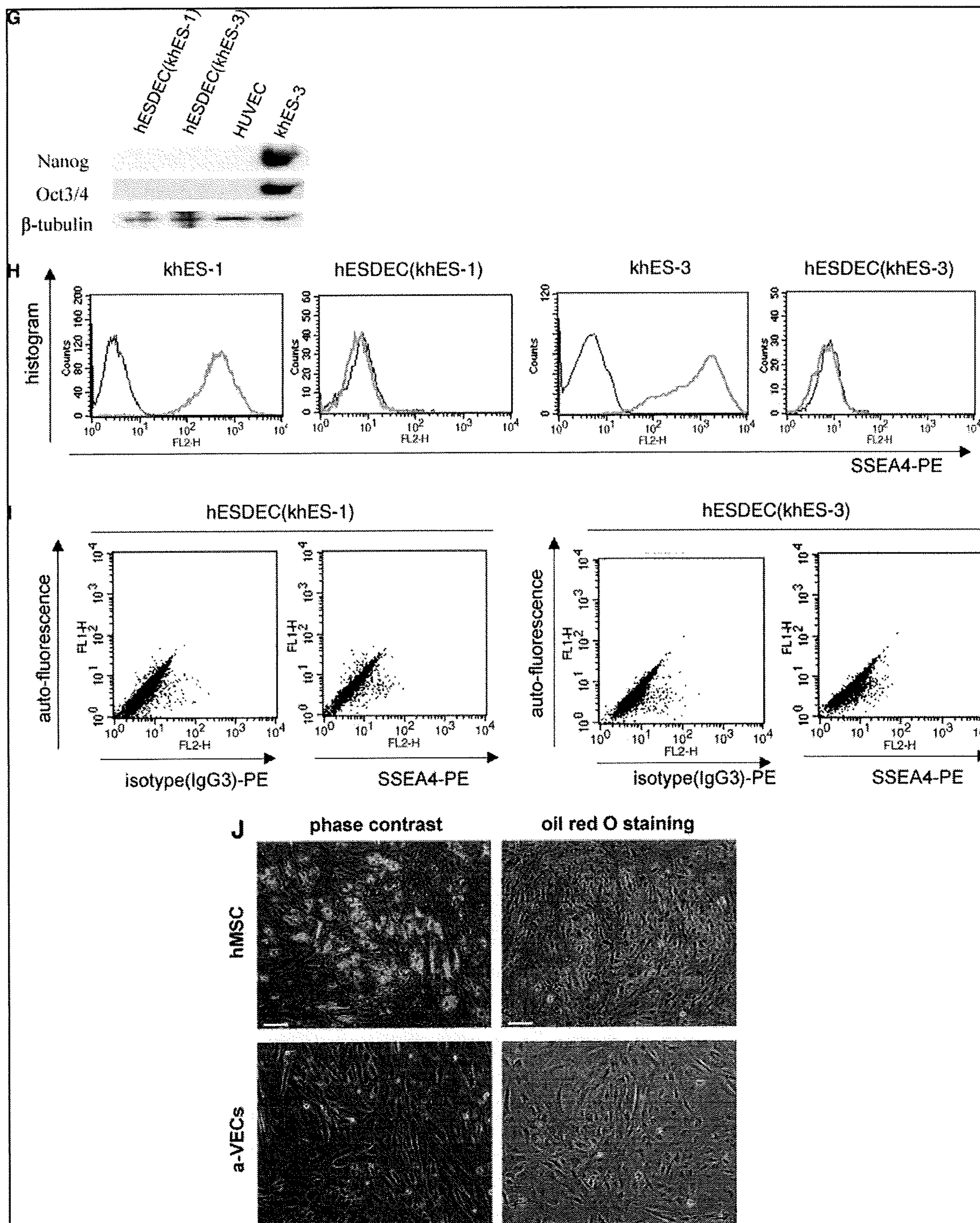

FIG. 3G–J. (*Continued*).

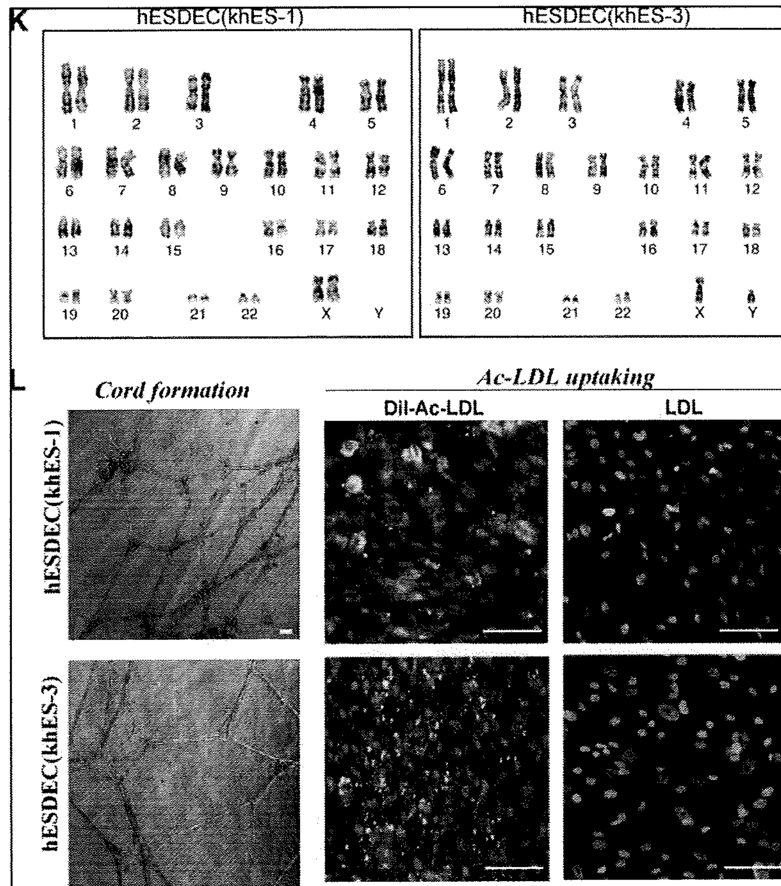

FIG. 3K–L. (*Continued*).

sion was detected by RT-PCR although its expression levels were considerably declined with an increment in passage number (Fig. 2D).

We further examined the expressions of other VEC markers including VEGF receptors, Tie-2, and CD34 in comparison with primary cultures of various human VECs including human glomerular endothelial cells (HGVEC), human dermal microvascular endothelial cells (HMVEC), human aortic endothelial cells (HAEC), and human umbilical vein endothelial cells (HUVEC). Tie-2 expression levels in khES-1-derived and khES-3-derived cells were higher than those of HMVEC and HGVEC. On the other hand, VEGF R2 expression was detectable only in minor populations of hESC-derived cells as shown by histogram analyses (Fig. 2E). Nevertheless, we could detect low-level expressions of VEGF R2 in majority of khES-1-derived and khES-3-derived cells by dot plot analyses (Fig. 2F). CD34 was absent in entire populations of khES-1-derived cells and in major parts of HGVEC, HMVEC, and HAEC, whereas it was expressed in about half populations of HUVEC and khES-3-derived cells. The expression levels of VEGF R1 were similar among all kinds of VECs (10–30%), while substantial variations were found in VEGF R3 expressions levels (10–70%). In general, hES-derived cells express various VEC markers, and variations in the expression levels of those markers between khES-1-derived and khES-3-derived cells were not significantly larger than those among human VECs derived from different tissues.

We next checked the contamination of hES-derived VECs by other lineage cells. Cogeneration of pericytes was ruled out by the absence of a mature smooth muscle cell marker of calponin-1 (Fig. 3A), a smooth muscle cell/macrophage maker of actin α-2 (Fig. 3B) and pericyte/mesenchymal stem cell marker of PDGF-Rβ (Fig. 3C). Coexistence of lymphatic endothelial cells was excluded by the absence of LYVE-1 and Prox-1 (Fig. 3D). Possibility that VE-cadherin/PECAM1-negative populations would be monocytic VEC progenitors was also excluded by the absence of a monocyte marker of CD14 (Fig. 3E) and a pan-leukocyte marker of CD45 (Fig. 3F). Contamination by immature hESCs was ruled out by the absence of immature hESC markers of Nanog (Fig. 3G), and Oct3/4 (Fig. 3G) as shown by Western blotting. This finding was further supported by flow cytometric analyses on SSEA-4 expressions: there was no significant differences between the isotype IgG3 control-staining pattern and the anti-SSEA-4 antibody-staining pattern in both khES-1-derived and khES-3-derived cells as demonstrated by histogram (Fig. 3H) and dot plots (Fig. 3I). Finally, the presence of mesenchymal stem cell-like populations was ruled out by the lack of adipocyte differentiation potentials of hES-derived

cells (Fig. 3J). All these findings together indicate that the khES-1-derived cells and khES-3-derived VE-cadherin/PECAM1-negative cells are a-VECs.

We also checked the chromosomal stability of the hESC-derived cells. As shown in Fig. 3K, no chromosomal abnormalities were detected in khES-1-derived cell or khES-3-derived cells, at least by karyotyping studies with G-banding.

Phenotypes of hESC-derived cells were stable during the subculture process: cord-forming activities and Ac-LDL-uptaking capacities at passage 10 (Fig. 3L) were comparable to those at passage 4 (Fig. 2A). Moreover, no significant morphological changes were observed until the cells entered senescence, when they became enlarged and flattened (data not shown). hESC-derived VECs were easily expanded by regular passages twice a week by 1:2 dilution. Accordingly, we could obtain 2×10^9 VECs from 6×10^6 hESCs after 10 passages.

From all these results, we concluded that our feeder-free differentiation method enabled pure production of VECs, which include c-VECs and a-VECs, from hESCs that were maintained under a feeder-free condition without inducing chromosomal abnormalities.

Evaluation of in vivo *functions of a-VECs*

Although the *in vitro* studies have confirmed the functional maturation of a-VECs, which bear cord-forming activities and Ac-LDL-uptaking capacities (Fig. 2A) and express vWF (Fig. 2B), eNOS (Fig. 2B), N-cadherin (Fig. 2C), and Tie-2

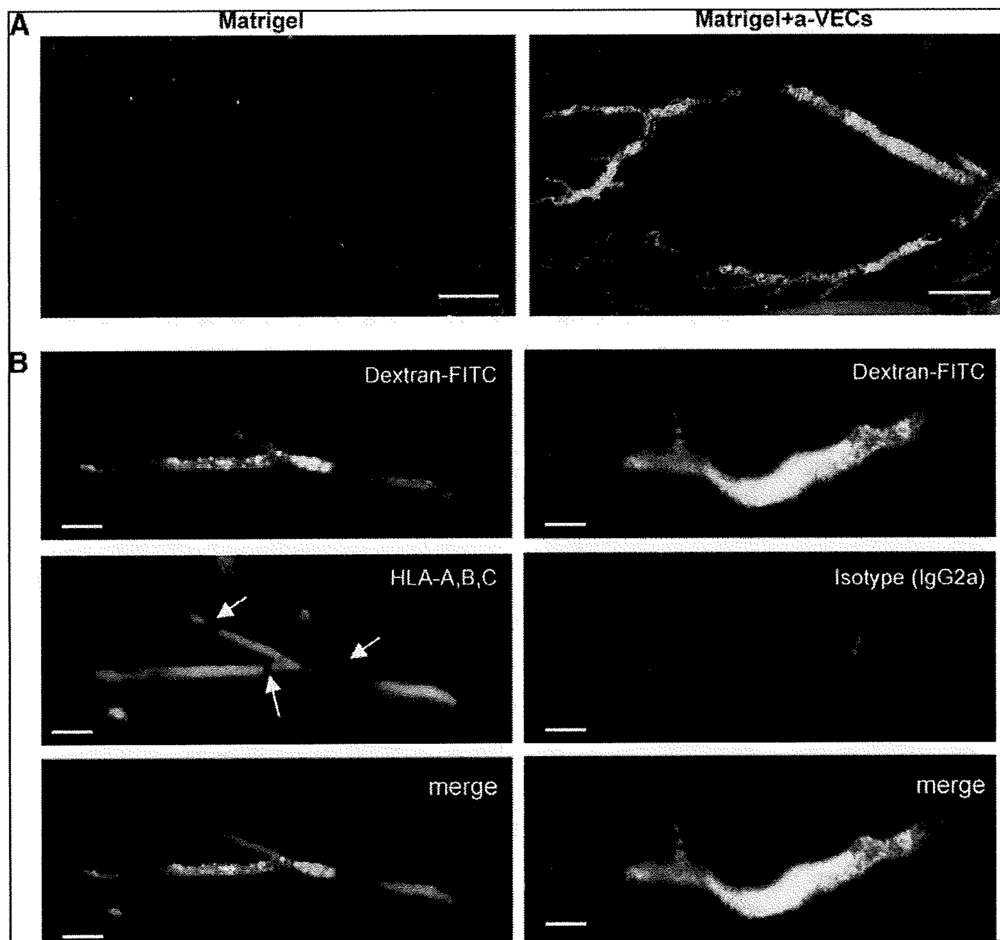

FIG. 4. Evaluation of neovascularity-forming activities of a-VECs in vivo. Matrigel plug assays. Matrigel plug assays were performed using khES-1-derived cells at passage 11 to passage 14 as described in Materials and Methods. (**A**) Detection of neovascularity. Effective neovascularization that was connected to a host circulation system was determined by the presence of FITC-dextran in the lumen (lower) while no neovascularity was detected by a sole Matrigel transplantation. Scale bars indicate 100 μm. (**B**) Detection of human cells. Immunostaining studies were performed using indicated antibodies. khES-1-derived cells were recognized by the staining using an anti-human HLA-A,B,C antibody. Note that majority of the areas were positively stained except for the regions shown by arrows. (**C**) PECAM1 expression. Matirgel plug tissues (upper three panels) or recipient mouse muscle tissues (lower tow panels) were subjected to immunostaining studies using anti-human PECAM1 antibody or anti-SMA antibody as indicated. Arrows indicate the autofluorescent images of erythrocytes. Scale bars indicate 50 μm.

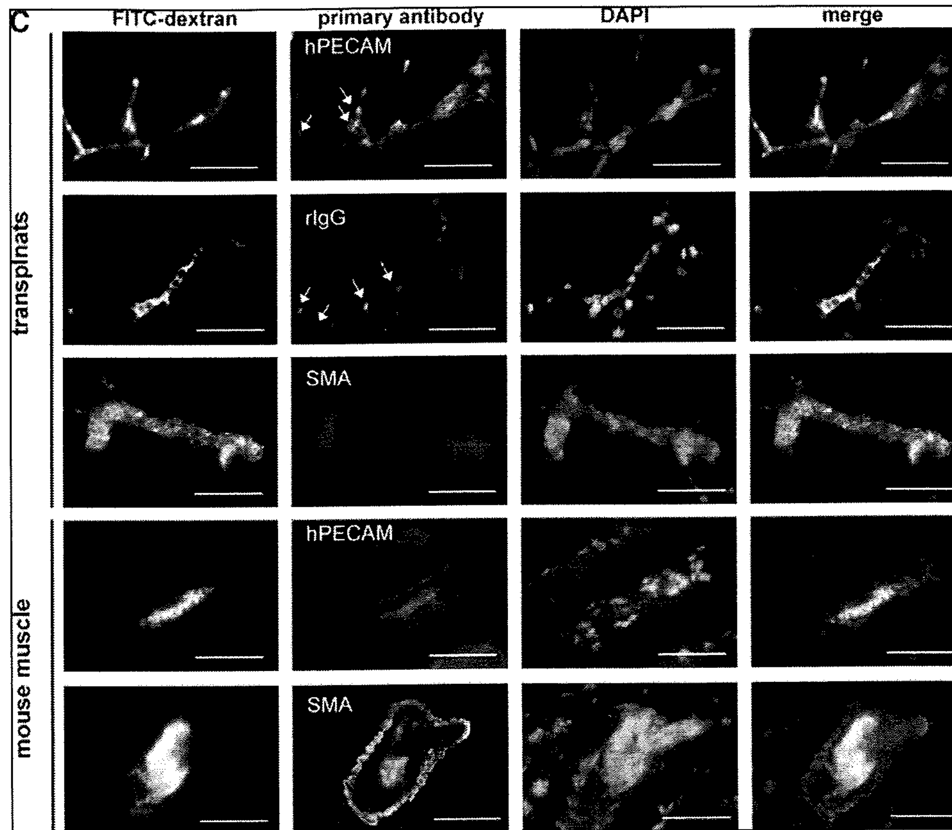

FIG. 4. (*Continued*).

(Fig. 2E), it is a crucial point whether they indeed demonstrate neovascular formation activities *in vivo* if we consider hESC-derived VECs as a tool of regenerative medicine.

To address this issue, we performed Matrigel plug assay using khES-1-derived a-VECs. As shown in Figure 4A, transplantation of khES-1-derived cells resulted in an effective formation of neovascularity that were linked with the host circulation system (Fig. 4A, lower). By contrast, transplantation of sole Matrigel did not induce a neovascular formation (Fig. 4A, upper). To confirm the recruitment of khES-1-derived cells into neovascularity, we determined the presence of human cells by immunostaining studies using an antihuman HLA-A, -B, -C antibody. We found that the majority of the cells lining at the neovascularity expressed human antigens as shown in a tangentially sectioned sample (Fig, 4B). The absence of the human HLA-A, -B, -C-staining in some areas (arrows in Fig. 4B, left middle) might be due to the recruitment of host murine VECs, which inevitably occurs during the connection between the khESC-1-derived neovascularity and the host circulation system. Unexpectedly, majority of the neovescularity-constituting cells were stained by human PECAM1 antibody (Fig. 4C, top panels). Because this antibody specifically recognizes human VECs and does not react with murine VECs (Fig. 4C, forth panelS from the top), it was suggested that a-VECs turned to express PECAM1 after transplantation. On the other hand, neovascular tissues were scarcely stained by anti-SMA antibody (Fig. 4C, third panels from the top), indicating that murine pericytes were not efficiently recruited into the neovascularity. Although this finding possibly raises an issue of impaired maturation of neovascularity, it at least guarantees that a-VECs would not transformed into pericytes even after transplantation. By contrast, VE-cadherin expression was not detected even after transplantation (data not shown).

Thus, hESC-derived a-VECs were functional *in vivo*, demonstrating effective recruitment into neovascularity in SCID mice.

Discussion

We reported our success in producing pure VECs from hESCs. Our system has three advantages. First, the process is completely free from xenogenic cells including an immature hESC-maintaining step. Second, it does not require cell-sorting techniques. Finally, the product does not contain immature hESCs or other lineage cells including pericytes and lymphatic endothelial cells. In our knowledge, there is no other method that satisfies all those conditions.

Our method had originally been established using cmESC. To apply this method to hESCs, we added a slight but technically critical modification to the original protocol. For sphere formation, cmESCs were incubated by a hanging drop culture. Although hanging drop culture has an advantage in generating homogenous sized spheres, it induced substantial

damages to hESCs. So, we tried simple floating culture using low attachment plates. Despite a considerable heterogeneity in sphere sizes, we successfully accomplished VEC differentiation of khES-3 with a comparable quality to cmESCs. Due to its higher feasibility, floating culture method has enabled us to handle larger amounts of hESCs at a time. The additional improvement is that we used the hESCs maintained under feeder-free conditions as starting materials, which is an advantage in view of clinical application.

By our system, both cell surface VE-cadherin/PECAM1-positive c-VECs and VE-cadherin/PECAM1-negative a-VECs were generated from primate ESCs. There is a large variation in the ratio of c-VECs to a-VECs among ESC lines: both cmESCs and khES-3 produces 20~30% of c-VECs, whereas khES-1 produced only a-VECs. Eventually, khES-1 scarcely produced c-VECs even on OP9 feeder layers (Fig. 1D). As for now, we do not know the reason for this variation. Recently, it was reported that there were marked differences in differentiation propensity among hESC lines (Osafune et al., 2008). Thus, an intrinsic character of each ESC line may affect the quality of VEC differentiation. As we reported previously, both cmESCs (Nakahara et al., 2008) and khES-3 (Saeki et al., 2009) effectively produced hematopoietic cells including functional neutrophils while khES-1 cells do not (Saeki et al., 2009). Common molecular signals are involved in the development of hematopoietic and endothelial cells (Lugus et al., 2005). It is widely accepted that adult hematopoietic cells are principally derived from VE-cadhein-positive hemogenic endothelial cells in mice (Lancrin et al., 2009; Taoudi et al., 2008), and the presence of hemogenic endothelial cells was also reported in human (Wu et al., 2007). As we showed in studies on cmESCs, hematopoietic cytokines have beneficial effects for an effective VEC differentiation (Saeki et al., 2008). From all those findings, we speculate that the hESC lines suited for hematogenesis are advantageous in producing c-VECs. Further studies will illuminate the molecular basis for the differentiation propensity.

Whether a-VECs have *in vivo* counterparts is an interesting and important issue. Our preliminary data indicate that primary cultures of human VECs including HUVEC and aortic endothelial cells (HAEC) contain minor fractions (<5%) of VE-cadherin-negative and/or PECAM1-negative populations, although the percentages of those fractions vary among lots. Eventually, a-VECs turned to express PECAM1 within 3 weeks after transplantation as we showed in Matrigel plug assays (Fig. 4D), suggesting that certain *in vivo* microenvironments induced the switching towards c-VECs. It might be possible that c-VECs and a-VECs could be interchangeable, depending on their microenvironments. Alternatively, there might be a spectrum between a-VECs and c-VECs. Further investigations are required for the evaluation of *in vivo* relevance of a-VECs.

Our preliminary data indicate that our differentiation method is applicable to human iPS cells (Gokoh, M., in preparation), providing a way to the realization of order-made regenerative medicine for vascular disorders. Before clinical application, however, the differentiation procedure must be improved to meet with the requirement of Good Manufacturing Practice. At present, we use following xenogenic materials: KSR™ and Matrigel™ Matrix during the maintenance culture of hESCs; FBS and porcine gelatin during the differentiation process. We have found that Matrigel™ Matrix can be substituted by a mixture of type IV collagen and laminin derived from human placenta (Nakahara, M., unpublished observations), and that FBS can be replaced by KSR™ although the expansion rates of VECs are diminished (Yogiashi, Y., and Gokoh, M., unpublished observations). Replacement of KSR™ by human serum and/or synthetic materials should be further tried in aim of clinical application.

Author Disclosure Statement

The authors declare no conflicting financial interests exist.

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