Molecular Strategies to Treat Vascular Diseases

— Circulating Vascular Progenitor Cell as a Potential Target for Prophylactic Treatment of Atherosclerosis —

Masataka Sata, MD

Atherosclerosis is responsible for more than half of all deaths in Western countries. Numerous studies have reported that accumulation of smooth muscle cells (SMCs) plays a principal role in atherogenesis, post-angioplasty restenosis and transplantation-associated vasculopathy. Although much effort has been devoted to targeting the migration and proliferation of medial SMCs, effective therapy to prevent occlusive vascular remodeling has not been established. Recently, it was suggested that bone marrow-derived precursors can give rise to vascular cells that contribute to the repair, remodeling, and lesion formation of the arterial wall under certain circumstances. This review highlights the recent findings on circulating vascular precursors and describes the potential therapeutic strategies for vascular diseases, targeting mobilization, homing, differentiation and proliferation of circulating progenitor cells. (*Circ J* 2003; 67: 983–991)

Key Words: Atherosclerosis; Bone marrow; Gene therapy; Progenitor; Smooth muscle cell

Exuberant accumulation of smooth muscle cells (SMCs) plays a principal role in the pathogenesis of vascular diseases.^{1–6} In atherosclerotic plaques, SMCs proliferate and synthesize extracellular matrix, thereby contributing to lesion formation. Percutaneous coronary interventions (PCIs) have been widely adopted for treatment of coronary atherosclerosis, but a significant number of these procedures fail because of post-angioplasty restenosis.⁷ Although the increasing use of new devices for dilatation of stenosed arteries has lowered the incidence of acute complications, restenosis still limits the long-term outcome of PCI.⁸ SMC hyperplasia is also a major cause of post coronary bypass surgery occlusion^{9,10} and furthermore, graft vasculopathy, a leading cause of graft failure and retransplantation after the first postoperative year,¹¹ results from SMC hyperplasia.¹¹ Therefore, much effort has been devoted to understanding the molecular pathways that regulate SMCs hyperplasia in order to prevent vascular diseases.^{1–4} However, the pathogenesis remains largely unknown and, consequently, effective therapy has not yet been established.⁵

Hypothesis of the Derivation of Neointimal Cells

There is a widely accepted view that atherosclerotic lesions result from an excessive, inflammatory–fibroproliferative response to various forms of insult to the endothelium and smooth muscle of the artery wall.^{4,12–14} In brief,

after endothelial injury, inflammatory cells infiltrate and secrete various cytokines, such as tumor necrosis factor α , interleukin-1 α , and interferon- γ , which trigger dedifferentiation of the medial SMCs that regulate vascular tone and blood flow under normal physiological conditions.^{1,4} Dedifferentiated SMCs are characterized by a large cell body containing synthetic and secretory organelles.^{1,5,15–24} It has been hypothesized that dedifferentiated SMCs migrate into the subendothelial space, proliferate, and synthesize extracellular matrix;² and it has been assumed that all of the neointimal cells in post-angioplasty restenosis and graft vasculopathy are derived from medial SMCs.³ Thus, numerous pharmacological and gene therapies have been proposed to target the dedifferentiation, migration and proliferation of medial SMCs.^{25–36} However, there are several phenomena that cannot be explained by the hypothesis.

First, very few studies have documented that SMCs migrate across the internal elastic lamina from the media into the subendothelial layer.^{1,4} On the other hand, many studies have shown that blood cells attach to the luminal side of the injured artery prior to the development of the neointimal hyperplasia that is composed exclusively of SMCs.^{37,38} Second, it has been observed that neointima can form after medial cells have been killed by severe injury (Fig 1).³⁷ That study noted that neointimal cells were negative for SMC markers and appeared to be hematopoietic rather than vascular cells at 1 week after injury. Third, many studies have reported that SMC hyperplasia can be prevented by blocking chemokines or adhesion molecules,^{39,40} which play a crucial role in recruiting blood cells, but have no effect on the migration and proliferation of differentiated SMCs.³⁹ Fourth, neointimal SMCs are quite distinct from medial SMCs in phenotypic and genetic expression patterns.^{41,42} Neointimal SMCs express a number of hematopoietic lineage markers, including FK506-binding protein 12, interferon regulatory factor, and proinflammatory proteins,^{41,42} suggesting that some of the neointimal cells might be derived from blood cells rather than medial

(Received September 22, 2003; revised manuscript received September 22, 2003; accepted September 24, 2003)

Department of Cardiovascular Medicine, University of Tokyo Graduate School of Medicine, Tokyo and PRESTO, Japan Science and Technology Agency, Kawaguchi, Saitama, Japan

Mailing address: Masataka Sata, MD, Department of Cardiovascular Medicine, University of Tokyo Graduate School of Medicine, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. E-mail: sata-2im@h.u-tokyo.ac.jp

Fig 1. Neointima formation in the absence of medial cells. A large wire was inserted into the femoral artery of C57BL/6J mice, and the injured arteries were harvested at the time points indicated. The lumen of the injured artery remained dilated because of thinning of the media. At 1, 3, and 7 days after the injury, the media contained only a few cells. At 1 week, thin neointimal formation was observed on the luminal side of the internal elastic lamina (arrows). The neointimal hyperplasia continued to grow for 3 or 4 weeks after which lesion formation ceased. Reproduced with permission from Elsevier Science (Reference 37).

Fig 2. Contribution of recipient cells to graft-vasculopathy. When wild-type hearts were transplanted into LacZ mice, about 90% of the neointima cells were LacZ-positive and thus originating from the recipient (Left; Bar=50µm). Conversely, LacZ-negative recipient cells formed neointima on the LacZ-positive coronary arteries after cardiac transplantation from LacZ-mice to wild-type mice (Right; Bar=25µm). Arrows in histological panels indicate internal elastic lamina. Reproduced with permission from Nature Publishing Company (Reference 43).

SMCs. Evidence in support of this hypothesis has been provided in several models of vascular diseases^{43–46}

Bone Marrow Cells

Contribution to Transplant-Associated Arteriosclerosis

The contribution of bone marrow cells (BMCs) was first

Fig 3. Contribution of bone marrow cells to healing and lesion formation after mechanical injury. A large wire was inserted into the femoral artery of wild-type mice whose bone marrow had been reconstituted with that of LacZ mice. Four weeks later there was neointima formation. Approximately 60% of the neointimal cells and 40% of the medial cells were LacZ-positive, and thus derived from the bone marrow. Arrows and arrowheads in histological panel indicate internal and external elastic lamina, respectively. Scale bar=50 μ m. Reproduced with permission from Nature Publishing Company (Reference 44).

investigated in graft vasculopathy, a robust form of atherosclerosis that develops rapidly in transplanted organs, leading to failure of the allograft.^{3,11} Heterotopic cardiac transplantation was carried out between wild-type mice and ROSA26 mice,^{43,44} which are knock-in mice expressing LacZ ubiquitously (LacZ-mice).^{47,48} Four weeks after transplantation, the allografts were harvested and stained with X-gal (5-bromo-4-chloro-3-indolyl -D-galactopyranoside) (Fig 2). The lumens of the large epicardial coronary arteries, their smaller branches and arterioles were found to have narrowed because of concentric neointimal hyperplasia, the majority of which was composed of recipient cells expressing LacZ.^{43,44} It was also observed that some of the medial SMCs, as well as endothelial cells (ECs), had been replaced by recipient cells. Immunofluorescence studies revealed that the LacZ-positive cells in the neointima expressed various markers for SMCs, including myosin heavy chain, calponin, α -caldesmon, and α -smooth muscle actin (α -SMA).⁴⁴ Conversely, when LacZ-positive hearts were transplanted into wild-type mice, LacZ-negative neointima developed on the LacZ-positive coronary arteries. Furthermore, in situ hybridization of the allografts from female to male mice revealed that most of the neointimal cells corresponded to cells from the recipients.⁴³ These results indicated that the majority of the neointimal cells were derived from the recipient cells, but not from the medial cells of donor origin. Consistent with these observations, others independently reported that recipient cells are a major source of graft vasculopathy in the aortic transplantation model.^{49–54} Moreover, it has been reported that in human transplant-associated arteriosclerosis after renal transplantation most of the neointimal cells and ECs are derived from the recipient.^{55,56}

To identify the potential source of recipient cells that contribute to allograft vasculopathy, bone marrow transplantation (BMT) was performed from LacZ mice to wild-type mice (BMT^{LacZ} wild mice). After 4–8 weeks, wild-type hearts were transplanted into the BMT^{LacZ} wild mice, and 4 weeks later most of the neointimal cells were found to be LacZ-positive. Similarly, when wild-type hearts were

transplanted into wild-type mice whose bone marrow had been reconstituted with that of transgenic mice that ubiquitously expressed enhanced green fluorescent protein (GFP mice) (BMT^{GFP} wild mice),⁴⁴ it was observed that GFP-positive cells accumulated on the luminal side of the graft coronary arteries. The immunofluorescence study revealed that some of the GFP-positive cells in graft vasculopathy expressed α -SMA. These results indicate that recipient BMCs may substantially contribute to neointimal formation in transplanted grafts.

Contribution to Vascular Remodeling After Mechanical Injury

BMCs can also contribute to the pathogenesis of lesion formation after mechanical vascular injury (Fig 3).^{44–46} The bone marrow of wild-type mice were replaced with that of LacZ-mice (BMT^{LacZ} wild mice) and it was found that transplanted LacZ BMCs had settled in bone marrow, spleen, and thymus, whereas LacZ-positive cells were not detected in the uninjured femoral arteries of the BMT^{LacZ} wild mice. Four to 8 weeks after BMT, a large wire was inserted into the femoral artery of the BMT^{LacZ} wild mice, being a new method of vascular injury that resembles balloon angioplasty.³⁷ This injury led to complete denuding of the endothelium and marked enlargement of the lumen (Fig 1).^{37,57,58} The cellularity of the medial layer decreased because of acute onset of SMC apoptosis.^{37,57,58} One week after the injury, the artery remained dilated and X-gal staining revealed that LacZ-positive cells were attached to the luminal side of the injured vessels. The LacZ-positive cells did not express the marker for SMCs (ie, α -SMA) or that for ECs (ie, CD31). The dilated lumen gradually narrowed because of neointimal hyperplasia, which was primarily composed of SMCs. A significant number of the neointimal and medial cells were LacZ-positive on X-gal staining.^{44,46} Immunofluorescence double-staining documented that some bone marrow-derived LacZ positive cells in the neointimal lesions expressed α -SMA or CD31.⁴⁶ These results indicate that BMCs may give rise to vascular cells, thereby contributing to the arterial remodeling after the

Fig 4. Bone marrow-derived smooth muscle cells (SMCs) in atherosclerotic plaques. Bone marrow transplantation was performed from GFP-mice to ApoE-deficient mice. After being fed a Western-type diet, the aortas of BMT^{GFP} ApoE^{-/-} mice were observed under a xenon fiber optic light source. Arrows indicate atherosclerotic plaques. A, aorta; V, vertebra. Cross-sections of the aortas of the BMT^{GFP} ApoE^{-/-} mice were stained with Cy3-conjugated anti-smooth muscle actin antibody (red) and Hoechst 33258 (blue). Arrows indicate internal elastic lamina. Arrowheads indicate GFP-positive cells (green) expressing α -smooth muscle actin (α SM actin, red) in atherosclerotic lesions. Scale bar = 50 μ m. Reproduced with permission from Nature Publishing Company (Reference 44).

wire-mediated endovascular injury.

Bone Marrow-Derived SMC-Like Cells in Atherosclerotic Plaques

To investigate the potential source of the SMCs observed in atherosclerotic plaques,⁴ the bone marrow of 8-week-old ApoE^{-/-} mice was replaced with that of GFP-mice (BMT^{GFP} ApoE^{-/-} mice) or ROSA26-mice (BMT^{LacZ} ApoE^{-/-} mice).⁴⁴ The recipient mice were fed a Western-type diet for 8 weeks, starting at 4 weeks after BMT.⁵⁹ GFP-positive cells accumulated in atherosclerotic plaques developing in the aortas of the BMT^{GFP} ApoE^{-/-} mice (Fig 4). Immunofluorescence study of cross sections of the vessels revealed that some of the GFP-positive cells expressed markers for SMCs (ie, α -SMA). Similarly, in the atherosclerotic lesions of the BMT^{LacZ} ApoE^{-/-} mice, some of the LacZ-positive cells expressed α -SMA. Furthermore, immunogold-labeling for LacZ identified bone marrow-derived SMC-like cells with muscle fibers in the atherosclerotic plaques of BMT^{LacZ} ApoE^{-/-} mice. The SMC-like cells displayed a typical 'synthetic' phenotype. These results suggest that some of the SMC-like cells observed in hyperlipidemia-induced atherosclerotic plaques may originate from bone marrow.

New Mechanism for SMC Accumulation in Vascular Lesions

Coronary angioplasty causes vessel wall injury and induces SMC proliferation with subsequent abundant production of extracellular matrix. Transplant-associated arteriosclerosis is also considered a consequence of an immunological attack against the allograft by the recipient.^{3,11}

Various atherogenic substances, such as oxidized low density lipoprotein,^{60–62} homocysteine,⁶³ angiotensin II⁶⁴ and lipopolysaccharides⁶⁵ have been reported to induce vascular cell apoptosis, presumably initiating the earliest phase of lesion development in atherosclerosis.⁶⁶ Therefore, neointima formation appears to be similar to the healing process in response to vascular injuries.⁴ In addition to the conventional assumption that damaged tissues are repaired by individual parenchymal cells, an accumulating body of evidence indicates that there exist somatic stem cells that are mobilized to remote organs, differentiate into required lineages and participate in organ repair and regeneration.^{67–72} It has been also reported that Flk-1 positive cells derived from mouse embryonic stem (ES) cells can act as vascular progenitor cells that contribute to fetal and adult neovascularization.^{73,74} Bone marrow might be an additional source of vascular cells that contribute to both vascular repair and the pathological remodeling at least in models of post-angioplasty restenosis, transplant-associated arteriosclerosis and hyperlipidemia-induced atherosclerosis.

Diverse Origins of Neointimal Cells

Of course, bone marrow could not be the only source of neointimal cells.⁴⁵ Numerous reports have shown that neointimal cells are heterogeneous and that the SMCs in vascular lesions are composed of cells of diverse origins.^{16,46,75} Recently, we also reported that the cellular constituents differ according to the type of vascular injury.⁴⁶ Three distinct types of mechanical injuries were induced in the same mouse whose bone marrow had been reconstituted with that of GFP- or LacZ- mice (Fig 5). After wire-mediated endovascular injury, a significant number of the neointimal and

Fig 5. Marked diversity in the contribution of bone marrow-derived cells to vascular remodeling. Three distinct types of mechanical injuries were induced in the same mouse whose bone marrow had been reconstituted with that of GFP- or LacZ- mice. Endovascular injury was induced by inserting a large wire into the left femoral artery (Wire-mediated expansion), a perivascular injury was induced by placing a polyethylene tube around the right femoral artery of the same mouse (Cuff-replacement) and a flow-restriction vascular injury was induced by ligating the left common carotid artery (Flow-restriction by ligation). The injured arteries of BMT^{GFP} *ApoE*^{-/-} mice were harvested after 4 weeks and observed with a GFP-lighting system and a cooled CCD camera (Macroscopic panels). Scale bar=1 mm. The injured arteries from the BMT^{LacZ} *Wild* mice were stained with X-gal to detect LacZ. Paraffin-embedded sections were rehydrated and stained with hematoxylin. Arrows indicate the internal elastic lamina. Arrowheads indicate LacZ⁺ cells (Histological panels). Scale bar=20 μm. Reproduced with permission from Lippincott Williams & Wilkins (Reference 46).

medial cells were found to be derived from bone marrow. In contrast, marker-positive cells were seldom detected in the lesion induced by perivascular cuff replacement. There were only a few bone marrow-derived cells in the neointima following ligation of the common carotid artery. These findings suggest that the mode of injury is crucial for the recruitment of bone marrow-derived cells to tissue remodeling.

Given the complexity of human atherosclerotic lesions, none of the vascular injury models would represent the exact pathogenesis. It has been suggested that BMCs would substantially contribute to lesion formation when arteries are subjected to severe injuries.⁴⁶ Advanced atherosclerotic lesions exhibit a higher incidence of internal elastic rupture and intimal medial interface damage,⁷⁶ both of which are associated with focal intraplaque microhemorrhage.⁷⁷ Angioplasty completely denudes the endothelium and mechanically dilates atherosclerotic lesions with a tear in the luminal surface.⁷⁸ It is likely that circulating progenitors substantially contribute to vascular remodeling in humans when arteries are subjected to severe injuries, such as PCI, transplantation and plaque rupture.^{44–46,57,58,79} Consistent with this notion, an analysis of sex-mismatched bone marrow transplant subjects revealed that SMCs throughout the atherosclerotic vessel wall are derived from the donor bone marrow.⁸⁰ Of interest is a finding that recruitment of bone marrow-derived SMCs was more extensive in diseased compared with undiseased segments.

Bone Marrow Cell Types Contributing to Vascular Remodeling

Pluripotent cells have been identified in bone marrow and are classified into hematopoietic stem cells (HSCs)⁸¹ and mesenchymal stem cells.⁸² Although it has been assumed that HSCs give rise only to blood cells of hematopoietic lineage, recent reports suggest that they may have the broader potential to differentiate into various cell types, including epithelial cells,⁸³ hepatocytes,^{84,85} and cardiomyocytes.^{68,86} To identify the BMCs that have the potential to generate vascular cells, a HSC-enriched fraction (c-Kit⁺, Sca-1⁺, Lin⁻) was isolated from the bone marrow of LacZ-mice by fluorescence-activated cell sorting.⁴⁴ Next, 3,000 cells were injected into lethally irradiated wild-type mice. Four weeks after bone marrow reconstitution, the femoral artery of the recipient mice was mechanically injured with a large wire³⁷ and 4 weeks later, the injured arteries showed neointimal hyperplasia. The neointima and the media contained many LacZ-positive cells.⁴⁴ Some LacZ-positive cells expressed α -SMA. LacZ-positive cells also contributed to endothelial regeneration.⁴⁴ These findings suggest that the c-Kit⁺, Sca-1⁺, Lin⁻ fraction of BMCs may have the potential to differentiate into either the SMCs or ECs that participate in vascular remodeling.

Recently, Wagers et al extensively analyzed uninjured organs of wild-type mice whose bone marrow had been reconstituted with a single HSC and concluded that trans-

Fig 6. Therapeutic strategies targeting putative vascular progenitor cells. Research findings provide the basis for the development of new therapeutic strategies for vascular diseases, targeting the mobilization, circulation, homing, differentiation and proliferation of bone marrow-derived vascular progenitor cells.

differentiation of HSCs into other lineages is an extremely rare event⁸⁷ In the aforementioned study⁴⁴ the recipient bone marrow was reconstituted with 3,000 cells of the c-Kit⁺, Sca-1⁺, Lin⁻ fraction, which substantially contributed to arterial remodeling after wire-mediated injury. The apparent discrepancy in the results of these 2 studies could merely derive from the analysis of non-injured versus injured tissues. Moreover, although the c-Kit⁺, Sca-1⁺, Lin⁻ fraction is supposed to be enriched in HSCs,⁸⁵ non-hematopoietic cells may potentially contaminate the population. It remains unclear whether a highly purified single HSC can give rise to neointimal cells as the c-Kit⁺, Sca-1⁺, Lin⁻ fraction does. It is possible that the contaminated non-hematopoietic (CD45-negative) cells rather than the hematopoietic (CD45-positive) cells contributed to neointimal formation after vascular injury. My group is currently determining whether a HSC can transdifferentiate into vascular cells by analyzing the vascular lesions produced in the mice whose bone marrow has been reconstituted by a single HSC.

Cell Fusion as a Possible Mechanism of BMC 'Differentiation'

Recent evidence suggests that there somatic stem cells or adult stem cells remain in the adult⁸⁸ Many animal experiments have documented that adult stem cells can transdifferentiate into other lineages^{72,83,89,90} In gender-mismatched human BMT, it has been reported that donor-derived hematopoietic stem cells participated in organ regeneration^{83,85} On the other hand, the results of recent studies suggest that adult stem cells adopt a tissue-specific phenotype by cell fusion, *in vitro*^{91,92} and *in vivo*^{93,94} but not by transdifferentiation. Consistent with this notion, polyploidization of vascular SMCs in response to mechanical and humoral stimuli has been documented⁹⁵ Thus, it is possible that cell fusion can account for, at least in part, the accumulation of bone marrow-derived SMC-like cells in vascular lesions;⁴⁵ although spontaneous cell fusion between recipient and donor-derived cells seems to be a rare event in a murine model of cardiac transplantation.⁹⁶

Therapeutic Strategies Targeting Circulating Vascular Progenitor Cells

Although a lot of effort has been devoted to targeting the migration and proliferation of medial SMCs,⁵ effective therapy to prevent vascular lesions has not yet been established. Our findings indicate that bone marrow-derived smooth muscle progenitor cells might become additional targets (Fig 6)⁴⁵ Consistent with this notion is a previous observation that transient myelosuppression inhibited SMC hyperplasia in balloon-injured coronary arteries.⁹⁷ Similar effects could be obtained by inhibition of a chemokine³⁹ or an adhesion molecule⁴⁰ which may play a crucial role in the recruitment and homing of putative SMC progenitors. Recently, a sirolimus-eluting stent has emerged as a promising strategy to inhibit post-PCI restenosis.^{98,99} In spite of increasing clinical enthusiasm, very little is known as yet about the mechanism by which the stent effectively prevents neointimal hyperplasia. Sirolimus is an immunosuppressive drug, which is supposed to have relatively little effect on medial SMCs.⁴¹ It is possible that sirolimus effectively inhibits accumulation of bone marrow-derived vascular precursors when delivered locally at the site of progenitor cell accumulation.

Possible Gene Therapy

Several gene therapies targeting putative vascular progenitors are being studied, particularly the potential utilities of the Fas/Fas ligand system.^{100,101} Fas is a death receptor that transmits an apoptosis-inducing signal when activated by its ligand, Fas ligand (FasL).^{102,103} Recent observations of the Fas system in the vasculature indicate that ectopic or augmented expression of FasL may be useful for treating vascular diseases.^{104–106} FasL induces apoptosis in Fas-bearing vascular SMCs,¹⁰⁴ whereas ECs are refractive to FasL overexpression.^{104,107,108} Thus, the local delivery of FasL to sites of balloon injury is predicted to reduce the number of SMCs by inducing apoptotic cell death, while not inhibiting the beneficial process of re-endothelialization at the same site.^{104,109} Moreover, local delivery of FasL should also kill bone marrow-derived progenitors as well as inflammatory cells at these lesions because these cells express Fas.^{106,107} Consistent with this notion, it was reported that ECs can be genetically modified by adenovirus-mediated gene transfer to overexpress functional, cell-surface FasL without undergoing self-destruction.¹⁰⁹ In a rodent model of transplant graft vasculopathy, endothelial overexpression of FasL attenuated homing of hematopoietic cells at 1 week post-transplantation¹⁰⁵ and the vessels also displayed reduced neointima formation at 1 and 2 months post-transplantation. These results indicate that endothelial cell-specific overexpression of FasL may have utility in the treatment of transplant arteriosclerosis by preventing accumulation of smooth muscle progenitors. Similar therapeutic strategies may be effective for prophylactic treatment of other types of vascular diseases.

Implications for Regenerative Medicine

There is an accumulating body of evidence that in adult there are somatic stem cells that can differentiate into various lineages.^{67,85,88,110} This has aroused enthusiasm for the potential use of somatic stem cells for 'cell transplantation therapy' and 'tissue engineering'.⁶⁷ However, given the pluripotency of adult stem cells, they may potentially differentiate into unfavorable cell types. In fact, there are a

number of similarities between stem cells and cancer cells¹¹ and there is evidence that somatic stem cells do participate in pathological remodeling in remote organs.^{43,44} For clinical use of adult stem cells, attention must be paid to potential adverse effects as well as beneficial aspects caused by the transplanted stem cells.

Future Directions

Further studies are needed to identify and characterize putative vascular progenitor cells. It has been observed that bone marrow-derived cells were negative for the markers of SMCs and ECs when they homed on the luminal side of the artery at 1 week after the mechanical injury⁴⁴ so it is likely that plastic, immature cells may be mobilized to the injured vessels, where they differentiate in response to mechanical and humoral stimuli. Consistent with that, it has been reported that blood cells contain progenitors that have the potential to differentiate into either ECs or SMCs in vitro according to the composition of the culture medium.^{79,80,112,113} Experiments that dissect the molecular mechanisms by which progenitors are recruited and differentiate at the site of injury are required.

Conclusions

In summary, the findings of research to date indicate that BMCs, including hematopoietic stem cells, contribute not only to the healing process of injured organs, but also to pathological remodeling. These findings provide the basis for the development of new therapeutic strategies for vascular diseases, targeting the mobilization, homing, differentiation and proliferation of bone marrow-derived vascular progenitor cells.

Acknowledgments

This study was supported in part by grants from Ministry of Education, Culture, Sports, Science and Technology (15039213, 15390241, and 15659180) and Ministry of Health, Labor and Welfare.

I thank Yoshie Maejima, Hiromi Kato-Nakajima, Fumiko Adachi, Takaaki Hasegawa, Yumi Sugawara, Mariko Kinoshita, Nobuko Nangi, Miwa Washida, Yukari Kaneda and Satoru Fukuda for their excellent technical assistance.

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