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# Prostacyclin Stimulated Integrin-Dependent Angiogenic Effects of Endothelial Progenitor Cells and Mediated Potent Circulation Recovery in Ischemic Hind Limb Model

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***Background:*** Prostacyclin ($PGI_2$) enhances angiogenesis, especially in cooperation with bone marrow (BM) derived endothelial progenitor cells (EPCs). However, the mechanisms of $PGI_2$ in EPC mediated angiogenesis in vivo remain unclear. The purpose of this study was to clarify the role of $PGI_2$ in EPC mediated angiogenesis using BM specific IP deletion mice.

***Methods and Results:*** Hind limb ischemia (HLI) was induced in wild type (WT) mice transplanted with IP deleted BM (WT/BM($IP^{-/-}$)). Recovery of blood flow (RBF) in WT/BM($IP^{-/-}$) was impaired for 28 days after HLI, whereas RBF in $IP^{-/-}$/BM(WT) was attenuated for up to 7 days compared with WT/BM(WT). The impaired RBF in WT/BM($IP^{-/-}$) was completely recovered by intramuscular injection of WT EPCs but not $IP^{-/-}$ EPCs. The impaired effects of $IP^{-/-}$ EPCs were in accordance with reduced formation of capillary and arterioles in ischemic muscle. An ex vivo aortic ring assay revealed that microvessel formation was enhanced by accumulation/adhesion of EPCs to perivascular sites as pericytes. $IP^{-/-}$EPCs, in which expression of integrins was decreased, had impaired production of angiogenic cytokines, adhesion to neovessels and their angiogenic effects. The small interfering RNA (siRNA) mediated knockdown of integrin $\beta1$ in WT EPCs attenuated adhesion to microvessels and their in vivo and in vitro angiogenic effects.

***Conclusions:*** $PGI_2$ may induce persistent angiogenic effects in HLI through adhesion of EPCs to perivascular sites of neovessels via integrins in addition to paracrine effects. (*Circ J* 2013; **77:** 1053–1062)

**Key Words:** Angiogenesis; Endothelial progenitor cells; Hind limb ischemia; Prostacyclin

Prostacyclin ($PGI_2$), which induces vasodilation and inhibition of platelet aggregation and vascular smooth muscle cell proliferation, is an important prostanoid for homeostasis of the cardiovascular system.[1] Accumulating evidence suggests that $PGI_2$ mediates angiogenesis, leading to significant improvement of ischemia.[2–5] Stable $PGI_2$ analogs such as beraprost have been widely used for treatment of peripheral arterial disease.[6,7] $PGI_2$ analogs protect ischemic tissues through augmentation of not only capillary formation[3,8] but also collateral growth.[5]

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Several mechanisms to explain the potent angiogenic effects of $PGI_2$ have been proposed. $PGI_2$ analogs mediate angiogenesis by the upregulation of angiogenic factors such as vascular endothelial growth factor (VEGF) in various cell types.[4,9] It is known that endothelial progenitor cells (EPCs) contribute to the growth of vessels and induce prolonged vascular recovery from ischemia.[10,11] Recently, it has been reported that $PGI_2$

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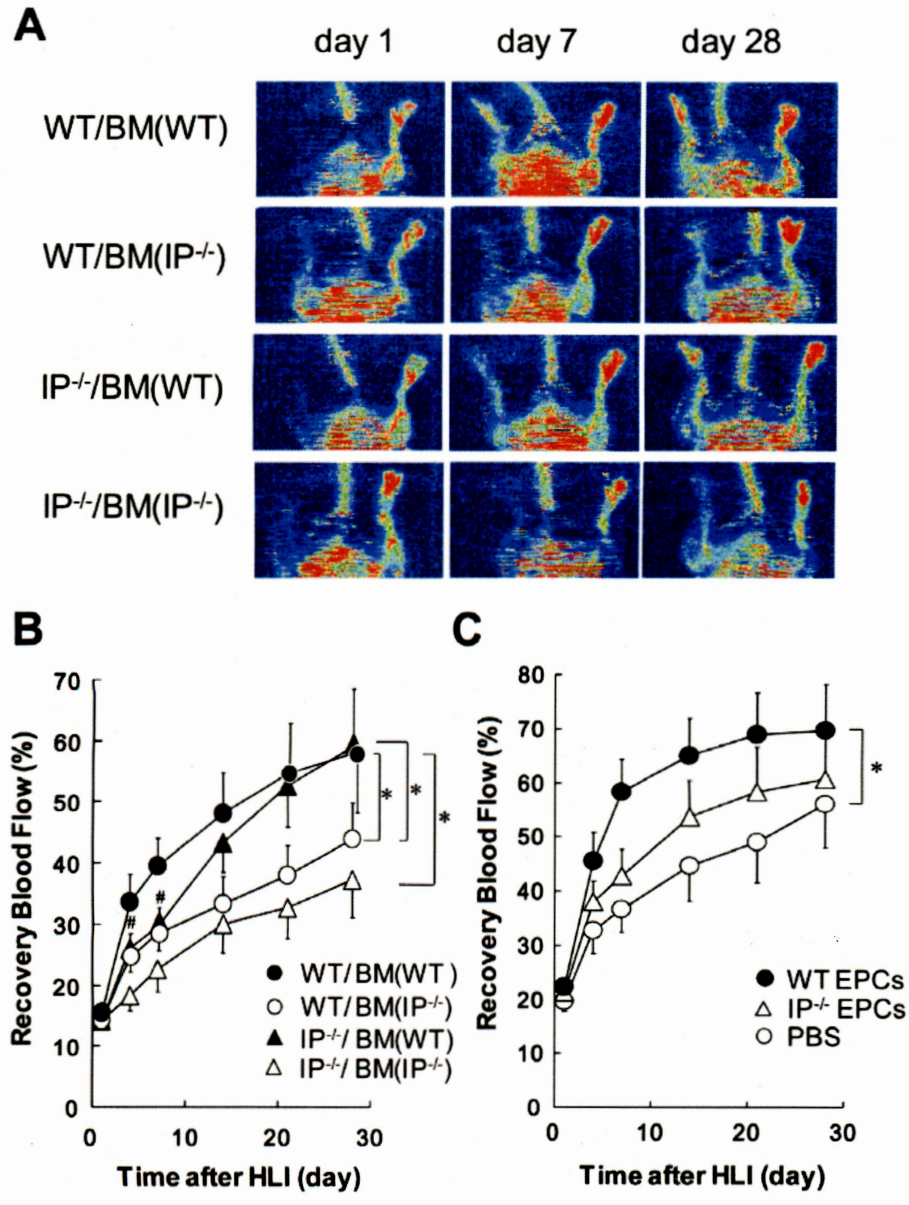


**Figure 1.** Effects of IP deletion in bone marrow (BM) and endothelial progenitor cells (EPCs) on perfusion recovery of hind limb ischemia (HLI) measured by laser Doppler peripheral blood flow images (LDPI), and the ischemic to non-ischemic LDPI ratio is expressed as recovery of blood flow (RBF). Values are mean±SEM. (**A**) Representative LDPI at indicated times after HLI surgery. WT or IP$^{-/-}$ mice were transplanted with BM of WT or IP$^{-/-}$: WT/BM(WT), IP$^{-/-}$/BM(WT), WT/BM(IP$^{-/-}$) and IP/BM(IP$^{-/-}$), respectively. (**B**) RBF in hind limbs of BM-transplanted mice at indicated times. (n=16–18, *P<0.05). (**C**) Isolated WT EPCs, IP$^{-/-}$ EPCs and the vehicle (PBS) were injected into the ischemic hind limb of WT/BM(IP$^{-/-}$) mice, and RBF was measured at indicated times (n=12, *P<0.05). IP, prostacyclin ($PGI_2$)-specific receptor; PBS, phosphate-buffered saline; PG, prostaglandin; WT, wild-type.

contributes to angiogenesis in cooperation with bone marrow (BM)-derived cells, including EPCs.[3,8] He et al showed that $PGI_2$ stimulated proangiogenic activities of EPCs by means of their endothelial-like properties, in vitro tube formation and in vivo capillary formation.[12] However, recent insights into the mechanisms of EPC-mediated vascular growth have indicated that EPCs promote neovascularization by their paracrine effects (ie, secretion of angiogenic cytokines stimulating the proliferation of resident mature endothelial cells (ECs)), rather than by incorporation of the cells into the neovascular endothelium.[13–15] Thus, the actions of $PGI_2$ on endothelium-like EPCs are insufficient to explain fully the potent angiogenic effects of $PGI_2$ in vivo, and the mechanisms of the angiogenic effects of $PGI_2$ through EPCs are still unclear.

Recent studies have reported that the angiogenic effects of EPCs are mediated by $PGI_2$ through IP-independent peroxisome proliferator-activated receptor (PPARs) nuclear signals.[12,16] In contrast, the importance of $PGI_2$/IP signals in the angiogenic

effect of EPCs is unclear. Our recent finding[17] that deletion of the $PGI_2$-specific receptor, IP, in EPCs causes dysfunction of EPCs prompted us to clarify the role of $PGI_2$ in EPC-mediated angiogenesis in hind limb ischemia (HLI) using mice with BM-specific deletion of IP. In the present study, we demonstrate that the $PGI_2$/IP system mediates the adhesion of EPCs to perivascular sites of neovessels in addition to their paracrine effects, and contributes to the prolonged perfusion recovery effects of EPCs in HLI.

## Methods

An expanded Methods section is provided in Data S1.

### Animals and BM Transplantation

All animal interventions were approved by the Animal Care and Use Committee of Asahikawa Medical College. All mice were fed normal chow. $IP^{-/-}$ mice with the genetic background of C57BL/6 were prepared as described previously.[18] For cell tracing experiments, green fluorescent protein (GFP) transgenic mice (C57BL/6, homozygous) donated by M. Okabe (Osaka University, Japan) were used. BM transplantation models in which BM mononuclear cells (BM-MNCs) from wild-type (WT) and $IP^{-/-}$ mice were injected into WT or $IP^{-/-}$ mice were established as described previously.[17]

### HLI Model

The mice (12–14 week-old males) were anesthetized (pentobarbital 50mg/kg i.p.) and the left femoral artery/vein and subcutaneous fatty tissues were gently removed. The blood flow in the limbs was measured by a laser Doppler perfusion imager (Moore, Axminster, UK). The calculated perfusion blood flow was expressed as a ratio of left (ischemic) to right (normal) limb.

### Preparation of EPCs

EPCs were isolated from BM-MNCs as described previously.[17] Lineage-negative and ckit/Flk1 double-positive cells were isolated from MNCs using a magnetic sorting system (lineage deletion kit, multisorting system; Miltenyi Biotec, Germany). These $Lin^-$/$ckit^+$/$Flk^+$ cells were used as EPCs for the in vitro angiogenesis assay and cellular transplantation experiments as described in Data S1.

### Statistical Analysis

Results are presented as mean±SEM unless otherwise noted. Significance between 2 measurements was determined by unpaired Student's t-test, and multiple comparisons were evaluated through the use of ANOVA, followed by Fisher's test. Values of $P<0.05$ were considered statistically significant.

## Results

### Blood Flow Recovery in Hind Limbs of IP-Deleted Mice

RBF in the hind limbs of $IP^{-/-}$ mice was reduced compared with WT mice (Figure 1). In order to determine whether the reduction of RBF was related to the lack of $PGI_2$-specific receptor IP deletion in either BM or non-BM tissues, including peripheral vessels, WT or $IP^{-/-}$ mice were transplanted with BMCs isolated from WT and $IP^{-/-}$ mice, WT/BM(WT) and WT/BM($IP^{-/-}$) or $IP^{-/-}$/BM(WT) and $IP^{-/-}$/BM($IP^{-/-}$), respectively, and subjected to hind limb surgery. As shown in Figure 1B, RBF in WT/BM(WT) mice had recovered to approximately 60% of the non-ischemic normal flow at 28 days after hind limb surgery. At 4–7 days after surgery, initial blood flow recovery was reduced in $IP^{-/-}$/BM(WT) and WT/BM($IP^{-/-}$) mice. Thereafter, RBF in WT/BM($IP^{-/-}$) mice remained severely depressed up to 28 days. In contrast, in $IP^{-/-}$/BM(WT) mice, RBF was temporarily attenuated for up to 7 days but had increased to a level similar to that in WT/BM(WT) mice at 14 days after HLI surgery (Figure 1B). These results suggest that the $PGI_2$/IP system in BM and that in non-BM tissues have different roles in post-ischemic RBF.

### Effects of IP Deletion in EPCs on In Vivo Angiogenesis

To determine the role of EPCs in reduced post-ischemic RBF in WT/BM($IP^{-/-}$) mice, we investigated whether the reduction in RBF subsided with the addition of exogenous EPCs. Transplantation of WT EPCs markedly improved RBF in the HLI of WT/BM($IP^{-/-}$) mice, and RBF was increased up to a level similar to that in WT/BM(WT) mice (Figure 1C). However, the effect of transplantation of $IP^{-/-}$ EPCs was reduced and RBF did not significantly increase compared with control mice given phosphate-buffered saline (PBS) injections (Figure 1C). Capillaries and arterioles in the muscles were detected by immunostaining with anti-CD31 and ▪-smooth muscle actin antibodies, respectively (Figure 2A). Quantitative analysis revealed that the capillary/muscle fiber ratio in the ischemic muscle was significantly increased by injections of WT EPCs compared with control mice (Figure 2B). Similarly, arteriolar area per section was also increased in the WT EPCs-injected group (Figure 2C). In accordance with the results for RBF, the angiogenic effect of $IP^{-/-}$ EPCs was significantly reduced compared with that of the WT EPCs (Figure 2). These results suggest that among BM-derived cells, EPCs contribute to the effects of the $PGI_2$/IP system in BM for persistent RBF in HLI and that $PGI_2$ is crucial for EPCs to mediate not only capillary formation but also arteriolar formation in ischemic tissues.

### Localization of Transplanted EPCs in Ischemic Skeletal Muscle

Immunofluorescent examination of the gastrocnemius muscle was performed at 14 days after transplantation of GFP-expressing EPCs in HLI. GFP-positive cells frequently colocalized with NG2-positive pericytes, and under confocal microscopy some were stained with CD31 antibodies (Figures 3A,B). Some of GFP-positive cells were also present around the Arterioles but not incorporated into the arteriolar endothelial layer (Figure 3C). The number of GFP-positive $IP^{-/-}$ EPCs within skeletal tissues was apparently reduced in parallel with reduced angiogenesis (data not shown). In order to estimate the amount of homing GFP-expressing EPCs within the ischemic muscle tissue, the expression level of GFP RNA within muscles was measured. As shown in Figure 3D, the GFP-expression level in $IP^{-/-}$ EPCs-transplanted muscles was significantly reduced compared with that of WT EPCs.

Hydrogen peroxide ($H_2O_2$, up to 500 ¶mol/L) did not significantly affect the viability of EPCs (Figure 3E). However, in the presence of COX inhibitor, indomethacin (Indo), or IP-selective antagonist, RO1138452 (RO1138), the viability of EPCs was significantly attenuated by $H_2O_2$, suggesting that paracrine $PGI_2$ protects EPCs from oxidative stress. The antioxidative effect of $PGI_2$ would contribute to the survival and/or accumulation of EPCs in ischemic tissues in order to induce angiogenesis.

### Production of Angiogenic Cytokines From EPCs by $PGI_2$

The paracrine effect has been proposed as an important mechanism for EPC-mediated angiogenesis in HLI.[13,19,20] To determine whether $PGI_2$ stimulated the production of angiogenic

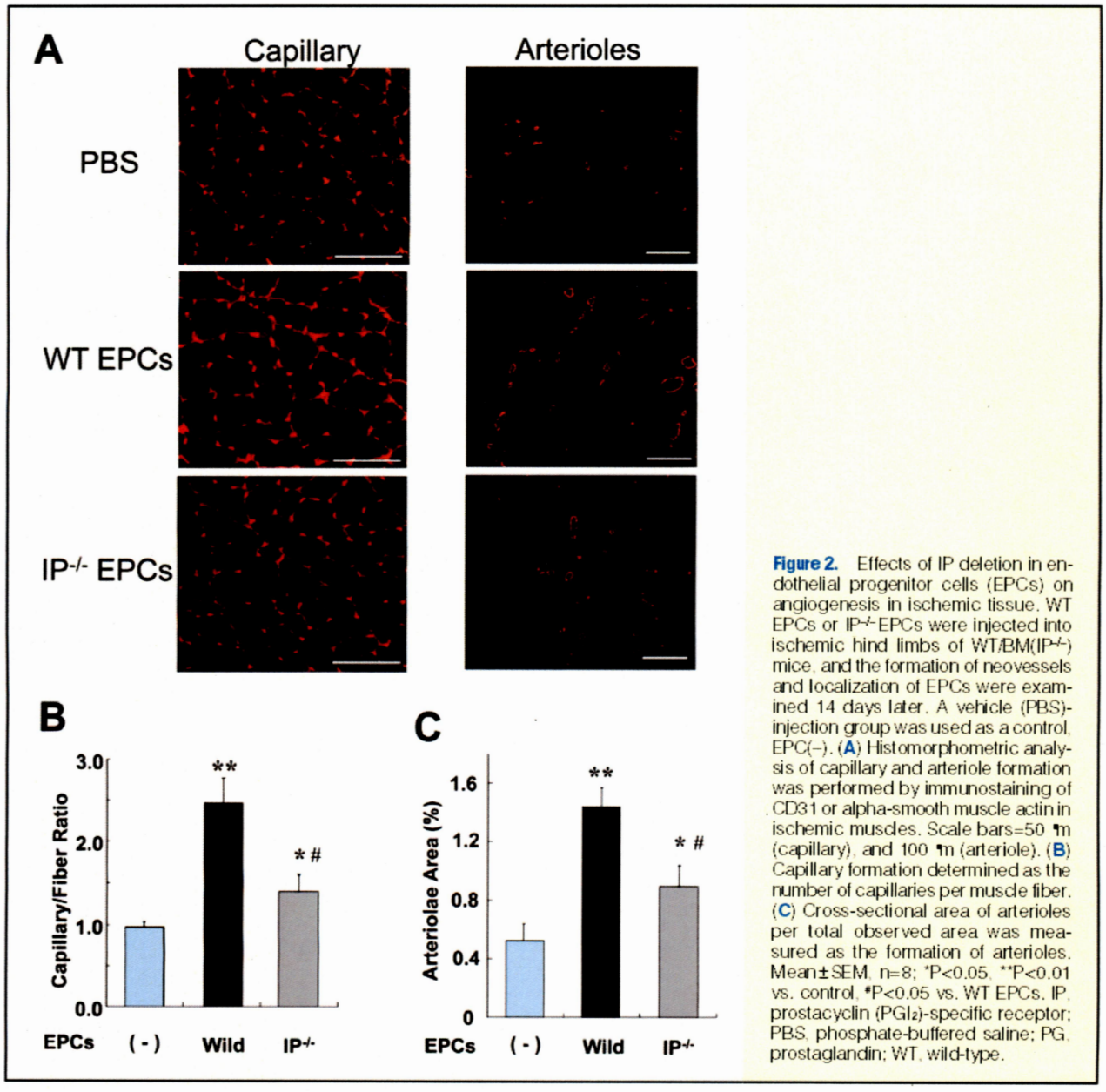


**Figure 2.** Effects of IP deletion in endothelial progenitor cells (EPCs) on angiogenesis in ischemic tissue. WT EPCs or IP$^{-/-}$ EPCs were injected into ischemic hind limbs of WT/BM(IP$^{-/-}$) mice, and the formation of neovessels and localization of EPCs were examined 14 days later. A vehicle (PBS)-injection group was used as a control, EPC(–). (**A**) Histomorphometric analysis of capillary and arteriole formation was performed by immunostaining of CD31 or alpha-smooth muscle actin in ischemic muscles. Scale bars=50 ¶m (capillary), and 100 ¶m (arteriole). (**B**) Capillary formation determined as the number of capillaries per muscle fiber. (**C**) Cross-sectional area of arterioles per total observed area was measured as the formation of arterioles. Mean±SEM, n=8; *P<0.05, **P<0.01 vs. control, #P<0.05 vs. WT EPCs. IP, prostacyclin ($PGI_2$)-specific receptor; PBS, phosphate-buffered saline; PG, prostaglandin; WT, wild-type.

cytokines by EPCs, we measured the levels of cytokines in a conditioned medium of EPC culture with and without beraprost. After confluent WT or IP$^{-/-}$ EPCs had been incubated in a serum-free medium for 1 day, the conditioned medium was used for an antibody array assay. Various angiogenic growth factors or cytokines, such as VEGF and granulocyte/macrophage colony-stimulating factor (GM-CSF), were produced by WT EPCs, and their production by EPCs was enhanced by beraprost. In IP$^{-/-}$ EPCs, the synthesis of these cytokines was clearly reduced and was not responsive to beraprost (**Table 1**).

### Effects of IP Deletion and IP Antagonist on EPC-Mediated Ex Vivo Angiogenesis

In order to clarify the mechanisms of the angiogenic effects of $PGI_2$ through the action of EPCs, we performed an ex vivo angiogenesis assay using aortic explants with GFP-expressing EPCs. Co-incubation with EPCs significantly enhanced angiogenesis, as assessed by the total length of sprouting microvessels (**Figures 4A,B**). The angiogenic effects of EPCs were significantly weaker in IP$^{-/-}$ EPCs than in WT EPCs (**Figures 4A,B**). We alttso examined the effects of RO1138 on EPC-mediated angiogenesis. In this experiment particularly, an IP$^{-/-}$ aortic ring was used to exclude the effects of an IP antagonist on aortic tissues. As shown in **Figures 4A,B**, RO1138 significantly decreased the effects of WT EPCs but did not alter the effects of IP$^{-/-}$ EPCs, which were already attenuated compared with WT EPCs. As shown in **Figures 4A,B**, the number of GFP-positive cells around perivascular sites increased in parallel with growing microvessels in WT EPCs. RO1138 decreased the number of EPCs in accordance with decreased angiogenesis. In the IP$^{-/-}$ EPCs, the number of GFP-positive EPCs was clearly less than that of WT EPCs, and an inhibitory effect of RO1138 was not observed.

We have previously demonstrated that EPCs produce sig-

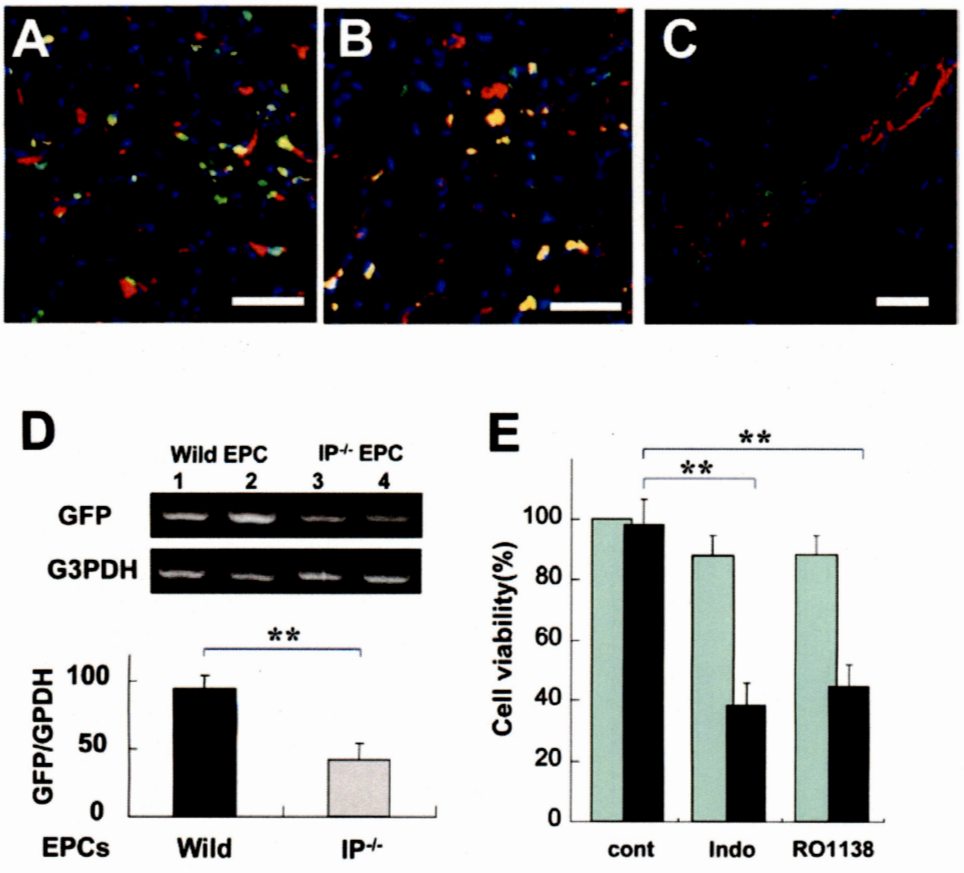


**Figure 3.** Localization of injected endothelial progenitor cells (EPCs) in ischemic muscle tissue. To estimate the localization of transplanted EPCs within the ischemic tissue, GFP-expressing EPCs were used. Transplanted EPCs (green) frequently colocalized with pericyte marker NG2 (**A**, arrows), and some cells stained as CD31+ endothelial cells (**B**, arrows). GFP-positive cells were also present around arterioles or collateral arteries, and the perivascular GFP-positive cells were in close contact with vascular walls but not in the CD31+ endothelial layer of the vessel (**C**, arrow). Nuclei were counterstained with Hoechst 33258 (blue). Scale bars=50 ¶m for **A** and **B** and 100 ¶m for **C**. (**D**) At 7 days after transplantation of GFP-expressing WT EPCs (lanes 1, 2) or $IP^{-/-}$ EPCs (lanes 3, 4) into ischemic skeletal muscles, the level of GFP RNA within muscle tissues was estimated by RT-PCR. The ratio of GFP to G3PDH was calculated. (**E**) EPCs pretreated with indomethacin (Indo; 10 ¶mol/L) or IP-selective inhibitor, RO1138452 (RO1138; 10 ¶mol/L) for 30 min, and exposed to hydrogen peroxide ($H_2O_2$ 500 ¶mol/L; closed bars) for 18 h. The cellular viability of EPCs was estimated by MTT reagents. Data are expressed as a percentage of those in the control non-treated group. Mean±SEM. n=6, **P<0.01). G3PDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; IP, prostacyclin ($PGI_2$)-specific receptor; PG, prostaglandin; RT-PCR, reverse transcription polymerase chain reaction; WT, wild-type.

nificant amounts of $PGI_2$ through COX1/2 activation.[17] In this study, the functions of EPCs were significantly reduced by either blockage of the $PGI_2$-IP signal or COX inhibition in EPCs (**Figures 3E,4B,S2**). These findings indicate that the cellular functions of EPCs are tightly regulated by auto/paracrine effects of $PGI_2$. As shown in **Figure 4C**, the production of $PGI_2$ by WT EPCs did not differ from that of $IP^{-/-}$ EPCs. Thus, the reduced effects of $IP^{-/-}$ EPCs may not be related to decreased production of $PGI_2$.

### Localization of EPCs Within Growing Neovessels

We observed the localization of EPCs in an ex vivo aortic ring angiogenesis system. Most of the GFP-expressing EPCs accumulated and were growing around endothelial sprouts (**Figure 5**). Some of these cells were attached to sprouting ECs, co-stained by a pericyte marker, NG2 (**Figure 5A**), and a few EPCs were incorporated into the microvasculature, stained as endothelial markers (ie, CD31+ cells; **Figure 5B**). Confocal microscopic analysis revealed that EPCs adhered around a tubular structure of vWF-positive ECs, forming a capillary-like structure (**Figure 5C**).

After GFP-expressing EPCs accumulated/adhered to growing microvessels, the EPCs were isolated for cell counts and quantitative polymerase chain reaction (PCR) analysis. In the presence of aortic rings, the proliferation of EPCs was enhanced in accordance with the growth of neovessels from the aortic rings. The proliferation of WT EPCs and that of $IP^{-/-}$ EPCs in the gel without aortic rings were not apparently different (data not shown). However, in the presence of aortic rings, the total number of $IP^{-/-}$ EPCs in the gel was significantly reduced compared with that of WT EPCs (**Table 2**), in parallel with their impaired angiogenic effects (**Figure 4B**). The levels of endothelial-related (VEGFR2, VE-cadherin and PDGFR ¶) and pericyte-related genes (NG2) in the EPCs were

**Table 1. Production of Angiogenic Cytokines by EPCs**

| | EPC | | |
|---|---|---|---|
| | WT | IP$^{-/-}$ | |
| Beraprost | + | − | + |
| L-selectin | 3.61 | 0.40 | 0.42 |
| IL-6 | 2.46 | 0.54 | 0.50 |
| IL-5 | 2.38 | 0.51 | 0.56 |
| VEGF | 2.05 | 0.75 | 0.82 |
| P-selectin | 1.72 | 0.34 | 0.22 |
| GM-CSF | 1.70 | 0.46 | 0.22 |
| M-CSF | 1.67 | 0.52 | 0.40 |
| TNF$\alpha$ | 1.67 | 0.87 | 0.92 |

Production of various angiogenic cytokines by EPCs was estimated by subquantitative array analysis. After WT or IP$^{-/-}$ EPCs were incubated for 1 day in a serum-free medium with or without beraprost (5nmol/L), the conditioned medium was applied for a cytokine antibody array. The value was expressed as a ratio to that of WT EPCs without beraprost.
EPCs, endothelial progenitor cells; GM-CSF, granulocyte/macrophage colony-stimulating factor; IL, interleukin; IP, prostacyclin ($PGI_2$)-specific receptor; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor; WT, wild-type.

increased by co-incubation with growing neovessels (Table 2). Interestingly, IP deletion in the EPCs did not affect the ratio of increase in these marker genes, whereas the total number of differentiated cells was significantly decreased (Table 2).

### $PGI_2$ Induces EPCs-Mediated Angiogenesis Through Integrins

We previously reported that expression levels of integrin family members such as 1, 3 and 5 were reduced in IP$^{-/-}$ EPCs and that adhesion to the extracellular matrix (ECM) and consequent proliferation of IP$^{-/-}$ EPCs on the ECM were decreased.[17] It has been reported that integrins mediate the homing or adhesion of EPCs to not only the ECM but also other living cells including ECs.[21] We therefore examined whether impairment of the angiogenic effects of IP$^{-/-}$ EPCs was related to defects in the expression of integrins. Expression of integrin 1 in EPCs was selectively reduced by silencing RNA (Figure 6A), and then these cells were applied to an ex vivo aortic ring angiogenesis assay. Knockdown of integrin 1 (Int 1-KD) in WT EPCs suppressed the adhesion of EPCs to sprouting microvessels and also their angiogenic effect (Figure 6B). As expected, in IP$^{-/-}$ EPCs in which the expression of integrin 1 was already decreased (Figure 6A), the reduced angiogenic effect was not further decreased by treatment with integrin 1 small-interfering RNA (siRNA) (Figure 6B).

We further examined the effects of integrin 1 siRNA on RBF in HLI. In order to estimate the amount of resident GFP-expressing EPCs within the ischemic muscle tissue, the expression level of GFP mRNA within the muscles was measured. The level of GFP-expression in Int 1-KD EPC-transplanted muscles was significantly reduced compared with control EPCs (Figure 6C). As shown in Figure 6D, Int 1-KD in WT EPCs, but not in IP$^{-/-}$ EPCs, significantly attenuated RBF in the HLI of IP$^{-/-}$ mice.

## Discussion

In the present study, we demonstrated that the $PGI_2$/IP system has different roles in BM and non-BM tissues in the improvement of ischemia by using a HLI model of marrow-specific IP-deletion chimera mice. The finding that a temporal insufficiency of RBF in IP-deleted non-BM tissues suggests that the $PGI_2$/IP system of the peripheral tissues has a role in the early stage of ischemic recovery. In contrast, deletion of IP specifically in BM induced persistent impairment of peripheral circulation recovery in HLI (Figure 1). This evidence suggests that the $PGI_2$/IP signal system of BM is required for persistent circulation recovery in peripheral ischemia. We demonstrated that the impairment of peripheral circulation recovery in WT/BM(IP$^{-/-}$) mice was completely rescued by implantation of WT EPCs, but was not completely rescued by implantation of IP$^{-/-}$ EPCs (Figure 1C). Therefore, these findings indicate that EPCs among BM cells might contribute to $PGI_2$/IP-mediated persistent improvement in ischemia.

Transplantation of EPCs stimulated not only the growth of capillaries but also the formation of arterioles in the ischemic hind limb, and these effects were dependent on the $PGI_2$/IP signal (Figure 2). The formation of matured vessels, such as arterioles, might contribute to the persistent angiogenic effects of $PGI_2$. Vascular maturation is complex and includes recruitment of pericytes around the immature endothelial tubes formed by either vasculogenesis or angiogenesis.[22] Although it is unclear how the $PGI_2$/IP signal mediates vessel maturation in addition to the formation of capillaries in ischemic tissues, our proposed effect of the $PGI_2$/IP signal on EPCs can explain in part the persistent improvement in ischemia by $PGI_2$.

Consistent with the results of previous studies,[13–15] transplanted GFP-expressing EPCs were frequently observed around neovessels or arterioles as NG-2-positive pericytes, but rarely as endothelial marker-positive cells within ischemic tissues (Figure 3). Similar to these observations in vivo, under the condition of co-incubation of EPCs with microvessels sprouting from aortic rings, the augmentation of EPCs at perivascular sites and the formation of a capillary-like vasculature were also attenuated in an IP-dependent manner (Figure 4). In general, the augmentation of EPCs is determined by the balance of gain (proliferation) and loss (apoptosis) of EPCs. Previously, we reported that $PGI_2$ stimulated the growth of WT EPCs, but not IP$^{-/-}$ EPCs, in a paracrine manner.[17] It is known that EPCs have more resistance to oxidative stress than vascular cells such as ECs.[23] $PGI_2$ acts against oxidative stress, and has anti-apoptotic effects in several cells including ECs.[24,25] We showed that $PGI_2$ protected EPCs from oxidative stress (Figure 3E) and mediated the angiogenic effects of EPCs (Figure S2) through its paracrine effects. Thus, autocrine $PGI_2$ contributes to the oxidative stress resistance of EPCs and may enhance the augmentation of EPCs within ischemic tissues through not only the EPCs' proliferative, but also their anti-oxidative stress effects.

We also found that the $PGI_2$/IP signal mediated the production of angiogenic growth factors and cytokines by EPCs (Table 1). Growth factors such as VEGF and basic fibroblast growth factor (bFGF) stimulate endothelial proliferation to induce angiogenesis, whereas growth factors stimulating proliferation of smooth muscle cells such as bFGF and GM-CSF also mediate arteriogenesis.[26] It is known that pericytes regulate the proliferation of adjacent ECs through paracrine effects and/or cell-to-cell adhesion signals and have crucial roles in vascular maturation, such as stability and enlargement of microvessels.[22,27] Thus, it is thought that the $PGI_2$/IP signal contributes to the formation of stable microvessels through not only the paracrine effects of EPCs but also the cellular adhesion of EPCs to neovessels as pericytes.

In our ex vivo angiogenesis assay, EPCs mostly accumu-

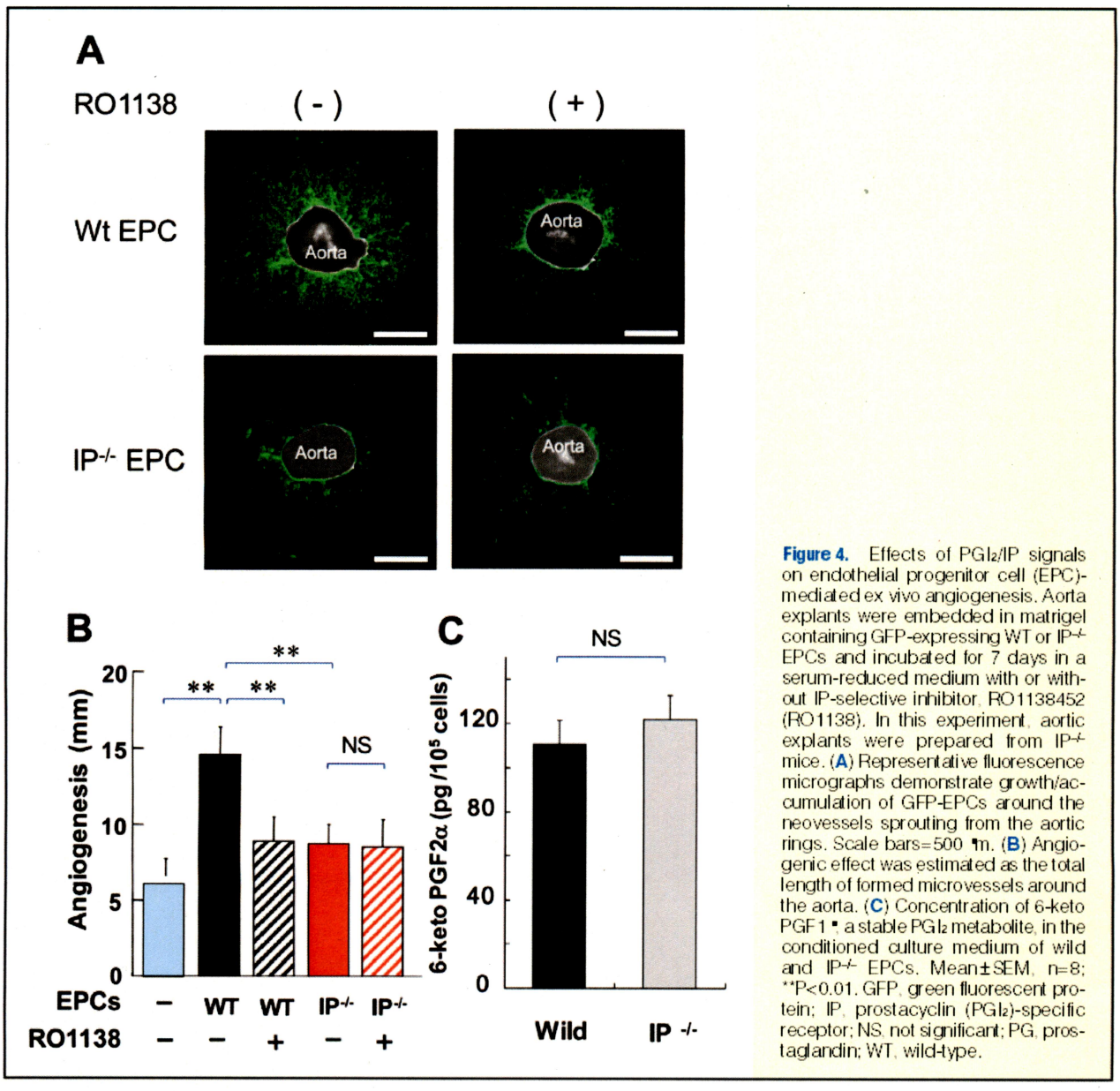


**Figure 4.** Effects of $PGI_2$/IP signals on endothelial progenitor cell (EPC)-mediated ex vivo angiogenesis. Aorta explants were embedded in matrigel containing GFP-expressing WT or $IP^{-/-}$ EPCs and incubated for 7 days in a serum-reduced medium with or without IP-selective inhibitor, RO1138452 (RO1138). In this experiment, aortic explants were prepared from $IP^{-/-}$ mice. (**A**) Representative fluorescence micrographs demonstrate growth/accumulation of GFP-EPCs around the neovessels sprouting from the aortic rings. Scale bars=500 ¶m. (**B**) Angiogenic effect was estimated as the total length of formed microvessels around the aorta. (**C**) Concentration of 6-keto PGF1 ¶, a stable $PGI_2$ metabolite, in the conditioned culture medium of wild and $IP^{-/-}$ EPCs. Mean±SEM, n=8; **P<0.01. GFP, green fluorescent protein; IP, prostacyclin ($PGI_2$)-specific receptor; NS, not significant; PG, prostaglandin; WT, wild-type.

lated around the growing microvessels and some were integrated into the microvessels as endothelial tubes (**Figure 5**). The accumulation of EPCs at perivascular sites was significantly attenuated by either deletion of IP in EPCs or an IP-selective inhibitor (**Figure 4**). Accordingly, the number of EPCs that differentiated into VE-cadherin-positive ECs or NG2-positive pericytes was also decreased by IP-deletion in EPCs (**Table 2**). However, $PGI_2$ might not directly contribute to the differentiation of EPCs into vascular cells, because the ratio of differentiated cells (ie, ratio of ECs or pericytes to total EPCs) was not different between WT and $IP^{-/-}$ EPCs (**Table 2**).

In the present study, we clearly demonstrated the role of integrin ¶1 in EPC-mediated effects, either in vitro angiogenesis or in vivo circulation recovery in HLI (**Figure 6**). However, integrin ¶1 might be just 1 of the adhesion molecules whose expression is regulated by $PGI_2$ and mediate EPC function. Integrins constitute a large family of ¶¶subunits heterodimeric cell surface proteins, and each ¶¶combination has its own binding specificity and signaling properties.[21] We previously reported that $PGI_2$ analogs enhance the expression of adhesion molecules, integrins such as ¶5, ¶1, ¶3 but not ¶4 in EPCs and thereby enhance their ECM-dependent proliferation and migration.[17] It is known that integrins such as ¶5, ¶1 and ¶2 are important for trafficking or homing of BMCs, including EPCs, to the endothelial layer.[21,28] Several subtypes of integrin mediate the interaction of pericytes with ECs during stabilization and maturation of neovessels.[29,30] It is known that the paracrine effects of EPCs also mediate the interaction of EPCs with neovessel ECs to form stable vessels. In particular, beraprost induced the production of L-selectin in WT EPCs but not in $IP^{-/-}$ EPCs (**Table 1**). L-selectin plays an important role in the initial events of vascular adhesion of inflammatory cells, mediating recruitment of leukocytes to the endothelial layer. L-selectin is expressed not only in leukocytes but also in other cells, including EPCs. It has been reported that the expression of L-selectin by EPCs functionally mediates the interaction

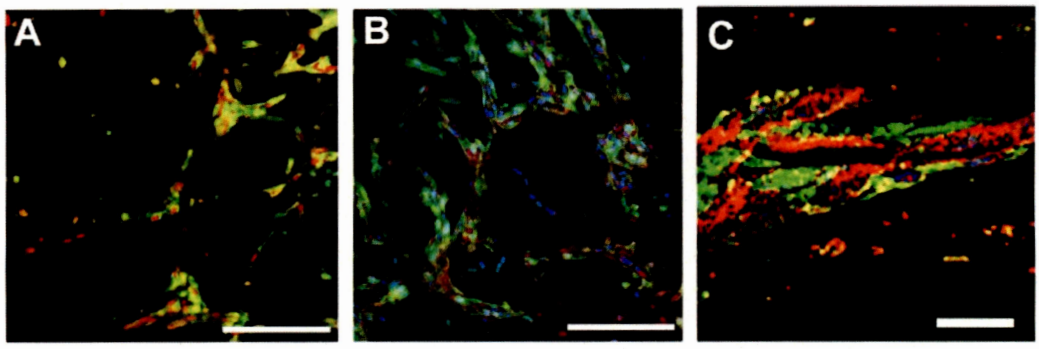


**Figure 5.** Localization of endothelial progenitor cells (EPCs) around neovessels sprouting from aortic explants. GFP-expressing EPCs were co-incubated with aortic rings in matrigel for 4–7 days and then immunostained. (**A**) Pericytes detected by staining with anti-NG2 antibody (red). GFP-EPCs (green) and NG2-positive pericytes adhere to sprouting vessels, and some of the EPCs are NG2-positive cells (yellow). (**B**) Endothelial cells (ECs) stained with anti-CD31 antibody (red). GFP-EPCs (green) accumulating on and adhering to sprouting endothelial tubes, but few have stained as $CD31^+$ cells. Nuclei counterstained with Hoechst 33258 (blue). (**C**) High-confocal immunostaining shows ECs stained with anti-vWF (red) forming a tubular structure, and EPCs (green) adhering to endothelial tubes. Scale bars=100 ¶m for **A** and **B**, and 20 ¶m for **C**. GFP, green fluorescent protein; IP, prostacyclin ($PGI_2$)-specific receptor; PG, prostaglandin.

**Table 2. Gene Typing in EPCs After Co-Incubation With Sprouting Microvessels**

| | EPC | | |
|---|---|---|---|
| | WT | IP$^{-/-}$ | |
| Aorta rings | + | − | + |
| No. of cells | 25.36±5.23 | 0.89±0.13 | 12.69±3.56 |
| IP | 1.55±0.51 | ND | ND |
| VEGFR2 (Kdr) | 3.02±0.62 | 1.07±0.26 | 3.41±0.67 |
| VE-cadherin | 4.73±1.44 | 0.83±0.24 | 5.07±1.25 |
| PDGFR$\beta$ | 12.20±4.81 | 1.60±0.37 | 10.91±2.81 |
| NG2 | 5.98±2.17 | 1.03±0.39 | 5.23±1.90 |

GFP-expressing EPCs ($5\times10^3$ cells) were incubated in matrigel with or without aortic rings. After 7 days of incubation, microvessels sprouted from aortic rings, and EPCs grew/accumulated around the neovessels. Complexes of microvessels and EPCs were separated from the gels and GFP-EPCs were selectively collected by fluorescence-activated cell sorting. The total number of EPCs in each gel was counted and gene expression in EPCs was evaluated by quantitative real-time PCR. Note that either WT EPCs or IP$^{-/-}$ EPCs alone did not proliferate in the gel without aortic rings, but grew in accordance with the formation of microvessels from the aortic ring. Total number of WT EPCs in each gel in the absence of aortic rings was $6.23+0.34\times10^3$. The value was expressed as a ratio to that of WT EPCs without aortic rings (n=4).
GFP, green fluorescent protein; ND, non-detected; PG, prostaglandin; PCR, polymerase chain reaction. Other abbreviations as in Table 1.

with ECs, contributing to the vascular homing of EPCs.[31] Therefore, it is thought that the $PGI_2$/IP signal promotes adhesion/proliferation of EPCs at perivascular sites through the expression of adhesion molecules such as integrins and finally contributes to the potent angiogenic effects of EPCs. In addition to integrin ¶1, other subtypes of integrins, and even other types of adhesion molecules, could also contribute to the $PGI_2$-mediated effects on EPCs, although the roles of those adhesion molecules have not been fully elucidated.

It has been reported that the effects of $PGI_2$ are mediated by the PPARs pathway in addition to the classical IP-cAMP signal pathway.[1,16] Angiogenic effects of $PGI_2$ are dependent on the activation of PPARs and are mediated by direct action on ECs or EPCs to induce endothelial tube formation and production of angiogenic factors.[32,33] Recently, He et al reported that $PGI_2$ stimulates endothelial-like EPCs to form endothelial tubes and capillaries in a PPAR ¶-dependent manner.[12] However, it has been recognized that EPC-induced vascular growth is mediated mainly by the paracrine effects of EPCs rather than by their incorporation into the neovascular endothelium.[13–15] Thus, the proposed mechanisms of endothelial-like EPCs that are prepared in long-term culture are still insufficient to explain $PGI_2$-mediated persistent circulation recovery, as well as the potent protective effects for cardiovascular diseases.[7] In this study, we have demonstrated for the first time the role of $PGI_2$/IP signal in the angiogenic effects of EPCs using in vivo HLI model of IP-deletion animal. In addition, we propose a new mechanism of $PGI_2$/IP signal-mediated angiogenesis, namely the accumulation of EPCs at perivascular sites in addition to their paracrine effects. This action would in part contribute to the prolonged and stable ischemic recovery effects of $PGI_2$ analogs and/or EPCs transplantation therapies.

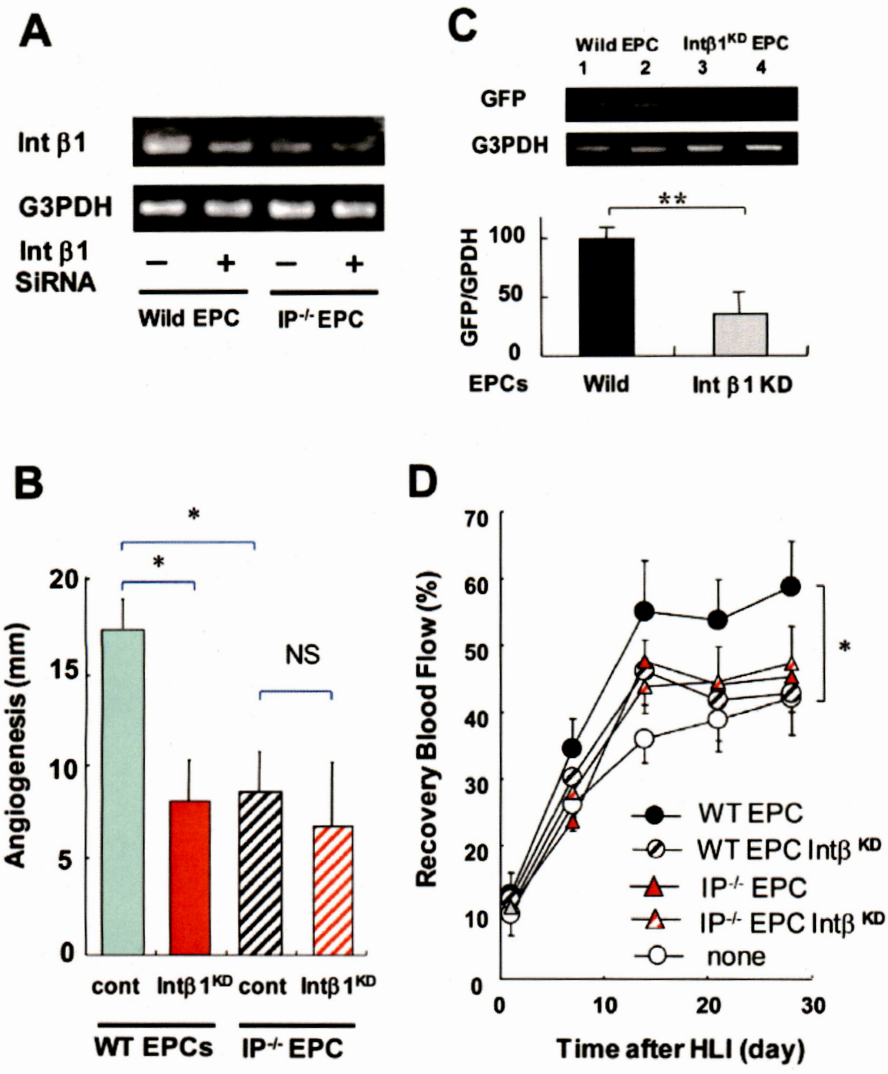


**Figure 6.** Effects of integrin β1 on endothelial progenitor cell (EPC)-mediated ex vivo and in vivo angiogenesis. Expression of integrin β1 in GFP-expressing EPCs was knocked down by integrin β1 small-interfering RNA (siRNA) (int β1-KD). Its scrambled siRNA was used as a control. (**A**) After 48 h of siRNA transfection, the expression level of integrin β1 mRNA was estimated by RT-PCR. G3PDH was used as the internal control. (**B**) Aortic ring ex vivo angiogenesis assay was performed under co-incubation with EPCs. The angiogenic effects of EPCs was estimated by the total length of sprouting vessels from the aortic ring. Mean±SEM, n=6–8; *P<0.05. (**C**) At 7 days after transplantation of GFP-expressing control EPCs (lanes 1, 2) or int β1-KD EPCs (lanes 3, 4) into ischemic skeletal muscle, the level of GFP RNA within muscle tissues was estimated by RT-PCR. The ratio of GFP to G3PDH was calculated. (**D**) WT EPCs and IP$^{-/-}$ EPCs were transfected with or without int β1 siRNA, then EPCs or PBS (none) were injected into the ischemic hind limbs of IP$^{-/-}$ mice, and reperfusion blood flow (RBF) was measured at the indicated times. Mean±SEM, n=10, *P<0.05. G3PDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; IP, prostacyclin ($PGI_2$)-specific receptor; NS, not significant; PBS, phosphate-buffered saline; PG, prostaglandin; RT-PCR, reverse transcription polymerase chain reaction; WT, wild-type.

## Conclusions

$PGI_2$ mediates the potent angiogenic effects of EPCs through the IP signal pathway in addition to the PPAR signal pathway, and may contribute to persistent recovery in ischemic tissues. For more effective EPC transplantation therapy against ischemic diseases, it is important to increase the functional quality of the EPCs in addition to their number. These findings suggest a new approach to improving the angiogenic effects of EPCs in cellular therapy.

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## Disclosures

Conflict of Interest: None declared.

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## Supplementary Files

### Supplementary File 1

**Data S1.** Methods

**Figure S1.** Level of 6-keto PGF1 $_{\alpha}$, stable PGI2 metabolite, in skeletal muscle tissues measured at 0 day (non-ischemia) and 7 days after surgical hind limb ischemia in wild-type and IP $^{-/-}$ mice.

**Figure S2.** Effects of COX inhibitor on endothelial progenitor cell (EPC)-mediated reperfusion blood flow (RBF) in hind limb ischemia model.

Please find supplementary file(s):
http://dx.doi.org/10.1253/circj.CJ-12-0897