Table 1
Characteristics of circulating stem/progenitor cells

Circulating stem/progenitor cells [Ref.]	%OCN	%CD34+	%CXCR4	Osteogenic/vasculogenic potential
BMPCs (human) [66]	N/T	<1% (e/a)	N/T	*In vitro* osteogenic potential
Circulating OB lineage cells (human) [13,14,26,42]	>95% (f)	>30% (f)	>80%	*In vitro* and *in vivo* osteogenic potential *In vitro* vasculogenic potential
Circulating CD34+ cells/EPCs (human) [1,2,31,39,41]	20% (f)	>95% (f)	5%, >50% (f, e/a)	*In vitro* vasculogenic potential *In vitro* and *in vivo* osteogenic potential *In vitro* and *in vivo* vasculogenic potential
Circulating MOPCs (mouse) [45,46]	N/T	<1% (f/i)	>60% (f/i)	*In vitro* and *in vivo* osteogenic potential

BMPC, blood mesenchymal precursor cell; OB, osteoblast; MOPC, bone marrow-derived osteogenic progenitor cell; N/T, not tested; e/a, expanded adherent mononuclear cells; f, freshly isolated cells; f/i, freshly isolated cells with osteogenic induction.

prior to healing by mapping this migration following the systemic administration of PB Sca+Lin-Green Fluorescent Protein (GFP)+ cells into an animal. These findings indicate that fracture may induce the mobilization of EPCs from the BM to the fracture sites, by way of transport through the PB as a way to augment neovascularization and ultimately bone healing. As supportive data, in mouse fracture or destruction osteogenesis model, another groups reported the mobilization of EPCs for bone healing [31,33]. Moreover, recent reports have noted that there is a larger quantity of CD34+/AC133+ cells in the PB of patients with fracture [32].

Prior to our above-noted reports, CD34+ and CD133+ cells were reported to be capable of differentiating into osteoblasts [4,8,60] and CD34+ osteoblastic cells were noted by Ford et al. to line cartilage cavities around the site of a tibial osteotomy in a rabbit model [15], while intra-articularly injected human PB EPCs reported migrate to ischemic zones of nude rats undergoing distraction osteogenesis and participate in angiogenesis [7]. The latter phenomenon has been confirmed via Laser Doppler imaging to assess blood flow along the distraction zone as well as by labeling EPCs with a fluorescent dye, DiI, to track cell migration. Subsequent to these reports, other researchers have confirmed that PB EPCs contribute to fracture healing via vasculogenesis/angiogenesis and/or osteogenesis [4,35]. By one report, it appears that the *in vitro* expansion of autologous EPCs enhances the healing of critical-sized bone defects in sheep [35]. These findings all indicated that EPCs heal large bone defects, and can potentially heal non-unions and delayed unions in both large and small animals, and thereby pave the way toward the clinical use of these cells to enhance fracture repairs.

From a clinical standpoint, PB cells are appealing because they can be isolated in a relatively minimally invasive, safe, and efficacious fashion. This is advantageous over BM mesenchymal stem cells (MSCs), which have been used for bone healing with promising clinical results [18,48,49], yet can only be isolated via BM aspiration under anesthesia and is considered a form of surgical intervention. By contrast, PB cell aspiration does not require anesthesia. While we recognize that PB cells have advantages in minimally invasive, safe, and easy procedure to gain cells, we need further investigation comparing their efficacy for bone healing.

Circulating stem/osteoprogenitor cells

Blood mesenchymal precursor cells (BMPCs) have been a central focus in regenerative medicine for bone regeneration ever since these cells were first discovered to exist in the circulation of healthy patients. BMPCs were discovered by Zvaifler et al., who showed that these cells adhere to plastic and glass and proliferated logarithmically in DMEM-20% fetal calf serum without growth factors, thereby suggesting that these cells are relatively easy to expand *in vitro* [66]. Following the addition of osteogenic supplements (e.g., dexamethasone, ascorbic acid, and beta-glycerophosphate) into culture, there is inhibition of fibroblast formation, and BMPCs then assume a more cuboidal shape, that being of an osteoblasts as confirmed by stains with alkaline phosphatase (AP) and osteocalcin. While the reproducibility of these experiments has been limited, there have been recent reports focusing on peripherally circulating skeletal-lineage cells from humans in which Eghbali-Fatourechi et al. demonstrate via flow cytometry that cells which positively stain with osteocalcin and alkaline phosphatase immunohistostaining are indeed present in this lineage of cells [13,26]. This group further showed that circulating osteocalcin positive cells also express deposit mineral *in vitro* and bone *in vivo* in immunodeficient mice [13]. They further report that circulating osteocalcin positive cells are predominantly small, round cells [14] which makes them phenotypically similar to the cells originally isolated from the non-adherent bone marrow population by Long et al. [36].

Given the osteogenic potential of circulating BM cells, exposing these cells to osteogenic factors is a potent stimulus for bone formation. Otsuru et al. have recently reported that osteoblast progenitor cells in circulation originated from BM and form ectopic bone after implanting these cells with BMP-2-containing collagen pellet into the skeletal muscle beds of mice [45]. When these pellets were implanted into GFP-transgenic mice, there was a significant number of GFP-positive osteoblastic cells engrafting into the ectopic bone after circulatory migration to the osteogenic site. In addition, they demonstrated that PB MNCs from the BMP-2-implanted mouse contained cells which could differentiate into OBs *in vitro*. In a follow-up study, they proved that circulating BM-derived OB progenitor cells (MOPCs) were recruited to the bone-forming site by the CXCR4/stromal cell-derived factor-1 (SDF-1) pathway [46]. In this novel report, they demonstrated that the MOPCs, which expressed CD44 and CXCR4 – receptors of osteopontin and stromal cell-derived factor-1 (SDF-1), respectively – were mobilized from intact bones to transiently occupy approximately 80% of the MNC population in the circulating blood by BMP-2-pellet implantation. In addition, the MOPCs isolated from the mouse PB acted as capable OBs *in vitro* and *in vivo*. Furthermore, they proved that circulating MOPCs efficiently migrated to the region of osteogenesis by chemoattraction directed by SDF-1 expressed in vascular endothelial cells and the de novo osteoblasts of the region.

Interestingly, these two novel circulating cell populations which have an osteogenic potential show similar characteristics to circulating EPCs (Table 1). As mentioned above, approximately 40% of osteocalsin-positive skeletal progenitors co-express CD34 [14]. These findings are consistent with our single cell RT-PCR analysis showing that 20% of CD34+ cells co-express osteocalcin [39]. Although just comparing human and mouse cells may cause some controversies, circulating EPCs, similar to MOPCs, have shown to originate from BM [1,40,57] and the mobilization and incorporation of EPCs have proved to be regulated by CXCR4/SDF-1 pathway [55,61,63]. Of note, the CXCR4 expression of MOPCs was reported to reach to approximately 60% by BMP2-pellet implantation [45], and that of EPCs was to 50% by endothelial induction [46]. In addition, 82% of circulating osteocalcin positive cells were reported to express CXCR4 [42].The findings showing little CD34

expression of MOPCs in contrast to circulating EPCs [45] may caused by the differences of cell condition; MOPCs were isolated under osteogenic induction, but circulating EPCs were freshly isolated. In fact, there exist the reports showing disappearing CD34 expression due to culture condition, resulting in being adherent [19,21]. Taken together, these three circulating cell populations may at least partly overlap their fraction, which provide future clinical application for bone repair.

Summary and perspective

There is an expanding focus on circulating stem/progenitor cells due to their ease of isolation in a safe and efficacious manner, and their great potential for bone repair. Among them, we have investigated the potential of human PB CD34+ cells for bone healing and discussed their clinical feasibility. Our findings that PB CD34+ cells have a therapeutic potential via vasculogenesis/angiogenesis and osteogenesis for bone healing will provide an attractive strategy in cell-based therapy. Circulating EPCs, circulating osteoblast-lineage cells, and MOPCs are each isolated via different procedures, with yet exhibit similar cell characteristics and behaviors for bone healing, indicating that there is some functional and perhaps phenotypic overlap of these populations. Taken together, recent advances in circulating stem/progenitor cells provide new clinical applications to patients suffering from fracture non-unions and delayed unions. In the future, to expand on our knowledge and clinical application of these cells and their therapeutic effects, we hope that further in-depth investigations will focus on their inter-relationships and molecular mechanisms.

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References

[1] Asahara T, Masuda H, Takahashi T, Kalka C, Pastore C, Silver M, et al. Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. Circ Res 1999;85:221–8.
[2] Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, et al. Isolation of putative progenitor endothelial cells for angiogenesis. Science 1997;275:964–7.
[3] Assmus B, Schachinger V, Teupe C, Britten M, Lehmann R, Dobert N, et al. Transplantation of progenitor cells and regeneration enhancement in acute myocardial infarction (TOPCARE-AMI). Circulation 2002;106:3009–17.
[4] Bick T, Rozen N, Dreyfuss E, Soudry M, Lewinson D. Osteogenic differentiation of circulating endothelial progenitor cells. J Bone Min Res 2007;22:S143.
[5] Blau HM, Brazelton TR, Weimann JM. The evolving concept of a stem cell: entity or function? Cell 2001;105:829–41.
[6] Bouletreau PJ, Warren SM, Spector JA, Peled ZM, Gerrets RP, Greenwald JA, et al. Hypoxia and VEGF up-regulate BMP-2 mRNA and protein expression in microvascular endothelial cells: implications for fracture healing. Plast Reconstr Surg 2002;109:2384–97.
[7] Cetrulo Jr CL, Knox KR, Brown DJ, Ashinoff RL, Dobryansky M, Ceradini DJ, et al. Stem cells and distraction osteogenesis: endothelial progenitor cells home to the ischemic generate in activation and consolidation. Plast Reconstr Surg 2005;116:1053–64 discussion 1065–7.
[8] Chen JL, Hunt P, McElvain M, Black T, Kaufman S, Choi ES. Osteoblast precursor cells are found in CD34+ cells from human bone marrow. Stem Cells 1997;15:368–77.
[9] Colnot CI, Helms JA. A molecular analysis of matrix remodeling and angiogenesis during long bone development. Mech Dev 2001;100:245–50.
[10] Doherty MJ, Ashton BA, Walsh S, Beresford JN, Grant ME, Canfield AE. Vascular pericytes express osteogenic potential in vitro and in vivo. J Bone Miner Res 1998;13:828–38.
[11] Dominici M, Pritchard C, Garlits JE, Hofmann TJ, Persons DA, Horwitz EM. Hematopoietic cells and osteoblasts are derived from a common marrow progenitor after bone marrow transplantation. Proc Natl Acad Sci U S A 2004;101:11761–6.
[12] Douek DC, Vescio RA, Betts MR, Brenchley JM, Hill BJ, Zhang L, et al. Assessment of thymic output in adults after haematopoietic stem-cell transplantation and prediction of T-cell reconstitution. Lancet 2000;355:1875–81.
[13] Eghbali-Fatourechi GZ, Lamsam J, Fraser D, Nagel D, Riggs BL, Khosla S. Circulating osteoblast-lineage cells in humans. N Engl J Med 2005;352:1959–66.
[14] Eghbali-Fatourechi GZ, Modder UI, Charatcharoenwitthaya N, Sanyal A, Undale AH, Clowes JA, et al. Characterization of circulating osteoblast lineage cells in humans. Bone 2007;40:1370–7.
[15] Ford JL, Robinson DE, Scammell BE. Endochondral ossification in fracture callus during long bone repair: the localisation of 'cavity-lining cells' within the cartilage. J Orthop Res 2004;22:368–75.
[16] Gerstenfeld LC, Cullinane DM, Barnes GL, Graves DT, Einhorn TA. Fracture healing as a post-natal developmental process: molecular, spatial, and temporal aspects of its regulation. J Cell Biochem 2003;88:873–84.
[17] Goodell MA, Rosenzweig M, Kim H, Marks DF, DeMaria M, Paradis G, et al. Dye efflux studies suggest that hematopoietic stem cells expressing low or undetectable levels of CD34 antigen exist in multiple species. Nat Med 1997;3:1337–45.
[18] Horwitz EM, Prockop DJ, Fitzpatrick LA, Koo WW, Gordon PL, Neel M, et al. Transplantability and therapeutic effects of bone marrow-derived mesenchymal cells in children with osteogenesis imperfecta. Nat Med 1999;5:309–13.
[19] Howson KM, Aplin AC, Gelati M, Alessandri G, Parati EA, Nicosia RF. The postnatal rat aorta contains pericyte progenitor cells that form spheroidal colonies in suspension culture. Am J Physiol Cell Physiol 2005;289:C1396–1407.
[20] Iwasaki H, Kawamoto A, Ishikawa M, Oyamada A, Nakamori S, Nishimura H, et al. Dose-dependent contribution of CD34-positive cell transplantation to concurrent vasculogenesis and cardiomyogenesis for functional regenerative recovery after myocardial infarction. Circulation 2006;113:1311–25.
[21] Kaiser S, Hackanson B, Follo M, Mehlhorn A, Geiger K, Ihorst G, et al. BM cells giving rise to MSC in culture have a heterogeneous CD34 and CD45 phenotype. Cytotherapy 2007;9:439–50.
[22] Kalka C, Masuda H, Takahashi T, Kalka-Moll WM, Silver M, Kearney M, et al. Transplantation of ex vivo expanded endothelial progenitor cells for therapeutic neovascularization. Proc Natl Acad Sci U S A 2000;97:3422–7.
[23] Karsenty G, Wagner EF. Reaching a genetic and molecular understanding of skeletal development. Dev Cell 2002;2:389–406.
[24] Kawamoto A, Gwon HC, Iwaguro H, Yamaguchi JI, Uchida S, Masuda H, et al. Therapeutic potential of ex vivo expanded endothelial progenitor cells for myocardial ischemia. Circulation 2001;103:634–7.
[25] Kawamoto A, Tkebuchava T, Yamaguchi J, Nishimura H, Yoon YS, Milliken C, et al. Intramyocardial transplantation of autologous endothelial progenitor cells for therapeutic neovascularization of myocardial ischemia. Circulation 2003;107:461–8.
[26] Khosla S, Eghbali-Fatourechi GZ. Circulating cells with osteogenic potential. Ann N Y Acad Sci 2006;1068:489–97.
[27] Kocher AA, Schuster MD, Szabolcs MJ, Takuma S, Burkhoff D, Wang J, et al. Neovascularization of ischemic myocardium by human bone-marrow-derived angioblasts prevents cardiomyocyte apoptosis, reduces remodeling and improves cardiac function. Nat Med 2001;7:430–6.
[28] Kollet O, Shivtiel S, Chen YQ, Suriawinata J, Thung SN, Dabeva MD, et al. HGF, SDF-1, and MMP-9 are involved in stress-induced human CD34+ stem cell recruitment to the liver. J Clin Invest 2003;112:160–9.
[29] Korbling M, Estrov Z. Adult stem cells for tissue repair — a new therapeutic concept? N Engl J Med 2003;349:570–82.
[30] Kudo FA, Nishibe T, Nishibe M, Yasuda K. Autologous transplantation of peripheral blood endothelial progenitor cells (CD34+) for therapeutic angiogenesis in patients with critical limb ischemia. Int Angiol 2003;22:344–8.
[31] Laing AJ, Dillon JP, Condon ET, Coffey JC, Street JT, Wang JH, et al. A systemic provascular response in bone marrow to musculoskeletal trauma in mice. J Bone Joint Surg Br 2007;89:116–20.
[32] Laing AJ, Dillon JP, Condon ET, Street JT, Wang JH, McGuinness AJ, et al. Mobilization of endothelial precursor cells: systemic vascular response to musculoskeletal trauma. J Orthop Res 2007;25:44–50.
[33] Lee DY, Cho TJ, Kim JA, Lee HR, Yoo WJ, Chung CY, et al. Mobilization of endothelial progenitor cells in fracture healing and distraction osteogenesis. Bone 2008;42:932–41.
[34] Lemoli RM, Catani L, Talarico S, Loggi E, Gramenzi A, Baccarani U, et al. Mobilization of bone marrow-derived hematopoietic and endothelial stem cells after orthotopic liver transplantation and liver resection. Stem Cells 2006;24:2817–25.
[35] Lewinson D, Rozen N, DBick T, Shemian B, Yayon A, Soudry M. Healing of critical-sized bone defects by endothelial progenitor cells. J Bone Min Res 2007;22:S234.
[36] Long MW, Williams JL, Mann KG. Expression of human bone-related proteins in the hematopoietic microenvironment. J Clin Invest 1990;86:1387–95.
[37] Losordo DW, Schatz RA, White CJ, Udelson JE, Veereshwarayya V, Durgin M, et al. Intramyocardial transplantation of autologous CD34+ stem cells for intractable angina: a phase I/IIa double-blind, randomized controlled trial. Circulation 2007;115:3165–72.
[38] Marsh D. Concepts of fracture union, delayed union, and nonunion. Clin Orthop Relat Res 1998:S22–30.
[39] Matsumoto T, Kawamoto A, Kuroda R, Ishikawa M, Mifune Y, Iwasaki H, et al. Therapeutic potential of vasculogenesis and osteogenesis promoted by peripheral blood CD34-positive cells for functional bone healing. Am J Pathol 2006;169:1440–57.
[40] Matsumoto T, Mifune Y, Kawamoto A, Kuroda R, Shoji T, Iwasaki H, et al. Fracture induced mobilization and incorporation of bone marrow-derived endothelial progenitor cells for bone healing. J Cell Physiol 2008;215:234–42.
[41] Mifune Y, Matsumoto T, Kawamoto A, Kuroda R, Shoji T, Iwasaki H, et al. Local delivery of granulocyte colony stimulating factor-mobilized CD34-positive progenitor cells using bioscaffold for modality of unhealing bone fracture. Stem Cells 2008 [Epub ahead of print].
[42] Moedder U, Khosla S. Non-adherent circulating progenitor cells with osteoblastic potential become adherent in the presence of calcium and are able to mineralize. J Bone Miner Res 2007;22:S241.
[43] Murohara T, Ikeda H, Duan J, Shintani S, Sasaki K, Eguchi H, et al. Transplanted cord blood-derived endothelial precursor cells augment postnatal neovascularization. J Clin Invest 2000;105:1527–36.

[44] Olmsted-Davis EA, Gugala Z, Camargo F, Gannon FH, Jackson K, Kienstra KA, et al. Primitive adult hematopoietic stem cells can function as osteoblast precursors. Proc Natl Acad Sci U S A 2003;100:15877–82.
[45] Otsuru S, Tamai K, Yamazaki T, Yoshikawa H, Kaneda Y. Bone marrow-derived osteoblast progenitor cells in circulating blood contribute to ectopic bone formation in mice. Biochem Biophys Res Commun 2007;354:453–8.
[46] Otsuru S, Tamai K, Yamazaki T, Yoshikawa H, Kaneda Y. Circulating bone marrow-derived osteoblast progenitor cells are recruited to the bone-forming site by the CXCR4/stromal cell-derived factor-1 pathway. Stem Cells 2008;26:223–34.
[47] Pardanaud L, Yassine F, Dieterlen-Lievre F. Relationship between vasculogenesis, angiogenesis and haemopoiesis during avian ontogeny. Development 1989;105:473–85.
[48] Petite H, Viateau V, Bensaid W, Meunier A, de Pollak C, Bourguignon M, et al. Tissue-engineered bone regeneration. Nat Biotechnol 2000;18:959–63.
[49] Quarto R, Mastrogiacomo M, Cancedda R, Kutepov SM, Mukhachev V, Lavroukov A, et al. Repair of large bone defects with the use of autologous bone marrow stromal cells. N Engl J Med 2001;344:385–6.
[50] Risau W, Sariola H, Zerwes HG, Sasse J, Ekblom P, Kemler R, et al. Vasculogenesis and angiogenesis in embryonic-stem-cell-derived embryoid bodies. Development 1988;102:471–8.
[51] Rodriguez-Merchan EC, Forriol F. Nonunion: general principles and experimental data. Clin Orthop Relat Res 2004:4–12.
[52] Simmons PJ, Torok-Storb B. Identification of stromal cell precursors in human bone marrow by a novel monoclonal antibody, STRO-1. Blood 1991;78:55–62.
[53] Sivan-Loukianova E, Awad OA, Stepanovic V, Bickenbach J, Schatteman GC. CD34+ blood cells accelerate vascularization and healing of diabetic mouse skin wounds. J Vasc Res 2003;40:368–77.
[54] Slack JM. Stem cells in epithelial tissues. Science 2000;287:1431–3.
[55] Smadja DM, Bieche I, Uzan G, Bompais H, Muller L, Boisson-Vidal C, et al. PAR-1 activation on human late endothelial progenitor cells enhances angiogenesis in vitro with upregulation of the SDF-1/CXCR4 system. Arterioscler Thromb Vasc Biol 2005;25:2321–7.
[56] Taguchi A, Soma T, Tanaka H, Kanda T, Nishimura H, Yoshikawa H, et al. Administration of CD34+ cells after stroke enhances neurogenesis via angiogenesis in a mouse model. J Clin Invest 2004;114:330–8.
[57] Takahashi T, Kalka C, Masuda H, Chen D, Silver M, Kearney M, et al. Ischemia- and cytokine-induced mobilization of bone marrow-derived endothelial progenitor cells for neovascularization. Nat Med 1999;5:434–8.
[58] Tei K, Matsumoto T, Mifune Y, Ishida K, Sasaki K, Shoji T, et al. Administrations of Peripheral Blood CD34-positive Cells Contribute to Medial Collateral Ligament Healing via Vasculogenesis. Stem Cells 2008;26:819–30.
[59] Terai S, Ishikawa T, Omori K, Aoyama K, Marumoto Y, Urata Y, et al. Improved liver function in patients with liver cirrhosis after autologous bone marrow cell infusion therapy. Stem Cells 2006;24:2292–8.
[60] Tondreau T, Meuleman N, Delforge A, Dejeneffe M, Leroy R, Massy M, et al. Mesenchymal stem cells derived from CD133-positive cells in mobilized peripheral blood and cord blood: proliferation, Oct4 expression, and plasticity. Stem Cells 2005;23:1105–12.
[61] Walter DH, Haendeler J, Reinhold J, Rochwalsky U, Seeger F, Honold J, et al. Impaired CXCR4 signaling contributes to the reduced neovascularization capacity of endothelial progenitor cells from patients with coronary artery disease. Circ Res 2005;97:1142–51.
[62] Weissman IL. Stem cells: units of development, units of regeneration, and units in evolution. Cell 2000;100:157–68.
[63] Yamaguchi J, Kusano KF, Masuo O, Kawamoto A, Silver M, Murasawa S, et al. Stromal cell-derived factor-1 effects on ex vivo expanded endothelial progenitor cell recruitment for ischemic neovascularization. Circulation 2003;107:1322–8.
[64] Yeh ET, Zhang S, Wu HD, Korbling M, Willerson JT, Estrov Z. Transdifferentiation of human peripheral blood CD34+-enriched cell population into cardiomyocytes, endothelial cells, and smooth muscle cells in vivo. Circulation 2003;108:2070–3.
[65] Zengin E, Chalajour F, Gehling UM, Ito WD, Treede H, Lauke H, et al. Vascular wall resident progenitor cells: a source for postnatal vasculogenesis. Development 2006;133:1543–51.
[66] Zvaifler NJ, Marinova-Mutafchieva L, Adams G, Edwards CJ, Moss J, Burger JA, et al. Mesenchymal precursor cells in the blood of normal individuals. Arthritis Res 2000;2:477–88.

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Original article

Lesion-targeted thrombopoietin potentiates vasculogenesis by enhancing motility and enlivenment of transplanted endothelial progenitor cells via activation of Akt/mTOR/p70S6kinase signaling pathway

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ABSTRACT

Thrombopoietin (TPO), a physiological regulator of megakaryocyte and platelet development, is a multifunctional positive regulator in early hematopoiesis by hematopoietic stem cells. In this study, we investigated the effect of TPO on endothelial progenitor cells (EPCs) for therapeutic vasculogenesis *in vitro* and *in vivo*, and the intracellular signaling mechanism exerting the activity of EPCs. 7-day culture-expanded EPCs derived from human peripheral blood mononuclear cells were applied to each assay. Flow cytometry demonstrated the expression of c-Mpl, the receptor of TPO, in cultured EPCs. *In vitro* experiments revealed enhanced migration and survival of cultured EPCs by TPO. *In vivo*, TPO was intramuscularly administered into the foci of ischemic hindlimbs in athymic nude mice, immediately followed by intravenous injection of cultured EPCs, to assess the booster effect of TPO on vascular regeneration. At day 4 post-transplantation, transplanted EPCs were 1.7-fold higher in TPO-treated animals compared to control. At day 28, blood perfusion was recovered in the TPO-treated group, accompanied by an increase in microvascular density. The signaling transduction pathway underlying TPO-mediated activities of cultured EPCs was assessed by Western blotting. TPO induced sequential phosphorylations of Akt to p70S6kinase through mTOR. Inhibition of the PI3-kinase/Akt/mTOR/p70S6kinase signaling pathway negated the biological functions of cultured EPCs, either migration (by LY294002 for PI3-kinase and Rapamycin for mTOR) or survival and tubulogenesis (by Rapamycin). These findings provide evidence that TPO possesses booster potential for therapeutic vasculogenesis, by activating the PI3-kinase/Akt/mTOR/p70S6kinase pathway crucial to the biological activities of EPCs.

1. Introduction

Postnatal vasculogenesis, contributed by bone marrow (BM) derived endothelial progenitor cells (EPCs), has been recognized as one of the key events in physiological or pathological neovessel formation, concurrent with angiogenesis by resident endothelial cells (ECs) [1]. Based on a new dogma of blood vessel formation in postnatal life, "therapeutic vasculogenesis" targeting ischemic diseases has been recently developed, consisting of transplantation of total mononuclear cells from bone marrow, cultured EPCs, or autologous EPC fractioned in G-CSF-mobilized CD34+ cells [2,3]. However, the scarcity of original EPCs and the impairment of biological functions in patients suffering from risk factors of hyperlipidemia, hypertension, cigarette smoking, diabetes, aging, etc. [4] limits the efficacy of EPC transplantation on vasculogenesis in ischemic diseases. Therefore, methods of improving the quality and quantity of EPCs are desired for therapeutic purposes. Recently, several hematological regulators such as erythropoietin [5], stromal cell derived factor-1 (SDF-1) [6], granulocyte-colony stimulating factor (G-CSF), and estrogen have been described as versatile agents affecting the biological activities of endothelial lineage cells, including EPCs.

Thrombopoietin (TPO) is a hepatocyte-derived growth factor consisting of a 332 amino acid residue polypeptide which serves as a ligand for the TPO receptor (TPO-R; c-Mpl; CD110), which stimulates the development of human megakaryocyte (HuMK) progenitors and increases circulating platelets. Furthermore, TPO has recently been shown to play a pivotal role in hematopoietic stem/progenitor cells as well. Murine and human hematopoietic stem/progenitor cells have been shown to highly express c-Mpl [7], in which TPO stimulates VEGF expression in an autocrine manner to promote self-

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renewability [8]. Also, various studies have already revealed the ability of TPO to enhance the survival of hematopoietic stem/progenitor cells [9]. On the other hand, patients with unstable angina show higher serum levels of TPO as compared to healthy subjects or patients with stable angina [10], along with other heamatopoietic and angiogenic regulators elevated in patients with acute myocardial infarction, such as VEGF, angiopoietins and erythropoietin [11], or G-CSF [12]. Given such accumulated findings, TPO is intriguingly hypothesized to possess a preferential effect on EPC bioactivity, leading to a booster effect when co-administered in EPC transplantation.

Numerous studies in diverse cell types have demonstrated that TPO gives rise to biological functions of motility, survival, proliferation etc., through the orchestration of intracellular signaling pathways p44MAPK, JAK-STAT, or PI3K/Akt [13]. The Akt pathway has been revealed to be a key mediator for vasculogenic functions, such as migration, survival, differentiation, and proliferation in EPCs as well as ECs, concomitantly with phosphorylation of eNOS [14]. The mTOR (mammalian target of rapamycin)/p70S6kinase pathway, as the downstream effector of Akt activation, has also been indicated to regulate various biological functions of ECs for angiogenesis [15]. TPO is speculated to augment vasculogenic functions of EPC through the activation of the Akt/mTOR/p70S6kinase pathway. TPO has also been indicated to have angiogenic potential, not only by activating migration and tubulogenesis of human umbilical vein ECs (HUVECs) expressing c-Mpl *in vitro*, via the JAK-STAT pathway, but also by enhancing neovessel formation *in vivo* [16]. However, the association of PI3K/Akt and/or mTOR/p70S6kinase with TPO in an endothelial lineage for activating angio/vasculogenesis has not been evidenced as yet. Furthermore, the importance of sequential activation of the PI3K/Akt/mTOR/p70S6kinase pathway for EPCs remains to be elucidated, despite evidence that the mTOR/p70S6kinase pathway essential for producing EPCs from circulating ancestral CD133+ stem cells [17].

In the present study, we not only investigated the capability of TPO as a preferential mediator to ameliorate the efficacy of EPC transplantation for therapeutic vasculogenesis, but also assessed the intracellular mechanisms underlying the activities of EPCs.

2. Materials and methods

2.1. Materials

The following materials and antibodies were used: recombinant human TPO (KIRIN, JAPAN), recombinant human SDF-1α (PEPROTECH, France), LY294002 (SIGMA, USA), wortmannin (SIGMA, USA), rapamycin (Calbiochem, Germany), for FACS analysis; monoclonal anti-VEGF receptor-2 (KDR) (SIGMA, USA), purified mouse anti-human CD110 (c-Mpl), fluorescein isothiocyanate (FITC)-conjugated anti-human CD31, FITC-conjugate anti-human CD45, and phycoerythrin (PE)-conjugated CD31, CD133 (BD Pharmingen, USA), for immunohistochemistry; rabbit anti-human c-Mpl antibody (H-300) (SANTA CRUZE USA), Isolectin GSIB4 Alexa Fluor-488, Isolectin GSIB4 Alexa Fluor 594 (Molecular Probes, USA), for Western blotting analysis; anti-Actin (SIGMA, USA), phospho-mTOR (Ser2448), phospho-p70 S6 kinase (Thr421/Ser424), phospho-Akt (Ser473), Akt antibody (Cell Signaling, USA).

2.2. Ex vivo preparation of human cultured EPCs

Human cultured EPCs were cultured as described previously [1,2]. Briefly, total human peripheral blood mononuclear cells (PBMNC) isolated from healthy human volunteers by density-gradient centrifugation were cultured in human fibronectin coated Primaria tissue culture dishes (BD Falcon, USA), using 5% FBS-EBM-2 medium with EGMV-2 growth factor supplement (Clonetics, USA). Following the removal of suspended cells with PBS at day 4, adherent cells were cultured for 3 more days.

2.3. Cellular staining

After 7 days, cultured EPCs were detected by fluorescent staining of double positive cells with FITC-labeled *Ulex europaeus* agglutinin (UEA)-1 (Vector Lab, USA) and 1,1′-dioctadecyl-3,3,3,3′,3′-tetramethylindocarbocyyanine (DiI-I) labeled acetylated low density lipoprotein (DiI-acLDL Biomedical Technologies, USA). Cells were first incubated with DiI-acLDL at 37 °C for 4 h and later fixed with 1% paraformaldehyde for 10 min. After washing twice, the cells were reacted with FITC-conjugated UEA-1 (10 μg/ml) for 1 h. After staining, cells were observed with a fluorescence microscope (Olympus IX70, Japan). Cultured EPCs were also stained by mouse anti-human CD110 (c-Mpl) antibody with anti-mouse IgG1 Alex 594 as second antibody.

2.4. FACS analysis of cultured EPCs

Cultured EPCs from six healthy volunteer underwent FACS analysis not only to confirm their endothelial lineage but also assess c-Mpl expression, using PBS containing 2 mM EDTA, 1% BSA, and 0.1% $NaNO_3$. Cultured EPCs were stained with propidium iodide (PI) in order to exclude dead cells. The monoclonal antibodies for FACS staining were as follows: KDR (VEGFR-2), CD110 (c-Mpl), FITC-conjugated CD31, CD45 and PE-conjugated CD31, CD133. The cells were analyzed by FACS Calibur (BD Biosciences USA).

2.5. Migratory activity assay of cultured EPCs

To investigate EPC migratory activity, a modified Boyden chamber assay was performed using a 24-well microchemotaxis chamber with an 8 μm pore sized polycarbonate membrane (Corning Inc, USA), as described elsewhere [18]. The protocol was described in SM-I.

2.6. Proliferative activity assay of cultured EPCs

The assay for EPC proliferative activity effected by TPO was performed, according to the detail description in SM-II.

2.7. Survival assay of cultured EPCs

The assay for EPC survival effected by TPO was performed, according to detail description in SM-III.

2.8. RT-PCR for endothelial gene expression in cultured EPCs

The protocol for RT-PCR assay was described in SM-IV.

2.9. Lesion-targeted administration of TPO along with EPC transplantation in vivo

The impact of local administration of TPO after EPC transplantation on therapeutic neovasculogenesis was assessed in a hindlimb ischemic model, using athymic nude mice (Clea Japan Inc.) [2] for a TPO group or control (PBS). Soon after operative ligation of one femoral artery, 1 μg recombinant human TPO (rhTPO) in PBS (total 12 μl) per mouse was intramuscularly injected into ischemic thigh and lower muscles (total 3points), followed immediately by intravenous transplantation of (2.0×10^5 cells/mouse) cultured EPCs. To survey the transplanted EPCs incorporated into neovasculatures in ischemic muscles, four mice per group were injected with EPCs labeled with Cell Tracker CM-DiI (Molecular probes, USA), according to the manufacturers' protocol.

2.10. Blood flow assessment of ischemic hindlimb in transplanted mice

Laser Doppler perfusion imaging (LDPI) (Moor Instrument, UK) was used to record serial blood flow measurements for 4 weeks after EPC transplantation, as previously described [2].

2.11. Histological assessment of ischemic hindlimb in transplanted mice

Cross-sectional tissue samples at 6–8 μm thickness were sliced from tissue blocks of the muscles of ischemic and contralateral limbs embedded into O.C.T compound (Tissue-Tek, USA) and frozen in liquid nitrogen at day 4 and day 28, then subjected to each assessment described below.

At day 4, the muscles from the thigh and lower leg muscles (tibialis anterior (TA), extensor digitorum lounges, soleus, plantaris, gastrocnemius (ESPG), and quadriceps femoris (QF)) of ischemic hindlimbs injected with DiI-labeled EPCs were harvested and stained with Isolectin GSIB4 Alexa Fluor 488 (Molecular Probes, USA) to assess the incorporation of transplanted EPCs into vasculatures of ischemic muscles concurrently delivered TPO. DiI-labeled EPCs in ischemic hindlimbs were counted all inside a section in 3 cross sections (proximal, middle, distal) from each mouse ($N=4$) under a fluorescence microscopy (Olympus IX70, Japan, ×200 magnification). Also the sections were stained with rabbit anti-human c-Mpl antibody to assess the c-Mpl expression on DiI-labeled EPCs.

On the other hand, as an independent experiment, microvascular density (MVD) was evaluated at day 28 by counting the capillaries stained with Isolectin GSIB4 Alexa Fluor 594 (Molecular Probes, USA) under fluorescence microscopy. A total 24 different fields were randomly selected (3 cross sections from each animal, each groups were consist with 8 animals $N=8$), and capillary were counted (×100 magnification).

2.12. Note on experiments in animal subjects

All animal experiments were performed in accordance with the institutional Animal Care and Use Committee of the Isehara Campus, Tokai University School of Medicine. The experimental animal protocols for making ischemic models or LDPI were performed under adequate anesthetization by intraperitoneal pentobarbital injection (50 mg/kg).

2.13. Western blotting to assess phosphorylation of intracellular signaling pathway of cultured EPCs

The protocol for Western blotting assay was described in SM-V.

2.14. In vitro angiogenesis assay of HUVECs cocultured with cultured EPCs using Matrigel

Cultured EPCs were incubated in EBM-2 medium containing 0.1% BSA for 4 h to render the cells quiescent, harvested, and then incubated with or without TPO at serial concentrations for 60 min, following preincubation of Rapa (50 nM) for 10 min. After washing with ice-cold PBS twice, cultured EPCs were suspended (1.0×10^3/50 μl) in EBM-2 with 2% FBS. HUVECs cultured in 10 cm culture dishes were harvested at subconfluent conditions with trypsin/EDTA, and suspended (1.5×10^4/50 μl) in the same medium. Fifty μl from each cell suspension was cocultured at 37 °C for 12 h in a 96 well tissue culture plate provided with an equal volume of Matrigel (BD Bioscience, USA) in advance. Tube formation in each well was observed at ×40 magnification by a light microscope.

2.15. Statistical analysis

All results are expressed as mean ± SEM. Statistical significance was evaluated using the unpaired Student t-test for comparisons between 2 means. Multiple comparisons between >3 groups were done by ANOVA. Probability value of $p< 0.05$ denoted statistical significance.

3. Results

3.1. c-Mpl expression in ex vivo cultured EPCs

Only EPCs cultured for 7 days were characterized as endothelial lineage cells by DiI-acLDL uptake and UEA-1 lectin-FITC binding (Fig. 1a) [2], but also by double positive of endothelial specific antigen such as CD31 and VEGF receptor-2 (KDR) (13.46 ± 1.41) by FACS analysis. The expression of c-Mpl on cultured EPCs were 30.1 ± 3.36% and 15.3 ± 3.63% of them were double positive for c-Mpl and CD31 (Fig. 1b). The expression of pan-leukocyte maker CD45 was 96.6 ± 1.08%. These data suggest that EPCs cultured for 7 days are similar to early EPC reported by Hur et al. [19]. The c-Mpl expression on cultured EPC was also confirmed by fluorescence immunohistochemistry, as described in supplemental Figure (SF)-1. About 62.4 ± 8.4% of cultured EPCs were stained with c-Mpl.

3.2. TPO-stimulated bioactivity of cultured EPCs

3.2.1. Migration

TPO enhanced the migratory potential of cultured EPCs in a dose-dependent manner (Migrated cells/100 × HPF = 9.4 ± 1.3 at 1 ng/ml; 12.9 ± 1.9 at 10 ng/ml, $p< 0.005$; 15.5 ± 1.9 at 100 ng/ml, $p< 0.0001$ vs. 6.7 ± 0.8 for control) (Fig. 2a).

3.2.2. Proliferation

The proliferation activity of cultured EPCs was not affected by low or middle doses of TPO and decreased with a high dose (O.D. 460 nm = 0.541 ± 0.014 at 1 ng/ml; 0.528 ± 0.008 at 10 ng/ml; 0.505 ± 0.015 at 100 ng/ml, $p< 0.01$ vs. 0.554 ± 0.009 for control) (Fig. 2b).

3.2.3. Apoptosis

The percentage of pyknotic nuclei in cultured EPCs was significantly decreased by TPO (% pyknotic nuclei = 19.0 ± 2.4 at 10 ng/ml, $p< 0.05$; 19.3 ± 3.78 at 100 ng/ml, $p< 0.05$ vs. 28.5 ± 1.7 for control) (Fig. 2c). Furthermore in the cell death detection assay, value at O.D. 405 nm in the TPO-treated group was significant smaller vs control (O.D. 405 nm = 0.118 ± 0.01 at 100 ng/ml TPO, $p< 0.05$ vs. 0.143 ± 0.006 for control) (Fig. 6c). These findings revealed that TPO potentiated the migration and survival of cultured EPCs *in vitro*, but not proliferation.

3.2.4. Gene expression

In day 7 cultured EPCs, endothelial gene expressions, such as von Willbrand Factor (vWF) and CD31 were detected. The expression of VE-cadherin on cultured EPCs was increased with 60 min stimulation of TPO (SF-II). This finding indicates that TPO stimulated EPCs differentiation.

3.3. Enhanced neovessel formation in vivo by EPC transplantation with lesion-targeted TPO co-administration

Histological examination disclosed that TPO significantly increased the number of DiI-labeled EPCs colocalized into vasculatures stained with Isolectin B4-Alex 488 in ischemic hindlimbs as compared with control (DiI-labeled EPCs No./section = QF 49.8 ± 10.2 in TPO, $p< 0.05$ vs 21.8 ± 2.1 in control; TA 87.7 ± 8.8 in TPO, $p< 0.05$ vs 60.2 ± 3.5 in control; ESPF 125.2 ± 7.0 in TPO, $p< 0.01$ vs 73.2 ± 11.8 in control;) (Figs. 3a,b). Some of the DiI stained transplanted EPCs incorporated into ischemic muscle, also expressing c-Mpl by fluorescence immunohistochemistry (Fig. 3c). This finding indicates that the recruitment potential of transplanted EPCs into the vasculature of ischemic hindlimbs was promoted by TPO co-administration. MVD was assessed by histological examination of tissue sections in ischemic hindlimbs

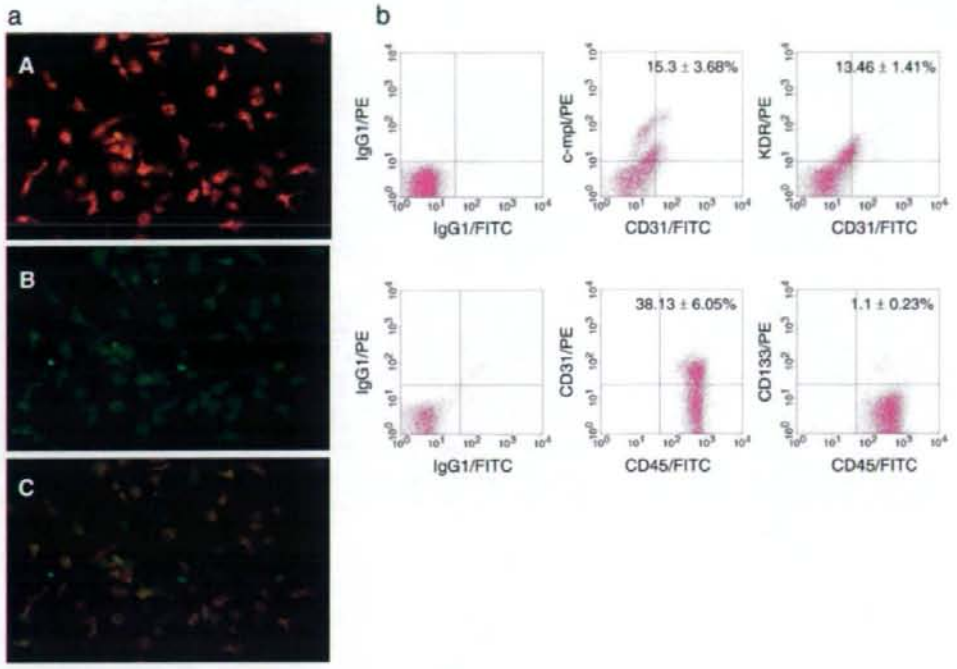

Fig. 1. Cultured EPCs expresses c-Mpl as well as endothelial specific antigens. (a) Cultured EPCs were shown to simultaneously endocytose DiI-acLDL (A) and bind FITC-UEA-1 (B) Mirror image (C). (b) Analysis of c-Mpl expression on cultured EPCs by flow cytometry. Cultured EPCs was analyzed for double positive of c-Mpl and CD31, KDR and CD31, CD31 and CD45, and CD133 and CD45.

stained with an endothelial marker, Isolectin B4-Alexa594, at day 28 (Fig. 3d). MVD was significantly augmented in the TPO-treated group as compared to the control (MVD/×100 HPF = 156.32 ± 50.824 in TPO, $p < 0.0001$ vs. 107.89 ± 55.95 in control) (Fig. 3e). Thus, neovessel formation by cultured EPC transplantation was augmented by lesion-targeted TPO co-administration.

3.4. Induced blood flow recovery of ischemic limb by TPO co-administration

At day 14 and day 28, the blood flow of ischemic hindlimbs in the TPO treatment group significantly improved, as compared with control (ischemic/contralateral perfusion ratio = at day 4: 0.15 ± 0.02 in TPO vs. 0.20 ± 0.05 in control; at day 7: 0.46 ± 0.06 in TPO vs. 0.31 ± 0.06 in control; at day 14: 0.86 ± 0.14 in TPO, $p < 0.05$ vs. 0.50 ± 0.09 in control; at day 28: 0.81 ± 0.07 in TPO, $p < 0.05$ vs. 0.55 ± 0.10 in control) (Figs. 4a,b). These results indicated that lesion-targeted TPO co-administration enhanced neovessel formation by EPC transplantation, leading to the functional improvement of blood flow in ischemic hindlimbs.

3.5. TPO activation of the Akt/mTOR/p70S6K signal pathway in cultured EPCs

We next examined the effect of TPO on signaling transduction pathways in cultured EPCs. Akt phosphorylation was induced by TPO in 5 min. and reached a peak at 30 min. (Figs. 5a,b). Furthermore, phosphorylation of mTOR and p70S6K in the mTOR/p70S6K pathway downstream of Akt was also induced by TPO (Figs. 5c,d) (in Fig. 5b: phospho Akt intensity ratio/0 min = 1.429 ± 0.092 at 5 min, $p < 0.05$;

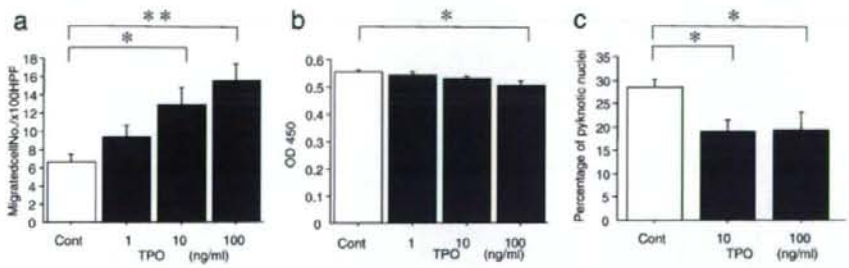

Fig. 2. TPO enhanced bioactivity of cultured EPCs. (a) TPO induced EPC migration. Migratory response of EPCs toward different dosages of TPO was measured by modified Boyden chamber migration assay. Cultured EPCs demonstrated a potent dose-dependent activity toward TPO ($*p < 0.005$, $**p < 0.0001$, $N = 10$). (b) Increment of proliferative activity of cultured EPCs in response to TPO ($*p < 0.01$, $N = 8$). (c) TPO attenuated cultured EPCs apoptosis ($*p < 0.05$, $N = 5$).

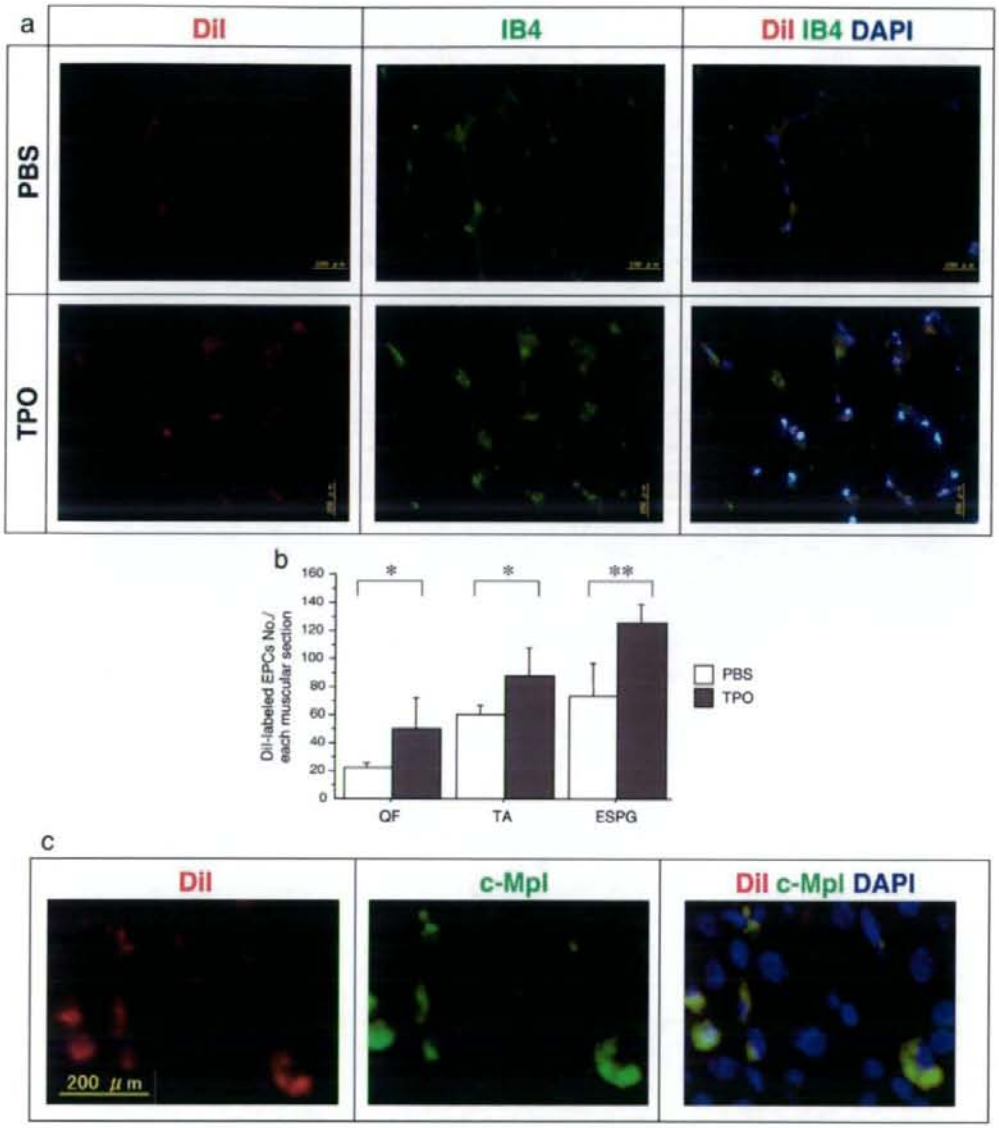

Fig. 3. Recruitment potential of transplanted EPC into vasculature of ischemic hindlimb was ameliorated by TPO co-administration at early time point. (a) Representative microscopic photographs of double fluorescence in ischemic muscles (tibialis anterior (TA), extensor digitorum lounges, soleus, plantaris, gastrocnemius (ESPG), and quadriceps femoris (QF) at day 4. Transplanted EPCs were labeled with a DiI fluorescence marker (red) in histological sections retrieved from ischemic muscles. Host mouse vasculatures were identified by isolectin B4 (green) in same tissue sections. Double positive cells were recognized as transplanted EPCs incorporate into mouse vasculature. The upper row: Ipsilateral hindlimb injected with PBS, The lower row: Ipsilateral hindlimb injected with TPO. (b) Quantitative analysis of incorporated EPCs in the muscle by counting number of DiI-labeled EPCs (red fluorescence) in PBS group at day 4 (*$p< 0.05$ **$p< 0.01$, $N=4$). (c) The c-Mpl expression (green) on DiI positive transplanted EPC (red). (d) Representative histology of microvasculature stained by Isolectin B4-Alexa594 in ischemic tissue at day 28. left: PBS group, right: TPO-treated group. (e) Quantitative analysis of MVD at 28 days (**$p< 0.0001$, $N=8$).

1.804±0.160 at 10 min, $p< 0.005$; 2.263±0.152 at 30 min, $p< 0.0001$ vs. 1.000±0.00 at 0 min; in Fig. 5d: phospho-mTOR intensity ratio/0 min = 1.448±0.049 at 5 min; 2.032±0.072 at 10 min, $p<0.0001$; 1.978±0.140 at 30 min, $p<0.0001$ vs. 1.000±0.00 at 0 min; in Fig. 5d: phosph p70S6k intensity ratio/0 min = 1.219±0.084 at 5 min; 2.957±0.267 at 10 min, $p< 0.005$; 3.559±0.310 at 30 min, $p< 0.0001$ vs. 1.000±0.00 at 0 min).

3.6. Essential role of the TPO-induced PI3K/Akt/mTOR/p70S6 kinase signaling pathway for migration and survival of cultured EPCs in vitro

To address the mechanism of the augmented effect of TPO on the *in vitro* bioactivity of cultured EPCs, we examined the effect of a phosphatidylinositol-3 kinesis (PI3K) inhibitor, Ly, and an mTOR inhibitor, Rapa, on augmented migration and survival capabilities

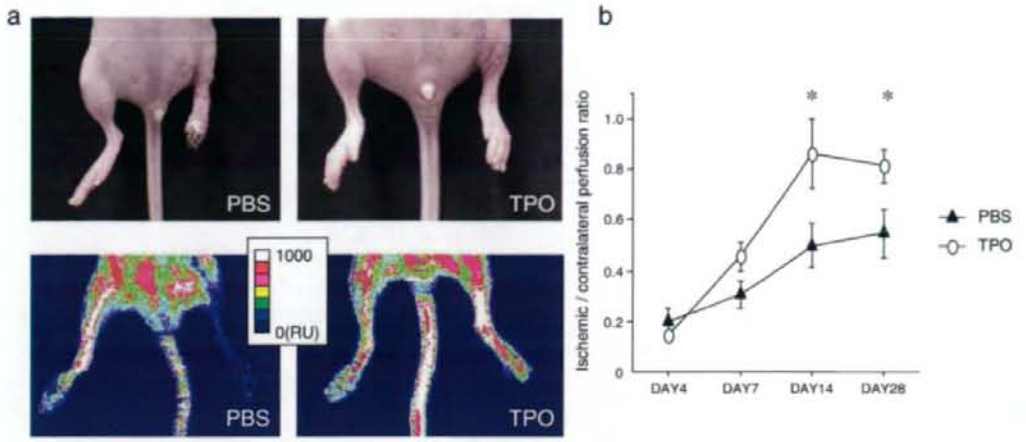

Fig. 4. Induced blood flow recovery of ischemic limb by TPO co-administration. (a) Representative photographs of hindlimbs in treated animals assessed by LDPI at 28 days after EPC transplantation. Color scale illustrates variation in blood flow from maximum perfusion (white) to minimal perfusion (dark blue). (b) Quantitative analysis of perfusion recovery measured by LDPI during the observation period. Open circle and closed triangle show TPO-treated and PBS groups, respectively (*$p< 0.05$, $N=8$).

of cultured EPCs induced by TPO. As shown in Fig. 6a, the inhibition of phosphorylation in the PI3K/Akt signaling pathway by Ly as well as another PI3K inhibitor, Wort, negated the upregulated migration potential effect of cultured EPCs induced by TPO. Furthermore, to investigate the essential role of the mTOR/p70S6K signaling pathway for the enhanced migration potential of cultured EPCs induced by TPO, we examined the effect of Rapa. Interestingly, Rapa also recovered the enhanced migration effect of cultured EPCs induced by TPO (Ratio of migrated cell No. vs cont = 1.00 ± 0.45 for control, $p< 0.01$; 1.04±0.13 for Ly, $p< 0.01$; 1.10±0.09 for Rapa, $p< 0.01$; 1.35±0.15 for Wort, $p< 0.05$ vs. 1.82±0.10 for TPO alone. 2.20±0.23 at SDF-1 as positive control) (Fig. 6a).

Alternatively, the anti-apoptotic effect of TPO on cultured EPCs was negated by Rapa in two kinds of experiments. (Ratio of pyknotic nuclei percentage vs. cont = 1.008±0.078 for control, $p< 0.01$; 0.868±0.089 for Rapa, $p< 0.05$ vs. 0.650±0.054 for TPO alone) (Fig. 6b), (O.D. 405 nm in Cell death detection assay = 0.143±0.006 for control, $p< 0.05$; 0.143±0.007 for Rapa, $p< 0.05$ vs. 0.118±0.010 for TPO alone. 0.150±0.018 at Rapa alone as negative control. 0.102±0.004 at SDF-1α 50 ng/ml as positive control.) (Fig. 6c).

Western blotting analysis showed the complete inhibition of phosphorylation of mTOR and p70S6kinase (located downstream of the PI3K/Akt pathway) by Rapa, which is an inhibitor of mTOR *per se*, as well as Ly or Wort (Fig. 6d).

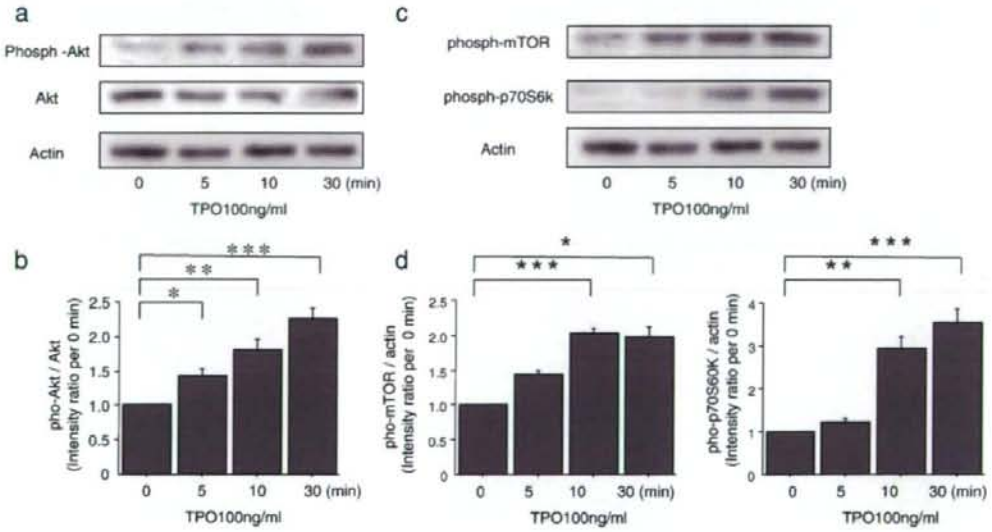

Fig. 5. TPO stimulated Akt/mTOR/p70S6kinase pathway in cultured EPCs. (a) Akt phosphorylation in TPO-stimulated EPCs by Western blotting. (b) Increment of Akt phosphorylation in TPO-stimulated EPCs at serial time points (*$p< 0.05$, **$p< 0.005$, ***$p< 0.0001$, $N=3$). (c) Phosphorylation of mTOR/p70S6kinase pathway in TPO-stimulated EPCs by Western blotting. (d) Increment of mTOR and p70S6kinase phosphorylation in TPO-stimulated EPCs at serial time points (*$p< 0.05$, **$p< 0.005$, ***$p< 0.0001$, $N=3$).

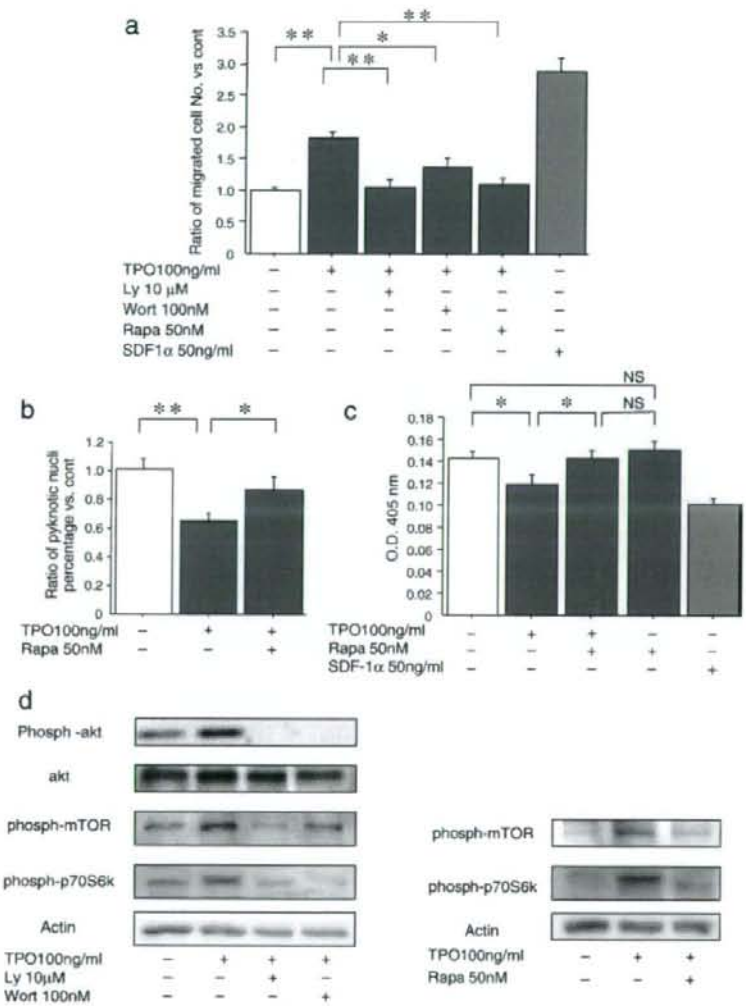

Fig. 6. The mTOR/p70S6kinase signaling pathway is required for migration and survival of cultured EPC stimulated by TPO. (a) Increment of migration of cultured EPCs treated with TPO is negated by inhibitors of the Akt/mTOR/p70S6kinase signaling pathway (*$p< 0.05$, **$p< 0.01$, $N=10$). (b) Anti-apoptotic activity of TPO in cultured EPCs, negated by inhibition of the mTOR/p70S6kinase signaling pathway. The ratio of pyknotic nuclei percentage vs. cont (*$p< 0.05$, $N=4$). (c) Similar results were obtained by cell death detection assay (*$p< 0.05$, $N=4$). (d) Left: Phosphorylation of the mTOR/p70S6kinase signaling pathway in TPO-treated cultured EPCs negated by inhibitors of the PI3K/Akt signaling pathway. Right: Inhibition of phosphorylation of the mTOR/p70S6kinase pathway in TPO-stimulated EPCs. Similar results were obtained in 3 independent experiments.

These results indicated that the PI3K/Akt/mTOR/p70S6kinase signaling pathway plays an essential role in the migration and anti-apoptotic effect on cultured EPCs augmented by TPO.

3.7. *Significance of the TPO-induced mTOR/p70S6K signaling pathway for in vitro vasculogenesis in cultured EPCs*

Matrigel tube formation assay for HUVECs cocultured with TPO-stimulated EPCs was performed to assess the involvement of the mTOR/p70S6K signaling pathway in the TPO-augmented vasculogenic potential of cultured EPCs. Of note, TPO-stimulated EPCs significantly enhanced the tube formation of HUVECs in Matrigel, as compared with unstimulated EPCs, which was inhibited by Rapa (tube number/×40 HPF=42.8±3.0 for TPO unstimulated EPCs, $p< 0.0001$; 46.8±3.6 for TPO-stimulated EPCs treated with Rap, $p< 0.001$; 44.500±1.916 for HUVEC alone, $p< 0.001$ vs. 62.1±3.1 for TPO-stimulated EPCs) although unstimulated EPCs possessed no effect on tube formation (Fig. 7). These findings also indicated that the mTOR/p70S6Kkinase signaling pathway plays a pivotal role in the TPO-stimulated vasculogenetic potential of cultured EPCs.

4. Discussion

In the present study, we investigated whether TPO, as a positive mediator of postnatal vasculogenesis, potentiates the vasculogenic capability of EPCs transplanted in acute ischemia. In *in vitro* experiments, TPO demonstrated the potential to enhance motility and survival of cultured EPCs, as well as tubulogenesis of HUVECs through TPO-stimulated EPCs. The functional activities of EPCs are considered to be conducted via the c-Mpl receptor of TPO expressed in EPCs (Figs. 1b,c). *In vivo*, the systemic transplantation of EPCs via tail

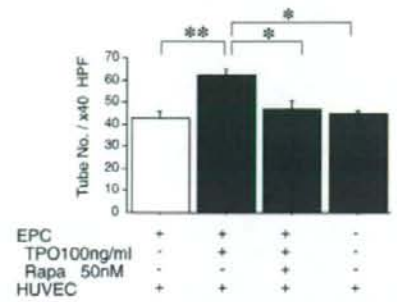

Fig. 7. The mTOR/p70S6kinase signaling pathway plays a pivotal role in vasculogenesis of cultured EPC stimulated by TPO *in vitro*. Serum starved cultured EPCs were pre-treated with TPO (100 ng/ml) and Rapa (50 nM), followed by co-culturing with HUVECs and in Matrigel-coated plates (*$p < 0.001$, **$p < 0.0001$, $N = 4$).

veins, along with intramuscular local administration of TPO, showed an augmentation of EPC homing concurrent with an increase of capillary densities in ischemic hindlimbs, compared with EPC transplantation alone. Some groups reported that the maximal concentration of rhTPO (1 μg/mouse) was reached at 2 h after subcutaneous injection. The terminal half-life was 10.8 h and rhTPO was excreted within 72 h after dosing [20]. Considering this report, injection ways were different from our experiments, TPO works specially in the early period of ischemic muscle and homed transplanted EPCs into the ischemic site (Figs. 3a,b). The effect of TPO on EPC homing into ischemic tissues is figured to be mediated via the motility promoted by the concentration gradient of locally delivered TPO in the ischemic tissue. This is indicated by the preferential effect of TPO on the migratory activity of EPCs shown in Figs. 2a, 3a,b, and in similar findings from a previous report regarding local delivery of SDF-1 in ischemic tissues [6]. However, the enhanced neovascularization is not only due to the accumulation of EPCs in the foci of ischemia, but also because of the elongation of lifespan as revealed by an *in vitro* apoptosis assay (Figs. 2c and 6c).

Interestingly, the *in vitro* migration and survival of cultured EPCs boosted by TPO is mediated through the activation of the PI3K/Akt/mTOR/p70S6K signaling pathway, which is abrogated by pathway inhibitors LY294002, Wortmannin, or Rapamycin (Figs. 5, 6). As previously reported, Akt is an essential intracellular signal mediator of angiogenesis, regulating migration, survival, proliferation, and tubulogenesis in vascular ECs [21,22]. Akt signaling also plays a crucial role in the activities of immature endothelial lineage cells and EPCs undergoing vasculogenesis. Previous reports demonstrate that statins (HMG-CoA reductase inhibitors) promote vasculogenesis in terms of enhanced mobilization, migration, survival, differentiation, or proliferation of EPCs via the activation of the Akt pathway [14]. Erythropoietin directly promotes EPC proliferation and differentiation through Akt phosphorylation [5,23]. Estrogen limits EPC senescence via the PI3K/Akt signaling pathway [24]. The importance *per se* of the downstream signal pathway, mTOR/p70S6K, has been already suggested for the proliferation and differentiation of immature EPCs given the inhibitory effect of Rapamycin on the activities of CD133+ cells in peripheral blood [17]. While the universal significance of PI3K/Akt/mTOR pathway in endothelial lineage cells is unclear, its effects are not equivalent between differentiated ECs, therapeutical EPCs, and immature EPCs, thereby suggesting different roles in each cell type.

Of note, in contrast to immature EPCs [17], the pathway is unlikely to be related to the proliferative activity of adherent EPCs, as reduced by TPO, despite mTOR/p70S6K pathway phosphorylation mediating cell growth and proliferation in diverse cell types [25–27] (Fig. 2b). Considering the data, the proliferative activity of EPCs might not be influenced by TPO, because of the possible suppression of the PI3K/Akt pathway following mTOR/p70S6K activation as a negative feedback mechanism [28,29]. Consequently, we have shown for the first time that TPO enhances the migration and survival of adherent EPCs for vasculogenesis through the activation of the PI3K/Akt/mTOR/p70S6kinase pathway via c-Mpl *in vitro*, and promotes the accumulation and incorporation of EPCs in ischemic tissues *in vivo*.

Our former study indicated that local transfection of adenoviral vector encoding TPO in ischemic muscles induces an increase in circulating platelets by HuMK stimulation earlier than systemic transfection [30]. Though there is a difference between bolus and continuous administration of TPO, we can readily predict that locally administered TPO will trigger an increase in platelet accumulation in ischemic tissues [31,32], thereafter activating angiogenic mediators such as VEGF [33,34], or angiogenic platelet-derived lipids such as sphingosine 1-phosphate, lysophosphatidic acid, and phosphatidate [16,35,36]. Especially, sphingosine 1-phosphate (S1P) is capable of inducing angiogenetic factors *in vitro*; for example EC chemotaxis, survival, proliferation, capillary morphogenesis, etc [35]. Moreover, S1P has recently been reported to induce phosphorylation of CXCR-4, a chemokine receptor of SDF-1 through S1P receptor 3, and is thereby involved in the SDF-1/CXCR-4 signaling pathway which is important for EPC recruitment in ischemic tissues [6,37].

Alternatively, locally administered TPO is thought to encourage *in situ* ECs via c-Mpl to secrete platelet activating factor (PAF) [16,38], a candidate potent angiogenic phospholipid, mediating the secretion of angiogenic factors including VEGF [39]. Considering the descriptions above, we conclude that TPO-augmented therapeutic vasculogenesis is exerted by orchestrating the direct and indirect functions of TPO on EPC biological activities *in vitro* and *in vivo* in ischemic hindlimb.

Despite the information concerning the role of TPO exerting biological actions on cancer cells via activation of c-Mpl and its downstream signaling [40], in terms of clinical applications, rhTPO intravenous administration for delayed platelet recovery after HSC transplantation has shown no serious adverse effects including cancer disease [41]. rhTPO administration has been reported to safely induce mobilization of BM derived CD34+ cells without thrombogenic events in patients with cancer undergoing high-dose chemotherapy and autologous PB CD34+ cells transplantation [42]. Therefore, TPO may be a favorable mediator to safely augment the efficacy of EPC transplantation, especially through local administration as medicated in the present study.

In conclusion, the present study demonstrates that TPO enhances the efficacy of EPC transplantation by upregulating migration and survival of EPCs via activation of the Akt/mTOR/p70S6kinase signaling pathway, making it a promising candidate to exert a booster effect in therapeutic vasculogenesis.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.yjmcc.2008.08.002.

References

[1] Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, et al. Isolation of putative progenitor endothelial cells for angiogenesis. Science 1997;275:964–7.

[2] Kalka C, Masuda H, Takahashi T, Kalka-Moll WM, Silver M, Kearney M, et al. Transplantation of ex vivo expanded endothelial progenitor cells for therapeutic neovascularization. Proc Natl Acad Sci U S A 2000;97:3422–7.

[3] Kawamoto A, Iwasaki H, Kusano K, Murayama T, Oyamada A, Silver M, et al. CD34-positive cells exhibit increased potency and safety for therapeutic neovascularization after myocardial infarction compared with total mononuclear cells. Circulation 2006;114:2163–9.

[4] Hill JM, Zalos G, Halcox JP, Schenke WH, Waclawiw MA, Quyyumi AA, et al. Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. N Engl J Med 2003;348:593–600.

[5] George J, Goldstein E, Abashidze A, Wexler D, Hamed S, Shmilovich H, et al. Erythropoietin promotes endothelial progenitor cell proliferative and adhesive properties in a PI 3-kinase-dependent manner. Cardiovasc Res 2005;68:299–306.

[6] Yamaguchi J, Kusano KF, Masuo O, Kawamoto A, Silver M, Murasawa S, et al. Stromal cell-derived factor-1 effects on ex vivo expanded endothelial progenitor cell recruitment for ischemic neovascularization. Circulation 2003;107:1322–8.

[7] Berardi AC, Wang A, Levine JD, Lopez P, Scadden DT. Functional isolation and characterization of human hematopoietic stem cells. Science 1995;267:104–8.

[8] Kaushansky K. Thrombopoietin and the hematopoietic stem cell. Ann N Y Acad Sci 2005;1044:139–41.

[9] Negrotto S, Pacienza N, D'Atri LP, Pozner RG, Malaver E, Torres O, et al. Activation of cyclic AMP pathway prevents CD34(+) cell apoptosis. Exp Hematol 2006;34:1420–8.

[10] Lupia E, Bosco O, Bergerone S, Dondi AE, Goffi A, Oliaro E, et al. Thrombopoietin contributes to enhanced platelet activation in patients with unstable angina. J Am Coll Cardiol 2006;48:2195–203.

[11] Namiuchi S, Kagaya Y, Ohta J, Shiba N, Sugi M, Oikawa M, et al. High serum erythropoietin level is associated with smaller infarct size in patients with acute myocardial infarction who undergo successful primary percutaneous coronary intervention. J Am Coll Cardiol 2005;45:1406–12.

[12] Leone AM, Rutella S, Bonanno G, Contemi AM, de Ritis DG, Giannico MB, et al. Endogenous G-CSF and CD34+ cell mobilization after acute myocardial infarction. Int J Cardiol 2006;111:202–8.

[13] Chanprasert S, Geddis AE, Barroga C, Fox NE, Kaushansky K. Thrombopoietin (TPO) induces c-myc expression through a PI3K- and MAPK-dependent pathway that is not mediated by Akt, PKCzeta or mTOR in TPO-dependent cell lines and primary megakaryocytes. Cell Signal 2006;18:1212–8.

[14] Llevadot J, Murasawa S, Kureishi Y, Uchida S, Masuda H, Kawamoto A, et al. HMG-CoA reductase inhibitor mobilizes bone marrow-derived endothelial progenitor cells. J Clin Invest 2001;108:399–405.

[15] Li W, Petrimpol M, Molle KD, Hall MN, Battegay EJ, Humar R. Hypoxia-induced endothelial proliferation requires both mTORC1 and mTORC2. Circ Res 2007;100:79–87.

[16] Brizzi MF, Battaglia E, Montrucchio G, Dentelli P, Del Sorbo L, Garbarino G, et al. Thrombopoietin stimulates endothelial cell motility and neoangiogenesis by a platelet-activating factor-dependent mechanism. Circ Res 1999;84:785–96.

[17] Butzal M, Loges S, Schweizer M, Fischer U, Gehling UM, Hossfeld DK, et al. Rapamycin inhibits proliferation and differentiation of human endothelial progenitor cells in vitro. Exp Cell Res 2004;300:65–71.

[18] Asahara T, Takahashi T, Masuda H, Kalka C, Chen D, Iwaguro H, et al. VEGF contributes to postnatal neovascularization by mobilizing bone marrow-derived endothelial progenitor cells. Embo J 1999;18:3964–72.

[19] Hur J, Yoon CH, Kim HS, Choi JH, Kang HJ, Hwang KK, et al. Characterization of two types of endothelial progenitor cells and their different contributions to neovasculogenesis. Arterioscler Thromb Vasc Biol 2004;24:288–93.

[20] Liu XW, Tang ZM, Song HF, Dou GF. Metabolism, distribution and excretion of recombinant human thrombopoietin in mice. Zhongguo Shi Yan Xue Ye Xue Za Zhi 2001;9:318–22.

[21] Dimmeler S, Fleming I, Fisslthaler B, Hermann C, Busse R, Zeiher AM. Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. Nature 1999;399:601–5.

[22] Maffucci T, Piccolo E, Cumashi A, Iezzi M, Riley AM, Saiardi A, et al. Inhibition of the phosphatidylinositol 3-kinase/Akt pathway by inositol pentakisphosphate results in antiangiogenic and antitumor effects. Cancer Res 2005;65:8339–49.

[23] Bahlmann FH, De Groot K, Spandau JM, Landry AL, Hertel B, Duckert T, et al. Erythropoietin regulates endothelial progenitor cells. Blood 2004;103:921–6.

[24] Imanishi T, Hano T, Nishio I. Estrogen reduces endothelial progenitor cell senescence through augmentation of telomerase activity. J Hypertens 2005;23:1699–706.

[25] Fingar DC, Blenis J. Target of rapamycin (TOR): an integrator of nutrient and growth factor signals and coordinator of cell growth and cell cycle progression. Oncogene 2004;23:3151–71.

[26] Hay N, Sonenberg N. Upstream and downstream of mTOR. Genes Dev 2004;18:1926–45.

[27] Brader S, Eccles SA. Phosphoinositide 3-kinase signalling pathways in tumor progression, invasion and angiogenesis. Tumori 2004;90:2–8.

[28] Harrington LS, Findlay GM, Lamb RF. Restraining PI3K: mTOR signalling goes back to the membrane. Trends Biochem Sci 2005;30:35–42.

[29] Pellegatta F, Catapano AL, Luzi L, Terruzzi I. In human endothelial cells amino acids inhibit insulin-induced Akt and ERK1/2 phosphorylation by an mTOR-dependent mechanism. J Cardiovasc Pharmacol 2006;47:643–9.

[30] Amano H, Hackett NR, Rafii S, Crystal RG. Thrombopoietin gene transfer-mediated enhancement of angiogenic responses to acute ischemia. Circ Res 2005;97:337–45.

[31] Massberg S, Enders G, Matos FC, Tomic LI, Leiderer R, Eisenmenger S, et al. Fibrinogen deposition at the postischemic vessel wall promotes platelet adhesion during ischemia-reperfusion in vivo. Blood 1999;94:3829–38.

[32] Roberts AM, Ovechkin AV, Mowbray JG, Robinson TW, Lominadze D. Effects of pulmonary ischemia–reperfusion on platelet adhesion in subpleural arterioles in rabbits. Microvasc Res 2004;67:29–37.

[33] Gunsilius E, Petzer A, Stockhammer G, Nussbaumer W, Schumacher P, Clausen J, et al. Thrombocytes are the major source for soluble vascular endothelial growth factor in peripheral blood. Oncology 2000;58:169–74.

[34] Kranz A, Rau C, Kochs M, Waltenberger J. Elevation of vascular endothelial growth factor-A serum levels following acute myocardial infarction. Evidence for its origin and functional significance. J Mol Cell Cardiol 2000;32:65–72.

[35] English D, Garcia JG, Brindley DN. Platelet-released phospholipids link haemostasis and angiogenesis. Cardiovasc Res 2001;49:588–99.

[36] Kim HK, Song KS, Chung JH, Lee KR, Lee SN. Platelet microparticles induce angiogenesis in vitro. Br J Haematol 2004;124:376–84.

[37] Walter DH, Rochwalsky U, Reinhold J, Seeger F, Aicher A, Urbich C, et al. Sphingosine-1-phosphate stimulates the functional capacity of progenitor cells by activation of the CXCR4-dependent signaling pathway via the S1P3 receptor. Arterioscler Thromb Vasc Biol 2007;27:275–82.

[38] Camussi G, Montrucchio G, Lupia E, De Martino A, Perona L, Arese M, et al. Platelet-activating factor directly stimulates in vitro migration of endothelial cells and promotes in vivo angiogenesis by a heparin-dependent mechanism. J Immunol 1995;154:6492–501.

[39] Ma X, Ottino P, Bazan HE, Bazan NG. Platelet-activating factor (PAF) induces corneal neovascularization and upregulates VEGF expression in endothelial cells. Invest Ophthalmol Vis Sci 2004;45:2915–21.

[40] Romanelli RG, Petrai I, Robino G, Efsen E, Novo E, Bonacchi A, et al. Thrombopoietin stimulates migration and activates multiple signaling pathways in hepatoblastoma cells. Am J Physiol Gastrointest Liver Physiol 2006;290:G120–128.

[41] Nash RA, Kurzrock R, DiPersio J, Vose J, Linker C, Maharaj D, et al. A phase I trial of recombinant human thrombopoietin in patients with delayed platelet recovery after hematopoietic stem cell transplantation. Biol Blood Marrow Transplant 2000;6:25–34.

[42] Linker C, Anderlini P, Herzig R, Christiansen N, Somlo G, Bensinger W, et al. Recombinant human thrombopoietin augments mobilization of peripheral blood progenitor cells for autologous transplantation. Biol Blood Marrow Transplant 2003;9:405–13.