Aberrant Kinetics of Bone Marrow–Derived Endothelial Progenitor Cells in the Murine Oxygen-Induced Retinopathy Model

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PURPOSE. Retinopathy of prematurity (ROP) causes serious blindness because of the vasculopathy that results from the abnormal oxygen dynamics. However, the systemic kinetics of bone marrow–derived endothelial progenitor cells (BM-derived EPCs) during the “postnatal vasculogenesis” of ROP has yet to be elucidated. Thus, the authors investigated the kinetics of BM-derived EPCs using a murine oxygen-induced retinopathy (OIR) model.

METHODS. OIR was induced in C57BL/6J mice by continual aeration with 75% oxygen from postnatal day (P) 7 to P12 that afterward returned to normal room air.

RESULTS. The frequency of circulating EPCs (Sca-1⁺/c-Kit⁺ cells in blood) in an OIR model estimated by FACS decreased immediately after the hyperoxic phase (P12) and then increased at the hypoxic phase (P17) compared with control. Further, EPC colony-forming assay of BM-Lin[−]/Sca-1⁺ (BM-LS) cells exhibited a conversion from the predominant primitive EPC colony production at P12 to the definitive EPC colony at P17. In the OIR retinas of BM-transplanted mice with BM-LS cells of EGFP transgenic mice, there was less incorporation of GFP⁺ cells into vascular structures at P12, whereas there was a drastic recruitment into the “tufts” and for the intact vasculature at P17. Moreover, the definitive EPC colony cells intravitreally injected into OIR significantly abrogated pathologic versus primitive vascular growth.

CONCLUSIONS. Taken together, these findings propose that the deviation of functional bioactivities of BM-derived EPCs contributing to intact vascular development under the abnormal oxygen dynamics may provide important mechanistic insight into pathologic vascular development in ROP. (*Invest Ophthalmol Vis Sci.* 2011;52:7835–7841) DOI:10.1167/iovs.10-5880

Retinopathy of prematurity (ROP) causes pathologic retinal vascularization, which frequently leads to irreversible visual acuity loss. In developed countries, ROP-induced blindness is a serious health problem in school-age children.¹ Use of experimental animal models has proven to be useful in understanding the pathogenesis of ROP.^{2–5} Smith et al.^{5,6} created an oxygen-induced retinopathy (OIR) model that can be easily prepared and used to determine quantitative changes. Studies using this murine model have shown that the pathophysiological course for ROP involves two phases. Phase 1 is the vaso-obliterative (hypovascular) phase caused by the delayed retinal vascular growth that is associated with premature births. Phase 2 is the proliferative (hypervascular) phase induced by aberrant oxygen dynamics that are responsible for converting the extrauterine relative hyperoxic state to the hypoxic state in the retina.^{7–9} Essential growth factors related to the pathologic progression of OIR that have been identified thus far include vascular endothelial growth factor (VEGF),^{10–12} angiopoietins,¹³ hypoxia inducible factor (HIF)-1 α ,¹⁴ insulin-like growth factor (IGF)-1,¹⁵ growth hormone,¹⁶ and pigment epithelium-derived factor.¹⁷ Aberrant oxygen dynamics in particular have been shown to distort physiological retinal vascular development by inhibiting VEGF expression during the vaso-obliterative phase. This subsequently causes promotion of the proliferative phase^{10,18} by HIF-1 α , which regulates the oxygen concentration in the retina.^{7,14}

Bone marrow (BM)-derived endothelial progenitor cells (EPCs) have recently been shown to contribute to the pathological or physiological neovascularization that is controlled postnatally by growth factors, cytokines, and hormones.^{19–21} Extrinsic transplantation of BM-derived hematopoietic stem/progenitor cells (HSPCs), the Lin[−] cells and Lin[−]/Sca-1⁺ (LS) cells, have been shown to have a revascularizing capability in induced retinal ischemia by causing differentiation of the stem/progenitor cells into endothelial cells.^{22–24} These findings suggest that BM-derived HSPCs might be therapeutically useful when applied in cases of retinal vascular ischemia caused by degenerative retinopathy. On the other hand, there have been very few reports in the literature on the endogenous kinetics of the BM-derived EPCs that contribute to the pathologic neovascularization in ischemic retinas. Yodoi et al.²⁵ reported finding attenuation of both the number and the endothelial colony-forming activity of the circulating CD34⁺ cells in human subjects with severe age-related macular degeneration.

Based on all the previous findings, we speculate that BM-derived EPCs in the circulation could play a critical role in pathologic neovascularization in ROP. Therefore, the aim of the present study was to determine the kinetics of the BM-EPCs through investigations of the oxygen dynamics in OIR.

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MATERIALS AND METHODS

Murine OIR Model

All experimental procedures were conducted in accordance with the guidelines developed by the Animal Care and Use Committee at Tokai University School of Medicine and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The murine OIR model was based on the protocol established by Smith et al.⁵ Briefly, C57BL/6J mice (Clea Japan, Inc., Tokyo, Japan) together with their nursing mothers were aerated with 75% oxygen from postnatal day (P)7 to P12. After the hyperoxic exposure, pups were returned to normal room air conditions until P17.

FACS Analysis of Circulating EPC Assessment

Whole blood (WB) was collected using needle aspiration of the left ventricle at P12, P14, and P17. Using a previously described method,¹⁵ WB-mononuclear cells (MNCs) at each time point were isolated by a density gradient centrifugation method that used a solution of polysucrose and sodium diatrizoate (Histopaque 1083; Sigma-Aldrich, St. Louis, MO), followed by hemolysis with ammonium chloride. In accordance with the method reported by Fadini et al.,²⁶ Sca-1⁺/c-Kit⁺ cells were used as the circulating EPCs in the WB-MNCs and then numerically evaluated by flow cytometry (FACSCalibur; Becton Dickinson Immunocytometry System, Mountain View, CA) by using monoclonal antibodies of FITC anti-mouse Ly-6A/E (Sca-1; eBioscience, San Diego, CA) and PE anti-mouse CD117 (c-Kit; BD PharMingen, San Diego, CA).

Measurement of Serum VEGF Concentration

Using the supplemental protocol of an immunoassay kit (Quantikine; R&D Systems, Minneapolis, MN), we measured the vascular endothelial growth factor (VEGF) concentrations in the WB serum at P12, P14, and P17.

BM-Transplanted Mice by HSPCs

To investigate the differentiation aspects of the BM-derived EPCs in the OIR model, we examined newborn BM-transplanted mice using BM-LS cells from EGFP mice. BM cells in femurs and tibias of EGFP mice (8–12 weeks old; C57BL/6Tg; SLC, Tokyo, Japan)²⁷ were hemolyzed using ammonium chloride and then washed by phosphate-buffered saline (PBS). Subsequently, cells were incubated with a cocktail of biotinylated monoclonal antibodies against B220 (RA3–6B2), TER-119 (Ly-76), CD3e (145–2C11), CD11b (M1/70), ZLy6G, and Ly-6C (RB6–8C5) (all from BD PharMingen) at 4°C for 30 minutes. After washing and suspending with MACS buffer, magnetic beads (Streptavidin MicroBeads; Miltenyi Biotec Inc., Auburn, CA) were added to the cell suspension and then incubated at 4°C for 20 minutes. Lin[−] cells were negatively selected for by using an automated magnetic cell sorter (autoMACS Separator; Miltenyi Biotec Inc.), followed by incubation with antibody (anti-Sca-1 MicroBeads; Miltenyi Biotec Inc.). Positive selection by the cell sorter was used for obtaining the LS cells. All C57BL/6J newborn mice were irradiated (500 cGy) within 24 hours after delivery. The faces of all the animals were covered with saturnine shields before the irradiation procedure.

Based on a modified protocol of Hisatomi et al.,²⁸ animals in the recipient litters received LS cells (10⁵ cells/7 μ L/mouse) through the temporal facial vein with a 31-gauge needle syringe (Ito Corporation, Tokyo, Japan) 1 day after irradiation. BM reconstitution in the recipient mice was confirmed by identification of GFP⁺ cells in the WB and BM.

Fluorescence microscopy (SZX12; Olympus, Tokyo, Japan) with a triple band (U-N61000v2; Chroma, Bell Falls, VT) and a single-band GFP filter (U-MGFPHQ; Olympus) was used to determine incorporation of the GFP⁺ cells into the retinal vasculature.²⁹

Immunohistochemistry of Retinal Vessels

Pups were euthanatized at P12 and P17 with pentobarbital sodium (60 μ g/g body weight) and then perfused with PBS followed by 4% para-

formaldehyde (PFA). Eye globes were carefully enucleated and fixed in 4% PFA. After blocking with PBS in 1% bovine serum albumin and 0.3% Triton X-100 (Sigma Aldrich, St. Louis, MO) at 4°C overnight, flat-mounted samples of excised retinas were incubated with rat anti-mouse monoclonal antibody, CD31 and CD11b (1:500, 1:1000; BD PharMingen), at 4°C overnight. After samples were washed with PBS, they were incubated with Alexa Fluor 594-conjugated goat anti-rat antibodies (1:1000; Molecular Probes, Eugene, OR) at 4°C for 4 hours. The retinal samples were examined by fluorescence microscopy. Retinal cross-sections were also examined. Enucleated eye globes were incubated with sucrose solutions at serial dilutions of 5%, 10%, 15%, 20%, and 25% in 0.01 M PBS. Subsequently, samples were embedded in OCT compound (Sakura Finetek Co., Ltd., Tokyo, Japan) and then quickly frozen in isopentane cooled by liquid nitrogen. The 8- μ m-thick sample slices were blocked with 10% goat serum for 1 hour. Sections were stained with rat anti-mouse CD31 monoclonal antibody (1:500; BD PharMingen) at 4°C overnight. Reactions were revealed by using Alexa Fluor 594-conjugated goat anti-rat antibody (1:1000; Molecular Probes) at room temperature for 2 hours. Cell nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI; Vectashield H-1500, Vector Laboratories, Inc., Burlingame, CA).

EPC Colony-Forming Assay

To determine whether there was an association between BM-LS cells and EPCs, we used a previously described procedure^{30,31} to perform an EPC colony-forming assay (EPC-CFA) using semisolid methylcellulose culture medium. The medium consisted of M3236 (Stem Cell Technologies, Vancouver, Canada) and was manually supplemented with various proangiogenic factors including VEGF (R&D Systems), stem cell factor, interleukin-3, basic fibroblast growth factor, epidermal growth factor (EGF), and IGF-1 (PeproTech, Rocky Hill, NJ).

Freshly isolated Lin[−]/Sca-1⁺ cells (2×10^3 cells/dish) were seeded into a 35-mm tissue culture dish (Primaria; Becton Dickinson Labware, Franklin Lakes, NJ) and cultured for 7 days.

The frequencies of the primitive and the definitive EPC-colony forming units (EPC-CFUs) were identified by morphologic features, which included small, round, and large spindle-like cells. All samples were assessed by two masked investigators. Normally, an intermediate type of colony appears at day 8 and later. Because this colony typically develops from primitive EPC-CFUs, we evaluated each EPC-CFU by day 7 to detect the initial colony formation. The endothelial phenotype of each colony was detected by observing the uptake of 1,1'-diiododecyl-3,3,3',3'-tetramethylindocarbocyanine-labeled acetylated low density lipoprotein (DiI-acLDL; Biomedical Technologies, Stoughton, MA), and the binding of isolectin GS-IB₄ (isolectin GS-IB₄ from *Griffonia simplicifolia*, Alexa Fluor 477 conjugate; Molecular Probes). In addition, we used fluorescence microscopy (IX70; Olympus) to determine immunohistochemical staining of endothelial nitric oxide synthase (eNOS; BD Biosciences, San Jose, CA) or affinity-purified anti-mouse Flk-1 (eBioscience).

Statistical Analysis

All values were expressed as mean $\pm$ SE. Results were statistically analyzed (StatView 5.0 software program; Abacus Concepts Inc., Berkeley, CA). The Mann-Whitney *U* test was used for comparisons between the two groups. Kruskal-Wallis test was used for multiple comparison tests. *P* < 0.05 was considered statistically significant.

RESULTS

Biphasic Aspects of Serum VEGF Concentration in OIR

Serum VEGF concentration in the OIR group was lower than in the age-matched control group during the early phase (P12) (70.30 $\pm$ 3.82 pg/mL for OIR vs. 81.70 $\pm$ 3.73 pg/mL for

FIGURE 1. The kinetics of circulating VEGF and MNCs in OIR. (a) Serum VEGF level. * $P < 0.05$, *** $P < 0.001$, $n = 12$. (b) MNC number per 100 μ L WB. * $P < 0.05$, $n = 9$.

control; $P = 0.043$). However, higher levels were noted during the later phase (P17) (85.03 ± 3.20 pg/mL for OIR vs. 70.60 ± 2.14 pg/mL for control at P17, $P = 0.0009$) (Fig. 1a).

Aberrant EPC Mobilization in OIR

Compared with the control group, there was a significant decrease in the number of WB-MNCs in OIR at P12, followed by a subsequent increase at P14 (cell number/100 μ L WB: $0.66 \pm 0.05 \times 10^5$ for OIR vs. $0.86 \pm 0.05 \times 10^5$ for control at P12, $P = 0.034$; $0.84 \pm 0.09 \times 10^5$ for OIR vs. $0.56 \pm 0.04 \times 10^5$ for control at P14, $P = 0.023$) (Fig. 1b). Although the number of Sca-1⁺/c-Kit⁺ cells that were observed as circulating EPCs in the 100 μ L WB gradually increased and reached significance at 1.34-fold of the control at P17 (cell number/100 μ L WB: $1.47 \pm 0.09 \times 10^5$ for control vs. $1.97 \pm 0.21 \times 10^5$

for OIR), the frequency of the cell fraction in WB-MNCs was not statistically different between the groups (Fig. 2). These findings demonstrate that it is the secondary hypoxia that is responsible for inducing mobilization of the EPCs from the BM into the circulation.

Identification of BM-LS Cells as the EPC Fraction by Using EPC-CFA

Both primitive and definitive EPC-CFUs, which were produced from the BM-LS cells, were found to have endothelial phenotypes with both acLDL-Dil uptake and isolectin B4-binding positivities. Furthermore, immunocytochemical analysis of the EPC colonies showed there was positive expression for the endothelial antigen Flk-1 or eNOS (Figs. 3a, 3b).

FIGURE 2. Numerical assessment of circulating BM-derived EPCs in OIR. (a, b) The scatter diagram and the frequency of circulating EPCs (Sca-1⁺/c-Kit⁺ cells) in WB-MNCs when evaluated by FACS. (c) EPC number per 100 μ L WB. * $P < 0.05$, $n = 6$.

FIGURE 3. Differentiation profile of BM-derived EPCs in OIR. The profiles of the EPC colonies (primitive and definitive EPC-CFUs) from the BM-LS cell fraction. (a) Doubly stained with DiI-acLDL (red) and isolectin B4-FITC (green). (b) Immunohistochemical analysis showing positively stained endothelial surface antigen, eNOS or Flk-1 (green) with DAPI (blue). (c) EPC differentiation profile from BM-LS cell fraction in EPC-CFA. * $P < 0.05$, ** $P < 0.01$, $n = 18$.

In Vivo EPC Differentiation in BM-LS Cells of OIR When Evaluated by EPC-CFA

The transitional oxygen exposure in OIR did not affect the total number of EPC-CFUs at any of the time points (Fig. 3c). Of note for the OIR, the frequency of EPC-CFUs was significantly greater in the primitive EPC-CFUs but inversely lower in the definitive EPC-CFUs compared with the control group at P12 immediately after hyperoxia (primitive EPC-CFUs number/dish: 16.58 ± 0.54 for OIR vs. 13.76 ± 0.60 for control, $P = 0.002$; definitive EPC-CFUs/dish: 8.60 ± 0.58 for OIR vs. 10.52 ± 0.56 for control, $P = 0.022$).

In contrast, during responsive hypoxia, the frequency for the definitive EPC-CFUs in OIR was higher during the late phase at P17 (definitive EPC-CFUs number/dish: 11.94 ± 0.60 for OIR vs. 9.88 ± 0.70 for control, $P = 0.032$). These findings demonstrate that the definitive EPC colony-forming activity of the BM-LS cells was especially synchronized with the serum VEGF levels, which indicates there was a systemic effect for VEGF in OIR.

Incorporation of BM-Derived EPCs into Pathological and Physiological Retinal Vessels of OIR

After bone marrow transplantation, BM-derived GFP⁺ cells were costained with the retinal vasculatures by CD31 immunohistochemistry in the retinal flatmounts and in the cross-sections regardless of OIR induction, thereby identifying the BM-derived EPCs (Figs. 4a, 4b; Supplementary Movie S1, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-5880/-/DCSupplemental>). On the other hand, the GFP⁺ cells that were located outside the retinal vessels were morphologically detected as having arborescent shapes and were costained with CD11b (Fig. 4c). In addition, no GFP⁺/glial fibrillary acidic protein (GFAP; DAKO, Cambridge, UK; 1:2000) cells were found in either the control or the OIR retinas (data not shown). The BM-derived EPCs numerically exhibited distinctive features at each time point (Fig. 4d). Although the frequency of the BM-derived EPCs per retina within the intact vascular network of OIR was attenuated

FIGURE 4. Contribution of BM-derived EPCs in pathologic retinal vascular development in OIR. **(a)** BM-derived EPCs incorporated into the retinal vasculatures in the OIR model of BMT/EGFP/LS mice. *Arrowhead*: vascular tuft. GFP, *green*; CD31, *red*. **(b)** Retinal cross-section in OIR⁺ reveals the overlapping feature of BM-derived EPCs with retinal vessels. GFP, *green*. CD31, *red*. DAPI, *blue*. Scale bar, 50 μ m. **(c)** GFP⁺ cells were also detected outside the retinal vessels that were costained with CD11b. *Asterisk*: BM-derived EPCs. *Arrows*: BM-derived GFP⁺ cells located outside the vessels. GFP, *green*. *Top*: CD31, *red*. *Bottom*: CD11b, *red*. **(d)** Numerical assessment of incorporated BM-derived EPCs in the retinal vasculature per eye. *** $P < 0.001$, $n = 10$.

compared with the control group at P12 (4.429 ± 0.58 in OIR vs. 20.148 ± 1.81 in control, $P < 0.0001$), it was drastically enhanced at P17 not only within the intact vascular network (35.8 ± 4.91 at P17 vs. P12, $P < 0.0001$) but also in the tufts (30.2 ± 3.066 at P17 vs. 0 at P12, $P < 0.0001$). Collectively, the frequency of the GFP⁺ cells that were incorporated into the whole vasculatures at P17 was significantly increased in OIR compared with the control group (66.0 ± 6.30 in OIR vs. 31.8 ± 6.91 in control, $P = 0.0018$).

DISCUSSION

In the present study, our results showed there was a delayed mobilization and differentiation of the BM-derived EPCs resulting in an abnormal vascular development. These results were in line with the data observed for the hyperoxic to hypoxic conditions in the murine OIR model.

A number of studies have recently reported evidence for the involvement of BM-derived stem/progenitor cells in retinal vascular formation.^{24,31,32} Although these findings demon-

strated recruitment and incorporation of exogenously transplanted cells into the vasculature, these studies did not examine the endogenous kinetics of the cells.

Alternatively, previous evidence has reported a BM-derived EPC incorporation into the foci of pathologic vasculatures in the choroidal neovascularization animal model.^{33–36} However, a definitive classification of BM-derived EPC kinetics in the circulation relating to the pathologic vascular development of ischemic ocular or retinal diseases, including ROP, has yet to be investigated.^{37–39}

The pathophysiological aspect of OIR can be divided into two phases, a primary vaso-obliterative phase that occurs under an extrauterine relative hyperoxic environment and a later phase that is caused by retinal ischemia induced-aberrant neovascularization.^{8,9} Subsequently, the secondary normoxic circumstances that take place after birth cause pathologic neovascularization. Actually, in our study, the serum level of VEGF, as one of causative secretory proteins triggering OIR, fluctuated during the observed period of the OIR model compared with control. Considering this, it is easily hypothesized that BM-derived EPC kinetics may be affected by the extraordinary

circumstance of such proangiogenic growth factors or cytokines in OIR pathogenesis.

In the previous studies, we developed a murine EPC colony-forming assay (EPC-CFA) of BM-c-Kit⁺/Sca-1⁺/Lin⁻ (KSL) or BM-LS cells that made it possible to quantitatively estimate EPC differentiation.^{30,40} Therein, we were able to quantify the differences in the endothelial marker positivities between the primitive and the definitive EPC-CFUs.⁴⁰ FACS analysis disclosed that definitive EPC-CFUs exhibited higher positivities than primitive EPC-CFUs when the staining used endothelial antibodies of Flk-1 or Tie-2 but also acLDL-DiI and isolectin-B4-FITC.

Similarly, in our study of in vitro FACS analysis of colony cells from primitive or definitive EPC-CFUs from BM-KSL cells, we detected some markers with the different positivities to define the individual types of EPC-CFUs. Both colony cells had high positivity of leukocyte/monocyte markers of CD45 and CD11b. On the other hand, endothelial surface marker expression of Flk-1, Tie-2, or von Willebrand factor (vWF) was up-regulated in definitive versus primitive EPC-CFUs. In particular, the percentage positivity of vWF increased 3.7-fold in definitive EPC-CFUs. Thus, endothelial features detected by FACS were outstandingly exhibited according to the differentiation (Supplementary Fig. S1, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10.5880/-/DCSupplemental>).

These findings suggested that definitive EPC-CFUs with larger cell sizes might exhibit a greater differentiation than the primitive EPC-CFUs with smaller cell sizes, which could potentially make it possible to quantify EPC differentiation by simply counting each EPC-CFU. Based on these findings, we designed the present study to use EPC-CFA of the BM-KSL cells in the murine OIR model to investigate the differentiation features of BM-EPCs.

Interestingly, EPC differentiation was abrogated at P12 and then inversely promoted at P17. This presumably reflects the effect of the growth factors, such as VEGF, that are secreted from the pathologic region into the circulation.

We also discovered that at P17 in OIR, there was a drastically augmented recruitment of BM-derived EPCs into the pathologic vasculature. As a result, the functional BM-EPC kinetics in circulation, mobilization (Fig. 2), differentiation (Fig. 3), and tissue recruitment (Fig. 4) was considered distorted by original aberrant oxygen dynamics. Next, the issue of whether BM-derived EPCs favorably contribute to intact vascular development or pathologic vascular growth might be resolved to an appreciable extent by the data in Supplementary Fig. S2 (<http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10.5880/-/DCSupplemental>). Injected colony cells of definitive EPC-CFUs predominantly abrogated pathologic vascular growth, compared with primitive EPC-CFUs, concurrent with the greater involvement of the cells in vascular structures. Regarding the present data, colony cells of definitive EPC-CFUs from human umbilical cord blood have been recently demonstrated to exhibit functional vascular regeneration potential using a murine ischemic hind limb model versus primitive EPC-CFUs.⁴¹

Given these findings, delayed EPC kinetics, especially the functional EPC differentiation contributing to intact retinal vascular development by nature, is considered an important mechanistic insight into OIR together with local retinal ischemia caused by the secondary hypoxic phase. According to EPC differentiation at P17, BM-derived EPCs, locally incorporated in vascular tufts as well as in intact vasculatures (Fig. 4), might contribute to convert the pathologic vasculatures to intact retinal vascular development, which is usually recognized at the later stage of OIR.⁵ Collectively, BM-derived EPC kinetics is suggested to contribute to intact retinal vascular development under normal oxygen dynamics, thereby indicat-

ing that the distortion may give rise to the pathologic vascular growth in OIR.

The present study also provides the foundation for the possible use of FACS, EPC-CFA, or both to quantitatively or qualitatively assess the circulating EPC kinetics. Therefore, in the future, this methodology may potentially be used clinically to determine pathophysiology or prognosis in ROP patients.

At present, photocoagulation, medication, and surgical treatments have been developed and are used as effective therapeutic strategies. In some cases they have successfully abrogated the progression of ROP.⁴²⁻⁴⁷ In addition, strict oxygen control after birth has also been proposed to be a preventive threshold against ROP and has been shown to lead to overall satisfactory results.⁴⁸ Furthermore, the EPC kinetics documented in the present study may hold the key to developing a way to improving distorted EPC kinetics and thus provide an even more effective therapy for use in ROP patients.

In conclusion, the dyskinesia of mobilization and the retinal incorporation of BM-derived EPCs due to irregular oxygen dynamics appear to play a considerable role in ROP pathophysiology because they not only provide diagnostic or prognostic information, they might also hold the key to a potential novel therapeutic cue in ROP patients.

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References

- Steinkuller PG, Du L, Gilbert C, Foster A, Collins ML, Coats DK. Childhood blindness. *J AAPOS*. 1999;3:26-32.
- Patz A. The role of oxygen in retrolental fibroplasia. *Trans Am Ophthalmol Soc*. 1968;66:940-985.
- Flower RW, McLeod DS, Luttly GA, Goldberg B, Wajer SD. Postnatal retinal vascular development of the puppy. *Invest Ophthalmol Vis Sci*. 1985;26:957-968.
- Penn JS, Tolman BL, Lowery LA. Variable oxygen exposure causes preretinal neovascularization in the newborn rat. *Invest Ophthalmol Vis Sci*. 1993;34:576-585.
- Smith LE, Wesolowski E, McLellan A, et al. Oxygen-induced retinopathy in the mouse. *Invest Ophthalmol Vis Sci*. 1994;35:101-111.
- Connor KM, Krah NM, Dennison RJ, et al. Quantification of oxygen-induced retinopathy in the mouse: a model of vessel loss, vessel regrowth and pathological angiogenesis. *Nat Protoc*. 2009;4:1565-1573.
- Banin E, Dorrell MI, Aguilar E, et al. T2-TrpRS inhibits preretinal neovascularization and enhances physiological vascular regrowth in OIR as assessed by a new method of quantification. *Invest Ophthalmol Vis Sci*. 2006;47:2125-2134.
- Smith LE. Pathogenesis of retinopathy of prematurity. *Semin Neonatol*. 2003;8:469-473.
- Chen J, Smith LE. Retinopathy of prematurity. *Angiogenesis*. 2007;10:133-140.
- Pierce EA, Avery RL, Foley ED, Aiello LP, Smith LE. Vascular endothelial growth factor/vascular permeability factor expression in a mouse model of retinal neovascularization. *Proc Natl Acad Sci USA*. 1995;92:905-909.
- Donahue ML, Phelps DL, Watkins RH, LoMonaco MB, Horowitz S. Retinal vascular endothelial growth factor (VEGF) mRNA expression is altered in relation to neovascularization in oxygen induced retinopathy. *Curr Eye Res*. 1996;15:175-184.
- Pierce EA, Foley ED, Smith LE. Regulation of vascular endothelial growth factor by oxygen in a model of retinopathy of prematurity. *Arch Ophthalmol*. 1996;114:1219-1228.

13. Takagi H, Koyama S, Seike H, et al. Potential role of the angiopoietin/tie2 system in ischemia-induced retinal neovascularization. *Invest Ophthalmol Vis Sci.* 2003;44:393–402.
14. Ozaki H, Yu AY, Della N, et al. Hypoxia inducible factor-1 α is increased in ischemic retina: temporal and spatial correlation with VEGF expression. *Invest Ophthalmol Vis Sci.* 1999;40:182–189.
15. Smith LE, Shen W, Perruzzi C, et al. Regulation of vascular endothelial growth factor-dependent retinal neovascularization by insulin-like growth factor-1 receptor. *Nat Med.* 1999;5:1390–1395.
16. Smith LE, Kopchick JJ, Chen W, et al. Essential role of growth hormone in ischemia-induced retinal neovascularization. *Science.* 1997;276:1706–1709.
17. Stitt AW, Graham D, Gardiner TA. Ocular wounding prevents pre-retinal neovascularization and upregulates PEDF expression in the inner retina. *Mol Vis.* 2004;10:432–438.
18. Werdich XQ, McCollum GW, Rajaratnam VS, Penn JS. Variable oxygen and retinal VEGF levels: correlation with incidence and severity of pathology in a rat model of oxygen-induced retinopathy. *Exp Eye Res.* 2004;79:623–630.
19. Maeng YS, Choi HJ, Kwon JY, et al. Endothelial progenitor cell homing: prominent role of the IGF2-IGF2R-PLC β 2 axis. *Blood.* 2009;113:233–243.
20. Asahara T, Takahashi T, Masuda H, et al. VEGF contributes to postnatal neovascularization by mobilizing bone marrow-derived endothelial progenitor cells. *EMBO J.* 1999;18:3964–3972.
21. Masuda H, Kalka C, Takahashi T, et al. Estrogen-mediated endothelial progenitor cell biology and kinetics for physiological postnatal vasculogenesis. *Circ Res.* 2007;101:598–606.
22. Otani A, Dorrell MI, Kinder K, et al. Rescue of retinal degeneration by intravitreally injected adult bone marrow-derived lineage-negative hematopoietic stem cells. *J Clin Invest.* 2004;114:765–774.
23. Dorrell MI, Otani A, Aguilar E, Moreno SK, Friedlander M. Adult bone marrow-derived stem cells use R-cadherin to target sites of neovascularization in the developing retina. *Blood.* 2004;103:3420–3427.
24. Grant MB, May WS, Caballero S, et al. Adult hematopoietic stem cells provide functional hemangioblast activity during retinal neovascularization. *Nat Med.* 2002;8:607–612.
25. Yodoi Y, Sasahara M, Kameda T, Yoshimura N, Otani A. Circulating hematopoietic stem cells in patients with neovascular age-related macular degeneration. *Invest Ophthalmol Vis Sci.* 2007;48:5464–5472.
26. Fadini GP, Sartore S, Schiavon M, et al. Diabetes impairs progenitor cell mobilisation after hindlimb ischaemia-reperfusion injury in rats. *Diabetologia.* 2006;49:3075–3084.
27. Okabe M, Ikawa M, Kominami K, Nakanishi T, Nishimune Y. 'Green mice' as a source of ubiquitous green cells. *FEBS Lett.* 1997;407:313–319.
28. Hisatomi T, Sonoda KH, Ishikawa F, et al. Identification of resident and inflammatory bone marrow derived cells in the sclera by bone marrow and haematopoietic stem cell transplantation. *Br J Ophthalmol.* 2007;91:520–526.
29. Tamaki T, Uchiyama Y, Okada Y, et al. Functional recovery of damaged skeletal muscle through synchronized vasculogenesis, myogenesis, and neurogenesis by muscle-derived stem cells. *Circulation.* 2005;2857–2866.
30. Kwon SM, Suzuki T, Kawamoto A, et al. Pivotal role of Ink adaptor protein in endothelial progenitor cell biology for vascular regeneration. *Circ Res.* 2009;104:969–977.
31. Tanaka R, Wada M, Kwon SM, et al. The effects of flap ischemia on normal and diabetic progenitor cell function. *Plast Reconstr Surg.* 2008;121:1929–1942.
32. Otani A, Kinder K, Ewalt K, Otero FJ, Schimmel P, Friedlander M. Bone marrow-derived stem cells target retinal astrocytes and can promote or inhibit retinal angiogenesis. *Nat Med.* 2002;8:1004–1010.
33. Sengupta N, Caballero S, Mames RN, Butler JM, Scott EW, Grant MB. The role of adult bone marrow-derived stem cells in choroidal neovascularization. *Invest Ophthalmol Vis Sci.* 2003;44:4908–4913.
34. Sengupta N, Caballero S, Mames RN, Timmers AM, Saban D, Grant MB. Preventing stem cell incorporation into choroidal neovascularization by targeting homing and attachment factors. *Invest Ophthalmol Vis Sci.* 2005;46:343–348.
35. Tomita M, Yamada H, Adachi Y, et al. Choroidal neovascularization is provided by bone marrow cells. *Stem Cells.* 2004;22:21–26.
36. Takahashi H, Yanagi Y, Tamaki Y, Muranaka K, Usui T, Sata M. Contribution of bone-marrow-derived cells to choroidal neovascularization. *Biochem Biophys Res Commun.* 2004;320:372–375.
37. Chang KH, Chan-Ling T, McFarland EL, et al. IGF binding protein-3 regulates hematopoietic stem cell and endothelial precursor cell function during vascular development. *Proc Natl Acad Sci USA.* 2007;104:10595–10600.
38. Lofqvist C, Chen J, Connor KM, et al. IGFBP3 suppresses retinopathy through suppression of oxygen-induced vessel loss and promotion of vascular regrowth. *Proc Natl Acad Sci USA.* 2007;104:10589–10594.
39. Machalinska A, Modrzejewska M, Kotowski M, et al. Circulating stem cell populations in preterm infants: implications for the development of retinopathy of prematurity. *Arch Ophthalmol.* 2007;125:1311–1319.
40. Yang J, Li M, Kamei N, et al. CD34 cells represent highly functional endothelial progenitor cells in murine bone marrow. *PLoS One.* 2011;6:e20219.
41. Masuda H, Alev C, Akimaru H, et al. Methodological development of a clonogenic assay to determine endothelial progenitor cell potential. *Circ Res.* 2011;109:20–37.
42. Early Treatment for Retinopathy of Prematurity Cooperative Group. Revised indications for the treatment of retinopathy of prematurity: results of the early treatment for retinopathy of prematurity randomized trial. *Arch Ophthalmol.* 2003;121:1684–1694.
43. Azuma N, Ishikawa K, Hama Y, Hiraoka M, Suzuki Y, Nishina S. Early vitreous surgery for aggressive posterior retinopathy of prematurity. *Am J Ophthalmol.* 2006;142:636–643.
44. Kong L, Mintz-Hittner HA, Penland RL, Kretzer FL, Chevez-Barrios P. Intravitreal bevacizumab as anti-vascular endothelial growth factor therapy for retinopathy of prematurity: a morphologic study. *Arch Ophthalmol.* 2008;126:1161–1163.
45. Zamora DO, Davies MH, Planck SR, Rosenbaum JT, Powers MR. Soluble forms of EphrinB2 and EphB4 reduce retinal neovascularization in a model of proliferative retinopathy. *Invest Ophthalmol Vis Sci.* 2005;46:2175–2182.
46. Spandau UH, Sauder G, Schubert U, Hammes HP, Jonas JB. Effect of triamcinolone acetonide on proliferation of retinal endothelial cells in vitro and in vivo. *Br J Ophthalmol.* 2005;89:745–747.
47. Kusaka S, Shima C, Wada K, et al. Efficacy of intravitreal injection of bevacizumab for severe retinopathy of prematurity: a pilot study. *Br J Ophthalmol.* 2008;92:1450–1455.
48. Wright KW, Sami D, Thompson L, Ramanathan R, Joseph R, Farzavandi S. A physiologic reduced oxygen protocol decreases the incidence of threshold retinopathy of prematurity. *Trans Am Ophthalmol Soc.* 2006;104:78–84.