml of atelocollagen (2% type I collagen extracted from bovine calf skin by pepsin digestion).# All four groups of the MSC-collagen gel and the atelocollagen alone were transplanted into the Class III furcations in all 12 dogs. The flaps were coronally repositioned and sutured with 4-0 interrupted silk sutures. Good oral hygiene was maintained postoperatively by brushing and swabbing with 0.2% povidone iodine.**

Tissue Preparation for Histological Analysis

One month after transplantation, anesthetized animals were perfused with 1% glutaraldehyde in sodium cacodylate buffer containing 0.05% calcium chloride (pH 7.3).

§ Hyclone, South Logan, UT.
‖ Invitrogen Corp., Carlsbad, CA.
¶ PeproTech EC Ltd., Rocky Hill, NJ.
# Koken Co., Ltd., Tokyo, Japan.
** Meiji-seika Co., Ltd., Tokyo, Japan.

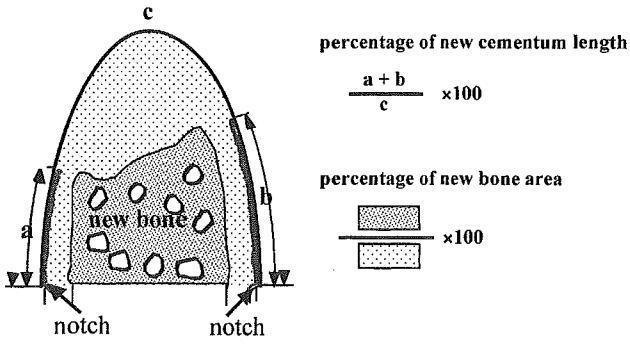

Figure 2.
Schematic drawing of morphometric analysis of percentage of new cementum length and percentage of new bone area.

The mandibles were dissected and immersed in the same fixative. After decalcification with hydrochloride for 3 to 5 days, the mandibles were dehydrated through graded ethanol, cleared with xylene, and embedded in paraffin. Serial sections (5 μm) were cut in the mesial-distal plane throughout the buccal-lingal extension of the tooth. The sections were stained with hematoxylin and eosin (H&E) or the Azan method, and observed using a light microscope. The most central section and the immediate section on either side were subjected to morphometric analysis.

Morphometric Analysis

The percentage of new cementum length and percentage of new bone area were measured on digitized photomicrographs captured in a computer. The lengths of new cementum formed along the denuded root surface on each specimen were added, and the percentage of the lengths to the total root surface length from one notch to the next notch was calculated. The area of new bone on each specimen was calculated as a percentage of the area surrounded with reference notches at mesial and distal root surfaces facing the bone defect. Since the periodontal ligament space is present in normal periodontal tissue, the percentage of bone area in untreated specimens was also calculated (Fig. 2). All data were statistically analyzed using the Mann-Whitney U test.

RESULTS

In the MSC-atelocollagen groups (experimental groups), significant amounts of new bone and adequate width of periodontal ligament were observed (Fig. 3). The denuded root surface was almost completely covered with new cementum, and regenerated periodontal ligament separated the new bone from the cementum (Fig. 4). On the denuded root surfaces of the furcation area, newly formed cementum covered the surface, and Sharpey's fibers inserted into the cementum were frequently observed (Fig. 5). However, complete alveolar bone reconstruction was not obtained. Epithelial cell invasion, bone ankylosis, and root resorption were not observed on the root surface. In the atelocollagen alone

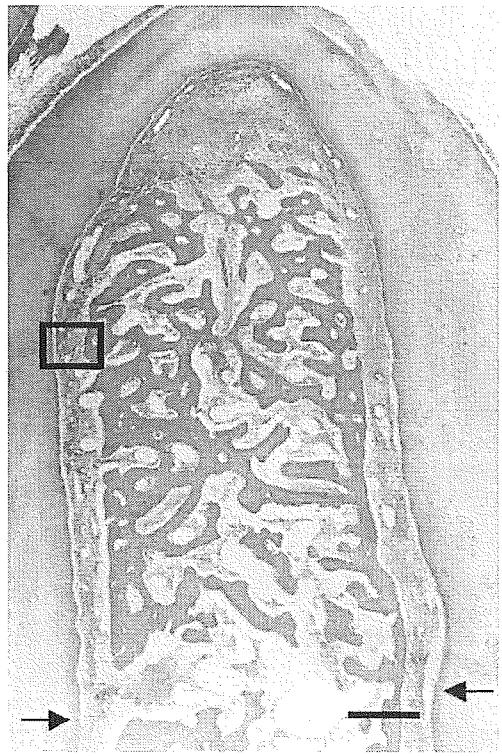

Figure 3.
Low-power view of Class III defects in the mesio-distal plane 1 month after transplantation of MSCs (2×10^7 cells/ml) in atelocollagen. Significant amounts of new bone and cementum, and adequate width of periodontal ligament can be observed. Arrows indicate apical border of the denuded root surface. The square area is shown in higher magnification in Figure 4. (H&E staining; bar = 200 μm.)

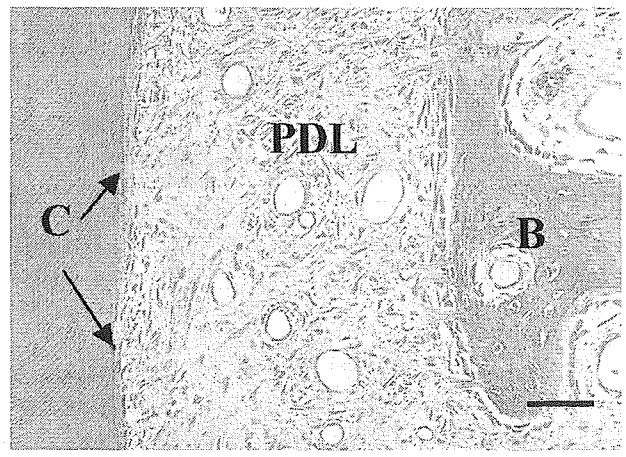

Figure 4.
A higher-power view of the square area in Figure 3 showing new cementum (C), bone (B), and regenerated periodontal ligament (PDL). (H&E staining; bar = 20 μm.)

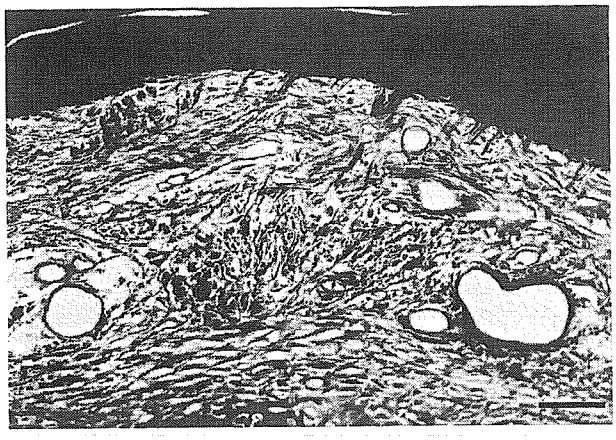

Figure 5.
A higher-power view of the furcation area in Figure 3 showing new cementum with Sharpey's fibers. (Azan staining; bar = 20 μm.)

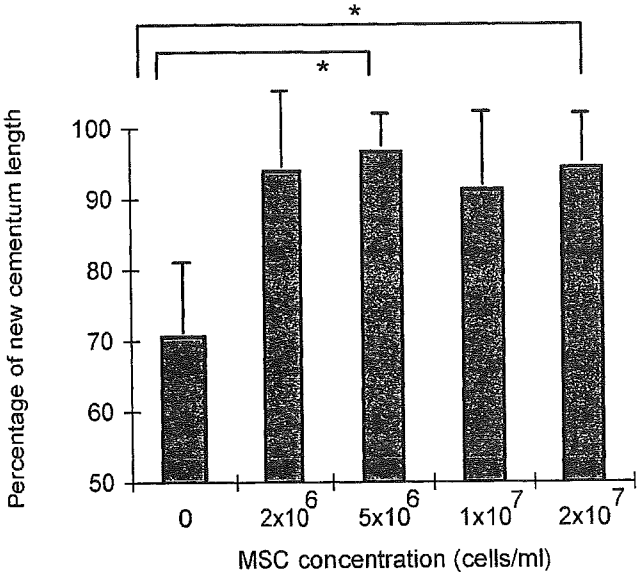

Figure 7.
*The percentage of new cementum length. The 5 × 10^6 and 2 × 10^7 cells/ml groups were significantly higher compared to the control group (*P <0.01).*

group (control group), epithelial cells invaded into the top of the furcation, and no cementum regeneration was observed in the area (Fig. 6). Less bone regeneration was observed in this group compared to the MSC-atelocollagen groups. Bone ankylosis and root resorption were not observed.

The percentages of new cementum length in the 2 × 10^6, 5 × 10^6, 1 × 10^7, and 2 × 10^7 cells/ml groups were 93.9% ± 14.3%, 96.7% ± 5.23%, 91.3% ± 12.3%, and 94.4% ± 8.27%, respectively, compared to 70.5% ± 12.0% in the control group (Fig. 7). However, a significant difference was observed only in the 5 × 10^6 and

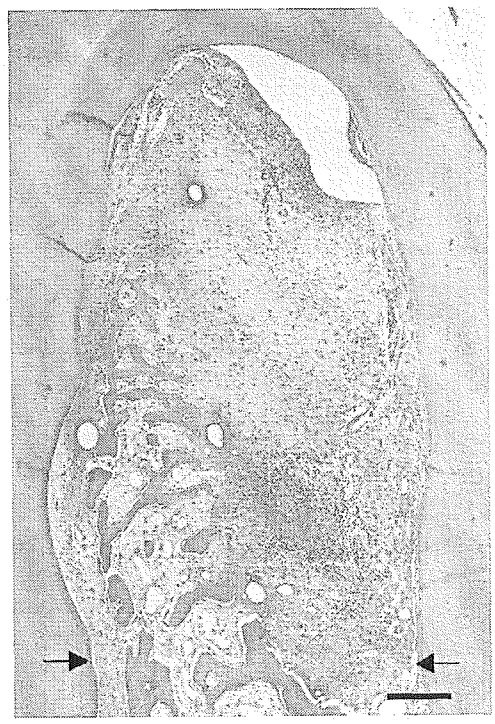

Figure 6.
Low-power view of Class III defect in the mesio-distal plane 1 month after implantation of atelocollagen alone. Migration of epithelial cells into the top of the furcation and cementum formation were not observed in the area. Note less bone regeneration compared to Figure 3. Arrows indicate apical border of denuded root surface. (H&E staining; bar = 200 μm.)

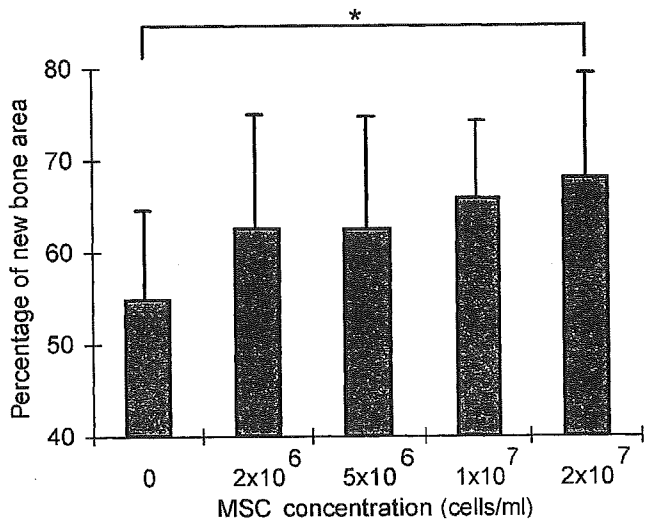

Figure 8.
*The percentage of new bone area. The 2 × 10^7 cells/ml group was significantly higher compared to the control group (*P <0.01).*

2 × 10^7 cells/ml groups (P<0.01). The percentages of new bone area in the experimental groups were 62.6% ± 14.6%, 62.5% ± 13.6%, 65.8% ± 9.62%, and 68.1% ± 10.7%, respectively, compared to 54.8% ± 10.7% in the control group (Fig. 8). In particular, the percent-

age of new bone area in the 2×10^7 cells/ml group was significantly ($P<0.01$) higher than in the control group. The percentage of bone area in the normal specimen was 73%.

DISCUSSION

The findings of this study suggest that auto-transplantation of bone marrow mesenchymal stem cells can promote periodontal tissue regeneration. We speculate that this was not solely due to stimulated regeneration from resident cells. It was reported that bone marrow MSCs can be induced to differentiate into osteoblasts, chondrocytes, tenocytes, adipocytes, etc.[9-15] If tissue regeneration can be accomplished by a single cell type, as in the case of bone or cartilage defects, transplantation of MSC-derived osteoblasts or chondrocytes may be effective. To promote chondrogenic differentiation, MSCs were cultured without serum and with transforming growth factor-β3.[9] To promote osteogenic differentiation, MSCs were cultured with dexamethasone, β-glycerophosphate, and ascorbate in the presence of 10% v/v fetal bovine serum.[10] However, plural cell types, including cementoblasts, osteoblasts, and periodontal ligament fibroblasts, were required for periodontal tissue regeneration. Recent studies reported that transplanted bone marrow MSCs differentiated into various connective tissue cells in vivo under the influence of host factors.[17,18] It was also proposed that injured tissues produce the appropriate cues necessary for engraftment. Thereafter, the local microenvironment stimulates the differentiation of transplanted cells into functional, specialized cells.[19] The findings in the current study showed that reconstruction of periodontal tissue, cementum, alveolar bone, and periodontal ligament occurred after bone marrow MSCs were transplanted into Class III defects. Although we do not know the precise mechanisms underlying periodontal tissue regeneration with MSCs, it might be possible that transplanted bone marrow MSCs expressed their multilineage differentiation potential in vivo and underwent site-specific differentiation into the appropriate periodontal cells. Thus, periodontal regeneration may have been accomplished by transplantation of bone marrow MSCs that had not received preinduction or treatment for differentiation.

One significant finding of this study was that cementum with extrinsic fibers formed along almost all of the denuded root surfaces after MSC transplantation. Previous studies showed that new cementum had increased thickness, greater cellularity, a predominance of intrinsic collagen fibers, and the investment of sparse, extrinsic Sharpey's fibers.[20-25] However, some histological studies showed that enamel matrix derivative (EMD) treatment and combined treatment with EMD and GTR induced the formation of an acellular type of cementum.[26-31] Discrepancies in these histological findings on cementum may reflect heterogeneity in both cellular activity and extracellular matrix events during periodontal healing. Tissue dynamics must be considered to understand these variations.

Once new cementum with extrinsic fibers covers the denuded root surface, epithelial cells may not be able to migrate on the surface. It has been hypothesized that the type of tissue which predominates in the healing wound determines whether the response is one of repair or regeneration.[32] Moreover, components of newly formed cementum matrix provide informational signals for recruitment, proliferation, and differentiation of periodontal cells and regulate regeneration of cementum in addition to adjacent periodontal components.[33] It is generally accepted that new cementum formation and restoration of soft tissue attachment to the cementum are the major goal of regenerative periodontal therapy.[1,4,33] Therefore, MSC transplantation may be useful for periodontal tissue regeneration.

The amount of regeneration observed in the control group was still quite significant, although admittedly less organized than that observed in the MSC-Atelocollagen groups. In this study, experimental defects were treated immediately. It has been reported that approximately 50% to 70% spontaneous regeneration can be expected in acute defect models.[34] Subsequent studies of MSC transplantation into a chronic, plaque-infected periodontal defect model and/or a large periodontal defect model would provide further information on periodontal regeneration.

Although the mechanism by which MSC transplantation induces formation of cementum is unknown, MSCs may attach to denuded root surfaces and cell-matrix interactions may promote their differentiation. A variety of chemotactic factors, adhesion molecules, growth factors, and extracellular matrix macromolecules could participate together in inducing differentiation of MSCs into cementoblasts or could recruit cementoblast progenitors.[33] A previous study showed deposition of cementum-like matrix when periodontal ligament cells were cultured on the root surface.[35] Thus, the local environment is an important regulator of cell activity.

We used atelocollagen as a biomaterial scaffold to hold MSCs in a temporary matrix, and to keep them suspended during transplantation and in the early period of regeneration. When considering clinical applications, a safe scaffold must be carefully selected. With atelocollagen, the antigenic determinants on the peptide chain of type I collagen (telopeptide) are removed, and thus it is often used clinically for the treatment of skin disorders.[36,37] Furthermore, autologous chondrocytes embedded in atelocollagen transplanted into cartilage defects were reported to promote repair.[38-40] Accordingly, atelocollagen is an appropriate scaffold material for MSC transplantation.

Cell density of MSCs is also an important factor for tissue regeneration. Sufficient tissue fluids and blood supply might be necessary for MSCs to survive after

transplantation and subsequent differentiation into periodontal cells. A high cell concentration of MSCs might disturb the infiltration of nutrients into the deeper areas of transplanted MSCs. Among the cell concentrations (2×10^6 to 2×10^7) used in the present study, no significant differences were observed in bone or cementum formation. Effective tissue regeneration was reported when the same range of cell concentrations was used for transplantation into various defects.[39-43] However, additional studies with different scaffold materials and various concentrations of cells will extend our knowledge on viable periodontal tissue engineering.

Polypeptide growth factors, including platelet-derived growth factor (PDGF), insulin-like growth factor (IGF), transforming growth factor (TGF), fibroblast growth factor (FGF), and bone morphogenetic protein (BMP), have been applied to periodontal defects.[4] In vivo and in vitro studies suggested that FGF, PDGF, and PDGF combined with IGF strongly stimulate periodontal tissue regeneration.[44-47] BMP has the potential for stimulating bone and cementum regeneration.[48,49] A human clinical trial in 1997 tested the safety and efficacy of several of these growth factors in the treatment of periodontal defects.[50] In the future, transplantation of MSCs with these growth factors will likely have even better beneficial effects on periodontal tissue regeneration. Growth factors play different roles depending upon the healing stage, target cells, and availability of matrix components.[4] The actions of these growth factors on residual periodontal cells and transplanted MSCs remain unknown.

In conclusion, the present findings suggest that autotransplantation of bone marrow MSCs is a useful option for periodontal tissue regeneration. Our culture system to expand bone marrow MSCs decreased the volume of bone marrow aspirates.[15]

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Enforced Expression of PU.1 Rescues Osteoclastogenesis from Embryonic Stem Cells Lacking Tal-1

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Key Words. Embryonic stem cells • Hematopoiesis • PU.1 • Transcription factor SCL/Tal-1 • Osteoclast

ABSTRACT

Transcription factor T-cell acute lymphocytic leukemia 1 (Tal-1) is essential for the specification of hematopoietic development. Mice lacking *Tal1* fail to generate any hematopoietic precursors. Using our co-culture system with stromal cells, we demonstrate that enforced expression of the transcription factor PU.1 under tetracycline control in *Tal1*-null embryonic stem (ES) cells rescues the development of osteoclasts and macrophage–like phagocytes. It was low efficiency compared with wild–type ES cells; other hematopoietic lineage cells of granulocytes, B cells, mast cells, megakaryocytes, and erythroid cells were not generated. Osteoclasts developed in this culture were multinucleated and competent for bone resorption. Their development depended on macrophage colony-stimulating factor and receptor activator of nuclear factor κB ligand. The majority of cells with the potential to differentiate into osteoclasts expressed fetal liver kinase 1 (Flk-1) and could be isolated using anti–Flk-1 antibody. These results suggest that the expression of PU.1 is a critical event for osteoclastogenesis and that Tal-1 may lie upstream of PU.1 in a regulatory hierarchy during osteoclastogenesis. STEM CELLS *2005;23:134–143*

INTRODUCTION

Hematopoiesis can be viewed as a hierarchy with hematopoietic stem cells (HSCs) at the top and progenitors and their descendents below. *Tal1* gene knockout (*Tal1*$^{-/-}$) mice succumb during embryogenesis due to a complete failure to produce blood cells [1, 2]. The product of the *Tal1* gene is believed to be essential for the initiation of hematopoietic development and the formation of HSCs. Consistent with the absence of hematopoietic cells and their descendants, transcription factor PU.1 (gene symbol, *Sfpi1*) is not detected in *Tal1*$^{-/-}$ embryonic stem (ES) cells induced to form hematopoietic cells in vitro [3] (in this report). PU.1

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controls development into myeloid and B-lymphoid cell lineages by regulating, in part, the receptors for macrophage colony-stimulating factor (M-CSF), Fms [4], and interleukin-7 (IL-7R) α [5], respectively. Myelopoiesis, B lymphopoiesis, and osteoclastogenesis are impaired in PU.1-deficient mice [6, 7]. Therefore, although it is not clear whether the expression of PU.1 is directly regulated by Tal-1, we wondered whether deficiency of Tal-1 might lead to failure to induce PU.1, resulting in the absence of some (or all) PU.1-dependent lineages.

Osteoclasts resorb bone matrices. They are ultimately derived from HSCs. Osteoclasts are distinguished by their multinuclearity and expression of tartrate-resistant acid phosphatase (TRAP) [8–10]. Osteoblasts and stromal cells support osteoclastogenesis by supplying essential factors, such as M-CSF [11] and receptor activator of nuclear factor κB ligand (RANKL) [12–14]. Previously we developed a culture system for osteoclastogenesis in which ES cells are co-cultured with stromal cells [15]. This system enables access to the entire program of osteoclastogenesis from undifferentiated ES cells to mature functional osteoclasts.

To assess the potential function of PU.1 downstream of Tal-1 in osteoclastogenesis, we have expressed exogenous PU.1 in *Tal1*$^{-/-}$ ES cells using the tetracycline (Tc)-off system [16, 17]. Surprisingly, we observe that enforced PU.1 expression induced osteoclast differentiation, even though the efficiency was lower than in wild-type ES cells. In this context, osteoclast development depended on M-CSF and RANKL, as in normal osteoclastogenesis. These findings suggest that PU.1 is a critical transcription factor for osteoclastogenesis; they are consistent with a role for PU.1 downstream of Tal-1 in a pathway culminating in osteoclastogenesis.

Materials and Methods

Cell Lines

A bone marrow–derived stromal cell line, ST2 [18], was maintained in RPMI-1640 (Roswell Park Memorial Institute: Gibco-Invitrogen Corp., Grand Island, NY, http://www.invitrogen.com), supplemented with 5×10^{-5} M 2-mercaptoethanol (2ME: Wako Pure Chemical Industries, Osaka, Japan; http://www.wako-chem.co.jp/english) and 5% fetal bovine serum (FBS: JRH Biosciences, Lenexa, KS; http://www.jrhbio.com). The OP9 stromal cell line [19] was cultured in minimum essential medium alpha (αMEM: Gibco-Invitrogen) supplemented with 20% FBS. ES cell lines J1 and *Tal1*$^{-/-}$ J1 [2], and *Sfp/1 PU1*-transfected *Tal1*$^{-/-}$ J1 ES cell lines were maintained in Dulbecco's modified essential medium (DMEM: Gibco-Invitrogen) supplemented with 10% knockout serum (Gibco-Invitrogen), 1% heat-inactivated FBS, 10^{-4} M 2ME, 1× nonessential amino acids (Gibco-Invitrogen), 2 mM L-glutamine (Gibco-Invitrogen), and leukemia inhibitory factor (LIF) equivalent to 1,000 U/ml on 0.1% gelatin-coated culture dishes. The RAW 264.7 macrophage cell line was maintained in αMEM supplemented with 10% FBS (JRH).

Constructs

Tal1$^{-/-}$ J1 ES cells were first transfected with Tc-regulated transactivator (tTA) driven by the CAG promoter [20] of the Tc-responsive promoter (CMV*-1)-puro^r (a gift from Dr. H. Niwa, RIKEN Kobe, Japan). These cells were cultured in the presence of 1 μg/ml puromycin and LIF for 8 days, and growing colonies were recovered. After Tc was added to the culture medium, clones sensitive to puromycin were chosen. These clones were secondarily transfected with a CMV*-1-ligated mouse *Sfpi1* cDNA sequence, followed by an internal ribosomal entry site (IRES) and green fluorescent protein (GFP) (CMV*-1-PU.1-IRES-EGFP, Fig. 1A). PSV2-*bsr* was simultaneously transfected. These cells were cultured with 1 μg/ml Tc and 3 μg/ml blastocidin S hydrochloride (blastocidin: Kaken Pharmaceutical Co., Tokyo, Japan; http://www.nni.nikkei.co.jp) for an initial 4 days, and during the following period, 0.5 μg/ml blastocidin was added to cultures. On day 12, colonies were picked up, and clones whose expression of GFP was regulated by Tc were chosen.

Differentiation of ES Cells

Undifferentiated ES cells were inoculated at 10^4 cells per well in six-well plates (Corning, NY; http://www.corning.com) on pre-seeded OP9 cells and cultivated in αMEM supplemented with 20% FBS (Thermo Trace, Melbourne, Australia; http://www.thermotrace.com.au/). On day 5, the cells were harvested and re-seeded at 10^5 cells per well in six-well plates onto confluent OP9 layers [19] in αMEM containing 20% FBS. Five days later, cells were harvested and osteoclasts were induced on ST2 stromal cells at 10^3 cells per 24-well plate (Corning) in αMEM/10% FBS (JRH) supplemented with 10^{-8} M 1α,25$(OH)_2D_3$ (Biomol Research Laboratories, Plymouth Meeting, PA; http://www.biomol.com) and 10^{-7} M dexamethasone (Dex: Sigma Chemical Corp., St. Louis, MO; http://www.sigma-aldrich.com). Six days later, TRAP staining was performed and TRAP$^+$ cells were counted under a microscope. These cultures are referred to as the osteoclast cultures (Fig. 1E) [21]. In the pit-formation assay, cells derived from ES cells were co-cultured with ST2 cells in the presence of 10^{-8} M 1α,25$(OH)_2D_3$, 10^{-7} M Dex, 10 μg/ml human M-CSF (provided by Dr. M. Takahashi, Otsuka Pharmaceutical, Tokyo), and 25 ng/ml human soluble RANKL (PeproTech, Rocky Hill, NJ; http://www.peprotech.com) on dentine slices (a gift from Dr. N. Udagawa, Matsumoto Dental University, Shiojiri, Japan).

Western Blot Analysis

Proteins were extracted from cultured cells by lysing the cells with lysis buffer containing EDTA and Triton X-100. Each sample (300 μg of protein) was separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane (Hybond ECL: Amersham Pharmacia Biotech, Piscataway, NJ; http://www.amershambiosciences.com). After the membrane was blocked with 5% skim milk (DIFCO Laboratories, Detroit, MI; http://www.vgdusa.com/DIFCO.htm), it was incubated with rabbit anti-mouse PU.1 antibody. PU.1 protein was visualized by using horseradish peroxidase (HRP)–conjugated goat anti-rabbit immunoglobulin-G (IgG) (Amersham Pharmacia Biotech) and the enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech). Protein extract from macrophage cell line RAW264.7 was used as positive control.

TRAP Staining

Cultured cells were fixed with 10% formaldehyde (Wako Pure Chemical Industries) for 10 minutes at room temperature and with ethanol/acetone (50:50 v/v: Wako) for 1 minute. After the cells were washed with PBS, they were stained with fast red violet LB-salt (Sigma) mixed with TRAP solution containing 59.3 M of sodium tartrate (Wako), 165.7 M sodium acetate (Wako), and 0.56 mg/ml naphthol AS-MX phosphate (Sigma) for 5 minutes at room temperature. Red-stained cells were visualized under a microscope and counted as $TRAP^+$ cells.

Reverse Transcription–Polymerase Chain Reaction (RT-PCR)

Total RNA was purified using ISOGEN (Nippon Gene, Toyama, Japan; www.nippongene.jp) and used as the template to synthesize cDNA using RevaTra Ace (Toyobo, Osaka, Japan; http://www.toyobo.co.jp/e) primed with oligo-dT from 1 μg of total RNA. Gene expression was analyzed by PCR using the following primers:

Tal1: 5'-CCT CCC CAT ATG AGA TGG AGA-3' and 5'-CCA TCC AGA GAG CTG CCA CA-3'

Both endogenous and exogenous *Sfpi1*:5' -GGA GAC AGG CAG CAA GAA AA-3' and 5' -GCG ACG GGT TAA TGC TAT GG-3').

Only endogenous *Sfpi1*: 5' -TTG ATC CCC ACC GAA GCA GG-3' and 5' -ATG TGG CGA TAG AGC TGC TG-3

Hbb (b-hemoglobin): 5'-CAC AAC CCC AGA AAC AGA CA-3' and 5'-CTG ACA GAT GCT CTC TTG GG-3'

Hbb-bh1 (ζ-hemoglobin): 5'-GCT CAG GCC GAG CCC ATT GG-3' and 5'-TAG CGG TAC TTC TCA GTC AG-3'

Pax5: 5'-CTA CAG GCT CCG TGA CGC AG-3' and 5'-TCT CGG CCT GTG ACA ATA GG-3'

Igb (Igβ/B29): 5'-GCA GCC CCA GGA ACT GGT CT-3' and 5'-CCT CCA TCC CAG CCT TGC CG-3'

Cpa3 (mast cell carboxypeptidase A precursors; mMC-CPA): 5'-CCG TGT GAA ACT CCA GAA TG-3' and 5'-GTG CAT GAA TGC CAC AGT CC-3'

Ppbp (platelet basic protein; PBP): 5'-ATG GGC TTC AGA

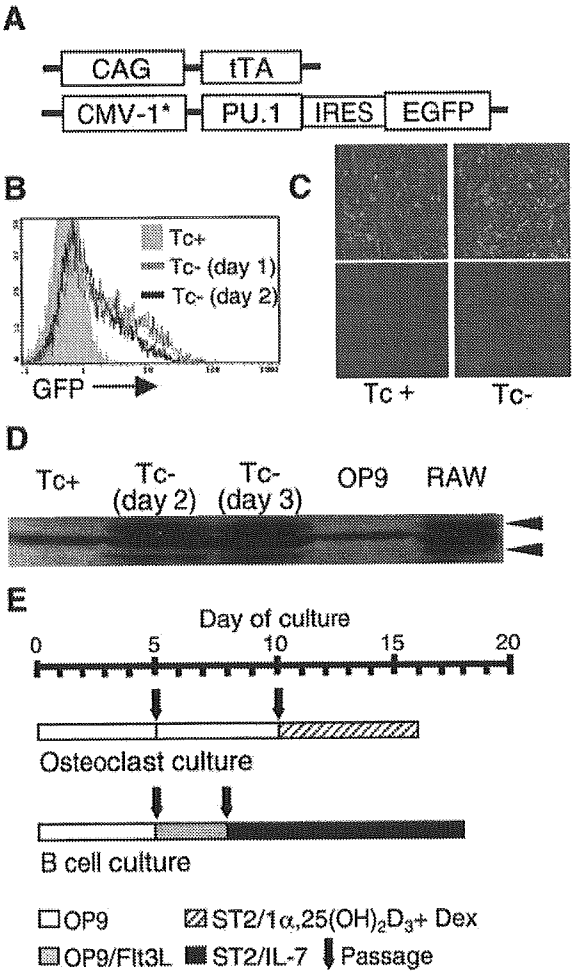

Figure 1. Establishment of Tc-controlled PU.1-expressing *Tal1*$^{-/-}$ES cell lines. **(A):** Constructs transfected into *Tal1*$^{-/-}$ ES cells (see Materials and Methods). **(B, C):** The expression of PU.1 was evaluated by monitoring the fluorescence of GFP. ES cells were cultured in the presence of LIF, and flow cytometric analysis was performed 1 or 2 days after Tc was withdrawn **(B)**. Microscopic analysis of phase contrast field and fluorescent microscopic fields of TUNE ES cells cultured with TC (Tc+) or without Tc (Tc-) TC for 2 days in upper and lower rows, respectively **(C)**. **(D):** PU.1 protein was detected in cell lysates prepared on day 2 or day 3 from TUNE ES cells cultured on OP9 cells after Tc was withdrawn starting on day 0, and from a macrophage cell line, RAW264.7 (RAW). Cell lysates from TUNE ES cells cultured in the presence of Tc throughout the culture period (Tc+) or from OP9 cells did not contain detectable PU.1 protein. **(E):** Schematic diagram of culture systems in this study.

CTC AGA CC-3' and 5'-GAC GAT TCT CTT GAC GCC AG-3'
Hprt: 5'-AGT TCT TTG CTG ACC TGC TG-3' and 5'-GCT TTG TAT TTG GCT TTT CC-3'
Kdr (Flk-1): 5'-ACC GCC TCT GTG ACT TCT TT-3' and 5'-TGT CCC CTG CAA GTA ATC TG-3'

PCR was performed under the following conditions: an initial cycle consisting of 94°C for 4 minutes, annealing at 60°C for 3 minutes, and 72°C for 1 minute, followed by 34 cycles or 44 cycles of 94°C for 1 minute, annealing at 60°C for 1 minute, and 72°C for 1 minute. cDNA prepared from cells expressing each gene tested was used as a positive control (Posi). Distilled water (DW) was used for negative control.

Detection of Phagocytes

After ES cells were cultured on OP9 layers for 5 days in αMEM supplemented with 20% FBS (Thermo Trace), phagocytes were induced on pre-seeded ST2 layers for 6 days in αMEM supplemented with 10% FBS (Thermo Trace) and M-CSF. Cells were incubated with fluoresbrites microspheres (Poly-Science, Niles, IL; http://www.polyscience.com) for 2 hours at 37°C. After washing, these cells were harvested by trypsinization. They were re-seeded into 100-mm culture dishes (Corning) to easily distinguish phagocytes from stromal cells. Under fluorescent microscopy, the number of phagocytes that had more than 20 fluorescent beads was counted. To observe appearances of phagocytes, the cells were recovered, cytospun, and stained with May-Grunwald-Giemsa solution.

Immunochemical Staining

Phagocytes were cultivated on cover glasses (Matsunami Glass Ind., Osaka, Japan; http://www.matsunami-glass.co.jp/e) and were incubated with latex beads (Sigma) for 2 hours at 37°C. After washing, cells were fixed with methanol for 15 minutes and then incubated with Bloking Ace (Snow Brand Milk Products, Co. Ltd., Tokyo, japan; http://www.snowbrand.co.jp) for 20 minutes at 4°C. Macrosialin was detected with rat anti-mouse macrosialin antibody (FA11; Serotec, Raleigh, NC; http://www.serotec.com), biotinylated-goat anti-rat IgG antibody (KPL, Kirkegaard & Perry Laboratories, Gaithersburg, MD; http://www.kpl.com), streptavidin-peroxidase (KPL), and DAB reagent set (KPL).

Results

Establishment of PU.1-Expressing *Tal1*$^{-/-}$ ES Cell Lines under the Tc-off System

To provide a system for studying the regulated expression of PU.1, cDNA was placed under the control of the Tc-off system and introduced into *Tal1*$^{-/-}$ ES cells (Fig. 1A). Three independent ES clones (designated TUNE-1 to TUNE-3) were thereby established. Expression of the exogenous PU.1 was inhibited completely by 100 ng/ml Tc, and expression was induced by withdrawal of Tc from the culture. The expression of PU.1 was evaluated by monitoring the fluorescence of GFP (Fig. 1B, C). The expression of exogenous PU.1 reached a maximum level 1 day after Tc withdrawal (Fig. 1B). All three clones behaved similarly; therefore, representative data from TUNE-1 ES cells are shown unless otherwise indicated.

We used a stepwise culture system to induce osteoclasts from ES cells (Fig. 1E, osteoclast culture). PU.1 protein was detected on days 2 and 3 in PU.1-expressing (PU.1-on) TUNE ES cells in the osteoclast cultures but not in TUNE ES cells cultured in the presence of Tc (PU.1-off) or in OP9 cells (Fig. 1D).

Osteoclastogenesis from PU.1-Expressing TUNE ES Cells

Myelopoiesis and osteoclastogenesis are impaired in PU.1-null mice. To determine if exogenous PU.1 could rescue osteoclastogenesis in TUNE ES cells, TUNE ES cells were cultivated in osteoclast cultures. Multinucleated TRAP$^+$ cells displaying more than six nuclei were induced from PU.1-on TUNE ES cells following Tc withdrawal on day 2 or 3 of culture. Although cells derived from TUNE ES cells existed, no TRAP$^+$ cells were generated when Tc was present (Fig. 2A). This is consistent with our previous report that no TRAP$^+$ cells were induced from *Tal1*$^{-/-}$ ES cells [22]. PU.1 expression was restricted in GFP$^+$ fraction (data not shown), and TRAP$^+$ cells were mainly induced from GFP$^+$ fraction. Non-specific toxicity of Tc could not account for these findings, as the number of TRAP$^+$ cells derived from parental J1 ES cells was not affected by the presence of Tc.

To determine whether the TRAP$^+$ cells were functional osteoclasts, a pit-formation assay was performed. On day 10 of the osteoclast culture, harvested cells were induced to form osteoclasts on dentine slices by co-culturing with ST2 cells supplemented with 1α, 25$(OH)_2D_3$, Dex, human M-CSF, and human RANKL. On day 39, pit formation was observed in cultures of PU.1-on TUNE ES cells, whereas no pits were formed under the PU.1-off condition (Fig. 2A). These results indicate that mature functional osteoclasts were induced from *Tal1*$^{-/-}$ ES cells following expression of PU.1.

M-CSF and RANKL are known to be critical for osteoclastogenesis. To investigate the requirement for these factors, an antagonistic anti-Fms antibody or a decoy receptor of RANKL, osteoprotegerin (OPG) [23] was added to the final phase of the osteoclast cultures on ST2 cells. Both factors completely inhibited osteoclastogenesis from PU.1-on TUNE ES cells (Fig. 2B). These results demonstrate that M-CSF and RANKL are also essential for the development of osteoclasts derived from PU.1-on TUNE ES cells.

Macrophage-Like Phagocytes Were Induced from PU.1-on TUNE Cells

PU.1 is an essential transcription factor for differentiation of macrophages. Since osteoclast precursors are thought to be derived from monocyte-lineage cells, it may be possible to induce monocyte and macrophage lineages by enforced expression of PU.1. Therefore, PU.1-on TUNE cells were cultivated on OP9 stromal layers for 5 days, then co-cultured with ST2 stromal cells supplemented with M-CSF for a further 6 days. Finally, these cells were incubated with fluorescent microspheres. Phagocytes that had more than 20 microspheres were counted under the fluorescent microscopy.

The numbers of phagocytes induced from TUNE-2 cells was one-fortieth compared with that from J1 wild type ES cells. Few cells with microspheres were observed in the cultures of *Tal1*$^{-/-}$ J1 ES cells and PU.1-off TUNE-2 cells (Fig. 3A). These cells were cytospun and stained with May-Grunwald-Giemsa solution to observe their appearances.

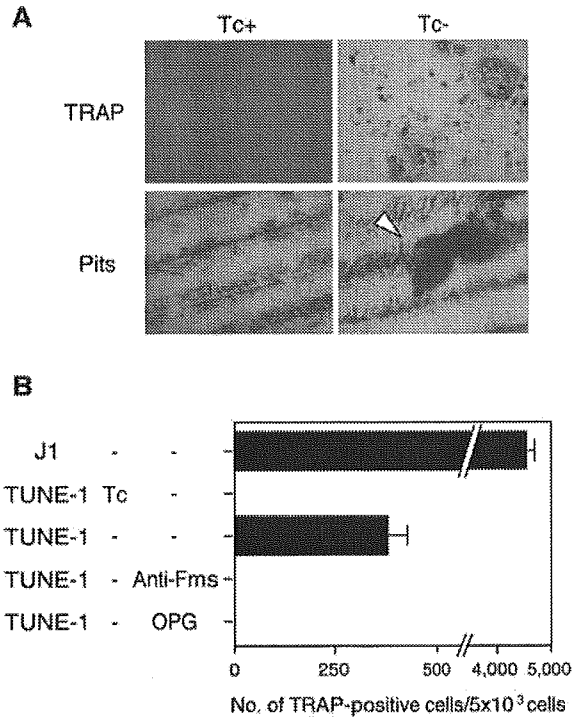

Figure 2. Enforced expression of PU.1-rescued osteoclastogenesis from *Tal1*$^{-/-}$ ES cells. TUNE ES cells were cultured on OP9 cells for 10 days. Tc was withdrawn from day 2 (Tc–). The cells harvested on day 10 were induced to form osteoclasts on ST2. **(A):** Osteoclasts were induced in the schedule as described in Figure 1E (osteoclast culture). TRAP–positive cells were detected as red cells by TRAP staining (upper row). Osteoclasts were induced on dentine slices by co-culturing with ST2 in the presence of $1\alpha,25(OH)_2D_3$, Dex, M-CSF, and RANKL for a further 29 days. Pits are indicated by an arrowhead (lower row). Neither osteoclast nor pits was observed in the presence of Tc (Tc+). **(B):** After ES cells were cultivated on OP9 cells for 10 days, the cells harvested from cultures of parental wild-type J1 ES cells, or TUNE ES cells continuously treated with Tc (Tc) or from which Tc was withdrawn on day 2(–) were co-cultured on ST2 layers in the presence of $1\alpha,25(OH)_2D_3$ and Dex for 6 days. TUNE ES cells from which Tc had been withdrawn were cultured with an anti-Fms antagonistic antibody (Anti-Fms) or OPG during the final 6 days. Each column represents the mean ± SD of triplicate cultures.

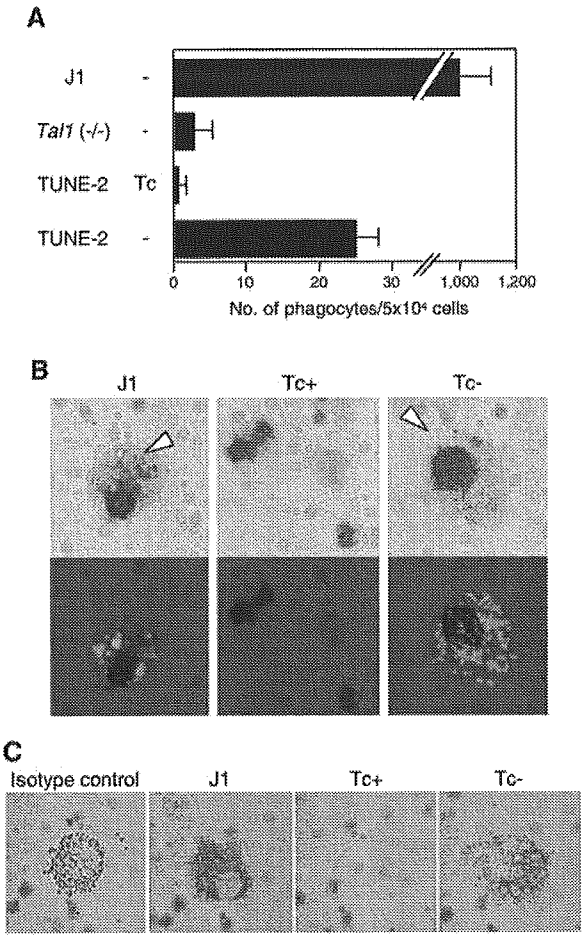

Figure 3. Macrophage-like phagocytes were induced from PU.1-on TUNE-2 cells. After ES cells were cultured on OP9 layers for 5 days, phagocytes were induced on ST2 layers supplemented with M-CSF for 6 days. Phagocytes with fluorescent beads **(B)** or nonfluorescent latex beads **(C)** were detected under microscopy. **(A):** The number of phagocytes was counted under fluorescent microscopy. J1: wild-type ES cells; *Tal1*$^{-/-}$: Tal-1-deficient J1 ES cells. **(B):** Cytospun cells were stained with May-Grunwald-Giemsa solution (upper row). Macrophage-like cells (arrowheads: upper row) with fluorescent microbeads (lower row). Tc–: cells cultured without Tc; Tc+: cells cultured with Tc. **(C):** Cells were stained with anti-macrosialin antibody. Macrosialin-positive cells were observed as brown. Isotype control: instead of anti-macrosialin antibody, anti-B220 antibody was used as the first antibody.

The bead-carrying cells from J1 ES and PU.1-on TUNE-2 cells looked like monocytes or macrophages (Fig. 3B). In contrast, almost all cells with microspheres in *Tal1*$^{-/-}$ ES and PU.1-off TUNE-2 cell cultures were the feeder stromal cells (Fig. 3B).

To further clarify their phenotype, phagocytes were stained with the antibody directed to macrosialin, which is macrophage-restricted antigen [24]. Macrosialin was detected in phagocytes with latex beads from cultures of both wild-type J1 and PU.1-on TUNE cells, while no macrosialin-positive cells were detected in cultures of *Tal1*$^{-/-}$deficient ES cells. This is accordance with observation that no hematopoiesis occurs from *Tal1*$^{-/-}$ ES. These results suggest that monocyte- and macrophage-like phagocytes were also induced from Tal-1–deficient ES cells by enforced expression of PU.1.

Erythrocytes, Mast Cells, and Megakaryocytes Were Not Induced from TUNE-1 ES Cells

To assess whether other hematopoietic lineages might be rescued by the expression of PU.1, transcripts for several lineage-associated genes were examined by RT-PCR. ES cells were cultured on OP9 cell layers, and on day 5, cDNAs were synthesized from total RNA of cultured cells. Exogenous PU.1, but not endogenous PU.1, was expressed. Lineage-related genes (β-globin and ζ-globin for erythrocytes, mMC-CPA for mast cells, and platelet basic protein [PBP] for megakaryocytes) were analyzed. Transcripts for these markers were detected in cultures of wild-type J1 ES cells; none, however, were observed in PU.1-on TUNE ES cells (Fig. 4). These data indicate that exogenous PU.1 fails to rescue the development of mast cells, erythrocytes, and megakaryocytes in *Tal1*$^{-/-}$ ES cells.

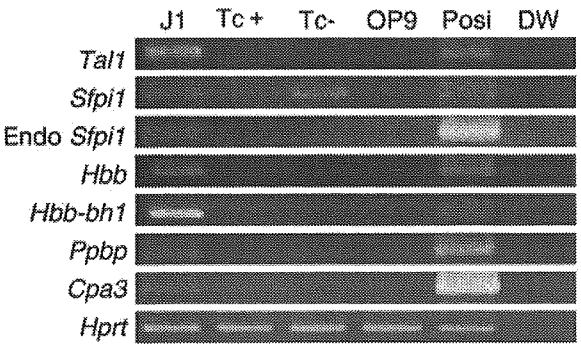

Figure 4. Several genes expressed in mast cells, erythrocytes, and megakaryocytes were not detected in PU.1-on TUNE-1 embryonic stem (ES) cells. After ES cells were cultured on OP9 cells for 5 days, mRNA was purified. The expression of several genes was analyzed by reverse transcription-polymerase chain reaction (RT-PCR). J1: cultured cells from wild-type J1 ES cell line; Tc+: TUNE ES cells cultured in the presence of tetracycline (Tc); Tc–: TUNE ES cells cultured with removal of Tc on day 2; OP9: stromal cells; Posi: positive controls.

Gene Expression of the B-Cell Lineage Was Not Detected in the Hematopoiesis from TUNE ES Cells

PU.1 is considered essential for B lymphopoiesis [25]. To assess whether exogenous PU.1 expression was able to rescue B lymphopoiesis from TUNE ES cells, the ES cells were cultured on OP9 stromal cells for 8 days, and then re-cultured on ST2 cells for an additional 10 days. Flt3-ligand (20 ng/ml) and IL-7 (20 U/ml) were added to the cultures during days 5–8, and days 8–18, respectively (Fig. 1E) [26].

Clusters of small cells were observed in parental J1 ES cell cultures on day 18, but no clusters appeared in cultures of TUNE ES cells with or without Tc from day 2 (Fig. 5A). B-lineage–expressed genes were monitored by RT-PCR. *Pax5* and *Igb* were not expressed on cells from TUNE ES cells even in the absence of Tc, although these genes were detected in the cultured cells from wild-type J1 ES cells (Fig. 5B). These results indicate that B-lineage cells are not induced from *Tal1*$^{-/-}$ ES cells with enforced expression of PU.1 under these culture conditions.

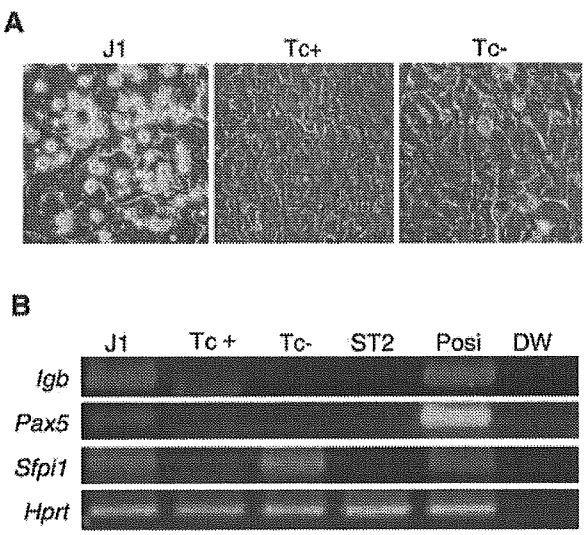

Figure 5. B-lymphocyte–related genes were not expressed in PU.1-on TUNE-1 embryonic stem (ES) cells. B-lineage cells were induced in the schedule as described in Figure 1E (B cell culture). **(A):** The appearance of each culture on day 18. **(B):** mRNA was prepared on day 18 of cultures, and the expressions of each gene were detected by reverse transcription-polymerase chain reaction (RT-PCR). J1: cultured cells from wild-type J1 ES cell line; Tc+: cells cultured in the presence of tetracycline (Tc); Tc–: cells cultured with removal of Tc on day 2; ST2: stromal cells; Posi: positive controls.

Efficiency of Osteoclastogenesis from *Tal1*$^{-/-}$ ES Cells Depended on the Timing of the Expression of PU.1

Since the Tc-off system permits temporal regulation of PU.1 expression in TUNE ES cells, we examined the relationship between the efficiency of osteoclastogenesis and the timing of PU.1 expression. After switching the culture conditions to induce differentiation on OP9 cells, Tc was withdrawn from cultures of TUNE-1 and TUNE-2 ES cells on successive days. Withdrawal of Tc on days 0–4 allowed osteoclastogenesis to take place more efficiently than withdrawal after 6 days (Fig. 6A). No osteoclasts were induced in cultures of either ES clone subjected to the continuous exposure to Tc. Some variability in efficiency was observed in each experiment and clone, but the trend that day 2 to day 4 was the appropriate timing to express PU.1 for induction of TRAP$^+$ cells from Tal-1–deficient ES cells was reproducible in all experiments and clones. These results indicate that the timing of expression of PU.1 is an important parameter in osteoclast induction, and day 2 to 4 is the appropriate timing. Since endogenous expression of PU.1 in cultures of undifferentiated wild-type J1 ES cells was first detected on day 5 rather than day 3 (data not shown), the timing of PU.1 expression for the rescue of osteoclastogenesis in *Tal1*$^{-/-}$ ES cells preceded the normal pattern by 1–2 days.

Efficiency of osteoclastogenesis from TUNE cells was significantly lower than that from wild-type J1 ES cells. To determine whether this resulted from the low expression of PU.1, semi-quantitative RT-PCR was performed on day 5 of osteoclast culture. Expression of the *Kdr* (Flk-1) gene was used as the control of amount of ES cell–derived mRNA because frequency of Flk-1–expressing cells was not different among J1, PU.1-on, and PU-1-off TUNE cells, and Flk-1 was not expressed in OP9 stromal cells. Among three TUNE clones, TUNE-2 cells were the most efficiently differentiated to osteoclasts, but the efficiency was less than one-hundredth of that from wild-type ES cells. Interestingly, the expression of PU.1 was quantitatively not different from J1 cells (Fig. 6B). These results suggested that an adequate amount of expression of PU.1 was not enough to completely rescue osteoclastogenesis from *Tal1*$^{-/-}$ ES cells.

Flk-1$^+$ Cells Efficiently Differentiated to Osteoclasts by PU.1 Expression

Based on the results presented above, we hypothesized that hematogenic cells—for example, hemangioblasts and mesodermal cells immediately before their commitment to the hematopoietic cell lineage—might differentiate into osteoclasts with enforced expression of PU.1. Hematopoietic cells appear in vivo on embryonic day 7.5, whereas Kit$^+$ blood cells are first observed on day 4 of ES cell culture [27]. At this time, Flk-1$^+$ cells containing hematopoietic precursors are present in ES cell cultures [28]. To assess the candidate cell populations with potential to differentiate into osteoclasts upon PU.1-expression, we compared the efficiency of osteoclastogenesis in Flk-1$^+$ and Flk-1$^-$ fractions.

J1 and TUNE ES cells were differentiated on OP9 cells. On day 3, Tc was withdrawn to allow for expression of PU.1. On day 4, the cells were harvested. No significant difference in the percentage of Flk-1$^+$ cells on day 4 was observed in cells derived from wild-type versus TUNE ES cells, either in the PU.1-off or -on condition (Fig. 7A). We separated the harvested cells into the column-bound (positive) and -passed

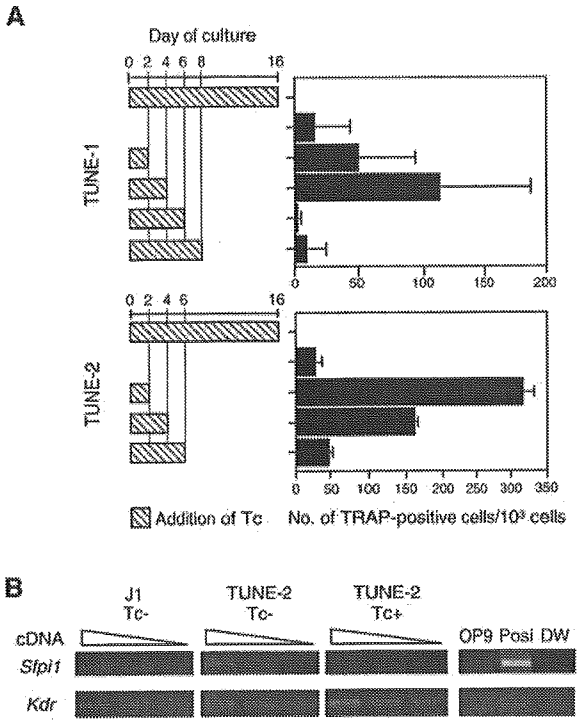

Figure 6. Different efficiencies of osteoclastogenesis from *Tal1*$^{-/-}$ ES cells depended on timing of expression of PU.1. **(A):** TUNE-1 and TUNE-2 ES cells were cultured in the schedule as described in Figure 1E, osteoclast culture. Shaded bars represent periods of addition of Tc. Each column represents the mean number ± SD of TRAP-positive cells in triplicate cultures. **(B):** The amount of gene expression of *Sfpi1* (PU.1) in TUNE-2 cells was not lower than that in cells derived from J1 ES cells. On day 5 of osteoclast culture, mRNA was purified, and semi-quantitative RT-PCR using sequential doses of cDNA (1, 1/2, 1/4) was performed. To estimate the amount of mRNA derived from ES cells, *Kdr* (Flk-1) RT-PCR products from serially doses of cDNA were indicated. Tc -: cells cultured without Tc; Tc +: cells cultured with Tc.

(negative) fractions by magnetic cell sorting using anti–Flk-1 antibody. The negative fraction contained 0% to 0.05% Flk-1$^+$ cells (Fig. 7A). The fractionated cell populations were induced to form osteoclasts on ST2 cells supplemented with 1α,25$(OH)_2D_3$ and Dex for 6 days. The number of TRAP$^+$ cells from the positive fraction of J1 ES cells was increased >200-fold over the number from the negative fraction. In TUNE ES cells expressing PU.1 from day 3, TRAP$^+$ cells developed from the positive fraction and not the negative fraction. Neither fraction of PU.1-off TUNE-1 ES cells generated TRAP$^+$ cells (Fig. 7B). These results suggest that Flk-1$^+$ cells on day 4 of osteoclast culture have the potential to differentiate to osteoclasts in response to PU.1 expression in the absence of Tal-1.

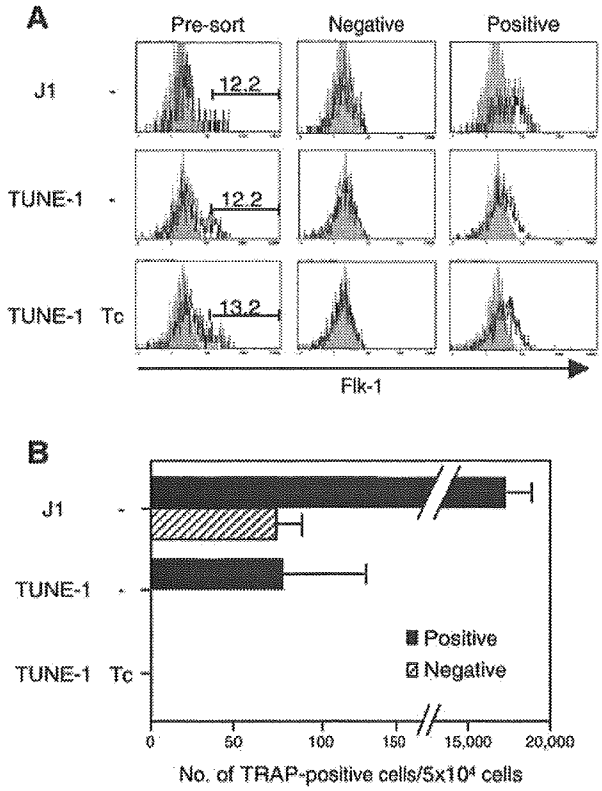

Figure 7. Flk-1$^+$ cells included osteoclast precursors of PU.1-on TUNE-1 embryonic stem (ES) cells. After ES cells were cultivated on the OP9 layer for 4 days, the cells were harvested and subjected to magnetic sorting using Flk-1 antibody. **(A):** The proportion of Flk-1$^+$ cells was analyzed by a flow cytometer. Negative: the column-passed fractions by the magnetic cell sorting using anti–Flk-1 antibody. Positive: the column-bound fraction. **(B):** Osteoclasts were induced from the sorted cells cultured on ST2 cells in the presence of 1α,25$(OH)_2D_3$ and dexamethasone (Dex) for 6 days. Each column represents the mean numbers ± SD of artrate-resistant acid phosphatase (TRAP)–positive cells in triplicate cultures. Tc: cells cultured in the presence of tetracycline (Tc).

Discussion

Tal1 is essential for the development of all hematopoietic lineages and HSCs [1, 2]. The events downstream in the regulatory hierarchy from Tal-1 to specific lineages are largely unknown. Presumably, Tal-1 participates in the transcriptional control of critical downstream factors, perhaps including PU.1. In this study, we demonstrated that enforced PU.1 expression rescues osteoclastogenesis from *Tal1*$^{-/-}$ ES cells, even though its efficiency was lower than that from J1 ES cells. These results suggest that PU.1 may serve as a critical factor for the osteoclast lineage. In addition, they raise the possibility that one role of Tal-1 may be to activate expression of PU.1 in a regulatory hierarchy. In these experiments, the expression of the endogenous *Sfpi1* locus was not activated by exogenous PU.1 (Fig. 4). These observations suggest that PU.1 does not participate in a positive autoregulatory loop; they also indicate that the chromatin structure surrounding the endogenous *Sfpi1* gene may be inaccessible before Tal-1 is expressed.

Macrophage-like phagocytes were also induced from PU.1-on TUNE cells. A small number of blood cell–like round cells appeared when PU.1-on TUNE cells were cultured in IL-3, GM-CSF, or G-CSF. These cells were cytospun and observed under microscopy. They looked like monocytes but not granulocytes (data not shown). We prefer the possibility that not only PU.1 but also other transcription factor(s) induced by Tal-1 were needed for granulopoiesis, even though we cannot rule out the possibility that it might be a result of the low sensitivity of granulocyte detection.

In our B-lymphoid culture system, the expression of B-cell–related genes was not detected from PU.1-expressing TUNE ES cells. As shown in Figure 5A, few, if any, hematopoietic-like cells were observed in PU.1-on TUNE ES cell cultures. Since GFP$^+$ cells, indicative of ES cell derivatives (data not shown), and PU.1 transcripts were detected by RT-PCR (Fig. 5B), ES-derived cells were present, though unable to undergo B lymphopoiesis. *Pax5*-deficient mice have pro-B cells, and the *Igb* gene is detected [29]. Neither gene was expressed in PU.1-on TUNE ES cell cultures. Thus, the number of B cells generated might have been very small because of the failure of proliferation, or because B-lineage differentiation might need PU.1 and other transcription factor(s). We have not excluded the possibility that the level or timing of PU.1 expression may not have been suitable for B lymphopoiesis under our conditions (Tc withdrawal on day 2).

The efficiency of osteoclastogenesis of PU.1-on TUNE ES cells was significantly lower than that of wild-type ES cells. On culture day 5, the frequency of osteoclast precursors from wild-type ES cells was 1/2,485, while that from PU.1-on TUNE-1 ES cells was 1/11,389 to 1/39,063. We had

expected the low dosage of endogenous PU.1 compared with J1 ES cells might be responsible for the incomplete rescue of osteoclastogenesis. However, a comparable amount of PU.1 was detected in TUNE cells (Fig. 6B). This suggests that differentiation of osteoclasts was induced by PU.1, but orchestration of Tal-1 or other genes induced by Tal-1 was needed for efficient osteoclastogenesis.

We cannot rule out the possibility that the timing of the expression of PU.1 may be inappropriate. We showed in Figure 6 that the timing was critical for osteoclastogenesis from PU.1-on TUNE-1 and TUNE-2 ES cells. By regulating the time of expression of PU.1, we found that days 2–4 were most appropriate for rescue of osteoclastogenesis from *Tal1*-null ES cells. The efficiency of osteoclastogenesis by removal of Tc from day 0 was lower than that from day 2–4. This might show that exogenous PU.1 expression from initiation of cultures affected other than hematopoietic cell lineages. Moreover, according to the decline of osteoclastogenesis by removal of Tc after day 4, these cells might not be maintained in these cultures. Since mesodermal derivatives or hemangioblasts were thought to appear on day 4 of our culture system, they might be candidates of cells competent to differentiate into osteoclasts. These cells might exist in the richest on day 4 of all culture days. Since osteoclasts are of hematopoietic origin, we infer that PU.1 expression in *Tal1*-null ES cells bypasses a developmental block in a mesodermal derivative or hemangioblast. This interpretation is consistent with our finding that Flk-1$^+$, rather than Flk-1$^-$, cells are the source of osteoclasts generated upon PU.1 expression. The development of Flk-1$^+$ cells in the absence of Tal-1 has been reported previously [30].

Tal-1 is indispensable for normal osteoclastogenesis [22]. The results presented here raise the possibility that Tal-1 may act during a brief time interval to establish the hematopoietic program. Once it has activated critical downstream transcription factors, its role in the generation of selected lineages might be dispensable. This is consistent with the observation that Tal-1 is largely dispensable for maintenance of the adult hematopoietic system, once it has been established, except for differentiation of erythroid precursors and megakaryocytes [31]. Ectopic expression of transcription factors, such as illustrated here, may provide a means to define critical factors acting downstream of *Tal1* in the hematopoietic regulatory hierarchy.

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Lipopolysaccharide-Induced Osteoclastogenesis in Src Homology 2-Domain Phosphatase-1-Deficient Viable Motheaten Mice

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Osteoclasts are hemopoietic cells that participate in bone resorption and remodeling. Receptor activator of nuclear factor-κB ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) are critical for development of osteoclasts. The Toll-like receptor (TLR) family shares some of the downstream signaling with RANK. The TLR4 ligand, lipopolysaccharide (LPS), is reported to accelerate bone lysis; however, signaling via TLRs has never been reported to induce osteoclastogenesis without RANKL. In this study we showed that significant numbers of mature osteoclasts were generated from protein tyrosine phosphatase Src homology 2-domain phosphatase-1-defective *Hcph*$^{me\text{-}v}$/*Hcph*$^{me\text{-}v}$ (*me*^{v}/*me*^{v}) bone marrow cells in the presence of M-CSF and LPS without addition of RANKL in culture. This M-CSF plus LPS-induced osteoclastogenesis was not inhibited by an anti-TNFα antagonistic antibody or by osteoprotegerin, a decoy receptor for RANKL. The replacement of RANKL by TLR ligands only occurred with LPS. Other ligands, a peptidoglycan for TLR2 or an unmethylated CpG oligonucleotide for TLR9, did not support osteoclast generation. The osteoclast precursors as well as RANKL-responsive osteoclast precursors were present in the Kit-positive cell-enriched fraction of bone marrow cells. Although *me*^{v}/*me*^{v} bone marrow cells required a comparable concentration of RANKL or TNFα as wild-type cells for the initiation of osteoclastogenesis, the numbers of multinucleated osteoclasts in *me*^{v}/*me*^{v} bone marrow cultures were significantly increased by the equivalent dose of RANKL or TNFα in the presence of M-CSF. These results indicate that a defect of Src homology 2-domain phosphatase-1 function not only accelerates physiological osteoclast development by RANKL/RANK, but also acquires a novel pathway for osteoclastogenesis by LPS. (*Endocrinology* 145: 2721–2729, 2004)

SIGNALING VIA MACROPHAGE-colony-stimulating factor (M-CSF) and receptor activator of nuclear factor-κB (NF-κB) ligand (RANKL) plays an essential role for the development of osteoclast precursors (OCPs) into tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells (MNCs) that resorb and remodel bones (1–4). Functional M-CSF-deficient *Csf1*^{op}/*Csf1*^{op} (*op/op*) mice lack mature osteoclasts, resulting in severe osteopetrosis (5, 6). The *op/op* mice carrying the *Bcl2* transgene are cured of the disease, and aged *op/op* mice show spontaneous reversal of osteopetrosis (7, 8). Therefore, signaling through the M-CSF receptor (Fms) is thought to function as a cell survival signal, and vascular endothelial growth factor partially replaces its function (9). Viable motheaten (*me*^{v}/*me*^{v}) mice mutated at the Src homology 2-domain phosphatase-1 (SHP-1) protein tyrosine phosphatase (*Hcph*) locus (10, 11) show accelerated osteoclastogenesis *in vitro* and *in vivo* (12, 13). Especially, numbers of TRAP^{+} MNCs were significantly increased (12, 13). In mice that are doubly homozygous for mutations at the *Csf1* and *Hcph* loci (*op/op me*^{v}/*me*^{v} mice) (12), partial, but significant, bone marrow (BM) formation was observed, indicating that the signaling via the receptor tyrosine kinases is regulated negatively by SHP-1 (10, 11).

RANK and its ligand, RANKL, are members of TNF receptor and TNF superfamilies, respectively (14, 15). Targeted mutation in either gene prevents the development of osteoclasts, resulting in the development of osteopetrosis (16, 17). Therefore, RANK/RANKL signaling is thought to be essential for osteoclastogenesis. However, several recent studies using RANK-knockout (RANK-KO) mice showed that osteoclastogenesis was induced without RANK/RANKL signaling (18, 19). Although it is still not clear whether RANK/RANKL signaling is totally absent (20), mouse TNFα was reported to induce osteoclastogenesis *in vivo* and *in vitro* in the presence of M-CSF (18, 21).

Signaling via members of the Toll-like receptor (TLR) superfamily shares some of the downstream pathways, such as TNF receptor-associated factor 6 (TRAF6), NF-κB, and

Abbreviations: Ab, Antibody; BM, bone marrow; FBS, fetal bovine serum; Hprt, hypoxanthine phosphoribosyl transferase; IRAK, IL-1 receptor-associated kinase; KO, knockout; LPS, lipopolysaccharide; M-CSF, macrophage-colony-stimulating factor; MNC, multinucleated cell; NF-κB, nuclear factor-κB; OCP, osteoclast precursor; ODN, oligonucleotide; OPG, osteoprotegerin; PE, phycoerythrin; PEC, peritoneal cavity cell; PGN, peptidoglycan; RANK, receptor activator of nuclear factor-κB; RANKL, receptor activator of nuclear factor-κB ligand; SHP, Src homology 2-domain phosphatase; TLR, Toll-like receptor; TRAF, TNF receptor-associated factor; TRAP, tartrate-resistant acid phosphatase; TREM, triggering receptor expressed on myeloid cell.

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