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Identification of Adult Mesodermal Progenitor Cells and Hierarchy in Atherosclerotic Vascular Calcification

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ABSTRACT

The nature of calcifying progenitor cells remains elusive. In this study, we investigated the developmental hierarchy and dynamics of progenitor cells. In vitro and in vivo reconstitution assays demonstrated that Sca-1+/PDGFRα– cells in the bone marrow (BM) are the ancestors of Sca-1+/PDGFRα+ cells. Cells of CD29 + Sca-1+/PDGFRα– lineage in the BM showed both hematopoietic potential with osteoclastic differentiation ability as well as mesenchymal stem cell-like properties with osteoblastic differentiation potential. Clonally-isolated BM-derived artery-infiltrated Sca-1+/PDGFRα– cells maintained osteoblastic/osteoclastic bipotency but lost hematopoietic activity. In hypercholesterolemic apolipoprotein-E-deficient (Apoe-/-) mice, the mobilization from BM to peripheral circulation, followed by migration into atherosclerotic plaques of Sca-1+/PDGFRα– cells, but not Sca-1+/PDGFRα+ cells, were significantly decreased, and Interleukin-1β (IL-1β) and Interleukin-5 (IL-5) mediated this response. Here, we demonstrated that Sca-1+/PDGFRα– cells are mesodermal progenitor cells in adults, and the dynamics of progenitor cells were regulated by atherosclerosis-related humoral factors. These results may contribute to better understanding of vascular homeostasis and assist in the development of novel therapies for atherosclerosis. STEM CELLS *2018;36:1075–1096*

SIGNIFICANCE STATEMENT

Mesodermal progenitor cells ([MPCs] Lin-CD29 + Sca-1+/PDGFRα– cells) are present in the adult BM. Lin-CD29 + Sca-1+/PDGFRα– cells have bidirectional (osteoblastic/osteoclastic differentiation) and hematopoietic potential, and are ancestors of Sca-1+/PDGFRα+ cells. MPCs mobilize into peripheral blood and infiltrate the artery under the influence of atherosclerosis-related cytokines. In contrast, Lin-CD29 + Sca-1+/PDGFRα+ cells have unidirectional (osteoblastic differentiation) potential and are not affected by the atherosclerotic milieu.

INTRODUCTION

Vascular disorders, such as atherosclerosis and calcification, present a significant clinical challenge. Intimal and medial calcifications are the two major forms of calcification that can be distinguished pathologically and exist in different clinical settings. Intimal calcification is usually associated with the atherosclerotic milieu, occurs under the injured endothelium, and is mediated by atherosclerosis-associated lipids and cytokines. In contrast, medial calcification is involved with accumulation of minerals in the smooth muscle layer and is associated with metabolic disorders [1]. In this study, we focused on atherosclerotic intimal calcification.

Recent studies have revealed that cellular components and their dynamics play a pivotal role in vascular disease, representing potential therapeutic targets for the treatment, or even reversal, of disease processes [2–4]. We recently identified vascular calcifying progenitor cells in arterial tissue, mainly the adventitia, using the cell surface markers stem cell antigen-1 (Sca-1) and platelet-derived growth factor receptor alpha (PDGFRα) [5, 6]. These cells were derived from the bone marrow (BM) and homed to inflamed atherosclerotic lesions. These BM-derived vessel-infiltrated calcifying progenitor cells were of two types: (a) Sca-1+/PDGFRα+ cells possessing a unipotent osteoblastic (OB) differentiation potential and (b) Sca-1+/PDGFRα– cells exhibiting bipotent (OB/osteoclastic [OC] differentiation (bidirectional) properties. We demonstrated that bipotent Sca-1+/PDGFRα– cells could be used to reduce vascular calcification by inducing them to differentiate into OC-like cells upon peroxisome proliferator-activated

receptor γ (PPARγ) activation. However, the nature of the progenitor cells remains elusive.

A widely accepted theory is that OBs are derived from cells of the mesenchymal lineage [7, 8]. Previous studies have identified the various types and origins of circulating and artery-resident calcifying cells as well as their multi-lineage potential and contribution to vascular calcification [9–11]. However, decalcifying OCs are considered as cell-cell fused multinucleated cells derived from hematopoietic progenitor cells in the bone and BM [12], which originate from a different lineage than OBs.

Therefore, the concept of OB/OC bipotent progenitor cells, especially in adults, is against the traditional concept and required further investigation. Here, we attempted to determine whether mesodermal progenitor cells (MPCs) exist in adults. We hypothesized that multipotent MPCs [13–16] generate both mesenchymal stem cells (MSCs) and hematopoietic stem cells (HSCs) and subsequently differentiate into OB- and OC-like cells. We investigated the developmental hierarchy of the two types of progenitor cells and investigated their in vivo dynamics in the atherosclerotic milieu as well as their role in the pathophysiology of the disease. Furthermore, we explored the role of the surface marker PDGFRα during differentiation. We observed that the multipotent MPC, which is the common ancestor of the HSC and MSC, exists in adult BM, and demonstrated that the dynamics of this progenitor cell are mainly regulated by atherosclerosis-related humoral factors. In addition, we demonstrated that PDGFRα, which is expressed as a surface marker, does not play a functional role during OB/OC differentiation.

Materials and Methods

The detailed materials and methods are described in the Supporting Information Experimental Methods section.

Results

Isolation of Two Populations of Calcifying Progenitor Cells from the Adult BM

We isolated calcifying progenitor mononuclear cells from the BM of Apoe-/- mice and used lineage cocktail and CD29 antibodies to delete mature hematopoietic-lineage cells and include mesenchymal-lineage cells [17, 18]. For identifying calcifying progenitor cells, we stained cells with Sca-1 and PDGFRα antibodies as described previously [5].

We compared the OB differentiation potentials of Sca-1/PDGFRα-sorted cells and Sca-1/PDGFRβ-sorted cells using PDGFRβ, a well-known MSC marker [19]. Sca-1+/PDGFRβ+ and Sca-1+/PDGFRβ- cells, as well as Sca-1+/PDGFRα+ and Sca-1+/PDGFRα– cells, demonstrated OB activity (Supporting Information Fig. S1). Despite similar trends of PDGFRα and PDGFRβ as markers, we selected PDGFRα for use in this study owing to experimental efficiency and clarity of data.

Using Sca-1 and PDGFRα markers, we isolated two different progenitor cell populations: Lin-CD29 + Sca-1+/PDGFRα+ and Lin-CD29 + Sca-1+/PDGFRα– (Fig. 1A). Under OB differentiation conditions, both Sca-1+/PDGFRα+ and Sca-1+/PDGFRα– cells expressed alkaline phosphatase (ALP). OB differentiation triggered PDGFRα expression on Sca-1+/PDGFRα– cells (Supporting Information Fig. S4 and Fig. 1B, lower panel; red box → blue box), and the Sca-1+/PDGFRα+ cells were apparently derived from Sca-1+/PDGFRα– cells. Under OC differentiation conditions, the Sca-1+/PDGFRα– cells expressed receptor activator of nuclear factor κB (RANK); however, the Sca-1+/PDGFRα+ cells did not express this marker (Fig. 1C). During OC differentiation, the majority of Sca-1+/PDGFRα– cells did not exhibit any changes in the expression of the surface markers, which was opposite to the trend observed during OB differentiation, suggesting that the milieu under OB condition could induce the differentiation of Sca-1+/PDGFRα– cells into Sca-1+/PDGFRα+ cells. Overall, these results showed that Lin-CD29 + Sca-1+/PDGFRα– cells are OB/OC bipotent, whereas Lin-CD29 + Sca-1+/PDGFRα+ cells are OB unipotent. These data were consistent with those reported in our previous study [5].

A previous study demonstrated that perivascular Gli1+ cells have mesenchymal characteristics and respond to Sonic hedgehog (Shh) stimulation [19]. Gli1 is an intracellular molecule downstream of Shh, the levels of which are increased by Shh stimulation [20–22]. Therefore, we examined Gli1 expression and Shh responsiveness in Sca-1+/PDGFRα+ and Sca-1+/PDGFRα– cells. Sca-1+/PDGFRα+ cells expressed Gli1 (Supporting Information Fig. S2), and Shh stimulation increased Gli1 expression (Supporting Information Fig. S3A). Interestingly, Lin-CD29 + Sca-1+/PDGFRα+ cells expressed more Gli1 than other types of BM cells but did not respond to Shh stimulation (Supporting Information Fig. S3B).

Lin-CD29 + Sca-1+/PDGFRα– Cells in Adult BM Are MPCs

To determine the hematopoietic potential and hierarchy of the two types of progenitor cells, we performed in vitro colony-forming assay in methylcellulose [23]. Lin-CD29 + Sca-1+/PDGFRα+ cells did not form colonies, whereas Lin-CD29 + Sca-1+/PDGFRα– cells formed various types of hematopoietic colonies, including colony forming unit-macrophage/granulocyte (CFUGM), colony forming unit-granulocyte/erythroid/macrophage/megakaryocyte (CFU-GEMM), and burst-forming unit-erythroid (BFU-E) colonies (Fig. 2A). We analyzed subfractions of the two types of progenitor cells to compare traditionally recognized HSCs (Fig. 2B). We observed that 10% Lin-CD29 + Sca-1+/PDGFRα+ cells expressed c-Kit, but did not form colonies, whereas 50% Lin-CD29 + Sca-1+/PDGFRα– cells expressed c-Kit and formed various types of colonies (Fig. 2C).

Next, we isolated Lin-/Sca-1+/c-Kit+ cells and co-stained them with various markers of repopulating and self-renewing HSCs [24] to confirm that the CD29 (a marker of mesenchymal lineage)-expressing Sca-1+/PDGFRα– cells have HSC potential. Both Lin-/Sca-1+/c-Kit+/CD34-/CD38- and Lin-/Sca-1+/c-Kit+/CD34-/CD135- cells were CD29+/PDGFRα–. Moreover, Lin-/Sca-1+/c-kit+/CD34-/CD150+ cells and Lin-/Sca-1 + c-Kit + CD34-CD90.1+ cells were also CD29+/PDGFRα–. Therefore, these results suggested that Lin-CD29 + Sca-1+/PDGFRα– cells exhibit hematopoietic potential (Fig. 2D).

To confirm the in vitro results, we investigated the in vivo hematopoietic activity using nonmyeloablative sublethal radiation [25], following which, we injected 1,000 green fluorescent protein (GFP) + Lin-CD29 + Sca-1+/PDGFRα+ or GFP + Lin-CD29 + Sca-1+/PDGFRα– cells isolated from the BM of GFP mice (Fig. 2E). Twelve weeks after cell infusion, GFP fluorescence was used to evaluate chimerism. The GFP + Lin-CD29 + Sca-1+/PDGFRα+ transplantation group showed less than 0.1% donor chimerism, whereas the GFP + Lin-CD29 + Sca-1+/PDGFRα– transplantation

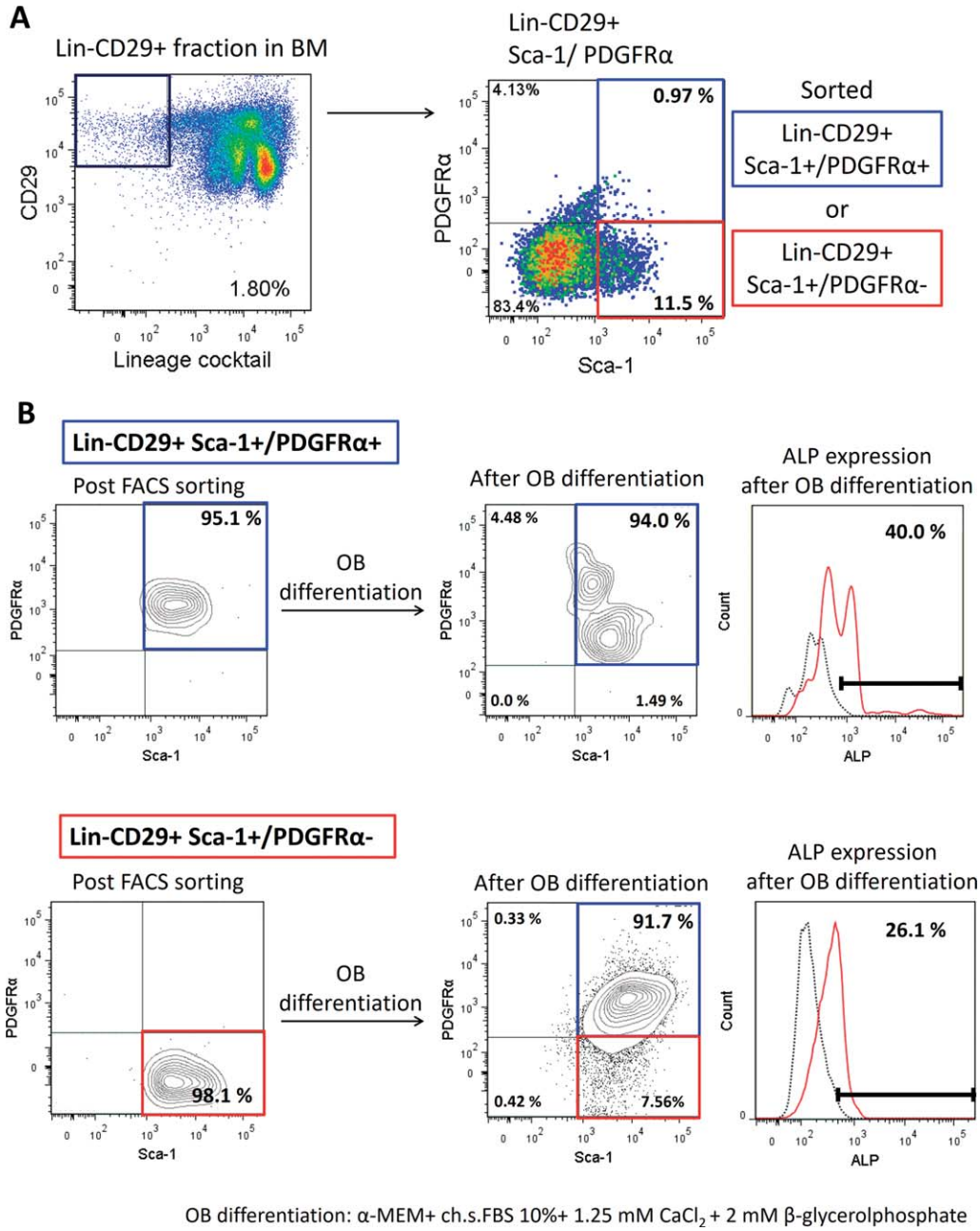
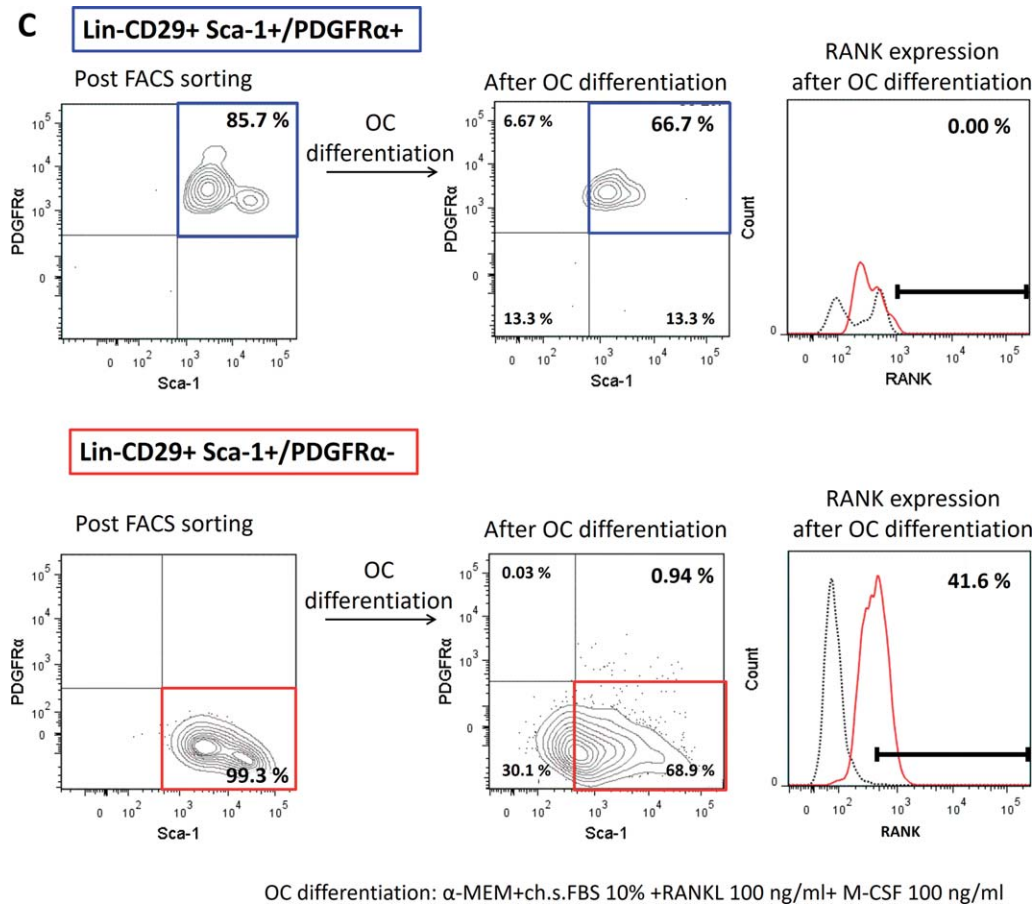


Figure 1. Isolation of two types of progenitor cells in adult BM and their differentiation potential. **(A):** Lineage cocktail (Lin) and CD29 antibodies were used to identify calcifying progenitor cells from the BM of Apoe-/- mice. Lin-CD29+ cells were further stained with Sca-1 and PDGFRα. Sca-1+/PDGFRα+ cells (blue box) and Sca-1+/PDGFRα– cells (red box) under the Lin-CD29+ gate. **(B):** Under OB differentiation conditions, both Lin-CD29 + Sca-1+/PDGFRα+ and Lin-CD29 + Sca-1+/PDGFRα– cells expressed the OB marker ALP. Lin-CD29 + Sca-1+/PDGFRα– cells turned into Lin-CD29 + Sca-1+/PDGFRα+ cells during OB differentiation (red box → blue box). **(C):** Under OC differentiation conditions, Lin-CD29 + Sca-1+/PDGFRα– cells (red box) expressed the OC marker RANK. Lin-CD29 + Sca-1+/PDGFRα+ cells (blue box) did not express RANK. All experiments were conducted at least in triplicate. Abbreviations: ALP, alkaline phosphatase; BM, bone marrow; OB, osteoblastic; PDGFRα, platelet-derived growth factor receptor alpha; Sca-1, stem cell antigen-1.

group showed 17% donor chimerism, indicating in vivo hematopoietic reconstitution potential (Fig. 2F). Furthermore, Sca-1+/PDGFRα– cells generated Sca-1-/PDGFRα–, Sca-1-/PDGFRα+, and Sca-1+/PDGFRα+ progeny cells, indicating that Lin-CD29 + Sca-1+/PDGFRα– cells represent the ancestors of Lin-CD29 + Sca-1+/PDGFRα+ cells (Fig. 2F). We additionally investigated the reconstituted BM using various HSC markers. We isolated GFP+/Lin-/Sca-1+/c-Kit+ cells from BM, which were CD29+ and PDGFRα– (Fig. 2G). Additionally, we co-stained GFP+/Lin-/Sca-1+/c-Kt+ cells with CD34, CD38, CD135, CD150, or CD90.1. All five types of repopulating HSCs [24] in the Lin-/Sca-1+/c-kit+ cell population of the reconstituted BM were CD29+/PDGFRα– (Supporting Information Fig. S5).

To validate the hematopoietic activity of the two cell types, we isolated reconstituted BM cells under GFP + Lin-CD29+ gates and then separated Sca-1+/PDGFRα+ and



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Sca-1+/PDGFRα– cells. Ex vivo colony-forming assay revealed that Sca-1+/PDGFRα– cells, but not Sca-1+/PDGFRα+ cells, formed numerous hematopoietic colonies (Fig. 2H). These results indicated that Lin-CD29 + Sca-1+/PDGFRα– cells possess hematopoietic potential. We also confirmed that the OB/OC differentiation potential of the colony formed by GFP + Lin-CD29 + Sca-1+/PDGFRα– cells (Fig. 2I). However, since the GFP + Lin-CD29 + Sca-1+/PDGFRα+ cells did not form any colonies, the differentiation potential of OB/OC could not be confirmed. In summary, the Sca-1+/PDGFRα– cells are MPCs in adult BM, which possess both hematopoietic and mesenchymal differentiation potential, whereas the Sca-1+/PDGFRα+ cells are OB progenitors (Fig. 2J).

Multipotent and Unipotent Progenitor Cells in the Artery

We reconstituted BM with GFP+ cells after lethal irradiation to trace BM-derived cells in the artery (Fig. 3A). In our previous study, we investigated the possibility of the direct incorporation of GFP+ cells into the artery when cells were infused intravenously for BMT. At 5 days after cell infusion, less than 0.5% of the arterial cells were GFP+ [5]. At least 90 days after BMT, we isolated mononuclear cells from the artery, and observed that 65% of the arterial cells were derived from BM (Fig. 3B). We further analyzed BM-derived artery-infiltrated cells under the GFP+ gate and observed that 10% of the GFP+ cells were Lin-CD29+, indicative of nonhematologic cells. The majority of Lin-CD29+ cells were Sca-1+/PDGFRα– (Fig. 3B, right panel, red box).

Next, we investigated the differentiation potential of the isolated progenitor cells. Both BM-derived Sca-1+/PDGFRα+ and Sca-1+/PDGFRα– cells expressed ALP and other OB-related genes under OB differentiation conditions (Fig. 3C, Supporting Information Figs. S12A, S14A). Sca-1+/PDGFRα– cells changed into Sca-1+/PDGFRα+ cells during OB differentiation (Fig. 3C, lower panel; red box → blue box). In addition, 60% of the Sca-1+/PDGFRα– cells changed into Sca-1+/PDGFRα+ cells (Supporting Information Fig. S6, red box → blue box) when cultured for more than 90 days after the isolation of progenitor cells. Under OC differentiation conditions, Sca-1+/PDGFRα– cells, but not Sca-1+/PDGFRα+ cells, expressed RANK and OC-related genes, indicative of OC-like differentiation (Fig. 3D and Supporting Information Fig. S14B). These results showed that BM-residing Sca-1+/PDGFRα– cells exhibit multipotency (Fig. 2B, 2C), whereas BM-derived artery-resident Sca-1+/PDGFRα– cells lose their hematopoietic potential (Fig. 3E, 3F).

Hierarchy Between BM-Derived Artery-Resident Progenitor Cells

We transplanted GFP + Lin-CD29 + Sca-1+/PDGFRα+ or GFP + Lin-CD29 + Sca-1+/PDGFRα– cells isolated from the BM of GFP+ mouse after nonmyeloablative sublethal irradiation to

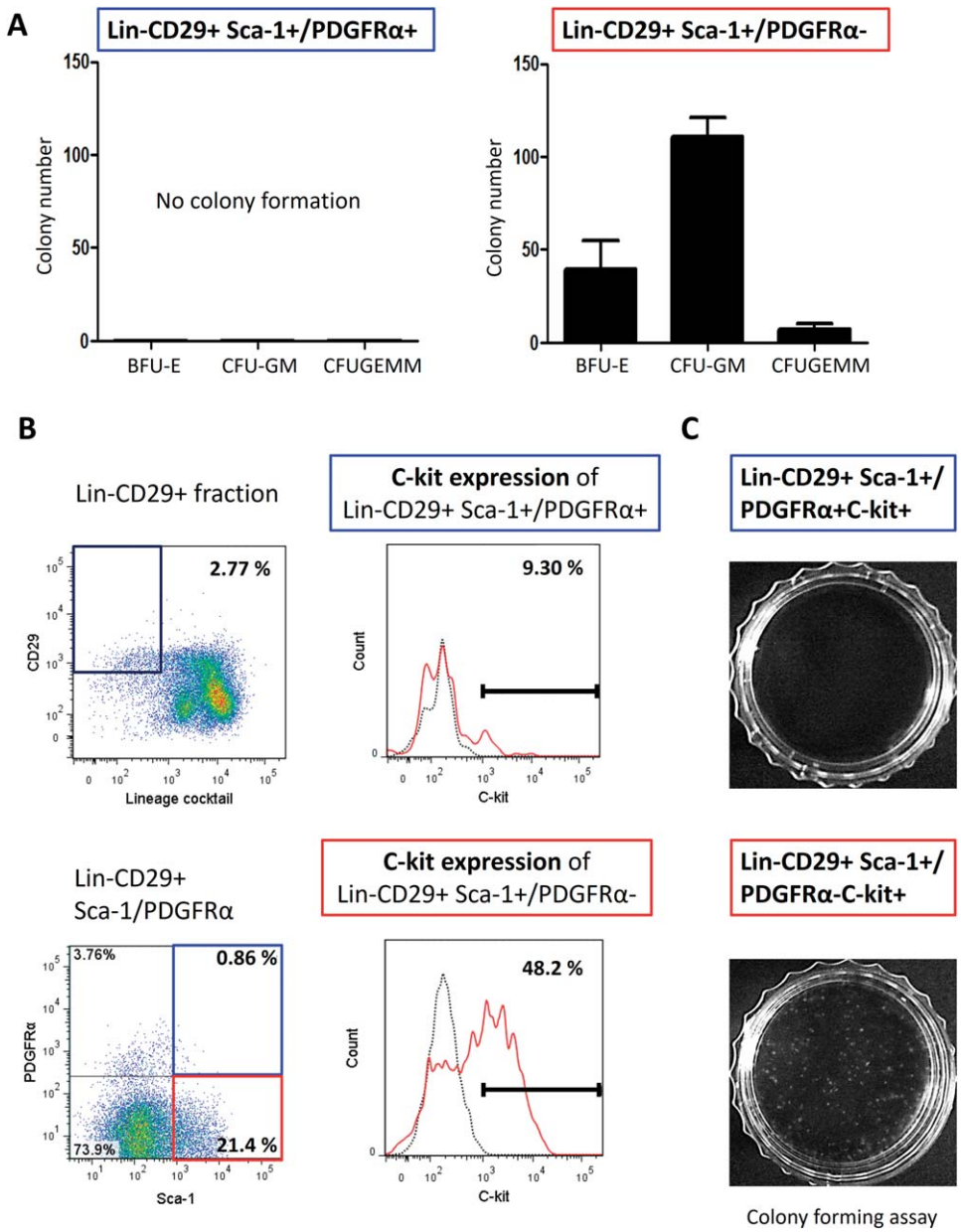
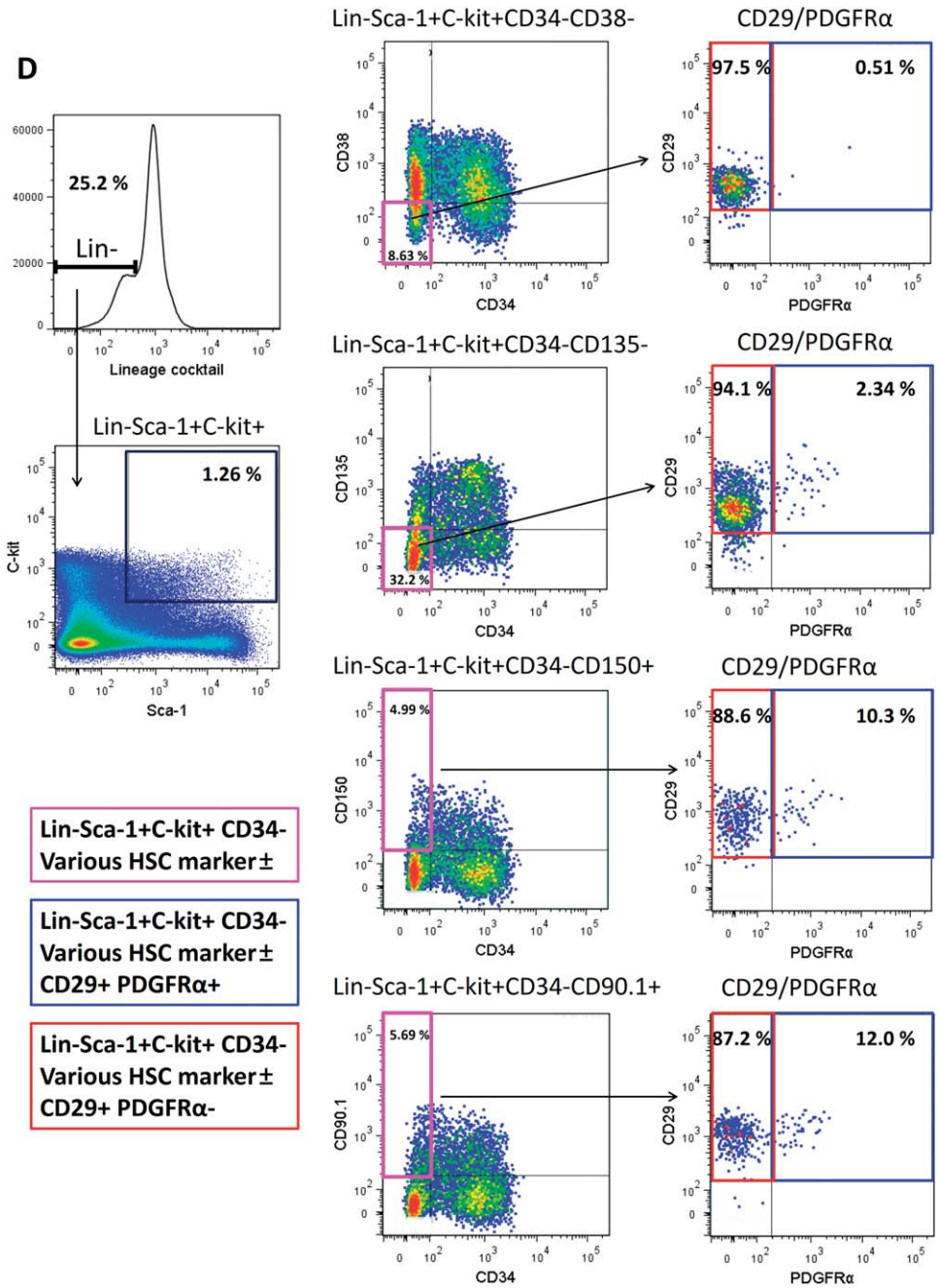


Figure 2. Lin-CD29 + Sca-1+/PDGFRα– cells from the BM possess hematopoietic and osteoblastic differentiation potential. **(A):** Hematopoietic colony-forming assay of two types of BM-derived progenitor cells ($n = 3$, each group). **(B):** c-Kit expression predominates in Lin-CD29 + Sca-1+/PDGFRα-cells (red box) compared with Lin-CD29 + Sca-1+/PDGFRα+ (blue box). **(C):** Lin-CD29 + Sca-1+/PDGFRα-c-kit+ cells formed numerous colonies. Lin-CD29 + Sca-1+/PDGFRα+c-Kit+ cells did not form colonies. **(D):** Repopulating and self-renewing HSCs among Lin-/Sca-1+/c-Kit+ (LSK) cells sorted by CD34-, CD38-, CD135-, CD150+, and CD90.1+ were positive for CD29 and negative for PDFGRα. **(E):** Schematic representation of BMT for investigating the in vivo hematopoietic activity of two types of progenitor cells ($n = 5$, each group). **(F):** FACS analysis of BM cells at 12 weeks after cell injection showed that Lin-CD29 + Sca-1+/PDGFRα+ cells did not reconstitute BM. Lin-CD29 + Sca-1+/PDGFRα– cell transplantation showed 17% donor chimerism, and generated Sca-1+/PDGFRα+ and Sca-1+/PDGFRα– progeny cells. **(G):** Subanalysis of GFP+ reconstituting BM cells demonstrated that Lin-CD29 + Sca-1+/PDGFRα– cells are the ancestors of repopulating HSCs. **(H):** Ex vivo hematopoietic colony-forming assay of reconstituting BM cells. **(I):** OB and OC differentiation potential of reconstituting Lin-CD29 + Sca-1+/PDGFRα– cells. **(J):** The proposed hierarchy between Lin-CD29 + Sca-1+/PDGFRα+ and Lin-CD29 + Sca-1+/PDGFRα– cells in adult BM. All experiments were conducted at least in triplicate. Abbreviations: BFU-E, burst-forming unit-erythroid; BM, bone marrow; CFU-GEMM, colony forming unit-granulocyte/erythroid/macrophage/megakaryocyte; CFUGM, colony forming unit-macrophage/granulocyte; HSCs, hematopoietic stem cells; OB, osteoblastic; OC, osteoclastic; PDGFRα, platelet-derived growth factor receptor alpha; Sca-1, stem cell antigen-1.

investigate the hierarchy between BM-derived Sca-1+/PDGFRα+ and Sca-1+/PDGFRα– cells. We divided mice into those fed with normal conventional diet and 3% high-cholesterol diet (Fig. 4A). The mobilization of GFP + Lin-CD29 + Sca-1+/PDGFRα+ cells in peripheral blood (PB) showed minimal GFP+ cells, indicating that hematopoietic reconstitution did not occur, whereas the mobilization of GFP + Lin-CD29 + Sca-1+/PDGFRα– cells demonstrated 20% donor chimerism and generated various progeny cells



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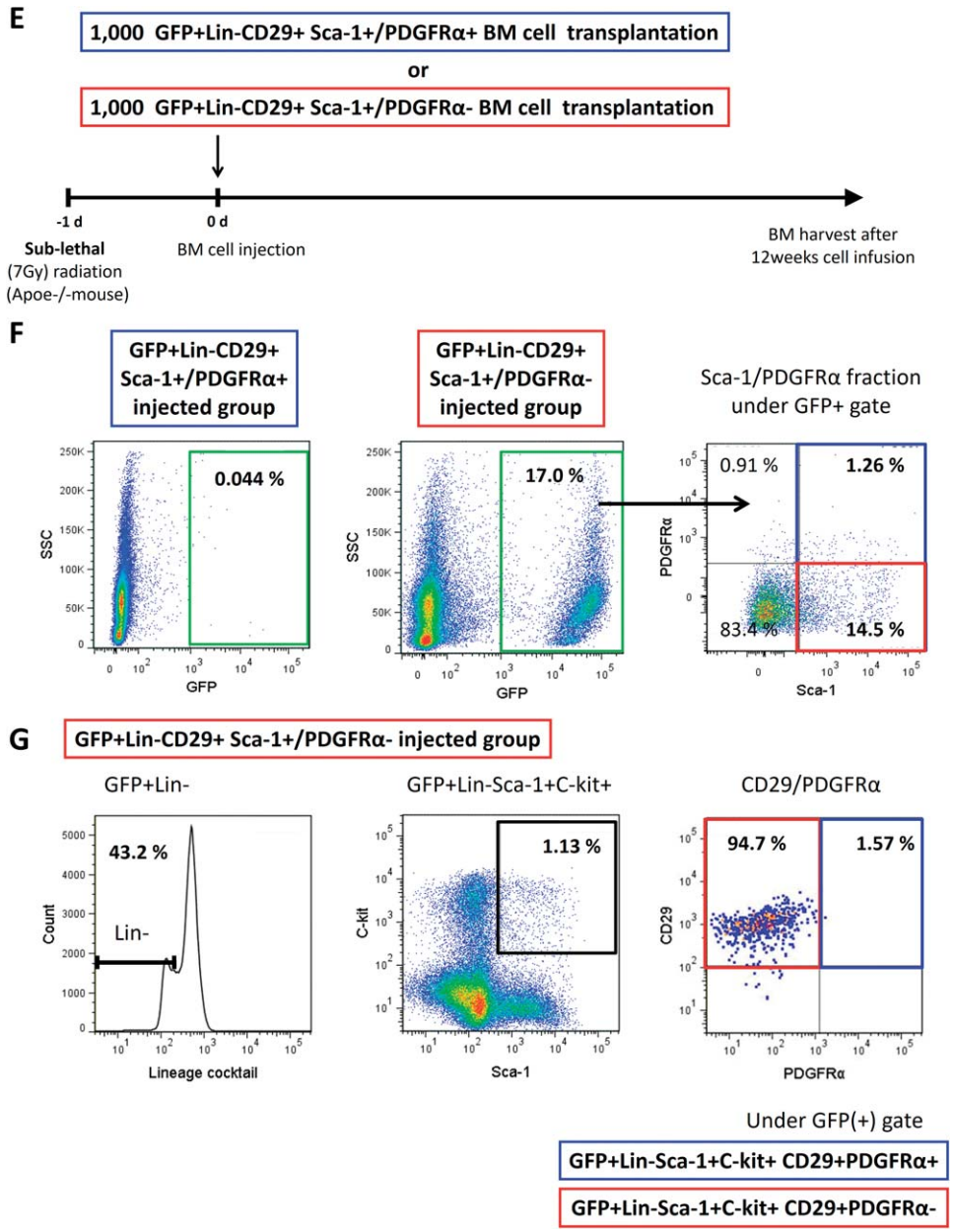
(Fig. 4B). The mobilized GFP + Lin-CD29 + Sca-1+/PDGFRα– cells from the BM were repopulating hematopoietic cells (Supporting Information Fig. S7). Next, we analyzed vessel-infiltrated mononuclear cells in the artery. The GFP + Lin-CD29 + Sca-1+/PDGFRα+ transplantation group showed negligible GFP signal, whereas 7.5% of the arterial cells were GFP+ in the GFP + Lin-CD29 + Sca-1+/PDGFRα– transplantation group. We further analyzed GFP+ cells in the artery, which were derived from Sca-1+/PDGFRα– cells. We observed that 70% of the GFP+ cells were Sca-1+/PDGFRα–, and 10% were Sca-1+/PDGFRα+, suggesting that Sca-1+/PDGFRα– cells differentiate into Sca-1+/PDGFRα+ cells in vivo, and that the latter are the progeny of the former (Fig. 4D). The number of mobilized (Fig. 4B, 4C) and infiltrated (Fig. 4D, 4E) Sca-1+/PDGFRα– cells was relatively lower in the high-cholesterol diet group than in the normal diet group.

Taken together, these results showed that Lin-CD29 + Sca-1+/PDGFRα– cells exhibited a high degree of developmental hierarchy and possessed OB and OC bipotency, but lost its hematopoietic activity in the artery. Lin-CD29 + Sca-1+/PDGFRα+ cells, the progeny of Sca-1+/PDGFRα– cells, showed OB unipotency (Fig. 4F). These observations formed the basis for the hypothesis that atherosclerotic inflammatory stimuli, such as a high-cholesterol diet, may induce the differentiation of Sca-1+/PDGFRα– cells into Sca-1+/PDGFRα+ cells and modify the dynamics of BM-derived progenitor cells in the PB and artery.



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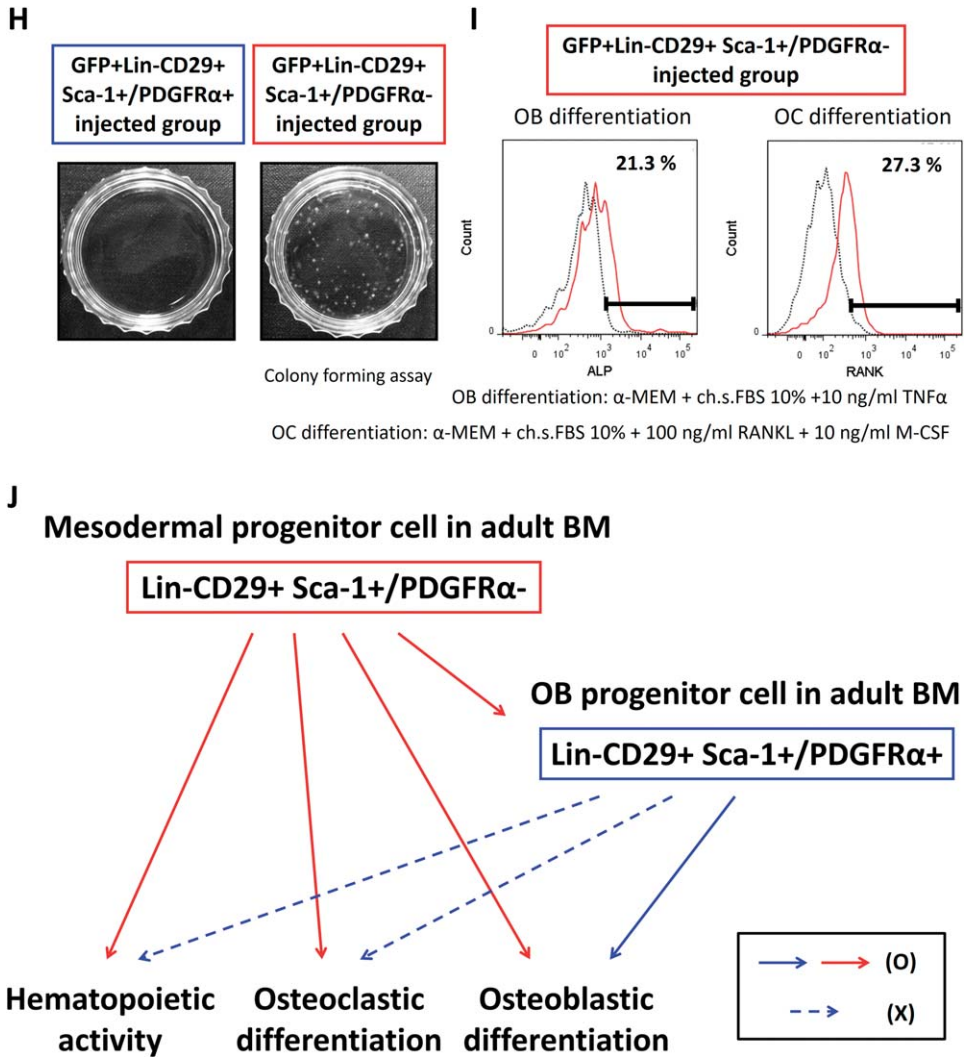
Dynamics of Two Types of Progenitor Cells in Atherosclerosis

We performed BM transplantation with GFP+ BM cells to trace whole BM-derived cells in the PB and artery. At 14 days after cell infusion, we divided mice into two groups, one fed with normal diet and the other with high-cholesterol diet (Fig. 5A) for at least 12 weeks, after which we harvested the BM, PB, and arteries. In the BM, the total white blood cell (WBC) count, measured using an automated complete blood count (CBC) machine that counted multinuclear and mononuclear as well as mature and immature cells, was higher in the high-cholesterol diet group than in the normal diet group, whereas no differences were observed between the two diet groups in the PB and artery (Fig. 5B). Next, to investigate the detailed progenitor cell components, we focused on mononuclear cells, and stained cells with Lin, CD29, Sca-1, and PDGFRα antibodies. Fluorescence-activated cell sorting (FACS) analysis showed that the two diet groups showed no significant differences in the BM subfractions (Fig. 5C). The mobilization of multipotent MPCs (Sca-1+/PDGFRα–) into PB was significantly lower in the high-cholesterol diet group than in the normal diet group (Fig. 5D). Moreover, the total number of GFP+ BM-derived cells infiltrating into the artery, was significantly lower in the high-cholesterol diet group than in the normal diet group (65% → 36%) (Fig. 5E, left panel). In other words, the infiltration of unipotent Sca-1+/PDGFRα+ cells was lower (6% → 24%), whereas that of multipotent Sca-1+/PDGFRα– cells was higher (76% → 61%) in the high-cholesterol diet group compared with that in the normal diet group (Fig. 5E, right panel).



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We stained the aorta with Sca-1 and PDGFRα antibodies to confirm the results obtained from FACS analysis and visualize the anatomic locations of the infiltrated cells. We used the ascending thoracic aorta in this study because in atherosclerotic calcification, the results obtained with the ascending thoracic aorta were clearer and more distinct than those obtained with other parts of the aorta [26]. We observed that GFP+ BM-derived cells were mainly present in the adventitia of the artery. The number of multipotent Sca-1+/PDGFRα– cells (red arrows) was higher in the normal diet group (Fig. 5F, upper panel), whereas that of unipotent Sca-1+/PDGFRα+ cells (white arrows) was higher in the high-cholesterol diet group (Fig. 5F, lower panel).

These results showed that multipotent MPCs (OB/OC) predominate under physiological conditions. However, under atherosclerotic inflammatory conditions, MPCs are retained in BM as their mobilization and infiltration into the artery is hindered. As a result, the number of unipotent OB progenitor cells increase in the artery under atherosclerotic conditions (Supporting Information Fig. S8).

The Migration of MPCs Is Regulated by Inflammatory and Anti-Inflammatory Cytokines

To investigate the mechanisms underlying the differences in the in vivo dynamics of the two progenitor populations, we first measured the levels of atherosclerosis-related cytokines in the serum and artery using a multiplex cytokine assay. Pro-inflammatory cytokine levels, such as Interleukin-1β (IL-1β) and IL-12, were significantly higher both in the serum and artery of the high-cholesterol diet group, whereas the levels of anti-inflammatory cytokines, such as Interleukin-5 (IL-5), were markedly higher in the normal diet group (Fig. 6A, 6B). Previous studies on atherosclerosis showed that TNFα is a well-known pro-inflammatory mediator [2, 27, 28]. However, in this study, significant differences in serum TNFα levels were not observed between the high-cholesterol and normal diet groups (Supporting Information Fig. S9). Therefore, we used TNFα as a positive control for experiments on cell migration. The level of IL-10, a known anti-inflammatory cytokine, was considerably increased in the serum and modestly increased in the artery of the high-cholesterol diet group, which may be interpreted as a compensatory increase under atherosclerotic conditions [27–29].

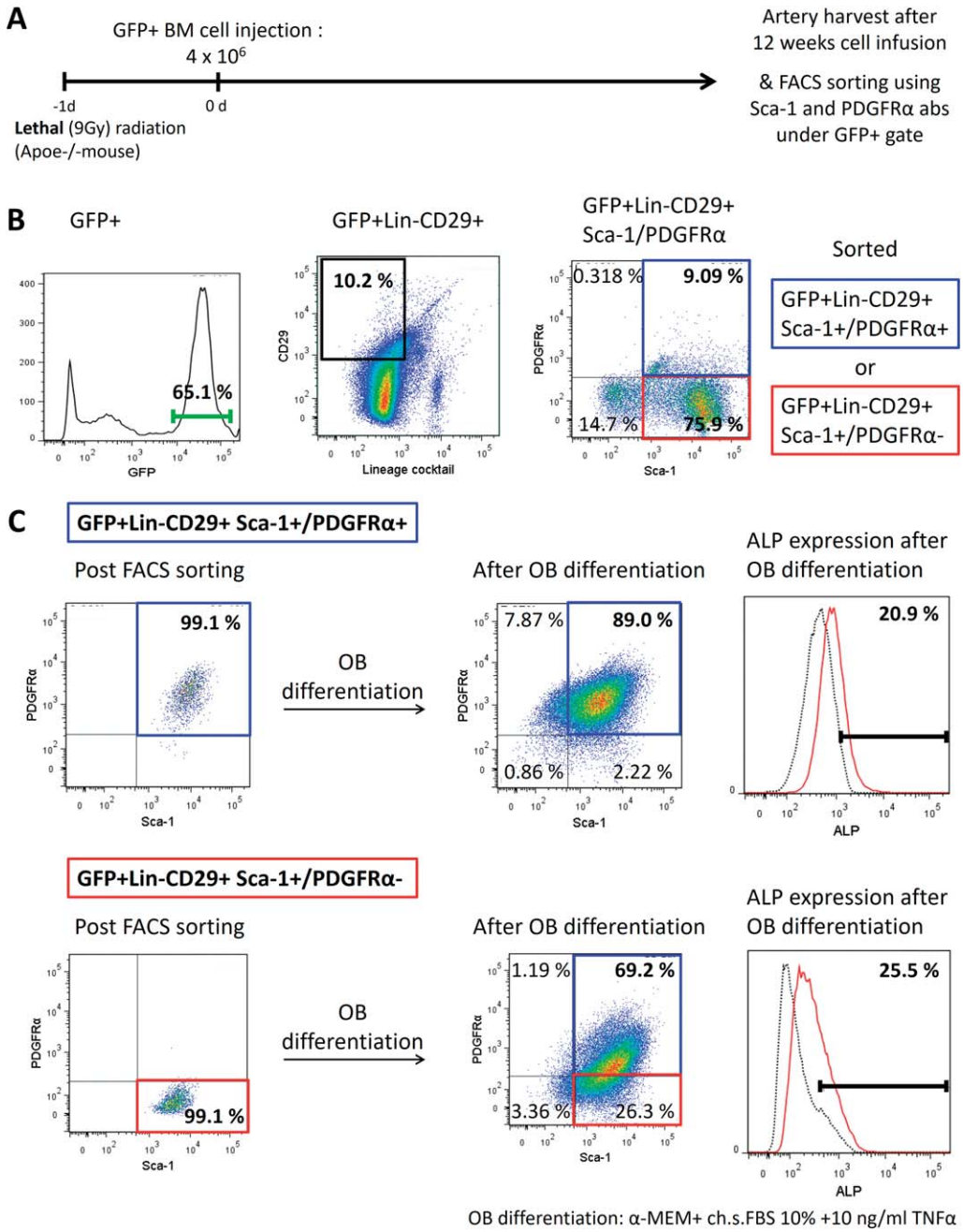
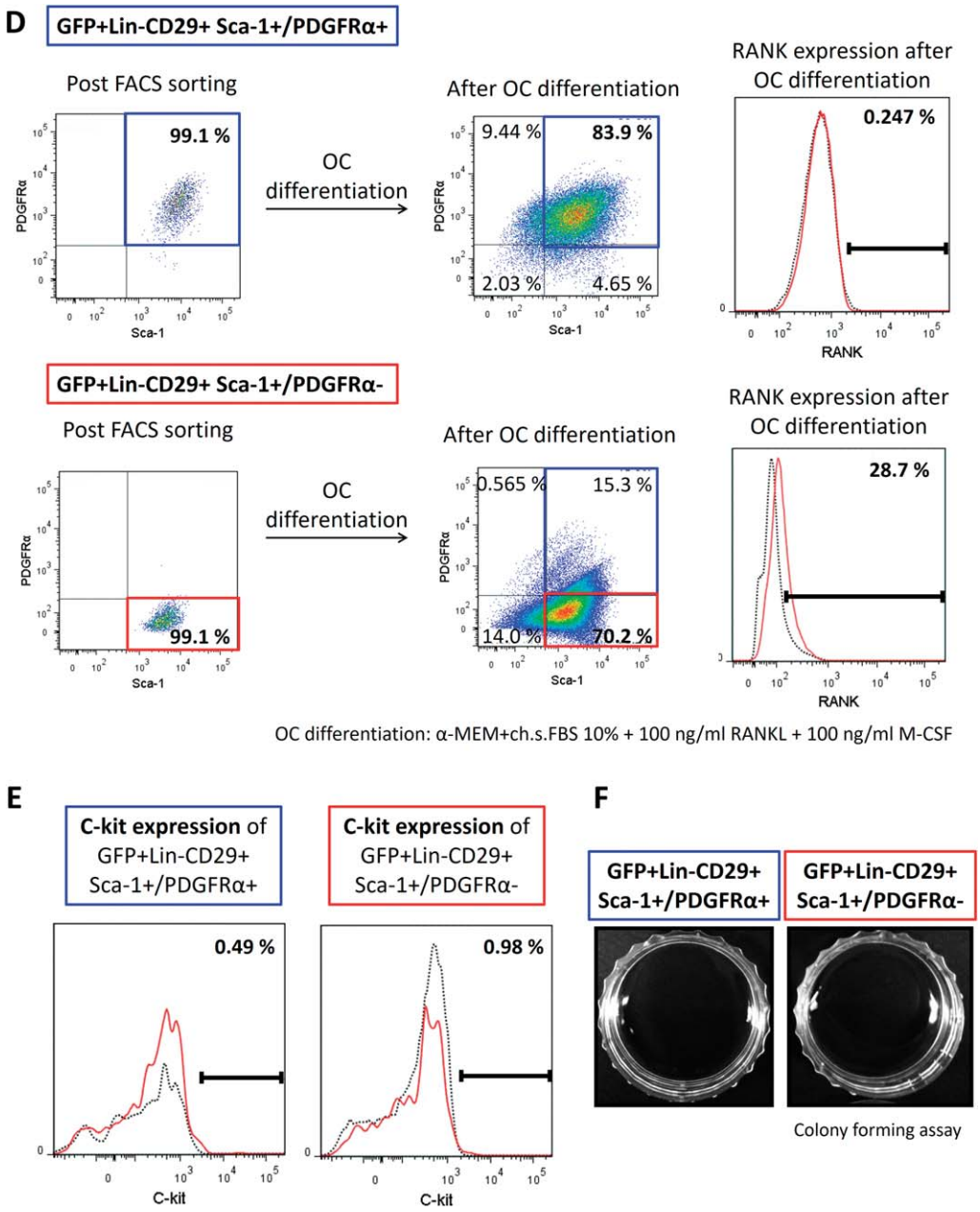


Figure 3. Multipotent and unipotent progenitor cells in the artery. **(A):** Schematic representation of experiments ($n = 10$) for tracing BM-derived cells in the artery. BM was reconstituted with GFP+ cells. **(B):** FACS analysis showed that 65% of the artery cells were derived from BM. The majority of infiltrated GFP + Lin-CD29+ progenitor cells were Sca-1+/PDGFRα– (red box). **(C):** OB differentiation of sorted cells. GFP + Lin-CD29 + Sca-1+/PDGFRα+ and Sca-1+/PDGFRα– cells expressed ALP. **(D):** OC differentiation of sorted cells. Only GFP + Lin-CD29 + Sca-1+/PDGFRα– cells expressed RANK. **(E, F):** Both GFP + Lin-CD29 + Sca-1+/PDGFRα+ and GFP + Lin-CD29 + Sca-1+/PDGFRα– cells were negative for c-Kit and did not exhibit hematopoietic colony-forming ability. All experiments were conducted at least in triplicate. Abbreviations: ALP, alkaline phosphatase; BM, bone marrow; OB, osteoblastic; OC, osteoclastic; PDGFRα, platelet-derived growth factor receptor alpha; Sca-1, stem cell antigen-1.

We investigated the biological effect of these cytokines on the migration of GFP + Lin-CD29 + Sca-1+/PDGFRα+ and GFP + Lin-CD29 + Sca-1+/PDGFRα– cells (Fig. 6C). IL-1β significantly inhibited the migration of Sca-1+/PDGFRα– cells by five-fold compared with tumor necrosis factor-alpha (TNFα) (positive control), and IL-5 substantially increased the migration to the same extent as TNFα. However, the migration of Sca-1+/PDGFRα+ cells was not significantly affected by atherosclerosis-related cytokines, except for TNFα (Fig. 6D). Upon comparing the ratio of cytokine-induced migration of the two types of progenitor cells, we observed that IL-1β increased the migration of Sca-1+/PDGFRα+ cells compared with that of Sca-1+/



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PDGFRα– cells, whereas IL-5 significantly increased the migration of Sca-1+/PDGFRα– cells (Fig. 6E). Overall, these results indicated that inflammatory cytokines such as IL-1β, the levels of which are high in the PB and artery in hypercholesterolemia, may increase the mobilization and infiltration of OB unipotent progenitor cells while inhibiting the mobilization and infiltration of bipotent MPCs that can differentiate into OCs. Our data provided novel mechanistic insights into the role of progenitor cells in vascular calcification during atherogenesis.

Role Of PDGFRα During OB and OC Differentiation

PDGFRα expression was induced during the OB differentiation of Sca-1+/PDGFRα– cells. PDGFRα is a tyrosine kinase receptor expressed on the cell surface and mediates downstream signaling. Therefore, we investigated the role of PDGFRα during differentiation and attempted to determine whether the stimulation of PDGFRα of Lin-CD29 + Sca-1+/PDGFRα+ cells by two B chains of PDGF (PDGF-BB) enhances OB differentiation. We used single colony-derived, clonally-expanded cells that were BM-derived (GFP+) and artery-infiltrated progenitor cells (Supporting Information Fig. S10). We also confirmed that these cells were not contaminated by monocytes or macrophages using Lin staining and exhibited MSC characteristics (Supporting Information Figs. S6, S10). The FACS analysis of ALP expression showed that the addition of PDGF-BB with FBS did not boost OB differentiation. The addition of TNFα along with FBS enhanced the OB differentiation of Lin-CD29 + Sca-1+/PDGFRα+ cells, whereas the addition of PDGF-BB with FBS and TNFα did not

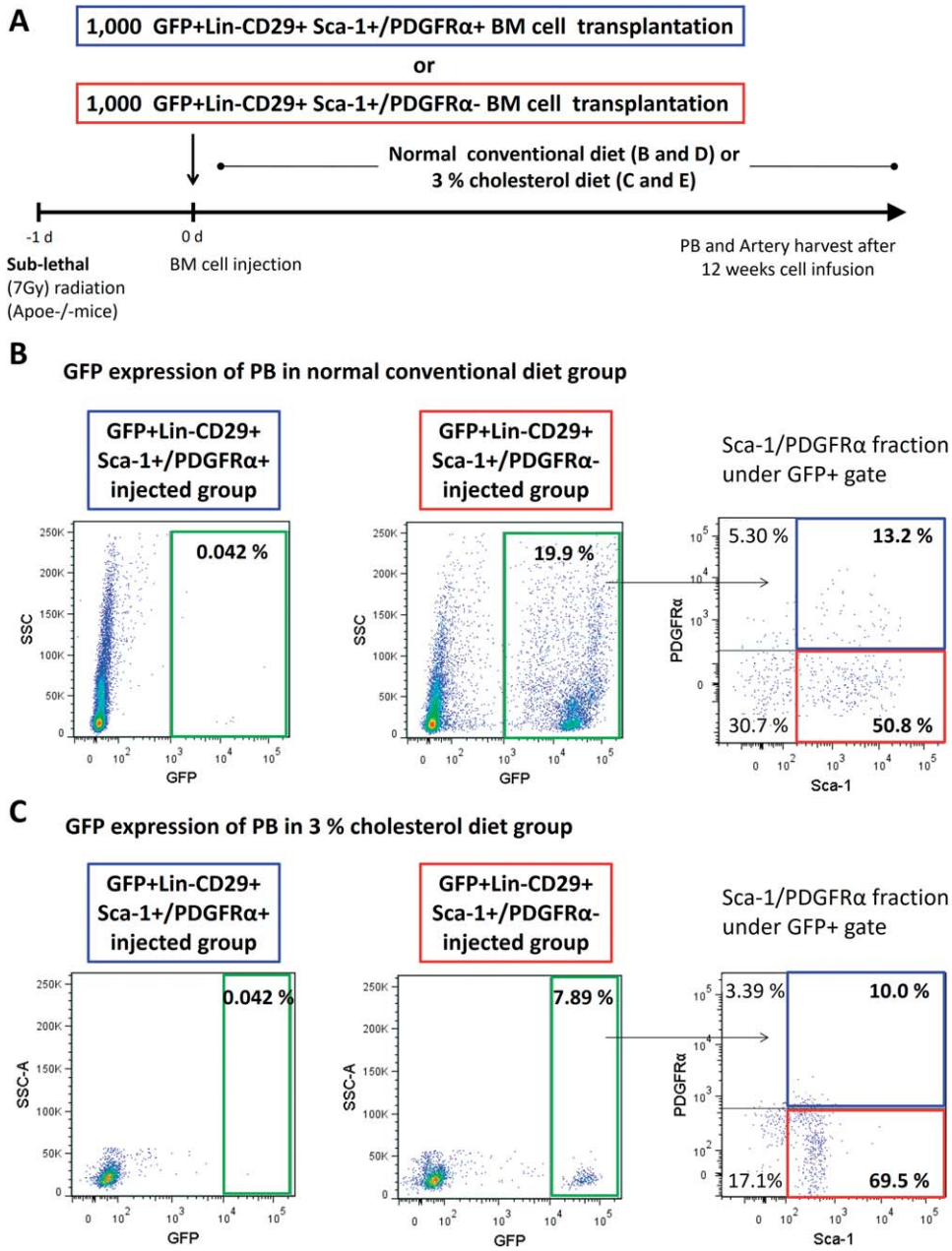
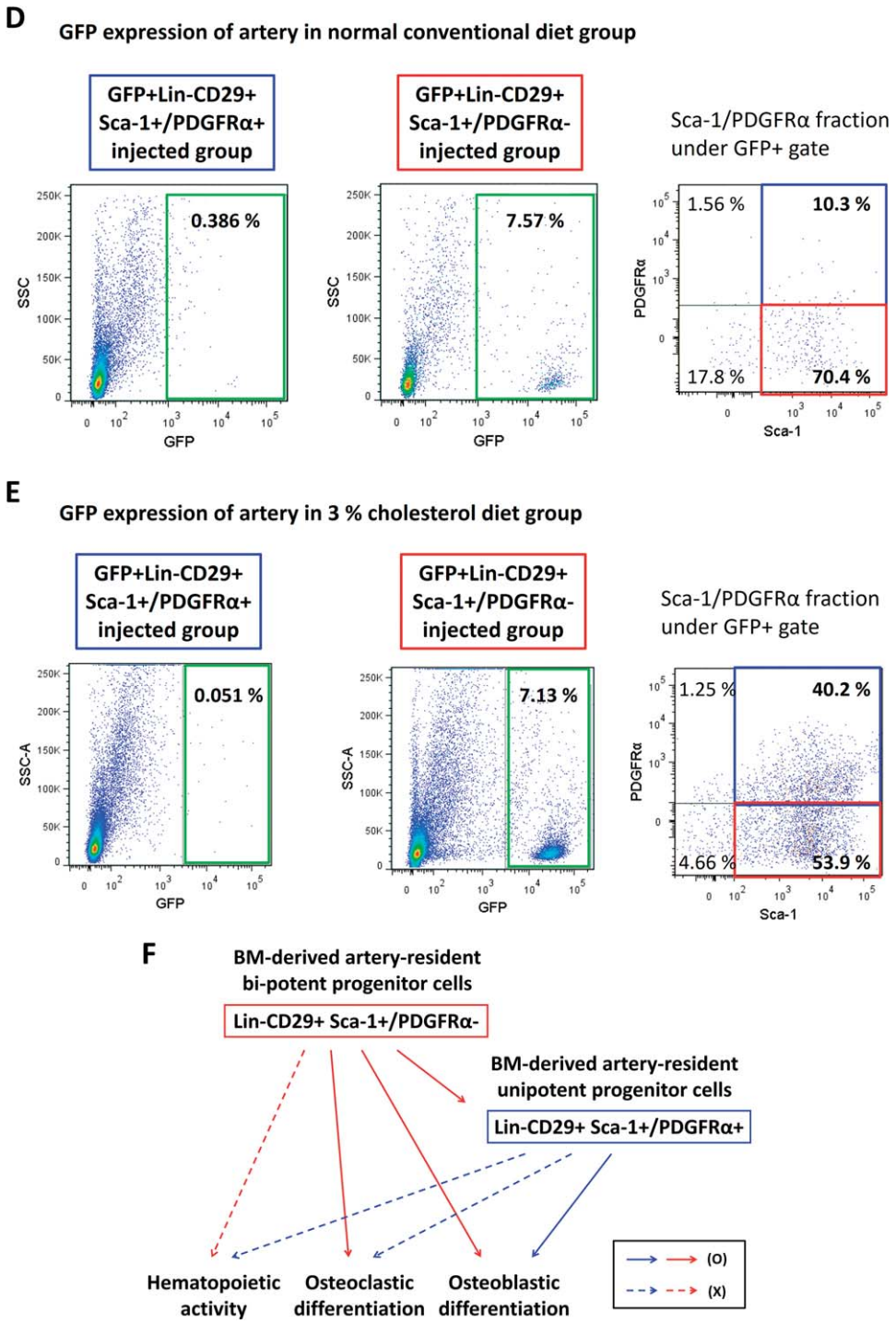


Figure 4. Hierarchy of BM-derived artery-infiltrated progenitor cells. **(A):** Schematic representation of experiments ($n = 5$, each group). **(B):** PB was analyzed at 12 weeks after the injection of two different types of progenitor cells. Few GFP+ cells were observed in the Lin-CD29 + Sca-1+/PDGFRα+ transplantation group. The Lin-CD29 + Sca-1+/PDGFRα– transplantation group exhibited 20% donor chimerism and generated Sca-1+/PDGFRα–, Sca-1+/PDGFRα+ progeny cells in normal diet group. **(C):** The Lin-CD29 + Sca-1+/PDGFRα– transplantation group generated various types of progeny cells in the PB, few GFP+ infiltrated cells were detected in the Lin-CD29 + Sca-1+/PDGFRα+ transplantation group, and 7.9% of arterial cells were GFP+ in the Lin-CD29 + Sca-1+/PDGFRα– transplantation subgroup of the high-cholesterol diet group. **(D):** In the artery of normal diet group, few GFP+ infiltrated cells were detected in the Lin-/CD29/+Sca-1+/PDGFRα+ transplantation group, and 7.5% of arterial cells were GFP+ in the Lin-/CD29+/Sca-1+/PDGFRα– transplantation group. Various types of progeny cells were generated. **(E):** In the artery, changes in the proportion of infiltrated Sca-1+/PDGFRα+ and Sca-1+/PDGFRα– cells were observed in the Lin-CD29 + Sca-1+/PDGFRα– transplantation group. High-cholesterol diet resulted in a relative increase of Sca-1+/PDGFRα+ cell numbers compared with normal diet. **(F):** The proposed hierarchy between BM-derived artery-infiltrated Lin-CD29 + Sca-1+/PDGFRα+ cells and Lin-CD29 + Sca-1+/PDGFRα– cells. All experiments were conducted at least in triplicate. Abbreviations: BM, bone marrow; PB, peripheral blood; PDGFRα, platelet-derived growth factor receptor alpha; Sca-1, stem cell antigen-1; SSC-A, side scatter area.

elicit any difference. PDGF-BB stimulation did not stimulate OC differentiation of Lin-CD29 + Sca-1+/PDGFRα+ cells as measured by RANK expression (Fig. 7A). Immunofluorescence staining of osteocalcin (OB marker) and cathepsin K (OC marker) also showed that PDGF-BB did not affect the differentiation of Sca-1+/PDGFRα+ cells (Supporting Information Fig. S11). The



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expression of OB-related genes increased in response to the differentiation conditions, whereas those of OC-related genes was not induced in the Sca-1+/PDGFRα+ cells. These profiles of gene induction were not affected by PDGF-BB. In addition, the expression of vascular smooth muscle cell (VSMC)-related genes did not change after PDGF-BB treatment (Supporting Information Fig. S12). Next, we stimulated PDGFRα in Lin-CD29 + Sca-1+/PDGFRα– cells using PDGF-BB, as Sca-1+/PDGFRα– cells gradually express PDGFRα on their surface during OB differentiation. Similar to Sca-1+/PDGFRα+ cells, PDGF-BB stimulation did not affect the OB differentiation of Sca-1+/PDGFRα– cells. The differentiation of Lin-/CD29+/Sca-1+/PDGFRα– cells into OC-like cells (cathepsin K+) was also not affected by PDGF-BB stimulation (Fig. 7B, Supporting Information Figs. S13, S14). We suppressed PDGFRα expression using shRNA lentivirus with previously confirmed transduction efficiency to perform loss-of-

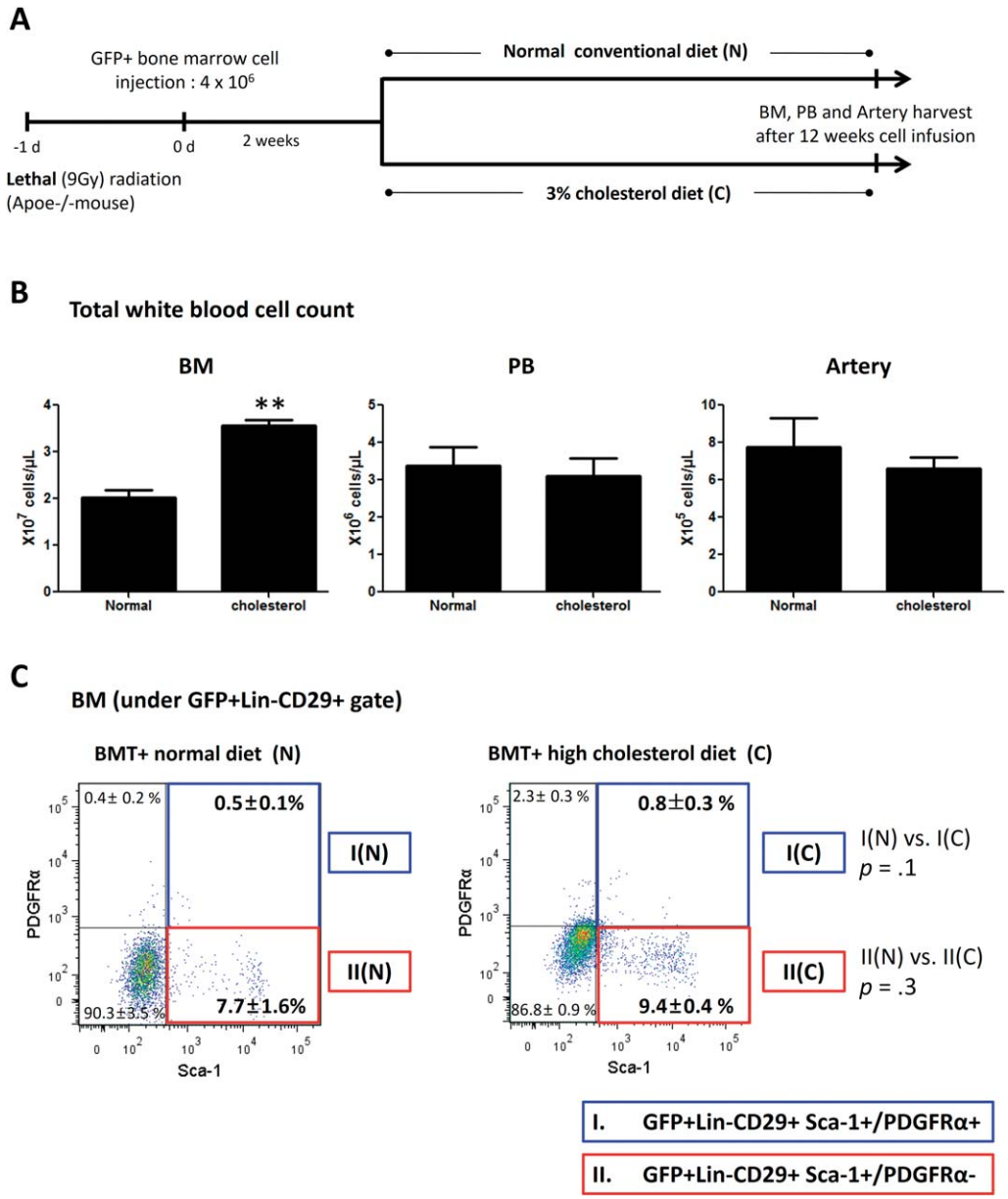
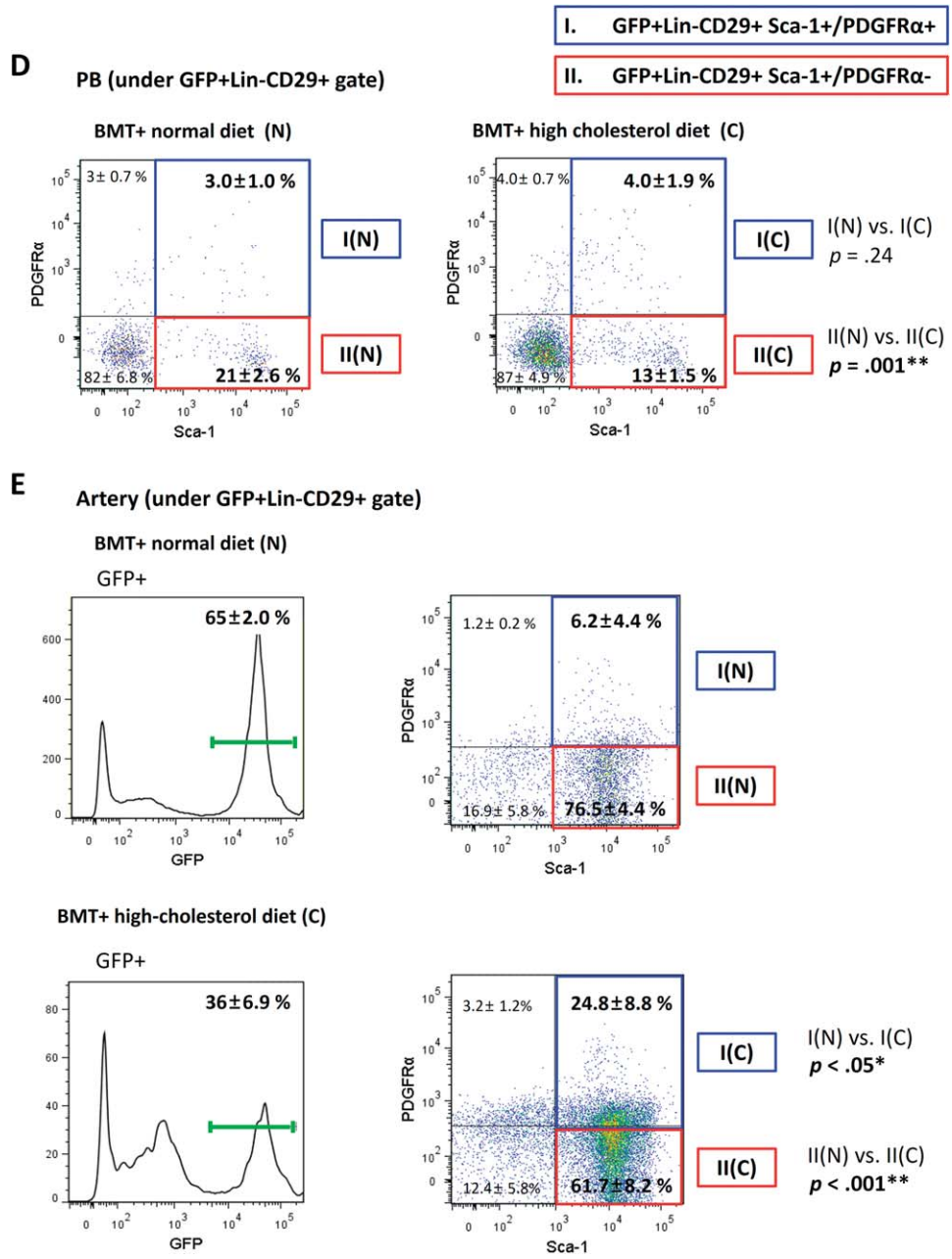


Figure 5. Dynamics of two types of progenitor cells in atherosclerosis. **(A):** Schematic representation of experiments for investigating the effects of atherosclerotic stimuli. **(B):** High-cholesterol diet increased the total number of WBCs in the BM, but no changes were observed in the PB and artery (**, $p < .01$ compared with normal diet in BM, $n = 3$, each group). **(C):** FACS analysis indicated no differences in BM sub-fractions between the two diet groups. **(D):** The mobilization of GFP + Lin-CD29 + Sca-1+/PDGFRα− cells (red box) from BM into the PB was significantly lower in the high-cholesterol diet group compared with the normal diet group. **(E):** The total number of BM-derived cells infiltrating into the artery was reduced in the high-cholesterol diet group compared with the normal diet group (GFP+, left panels). The proportion of Sca-1+/PDGFRα+ cells (blue box) was relatively higher, and that of Sca-1+/PDGFRα− cells (red box) was lower in the high-cholesterol diet group compared with the normal diet group (right panels). **(F):** The aorta, including adventitia, was harvested ($n = 10$, each group), and representative immunofluorescence staining was performed. White-dashed lines, media (M), lumen of artery (L), and adventitia (A); White arrows, infiltrated GFP + Sca-1+/PDGFRα+ cells; Red arrows, GFP + Sca-1+/PDGFRα− cells. Yellow box, magnified area; GFP, BM-derived cells; DAPI, nuclei; White bars, 50 μm; Yellow bars, 20 μm. All experiments were conducted at least in triplicate. Abbreviations: BM, bone marrow; BMT, bone marrow transplantation; PB, peripheral blood; PDGFRα, platelet-derived growth factor receptor alpha; Sca-1, stem cell antigen-1.

function studies (Supporting Information Fig. S15). Compared with the control shRNA lentivirus transduction in Lin-CD29 + Sca-1+/PDGFRα+ cells, PDGFRα shRNA lentivirus significantly decreased PDGFRα expression during differentiation (90% → 30%, Fig. 7C, Supporting Information Figs. S15B, S17A). The physiological inhibition of PDGFRα in Sca-1+/PDGFRα+ cells did not affect OB differentiation induced by FBS and TNFα or OC differentiation as shown by the FACS analysis of ALP or RANK, immunofluorescence staining of osteocalcin or cathepsin K, and the analysis of OB- or OC-related gene expression (Fig. 7C, Supporting Information Figs. S16, Fig. S17). PDGFRα was expressed during OB differentiation in Lin-CD29 + Sca-1+/PDGFRα− cells, and the



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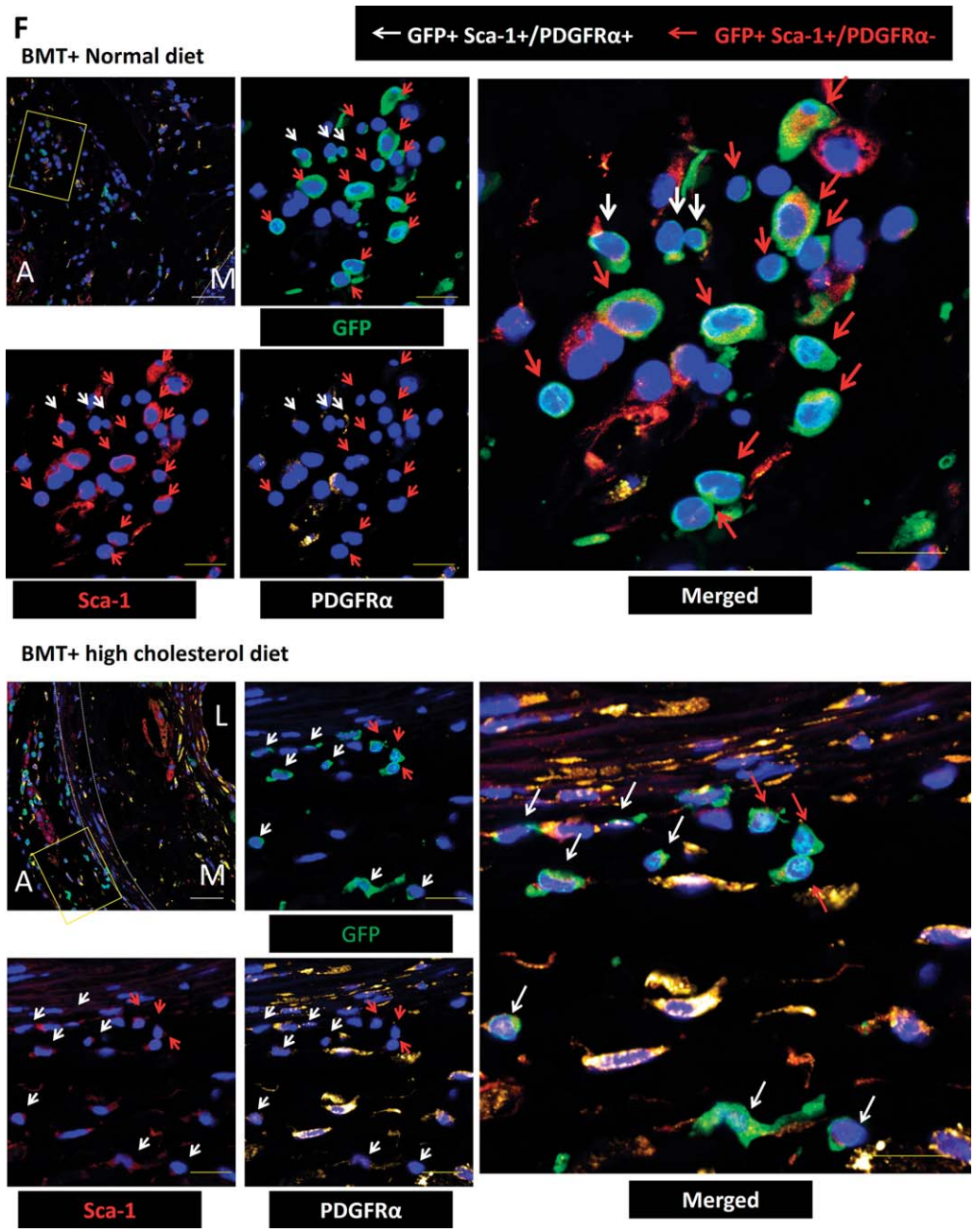
PDGFRα shRNA lentivirus effectively suppressed the emergence of PDGFRα on these cells (70% → 30%, Fig. 7D and Supporting Information Fig. S19A); however, the OB differentiation potential did not change. No difference was observed in the efficacy of OC differentiation between control shRNA- and PDGFRα shRNA-transduced cells. Immunofluorescence staining and real-time polymerase chain reaction (PCR) analysis yielded results similar to FACS (Fig. 7D, Supporting Information Figs. S18, S19). Collectively, these results showed that the stimulation or inhibition of PDGFRα did not modify the OB or OC differentiation of Sca-1+/PDGFRα− cells and did not alter the OB differentiation of Sca-1+/PDGFRα+ cells, indicating that PDGFRα serves as a surface marker but not as a functional player.

Discussion

We demonstrated that Sca-1+/PDGFRα− cells in adult BM are MPCs that possess bipotent hematopoietic and mesenchymal differentiation potential. In terms of developmental hierarchy, the Sca-1+/PDGFRα+ cells, which are the progeny of the Sca-1+/PDGFRα− progenitor cells, possess unipotent potential to differentiate into cells of mesenchymal lineage such as OBs. Under atherosclerotic conditions, the mobilization and infiltration of multipotent Sca-1+/PDGFRα− progenitor cells into the PB and artery were decreased because of inhibition of their mobility by IL-1β (high in hypercholesterolemia), whereas it was enhanced by IL-5 (low in hypercholesterolemia). In contrast, the



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dynamics of unipotent Sca-1+/PDGFRα+ progenitor cells were not significantly influenced by atherosclerosis-related cytokines. These results suggested that MPCs, reported as a key regulator of vascular calcification in our previous study [5], also play an important role during atherogenesis in hypercholesterolemia and act as a source of new therapeutic targets in the treatment of vascular calcification during atherosclerosis.

MPCs generate hemangioblasts and are involved in the development of various skeletal components such as the bone. MPCs are common ancestors of HSCs and MSCs during the early stages of embryonic development [13, 14, 30]; however, in adulthood, they are considered as two distinct populations and exist as separate entities, even though previous studies have suggested the concept of MPCs in adults [15, 16]. In the present study, we hypothesized that MPCs, the common ancestor of HSCs and MSCs, exist in the adult BM and generate both HSC and MSC populations, which subsequently differentiate into OBs and OCs, respectively.

To define MPCs in adult mice, we used multiple cell surface markers such as Sca-1 and PDGFRα, which are essential indicators for distinguishing bipotent progenitor cells from unipotent progenitor cells. Sca-1 is a glycosyl phosphatidylinositol (GPI)-linked protein expressed on the surface of various murine stem

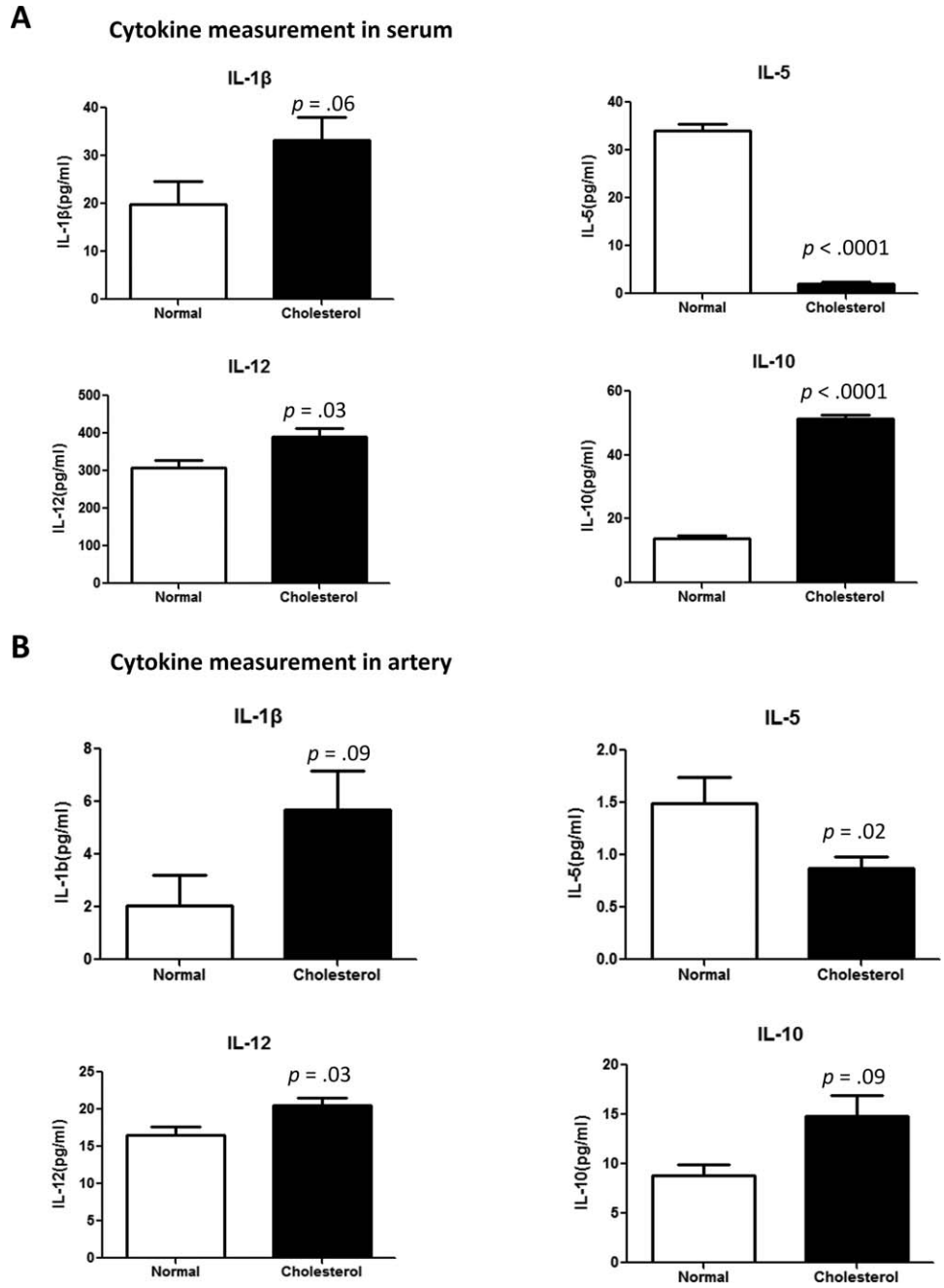
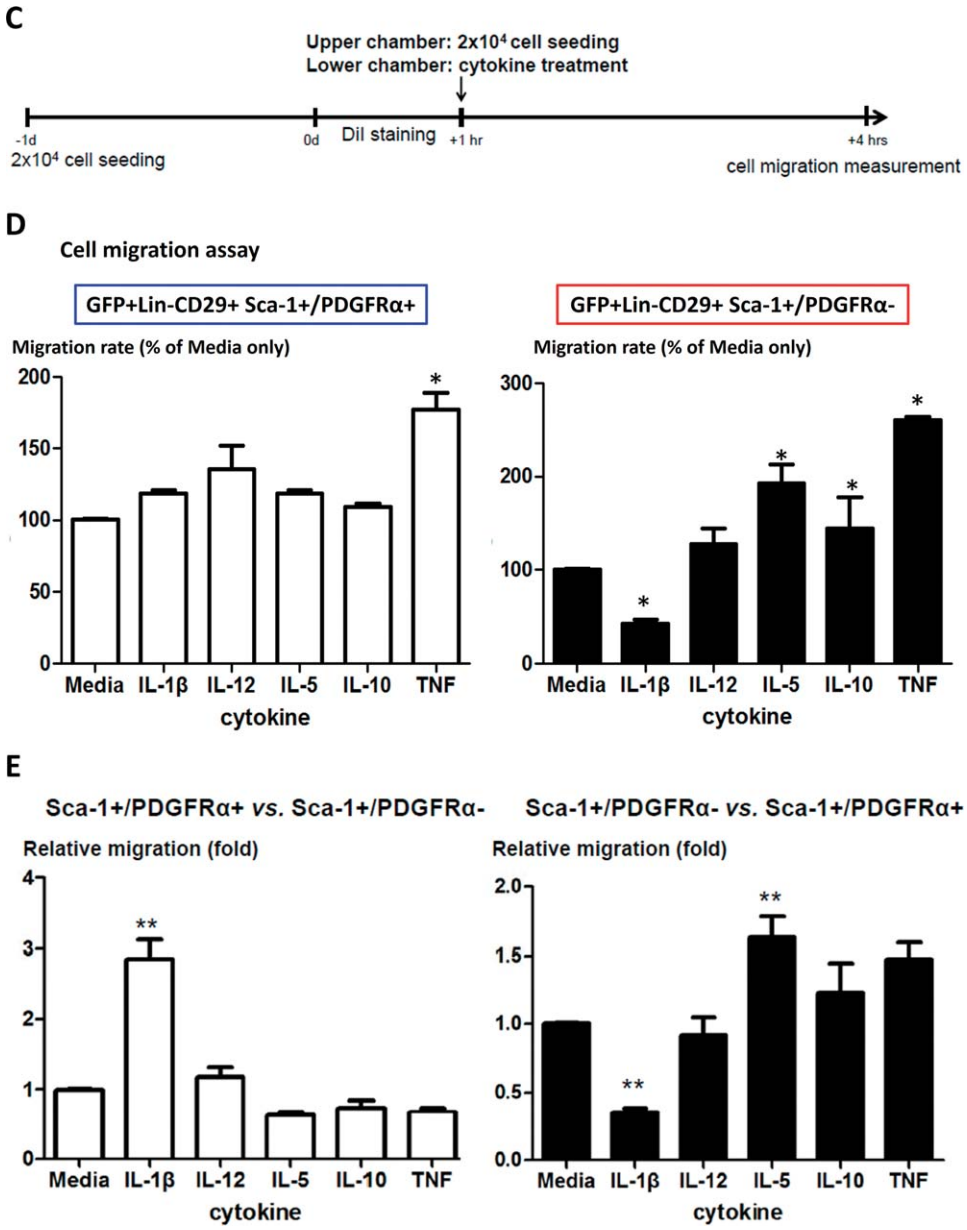


Figure 6. The migration of mesodermal progenitor cells is regulated by inflammatory and anti-inflammatory cytokines. **(A, B):** Serum and arterial tissues harvested from the high-cholesterol diet group and the normal diet group. Cytokine levels were measured using multiplex cytokine assay. **(C):** Schematic graph for in vitro transwell migration assay. **(D):** Cells were treated with various cytokines and the cell migration rates were measured. TNFα treatment was used as a positive control for cell migration (*, $p < .05$ compared with alpha minimum essential medium [α-MEM]; $n = 5$, each group). All experiments were conducted at least in triplicate. **(E):** Relative values for the cytokine-induced migration of the two types of progenitor cells, Lin-CD29 + Sca-1+/PDGFRα+ and Sca1+/PDGFRα– (**, $p < .01$ compared with α-MEM Media). All experiments were conducted at least in triplicate. Abbreviations: PDGFRα, platelet-derived growth factor receptor alpha; Sca-1, stem cell antigen-1; TNFα, tumor necrosis factor-alpha.

cells such as HSCs and MSCs [31, 32]. PDGFRα, a receptor tyrosine kinase, is known as a marker of cells of the mesenchymal lineage such as MSCs and VSMCs. Murine adult HSCs do not express PDGFRα on their surface [33]. Thus, it is possible that Sca-1+/PDGFRα– cells encompass HSCs and other progenitor cells, and that Sca-1+/PDGFRα+ cells do not possess hematopoietic potential. For a more specific discrimination between bipotent and unipotent progenitor cells, we used additional markers, namely CD29 and the lineage cocktail. We assumed that CD29-positive cells would include MSCs [34, 35], and that Lin-negative cells would exclude differentiated WBCs, which might generate OCs [33]. We expected that Lin-



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CD29 + Sca-1+/PDGFRα– cells in the BM would represent progenitor cells, which retain both their hematopoietic ability and mesenchymal-lineage differentiation potential. Our in vitro, ex vivo, and in vivo data supported that Lin-CD29 + Sca-1+/PDGFRα– cells are bipotent MPCs that act as repopulating HSCs and differentiate into OBs, whereas Lin-CD29 + Sca-1+/PDGFRα+ cells are unipotent OB progenitor cells.

We observed that PDGFRα is a crucial marker for distinguishing between bipotent and unipotent progenitor cells. Cells expressing PDGFRα had lost their hematopoietic potential. Moreover, PDGFRα expression was activated during OB differentiation of Lin-CD29 + Sca-1+/PDGFRα– cells. Previous studies have reported that PDGFRα signaling facilitates vessel formation and hematopoiesis [36, 37]. Therefore, we presumed that PDGFRα downstream signaling might play an important role in the lineage-specific differentiation of MPCs. To investigate this hypothesis, we stimulated downstream signaling by PDGF-BB, or suppressed its surface expression using shRNA lentivirus prior to the induction of differentiation. Our data showed that the manipulation of PDGFRα did not change the fate of Sca-1+/PDGFRα– or Sca-1+/PDGFRα+ cells. Therefore, we concluded that PDGFRα is a distinct surface marker in bipotent and unipotent progenitor cells, but not a functional upstream mediator.

We recently characterized calcifying progenitor cells in wild-type C57 mice [5] and demonstrated that the activation of PPARγ results in the tendency of Sca-1+/PDGFRα– cells to acquire a decalcifying phenotype. These results suggested that certain types of progenitor cells that are amenable to reprogramming might represent therapeutic targets for the

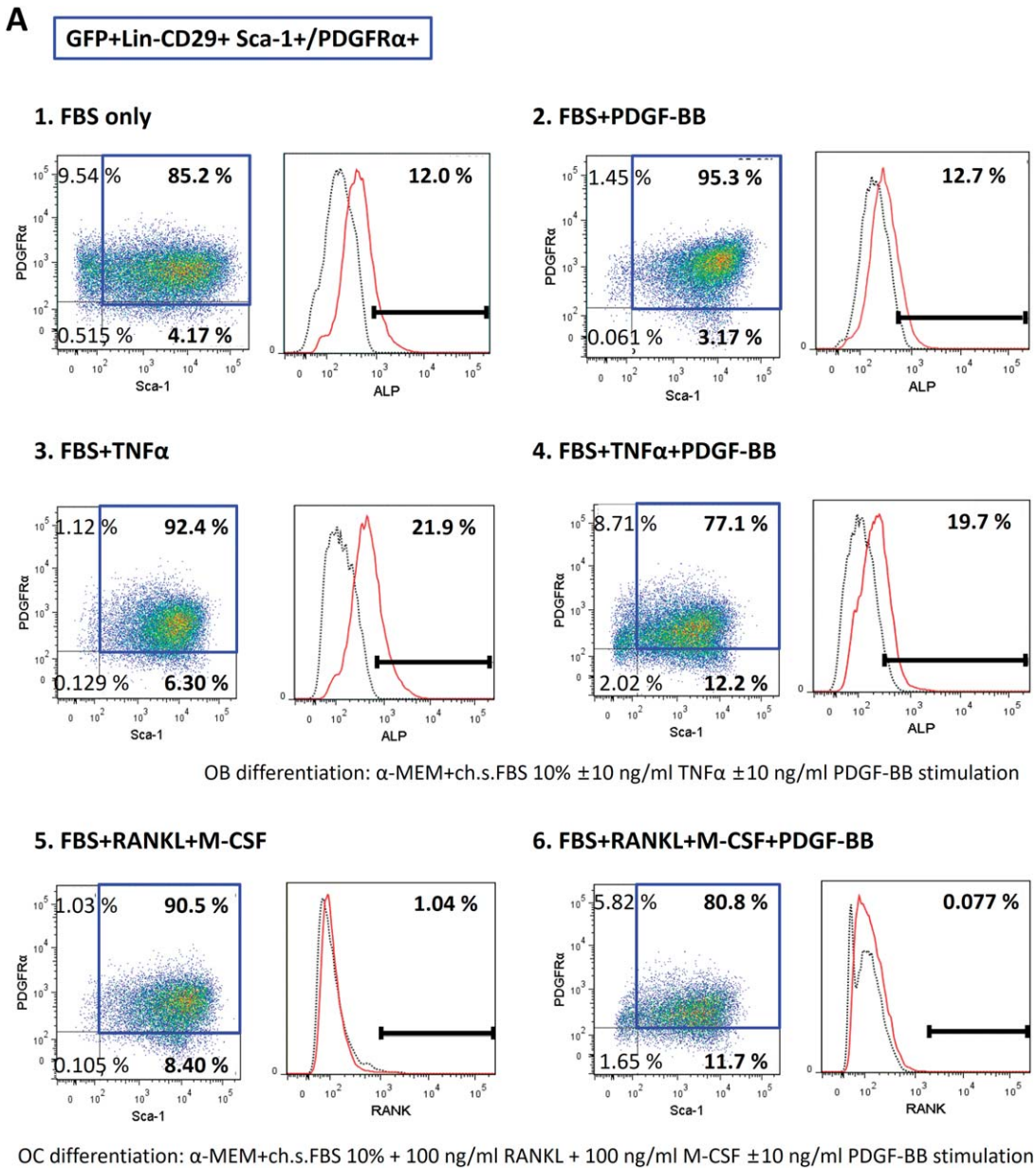
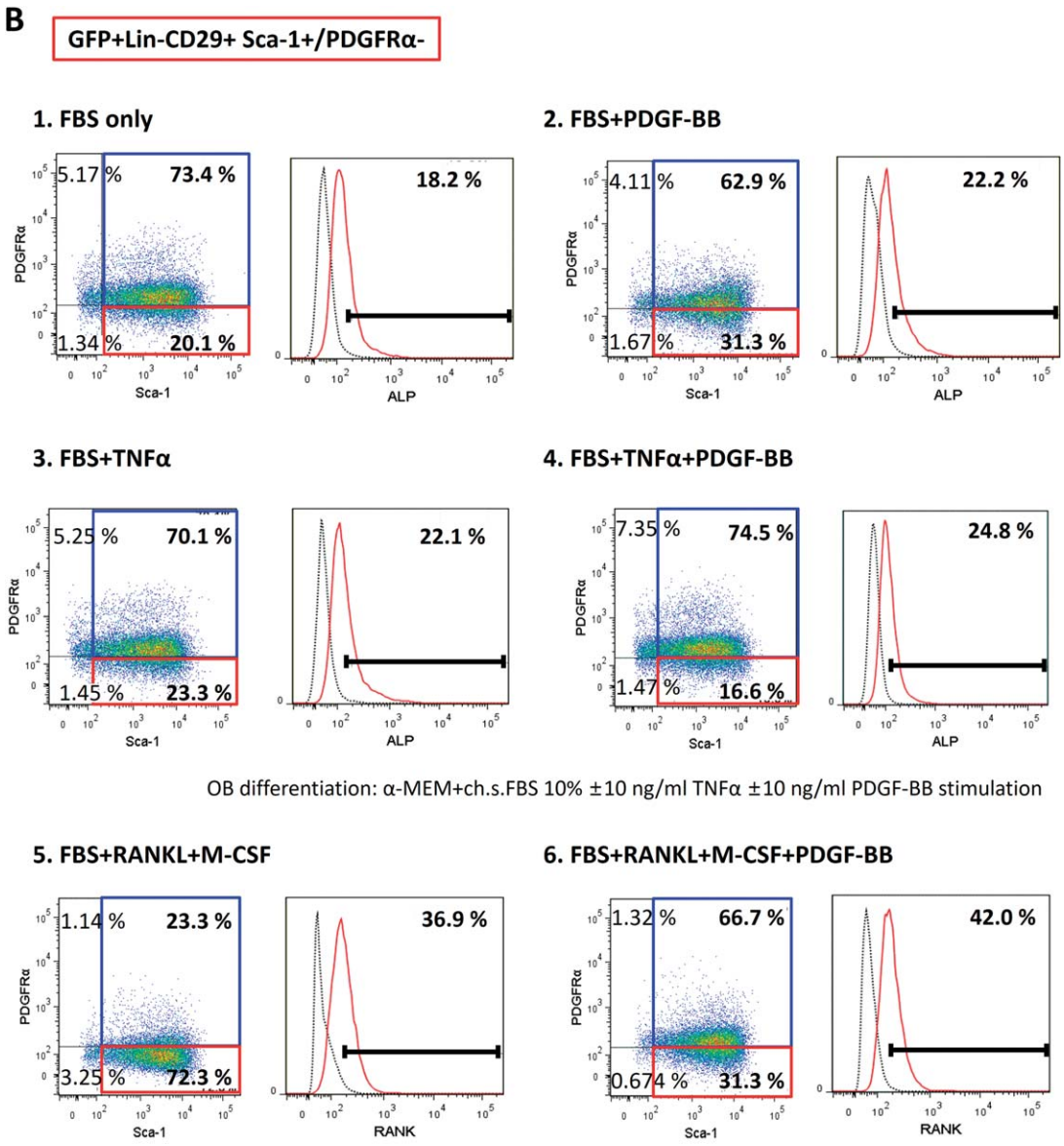


Figure 7. Stimulation or knockdown of PDGFRα does not alter the OB or OC differentiation of progenitor cells. We used single colony-derived, clonally-expanded progenitor cells that were BM-derived and artery-infiltrated. **(A, B):** Under OB/OC differentiation conditions, we stimulated the PDGFRα in GFP + Lin-CD29 + Sca-1+/PDGFRα+ and GFP + Lin-CD29 + Sca-1+/PDGFRα– cells with PDGF-BB. **(C, D):** To perform loss-of-function studies, the expression of PDGFRα was knocked down using shRNA-lentivirus transduction. PDGFRα shRNA transduction effectively hindered the expression of PDGFRα during differentiation. All experiments were conducted at least in triplicate. Abbreviations: M-CSF, macrophage colony-stimulating factor; PDGFRα, platelet-derived growth factor receptor alpha; Sca-1, stem cell antigen-1; TNFα, tumor necrosis factor-alpha.

reversal of vascular diseases. To further investigate this therapeutic potential, we studied the in vitro and in vivo behavior of two types of progenitor cells in Apoe-/- mice in an atherogenic environment and compared it with those observed under normal conditions. We demonstrated that the dynamics and fate of bipotent MPCs, but not of unipotent calcifying progenitor cells, were considerably affected by atherogenic signals.

Atherosclerotic conditions result in the retention of multipotent MPCs in the BM and hinder the mobilization and infiltration of these cells into the artery. In the present study, we investigated the regulatory mechanism of mobilization and showed that the serum and tissue levels of IL-5, an anti-inflammatory cytokine, were markedly reduced in the high-cholesterol diet group compared with that in the normal diet group. The in vitro assay revealed that IL-5 enhances the migration of bipotent Sca-1+/PDGFRα– MPCs, whereas the serum and tissue level of IL-1β, an inflammatory cytokine, were high in hypercholesterolemic conditions. Therefore, IL-1β was a potent inhibitor of the migration or mobilization of



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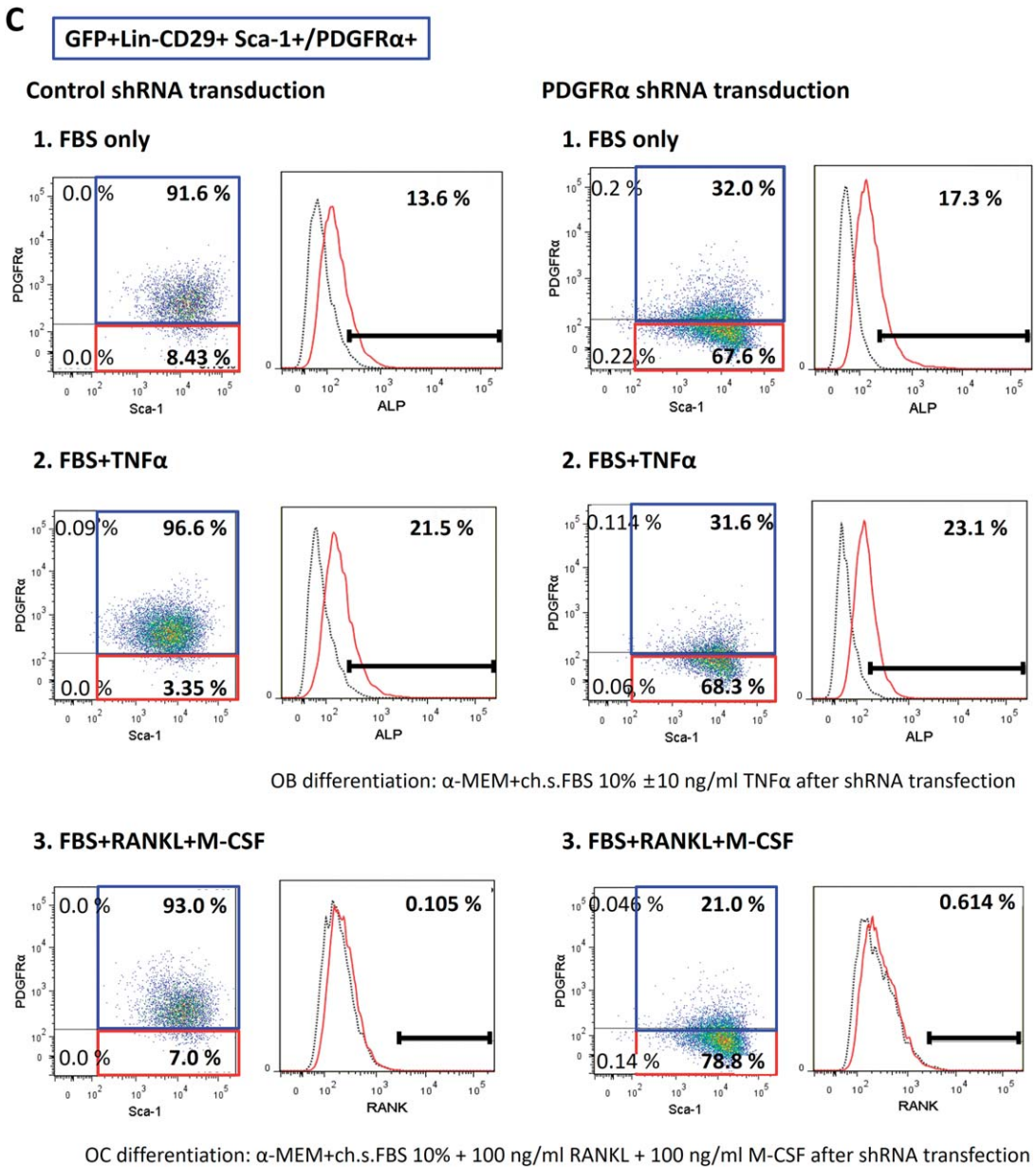
MPCs. These results indicated that the balance between inflammatory and anti-inflammatory humoral factors is a central regulator of the dynamics of bipotent MPCs and vascular calcification, whereas the migration of unipotent Sca-1+/PDGFRα+ calcifying progenitor cells was not significantly affected by various atherosclerosis-related cytokines. As a result, these inflammatory cytokines increased the migration of Sca-1+/PDGFRα+ cells compared with that of Sca-1+/PDGFRα– cells. Overall, our data indicated that Sca-1+/PDGFRα– MPCs play a key protective role during the progression of atherosclerosis and may represent valuable therapeutic targets for vascular protection or even disease reversal.

The present study has several limitations. Since we focused only on atherosclerotic calcification that is mediated by lipids and cytokines and is associated with inflammatory responses of endothelial cells and smooth muscle cells, our results might not be applicable to other types of calcification, such as medial calcification that is mediated by metabolic derangements. Therefore, further studies on other types of vascular calcifications are warranted [1, 38]. We determined the presence of MPCs in the adult BM and artery. Lin-CD29 + Sca-1+/PDGFRα– cells in the BM acted as repopulating hematopoietic cells and differentiated into OBs and OCs. However, BM-derived artery-infiltrated Lin-CD29 + Sca-1+/PDGFRα– cells lost their hematopoietic potential, even though they still possessed the OC potential. The milieu and microenvironment, including specific humoral factors, differ considerably between the BM and artery, which may alter the cellular fate and plasticity of multipotent progenitor cells.



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In addition, we investigated whether the results of murine experiments could be extrapolated to human progenitor cells. We investigated human surface markers that were equivalent to those in mice to address the question of mouse-to-human translatability. The human equivalent of Sca-1 is yet to be determined. Studies in humans have showed that osteocalcin+ cells circulate under physiological conditions [39], and that CD34 + osteocalcin+ cells in human PB exhibit the OB phenotype with endothelial differentiation potential [40], suggesting that multipotent progenitor cells could exist in humans. Furthermore, the mechanisms underlying the effects of the atherosclerotic milieu on the fate and dynamics of human multipotent progenitor cells require further investigation. A previous study has demonstrated that patients with coronary atherosclerosis exhibited higher number of osteocalcin+ cells in their PB than control patients [3], suggesting that the dynamics of human calcifying progenitor cells could be affected by the atherosclerotic stimuli.

PDGFRα may be used for the specific identification of adult human MPCs. Our murine experiments demonstrated that MPCs in the adult BM do not express PDGFRα, whereas OB progenitor cells strongly express this receptor. Recent studies in humans have revealed that fetal BM-MSCs [41] express PDGFRα, whereas adult BM-MSCs [42] do not. The reverse is observed for the hierarchy of progenitor cells in mice. These results suggested that PDGFRα expression is downregulated during development in humans. The physiological role of PDGFRα requires further investigation in both mouse and human cells.

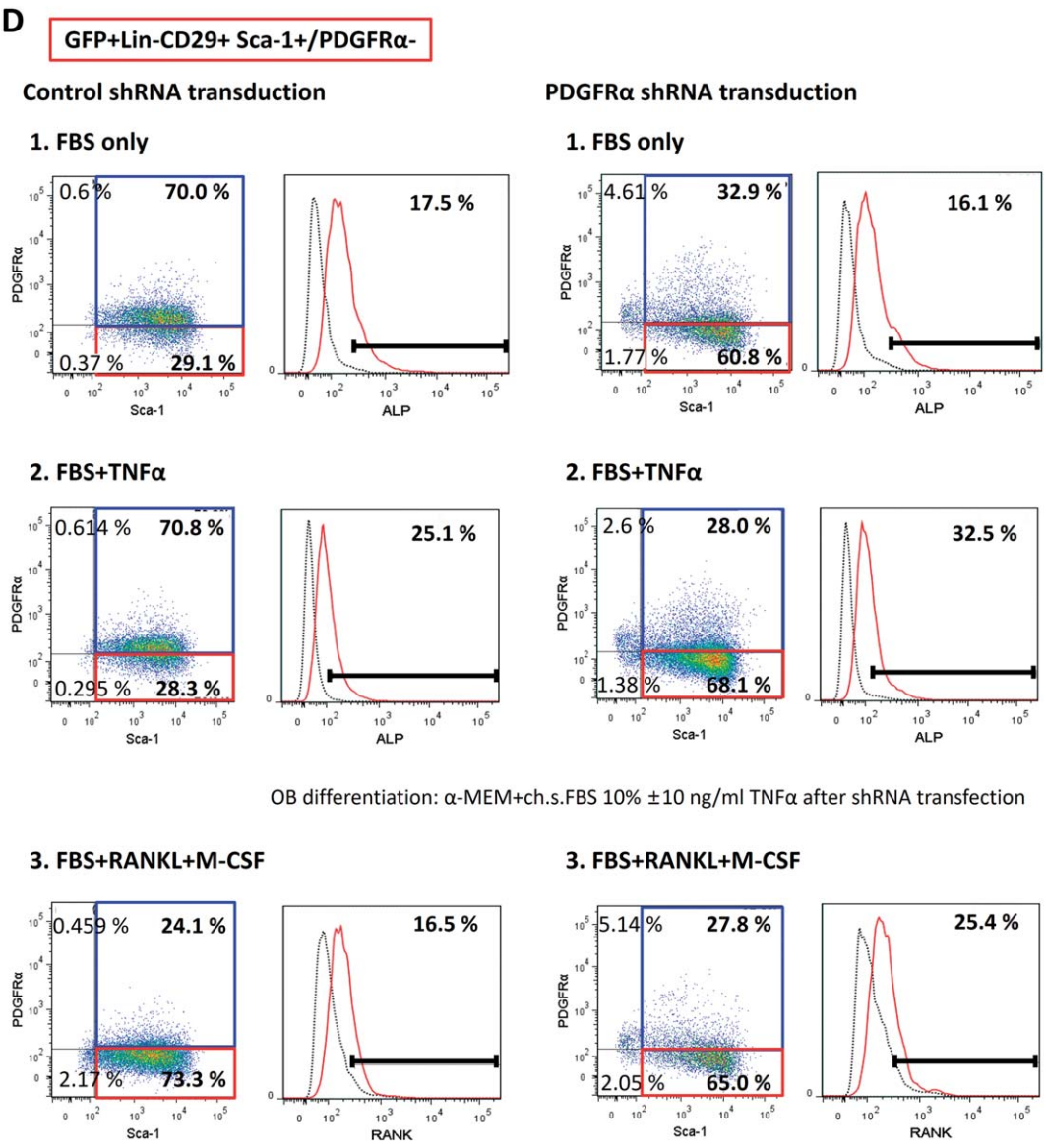

Figure 7. (Continued)

Conclusion

In summary, we showed that MPCs, the common ancestors of HSCs and MSCs, mobilize into PB and infiltrate the artery. MPC migration is regulated by atherosclerosis-related pro-inflammatory and anti-inflammatory cytokines. The present study provides valuable insights into the pathophysiology of atherosclerosis and vascular calcification, and might enable the identification of novel therapies for the treatment of vascular diseases. Future studies will extrapolate the results of murine experiments to human progenitor cells and translate research data to the clinic.

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Author Contributions

Hyun-Jai Cho: conception and design, financial support, collection and/or assembly of data, data analysis and interpretation, and manuscript writing; J.W.L.: collection and/or assembly of data, data analysis and interpretation, and manuscript writing; Hyun-Ju Cho: conception and design, collection and/or assembly of data, and data analysis and interpretation; C.S.L: collection and/or assembly of data and data analysis and interpretation; H.S.K.: conception and design, financial support, manuscript writing, and final approval of the manuscript.

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Disclosure of Potential Conflicts of Interest

The authors indicated no potential conflicts of interest.

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