Peroxisome proliferator-activated receptor-δ activates endothelial progenitor cells to induce angio-myogenesis through matrix metallo-proteinase-9-mediated insulin-like growth factor-1 paracrine networks

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Aims
The roles of peroxisome proliferator-activated receptor (PPAR)-δ in vascular biology are mainly unknown. We investigated the effects of PPAR-δ activation on the paracrine networks between endothelial progenitor cells (EPCs) and endothelial cells (ECs) and endothelial cells (ECs)/skeletal muscle.

Methods and results
Treatment of EPCs with GW501516, a PPAR-δ agonist, induced specifically matrix metallo-proteinase (MMP)-9 by direct transcriptional activation. Subsequently, this increased-MMP-9 broke down insulin-like growth factor-binding protein (IGFBP)-3, resulting in IGF-1 receptor (IGF-1R) activation in surrounding target cells. Treatment of conditioned medium from GW501516-stimulated EPCs enhanced the number and functions of human umbilical vein ECs and C2C12 myoblasts via MMP-9-mediated IGF-1R activation. Systemic administration of GW501516 in mice increased MMP-9 expression in EPCs, and augmented IGFBP-3 degradation in serum. In a mouse hindlimb ischaemia model, systemic treatment of GW501516 or local transplantation of GW501516-treated EPCs induced IGF-1R phosphorylation in ECs and skeletal muscle in the ischaemic limbs, leading to augmented angiogenesis and skeletal muscle regeneration. It also enhanced wound healing with increased angiogenesis in a mouse skin punch wound model. These pro-angiogenic and muscle-regenerating effects were abolished by MMP-9 knock-out.

Conclusion
Our results suggest that PPAR-δ is a crucial modulator of angio-myogenesis via the paracrine effects of EPCs, and its agonist is a good candidate as a therapeutic drug for patients with peripheral vascular diseases.

Keywords
Peroxisome proliferator-activated receptor-δ • Endothelial progenitor cell • Angio-myogenesis

Introduction
Peroxisome proliferator-activated receptors (PPARs) belong to the nuclear hormone receptor superfamily. Among the three subtypes of PPARs, PPAR-δ is the least understood. Peroxisome proliferator-activated receptor-δ has been shown to perform key roles in lipid homeostasis, carbohydrate metabolism, and energy expenditure. However, the roles of PPAR-δ in vascular biology remain largely unexplored.

We showed that PPAR-δ activation directly modulates ‘early’ endothelial progenitor cells (EPCs), and leads to enhanced

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'vasculogenesis'.

Another group showed the pro-vasculogenic effects of PPAR-δ on 'late' EPCs. Recent studies have demonstrated that PPAR-δ activation inhibits apoptosis of endothelial cells (ECs) and promotes 'angiogenesis' through direct effects on ECs. But in 'real-world' clinical settings, neovascularization alone does not suffice for the treatment of vascular diseases. For example, limb ischaemia is the complicated process where several components such as EPCs, ECs, and skeletal muscle should be involved. Naturally, we need to pay attention to the relationship among these components.

Insulin-like growth factor-1 (IGF-1) is a potent mitogen that induces angiogenesis and skeletal muscle regeneration. Six plasma IGF-1-binding proteins (IGFBPs) (IGFBP-1 to IGFBP-6) modulate the biological effects of IGF-1, which sequestrate IGF-1 and inhibit the interaction between IGF-1 and IGF-1 receptor (IGF-1R). Among IGFBPs, IGFBP-3 is the most abundant in human plasma. Proteolysis of IGFBPs by several proteases can release IGF-1 from the complex and activate IGF-IR.

Matrix metallo-proteinases (MMPs) are zinc-dependent proteinases, responsible for proteolytic processing of the extracellular matrix and associated with mechanisms of angiogenesis and cell migration. Matrix metallo-proteinases also cleave various regulatory molecules and activate growth factors and cytokines. Matrix metallo-proteinases-1,-2,-3,-7, and -9 have been known to degrade abundant secreted from 'early' EPCs. Among these, MMP-9 is known to be the most abundantly secreted from 'early' EPCs.

Based on the above facts, we supposed that the paracrine networks could exist between 'early' EPCs and ECs/skeletal myocytes linked through MMP-9-mediated IGFBP-3 degradation. In this study, we found that PPAR-δ activation significantly increased MMP-9 secretion from EPCs through direct transcriptional regulation. Subsequently, we assessed the potential in vitro effects of MMP-9 from PPAR-δ-activated EPCs on the IGF-1-signalling pathway in ECs and skeletal myocytes. Ultimately, we revealed its in vivo effects on angiogenesis and muscle regeneration.

**Methods**

**Early endothelial progenitor cells culture**

The protocol for use of human blood samples was approved by the Institutional Review Board at the Seoul National University Hospital. Early EPCs were isolated following our established protocol. Briefly, mononuclear cells (MNCs) were isolated from healthy volunteers' blood using Ficoll. 1 × 10⁶ MNCs/well were seeded on 2% fibronectin-coated plates and cultured using EGM-2MV BulletKit system (Clonetics) consisting of endothelial basal medium, 5% FBS, human EGF, VEGF, human FGF-B, IGF-1, ascorbic acid, and heparin in a 5% CO₂ incubator at 37°C. First media change was performed ~5 days after plating, and thereafter, media were changed every 3 days. In accordance with the status of cells, 7–10 days cultured early EPCs were used for experiments. Endothelial progenitor cell designates 'early' EPC hereafter.

**Statistical analysis**

All data are presented as the mean ± SEM. Student’s t-test or one-way ANOVA (post hoc analysis by Tukey) was performed for intergroup comparisons. Data taken at different time points were analysed by repeated-measures ANOVA (post hoc analysis by Tukey). SPSS version 16.0 was used for the analysis, and P < 0.05 was considered statistically significant.

Expanded methods are available in Supplementary material online.

**Results**

**Peroxisome proliferator-activated receptor-δ activation in endothelial progenitor cells specifically increases matrix metallo-proteinase-9 expression, secretion, and its activity**

We screened the mRNA change of several types of MMPs and tissue inhibitor of metallo-proteinases (TIMPs) by PPAR-δ activation in EPCs. Among these, only MMP-9 mRNA expression was significantly up-regulated in EPC cells after a 24 h-treatment with GW501516 (1 μM), a potent and highly specific PPAR-δ agonist. The mRNA expression of other MMPs (MMP-1,-2,-3,-7, and -14), TIMP-1, and TIMP-2 did not change on GW501516 treatment. The secretion of MMP-9 protein into culture medium was assessed by western blot assays using conditioned medium (CM). It demonstrated that GW501516 treatment (1 μM) significantly increased MMP-9 secretion from EPCs. The gelatinolytic activity by MMP-9 also increased after GW501516 treatment (1 μM). However, the secretion and gelatinolytic activity of MMP-2, which is another major MMP from EPCs, did not change. We also evaluated the effects of PPAR-δ activation on MMP-9 expression in the other cells constituting the important components in the environment of peripheral vascular disease, such as late EPCs, ECs, and C2C12 cells. C2C12 is a mouse myoblast cell line which differentiates rapidly, forms contractile myotubes, and produces characteristic muscle proteins. Intriguingly, in these other cells, there was no significant change in MMP-9 mRNA expression, its protein secretion into culture medium, and the enzymatic activity of secreted MMP-9 after GW501516 treatment (Supplementary material online, Figure S1). Accordingly, we focused on PPAR-δ-mediated MMP-9 regulation in EPCs thereafter.

**Peroxisome proliferator-activated receptor-δ activation in endothelial progenitor cells induces matrix metallo-proteinase-9 expression through direct transcriptional activation**

After activated by ligands, PPAR-δ, acting as a transcription factor, binds to the promoters of target genes that contain peroxisome proliferator-activated receptor response elements (PPREs). A PPRE is a direct repeat of the consensus sequence, AGGTCA, located at (-1972) 76.9%, (-245) 69.2% and (-74) 69.2% (homology to consensus sequence) (Supplementary material online, Figure S2). Choromatin immunoprecipitation assays using EPCs treated with GW501516 (1 μM) for 5 h verified...
in vivo binding of PPAR-δ to each three putative PPREs in a ligand-dependent manner (Figure 1D). To determine whether three putative PPREs actually function for MMP-9 promoter activity, luciferase promoter assays using sequential 5′-deletion mutations of the promoter were performed. GW501516 treatment (1 μM) significantly increased the full-length promoter activity. After sequential deletion of the putative PPREs, the PPAR-δ-mediated MMP-9 promoter activity decreased in order of precedence (Figure 1E). After deletion of all PPREs, the response to GW501516 treatment was totally abolished. These data suggested that PPAR-δ activation induced MMP-9 expression through direct transcriptional activation.
Peroxisome proliferator-activated receptor-δ activation in endothelial progenitor cells enhances matrix metallo-proteinase-9-mediated insulin-like growth factor-binding protein-3 proteolysis, leading to insulin-like growth factor-1R phosphorylation in target cells via a paracrine manner

To evaluate the possibility that the enhanced MMP-9 expression by PPAR-δ activation affects the IGF-1-signalling pathway, we determined whether MMP-9 in the CM from EPCs could degrade IGFBP-3 and led to activation of the IGF-1 receptor in surrounding target cells via a paracrine manner. Conditioned medium from EPCs treated for 72 h with GW501516 (1 μM) or vehicle was harvested and incubated with recombinant human (rh) IGFBP-3 (1 μg/mL). Conditioned medium from GW501516-treated EPCs more extensively degraded rhIGFBP-3 compared with that from vehicle-treated EPCs (Figure 2A). For more clarification of the effect of MMP-9 on IGFBP-3 degradation, MMP-9 was selectively collected from the CM of GW501516-treated EPCs by immunoprecipitation. The purified MMP-9 effectively degraded rhIGFBP-3 (Figure 2B).

After IGFBP-3 is degraded, IGF-1 is released from IGFBP-3 and may result in IGF-1R activation in the surrounding cells. To

**Figure 2** Matrix metallo-proteinase in the conditioned media from peroxisome proliferator-activated receptor-δ-activated endothelial progenitor cells degraded rhinsulin-like growth factor-binding protein-3, resulting in the activation of insulin-like growth factor-1R in endothelial cells. (A) Western blot assays for rhinsulin-like growth factor-binding protein-3 after incubation with the conditioned medium from GW501516-treated-(1 μM) (GW-CM) or vehicle-treated endothelial progenitor cells (vehicle-conditioned medium). The 46 kDa band indicates intact rhinsulin-like growth factor-binding protein-3, and the lower bands indicate its proteolytic fragments. (B) rhinsulin-like growth factor-binding protein-3 degradation by matrix metallo-proteinase-9 protein purified using immunoprecipitation from GW-CM. (C) Phosphorylated insulin-like growth factor-1R in human umbilical vein endothelial cells after GW-CM treatment. Total insulin-like growth factor-1R was used as an internal control. GM indicates GM6001. GM6001 reversed degradation of insulin-like growth factor-binding protein-3 and activation of insulin-like growth factor-1R, which were induced by peroxisome proliferator-activated receptor-δ activation, n = 3 in (A–C). All these immunoblot assays were performed in triplicate and each representative figure is shown.
examine this, IGF-1R tyrosine phosphorylation was assessed in the
target cells surrounding EPCs. We selected human umbilical vein
endothelial cells (HUVECs) as one of the representative target
cells. When the CM from GW501516-treated EPCs was treated
to HUVECs, tyrosine phosphorylation of IGF-1R in the HUVECs
was significantly increased (Figure 2C). Pretreatment with MMP
inhibitor, GM6001 (10 μM), inhibited rhIGFBP-3 degradation and
IGF-1R phosphorylation by PPAR-δ activation, demonstrating
their dependence on MMP. In contrast, CM of EPCs from
MMP-9 knock-out (KO) mice did not show the above effects of
IGFBP-3 degradation or IGF-1R activation by GW501516 treat-
ment (Figure 2A and C). It indicated MMP-9 specificity in the regu-
lation of IGF-1 signalling by PPAR-δ (Supplementary material
online, Figure S3).

Figure 3 Modulation of endothelial cells and skeletal myocytes by the conditioned medium from peroxisome proliferator-activated
receptor-δ-activated endothelial progenitor cells. (A) Quantitative graph of BrdU incorporation assay in human umbilical vein endothelial
cells. Representative figures and quantitative graphs of human umbilical vein endothelial cells migration (B) and tube formation (C). The
black line indicates the wound edge and the red line indicates the migrated cell margin (B). (D) Quantitative graph of BrdU incorporation
assay in C2C12 cells. (E) Quantitative graph of WST-1 assay under serum-starvation condition, n = 5 in (A), (D), and (E), and 3 in (B) and
(C). *P < 0.05 vs. Veh-CM, GW-CM + GM, and GW-CM + αIR3. Bars = 100 μm.
**Peroxisome proliferator-activated receptor-δ-activation in endothelial progenitor cells up-regulates the number and the angiogenic functions of endothelial cells through matrix metallo-proteinase-9-mediated insulin-like growth factor-1R activation**

Then, we evaluated the in vitro effects of MMP-9-mediated IGF-1R activation in surrounding ECs. Conditioned medium from GW501516 (1 μM)- or vehicle-treated EPCs was incubated with a mixture of IGF-1 and rIGFBP-3. Then we treated the resultant CM to HUVECs for the following bioassays. In a BrdU incorporation assay, CM from GW501516-treated EPCs significantly increased BrdU incorporation into HUVECs, indicating enhanced proliferation of ECs (Figure 3A). It also enhanced the angiogenic functions of ECs. It induced a strong migratory response of HUVECs in a scratch wound migration assay (Figure 3B) and augmented in vitro capillary formation by HUVECs in a Matrigel tube formation assay (Figure 3C). Pretreatment of the mixture with MMP inhibitor GP6601 (10 μM) or IGF-1R neutralizing antibody (αIR3) (1 μg/mL) reversed all these effects, showing their dependence on MMP and IGF-1R (Figure 3A–C).

**Peroxisome proliferator-activated receptor-δ-activated endothelial progenitor cells induce proliferation and enhance viability of skeletal myocytes**

As another important surrounding target cell, skeletal myocytes were selected because skeletal muscle regeneration is also an important component in peripheral vascular diseases. In a BrdU incorporation assay, CM from GW501516-treated EPCs increased the proliferation of C2C12 cells compared with that from vehicle-treated EPCs (Figure 3D). In a WST-1 assay performed under serum-starvation condition, it enhanced the survival of C2C12 cells (Figure 3E). These proliferating and pro-survival effects were also dependent on MMP and IGF-1R (Figure 3D and E).

GSK0660 (1 μM), a PPAR-δ antagonist, co-treatment did not affect the above results, showing that the effects of CM were not due to the residual PPAR-δ agonist (Supplementary material online, Figure S4A–E).

**Systemic administration of peroxisome proliferator-activated receptor-δ agonist increases matrix metallo-proteinase-9 production from endothelial progenitor cells in vivo and results in the enhanced serum insulin-like growth factor-binding protein-3 proteolysis in mice**

To investigate the in vivo effect of PPAR-δ agonist on MMP-9 in EPCs, GW501516 (10 mg/kg/day) was intraperitoneally (i.p.) injected to wild-type (WT) or MMP-9 KO mice. After 1 week of daily systemic administration, mouse EPCs were cultured from bone marrow (BM) MNCs and spleen MNCs were also isolated. A western blot assay showed augmented MMP-9 protein production in BM-derived EPCs and spleen MNCs of GW501516-treated WT mice (Figure 4A). No MMP-9 production was observed in MMP-9 KO mice regardless of GW501516 administration. Sera were isolated from the whole blood of the same mice. Immunoprecipitation and subsequent immunoblot assay showed the enhanced degradation of IGFBP-3 in the sera of GW501516-treated WT mice (Figure 4B). This effect was not observed in MMP-9 KO mice, showing its dependence on MMP-9 activity.

**Systemic administration of peroxisome proliferator-activated receptor-δ agonist activates the insulin-like growth factor-1R in endothelial cells and skeletal muscle of the mouse ischaemic hindlimb**

To determine the in vivo effects of PPAR-δ activation in EPCs on the IGF-1-signalling pathway of ECs and skeletal muscle, we performed a mouse hindlimb ischaemia model after bone marrow...
transplantation (BMT). Wild-type mice received BMT from WT or MMP-9 KO mice. After 4 weeks of engraftment, left femoral arteries of the recipient mice were excised. From 2 days before to 7 days after the operation, GW501516 (10 mg/kg/day) or vehicle was continuously infused through i.p. pump. To confirm the role of IGF-1-signalling pathway, IGFBP-3 (60 μg/kg/h) was infused together with GW501516 in some mice. An immunofluorescent staining of the ischaemic skeletal muscle on Day 7 showed significantly enhanced IGF-1R phosphorylation in ECs of capillaries in GW501516-treated WT/WT (donor/recipient) mice (Figure 5). Furthermore, GW501516-treated WT/WT mice (WT/WT/GW) showed stronger diffuse fluorescence over the skeletal muscle, showing that PPAR-α agonist also induced IGF-1R activation in skeletal muscle. In contrast, IGF-1R phosphorylation was not evident in both ECs and skeletal muscle regardless of GW501516 treatment in KO/WT mice, demonstrating that the phosphorylation was mainly dependent on MMP-9 from BM-derived cells. Insulin-like growth factor-binding protein-3 infusion inhibited the phosphorylation of IGF-1R in WT/WT mice receiving GW501516 treatment (WT/WT/GW + BP3).

**Systemic administration of peroxisome proliferator-activated receptor-α agonist increases regenerating myotubes in skeletal muscle of a mouse ischaemic hindlimb**

To assess the ultimate effects of PPAR-α activation in EPCs on in vivo angio-myogenesis, GW501516 (10 mg/kg/day) was systemically administered through peritoneum for 2 weeks in another mouse ischaemic hindlimb model with BMT from WT or MMP-9 KO mice. Subsequently to IGF-1R activation, skeletal muscle regeneration occurred. GW501516 treatment for 2 weeks increased the

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**Figure 5** Systemic administration of peroxisome proliferator-activated receptor-α agonist in a mouse hindlimb ischaemia model with bone marrow transplantation-induced insulin-like growth factor-1R phosphorylation in endothelial cells and skeletal muscle. Phosphorylated insulin-like growth factor-1R was labelled with anti-phospho-insulin-like growth factor-1R antibody (green) and endothelial cells were labelled with anti-CD31 antibody (red). Diffuse green fluorescence indicated insulin-like growth factor-1R phosphorylation in the skeletal muscle of ischaemic limbs. Bright green dots were co-localized with red fluorescence, indicating insulin-like growth factor-1R phosphorylation in endothelial cells. A greater number of the double positive cells were observed in WT/WT/GW (donor/recipient/treatment) mice. Insulin-like growth factor-binding protein-3 reversed the effects of GW501516, \( n = 4 \) in each group. Each representative figure is shown. Bars = 100 μm.
number of regenerating myotubes in ischaemic limbs of WT/WT mice, which have characteristic central nuclei and small caliber. This augmented muscle regeneration by PPAR-δ activation did not occur in KO/WT mice (Supplementary material online, Figure S5A and B). Interestingly, the total IGF-1R expression, including unphosphorylated and phosphorylated forms was mainly observed in regenerating myotubes, highlighting the importance of the IGF-1-signalling pathway in regenerating skeletal muscle (Supplementary material online, Figure S5C).

Systemic administration of peroxisome proliferator-activated receptor-δ agonist promotes angiogenesis and eventually protects skeletal muscle from ischaemic damage in a mouse ischaemic hindlimb

Serial examinations using laser Doppler perfusion imager revealed that the blood flow recovery more rapidly in the ischaemic limbs of GW501516-treated WT/WT mice. However, it was not improved by GW501516 administration in KO/WT mice (Supplementary material online, Figure SSD and E). The capillaries in the ischaemic muscle were identified by CD31 immunofluorescent staining, and then their number was normalized by skeletal myocytes. The capillary-to-myocyte ratio was significantly higher in GW501516-treated WT/WT mice than vehicle-treated mice. In KO/WT mice, fewer capillaries were observed regardless of GW501516 treatment (Supplementary material online, Figure SSF and G). The proliferation of ECs and skeletal myocytes by PPAR-δ activation in this model was verified by Ki-67 immunostaining (Supplementary material online, Figure S6A–D), whereas the decrease of their apoptosis was shown by TUNEL staining (Supplementary material online, Figure S6E–H). The neovascularization through this EPC paracrine networks potentiated by PPAR-δ was mainly angiogenesis, not arteriogenesis (Supplementary material online, Figure S7).

Enhanced muscle regeneration and increased angiogenesis by PPAR-δ agonist administration ultimately protected an ischaemic limb from hypoxic damage. In Masson’s trichrome stain, muscle architecture was preserved more intact and fibrosis was not observed in GW501516-treated WT/WT mice (Supplementary material online, Figure SSJ). Insulin-like growth factor-binding protein-3 infusion reversed the above effects of PPAR-δ agonist, demonstrating that IGF-1R activation was required for the angio-myogenic action of PPAR-δ activation (Supplementary material online, Figures SSA and B, D–H, SSA–H).

Transplantation of ex vivo peroxisome proliferator-activated receptor-δ-activated endothelial progenitor cells to ischaemic limb activates the insulin-like growth factor-1R, and induces angio-myogenesis

To confirm the specific role of EPCs in PPAR-δ-induced angiomyogenesis, PPAR-δ-activated EPCs were transplanted to ischaemic hindlimbs of athymic nude mice. Endothelial progenitor cells were isolated from WT or MMP-9 KO mice, then injected to ischaemic hindlimbs after 3 days ex vivo treatment of GW501516 (1 µM). Like systemic administration of GW501516, transplantation of GW501516-treated WT EPCs increased IGF-1R phosphorylation in ECs and skeletal muscle 1 week after the transplantation (Supplementary material online, Figure S8). It induced muscle regeneration, and enhanced limb perfusion and capillary density after 2 weeks (Supplementary material online, Figure S9A–F). This resulted in skeletal muscle preservation (Supplementary material online, Figure S9G). The above effects of ex vivo PPAR-δ activation were not observed in MMP-9 KO EPCs transplanted mice (Supplementary material online, Figure S9A–G).

Systemic administration of peroxisome proliferator-activated receptor-δ agonist promotes rapid wound healing with angiogenesis in a mouse skin punch wound model

To confirm the effects of PPAR-δ activation on angiogenesis, we used another in vivo model where angiogenesis plays a significant role, a mouse skin punch wound model. From 2 days before to 7 days after making 8-mm sized circular skin punch wounds in the back (n = 10 in each group), WT and MMP-9 KO mice received daily i.p. injection of GW501516 (10 mg/kg/day) or vehicle. GW501516 systemic administration induced rapid wound closure in WT mice (Supplementary material online, Figure S10A and B). It also increased MMP-9 protein expression in the skin wounds of WT mice (Supplementary material online, figure S1C). Immunofluorescent staining of wound sections showed that GW501516 injections significantly enhanced IGF-1R phosphorylation in capillary ECs (Supplementary material online, Figure S1D). In H&E staining, more capillaries containing red blood cells were observed in the skin wounds of WT mice that received GW501516, demonstrating enhanced angiogenesis (Supplementary material online, Figure S10E and F). All these effects were not observed in MMP-9 KO mice (Supplementary material online, Figure S10A–F).

Discussion

Neovascularization is generally classified as vasculogenesis by EPCs and angiogenesis by pre-existing ECs. In the previous study, we found that PPAR-δ directly regulates the number and functions of EPCs through the Akt pathway and induces vasculogenesis (Figure 6A). In contrast, in this study, we elucidated the role of PPAR-δ in the paracrine networks between EPCs and ECs or skeletal muscle cells, leading to angio-myogenesis (Figure 6B and C). Until now, the studies on EPCs have focused on neovascularization. Moreover, although the contribution of BM-derived cells to skeletal muscle regeneration was reported, the transdifferentiation of BM-derived stem cells into skeletal myocytes was suggested as a main mechanism. Our study demonstrated that EPCs or BM-derived circulating MNCs may home to ischaemic limb and play a pivotal role to regenerate skeletal muscle via a paracrine mechanism.

In the aspect of EPC biology, we and other investigators have demonstrated the heterogeneity of EPC that includes not only
Firstly reported by Asahara,23 but also ‘late’ EPCs15/endothelial outgrowth cells 24/endothelial colony-forming cells. 25 Recently, much attention is paid to paracrine effects rather than direct incorporation into endothelial monolayer to explain the mechanism of neovascularization by ‘early’ EPCs. In this regard, our study provides new evidence supporting the role of ‘early’ EPC as a key player in angio-myogenic paracrine networks.

Interestingly, though PPAR-δ is expressed also in ECs and skeletal muscle,3,5,26 the extent of MMP-9 up-regulation by PPAR-δ activation in ECs and C2C12 cells was negligible. Furthermore, the basal expression level of MMP-9 in these cell types was minimal compared with EPCs. Therefore, in the setting of EPC-mediated angio-myogenesis where EPCs, ECs, and skeletal myocytes take part, MMP-9-mediated IGF-1 pathway activation by PPAR-δ is considered to be specific to EPCs.

Figure 6 Peroxisome proliferator-activated receptor-δ agonist potentiates paracrine networks between endothelial progenitor cells and endothelial cells/skeletal muscle through matrix metallo-proteinase-9-mediated activation of insulin-like growth factor-1 pathway; augmented angiogenesis and skeletal muscle regeneration by peroxisome proliferator-activated receptor-δ activation. Peroxisome proliferator-activated receptor-δ activation enhances vasculogenesis by directly regulating endothelial progenitor cells through the activation of Akt pathway (A).2 Peroxisome proliferator-activated receptor-δ activation also induces matrix metallo-proteinase-9 secretion from endothelial progenitor cells, leading to insulin-like growth factor-binding protein-3 degradation in serum. Then, the free insulin-like growth factor-1 can activate insulin-like growth factor-1R in the surrounding endothelial cells and skeletal muscle. These potentiated paracrine networks induce angiogenesis (B) and muscle regeneration (C).
Peroxisome proliferator-activated receptor-\(\alpha\) activation is known to induce VEGF expression in ECs\(^{5}\) and colon carcinoma cells.\(^{27}\) In EPCs as shown in our experiments, however, PPAR-\(\alpha\) agonist treatment did not change the expression of angiogenic cytokines, such as IL-8 and VEGF (data not shown). We also evaluated the change of IGF-1 itself. Treatment of EPCs with PPAR-\(\alpha\) agonist did not change IGF-1 mRNA in vitro and systemic administration of PPAR-\(\alpha\) agonist did not elevate serum IGF-1 protein level in vivo. (Supplementary material online, Figure S11A and B). Therefore, the effects of PPAR-\(\alpha\) activation on paracrine networks of EPCs are mainly attributable to MMP-9. The secretion of MMP-9 is known to be induced by various cytokines, such as SDF-1.\(^{28}\) MMP-9 expression and activity in EPCs through direct regulation of PPAR-\(\alpha\) played a role of negative regulator in the PPAR-\(\alpha\) agonist action.\(^{29}\) However, the modulation of MMP-9 by PPAR-\(\alpha\) activation in EPCs has not been reported. In this study, we showed for the first time that PPAR-\(\alpha\) activation increased MMP-9 expression and activity in EPCs through direct regulation at the transcription level.

Interestingly, we found that IGF-1 receptors were predominantly expressed in regenerating skeletal myotubes. Although it is known that IGF-1 plays a crucial role in skeletal muscle regeneration,\(^{7}\) the difference in the pattern of expression of IGF-1R between mature myocytes and regenerating myotubes has not been reported. While much remains to be learned about the regulation of the IGF-1R expression in regenerating myotubes, our results highlight the significance of the IGF-1 pathway in skeletal muscle regeneration.

Recently a few studies demonstrated two different action mechanisms of IGFBP-3. Traditionally IGFBP-3 is a significant inhibitor of cell growth and enhancer of apoptosis via inhibition of the action of IGF-1. On the other hand, the direct positive effects of IGFBP-3 on vessel formation, independent of IGF-1/IGF-1R axis, take a growing interest.\(^{30}\) In order to clarify the controversy on the role and action mechanism of IGFBP-3 depending on the different situations, we adopted more physiological experimental design and treated HUVECs or C2C12 cells with both IGFBP-3 and IGF-1 in in vitro assays of this study. We believe that this experimental condition better reflects the in vivo physiological environment than IGFBP3 treatment alone, because \(\sim 98\%\) of IGF-1 is always bound to IGFBPs, and IGFBP-3 accounts for 75–90% of circulating IGF-1 binding.\(^{30}\) In our study, IGFBP-3 played a role of negative regulator in the PPAR-\(\alpha\) -induced angiomyogenesis through inhibition of IGF-1 signalling. Peroxisome proliferator-activated receptor-\(\alpha\) agonist treatment induced IGF-1R phosphorylation in vitro and in vivo experiments, and angiomyogenic effects of PPAR-\(\alpha\)/MMP-9-mediated IGFBP3 degradation were reversed by IGF-1R neutralizing antibody. These findings suggest that the mechanism of angiomyogenic effect of IGFBP3 degradation is mediated by IGF-1/IGF-1R axis in the environment of EPC-mediated angiomyogenesis.

In this study, we used two different in vivo models: ischaemic limb and skin wound. With these models, we confirmed the pro-angiomyogenic effects of the PPAR-\(\alpha\) agonist which was mediated by the paracrine networks between EPCs and ECs/skeletal muscle cells. Based on our results, we can reasonably expect a PPAR-\(\alpha\) agonist to possess therapeutic potential in peripheral vascular diseases, including diabetic foot, in which not only new vessel formation, but also skin wound healing and/or muscle regeneration is essential in the treatment.

PPAR-\(\alpha\) is widely expressed in various types of cells including ECs, skeletal myocytes, and keratinocytes. Therefore, systemic administration of PPAR-\(\alpha\) agonist may exert its direct effects on these cells and these effects may contribute to angiogenesis and skin wound healing. On the other hand, MMP-9-mediated IGF-1R activation may also occur in other types of cells on which we did not focus such as keratinocytes and smooth muscle cells. However, to reveal the effects of PPAR-\(\alpha\) or IGF-1R activation on every type of cells existing in our in vivo models was beyond the scope of this study and may require further research.

In conclusion, our data suggest that EPCs are crucial players in angiomyogenic paracrine networks with ECs and skeletal muscle through MMP-9-mediated IGF-1-signalling pathway, and PPAR-\(\alpha\) is a key molecule modulating the networks.

**Supplementary material**

Supplementary material is available at European Heart Journal online.

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