[2] Yamashita, J., Itoh, H., Hirashima, M., Ogawa, M., Nishikawa, S., Yurugi, T., Naito, M., Nakao, K., Nishikawa, S., et al. (2000) Flk1-positive cells derived from embryonic stem cells serve as vascular progenitors. Nature 408, 92–96.
[3] Sone, M., Itoh, H., Yamashita, J., Nakao, K., et al. (2003) Different differentiation kinetics of vascular progenitor cells in primate and mouse embryonic stem cells. Circulation 107, 2085–2088.
[4] Sone, M., Itoh, H., Nakao, K., et al. (2007) Pathway for differentiation of human embryonic stem cells to vascular cell components and their potential for vascular regeneration. Arterioscler. Thromb. Vasc. Biol. 27, 2127–2134.
[5] Yamahara, K., Sone, M., Itoh, H., Nakao, K., et al. (2008) Augmentation of Neovascularizaition in hindlimb ischemia by combined transplantation of human embryonic stem cells-derived endothelial and mural cells. PLOS One 3 (2), e1666.
[6] Takahashi, K. and Yamanaka, S. (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126, 663–676.
[7] Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K. and Yamanaka, S. (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131, 861–872.
[8] Yu, J., Vodyanik, M.A., Smuga-Otto, K., Antosiewicz-Bourget, J., Frane, J.L., Tian, S., Nie, J., Jonsdottir, G.A., Ruotti, V., Stewart, R., Slukvin II and Thomson, J.A. (2007) Induced pluripotent stem cell lines derived from human somatic cells. Science 318, 1917–1920.
[9] Nakagawa, M., Koyanagi, M., Tanabe, K., Takahashi, K., Ichisaka, T., Aoi, T., Okita, K., Mochiduki, Y., Takizawa, N. and Yamanaka, S. (2008) Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts. Nat. Biotechnol. 26, 101–106.
[10] Ebihara, K., Kusakabe, T., Masuzaki, H., Kobayashi, N., Tanaka, T., Chusho, H., Miyanaga, F., Miyazawa, T., Hayashi, T., Hosoda, K., Ogawa, Y. and Nakao, K. (2004) Gene and phenotype analysis of congenital generalized lipodystrophy in Japanese: a novel homozygous nonsense mutation in seipin gene. J. Clin. Endocrinol. Metab. 89 (5), 2360–2364.
[11] Ebihara, K., Masuzaki, H. and Nakao, K. (2004) Long-term leptin-replacement therapy for lipoatrophic diabetes. New Engl. J. Med. 351 (6), 615–616.
[12] Ebihara, K., Kusakabe, T., Hirata, M., Masuzaki, H., Miyanaga, F., Kobayashi, N., Tanaka, T., Chusho, H., Miyazawa, T., Hayashi, T., Hosoda, K., Ogawa, Y., DePaoli, AM., Fukushima, M. and Nakao, K. (2007) Efficacy and safety of leptin-replacement therapy and possible mechanisms of leptin actions in patients with generalized lipodystrophy. J. Clin. Endocrinol. Metab. 92 (2), 532–541.
[13] Ebihara, K., Ogawa, Y., Masuzaki, H., Shintani, M., Miyanaga, F., Aizawa-Abe, M., Hayashi, T., Hosoda, K., Inoue, G., Yoshimasa, Y., Gavrilova, O., Reitman, ML. and Nakao, K. (2001) Transgenic overexpression of leptin rescues insulin resistance and diabetes in a mouse model of lipoatrophic diabetes. Diabetes 50 (6), 1440–1448.
[14] Fujioka, T., Yasuchika, K., Nakamura, Y., Nakatsuji, N. and Suemori, H. (2004) A simple and efficient cryopreservation method for primate embryonic stem cells. Int. J. Dev. Biol. 48, 1149–1154.
[15] Dani, C., Smith, A.G., Ailhaud, G., et al. (1997) Differentiation of embryonic stem cells into adipocytes in vitro. J. Cell. Sci. 110, 1279–1285.
[16] Xiong, C., Xie, C.Q., Chen, Y.E., et al. (2005) Derivation of adipocytes from human embryonic stem cells. Stem Cells Dev. 14, 671–675.
[17] van Harmelen, V., Astrom, G., Ryden, M., et al. (2007) Differential lipolytic regulation in human embryonic stem cell-derived adipocytes. Obesity 15, 846–852.
[18] Barberi, T., Willis, L.M., Socci, N.D. and Studer, L. (2005) Derivation of multipotent mesenchymal precursors from human embryonic stem cells. PLoS Med. 2, e161.
[19] Olivier, E.N., Rybicki, A.C. and Bouhassira, E.E. (2006) Differentiation of human embryonic stem cells into bipotent mesenchymal stem cells. Stem cells 24, 1914–1922.
[20] Arner, P. (2005) Resistin: yet another adipokine tells us that men are not mice. Diabetologia 48, 2203–2205.
[21] Nakao, K. (2009) Adiposcience and adipotoxicity. Nat. Clin. Pract. Endocrinol. Metab. 5 (2), 63.
[22] Rosen, E.D. and Spiegelman, B.M. (2000) Molecular regulation of adipogenesis. Annu. Rev. Cell. Dev. Biol. 16, 145–171.
[23] Bernlohr, D.A., Bolanowski, M.A., Kelly Jr., T.J. and Lane, M.D. (1985) Evidence for an increase in transcription of specific mRNAs during differentiation of 3T3-L1 preadipocytes. J. Biol. Chem. 260, 5563–5567.
[24] Flier, J.S. (2004) Obesity wars: molecular progress confronts an expanding epidemic. Cell 116, 337–350.
[25] Zuk, P.A., Zhu, M., Mizuno, H., Huang, J., Futrell, J.W., et al. (2001) Multilineage cells from human adipose tissue: Implications for cell-based therapies. Tissue Eng. 7, 211–228.
[26] Zhu, Y., Liu, T., Song, K., Fan, X., Ma, X. and Cui, Z. (2008) Adipose-derived stem cell: a better stem cell than BMSC. Cell Biochem. Funct. 26, 664–675.

Induction and Isolation of Vascular Cells From Human Induced Pluripotent Stem Cells—Brief Report

Daisuke Taura, Masakatsu Sone, Koichiro Homma, Naofumi Oyamada, Kazutoshi Takahashi, Naohisa Tamura, Shinya Yamanaka, Kazuwa Nakao

Objective—Induced pluripotent stem (iPS) cells are a novel stem cell population derived from human adult somatic cells through reprogramming using a defined set of transcription factors. Our aim was to determine the features of the directed differentiation of human iPS cells into vascular endothelial cells (ECs) and mural cells (MCs), and to compare that process with human embryonic stem (hES) cells.

Methods and Results—We previously established a system for differentiating hES cells into vascular cells. We applied this system to human iPS cells and examined their directed differentiation. After differentiation, TRA1-60$^-$ Flk1$^+$ cells emerged and divided into VE-cadherin–positive and –negative populations. The former were also positive for CD34, CD31, and eNOS and were consistent with ECs. The latter differentiated into MCs, which expressed smooth muscle α-actin and calponin after further differentiation. The efficiency of the differentiation was comparable to that of human ES cells.

Conclusions—We succeeded in inducing and isolating human vascular cells from iPS cells and indicate that the properties of human iPS cell differentiation into vascular cells are nearly identical to those of hES cells. This work will contribute to our understanding of human vascular differentiation/development and to the development of vascular regenerative medicine. (*Arterioscler Thromb Vasc Biol.* **2009;29:1100-1103.**)

Key Words: angiogenesis ■ stem cells ■ vascular biology ■ endothelium ■ differentiation

Pluripotent embryonic stem (ES) cells are thought to represent a potentially unlimited pool from which to derive cells for new treatments in the area of regenerative medicine and for investigation of cell development/differentiation. We previously described the process by which mouse, monkey and human embryonic stem (ES) cells differentiate into vascular cells.[1,2,3] In addition, we used the hindlimb ischemia model with immunodeficient mice to demonstrate that transplanted vascular endothelial cells (ECs) and mural cells (MCs) derived from human (h)ES cells could be successfully incorporated into the host vasculature and significantly accelerate improvements in local blood flow.[3,4] However, immunologic and ethical problems remain to be overcome before clinical application.

Recently, novel ES cell-like pluripotent cells were generated from mouse skin fibroblasts by introducing 4 transcription factors.[5] Termed induced pluripotent stem (iPS) cells, they were subsequently generated from human skin fibroblasts.[6,7] At present, the properties of human iPS cell differentiation into vascular cells remain unknown. To address that issue, we investigated the differentiation of human iPS cells into ECs and MCs using our differentiation system previously developed for hES cells.

Materials and Methods

Cell Culture

hES, human iPS, and OP9 feeder cells were all established and maintained as described previously.[6,8,9] To induce differentiation, hES or iPS cells were cultured on an OP9 feeder layer as described previously.[3]

Flow Cytometry and Cell Sorting

Flow cytometric analysis and cell sorting were performed as described previously.[1,9]

Immunohistochemistry

Cultured cells were stained with various monoclonal antibodies as described.[1,9]

For details regarding cell culture, RT-PCR, and the antibodies used in flow cytometry and immunohistochemistry, please see the supplemental material (available online at http://atvb.ahajournals.org).

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From the Department of Medicine and Clinical Science (D.T., M.S., K.H., N.O., N.T., K.N.), Kyoto University Graduate School of Medicine; the Department of Stem Cell Biology (K.T., S.Y.), Institute for Frontier Medical Sciences, Kyoto University; and the Center for iPS Cell Research and Application (CiRA) (K.T., S.Y.), Institute for Integrated Cell-Material Sciences, Kyoto University, Japan.

Correspondence to Masakatsu Sone, MD, PhD, 54 Shogoin Kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan. E-mail sonemasa@kuhp.kyoto-u.ac.jp

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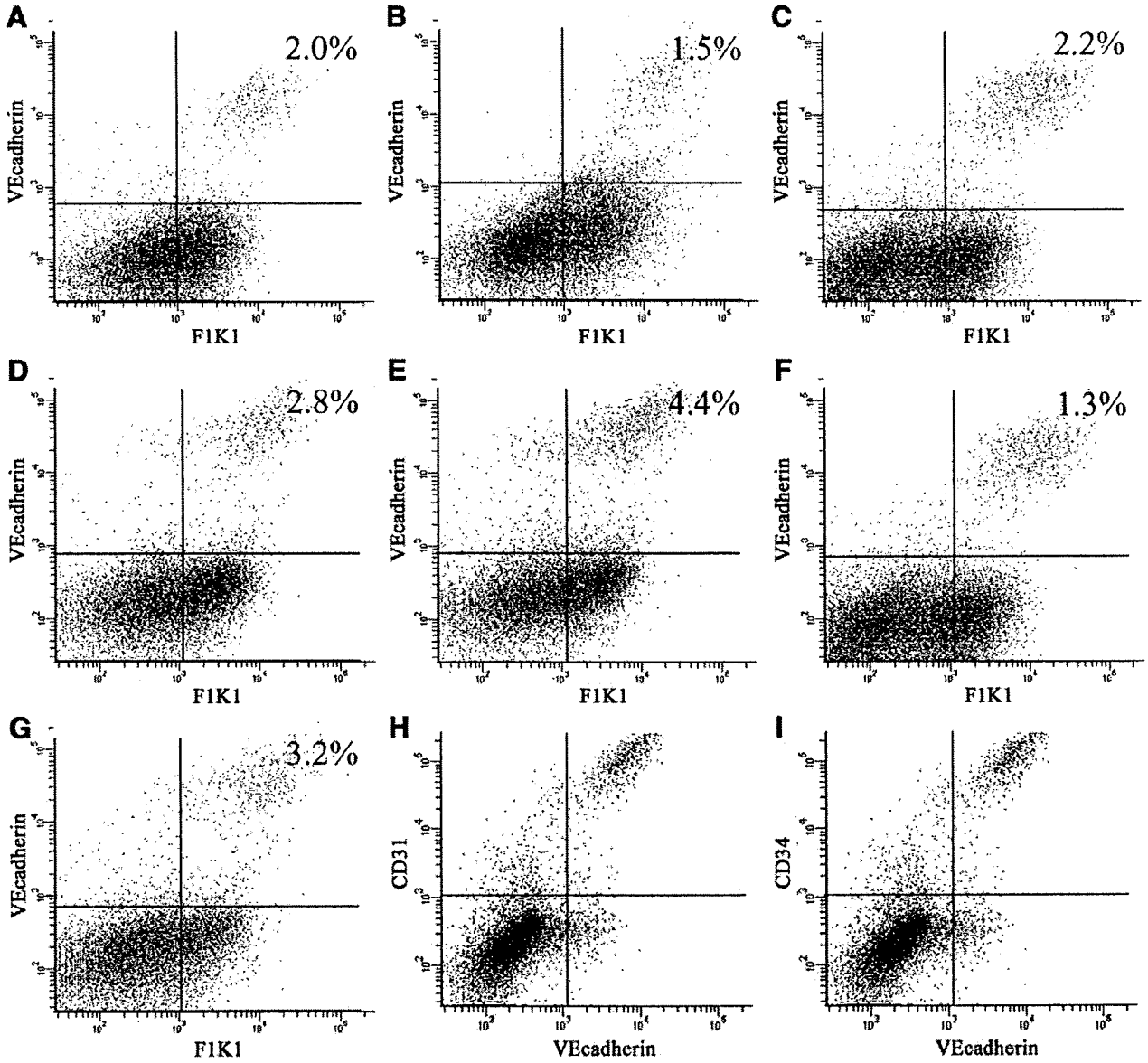

Figure 1. Flow cytometric analysis of hES-derived and iPS-derived cells on day 10 of differentiation. A through C, hES-derived cells (A, H9; B, HES3; C, KhES-1). D through G, iPS-derived cells (D, B6; E, B7; F, G1; G, G4). H and I, Analysis of human iPS-derived cells with other EC markers.

Results

We investigated 3 hES cell lines (H9, HES3, and KhES1) and 4 iPS cell lines (201B6, 201B7, 253G1, and 253G4).[5,6] 201B6 (B6) and 201B7 (B7) cells were generated from human skin fibroblasts by transfection with 4 transcription factors (Oct3/4, Sox2, Klf4, c-Myc), whereas 253G1 (G1) and 253G4 (G4) were generated using only 3 factors (c-Myc was omitted).[10] The morphology of these 4 lines did not differ from hES cells, and they were also positive for hES cell markers (supplemental Figure I). We induced differentiation of these iPS cell lines in an in vitro 2D culture system previously established for differentiation of hES cells into vascular cells.[3] After 10 days of differentiation, cells positive for Flk1 (also designated VEGF receptor-2) and the EC marker VE-cadherin emerged and accounted for 1% to 5% of the cells (Figure 1A through 1G). We noted no differences in the differentiation of the B and G lines, and both were comparable to the hES lines (Figure 1A through 1G). The $Flk1^+$ VE-$cadherin^+$ cell population was also positive for CD31 and CD34 (Figure 1H and 1I), and negative for the ES cell marker tumor rejection antigen 1–60 (TRA1–60). We sorted those cells and recultured with VEGF, and found that they formed a network-like structure on Matrigel, in vitro (Figure 2A), and had a cobblestone appearance when confluent on collagen IV-coated dishes (Figure 2B). Immunofluorescent staining for CD31 produced a characteristic marginal staining pattern (Figure 2C), and staining for endothelial NO synthase produced a cytoplasmic pattern (Figure 2D). Based on these observations, the cells were consistent with ECs. Subsequent RT-PCR analysis of EC markers revealed that both human iPS-derived and hES-derived ECs expressed

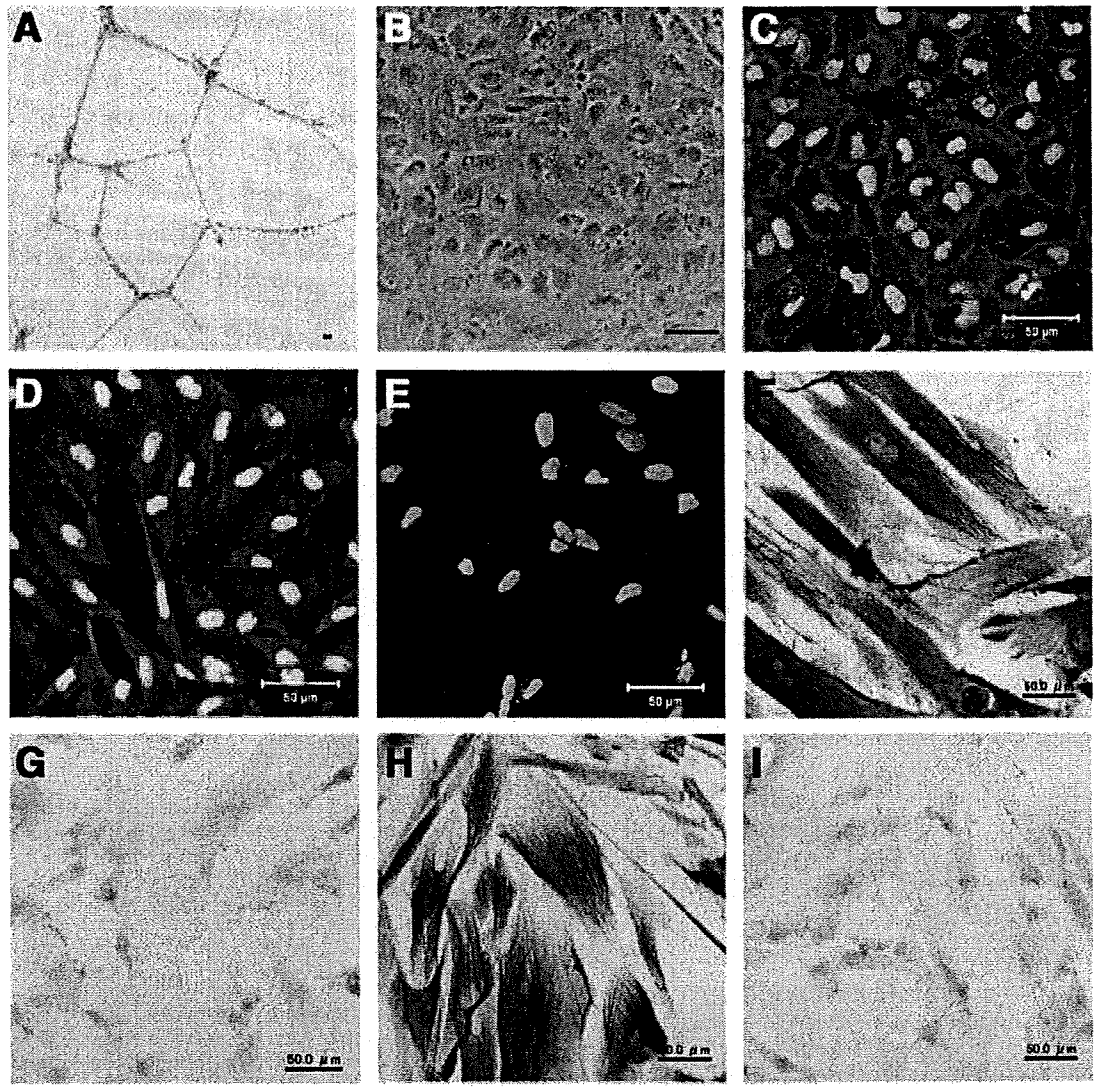

Figure 2. A, Network formation by iPS-derived VE-cadherin$^+$ cells after 24 hours of culture on Matrigel. B, Phase-contrast photomicrograph of iPS-derived VE-cadherin$^+$ cells. C through E, Immunostaining of VE-cadherin$^+$ cells: red, CD31 (C) or eNOS (D) or control mouse IgG1 (E; as negative control for C and D); green, nuclei. F through I, Immunostaining of VE-cadherin$^-$ Flk1$^+$ cells for MC markers: F, αSMA; G, control mouse IgG2 (negative control for F); H, calponin; I, control mouse IgG1 κ (negative control for H). Scale bars=50 μm.

VE-cadherin, CD31, von Willebrand factor (vWF), and CD34 at levels similar to those seen in adult ECs (supplemental Figure II).

We next sorted for Flk1$^+$ VE-cadherin$^-$ TRA1–60$^-$ cells on day 10 of differentiation and then induced differentiation into MCs using PDGF-BB as described previously.[3,4] Once differentiated, these cells stained positively for αSMA and calponin and were therefore consistent with MCs (Figure 2E and 2F). Both human iPS-derived and hES-derived MCs expressed the vascular smooth cell markers at levels similar to those seen in adult vascular smooth muscle cells (supplemental Figure II).

Discussion

The establishment of iPS cells opened a new avenue for regenerative medicine and stem cell biology. The directional differentiation of mouse iPS cells into vascular cells was recently reported.[11] In the present study, we have shown that human iPS cells can be directionally differentiated into vascular ECs and MCs by applying the same methods we established for hES cells.[3] We previously reported that the differentiation kinetics of primate ES cells to vascular cells is not equal to that of mouse ES cells.[2,3] To further clarify the differentiation process in human beings and to determine the possible clinical application of iPS cells, investigation of human iPS cells is essential because some characters were significantly different between mouse and human iPS cells as ES cells.

In contrast to human ES cells, iPS cells can be established from every human being irrespective of their genetic backgrounds. The establishment of in vitro differentiation system of human vascular cells from human iPS cells should make it possible to dissect out cellular mechanisms

in human vascular development and diseased states such as arteriosclerosis. The establishment of iPS cell lines from patients with inherited diseases presenting vascular abnormality should enable clarification of their pathogenesis. In addition, because they overcome the immunologic and ethical problems associated with human ES cells, our study should also contribute to the development of novel patient-specific cell based vascular regenerative therapies.

Several issues remain to be resolved before human iPS-derived vascular cells can be administered to humans, however. Although we observed no reappearance of undifferentiated or tumor cell-like structures in the in vitro cultures, when we examined the mRNA expression of the transgenes during differentiation experiments, we occasionally observed upregulation of the transgenic mRNA (supplemental Figure III). The safety of iPS cells needs to be confirmed for each iPS cell line, both in vitro and in vivo.

In conclusion, we succeeded in inducing and isolating human vascular cells from iPS cells and indicate that the properties of differentiation are nearly identical to those of hES cells. This work will contribute to our understanding of human vascular differentiation/development and to the development of vascular regenerative medicine.

We described additional discussions about the safety of iPS cells in the supplemental material.

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Disclosures

None.

References

1. Yamashita J, Itoh H, Hirashima M, Ogawa M, Nishikawa S, Yurugi T, Naito M, Nakao K, Nishikawa S. Flk1-positive cells derived from embryonic stem cells serve as vascular progenitors. *Nature*. 2000;408:92–96.
2. Sone M, Itoh H, Yamashita J, Yurugi-Kobayashi T, Suzuki Y, Kondo Y, Nonoguchi A, Sawada N, Yamahara K, Miyashita K, Park K, Shibuya M, Nito S, Nishikawa S, Nakao K. Different differentiation kinetics of vascular progenitor cells in primate and mouse embryonic stem cells. *Circulation*. 2003;107:2085–2088.
3. Sone M, Itoh H, Kenichi Yamahara, Yamashita J, Yurugi-K T, Nonoguchi A, Suzuki Y, Chao TH, Sawada N, Fukunaga Y, Miyashita K, Park K, Oyamada N, Sawada N, Taura D, Tamura N, Kondo Y, Nito S, Suemori H, Nakatsuji N, Nishikawa N, Nakao K, Pathway for differentiation of human embryonic stem cells to vascular cell components and their potential for vascular regeneration. *Arterioscler Thromb Vasc Biol*. 2007;27:2127–2134.
4. Yamahara K, Sone M, Itoh H, Yamashita J, Yurugi-K T, Homma K, Chao TH, Miyashita K, Park K, Oyamada N, Sawada N, Taura D, Fukunaga Y, Tamura N, Nakao K. Augmentation of Neovascularizaition in hindlimb ischemia by combined transplantation of human embryonic stem cells-derived endothelial and mural cells. *PLOS one*. 2008;3:e1666.
5. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell*. 2006;126:663–676.
6. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell*. 2007;131:861–872.
7. Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, Nie J, Jonsdottir GA, Ruotti V, Stewart R, Slukvin II, Thomson JA. Induced pluripotent stem cell lines derived from human somatic cells. *Science*. 2007;318:1917–1920.
8. Fujioka T, Yasuchika K, Nakamura Y, Nakatsuji N, Suemori H. A simple and efficient cryopreservation method for primate embryonic stem cells. *Int J Dev Biol*. 2004;48:1149–1154.
9. Hirashima M, Kataoka H, Nishikawa S, Matsuyoshi N, Nishikawa S. Maturation of embryonic stem cells into endothelial cells in an in vitro model of vasculogenesis. *Blood*. 1999;93:1253–1263.
10. Nakagawa M, Koyanagi M, Tanabe K, Takahashi K, Ichisaka T, Aoi T, Okita K, Mochiduki Y, Takizawa N, Yamanaka S. Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts. *Nat Biotechnol*. 2008;26:101–106.
11. Narazaki G, Uosaki H, Teranishi M, Okita K, Kim B, Matsuoka S, Yamanaka S, Yamashita J. Directed and systematic differentiation of cardiovascular cells from mouse induced pluripotent stem cells. *Circulation*. 2008;118:498–506.

KOKUYO セホ-50M