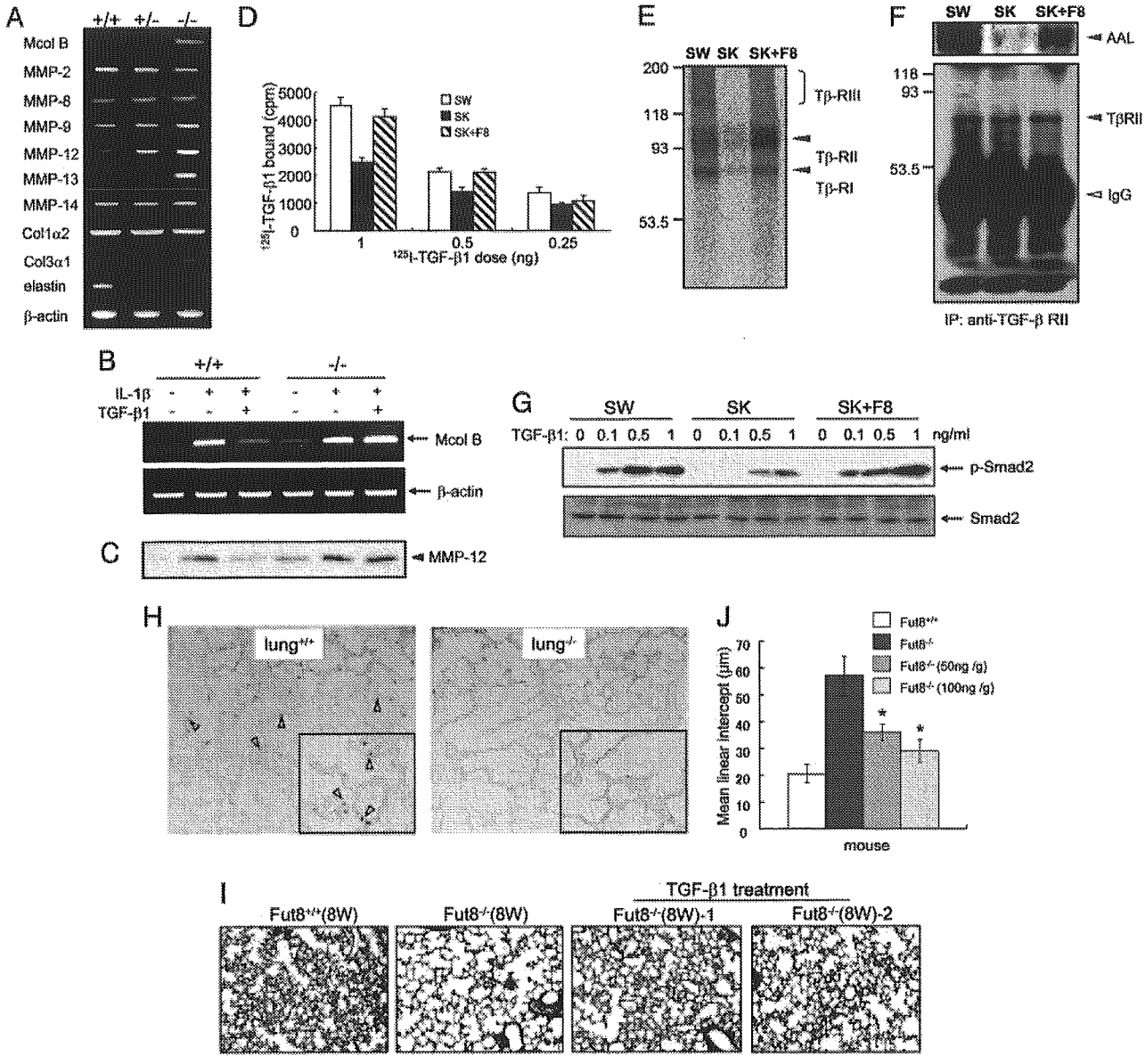

Fig. 3. Enhancement of some MMPs expression through down-regulation of TGF-β-receptor-mediated signaling in *Fut8*$^{-/-}$ lung and embryonic fibroblasts. (*A*) RT-PCR analysis of emphysema-relating genes (see Table 1, which is published as supporting information on the PNAS web site). Total RNAs from 18-day-old *Fut8*$^{+/+}$ (+/+), *Fut8*$^{+/-}$ (+/−), and *Fut8*$^{-/-}$ (−/−) lungs were used as template. β-Actin RNA is shown as a loading control. (*B*) Effects of IL-1β and TGF-β1 on McolB expression. These fibroblasts were preincubated with or without TGF-β1 (1 ng/ml) for 3 h and then further incubated with or without IL-1β (2 ng/ml) for 24 h. Total RNA was isolated and used as template. (*C*) Effects of IL-1β and TGF-β on protein expression of MMP-12 secreted into culture media. These fibroblasts were incubated with or without IL-1β and TGF-β1 in the absence of FCS. After incubation for 24 h, the conditioned media were concentrated and subjected to electrophoresis for Western blot. (*D*) Binding of ^{125}I-TGF-β1 to its receptors on the cell surface. These cells from *Fut8*$^{+/+}$ (+/+) and *Fut8*$^{-/-}$ (−/−) primary fibroblasts immortalized with SV40 large T, SW, and SK, respectively, or SK restored with *Fut8* (SK+F8), were incubated with different amounts of radiolabeled TGF-β1 as indicated and 10 ng of cold TGF-β1 for 2 h on ice. Cell lysate radioactivity was measured. (*E*) ^{125}I-TGF-β1 was bound and cross-linked to its receptors on cell surface. The cultured cells were incubated with 250 pM ^{125}I-TGF-β1 for 2 h at 4°C, and then cross-linked with reagent BS^3. (*F*) Analysis of fucosylation levels on TGF-β receptor II. TGF-β1 receptor II was immunoprecipitated from whole-cell lysates and then subjected to electrophoresis on 8% SDS/PAGE. After electroblotting, blots were probed by ALL lectin (*Upper*) and anti-TGF-β receptor II (*Lower*). (*G*) Effects of phosphorylated Smad2 levels on TGF-β1 stimulation. Serum-starved cells were treated with or without TGF-β1 at the indicated concentrations for 5 min and solubilized in lysis buffer as described in *Materials and Methods*. The cell lysates were detected by immunoblotting of anti-phospho-Smad2 antibody (*Upper*) and anti-Smad2 antibody (*Lower*). (*H*) The lung sections of 4-day-old mice were pretreated with hydroxygen blocking for 10 min at 37°C and then incubated with rabbit anti-human P-Smad2 antibody for 16 h at 4°C. The arrowheads indicate positive staining in *Fut8*$^{+/+}$ lung. (*I*) Therapeutic administration of recombinant TGF-β1 rescues emphysema-like changes in *Fut8*$^{-/-}$ mice. The surviving postnatal 18-day-old *Fut8*$^{-/-}$ mice were treated with or without recombinant TGF-β1 (50 or 100 ng/g of mouse body weight) for 20 times of injection every 2 days, and then the lung sections were subjected to hematoxylin/eosin staining. (*J*) Quantitative analyses of the pulmonary alveolar sizes were performed by mean linear intercept as described above. The diameters of the pulmonary alveoli were shown as the mean ± SD from three independent experiments. Statistical analysis was performed by using Student's *t* test. *, $P < 0.01$ (*Fut8*$^{-/-}$ mice treated with TGF-β versus the matched age of mice without treatment).

detected by AAL lectin in the TGF-β type II receptor, which is the primary binding subunit for TGF-β1 (30–32), were abolished in *Fut8*$^{-/-}$ cells, whereas they were recovered by restoring *Fut8* (Fig. 3*F*). The TGF-β1 signaling via receptors to intracellular mediators of the Smad family was suppressed significantly in *Fut8*$^{-/-}$ cells (Fig. 3*G*). Smad2 is a direct substrate for the activated TGF-β type

I receptor. In addition, Smad2 phosphorylation at C-terminal serine residues is required for its nuclear translocation (33). The down-regulation of Smad2 phosphorylation levels in *Fut8*$^{-/-}$ cells was rescued by reintroducing *Fut8* (Fig. 3*G*). Consistently, immunohistochemical analysis of the phosphorylation levels of Smad2 in lung tissues revealed that P-Smad2 levels were greatly suppressed in *Fut8*$^{-/-}$ mice, compared with that in *Fut8*$^{+/+}$ mice (Fig. 3*H*). Taken together, these results demonstrate that core fucosylation plays an important role in the regulation of TGF-β1 receptor function. Therefore, we assume that *Fut8*$^{-/-}$ lungs are committed to overexpressing MMPs, probably because they escape from the TGF-β1 suppressor mechanism, which operates in wild-type lungs, although other functions of core fucosylation of *N*-glycan-bearing glycoproteins might also be involved in the development of emphysema.

Exogenous TGF-β1 Treatment Rescued Emphysema-Like Changes in *Fut8*$^{-/-}$ Mice. We have performed rescue experiment with i.p. injection of exogenous TGF-β1 to postnatal-day-18 Fut8 knockout mice. Importantly, exogenous TGF-β1 resulted in a significant rescue of the emphysema-like phenotype (Fig. 3 *I* and *J*), stimulated the formation of elastin fiber (data not shown), and concomitantly reduced MMP-12 expression (Fig. 6*H*) in *Fut8*$^{-/-}$ lung. These data strongly support our hypothesis that the TGF-β1-mediated signaling pathway is down-regulated in *Fut8*$^{-/-}$ lungs. We do not exclude the possibility that aberrant regulation of other receptors may contribute partly to the emphysema-like changes.

In contrast to the mild and gradual formation of emphysema in integrin β6 knockout mice, which causes a local deficiency in active TGF-β1, the *Fut8* deficiency as well as the induction of cytokines such as IL-13, TNF-α, and IFN-γ (25, 34, 35) results in the severe and rapidly progressive development of emphysema. Interestingly, the absence of β6 integrin leads mainly to MMP-12 overexpression in the lungs of mutant mice, whereas in *Fut8*$^{-/-}$ lungs, MMP-13 is also induced at even larger amounts than MMP12, as assessed by real-time PCR quantitative analysis (Fig. 6*A*). This fact, together with the wide substrate specificity of MMP-13, could contribute to the explanation of the differences in the severity of emphysema phenotypes between integrin β6- and *Fut8*-deficient mice. It has been reported that disruption of the latent TGF-β-binding protein 4 (LTBP-4), which regulates TGF-β targeting to ECM and TGF-β-mediated signaling, causes abnormal lung development (36). Using antibodies specific for surfactant protein C (SP-C), a marker of differentiated type-II alveolar epithelial cells, we found that expression levels of SP-C protein at each stage were slightly weaker in *Fut8*$^{-/-}$ lungs than in *Fut8*$^{+/+}$ lungs (see Fig. 7, which is published as supporting information on the PNAS web site), suggesting that lung development was also disturbed by the loss of core fucosylation. The retarded alveolar epithelial cell differentiation may also contribute partly to emphysema-like changes of *Fut8*$^{-/-}$ lung from postnatal day 7 (Fig. 2*B*). Indeed, given the fact that alveolarization continues past day 7, at least part of the phenotype is related to abnormal lung development. Nevertheless, because it continues to increase after lung development, we conclude that both alveolar development and progressive (destructive) emphysema occur in the absence of *Fut8*.

TGF-β activation also leads to emphysema formation. Marfan syndrome is a human autosomal dominant disorder of connective tissue caused by mutations in fibrillin-1. Fibrillin-1 usually functions to limit the activation of TGF-β, although the precise mechanism by which fibrillin-1 controls TGF-β activation is still unknown. Studies with mutant mice have revealed that fibrillin deficiency causes a pronounced TGF-β activation that triggers the developmental inhibition of alveolarization, induces apoptosis in the developing lung, and finally results in destructive emphysema (37). Nevertheless, very recent results have demonstrated that Marfan syndrome can also be caused by loss of TGF-β signaling function due to TGF-β receptor type II mutations in a group of patients lacking mutations in fibrillin (38). These findings emphasize the idea that the TGF-β signaling pathway plays an important role in lung integrity, and consequently, there is an absolute need to maintain the precise levels of all components of this complex pathway. Our finding that defects in core fucosylation profoundly dysregulate TGF-β activation and signaling in *Fut8*$^{-/-}$ mice adds a level of control to this pathway and opens the possibility that similar defects could be found in some cases of human emphysema.

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Ascorbic acid promotes osteoclastogenesis from embryonic stem cells

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Abstract

Ascorbic acid (AA) is known to regulate cell differentiation; however, the effects of AA on osteoclastogenesis, especially on its early stages, remain unclear. To examine the effects of AA throughout the process of osteoclast development, we established a culture system in which tartrate-resistant acid phosphate (TRAP)-positive osteoclasts were induced from embryonic stem cells without stromal cell lines. In this culture system, the number of TRAP-positive cells was strongly increased by the addition of AA during the development of osteoclast precursors, and reducing agents, 2-mercaptoethanol, monothioglycerol, and dithiothreitol, failed to substitute for AA. The effect of AA was stronger when it was added during the initial 4 days during the development of mesodermal cells than when it was added during the last 4 days. On day 4 of the culture period, AA increased the total cell recovery and frequency of osteoclast precursors. Magnetic cell sorting using anti-Flk-1 antibody enriched osteoclast precursors on day 4, and the proportion of Flk-1-positive cells but not that of platelet-derived growth factor receptor α-positive cells was increased by the addition of AA. These results suggest that AA might promote osteoclastogenesis of ES cells through increasing Flk-1-positive cells, which then give rise to osteoclast precursors.

Keywords: Osteoclasts; Embryonic stem cells; Ascorbic acid

Ascorbic acid (AA) is an essential regulatory agent for various types of cell differentiation [1–3]. It functions as a reducing agent and a coenzyme for collagen synthesis, and the lack of AA results in scurvy [4] in primates, because it is also critical to maintain normal blood vessels. In skeletal development, AA has been clearly shown to play an important role in the development of the osteoblast lineage [5–8], but its influence on the bone-resorbing osteoclast lineage has not been thoroughly examined [7,9].

Osteoclasts are multi-nucleated cells that are involved in bone remodeling through resorbing bone matrices [10–12]. They are included in the myeloid lineage and their precursors arise from hematogenic mesodermal cells as other hematopoietic precursors do [13,14]. These mesodermal cells characteristically express Flk-1 [14,15] in embryonic and ES cell cultures. Since the developmental pathway of osteoclasts is similar to that of other hematopoietic lineages in terms of passing through an Flk-1-expressing stage, studies of osteoclastogenesis can yield valuable information about various aspects of hematopoiesis.

Previous research, however, focused on osteoclastogenesis from bone marrow cells or spleen cells. Studies of the early stage of osteoclastogenesis have not been reported yet. Moreover, Maeda et al. [16] reported that

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mice deficient in an enzyme required for AA synthesis, L-gulono-γ-lactone oxidase, are anemic unless provided with dietary AA. Considering that osteoclasts are derived from hematopoietic stem cells, it is possible that AA may play a role in osteoclastogenesis. However, mice lacking AA transporter, Slc23a1, suffer respiratory failure and die within a few minutes after birth [17]. Thus, it is difficult to assess the role of AA in early osteoclast development in vivo.

To overcome such problems, we previously established a culture system in which osteoclasts were induced from ES cells co-cultured with stromal cell lines [18–20]. To mimic in vivo osteoclastogenesis, we also reported a culture system in which osteoclasts could be induced from ES cells alone without stromal cell lines [13] in αMEM (which contained AA). In this study, osteoclasts were induced in modified MEM without ascorbic acid. Using this culture system, we investigated the effects of AA on osteoclastogenesis, especially on the early stage of osteoclast development. AA may accelerate osteoclastogenesis by increasing the induction of Flk-1-positive hematogenic cells from undifferentiated ES cells as well as the maturation of Flk-1-positive cells.

Materials and methods

Cell lines. ES cell line D3 [21] and J1 [22] was maintained in Dulbecco's modified essential medium (DMEM: Gibco-BRL, Grand Island, NY) supplemented with 10% knockout serum (Gibco-BRL), 1% heat-inactivated fetal bovine serum (FBS: JRH Biosciences, Lenexa, KS), 10^{-4} M 2-mercaptoethanol (2ME), 1× non-essential amino acids (Gibco-BRL), 2 mM L-glutamine (Gibco-BRL), and leukemia inhibitory factor (LIF) equivalent to 1000 U/ml on 0.1% gelatin-coated culture dishes.

Differentiation of ES cells. Undifferentiated ES cells were inoculated at 2×10^5 cells per well in 24-well plates (Corning-Costar, Corning, NY) in MEM (Gibco-BRL) supplemented with 15% FBS (Thermo Trace, Melbourne, Australia), 10 mg/L of adenosine (Sigma, St. Louise, MO), cytidine (Sigma), guanosine (Sigma), uridine (Sigma), 2′-deoxyadenosine (Sigma), 2′-deoxyguanosine (Sigma), and thymidine (Sigma), 11 mg/L of 2′-deoxycytidine (Sigma), 110 mg/L sodium pyruvate (Sigma), 1.4 mg/L vitamin B_{12} (Wako), 0.2 mg/L lipoic acid (Sigma), and 0.1 mg/L biotin (Sigma). On day 8, osteoclasts were induced by addition of 10^{-8} M 1α,25-dihydroxyvitamin D_3 [1α, 25$(OH)_2D_3$] (Biomol Research Laboratories, Plymouth Meeting, PA) and 10^{-7} M dexamethasone (Dex; Sigma). To induce osteoclasts directly, 10 ng/ml human macrophage colony-stimulation factor (M-CSF) (kindly provided by Dr. M. Takahashi, Otsuka Pharmaceutical, Tokushima, Japan) and 25 ng/ml human soluble receptor activator of nuclear factor κB ligand (RANKL) (Peprotech EC, London, UK) were added from day 8 to 14. On day 14, tartrate-resistant acid phosphatase (TRAP) staining was performed and TRAP-positive cells were counted under a microscope. AA was added to culture media at 50 μg/ml. Reducing agents, 2-mercaptoethanol (2ME, Wako, Osaka, Japan), monothioglycerol (MTG; Wako), and dithiothreitol (DTT; BioChemika, Steinheim, Switzerland), were added in the range of 10^{-9} to 10^{-2} M from day 0 to day 4.

For assessment of the factor dependency of osteoclastogenesis (Fig. 1), 10 μg/ml anti-mouse M-CSF receptor (Fms) antagonisitic antibody (AFS98; kindly provided by Dr. Nishikawa, Riken, Kobe, Japan) [23] or 50 ng/ml human osteoprotegerin (OPG: Peprotech EC) was added on day 8–14.

TRAP staining. Cultured cells were fixed with 10% formaldehyde (Wako) for 10 min and with ethanol/acetone (50:50 v/v: Wako) for 1 min at 25 °C. After the cells were washed with 1× PBS, they were stained with fast red violet LB-salt (Sigma) mixed with TRAP solution containing 59.3 M sodium tartrate (Wako), 165.7 M sodium acetate (Wako), and 0.56 mg/ml naphthol AS-MX phosphate (Sigma) for 5 min at 25 °C [13]. Red stained cells were visualized under a microscope and counted as TRAP-positive cells.

Frequency analysis. The frequency of osteoclast progenitors was estimated by a limiting dilution assay. Various numbers of cells harvested on day 4 were inoculated into the wells of 96-well plates (Corning) containing monolayers of ST2 cells [24] and cultured for 6 days in αMEM supplemented with 10^{-8} M 1α,25$(OH)_2D_3$ and 10^{-7} M

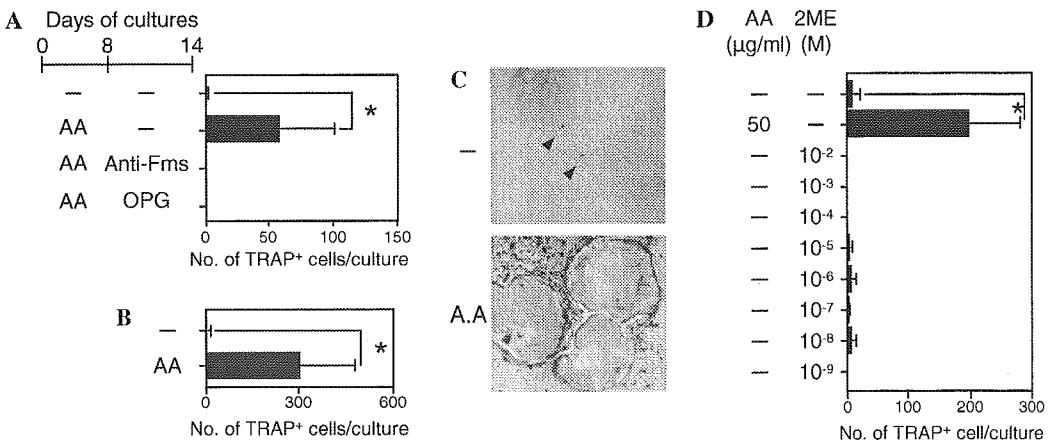

Fig. 1. Osteoclastogenesis of ES cells was strongly promoted by AA. (A) Undifferentiated ES cells were cultured on culture plates supplemented with AA for the initial 8 days, and osteoclasts were induced by addition of 1α,25$(OH)_2D_3$ and Dex on day 8–14 without or with anti-Fms antagonistic antibody (Anti-Fms) or osteoprotegerin (OPG). (B–D) Osteoclasts were induced from ES cells in the presence of M-CSF and RANKL on day 8–14. (B) AA was added in the initial 8 days. (C) The TRAP-staining of cultures on day 14 induced in the absence (top) or the presence (bottom) of AA. TRAP-positive multi-nucleated cells were observed (bottom). A few TRAP-positive cells were observed and are indicated by arrowheads (top). (D) AA or 2ME was added in the initial 4 days. Significant differences are indicated by an asterisk ($*p < 0.05$). Each column represents the mean ± SD of triplicate cultures.

Dex for efficient induction of osteoclastogenesis. The presence or absence of osteoclasts in each well was determined by TRAP staining. The frequency of osteoclast precursors was calculated by using the following formula: $1/\text{frequency} = N/\{\ln[T/(T-P)]\}$, where N is the number of cells seeded in a well, T is the number of wells per group, and P is the number of positive wells per group [25].

Immunostaining. Cells on day 4 were harvested and labeled with anti-Flk-1 (Bay Bioscience, Kobe, Japan), anti-CD31 (BD Pharmingen, San Diego), and anti-PDGFRα (APA5; kindly provided by Dr. Nishikawa) [26]. The proportion of these cells was analyzed using a flow cytometer (EPICS XL; Coulter, Palo Alto, CA).

Reverse transcription-polymerase chain reaction. Total RNA was purified using ISOGEN (Nippon Gene, Toyama, Japan) and used as the template to synthesize cDNA using RevaTra Ace (Toyobo, Osaka, Japan) primed with oligo(dT) from 1 μg of total RNA. Gene expression was analyzed by PCR using the following primers: *Fgf2* (basic fibroblast growth factor, bFGF): 5′-AAG CGG CTC TAC TGC AAG AA-3′ and 5′-TGG CAC ACA CTC CCT TGA TA-3′; *Inhba* (activin A): 5′-GAT CAT CAC CTT TGC CGA GT-3′ and 5′-GCC CAG AAG CAC TAG ACT GG-3′; *Bmp2* (bone morphogenic protein 2, BMP2): 5′-AGA TCT GTA CCG CAG GCA CT-3′, and 5′-GTC GAA GCT CTC CCA CTG AC-3′ and 5′-GTC GAA GCT CTC CCA CTG AC-3′; *Bmp4* (BMP4): 5′-TGA GCC TTT CCA GCA AGT TT-3′ and 5′-CTT CCC GGT CTC AGG TAT CA-3′; and *Hprt* (hypoxanthine-guanine phosphoribosyltransferase, HPRT): 5′-AAT GAT CAG TCA ACG GGG GAC A-3′ and 5′-CCA GCA AGC TTG CAA CCT TAA CCA-3′. PCR was performed under the following conditions: an initial cycle consisting of 94 °C for 4 min, annealing at 60 °C for 3 min, and 72 °C for 1 min, followed by 34 cycles or 44 cycles of 94 °C for 1 min, annealing at 60 °C for 1 min, and 72 °C for 1 min. cDNA prepared from fetal brain was used as a positive control. Distilled water was used as a negative control. For semi-quantitative RT-PCR, diluted cDNA (1/5, 1/25; equivalent to 10, 2 ng of total RNA, respectively) was used.

Magnetic cell sorting. Cells harvested using 10 mM EDTA were labeled with biotin-conjugated anti-Flk-1 and streptavidin-PE (Southern Biotechnology Associates, AL), and then these cells were incubated with anti-phycoerythrin (PE) microbeads (Miltenyi Biotec, Auburn, CA) at 4 °C for 15 min. The Flk-1-positive microbead-conjugated cells were passed through a column with a magnetic field to enrich these cells following the magnetic cell sorting (MACS) method (Miltenyi Biotec). The pre-sorting cells, column-bound cells, and column-passed cells were analyzed by flow cytometry to determine the proportion of Flk-1-positive cells in each cell fraction.

Results

Ascorbic acid promoted osteoclastogenesis of ES cells

In the previously reported culture system, undifferentiated D3 ES cells were cultivated with αMEM in culture grade plates for 8 days without any manipulation, and then gave rise to osteoclasts after $1\alpha,25(OH)_2D_3$ and Dex were added so as to induce the production of osteoclastogenic factors M-CSF [27] and RANKL [28–30], and also to inhibit the production of a decoy receptor for RANKL referred to as OPG [31,32]. On day 14, a few TRAP-positive cells appeared. These results showed that both osteoclast precursors and osteoblast-like cells supplying M-CSF and RANKL might be induced under the same culture conditions [13].

Since αMEM contained AA, to determine whether AA is required in this culture system, the differentiation of ES cells was induced in modified MEM without AA. Under these conditions, a few TRAP-positive cells were generally observed, and sometimes no TRAP-positive cells were detected (Fig. 1A, lane 1). However, osteoclasts were efficiently induced by the addition of AA from day 0 to 8 (Fig. 1A, lane 2). This induction was completely inhibited by the addition of an anti-M-CSF receptor antagonistic antibody or OPG during the last 6 days of culture (Fig. 1A, lanes 3 and 4). These data suggest that AA promotes osteoclastogenesis of ES cells that is dependent on M-CSF and RANKL, as is in vivo osteoclastogenesis.

It has been difficult to perform detailed analyses of early osteoclastogenesis because of the inaccessibility of embryonic cells and therefore it has not been reported whether AA affects early osteoclastogenesis during the development of osteoclast precursors from ES cells. To investigate the influence of AA on osteoclast precursors, the production of osteoclasts was directly induced by the addition of M-CSF and RANKL from day 8 to 14 of culture. This enabled us to assess the effects of AA on the development of osteoclast precursors. The number of TRAP-positive cells was increased by the addition of AA from day 0 to 8 (Fig. 1B) compared with that in cells cultured without AA. TRAP-positive multi-nucleated cells were induced in the presence of AA (Fig. 1C, bottom), while only a few TRAP-positive cells were observed in the absence of AA (Fig. 1C, top). Similar results were obtained using another ES cell line, J1 (data not shown).

AA is known to be an antioxidant and its reducing activity may have a critical effect on osteoclastogenesis. Therefore, the effects of other reducing agents were assessed. We added 2ME to the culture in the range of 10^{-9}–10^{-2} M (Fig. 1D), but efficient osteoclastogenesis was not observed. No significant effect was observed using either MTG or DTT as reducing agent (data not shown). These results imply that some activity of AA other than its reducing activity might be required for osteoclastogenesis.

Optimum conditions for increase of osteoclastogenesis by AA

To assess the period during which AA is required for the differentiation of osteoclast precursors in culture, we tested the effect of the addition of AA during various periods (Fig. 2). The presence of AA in any period we tested increased the number of TRAP-positive cells compared with cultures without AA (lane 1). Compared with the continuous addition of AA from the initiation (lane 2, 0–8 days), similar numbers of TRAP-positive cells were observed with addition of AA during days 0–4 (lane 3) and days 2–4 (lane 5) of culture. AA addition after the fourth day of culture (lanes 6 and 7) and between the third to fifth day also induced TRAP-posi-

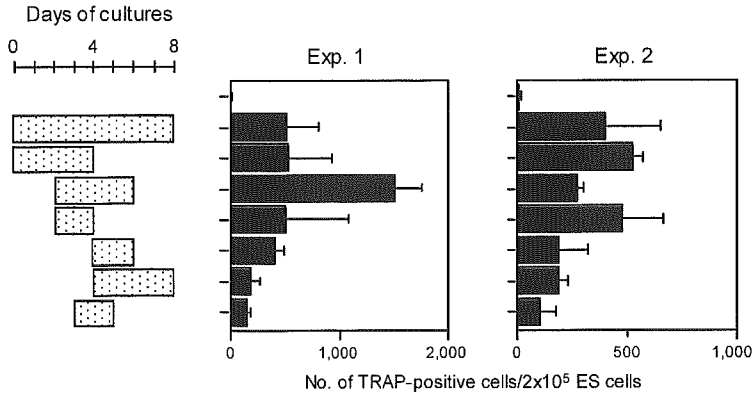

Fig. 2. Efficiency of osteoclastogenesis was affected by the timing of AA addition. Osteoclasts were induced from ES cells in the presence of M-CSF and RANKL on day 8–14. Dotted boxes represent the period with addition of AA. Exp. 1 and Exp. 2 were independently performed. Each column represents the mean ± SD of triplicate cultures.

tive cells; however, the number was lower than with the continuous addition. The efficiency of osteoclastogenesis varied between Exp. 1 and 2 with regard to the addition of AA during day 2–6 (lane 4). These results suggest that AA affects osteoclastogenesis from the initiation of the differentiation of ES cells, and it is likely that the addition of AA in the early phase is more effective than that in the late phase in this culture system.

To investigate the frequency of osteoclast precursors on day 4, a limiting dilution assay was performed. In the presence of AA in culture, the total number of cells recovered on day 4 increased 1.7-fold (7.5×10^5 with AA vs. 4.5×10^5 without AA) and the frequency of osteoclast precursors increased 3.5-fold (1/6,117 with AA vs. 1/21,672 without AA). Therefore, approximately 6-fold (122.6/culture with AA vs. 20.8/culture without AA) more osteoclast precursors developed from ES cells with the addition of AA (Table 1). There was no significant difference in the number of TRAP-positive cells per TRAP-positive cell-containing well. A majority of the TRAP-positive cells in a well might be derived from a single osteoclast precursor, because the number of seeded cells per well was 4500 and the frequency was 1/6117, and thus the number of seeded cells per well does not exceed the frequency. These results suggest that AA may increase the number of osteoclast precursors but may not affect their proliferative ability during their maturation.

Table 1
AA increases the number of osteoclast progenitors

	Without AA	AA
No. of cell recovery ($\times 10^5$)	4.5	7.5
Frequency of OCPs	1/21,672	1/6117
Total no. of OCPs	20.8	122.6
No. of $TRAP^+$ cells in positive wells	11.7 ± 14.0	7.6 ± 7.1

Undifferentiated ES cells were cultivated with or without ascorbic acid. Cells were harvested on day 4 and seeded at several numbers per well on pre-seeded ST2. The frequency of osteoclasts was calculated as described under Materials and methods. No. of $TRAP^+$ cells was counted and represented as the mean ± SD of triplicate cultures. OCPs, osteoclast precursors.

Increase of Flk-1-positive cells on day 4 by AA addition

Although the addition of AA enhanced the osteoclastogenesis from ES cells in any period we tested (Fig. 2), the initial 4 days after the induction of differentiation of ES cells may be critical to increase osteoclast precursors. Since osteoclasts are hematopoietic cells derived from mesodermal cells, it is possible that AA may bias the differentiation of ES cells toward mesodermal cells. It was reported that the development of mesodermal cells was affected by activin A [33], bFGF [33–35], BMP2 [36], and BMP4 [33,37]. To investigate whether these factors were involved in the increase, RT-PCR was performed using mRNA purified from cells on day 4. No expression of activin A or BMP2 was observed. BMP4 and bFGF were expressed, although weakly (Fig. 3A). However, semi-quantitative RT-PCR for these two genes showed no difference between the levels with or without AA (Fig. 3B).

PDGFRα is thought to be one of the mesodermal markers, but is preferentially expressed in paraxial mesoderm [15,38], which does not give rise to hematopoietic cell progenitors [39]. We assessed the proportion of PDGFRα-positive cells, but no effect of AA was observed (Fig. 3C). These results suggest that AA may not accelerate the production of mesoderm-inducing factors or the induction of PDGFRα-positive cells.

Hematopoietic precursors are derived from the Flk-1-expressing population on embryonic day (E) 8.5, and on day 4 of ES cell culture [14], and osteoclast precursors are also included in Flk-1-expressing cells [14,15,40]. To investigate the effects of AA on early osteoclastogenesis during the differentiation from ES cells to Flk-1-expressing cells, the proportion of Flk-1-positive cells was assessed by flow cytometry. Significantly higher numbers of Flk-1-expressing cells were observed with

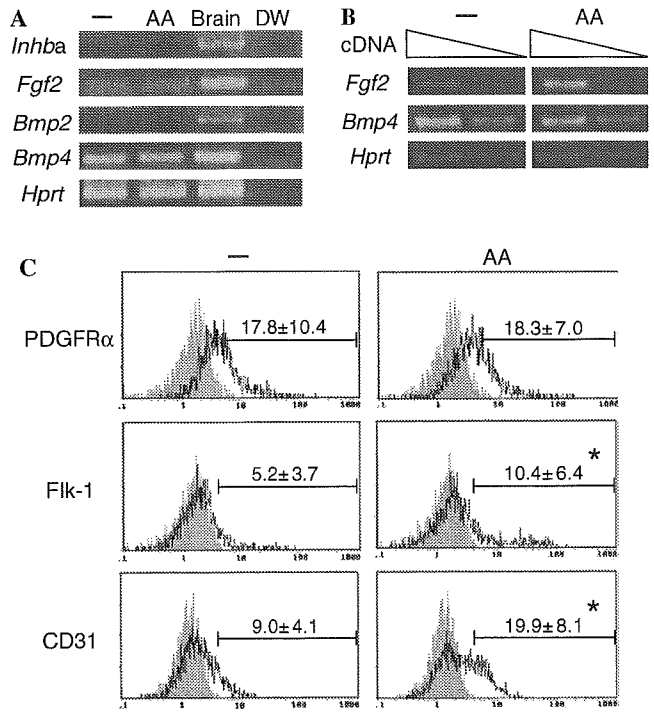

Fig. 3. AA increased Flk-1- and CD31-positive cell populations. (A) RT-PCR was performed using cDNA prepared from undifferentiated ES cells cultivated with or without AA on day 4. (B) Semi-quantitative RT-PCR was performed using diluted cDNA [1/5, 1/25 of (A)] as templates. cDNA from fetal brain (brain) and distilled water (DW) were used as positive control and negative control, respectively. (C) Cells were stained with anti-Flk-1, anti-CD31, or anti-PDGFRα antibody. The proportion was represented as the mean ± SD of triplicate cultures. Significant differences are indicated by an asterisk ($*p < 0.05$). (−) Without AA.

addition of AA from day 0 to day 4 compared to the number without AA (Fig. 3C). Cells in this population also give rise to endothelial cells [41]. To assess the effect of AA on these cells, the proportion of CD31-expressing cells was estimated because CD31 is expressed on an endothelial cell lineage [36,42]. CD31-positive cells were also significantly increased in cultures on day 4. Since the ratio of PDGFRα-expressing cells did not depend on AA addition, these results imply that AA affects particular populations including not only osteoclast precursors but also other derivatives.

Flk-1-positive cell population is enriched in osteoclast precursors

We previously determined that Flk-1-positive cells from ES cells differentiated into osteoclast precursors on day 4 using the co-culture system with OP9 stromal cells [40]. In this study, Flk-1-positive cells were also enriched by using anti-Flk-1 antibody and the MACS method on day 4 in the presence of AA, and the efficiency of osteoclastogenesis was estimated. Flk-1-positive cells were accumulated in the column-bound fraction compared with the column-passed fraction (Fig. 4A), and a higher number of osteoclasts were induced from the column-bound fraction than from the column-passed fraction (Fig. 4B). Flk-1-positive cells on day 4 may preferentially produce osteoclast precursors, as we have previously reported. This may mean that the increase of this cell population in culture by the addition of AA is responsible for the promotion of osteoclastogenesis.

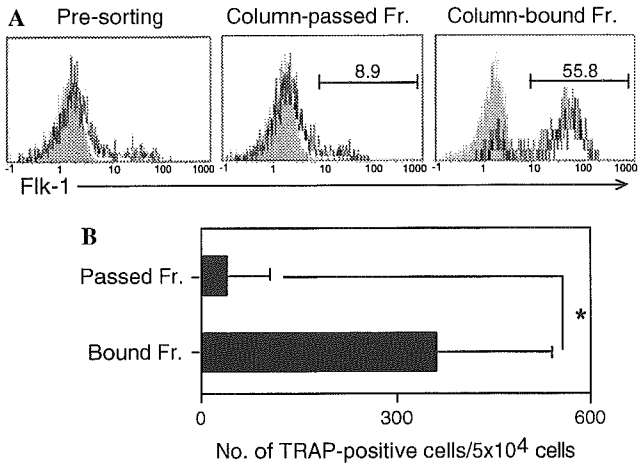

Fig. 4. Osteoclast precursors were increased by AA. Undifferentiated ES cells were cultured with or without AA for 4 days. (A) Harvested cells were labeled with anti-Flk-1 antibody and magnetic beads, and labeled cells were enriched by passing them through magnetic columns. Cells among column-bound fraction (Fr.) and column-passed Fr. were analyzed by flow cytometry. Gray histograms represent the control without first antibody. (B) After the enrichment of Flk-1-positive cells, osteoclasts were induced on ST2 in αMEM in the presence of $1\alpha,25(OH)_2D_3$ and Dex for 6 days. Each column represents the mean ± SD of triplicate cultures. Significant differences are indicated by an asterisk ($*p < 0.05$).

Discussion

In this study, to investigate the role of AA in osteoclastogenesis, we established a culture system in which TRAP-positive osteoclasts were induced from ES cells without co-culturing with stromal cell lines. Using this system, we showed that AA might accelerate osteoclastogenesis by increasing the induction of Flk-1-positive hematogenic cells from undifferentiated ES cells.

In our culture, TRAP-positive cells were induced by the addition of $1\alpha,25(OH)_2D_3$ and Dex, and OPG and antagonistic anti-Fms antibody inhibited the development of TRAP-positive cells (Fig. 1A). These data implied that osteoblast-like cells that could supply RANKL were induced in this culture, since osteoblasts or osteoblastic stromal cell lines that do so are known to respond to $1\alpha,25(OH)_2D_3$ and Dex. In fact, cells expressing alkaline phosphatase, which osteoblasts express, were also observed in this culture on day 8 (Tsuneto, unpublished data). However, it is not likely that

the promotion of osteoclastogenesis resulted from the enhancement of osteoblastogenesis. Cbfa1 is a critical transcription factor for osteoblast differentiation [43,44], and its first expression was observed on embryonic day (E) 12.5 in mice [43]. Considering that the ES cells used were established from the inner cell mass of a blastocyst on E3.5, E12.5 might correspond to day 9 of ES cell culture. Since the frequency of osteoclast precursors and the proportion of hematogenic Flk-1-positive cells on day 4 of culture were increased by AA (Table 1, Fig. 3C), these events might occur prior to the development of osteoblasts.

AA was required for the efficient differentiation of osteoclast precursors during days 0–8, and the addition of AA in the initial half period (days 0–4) promoted osteoclastogenesis more effectively than AA addition during the latter half (Fig. 2). These results imply that AA might influence the development of osteoclast precursors, resulting in increases of the frequency and total number of osteoclast precursors (Table 1), but more detailed experiments might be also needed to understand how the lack of AA in early osteoclastogenesis consequentially influences the bone metabolism. We previously demonstrated that Flk-1-positive cells on day 4 of ES cell cultures were hematogenic and almost all of osteoclast precursors were derived from this cell population [40]. In this study, the majority of osteoclasts were also derived from the corresponding cell population and this cell population was increased in the presence of AA in culture.

The ratio of the PDGFRα-expressing cell population on day 4 was not decreased even in the absence of AA, and the proportions of Flk-1-expressing cells and CD31-expressing cells were increased in the presence of AA. Since it has been reported that PDGFRα, Flk-1, and CD31 are expressed in paraxial-type mesoderm [15,38], lateral plate-type mesoderm [45], and endothelial cells [36,42], respectively, AA might preferentially affect the induction of lateral plate-type mesoderm, including hemangioblasts which give rise to both hematopoietic cells and endothelial cells, rather than paraxial-type mesoderm.

Reducing potential is an important property of AA and this function protects cells from oxidative stress [46,47]. Representative reducing agents, such as 2ME, MTG, and DTT, also play roles as anti-oxidants [48,49]. However, no effect on osteoclastogenesis was observed upon the addition of these reducing agents over a broad range of concentrations. Bergethon et al. [50] reported that DTT could not replace AA for enhancing the accumulation of elastin on muscular cells, supporting the notion that AA has a specific role that cannot be played by other reducing agents.

The mechanisms of AA in the increase of Flk-1-expressing cells remained to be unclear, even though the expression of activin A, bFGF, BMP2, and BMP4 that are involved in induction of Flk-1-expressing cells was not affected by AA (Fig. 3A and B). Semi-quantitative RT-PCR showed that the level of gene expression of mesoderm-inducing factors was comparable in the presence and absence of AA. Recently, Carcamo et al. [51] reported that AA inhibited signal transduction mediated by reactive oxygen species (ROS). It is possible that AA inhibits some yet unknown negative signaling pathway for the induction of Flk-1-positive cells.

Although osteoclastogenesis was significantly enhanced in the presence of AA, it is notable that a few osteoclasts were induced by M-CSF and RANKL without AA (Figs. 1A and B). Guinea pigs and humans are known to depend entirely on AA from the diet, but mice have L-gulono-γ-lactone oxidase for the synthesis of AA [52–54]. Although the biosynthesis of AA is observed mainly in liver, a variety of cell lineages are contained in our ES cell culture system [13,55,56]. Therefore, it is possible that insufficient, but significant de novo, AA biosynthesis may occur in our culture.

In conclusion, we demonstrated that AA enhances the osteoclastogenesis from undifferentiated ES cells, and that the early stage of osteoclastogenesis was preferentially promoted by AA. Our current data support the notion that AA may play a critical role in bone metabolism through regulating osteoclastogenesis.

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Original Article

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 *Sfpi1 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

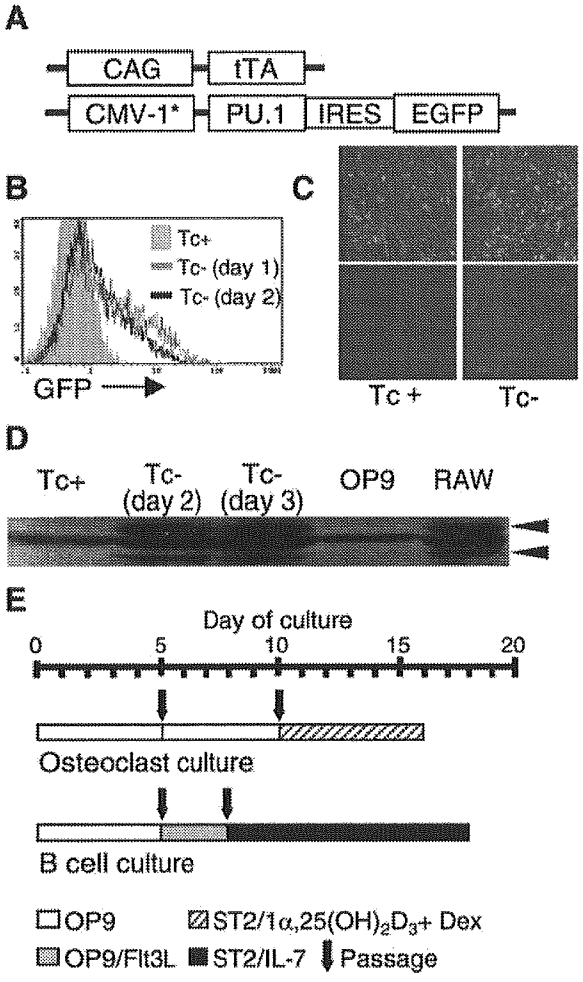

Figure 1. Establishment of Tc-controlled PU.1-expressing *Tal1*$^{-/-}$ES cell lines. **(A):** Constructs transfected into *Tal1*$^{-/-}$ES cells (see Materials and Methods). **(B, C):** The expression of PU.1 was evaluated by monitoring the fluorescence of GFP. ES cells were cultured in the presence 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.

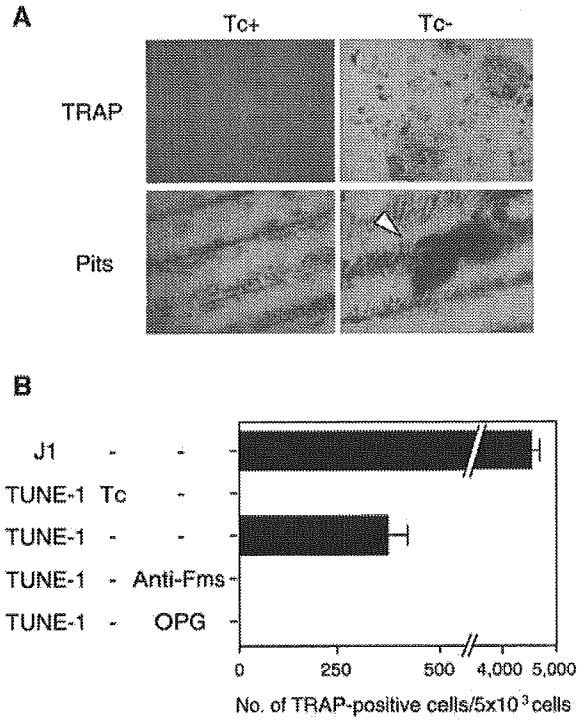

Figure 2. Enforced expression of PU.1-rescued osteoclastogenesis from *Tal1*$^{-/-}$ ES cells. TUNE ES cells were cultured on OP9 cells for 10 days. Tc was withdrawn from day 2 (Tc–). The cells harvested on day 10 were induced to form osteoclasts on ST2. **(A):** Osteoclasts were induced in the schedule 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.

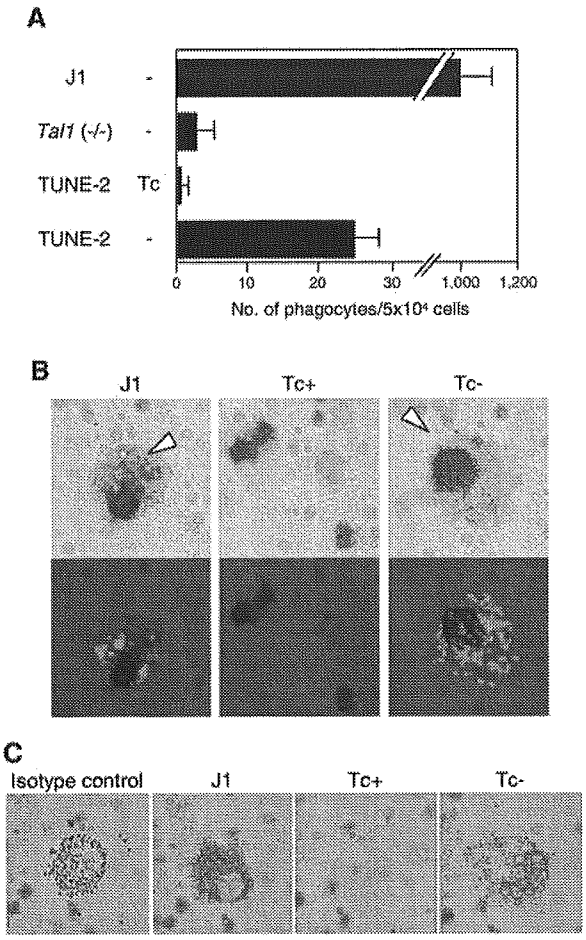

Figure 3. Macrophage-like phagocytes were induced from PU.1-on TUNE-2 cells. After ES cells were cultured on OP9 layers for 5 days, phagocytes were induced on ST2 layers supplemented with M-CSF for 6 days. Phagocytes with fluorescent beads **(B)** or nonfluorescent latex beads **(C)** were detected under 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.

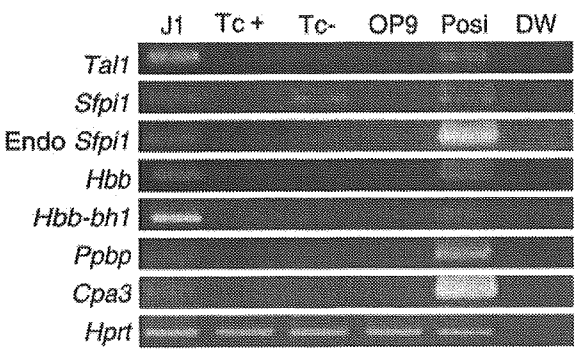

Figure 4. Several genes expressed in mast cells, erythrocytes, and megakaryocytes were not detected in PU.1-on TUNE-1 embryonic stem (ES) cells. After ES cells were cultured on OP9 cells for 5 days, mRNA was purified. The expression of several genes was analyzed by reverse transcription-polymerase chain 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.

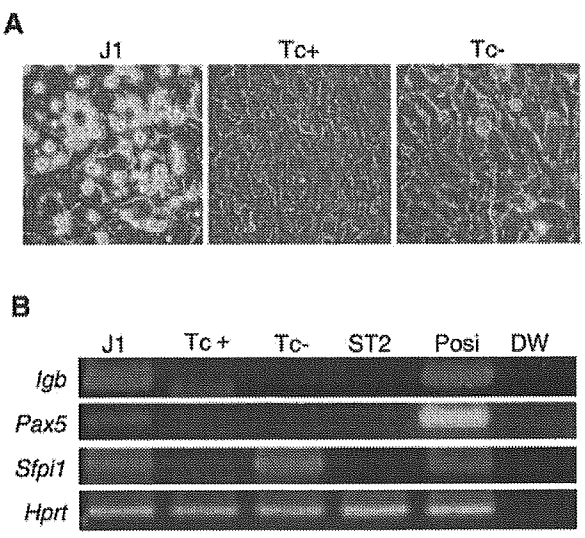

Figure 5. B-lymphocyte–related genes were not expressed in PU.1-on TUNE-1 embryonic stem (ES) cells. B-lineage cells were induced in the schedule as described in Figure 1E (B cell culture). **(A):** The appearance of each culture on day 18. **(B):** mRNA was prepared on day 18 of cultures, and the expressions 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$^+$ andFlk-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

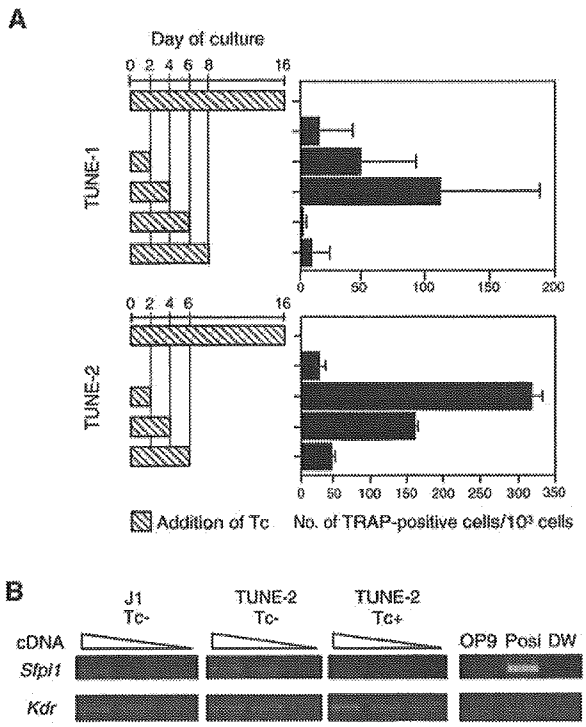

Figure 6. Different efficiencies of osteoclastogenesis from *Tal1*$^{-/-}$ ES cells depended on timing of expression of PU.1. **(A):** TUNE-1 and TUNE-2 ES cells were cultured in the schedule as described in Figure 1E, osteoclast culture. Shaded bars represent periods of addition of Tc. Each column represents 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.

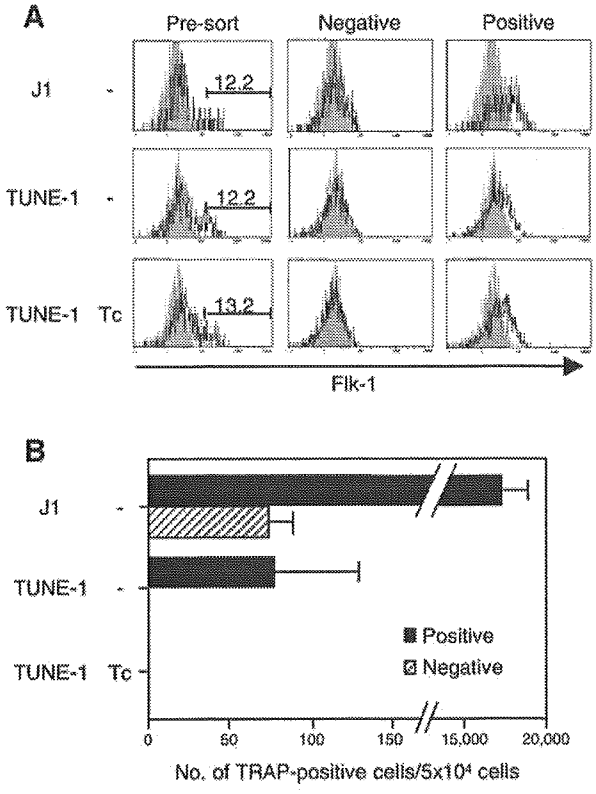

Figure 7. Flk-1$^+$ cells included osteoclast precursors of PU.1-on TUNE-1 embryonic stem (ES) cells. After ES cells were cultivated on the OP9 layer for 4 days, the cells were harvested and subjected to magnetic sorting using Flk-1 antibody. **(A):** The proportion of Flk-1$^+$ cells was analyzed by 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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