Origins and Properties of Dental, Thymic, and Bone Marrow Mesenchymal Cells and Their Stem Cells

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Abstract
Mesenchymal cells arise from the neural crest (NC) or mesoderm. However, it is difficult to distinguish NC-derived cells from mesoderm-derived cells. Using double-transgenic mouse systems encoding P0-Cre, Wnt1-Cre, Mesp1-Cre, and Rosa26EYFP, which enabled us to trace NC-derived or mesoderm-derived cells as YFP-expressing cells, we demonstrated for the first time that both NC-derived (P0- or Wnt1-labeled) and mesoderm-derived (Mesp1-labeled) cells contribute to the development of dental, thymic, and bone marrow (BM) mesenchyme from the fetal stage to the adult stage. Irrespective of the tissues involved, NC-derived and mesoderm-derived cells contributed mainly to perivascular cells and endothelial cells, respectively. Dental and thymic mesenchyme were composed of either NC-derived or mesoderm-derived cells, whereas half of the BM mesenchyme was composed of cells that were not derived from the NC or mesoderm. However, a colony-forming unit-fibroblast (CFU-F) assay indicated that CFU-Fs in the dental pulp, thymus, and BM were composed of NC-derived and mesoderm-derived cells. Secondary CFU-F assays were used to estimate the self-renewal potential, which showed that CFU-Fs in the teeth, thymus, and BM were entirely NC-derived cells, entirely mesoderm-derived cells, and mostly NC-derived cells, respectively. Colony formation was inhibited drastically by the addition of anti-platelet-derived growth factor receptor-β antibody, regardless of the tissue and its origin. Furthermore, dental mesenchyme expressed genes encoding critical hematopoietic factors, such as interleukin-7, stem cell factor, and cysteine-X-cysteine (CXC) chemokine ligand 12, which supports the differentiation of B lymphocytes and osteoclasts. Therefore, the mesenchymal stem cells found in these tissues had different origins, but similar properties in each organ.

Introduction

All organs consist of layers of epithelial cells derived from one of the germ layers and mesenchymal cells derived from the neural crest (NC) or mesoderm. NC cells emerge from the dorsal region of the neural tube during embryogenesis and differentiate into melanocytes, neurons, glia, and mesenchymal cells, including osteoblasts, chondrocytes, adipocytes, odontoblasts, and perivascular cells [1,2]. NC cells participate in the organogenesis of the craniofacial area, including the tooth, heart, thymus, and bone marrow (BM) [2–7]. In particular, the cephalic NC supplies perivascular cells to the craniofacial area and thymus [8–10].

Mesoderm-derived cells have the potential to differentiate into osteoblasts, chondrocytes, and adipocytes, and contribute to the mesenchymal cells in the heart, thymus, and BM [2,4,11]. The craniofacial skeleton, including the mandible and maxilla, mainly develops from NC-derived cells; the skeleton outside this region mainly develops from mesoderm-derived cells [2,12]. Some mesoderm-derived cells contribute to the bones and cartilage of the cranial base and head muscles [13,14]. Mouse neck and shoulder skeleton is derived from mesenchymal cells that develop from both mesoderm-derived and NC-derived cells [15]. However, it is difficult to distinguish between NC-derived and mesoderm-derived cells.

Mesenchymal stem cells (MSCs) are long-term self-renewing cells, giving rise to one or more specialized cell types [16]. Friedenstein et al. first identified MSCs in vitro and termed them fibroblast colony-forming units (CFU-F) [17]. They defined CFU-Fs as a BM cell population grown in a serum-containing medium that produces colonies of adherent fibroblast-like cells, which can differentiate into osteoblasts, chondrocytes, and adipocytes [17]. Although the origin of MSCs is unclear, they are present in both embryonic and adult tissues in mice and humans [16,18–20]. NC-derived multipotent cells in rodents can differentiate into neurons, glia, and myofibroblasts in the gut and sciatic nerve [21–23]; they have potentials similar to MSCs in the skin and BM [6,24–26].

To distinguish NC-derived cells from mesoderm-derived cells, we used double-transgenic mouse systems encoding P0-Cre, Wnt1-Cre, Mesp1-Cre, and Rosa26EYFP, which enabled us to trace NC- or mesoderm-derived cells as YFP-expressing cells [27–32]. Wnt1 and P0 are expressed in early migratory NC [5,30], and Mesp1, a transcription factor, is first observed at E6.5 (early gastrulation...
stages), specifically in nascent mesoderm-derived cells [28]. In this study, we investigated the contributions of NC-derived and mesoderm-derived cells to the teeth, thymus, and BM using three transgenic mouse lines to establish the origin and properties of dental, thymic, and BM MSCs. CFU-F assays indicated that dental, thymic, and BM CFU-Fs comprise NC-derived and mesoderm-derived cells. We clarified the presence of cells in CFU-F progeny with the capacity for repeatable colony formation and retained multipotency.

**Results**

**Contributions of NC-derived and mesoderm-derived cells to dental mesenchyme**

We used Wnt1-Cre, P0-Cre, and Mesp1-Cre mice crossed with Rosa26EYFP mice (i.e., Wnt1/YFP and P0/YFP, and Mesp1/YFP mice, respectively) to investigate the contribution of NC-derived and mesoderm-derived cells to dental mesenchyme. Initially, we isolated hematopoietic cell-deprived YFP+ and YFP− cells and examined the gene expression associated with the NC or mesoderm. Approximately two-thirds of YFP+ cells from E9.5 Wnt1/YFP or P0/YFP embryos (i.e., Wnt1/YFP+ and P0/YFP+ cells) expressed p75NGFR (Fig. S1A). E9.5 Wnt1/YFP+ (P0/YFP+) and Mesp1/YFP− cells expressed NC-associated genes such as Ap2 and Sox10 (Fig. S1B). Wnt1/YFP+ cells in the dental mesenchyme, which were isolated from E13.5 and two-day-old mice, expressed NC-associated genes such as p75, Sox10, and Krox20, whereas Wnt1/YFP− cells expressed Brachyury (T), a mesodermal gene (Fig. S1C). Therefore, we concluded that Wnt1-Cre and P0-Cre identified NC-derived cells.

To assess the proportion of Wnt1/YFP+ cells in the dental mesenchymal cells, we prepared samples from mice that were devoid of blood cells. We found that approximately 90% of dental mesenchymal cells from E13.5 or two-day-old mice were Wnt1/YFP+, whereas only approximately 7% were Mesp1/YFP− (Fig. 1A). This difference of approximately 10-fold was observed despite the presence of both NC-derived and mesoderm-derived cells in dental mesenchyme. Large numbers of E13.5 or two-day-old Wnt1/YFP+ cells were observed in histological sections of the dental mesenchymal layer around the enamel organ and dental pulp, and Wnt1/YFP+ cells were distributed throughout the mesenchyme, whereas only small numbers of Mesp1/YFP− cells were found in these locations (Fig. 1B, C).

**Characteristics of dental mesenchymal cells and the origins of their CFU-Fs**

We fractionated dental mesenchymal cells using three markers to compare their origins: CD31 (an endothelial marker), platelet-derived growth factor receptor-α (PDGFRα) (a mesenchymal cell marker), and PDGFRβ (a mesenchymal cell or perivascular cell marker). Among the E13.5 dental mesenchymal cells, Mesp1/YFP− expressed CD31 but Wnt1/YFP+ cells rarely expressed it. In contrast, Wnt1/YFP+ cells expressed PDGFRα and PDGFRβ but Mesp1/YFP− rarely expressed these markers (Fig. 1A). Wnt1-Cre and Mesp1-Cre were indicators of reciprocally separable cell populations. PDGFRα- and PDGFRβ-expressing cells were found only in the Mesp1/YFP− cell fraction. Dental pulp cells from two-day-old and four-week-old mice produced similar results (Fig. 1A).

We also examined the expression of the endothelial cell markers CD34, FLK1, and Sca1 (an MSC marker). Sca1 was expressed in Mesp1/YFP− cells from two-day-old mice (Fig. 1A). All four-week-old Mesp1/YFP− cells expressed CD31, whereas 42% and 53% expressed CD34 and FLK1, respectively (Fig. S2). Similarly, histological sections revealed that Wnt1/YFP+ cells in the perivascular lining of two-day-old mice expressed α-SMA, but not CD31 (Fig. 1D−D”). In two-day-old mice, Mesp1/YFP+ dental mesenchymal cells were located in the inner layer of blood vessels and expressed CD31, but not α-smooth muscle actin (α-SMA) (Fig. 1E−E”). Thus, NC-derived and mesoderm-derived cells may contribute to α-SMA+ perivascular cells and CD31+ endothelial cells, respectively.

We performed CFU-F assays to determine the origin of dental MSCs, which are functional assays for measuring MSCs in vivo (Fig. 2A). We used unfractionated cells, including YFP+ and YFP−
cells from E13.5 Wnt1/YFP or Mesp1/YFP embryos, but all colonies comprised Wnt1/YFP or Mesp1/YFP cells (Fig. 2B, C). Using unfractionated dental pulp cells from two-day-old mice, we found that all colonies were Wnt1/YFP, except one, and that all consisted of Mesp1/YFP cells (Fig. 2C). Four-week-old Mesp1/YFP and Wnt1/YFP mice yielded similar results (Table S1).

To estimate the self-renewal activity of CFU-Fs, we examined the capacity for repeatable colony formation (secondary or tertiary CFU-F assays). Cells from primary colonies were used to detect secondary CFU-Fs. The frequency of secondary colony formation (0.37%–2.00%) was approximately 10 times higher than that of primary colony formation (0.06%–0.29%) (Table S1). These results suggest that dental CFU-Fs contain self-renewing MSCs. All secondary colonies were Wnt1/YFP+, but only one secondary colony from four-week-old Mesp1/YFP mice was composed of Mesp1/YFP+ cells in one of two independent experiments (Table S1, Exp. 1). The YFP+ cells from Mesp1/YFP mice exhibited proliferative capacity. However, the frequency of Mesp1/YFP+ colony formation was very low in the tertiary CFU-F assays (0.1%; 2/2,000 cells) compared with Wnt1/YFP colonies (Fig. S3A).

**Figure 2.** CFU-F assays and differential potential of dental mesenchymal cells of Wnt1-Cre/YFP and Mesp1-Cre/YFP mice. (A) Protocol of the CFU-F assays and fibroblastic colonies obtained from Wnt1-Cre/YFP mice. Scale bars, 200 μm. (B) Numbers of colonies induced from dental mesenchymal cells including both YFP+ and YFP− cells of E13.5 and 2-day-old Wnt1-Cre/YFP and Mesp1-Cre/YFP mice. Values represent the mean (SD) of triplicate cultures of two independent experiments. Y+, YFP+ colonies; Y−, YFP− colonies. (C) Percentages of YFP+ and YFP− colonies induced from dental mesenchymal cells of Wnt1-Cre/YFP and Mesp1-Cre/YFP mice. Figures within parentheses indicate the number of YFP+ colonies of the total number of colonies. Values represent percentages and colony numbers of triplicate cultures of two independent experiments (n=6/group). (D) Differentiation potential of NC-derived and mesoderm-derived dental mesenchymal cells into adipocytes, osteoblasts, and chondrocytes. Cells from YFP+ or YFP− colonies using dental mesenchymal cells from Wnt1-Cre/YFP or Mesp1-Cre/YFP mice were collected using a cell sorter. YFP expression in each cell preparation is shown in the lower panel. The cultured cells were stained with oil red O (OilR), alizarin red (ALZ), and anti-type II collagen (Col II) antibody to detect adipocytes, osteoblasts, and chondrocytes, respectively. ST2 and ATDC5 cells were the positive controls. The experiments were repeated twice and one representative experiment is presented. Scale bars, 200 μm in OilR and Col II and 1 mm in ALZ.

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Mesp1/YFP<sup>+</sup> cells recovered from primary or secondary colonies expressed PDGFRβ, but scarcely expressed CD31, which was similar to Wnt1/YFP<sup>+</sup> cells (Fig. S3B, C). Thus, CFU-Fs containing dental MSCs in the dental mesenchyme were generated mainly from NC-derived cells and rarely from mesoderm-derived cells.

Dental CFU-Fs with the potential for differentiation into osteoblasts, adipocytes, and chondrocytes are derived from NC

CFU-Fs are defined as cells with the potential for differentiation into osteoblasts, adipocytes, and chondrocytes in vitro. To assess whether colonies induced from dental mesenchymal cells could differentiate into these types, we performed CFU-F assays using two-day-old or four-week-old Wnt1/YFP and Mesp1/YFP mice. Cell suspensions prepared from YFP<sup>+</sup> colonies were cultured using reagents to induce their differentiation. Because Mesp1/YFP dental mesenchymal cells formed few colonies in the CFU-F assays, Mesp1/YFP<sup>+</sup> cells were sorted and cultured. The cultured cells were stained with ALZ, OilR<sup>+</sup>, or anti-type II collagen (Col II) antibody after 2–3 weeks to detect osteoblasts, adipocytes, and chondrocytes, respectively. Large numbers of ALZ<sup>+</sup>, OilR<sup>+</sup>, and Col II<sup>+</sup> cells were induced from Wnt1/YFP<sup>+</sup> dental mesenchymal cells (Fig. 2D). PO/YFP<sup>+</sup> dental mesenchymal cells also produced similar results. However, OilR<sup>+</sup> cells and a small number of ALZ<sup>+</sup> cells, but no Col II<sup>+</sup> cells, were induced from Mesp1/YFP<sup>+</sup> dental mesenchymal cells. In contrast, large numbers of ALZ<sup>+</sup> and OilR<sup>+</sup> cells were induced from Mesp1/YFP<sup>+</sup> cells (Fig. 2D). These results indicate that dental CFU-Fs with MSC properties were present in the dental pulp and were derived only from NC.

Roles of PDGFRs in CFU-Fs from dental mesenchymal cells

It is known that PDGFRs are expressed on MSCs, and PDGFRα<sup>+</sup> and PDGFRβ<sup>+</sup> cells were found among Wnt1/YFP<sup>+</sup> dental mesenchymal cells. PDGF is related to the CFU-F colony size when culturing BM cells in serum-deprived conditions [33], but the roles of PDGFRs in dental mesenchymal cells are unclear. We assessed their roles in colony formation using inhibitory antibodies against PDGFRα (anti-PDGFRα) and/or PDGFRβ (anti-PDGFRβ). We classified the colonies as either large (>50 cells) or small (approximately 8–50 cells). Anti-PDGFRα alone had little effect on colony formation, whereas anti-PDGFRβ decreased the number of large colonies to 15% of that observed in the presence of the isotype control or anti-PDGFRα in the primary CFU-F assay (Table S2, Fig. 3A). The total colony number observed in the presence of anti-PDGFRβ was 55% of that observed in the presence of anti-PDGFRα or the isotype control (Table S2, Fig. 3A). Similarly, in the secondary CFU-F assay, the number of total and large colonies formed in the presence of anti-PDGFRβ were 65% and 33%, respectively, of those observed in the presence of the isotype control or anti-PDGFRα (Fig. 3B). Thus, anti-PDGFRβ signaling alone had little effect on colony formation, whereas anti-PDGFRβ decreased the number of large colonies to 15% of that observed in the presence of the isotype control or anti-PDGFRα in the primary CFU-F assay (Table S2, Fig. 3A). The total colony number observed in the presence of anti-PDGFRβ was 55% of that observed in the presence of anti-PDGFRα or the isotype control (Table S2, Fig. 3A). Similarly, in the secondary CFU-F assay, the number of total and large colonies formed in the presence of anti-PDGFRβ were 65% and 33%, respectively, of those observed in the presence of the isotype control (Fig. 3B). Thus, signaling by PDGFRβ is important for maintaining dental CFU-Fs. However, we cannot rule out the possibility that PDGFRβ signaling promoted the proliferation of CFU-F descendants (Fig. 3A, B), because anti-PDGFRβ affected the number of large colonies rather than the total number of colonies. To clarify this issue, we performed a primary CFU-F assay in the presence of anti-PDGFRβ and/or anti-PDGFRα, and a secondary CFU-F assay in the absence of these antibodies. Cells prepared from colonies in the primary CFU-F assay treated only with anti-PDGFRβ or with both anti-PDGFRα and anti-PDGFRβ produced 50% or 0% of cells prepared from colonies observed in the presence of the

Figure 3. Effects of inhibitory antibodies against PDGFRα and/or PDGFRβ in CFU-F assays using dental mesenchymal cells. (A) Numbers of primary colonies (1st CFU-F) induced from dental mesenchymal cells from 4-week-old mice in the presence of anti-PDGFRα (APAS) and/or anti-PDGFRβ (APBS). None, no antibody. Numbers of large (L, >50 cells), small (clusters) (S, 8–50 cells), and total colonies (T, L+S colonies) are shown. (B) Numbers of secondary colonies (2nd CFU-F) induced from primary colonies of 4-week-old mice in the presence of anti-PDGFRα and/or anti-PDGFRβ. The primary CFU-F assay was performed in the absence of these antibodies. Numbers of large, small, and total colonies are shown. (C) Numbers of secondary colonies (2nd CFU-F) induced from primary colonies from 4,000 dental mesenchymal cells of 4-week-old mice in the presence of anti-PDGFRα and/or anti-PDGFRβ. The secondary CFU-F assay was performed in the
isotype control in the secondary CFU-F assay (Fig. 3C). Thus, PDGFRβ signaling is important for maintaining self-renewal of dental CFU-Fs, rather than proliferation of CFU-F descendants.

Origins and characteristics of thymic and BM mesenchymal cells

Previously, we reported that multipotent NC-derived cells participate in the formation of fetal thymic and dental mesenchyme [31,32]. However, the origins and characteristics of BM and thymic mesenchymal cells and their MSCs remain unclear. Thus, we examined whether NC-derived and mesoderm-derived cells contributed to BM and thymic mesenchyme from the fetal stage to the adult stage.

In the BM, both Mesp1/YFP+ and P0/YFP+ (Wnt1/YFP+) cells contributed to mesenchyme in E14.5 or E15.5 embryos, two-day-old mice, and adult mice (Fig. 4A, B). However, half of the BM mesenchyme was composed of cells other than P0/YFP+ (Wnt1/YFP+) and Mesp1/YFP+ cells (Fig. 4A-C). Immunohistochemistry detected two-day-old P0/YFP+ and Mesp1/YFP+ BM mesenchymal cells in trabecular and cortical bone (Fig. 4B). Subsequently, we reported that fetal thymic and dental mesenchymal cells have the potential to differentiate into melanocytes (a highly reliable marker of NC-derived cells) [31,32]. We confirmed the presence of NC-derived cells in BM, by testing whether these mesenchymal cells differentiate into melanocytes. Pigmented melanocytes were induced from YFP+ cells in BM and YFP+ cells from the skin of E17.5 P0/YFP+ embryos (Fig. 4D). The results suggested that NC-derived cells were present in the BM.

Next, we tested the expression of PDGFRs in BM mesenchymal cells. Unlike dental mesenchyme, PDGFRα- and/or PDGFRβ-expressing cells were present in the YFP+ and YFP− fractions of BM mesenchymal cells from two-day-old Mesp1/YFP and P0/YFP mice (Fig. 4C). The sections showed that two-day-old P0/YFP BM mesenchymal cells around the blood vessels expressed α-SMA (Fig. 5A). Furthermore, Mesp1/YFP BM mesenchymal cells of the same age expressed CD31 in blood vessels (Fig. 5B). Thus, NC-derived and mesoderm-derived cells in the BM may contribute to α-SMA+ perivascular cells and CD31+ endothelial cells, respectively.

In the thymus, most E13.5 thymic mesenchymal cells (except MHC class II+ thymic epithelial cells) consisted of either Wnt1/YFP+ or Mesp1/YFP+ cells (Fig. 4E), while 86% of CD45−Ter119− thymic mesenchymal cells from two-day-old mice were composed of either Wnt1/YFP+ or Mesp1/YFP+ cells (Fig. 4F). Similar to BM, PDGFRα- and/or PDGFRβ-expressing cells were observed in the YFP+ and YFP− fractions of thymic mesenchymal cells from two-day-old Mesp1/YFP and Wnt1/YFP mice (Fig. 4F). The sections indicated that two-day-old Wnt1/YFP+ thymic mesenchymal cells around the blood vessels expressed α-SMA, whereas Mesp1/YFP+ thymic mesenchymal cells of the same age expressed CD31 in blood vessels (Fig. 5C, D). Thus, NC-derived and mesoderm-derived cells in the thymus may contribute to α-SMA+ perivascular cells and CD31+ endothelial cells, respectively.

Origins of BM and thymic CFU-Fs and roles of PDGFRs in these CFU-Fs

The observed discrepancy between PDGFR-expressing cells in the dental mesenchyme, the thymus, and the BM mesenchyme necessitated an assessment of CFU-F origins in the thymus and BM mesenchyme. We performed CFU-F assays using BM mesenchymal cells from two-day-old Wnt1/YFP (P0/YFP) and Mesp1/YFP mice. Unlike the dental mesenchymal cells, Mesp1/YFP+ and Wnt1/YFP+ (P0/YFP+) BM mesenchymal cells exhibited colony-forming capacity (Fig. 6A). Unlike dental and thymic mesenchymal cells, it was unclear whether Mesp1/YFP+ cells were Wnt1/YFP+ (P0/YFP+) NC-derived cells or whether Wnt1/YFP+ (P0/YFP+) cells were Mesp1/YFP+ mesoderm-derived cells, because the mesenchymal cells comprised 1% Wnt1/YFP+, 15% P0/YFP+, and 27% Mesp1/YFP+ cells, indicating that 57% of cells were not Wnt1/YFP+ (P0/YFP+) NC-derived cells or Mesp1/YFP+ mesoderm-derived cells. Therefore, Wnt1/YFP+ (P0/YFP+) and Mesp1/YFP+ mesoderm-derived cells may be present in BM (Figs. 4C, 6A). Nevertheless, Wnt1/YFP+ and P0/YFP+ cells comprised 44% and 83% of BM CFU-Fs, respectively (Fig. 6A, B). P0/YFP+ BM mesenchymal cells from seven-month-old mice continued to exhibit a colony-forming capacity, and cells prepared from these colonies expressed PDGFRα and PDGFRβ (Fig. S4).

A secondary CFU-F assay was performed to investigate the self-renewal activity of BM CFU-Fs. Secondary CFU-Fs in the BM comprised 80% Wnt1/YFP+ and 20% Wnt1/YFP− cells, or 100% Mesp1/YFP+ cells (Fig. 6C, D). These results suggest that BM MSCs are derived mainly from NC and that they are maintained in older mice.

BM CFU-Fs were present in PDGFRα- and PDGFRβ- cells. Because the role of PDGFRs in BM CFU-Fs remained unclear; therefore, we examined the effects of anti-PDGFR antibodies on colony formation. Similar to dental CFU-Fs, the number of CFU-Fs induced from BM cells decreased with the addition of anti-PDGFRα and anti-PDGFRβ to the culture (Fig. 6A, B). The number of YFP+ and YFP− colonies generated from BM mesenchymal cells of P0/YFP, Wnt1/YFP and Mesp1/YFP mice decreased (Fig. 6A, B). To assess the effects of these antibodies on the self-renewal capacity of CFU-Fs, we performed a primary CFU-F assay with anti-PDGFRα and anti-PDGFRβ antibodies, and a secondary CFU-F assay was performed without either antibody. In the BM mesenchymal cells from Wnt1/YFP mice, we found that the number of secondary colonies treated with both antibodies was 40% of that observed in the presence of the isotype control (Fig. 6C). The number of YFP+ secondary colonies induced from the BM of Wnt1/YFP and Mesp1/YFP mice were reduced in the presence of both antibodies (Fig. 6C, D). Irrespective of the origins of cells, it was clear that PDGFRβ signaling is important for maintaining self-renewal of BM CFU-Fs.

Next, we performed CFU-F assays using thymic mesenchymal cells from two-day-old Wnt1/YFP and Mesp1/YFP mice. Mesp1/YFP+ and Wnt1/YFP+ thymic mesenchymal cells exhibited colony-forming capacity (Fig. 6A). In particular, secondary CFU-Fs in the thymus were derived almost entirely from Mesp1/YFP+ or Wnt1/YFP+ cells (Fig. 6C, D). Similar to the BM CFU-Fs, the number of CFU-Fs induced from thymic cells decreased with the addition of anti-PDGFRα and anti-PDGFRβ to the culture (Fig. 6A, B). The number of YFP+ and YFP− colonies generated using thymic mesenchymal cells from Wnt1/YFP and Mesp1/YFP mice decreased (Fig. 6A, B). Furthermore, thymic mesenchymal cells from Wnt1/YFP mice and Mesp1/YFP mice treated with anti-PDGFRα and anti-PDGFRβ antibodies
Figure 4. Origins and characteristics of mesenchymal cells in BM and thymus. (A) Contributions of YFP+ mesenchymal cells from Mesp1-Cre/YFP, P0-Cre/YFP, and Wnt1-Cre/YFP embryos to BM. (B) YFP+ cells from BM mesenchyme of P0-Cre/YFP and Mesp1-Cre/YFP mice from neonatal to adult stages. The mean of two independent experiments is shown. W, week; M, month. Immunohistochemistry for YFP in BM of 2-day-old P0-Cre/YFP and Mesp1-Cre/YFP mice (lower panel). White arrowheads indicate YFP+ cells in the trabecular and cortical bone. (C) Expression of YFP, PDGFRα, PDGFRβ, and CD31 on BM mesenchymal cells prepared from P0-Cre/YFP, Wnt1-Cre/YFP, and Mesp1-Cre/YFP mice. (D) Induction of melanocytes from the sorted E17.5 P0/YFP+ BM (6.5 x 10^3). The P0/YFP+ cells from the skin of the same mice (P0/YFP+ skin) and C57BL/6 mice were the controls. White arrows indicate pigmented and YFP+ melanocytes. Scale bars, 200 μm. (E) Contributions of YFP+ mesenchymal cells from Mesp1-Cre/YFP and Wnt1-Cre/YFP embryos to thymus. (F) Expression of YFP, PDGFRα, PDGFRβ, and CD31 on thymic mesenchymal cells prepared from Wnt1-Cre/YFP, and Mesp1-Cre/YFP mice. The experiments were repeated and one representative experiment is presented.

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Cre/YFP. YFP chondrocytes (Fig. 6E). Unlike dental mesenchymal cells, Mesp1/mesenchymal cells differentiated into osteoblasts, adipocytes, and each antibody to indicate the presence or absence of YFP.

Figure 5. Immunohistochemistry of the BM or thymus of P0-Cre/YFP, Mesp1-Cre/YFP, and Wnt1-Cre/YFP mice. (A–D) Immunohistochemistry for YFP, CD31, and α-SMA in the BM (A, B) or thymus (C, D) of 2-day-old P0-Cre/YFP (A), Mesp1-Cre/YFP (B, D), and Wnt1-Cre/YFP (C) mice. High-magnification views (A′–D′) of the boxed areas in (A–D), respectively. Yellow and white arrowheads indicate positive cells for each antibody to indicate the presence or absence of YFP+ cells, respectively. Scale bars, 50 μm. The experiments were repeated and one representative experiment is presented.

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exhibited an unusual capacity for secondary colony formation (Fig. 6C, D). Similar to the BM, PDGFRβ signaling is important for maintaining thymic CFU-Fs containing self-renewal MSCs, irrespective of their origin.

BM and thymic mesenchymal cells can differentiate into osteoblasts, adipocytes, and chondrocytes

We cultured CFU-F progeny to assess the differentiation potential of BM and thymic mesenchymal cells and then found that CFU-F progeny from Wnt1/YFP+/P0/YFP+ BM and thymic mesenchymal cells differentiated into osteoblasts, adipocytes, and chondrocytes (Fig. 6E). Unlike dental mesenchymal cells, Mesp1/YFP+ BM and thymic mesenchymal cells had the potential to differentiate into osteoblasts and adipocytes (Fig. 6E). Thus, CFU-Fs with MSG properties develop mainly from NC and the mesoderm in the BM and thymic mesenchyme.

Dental mesenchymal cells support B lymphopoiesis and osteoclastogenesis

Dental and BM mesenchymal cells have many similarities. BM mesenchymal cells support the differentiation of B lymphocytes and osteoclasts [34-35]. However, it is unclear whether dental mesenchymal cells support the differentiation of these cells. Thus, we prepared dental and BM mesenchymal cells from three-day-old or nine-week-old Wnt1/YFP mice. First, we examined the expression of genes encoding the critical hematopoietic factors stem cell factor (SCF), interleukin-7 (IL-7), and CXC chemokine ligand 12 (CXCL12) [36]. Interestingly, dental mesenchyme expresses Dentin sialophosphoprotein (Dsp, which is an odonoblast-specific gene), Mgf, Il7, and Cxcl12, as does BM mesenchyme (Fig. 7A). To determine whether dental mesenchyme supported the differentiation of B lymphocytes, we prepared dental and BM mesenchymal cells from Wnt1/YFP mice. Two weeks later, 200 c-Ki67+ ScalLineage+ (KSL) cells were isolated from the BM of C57BL/6 mice were cultured in purified YFP+ dental mesenchyme, which contained 94% YFP+ cells, and unfractinated BM mesenchymal cells, which contained 18% YFP+ cells with rmIL-7 (Fig. 7B). Two weeks later, we found that a large number of CD19+ cells (B lineage cells) were induced on Wnt1/YFP+ dental mesenchymal cells (YTooth), BM mesenchymal cells (unfractinated BM), and ST2 cells, which support the differentiation of B lymphocytes and osteoclasts (Fig. 7B). After six days, we induced tartrate-resistant acid phosphatase (TRAP+ multynucleated osteoclasts on Wnt1/YFP+ dental mesenchyme, BM mesenchymal, and ST2 cells (Fig. 7C). We found that 94% of these dental mesenchymal cells were Wnt1/YFP+ NC-derived cells, which indicated that NC-derived dental mesenchymal cells had similar properties to BM mesenchymal cells in terms of their support of B lymphopoiesis and osteoclastogenesis.

Discussion

This study aimed to investigate the origin and characteristics of dental, thymic, and BM mesenchymal cells and MSCs. We used Wnt1/YFP and P0/YFP mice as markers of NC-derived cells and Mesp1/YFP+ mice as markers of mesoderm-derived cells. Most dental and thymic mesenchymal cells are composed of Wnt1/YFP+ NC-derived cells or Mesp1/YFP+ mesoderm-derived cells (Fig. 8). However, 57% BM mesenchyme comprises cells other than Wnt1/YFP+ (P0/YFP+) NC-derived cells or Mesp1/YFP+ mesoderm-derived cells (Fig. 8). The lateral plate mesoderm (LPM) generates the limb skeleton [37]. Moreover, the Mesp1 gene is expressed mainly in LPM-derived head mesenchyme and cardiac mesoderm. Therefore, expression of Mesp1 gene may be insufficient to allow Cre-mediated recombination in LPM, or possibly other mesoderm-derived cells such as the paraxial mesoderm may contribute to LPM. The origin of BM mesenchymal cells may differ from that of dental and thymic mesenchymal cells.

Approximately similar numbers of P0/YFP+ and Wnt1/YFP+ cells were observed in the tooth and thymus, whereas larger numbers of P0/YFP+ cells than Wnt1/YFP+ cells were detected in BM. Although we cannot explain the difference between the numbers of YFP+ cells, the fact that YFP+ BM cells of P0-Cre/Floxed-EGFP and Wnt1-Cre/Floxed-EGFP mice differentiate into peripheral neurons and glia developing from NC [6], along with our observation that P0/YFP+ and Wnt1/YFP+ BM mesenchymal cells differentiate into melanocytes, suggests that P0/YFP+ and Wnt1/YFP mice mark NC-derived cells. Because Wnt1-Cre is expressed in early migratory NC [5,38], whereas P0-Cre is expressed in the early stage of NC cells and in the glial subset of NC cells [30,39], P0/YFP may also label other populations of NC-derived cells in addition to the common population of NC-derived cells labeled by both P0/YFP and Wnt1/YFP mice in BM. Alternatively, the discrepancy between P0/YFP and Wnt1/YFP mice may be attributed to a Cre-switching efficiency. However, YFP+ BM mesenchymal cells in Wnt1/YFP demonstrated a marked capacity for colony formation, indicating that NC contributes to BM mesenchyme and its MSC. However, ectopic expression of the P0-Cre gene occurs in BM cells, because P0/YFP+ cells were partially detected in hematopoietic cells and marked some non-NC-derived cells, including a minor population of endothelial cells in adult BM [40]. On the other hand, YFP+ cells in Sox1-Cre/YFP mice, which help in tracing neuroepithelial cells, contribute to BM mesenchyme and BM MSCs [7]. The percentage of YFP+ cells in BM mesenchyme of Sox1-Cre/YFP...
Figure 6. Origins and characteristics of BM and thymic CFU-Fs and roles of PDGFRs in these CFU-Fs. (A) Contributions of YFP+ mesenchymal cells (CD45-Ter119-cells) from 2-day-old Wnt1-Cre/YFP and Mesp1-Cre/YFP mice to BM and thymus. BM and thymic CFU-F assays were performed in the presence of anti-PDGFRb (APB5) and anti-PDGFRa (APA5) or isotype-matched control antibody. (B) Numbers of colonies induced from BM mesenchymal cells from 2-day-old P0-Cre/YFP mice in the presence of the anti-PDGFRb and anti-PDGFRa. (C, D) The secondary CFU-F assay using cells from primary colonies induced from 1,000 BM (C) and thymic (D) mesenchymal cells of 2-day-old Wnt1-Cre/YFP and Mesp1-Cre/YFP mice in the presence of anti-PDGFRa and anti-PDGFRb antibodies. The secondary CFU-F assay was performed in the absence of antibodies. T, total colonies; Y+, YFP+ colonies; Y−, YFP− colonies. Values represent the mean (SD) of triplicate cultures. Asterisks indicate a significant difference in the number of colonies compared with that of the isotype-matched control antibody (p<0.05). (E) Differentiation potential of thymic and BM mesenchymal cells from Wnt1-Cre/YFP, P0-Cre/YFP, and Mesp1-Cre/YFP mice into adipocytes, osteoblasts, and chondrocytes. YFP expression in each cell preparation is shown in the lower panel. The cultured cells were stained with oil red O (OilR), alizarin red (ALZ), and anti-type II collagen (Col II) antibody to detect adipocytes, osteoblasts, and chondrocytes, respectively. ST2 and ATDC5 cells were the positive controls. The experiments were repeated twice and one representative experiment is presented. Scale bars, 200 μm in OilR and Col II in (E), and 1 mm in (ALZ in E). White arrows indicate positive cells against Col II antibody.

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Ter119 mice were cultured. Percentages of YFP\textsuperscript{Cre}/YFP\textsuperscript{+} week-old mice; 9WBM, CD45\textsuperscript{+} cells from 3-day-old mice; 9WTm, dental mesenchymal cells from 9-

electon transfectase (Hprt\textsuperscript{+}) was the positive control. D3Tm, dental mesenchymal cells and dental mesenchymal cells can differentiate into odontoblasts, chondrocytes, adipocytes, neurons, and endothelial cells [41]. These multipotent SP cells express CD31, CD34, FLK1, and CD105 (endothelial markers), but scarcely express \(\alpha\)-SMA or NG2 (perivascular cell markers). Because endothelial cells are of mesodermal origin [42], these SP cells may be more immature than NC-derived cells or may comprise both NC-derived and mesoderm-derived cells.

Wnt1/YFP\textsuperscript{+}, unlike Mesp1/YFP\textsuperscript{+} dental mesenchymal cells, exhibited marked potential for colony formation in the CFU-F assays. Because dental pulp includes small numbers of Mesp1/YFP\textsuperscript{+} mesoderm-derived cells, we calculated the ratio of the number of YFP\textsuperscript{+} mesenchymal cells in the CFU-F assays. Although Mesp1/YFP\textsuperscript{+} thymic and BM mesenchymal cells differentiate into adipocytes and osteoblasts, Mesp1/YFP\textsuperscript{+} dental mesenchymal cells may rarely possess colony-forming capacity in the CFU-F assays. Unlike dental CFU-Fs, BM and thymic mesenchymal cells can differentiate into osteoblasts and chondrocytes in vitro, even when they successfully proliferate. Mesp1/YFP\textsuperscript{+} mesoderm-derived cells in the dental mesenchyme may already have lost the potential to differentiate into osteoblasts and chondrocytes or be committed to endothelial cells.

Human dental pulp stem cells or SHEDs are similar to BM MSCs with regard to the expression of cell-surface antigens such as STRO1\textsuperscript{+} and \(\alpha\)-SMA\textsuperscript{+} (perivascular cell markers) and CD146\textsuperscript{+} (a perivascular cell and endothelial cell marker) [43]. In mouse dental pulp and BM cells, PDGFR-expressing cells have shown colony-forming capacity in vitro. However, unlike dental CFU-Fs, BM and thymic CFU-Fs comprise both Wnt1/YFP\textsuperscript{+} (PO/YFP\textsuperscript{+}) NC-derived cells and Mesp1/YFP\textsuperscript{+} mesoderm-derived cells. Furthermore, self-renewing CFU-Fs, including MSCs, consist entirely of Wnt1/YFP\textsuperscript{+} (PO/YFP\textsuperscript{+}) NC-derived cells in the teeth, and mostly Wnt1/YFP\textsuperscript{+} (PO/YFP\textsuperscript{+}) NG-derived cells in BM. We also showed that NC-derived dental mesenchymal cells expressed genes encoding critical hematopoietic factors such as IL-7, SCF, and CXCL12, and they supported the differentiation of B lymphocytes and osteoclasts [36,44]. Although it is unclear whether NC-derived and/or mesoderm-derived BM mesenchymal cells support B lymphopoiesis and osteoclasts, it is well known that cotransplantation of BM mesenchymal cells with hematopoietic cells promotes the reconstitution of hematopoiesis [45]. Because dental mesenchymal cells facilitate the easy preparation of MSCs compared to unfractionated BM mesenchymal cells in the presence of rmIL-7. Mac1 and CD19 were used as Myeloid cell lineage and B cell lineage markers. (C) Osteoclast induction. One hundred KSL cells were cultured on unfractionated BM and YFP\textsuperscript{+} dental mesenchymal cells (Y\textsuperscript{+} Tooth) prepared from the same mice in the presence of 1\(\text{a}_{25}(\text{OH})_{2}\text{D}_{3}\), and DEX. TRAP staining was performed to detect osteoclasts. ST2 stromal cells were used as the positive control to support the differentiation of B lymphocytes and osteoclasts. Each experiment with BM or dental mesenchyme is shown with ST2 as the control (B, C). Each experiment was repeated twice and one representative experiment is presented. Scale bars, 200 \(\mu\text{m}\).

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Figure 7. NC-derived dental mesenchymal cells support B lymphopoiesis and osteoclastogenesis. (A) Expression of genes encoding hematopoietic factors on CD45\textsuperscript{+} and Ter119\textsuperscript{+} BM mesenchymal cells and dental mesenchymal cells. RT-PCR was performed using RNA from these cells. Hypoxanthine guanine phosphoribosyl transferase (Hprt\textsuperscript{+}) was the positive control. D3Tm, dental mesenchymal cells from 3-day-old mice; 9WTm, dental mesenchymal cells from 9-week-old mice; 9WBM, CD45\textsuperscript{+} and Ter119\textsuperscript{+} BM mesenchymal cells from 9-week-old mice. (B) Induction of B lineage cells. Unfractionated BM and purified YFP\textsuperscript{+} dental mesenchymal cells (Y\textsuperscript{+} Tooth) from 3-day-old Wnt1-Cre/YFP mice were cultured. Percentages of YFP\textsuperscript{+} cells in the CD45\textsuperscript{+} and Ter119\textsuperscript{+} fractions after 2 weeks’ culture (upper panels). BM-derived 200 KSL cells were then cultured on purified YFP\textsuperscript{+} dental mesenchymal cells (Y\textsuperscript{+} Tooth) or unfractionated BM mesenchymal cells in the presence of rmIL-7. Mac1 and CD19 were used as Myeloid cell lineage and B cell lineage markers. (C) Osteoclast induction. One hundred KSL cells were cultured on unfractionated BM and YFP\textsuperscript{+} dental mesenchymal cells (Y\textsuperscript{+} Tooth) prepared from the same mice in the presence of 1\(\text{a}_{25}(\text{OH})_{2}\text{D}_{3}\), and DEX. TRAP staining was performed to detect osteoclasts. ST2 stromal cells were used as the positive control to support the differentiation of B lymphocytes and osteoclasts. Each experiment with BM or dental mesenchyme is shown with ST2 as the control (B, C). Each experiment was repeated twice and one representative experiment is presented. Scale bars, 200 \(\mu\text{m}\).

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mice was almost similar to that of YFP\textsuperscript{+} cells from Wnt1/YFP mice.

The origins of dental pulp stem cells and stem cells from human exfoliated deciduous teeth (SHEDs) are unclear [16,19]. Because odontoblasts generally develop from NC, SHEDs that can differentiate into odontoblasts, osteoblasts, and neurons may develop from NC-derived cells. Contrary to our results, Iohara et al. reported that side population (SP) cells of adult porcine dental mesenchymal cells can differentiate into odontoblasts, chondrocytes, adipocytes, neurons, and endothelial cells [41]. These multipotent SP cells express CD31, CD34, FLK1, and CD105 (endothelial markers), but scarcely express \(\alpha\)-SMA or NG2 (perivascular cell markers). Because endothelial cells are of mesodermal origin [42], these SP cells may be more immature than NC-derived cells or may comprise both NC-derived and mesoderm-derived cells.

Wnt1/YFP\textsuperscript{+}, unlike Mesp1/YFP\textsuperscript{+} dental mesenchymal cells, exhibited marked potential for colony formation in the CFU-F assays. Because dental pulp includes small numbers of Mesp1/YFP\textsuperscript{+} mesoderm-derived cells, we calculated the ratio of the number of YFP\textsuperscript{+} mesenchymal cells in the CFU-F assays. The frequency of CFU-Fs within YFP\textsuperscript{+} dental mesenchymal cells was lower than 1/2,040 (1/1,080) and 1/1,178 (1/728) in E13 (2-day-old) Mesp1/YFP and Wnt1/YFP mice, respectively. Mesp1/YFP\textsuperscript{+} dental mesenchymal cells may rarely possess colony-forming capacity in the CFU-F assays. Although Mesp1/YFP\textsuperscript{+} thymic and BM mesenchymal cells differentiate into adipocytes and osteoblasts, Mesp1/YFP\textsuperscript{+} dental mesenchymal cells rarely differentiate into osteoblasts and chondrocytes in vitro, even when they successfully proliferate. Mesp1/YFP\textsuperscript{+} mesoderm-derived cells in the dental mesenchyme may already have lost the potential to differentiate into osteoblasts and chondrocytes or be committed to endothelial cells.

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with BM stromal cells, dental mesenchyme may represent a useful resource in improving hematopoiesis in patients with hematopoietic disorders. However, it is still not clear the role of NC-derived cells or mesoderm-derived cells in the BM and thymic lymphohematopoiesis. Further examination is desire to elucidate these roles.

We first indicated that an inhibitory antibody against PDGFRβ decreased the CFU-F count. Retention of 15% CFU-Fs generated in the control culture in the presence of anti-PDGFRβ implies that both PDGFRβ-dependent and PDGFRβ-independent CFU-Fs are present in the dental pulp, BM, and thymus. Simultaneous addition of anti-PDGFRα and anti-PDGFRβ more effectively blocked secondary colony formation in dental and thymic CFU-F progeny. Because PDGFRα is upregulated in response to Pdgfb mutation [46], anti-PDGFRβ–treated cells may increase PDGFRα expression and become sensitive to anti-PDGFRα.

Conclusion
This is the first report to demonstrate that both NC-derived and mesoderm-derived cells with CFU-F capacity contribute to the dental pulp, thymus, and BM from the fetal stage to the adult stage. Although the origin of self-renewing CFU-Fs differs by tissue, these CFU-Fs are dependent on PDGFRβ irrespective of their origin.

Materials and Methods

Animals and Ethics Statement
P0-Cre, Wnt1-Cre, and Rosa26EYFP mice were provided by Drs. K. Yamamura (Kumamoto University), H. Sucov (Southern California University), and H. Enomoto (RIKEN Kobe), respectively [47]. Mesp1-Cre and C57BL/6 mice were obtained from the Riken Bioresource Center and Clea Japan, Inc., respectively. All mice were maintained at the Institute of Laboratory Animals, Mie University; all experimental procedures were approved by the Institutional Animal Care and Use Committee of Mie University (approval number 20–22), and were performed according to the Mie University guidelines for laboratory animals.

Preparation of single-cell suspensions
The mandibular molar tooth buds and dental pulp of the lower incisors were incubated with 2.4 U dispase II (Roche) in 10% FBS/HBSS for 30 min at 4°C and 1 mg/mL collagenase D (Roche) in 10% FBS/HBSS for 2 h at 37°C. Thymi were incubated with 1 mg/mL collagenase D in 10% FBS/HBSS for 1 h at 37°C. Femora and tibia were minced and were incubated with 2.0 U dispase II and 0.1 mg/mL collagenase D in 10% FBS/HBSS for 1 h at 37°C, and then in 2% FBS/HBSS for 1 h at 37°C. The dorsal regions of E9.5 embryos were dissected and incubated with 2.4 U dispase II in 10% FBS/HBSS at 4°C for 1 h, minced, and then incubated with 1 mg/mL collagenase D in
RNA isolation and RT-PCR

Total RNA was prepared using Trizol (Invitrogen). cDNA synthesis was carried out using reverse transcriptase (ReverTraAce; Toyobo) and oligo (dT) primers (Toyobo). PCR using cDNA was performed with rTag polymerase (Toyobo) and the forward primers and reverse primers were as follows: AP2: 5′-AGGGACTTTGGGTACGTGTG-3′, 5′-GAGATAGTT-3′, p75(NGRF): 5′-TGCTGCTGCTGCTGCTGCTGCTGCT-3′, 5′-GGGGCTTTTCC-3′, 4 min; 35 cycles at 93 °C for 1 min, and 72 °C for 1 min, and 72 °C for 7 min.

Antibodies

The following antibodies were used: biotin-conjugated PDGFRβ (AP5; eBioscience); Pacific Blue-conjugated streptavidin (eBioscience); APC-conjugated PDGFRα (AP5; eBioscience), PDGFRβ (AP5), CD31 (PECAM1) (MEC13.3; Biolegend), Mac1 (M1/70), Gr1 (8C5), and c-Kit (2B8; eBioscience); Cy7-PE-conjugated CD45 (30-F11; eBioscience), Ter119 (eBioscience), Mac1 (M1/70; Biolegend), and c-Kit (2B8; eBioscience); and/ or PDGFRβ (anti-PDGFRβ, AP5) [49]; antibodies against c-Kit (ACK4) and IL-7Rα (7R) were used as isotype-matched controls in BM and thymic cultures, respectively. Inhibitory antibodies against c-Fms (AP96) were used to inhibit macrophage proliferation. Two weeks later, large (>50 cells) and small colonies (clusters <50 cells) and YFP+ and YFP− colonies were scored as primary CFU-Fs [17]. For secondary CFU-F assays to detect self-renewing CFU-Fs, cells from primary cultures were cultured in the presence/absence of antibodies. Selected YFP+ /YFP− primary colonies were cultured to establish YFP+ clones.

Induction of adipocytes, osteoblasts, and chondrocytes

For osteoblast induction, 100,000 cells were cultured in DMEM medium (Gibco) supplemented with 10% FBS, 10−7 M Dexamethasone (DEX; Sigma), 40 nM human ascorbic acid 2-phosphatase (Sigma), 1 nM BMP4 (Neomarker), and 10 mM β-glycerophosphate (Sigma) [32,50]. For adipocyte induction, 100,000 cells were cultured in α-MEM supplemented with 10% FBS, 0.25 μM DEX, 0.5 mM 3-isobutyl-1-methylxanthine (Sigma), 1 μM Triiodo (Sigma), and 0.2 μM insulin (Sigma) [51]. For chondrocyte induction, 200,000 cells (1×105/mL) were cultured in α-MEM supplemented with 10% FBS, 10−7 M DEX, 40 nM ascorbic acid-2-phosphatase, and 1 nM TGFβ3 or BMP2 (Neomarker) [49]. After 2–3 weeks, cells were stained with oil-red-O (OIR), alizarin red (ALZ), and mouse anti-type II collagen antibody (6B3, Neomarker) and Cy5-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratory) to detect adipocytes, osteoblasts, and chondrocytes, respectively. ST2 and ATDC5 cells were the positive controls for osteogenesis and adipogenesis, and chondrogenesis, respectively [32].

Induction of B lymphocytes and osteoclasts

Dental and BM mesenchymal cells were prepared from 3-day-old Wnt1-Cre/YFP mice. Femoral c-Kit+ ScAl2 (KSL) cells were isolated from 8-week-old C57BL/6 mice. For B lymphocyte induction, 200 KSL cells were cultured on purified YFP+ dental mesenchymal cells or unfractonated BM mesenchymal cells in RPMI-1640 (Gibco) supplemented with 10% FBS, 5×10−3 M 2ME, and 10 ng/mL rmIL-7 (Invitrogen). After 2 weeks, cells collected were analyzed by FACs. For osteoclast induction, 100 KSL cells were cultured on these mesenchymal cells in α-MEM supplemented 10% FBS, 10−7 M DEX, and 10−7 M 1α, 25-dihydroxyvitamin D (1α,25(OH)2D3) (Biomol Research Laboratory). After 6 days, TRAP activity of cells was studied to detect osteoclasts [34]. ST2 cells were the positive control used for the differentiation of B lymphocytes and osteoclasts [34,35].

Induction of melanocytes from BM mesenchymal cells

Single-cell suspensions from BM of P0-Cre/YFP embryos were prepared. Sorted YFP+ BM mesenchymal cells from P0-Cre/YFP embryos (20,000) were cultured on ST2 cells in α-MEM containing 10% FBS with 10−7 M DEX, 20 pM rHGF (R&D Systems), 10 pM cholera toxin (Sigma), and 40 nM rHET3.
(Peptide Institute) [31]. Skin YFP+ cells of the same mice were used as a positive control. The pigmented melanocytes were microscopically examined after 3 weeks.

Statistical analysis
Data are expressed as means (SD). Statistical significance was assessed using Student’s t-test.

Supporting Information

Figure S1 Expression of NC- and mesoderm-associated genes on cells from P0-Cre/YFP, Wnt1-Cre/YFP, and Mesp1-Cre/YFP mice. (A) Expression of p75NGFR on cells in the CD45− and Ter119− fractions from E9.5 P0-Cre/YFP and Wnt1-Cre/YFP embryos. Empty means secondary antibody only (Alexafluor 405-conjugated goat anti-rabbit IgG) without primary antibody. (B) Expression of NC-associated genes on cells from E9.5 P0-Cre/YFP, Wnt1-Cre/YFP, and Mesp1-Cre/YFP embryos (n = 4/group). (C) Expression of NC- and mesoderm-associated genes in dental mesenchymal cells from Wnt1-Cre/YFP mice (n = 4/group). YFP+ and YFP− cells were isolated using a cell sorter. RT-PCR was performed using RNA from these cells. Hypoxanthine guanidine phosphoribosyl transferase (Hprt) was the positive control; no expression was detected without a template (data not shown).

(TIF)

Figure S2 Expression of cell-surface antigens related to endothelial cells on dental mesenchymal cells from 4-week-old Wnt1-Cre/YFP and Mesp1-Cre/YFP mice. Expression of cell-surface antigens related to endothelial cells on YFP+ or YFP− dental mesenchymal cells in CD45− and Ter119− fractions from 4-week-old Wnt1-Cre/YFP and Mesp1-Cre/YFP mice. The experiments were repeated twice and one representative experiment is presented.

(TIF)

Figure S3 Expression of PDGFR and CD31 on dental mesenchymal cells from CFU-F progenies of 4-week-old Mesp1-Cre/YFP or Wnt1-Cre/YFP mice. (A) Number of colonies induced from BM mesenchymal cells prepared from 4-week-old Wnt1-Cre/YFP mice. Values represent the means (SD) of triplicate cultures. Asterisks indicate a significant difference (p<0.05). The experiments were repeated twice and one representative experiment is presented.

(TIF)

Figure S4 CFU-Fs of BM mesenchymal cells from 7-month-old P0-Cre/YFP mice. (A) Numbers of colonies induced from BM mesenchymal cells from 7-month-old P0-Cre/YFP mice. (B) Expression of YFP, PDGFRα, and PDGFRβ on cells from these colonies. Values represent the mean (SD) of triplicate cultures. The experiments were repeated twice and one representative experiment is presented.

(TIF)

Table S1 The number of primary and secondary colonies and the origin of colony-forming cells in dental mesenchymal cells. Numbers of primary colonies (1st CFU-F, >50 cells) induced from 4×103 dental mesenchymal cells from 4-week-old Wnt1-Cre/YFP and Mesp1-Cre/YFP mice. Numbers of secondary colonies (2nd CFU-F, >50 cells) induced from 1×103 primary colonies of 4-week-old mice. Values represent the means (SD) of triplicate cultures. Asterisks indicate total number of colonies obtained from triplicate cultures of two independent experiments.

(DOC)

Table S2 Effects of inhibitory antibodies against PDGFRα in CFU-F assays using dental mesenchymal cells. Numbers of colonies induced from 3×103 dental mesenchymal cells prepared from 4-week-old Wnt1-Cre/YFP mice in the presence of inhibitory antibody against PDGFRα (AP5) and/or inhibitory antibody against PDGFRβ (APB5). No add means no antibody, and control means isotype-matched control antibody (ACK4). All antibodies were used in 10 mg/ml. Numbers of large (L, >50 cells), small (clusters) (S, <50 cells), and total colonies (T, L+S colonies) are shown. Values represent the means (SD) of triplicate cultures. Asterisks indicate a significant difference from the number of colonies in the presence of the isotype-matched control antibody (p<0.05). The experiments were repeated twice and one representative experiment is presented.

(DOC)

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Author Contributions
Conceived and designed the experiments: HY TY. Performed the experiments: YK DK KI HY. Analyzed the data: HY TY. Contributed reagents/materials/analysis tools: NT. Wrote the paper: HY TY SH.

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