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

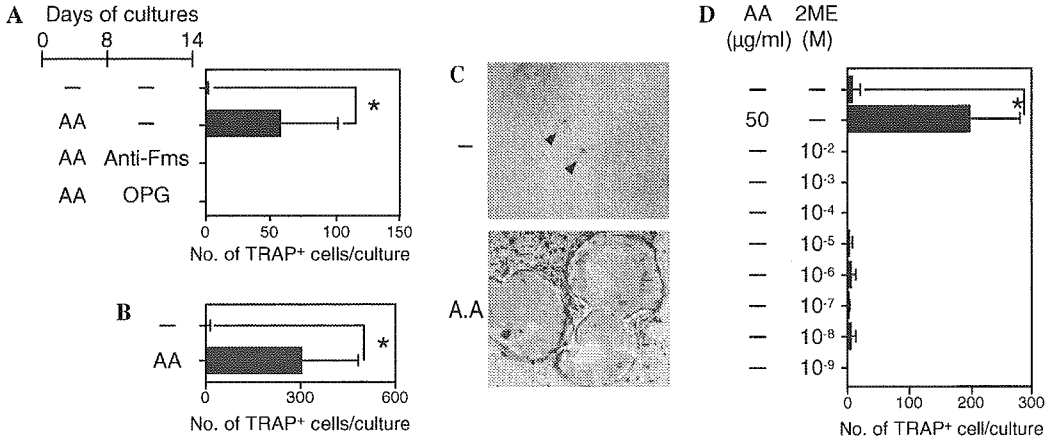

Fig. 1. Osteoclastogenesis of ES cells was strongly promoted by AA. (A) Undifferentiated ES cells were cultured on culture plates supplemented with AA for the initial 8 days, and osteoclasts were induced by addition of 1α,25$(OH)_2D_3$ and Dex on day 8–14 without or with anti-Fms antagonistic antibody (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-

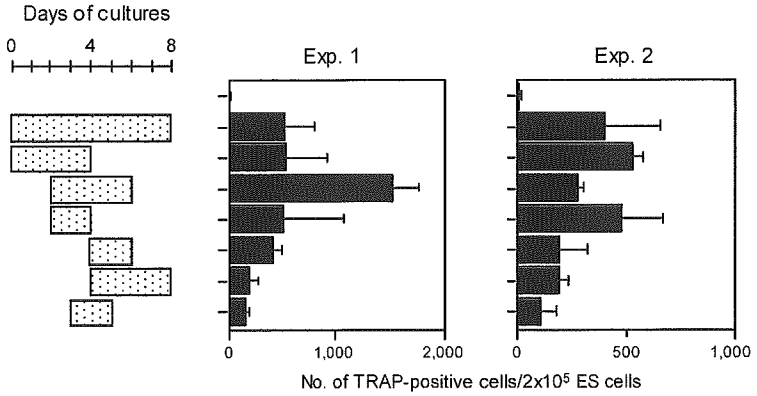

Fig. 2. Efficiency of osteoclastogenesis was affected by the timing of AA addition. Osteoclasts were induced from ES cells in the presence of M-CSF and RANKL on day 8–14. Dotted boxes represent the period with addition of AA. Exp. 1 and Exp. 2 were independently performed. Each column represents the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

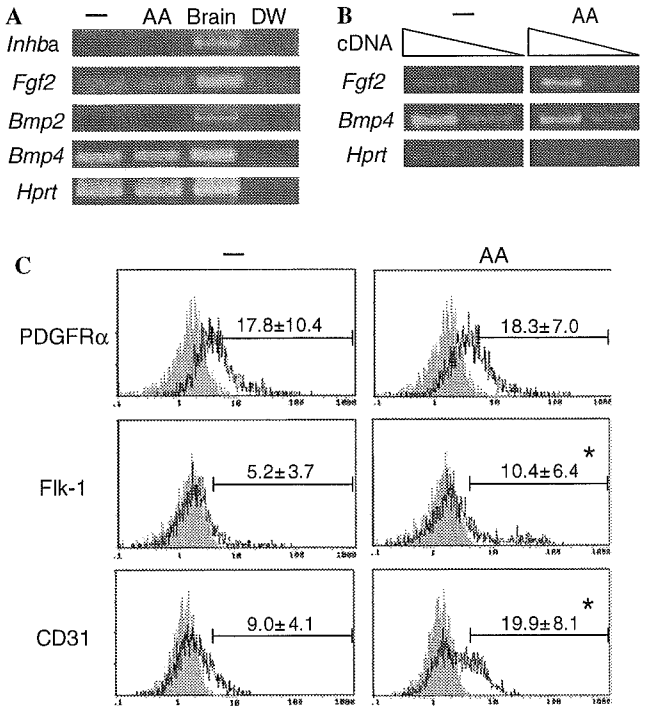

Fig. 3. AA increased Flk-1- and CD31-positive cell populations. (A) RT-PCR was performed using cDNA prepared from undifferentiated ES cells cultivated with or without AA on day 4. (B) Semi-quantitative RT-PCR was performed using diluted cDNA [1/5, 1/25 of (A)] as templates. cDNA from fetal brain (brain) 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.

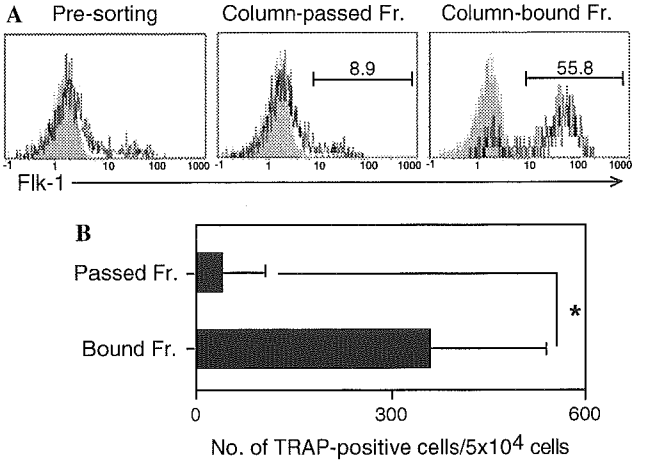

Fig. 4. Osteoclast precursors were increased by AA. Undifferentiated ES cells were cultured with or without AA for 4 days. (A) Harvested cells were labeled with anti-Flk-1 antibody and magnetic beads, and labeled cells were enriched by passing them through magnetic columns. Cells among column-bound fraction (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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Presence and distribution of neural crest-derived cells in the murine developing thymus and their potential for differentiation

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Abstract

Neural crest (NC) cells are multipotent cells that can differentiate into melanocytes, neurons, glias and myofibroblasts. They migrate into the fetal thymus on embryonic day (E) 12 in mice and may participate in thymic organogenesis. Although the abnormality of migration and distribution of NC cells in the thymus results in immunodeficiency, the spatial and temporal presence of their progeny cells has not been defined in detail. In this study, we traced NC-derived cells based on the myelin protein zero gene promoter-*Cre*-mediated excision. We demonstrated that large numbers of NC-derived cells in the thymus were detected on E11.5 to E16.5 but rarely on E17.5. A colony formation assay of single thymic cells demonstrated that multipotent cells with the potential to differentiate into melanocytes, neurons and/or glias were present in the E14.5 and E15.5 but not in the E17.5 fetal thymus. Furthermore, we confirmed that these multipotent cells were NC-derived cells. Taken together, these findings imply that multipotent NC-derived cells are present in the developing thymus, but rarely in this organ at a later stage, suggesting that NC-derived cells may play roles in thymic organogenesis at an early embryonic stage.

Introduction

Neural crest (NC) cells migrate and differentiate into a variety of cell lineages such as melanocytes, neurons, glial cells, myofibroblasts, chondrocytes and osteoblasts (1–5). NC cells migrating in the pharyngeal arch are thought to participate in the organogenesis of the craniofacial area, thyroid gland, heart and thymus (6–10). The failure or aberrant migration and abnormal distribution of NC cells result in a condition known as DiGeorge syndrome. It is characterized by defective aortic arch patterning, a conotruncal heart, thymic and parathyroid aplasia/hypoplasia including immunodeficiencies and craniofacial anomalies (11, 12). These reports suggest that NC-derived cells may contribute to thymic organogenesis. Using avian systems, Le Douarin and her colleagues proposed the participation of avian NC cells in thymic organogenesis (4, 8). Using *Wnt1-Cre* reporter mice, Jiang *et al.* reported that murine cardiac NC-derived cells were detected around the thymus (13). However, in the murine thymus, the distribution or potential for differentiation of NC-derived cells has been rarely substantiated.

The thymus is composed of three major cell lineages, i.e. hematopoietic cells (including T lymphocytes), epithelial cells

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that support T lymphocyte differentiation and mesenchymal cells surrounding the thymus (6, 14, 15). Experiments using chick and quail inter-species chimeras indicate that NC-derived cells differentiate into inter-lobular and outer thymic mesenchymal cells but not into hematopoietic cells or thymic epithelial cells (7, 8). Furthermore, ablation of NC cells by the microcautery of neural folds induces thymic abnormalities that reduce the numbers of both mesenchymal and epithelial cells (16, 17).

In this study, we employed the expression of the myelin protein zero (*P0*) gene, an indicator of NC-derived cells (18–21), and we chased NC-derived cells and assessed their presence, distribution, expression of adhesion molecules and potential in the murine thymus. It has been thought that melanocyte precursors might be distinct from neuronal and glial cell precursors or lose the potential to differentiate into neuron or glial cell lineages before or soon after the initiation of NC cell migration (22–27). Therefore, the demonstration of NC-derived cells that maintain the potential to differentiate into not only melanocytes but also neurons and glial cells would indicate the presence of NC-derived cells including multipotent NC cells. To examine the potential of NC-derived cells in the thymus, we developed a colony formation assay using single thymic cells incubated in the presence of endothelin 3 (ET3). ET3 is known to promote the survival and differentiation of not only melanocytes but also glial cells in avian cultures (28–30). Using these two types of analyses, we showed that at least double- or triple-potent NC-derived cells exist during early thymogenesis, and observed a severe reduction in their number at the late embryonic stages of thymus development in mice.

Methods

Mice

Mice carrying *Cre* recombinase driven by the protein 0 (*P0*) promoter were produced as described (19), and *Rosa26R* mice were obtained from Kumamoto University (18). C57BL/6 mice were purchased from Japan Clea (Tokyo, Japan).

Determination of genotypes of transgenic mice

Genomic DNA was prepared, and transgenes were detected by use of PCR. The respective sense and anti-sense primers used for PCR were as follow—*LacZ*: 5′-GGT AGC AGA GCG GGT AAA CT-3′/5′-ATC TGA CGG GCT CCA GGA GT-3′ and *Cre*: 5′-GGA CAT GTT CAG GGA TCG CCA GGC G-3′/5′-GCA TAA CCA GTG AAA CAG CAT TGC TG-3′. PCR was performed by incubation at 94°C for 4 min, followed by 35 cycles of incubation at 93°C for 1 min, 58°C for 1 min and 72°C for 1 min and a final extension at 72°C for 7 min.

Histological analysis

For detection of LacZ activity, whole embryos and tissues were fixed in PBS solution (pH 7.4) containing 2% formaldehyde (Wako), 0.2% glutaraldehyde (Wako) and 0.02% NP-40 (Sigma). After washing, samples were stained with a solution containing Bluo-Gal (GIBCO-BRL) in *N,N′*-dimethylformamide (Wako) until the desired color intensity had been obtained. For preparation of tissue sections, thymi were embedded in a polyester wax (BDH Laboratory Supplies). Sections were prepared at a 7-μm thickness, and stained with hematoxylin and eosin.

Fluorescein di-β-D-galactopyranoside loading and flow cytometric analysis

Single-cell suspensions from thymi of embryonic day (E) 12.5 to 3.5-day-old mice were prepared by digestion with collagenase D (Roche), Dispase II (Roche) and trypsin/EDTA (GIBCO-BRL). Fluorescein di-β-D-galactopyranoside (FDG) staining was carried out essentially as described (31). To reduce background fluorescence, we incubated the cells in FDG staining medium [4% fetal bovine serum (FBS; JRH)/10 nM HEPES (pH 7.3)/PBS] containing 1 mM chloroquine for 30 min at 37°C, 5% CO_2. Cells were then loaded with FDG (Molecular Probe, Eugene, OR, USA) by osmotic shock. Briefly, after the cells had been allowed to equilibrate in a water bath at 37°C for 10 min, an equal volume of pre-warmed 2 mM FDG in sterile water was rapidly mixed with the cell suspension. After exactly 2 min of incubation at 37°C, the FDG loading was stopped; and cells were suspended in ice-cold staining medium containing 10 μg ml^{-1} propidium iodide for 5 min at 4°C. Then, cells were blocked with rabbit serum, and stained with biotin-conjugated rat anti-mouse mAbs against CD45 (30-F11; BD PharMingen) and integrins α4 (P/S-2; a gift from K. Miyake, Tokyo University), α5 (5H10-27; BD PharMingen), αV (RMV-7; BD PharMingen), β1 (KMI-6; a gift from K. Miyake, Tokyo University) and β3 (2C9.G2; BD PharMingen). The stained cells were further incubated with *R*-PE-labeled streptavidin (Southern Biotech Associate, Inc.). The stained cells were analyzed by using an EPICS-XL flow cytometer (Coulter).

Induction of melanocytes from thymic cells

Single-cell suspensions from thymi of E12.5 to 3.5-day-old mice were prepared as described above. The prepared cells were inoculated into 24-well plates (Corning Costar) with ST2 stromal cells (32), and cultured in α-MEM (GIBCO-BRL) containing 10% FBS (Hyclone), supplemented with the following reagents: 10^{-7} M dexamethasone (DEX, Sigma), 40 nM human recombinant ET3 (Peptide Institute) and 1 nM BQ788, an antagonist of endothelin receptor B (ETR-B; Phoenix Pharmaceuticals, Inc.). Cultures were fed every third day by replacing the medium with 2 ml of fresh medium. After 3 weeks, the cells were harvested by treatment with 0.25% trypsin/EDTA, and the number of melanocytes was counted.

Induction of melanocytes, neurons and glias in thymic clonal cell cultures

Thymic cell suspensions from E14.5, E15.5 and E17.5 embryos were prepared as described above. Then, 1–2 × 10^5 cells were inoculated into six-well plates (Corning Costar) with ST2 stromal cells (32), and cultured in α-MEM supplemented with 10% FBS, 10^{-7} M DEX, 40 nM human ET3, 1 nM heregulin (Phoenix Pharmaceuticals, Inc.) and 1 nM forskolin (Phoenix Pharmaceuticals, Inc.) for the simultaneous induction of melanocytes, neurons and glia. Cultures were fed every third day by replacing the medium with 2 ml of fresh medium. After the cells had been cultured for 14 days, they were stained

with LacZ to confirm the presence of NC-derived cells and with specific antibodies to identify the types of colonies. For detection of LacZ activity, the cells were fixed in PBS solution (pH 7.4) containing 0.25% glutaraldehyde. After having been washed, they were stained with a solution containing Bluo-Gal (GIBCO-BRL) in *N,N'*-dimethylformamide for 8 h.

Antibodies and immunohistochemistry

On serial days after induction of the differentiation of thymic cells, the cultured cells were stained separately with the polyclonal rabbit anti-mouse dopachrome tautomerase (Dct) antibody (provided by V. Hearing; Laboratory of Cell Biology, National Institutes of Health, Bethesda, MD, USA; 33) or with the following mAbs: rat anti-mouse nerve growth factor receptor p75 (p75) (AB-N02; ATSBIO, Inc.), mouse anti-mouse β-tubulin III (TUJI; Babco), mouse anti-porcine glial fibrillary acidic protein (GFAP) (GA-5; Neomarker, Inc.), rat anti-mouse erythroid lineage cells (TER119; PharMingen) or mouse anti-human HLA-DQ (Neomarker, Inc.). The cultured cells were fixed with 2% PFA. After a washing, the endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide, and the cells were then incubated with the first antibody. The bound antibody was visualized by subsequent incubation with biotin-conjugated goat antibodies against mouse, rat or rabbit IgG followed by streptavidin–HRP (HistoMark™ Streptavidin–HRP Kit). After having been washed, the cells bearing immunocomplexes were visualized by using a Diaminobenzidine Reagent Set (Kirkegaard and Perry Laboratories, Inc.).

Statistical analysis

Data were presented as mean ± SD. Statistical significance was assessed by using Student's *t*-test.

Results

Tracing NC-derived cells in the fetal thymus by using the P0-Cre/Rosa26R mouse system

To assess the presence of NC-derived cells in the thymus, we constructed mice carrying the *Cre* gene under the control of the *P0* promoter (*P0-Cre*), which expresses the *Cre* gene in the NC cell lineage (19). Crossing *P0-Cre* mice and *Rosa26R* mice, which carry *LoxP-LacZ* sequences, enabled us to trace NC-derived cells that had expressed the *P0* gene as LacZ-expressing cells. LacZ$^+$ cells were detected in organs including the mandible and heart, in which NC-derived cells were previously reported to be present, and in the thymus of E14.5 *P0-Cre/Rosa26R* double-transgenic (*Tg*) embryos (Fig. 1B). However, no LacZ$^+$ cells were detected in age-matched *Rosa26R* embryos that did not carry *P0-Cre Tg* (Fig. 1A).

Temporal differences in the presence of NC-derived cells in the thymus

To clarify the spatial and temporal presence of NC-derived cells in the developing thymus, we stained *P0-Cre/Rosa26R* mice or *Rosa26R* mice from E11.5 to E18.5 for LacZ. As shown in Fig. 2, large numbers of LacZ$^+$ (NC-derived) cells were detected in E11.5, E12.5, E14.5, E15.5 and E16.5 thymi, but these cells were rarely detected in the thymus beyond E17.5 in

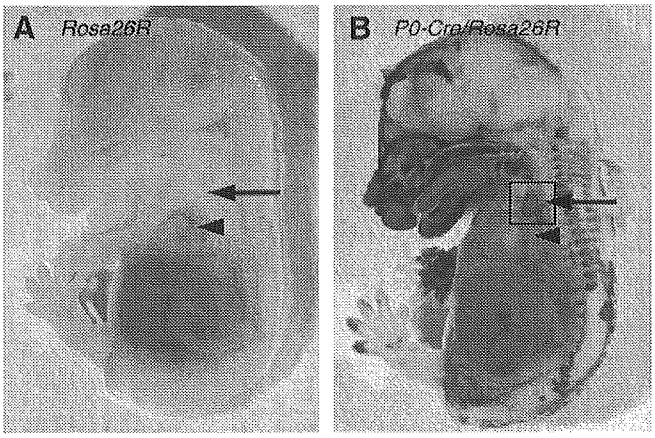


Fig. 1. Presence of NC-derived cells in the fetal thymus. Cells that expressed the *P0* gene were detected by staining of E14.5 *P0-Cre/Rosa26R* double-*Tg* mice for LacZ (B). LacZ$^+$ cells were present in the thymus (arrow) as well as in the face and heart (arrow head). No LacZ$^+$ cells were present in any tissues of age-matched *Rosa26R-Tg* mice (A).

P0-Cre/Rosa26R embryos. As NC-derived cells were still detected in the heart and craniofacial region of E18.5 *P0-Cre/Rosa26R* embryos, the present duration of NC-derived cells in the thymus might be distinct from that in other organs (Fig. 2B).

To confirm the results obtained by LacZ staining, we performed flow cytometric analysis using FDG, which allows LacZ$^+$ cells to be detected as living cells. Approximately 15% of the total thymic cells on E14.5, but only 2% on E18.5, were LacZ$^+$ cells (Fig. 3A). These results agree with the data on whole-mount embryos stained for LacZ and suggest that the number of NC-derived cells decreased in the developing thymus as it became older. Furthermore, we could rarely detect NC-derived cells in the post-natal thymus (data not shown).

Spatial distribution of NC-derived cells in the thymus

To assess the distribution of NC-derived cells in the thymus, we prepared E14.5 and E18.5 thymic sections and stained them with hematoxylin and eosin and for LacZ. In E14.5 sections, the fetal thymus was surrounded by LacZ$^+$ NC-derived cells (Fig. 3B). The region containing LacZ$^+$ cells corresponded to that of mesenchymal cells surrounding the thymus. However, only a few NC-derived cells were present in the E18.5 thymus, and their location was restricted to marginal sites close to the heart (Fig. 2B').

One-third (32%) of E13.5 total thymic cells were NC-derived ones (Fig. 3C, left graph). To assess the distribution of NC-derived cells at the marginal sites of the thymus, we treated E13.5 thymi with Dispase II to divide them into the mesenchymal and epithelial regions. It is known that digestion with Dispase II keep the basement membrane intact. Single-cell suspensions were then prepared from each region, and flow cytometric analysis was performed on them. When we used cells from the mesenchymal region surrounding the thymus, nearly half of the cells expressed LacZ. In contrast, the proportion of NC-derived cells was decreased in the population from the epithelial region (Fig. 3C). These results suggest

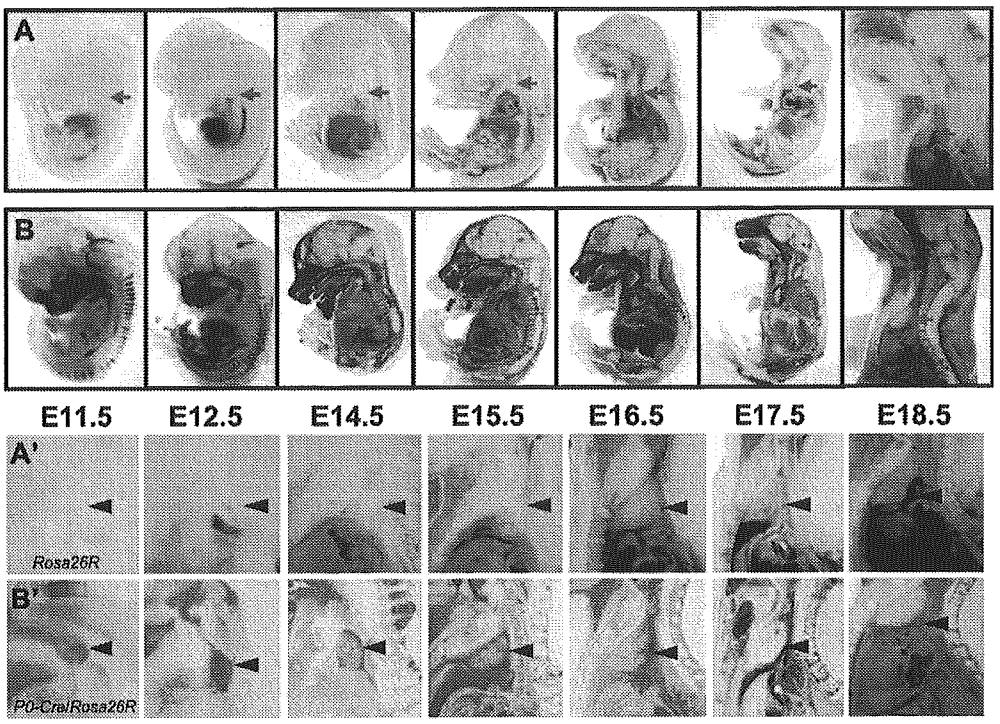


Fig. 2. Presence of NC-derived cells in the fetal thymus from E11.5 to E18.5. The thymi of E11.5 to E18.5 *Rosa26R-Tg* [A, A'(magnified)] and *P0-Cre/Rosa26R* double-Tg [B, B'(magnified)] mice were stained for LacZ (red arrows). Large numbers of $LacZ^+$ NC-derived cells were detected in the *P0-Cre/Rosa26R* double-*Tg* thymus; however, their number was reduced beyond E17.5. The arrows in (A) and (B) and the arrowheads in (A') and (B') point to the thymus.

that a majority of the NC-derived cells were distributed in the mesenchyme surrounding the thymus, although a few of them had invaded the thymic epithelium.

Characterization of NC-derived cells in the fetal thymus

To characterize NC-derived cells in the thymus, we examined the expression of adhesion molecules on NC-derived cells of *P0-Cre/Rosa26R* mice using flow cytometric analysis. First, we examined the expression of CD45 on E13.5 mesenchymal cells surrounding thymi. $LacZ^+$ cells rarely express CD45, indicating that NC do not contribute to hematopoietic cells in the thymus (data not shown). A majority of $LacZ^+$ cells in the E13.5 mesenchymal cells surrounding thymi express integrin α5, αV, β1 and β3, and half of the $LacZ^+$ cells express integrin α4 (Fig. 3D). However, larger numbers of LacZ-negative cells also express these molecules on the cell surface (Fig. 3D). It is known that mesenchymal cells are derived from both NC cells and mesodermal cells. These results may suggest that it is difficult to distinguish NC-derived mesenchymal cells from mesoderm-derived mesenchymal cells by the expression of adhesion molecules such as integrin family.

Presence of cells with the potential to differentiate into the melanocyte lineage in the fetal thymus

It is thought that melanocyte precursors might be distinct from neuronal and glial cell precursors or lose their potential to differentiate toward neuron or glial cell lineages before or soon after the initiation of NC cell migration (24, 26). In fact, mature pigmented melanocytes are not normally present in the thymus. If NC-derived cells in the thymus give rise to melanocytes, they may be multipotent cells before committing to the melanocyte lineage. Therefore, to assess the potential of NC-derived cells in the murine thymus, we first investigated whether these cells could differentiate into melanocytes. Cells from C57BL/6 wild-type fetal and neonatal thymi were cultured on ST2 stromal cells with ET3 and DEX for 3 weeks (34). Pigmented melanocytes could be induced from E12.5, E14.5 and E15.5, but not from E18.5 or 3.5-day-old thymi (Table 1, Fig. 4A). In cultures of E14.5 thymic cells, the number of melanocytes was significantly decreased in the presence of BQ788, an antagonist of ETR-B (Fig. 4A).

Subsequently, using thymic cells from *P0-Cre/Rosa26R* double-*Tg* mice or *Rosa26R* mice, we performed melanocyte induction by using the same experimental protocol. Large numbers of colonies in the cultures from *P0-Cre/Rosa26R* double-*Tg* mice consisted of $LacZ^+$ cells, and the majority of the cells with melanin granules expressed LacZ (Fig. 4C), whereas the *Rosa26R* cultures contained pigmented cells, but none was $LacZ^+$ (Fig. 4B). Therefore, almost all cells that differentiated into melanocytes had expressed the *P0-Cre* gene. The frequency of $LacZ^+$ cells forming colonies with pigmented melanocytes was ~1 per 10^4 thymic cells (Table 3). Thus, a small but significant number of NC-derived cells in the fetal thymus were able to differentiate into melanocytes. The period when $LacZ^+$ cells were present in the fetal thymus was

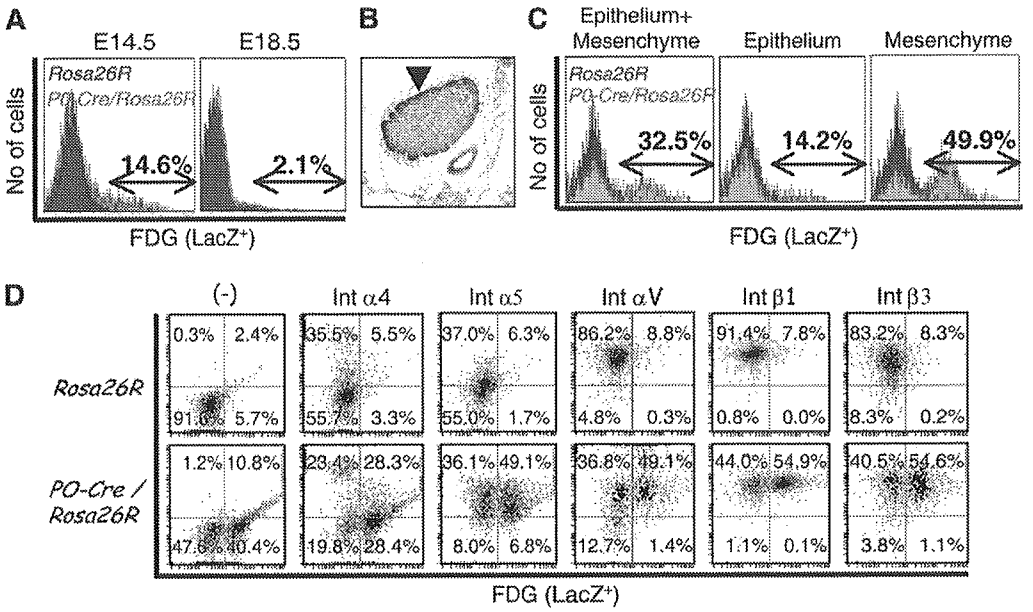


Fig. 3. Flow cytometric analysis of NC-derived cells in the thymus using FDG. (A) Cells from E14.5 or E18.5 thymi of *P0-Cre/Rosa26R* mice or *Rosa26R* mice were dissociated, and flow cytometric analysis was performed on the dissociated cells. Sections of E14.5 (B) and E18.5 (data not shown) thymi of *P0-Cre/Rosa26R* double-*Tg* mouse were stained for LacZ and also stained with hematoxylin and eosin. $LacZ^+$ cells were present in the area surrounding the thymus (B: arrowhead). No $LacZ^+$ cells were observed in the thymi of *Rosa26R* mice (data not shown). Next, E13.5 thymi were divided into mesenchyme and epithelium by treatment with Dispase II. (C) Cells from mesenchyme, epithelium and both were dissociated into single cells, and flow cytometric analysis was then performed on them. (D) The expression of adhesion molecules such as α4, α5, αV, β1 and β3 integrins on the mesenchymal cells surrounding thymi of E13.5 *P0-Cre/Rosa26R* mice. First, cells were stained with FDG to detect NC-derived cells, and then stained with antibodies against integrins.

mostly consistent with that when melanocyte precursors were present (Table 1). These results further confirm that $LacZ^+$ cells in the thymus of *P0-Cre/Rosa26R* mice are derived from NCs.

Next, to detect immature melanocyte lineage cells, we stained cultured cells with an antibody directed against Dct, which is specifically expressed on melanocyte precursors or 'melanoblasts' (33). Both Dct^+ melanoblasts and pigmented melanocytes were induced in the presence of ET3 and DEX (Fig. 4G), but neither of these types of cells was induced with DEX only (Fig. 4D and E). As neither Dct^+ cell (data not shown) nor pigmented melanocyte was detected in the E14.5 thymus, NC-derived cells prior to expressing the *Dct* gene might be stimulated by mainly ET3–ETR-B signaling to give rise to Dct^+ melanoblasts and pigmented melanocytes.

Table 1. Induction of melanocytes from fetal and post-natal C57BL/6 thymi in culture

	No. of melanocyte-containing cultures/No. of cultures examined				
Cells from +/+	E12.5 2/5[a]	E14.5 2/4[a]	E15.5 4/8	E18.5 0/6	D3.5 0/6

Two hundred thousand cells from fetal to 3.5-day-old (D3.5) thymi of C57BL/6 (+/+) mice were cultured with ST2 cells in the presence of ET3 and DEX. After 3 weeks, pigmented melanocytes were observed under a light microscope.
[a]Several thymic lobes were mixed and cultured in the case of E12.5 and E14.5 embryos to obtain 2×10^5 thymic cells, while only a pair of thymic lobes were cultured in the case of embryos from E15.5 or thereafter. Similar results were obtained in another independent experiment.

Presence of NC-derived cells in the fetal thymus with the potential to differentiate into multi-lineage cells

To determine whether NC-derived cells with the potential to differentiate into lineages other than the melanocyte lineage were present in the fetal thymus, we performed *in vitro* colony assays in the presence of ET3 with or without heregulin and forskolin, which strongly direct the differentiation of NC cells toward the glial cell lineage (35). Single cells from C57BL/6 thymi proliferated and formed a colony on day 14 of culture. The colonies from E14.5 or E15.5 thymic cells were characterized by the expression of p75 (a neuron and/or glial marker), β-tubulin III (a neuron-specific marker) or GFAP (a glia-specific marker) (Fig. 5). In some experiments, colonies were stained with a mixture of three antibodies against p75, β-tubulin III and GFAP (Fig. 5G and H). Colonies lacking both pigmented melanocytes and cells stained with antibodies directed against neurons and/or glias were omitted from the counting.

We identified three types of colonies: (i) pigmented melanocytes with melanin granules but no cells expressing neuronal or glial cell markers (named melanocyte colonies, Fig. 5A), (ii) $p75^+$ (Fig. 5B), β-tubulin III^+ or $GFAP^+$ cells (data not shown) but no melanocytes (neuron and/or glial colonies) and (iii) melanocytes and $p75^+$ (Fig. 5C), β-tubulin III^+ (Fig. 5D) or $GFAP^+$ cells (Fig. 5E) or cells stained by any three of the antibodies (Fig. 5H) (mixed colonies). As shown in Table 2, 4.5% (1 of 23 total colonies generated) of the colonies from

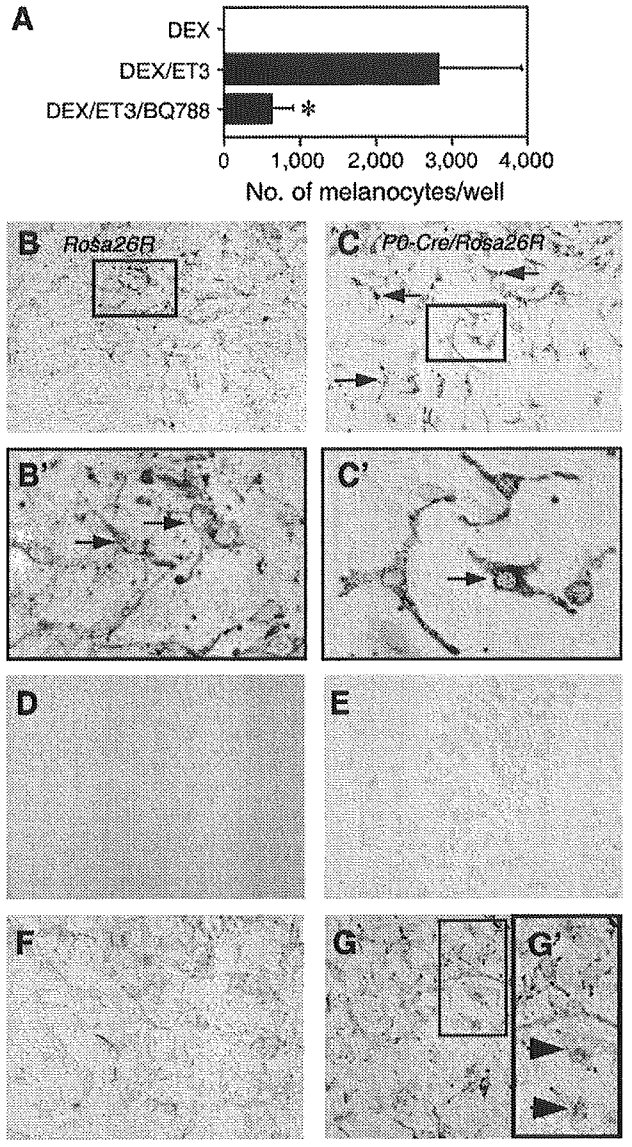


Fig. 4. Presence of cells with the potential to differentiate into melanocytes in the E14.5 thymus. (A) C57BL/6 E14.5 fetal thymic cells (2×10^5 per well) were cultured on ST2 stromal cells with DEX, DEX plus ET3 or DEX plus ET3 plus BQ788 for 3 weeks. The number of pigmented melanocytes (black cells) was then counted. Data are expressed as the mean ± SD of triplicate cultures. Asterisk indicates those significantly different from cultures with DEX + ET3 ($P < 0.05$). Next, E14.5 thymic cells from *P0-Cre/Rosa26R* double-*Tg* (C) and *Rosa26R-Tg* (B) mice were cultured in the presence of ET3. After 14 days, the cultured cells were stained for LacZ. The *P0-Cre/Rosa26R-Tg* cultures contained cells that were both pigmented (black) and LacZ$^+$ (blue) [C, C'(magnified); arrows], whereas the *Rosa26R* cultures contained pigmented cells, but none was LacZ$^+$ [B, B'(magnified); arrows]. Cultured cells treated with DEX (D, E) or with DEX + ET3 (F, G) were stained with rabbit anti-Dct antibody [E, G, G'(higher magnification)] to detect melanocyte precursors (melanoblasts: arrows) or with rabbit serum (D, F) as a control.

fetal thymi were p75$^+$ neuronal and/or glial colonies without melanocytes (Fig. 5B). Approximately 79% of the colonies from thymic cells (18/23) were melanocyte colonies, and 16% (4/23) were mixed colonies (Table 2). The formation of these mixed colonies of either melanocytes and neurons or melanocytes and glial cells thus indicate the presence of multipotent NC-derived cells (5, 36–38).

Using *P0-Cre/Rosa26R* double-*Tg* mice, we confirmed that the mixed colony-forming cells were NC-derived cells that expressed the *P0* gene (Fig. 5H). In a single colony (Fig. 5F), LacZ$^+$ cells with melanin granules (arrows in Fig. 5H) and cells stained by the mixture of antibodies directed against neurons and/or glias (arrowhead in Fig. 5H) co-existed. Pigmented melanocytes did not express p75, β-tubulin III or GFAP (Fig. 5C–E and H). These results indicate that multipotent NC-derived cells were present in the fetal thymus. In this culture, half of the colonies were melanocyte colonies, as was the case for cultures from wild-type mice, 15% of them were mixed colonies containing both p75/β-tubulin/GFAP$^+$ and pigmented cells and 30% of the colonies were neuronal and/or glial colonies containing p75/β-tubulin/GFAP$^+$ (Table 3).

NC-derived colony-forming cells were rarely detected in the E17.5 thymus

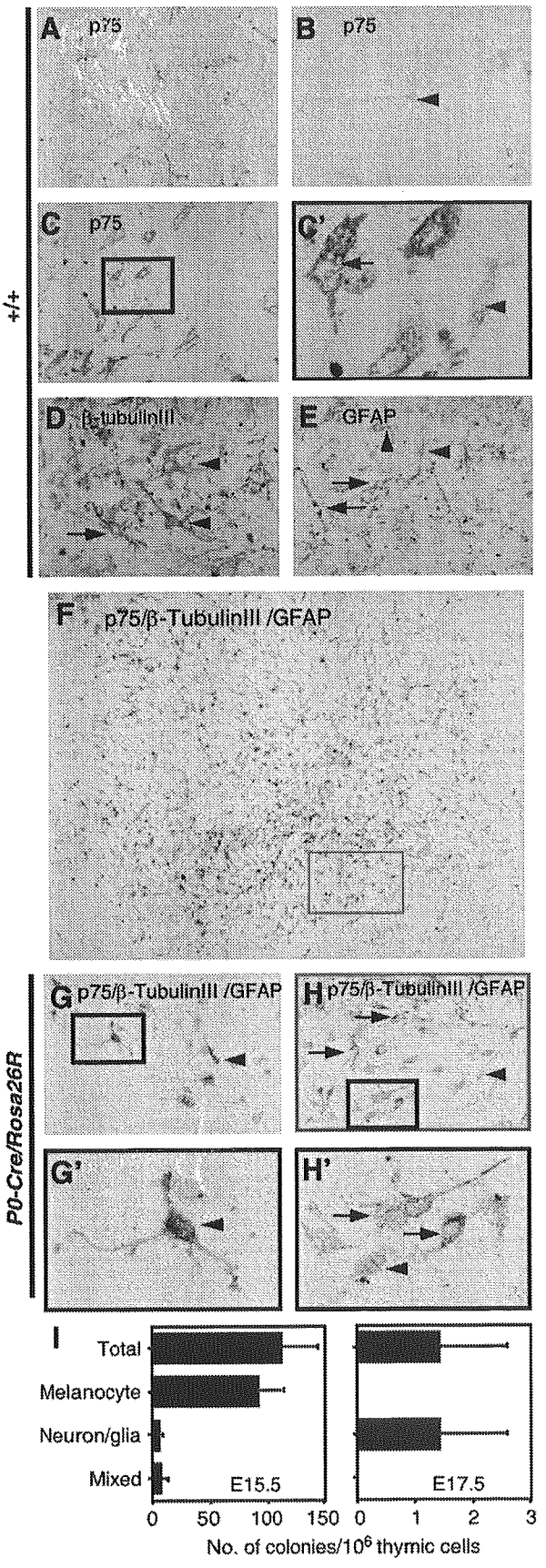
We showed that multipotent NC-derived cells were present in the E14.5 and E15.5 thymi (Fig. 5). When we assessed whether multipotent NC-derived cells were present in the thymus at late embryonic stages, few colonies were detected in cultures from the E17.5 thymus (Fig. 5I), confirming the rarity of NC-derived cells in the thymus after E17.5, as shown from the analysis of the *P0-Cre/Rosa26R* mice (Fig. 2).

Discussion

In this study, we showed that *P0-Cre/Rosa26R* mice were useful to trace NC-derived cells not only *in vivo* but also *in vitro*. Using these systems, we showed that NC-derived cells were mainly present in the area surrounding the thymus during E11.5 to E16.5 but were decreased in number beyond E17.5 and that NC-derived cells with the potential to differentiate into melanocyte, neuron and glia cell lineages were detected only during this same period.

Tracing NC-derived cells by using P0 promoter sequences

There is no specific marker for murine NC cells, unlike the case of HNK-1 for chickens and rats (39). Therefore, the promoters of *Pax3*, *Conexin43* and *Wnt1* genes have been used to detect the cranial NC cells containing cardiac NC cells (13, 40, 41). *Pax3-LacZ* and *Conexin43-LacZ* mice have provided the most reliable markers for the study of cardiac NC cell fate. Using mice bearing these markers, Waldo *et al.* detected NC cells in the E14 heart and thymus, but the expression of these transgenes was extinguished in mid- to late gestation (40). To trace the presence of NC-derived cells, we employed the promoter sequence of the *P0* gene, which promotes expression in migrating NC cells. The *P0* promoter sequence-driven *Cre* system as well as the human plasminogen activator–*Cre* system is useful for detecting not only cranial but also trunk NC-derived cells (19–21, 42), whereas the *Wnt1* promoter system allows the tracing of cranial NC cells only (13). We detected a large number of LacZ$^+$ cells in the E12.5 to E15.5 thymi of *P0-Cre/Rosa26R* mice, whereas the number was significantly decreased in E17.5 thymi and thereafter. Flow cytometric analysis confirmed the presence of LacZ$^+$ cells



(~15%) in the E14.5 thymus and showed a significant reduction in their number (to ~2%) in the E18.5 thymus (Fig. 3). This finding is not unique to *P0-Cre/Rosa26R* mice, because similar observations were made when *Wnt1-Cre/Rosa26R* mice were examined (13). These results indicate that NC-derived cells contribute to thymic organogenesis and might explain why no melanocytes were induced in the E18.5 thymi of wild-type embryos (Table 1). However, a few NC-derived cells were still present in the E18.5 thymus; thus, it remains possible that they might have lost their potential to differentiate into melanocytes.

Presence of multipotent NC-derived cells in the fetal thymus

The melanocyte lineage has been thought to be derived only from the migrating NC cells through dorsolateral pathways (43). In contrast, posterior rhombencephalic NC cells that have migrated into posterior visceral arches have the potential to differentiate into neuronal cells, but not into melanocytes (3). Therefore, the commitment to the melanocyte lineage might occur in an early differentiation phase (3, 23, 24, 27). However, it is also reported that NC cells consist of heterogenous populations with the potential for differentiation (3, 4, 27). Therefore, there still remains the possibility that NC-derived cells in the developing thymus have a potential to differentiate only into melanocytes and neurons, or melanocytes and glial cells rather than multipotent NC-derived cells.

Recently, the presence of 'NC stem cells' that differentiate into glial cells, neurons and myofibroblasts in the sciatic nerve and gut of fetal rats was reported (5, 44). These stem cells were identified based on their expression of p75 molecules but not on that of other lineage markers for glial cells, neurons or myofibroblasts. However, these studies did not demonstrate the potential of these stem cells to differentiate into melanocytes. The culture conditions might not have permitted melanogenesis, or, alternatively, the stem cells isolated might have lacked melanogenic potential. In our culture system, we could not detect multipotent NC-derived cells with the potential to differentiate into myofibroblasts in the thymus. Our culture conditions might not have permitted such differentiation.

Fig. 5. Presence of multipotent NC-derived cells able to differentiate into melanocytes, neuronal and/or glial precursors in the fetal thymus. Colony formation by E15.5 (A–E) or E14.5 (F–H) fetal thymic cells was induced by culturing the cells on ST2 for 14 days in the presence of ET3, DEX, heregulin and forskolin. Colonies were stained with anti-p75 (A–C), anti-β-tubulin III (D), anti-GFAP (E) or a mixture of anti-p75, anti-β-tubulin III and anti-GFAP antibodies (F–H). Cells reactive with each antibody are stained brown (arrowheads), and black-pigmented cells (arrows) are melanocytes. (F) An NC-derived mixed colony with both melanocytes and neurons and/or glia cells. Colonies lacking both pigmented melanocytes and cells stained by antibodies directed against neurons and/or glia were omitted from the counting. NC-derived cells were detected as $LacZ^+$ cells that had expressed *P0-Cre Tg* by using *P0-Cre/Rosa26R-Tg* mice (F–H). Higher magnifications of the boxed regions in (G) and (H) are shown in (G') and (H'), respectively. Rat and mouse mAbs against irrelevant molecules were used as controls, and no positive cells were observed in the colonies (data not shown, see Methods). Two hundred thousand cells from E15.5 or E17.5 (I) fetal thymi from C57BL/6 (+/+) mice were cultured and stained as described. 'Mixed colonies' contained both melanocytes and $p75^+$ cells. This graph shows the number of colonies per 10^6 cells. Similar results were obtained in another independent experiment.

Table 2. Detection of melanocytes, neurons and/or glias in the colonies from fetal thymic cells

Colony type	No. of colonies/2 × 10^5 thymic cells
	+/+
Total colonies	22.7 ± 6.7 (100%)
Melanocyte colonies	18.0 ± 4.4 (79%)
Neuronal and/or glial colonies	1.0 ± 0.7 (4%)
Mixed colonies	3.7 ± 1.5 (16%)

Two hundred thousand cells from E15.5 fetal thymi from C57BL/6 (+/+) mice were cultured on ST2 in the presence of ET3, DEX, heregulin and forskolin. After 14 days, the cells were stained with anti-p75 mAb to identify the type of colonies (see caption of Fig. 5). A 'mixed colony' means that the colony contained both melanocytes and $p75^+$ cells. Similar results were obtained in another independent experiment.

Table 3. Detection of melanocytes, neurons and/or glias in the colonies from *P0-Cre/Rosa26R* E14.5 thymic cells

Colony type	No. of $LacZ^+$colonies/1 × 10^5 thymic cells
Total colonies	11.1 ± 5.3 (100%)
Melanocyte colonies	5.6 ± 2.7 (50%)
Neuronal and/or glial colonies	3.4 ± 1.4 (31%)
Mixed colonies	1.7 ± 1.9 (15%)
Others	0.4 ± 0.8 (4%)

One hundred thousand cells from E14.5 fetal thymi from *P0-Cre/Rosa26R* mice were cultured on ST2 in the presence of ET3, DEX, heregulin and forskolin. After 14 days, the cells were stained with a mixture of mAbs against p75/β-tubulin III/GFAP to identify the type of colonies (see caption of Fig. 5). A 'mixed colony' means that the colony contained both melanocytes and p75/β-tubulin III/$GFAP^+$ cells. The designation 'others' means $LacZ^+$ colonies melanocytes or p75/β-tubulin III/$GFAP^+$ cells. Similar results were obtained in another independent experiment.

Although large numbers of NC-derived cells were present in the thymus, they were rarely stained by specific antibodies directed against neurons, glias or melanocytes (data not shown). The majority of cells that expressed *P0* may have differentiated into cells of other lineages, or may have remained multipotent. The absence of melanocytes, neurons and glias in the normal thymus may result from the lack of proper microenvironmental factors for differentiation into these lineages. Here, we assessed only three cell lineages, namely melanocytes, neurons and glias. Analysis of additional cell lineages may reveal the precise roles of NC-derived cells in the thymus.

NC-derived cells and thymic organogenesis

The thymus is composed of both mesenchymal and epithelial cell components (6, 15). The thymic epithelium and mesenchyme are thought to be derived from the endoderm and/or ectoderm, and the mesoderm and/or NC cells, respectively (45–47). Thymic organogenesis is divided temporally into three stages, i.e. early (E9.5–E11), middle (E11.5–E15) and late (E15.5 to birth) (48). The early-stage-initiating thymic organogenesis is thought to be regulated by interactions between epithelial and mesenchymal cells (49–51).

In the middle stage, the patterning and initial epithelial differentiation occur in thymic rudiments, and T lymphocyte progenitors immigrate there (52–54). NC-derived cells are known to contribute to blood vessel formation, and they differentiate into pericytes surrounding vascular endothelial cells derived from the mesoderm (4, 55). Although in the middle stage, blood vessels have not yet formed in the thymus, mesenchymal cells surrounding the fetal thymus express the adhesion molecules that control migration and homing of hematopoietic cells. NC-derived cells are already present in the E11.5 thymus, meaning that cells of this lineage migrate into the thymus before hematopoietic cells. As shown in this study, NC-derived cells are mainly detected in the mesenchymal region surrounding the thymus, suggesting that NC-derived cells might help the immigration of T lymphocyte progenitors into the thymus (6, 8, 56).

Furthermore, we showed that integrin family members, such as integrin α4, α5, αV, β1 and β3 (Fig. 3), and CD44 (data not shown) were expressed on the cell surface of $LacZ^+$ cells (NC-derived cells) in the thymus. Previously, it was reported that signals via interaction between integrin receptors on mesenchyme and T progenitors are important for immigration of T progenitors into the thymus (57–59). These data suggest that NC-derived cells might play an important role in the immigration of T progenitor cells. Approximately 60% of the mesenchymal cells surrounding the E13.5 thymi express PDGFRα, and 70% in these $PDGFR\alpha^+$ cells are $LacZ^+$ cells (derived from NC-derived cells) (H.Y., unpublished data). $PDGFR\alpha^+$ cells are known to be important for thymic organogenesis because the mutation of this gene results in abnormalities of both the thymus and the heart (60, 61). As PDGFRα is expressed on both mesoderm-derived cells and NC-derived ones (62), it is not clear whether $PDGFR\alpha^+$ NC-derived cells are important for thymic organogenesis (62).

NC-derived cells and the late stages of thymic organogenesis

In the late stage, thymic epithelial cells have acquired the functional competence for supporting T lymphocyte development. In this stage, NC-derived cells are rarely detected, and T progenitors immigrate into the thymus through blood vessels in the cortical–medullar junction (63), suggesting that NC-derived cells might not be required during this stage. It is thought that these blood vessels are derived from the mesoderm and not from NC-derived cells. Petrie proposed that NC-derived cells have a minor role in thymic organogenesis after birth because few NC-derived cells were observed in the *Wnt1-Cre/Rosa26R* thymus at that time (13, 64).

Le Douarin and Jotereau (8) reported that avian NC cells contribute to thymic connective tissues, which are especially located in interlobular sites in the cortex and lining blood vessels in the cortex and medulla of the gland (65). Considering these previous reports, thymic NC-derived cells might likely be considered to be detected in the thymic cortex and medulla of murine embryos at a late stage. However, in E17.5 and E18.5 *P0-Cre/Rosa26R* mice as well as in *Wnt1-Cre/Rosa26R* mice, only a few NC-derived cells were present, and their location was restricted to the epithelial marginal sites of the thymic lobes (Figs 2 and 3). However, the possibility

cannot be ruled out that these promoters might not label NC cells completely. Alternatively, NC-derived cells that do not express *P0* or *Wnt1* might contribute to the thymic organogenesis at late embryonic stages. The roles of NC-derived cells may not be identical in avian and murine systems.

Conclusions

In this study, to assess both spatially and temporally the presence and the potential of NC-derived cells in the thymus, we traced NC-derived cells and developed a colony formation assay system. It is likely that NC-derived cells in the thymus retain multipotency, but are only present during a restricted period of thymogenesis.

In the heart and craniofacial area, NC-derived cells are continuously present and contribute to the organogenesis from the embryonic stage to adult life (13). However, the fact that NC-derived cells are only present in the thymus of early embryos may indicate the existence of an as yet unknown system that contributes to the organogenesis. Further studies using our system will yield important information on the role of NC-derived cells in thymic organogenesis.

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Abbreviations

Dct	dopachrome tautomerase
DEX	dexamethasone
E	embryonic day
ET3	endothelin 3
ETR-B	endothelin receptor B
FBS	fetal bovine serum
FDG	fluorescein di-β-D-galactopyranoside
GFAP	glial fibrillary acidic protein
HNK	Human natural killer
NC	neural crest
P0	myelin protein zero
p75	nerve growth factor receptor p75
Tg	transgenic

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Down-regulation of osteoprotegerin production in bone marrow macrophages by macrophage colony-stimulating factor

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Abstract

Macrophage colony-stimulating factor (M-CSF) and receptor activator of NF-κB ligand (RANKL) induce the differentiation of bone marrow macrophages (BMMs) into osteoclasts. To delineate mechanisms involved, the effect of M-CSF on the production of osteoprotegerin (OPG), decoy receptor of RANKL, in BMMs was investigated. Mouse bone marrow cells were cultured with M-CSF for 4 days and adherent cells formed were used as BMMs. BMMs were cultured with or without M-CSF, and analyzed for expression of OPG and receptor activator of NF-κB (RANK; receptor for RANKL) mRNAs by real-time polymerase chain reaction and secretion of OPG by enzyme-linked immunosorbent assay. BMMs expressed macrophage markers, CD115 (c-fms), Mac-1 and F4/80, and showed phagocytotic activity. In addition, BMMs expressed OPG mRNA and secreted OPG into medium. M-CSF inhibited both the OPG mRNA expression and the OPG secretion dose-dependently and reversibly. The expression of RANK mRNA was not significantly affected by M-CSF. The results showed that M-CSF suppresses the OPG production in BMMs, which may increase the sensitivity of BMMs to RANKL.

Keywords: Macrophage colony-stimulating factor; Osteoprotegerin; Bone marrow macrophage; Osteoclastogenesis

1. Introduction

Osteoclasts, multinucleated cells that resorb bone, are generated from hemopoietic cells of the monocyte/macrophage lineage [1–3]. In this process, bone marrow stromal cells or osteoblasts play a pivotal role through cell-to-cell interaction [3–6]. Stromal cells/osteoblasts express receptor activator of NF-κB ligand (RANKL) on their surface and secrete macrophage colony-stimulating factor (M-CSF). RANKL and M-CSF bind to respective receptors, receptor activator of NF-κB (RANK) and M-CSF receptor (CD115, c-fms), expressed on osteoclast progenitors to induce their differentiation [3,5–12]. Stromal cells/osteoblasts also secrete osteoprotegerin (OPG), a soluble glycoprotein of the tumor necrosis factor receptor superfamily, that acts as a decoy receptor for RANKL competing against RANK and thereby inhibiting osteoclast differentiation [3,8–12]. Various cytokines or hormones exert their effects on osteoclast differentiation by regulating expression of RANKL, M-CSF and OPG in stromal cells or osteoblasts [9,10,12].

Kobayashi et al. [13] have shown that incubation of bone marrow cells with M-CSF in vitro generates adherent cells that express specific macrophage markers

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