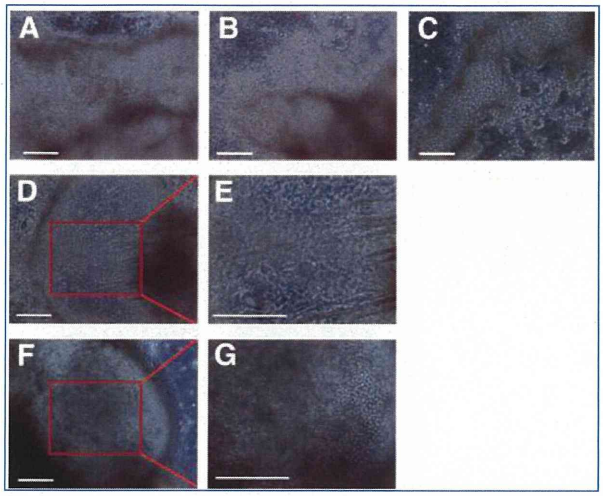

FIG. 3. Phase contrast microscopic observation during hematopoietic differentiation of hiPSCs. (**A–C**) Micrographs of the sac-like structures generated from 253G1 (**A**), 201B7 (**B**), and #25 (**C**). (**D**, **E**) Micrographs of the sac-like structures of 253G1 several days after manually puncturing the sac walls and releasing the inner hematopoietic cells. Note that the sac remained empty (**E**). (**F**, **G**) Micrographs of the sac-like structures of #25 several days after manually puncturing the sac walls and releasing the inner hematopoietic cells. Note that the sacs were refilled with hematopoietic cells (**G**). Scale bars indicate 200 μm.

sox2, klf-4, and c-my (OSKM) (Takahashi et al., 2007) and #25, which were established from human embryonic lung fibroblasts of MRC-5 by introducing retroviral transgenes of OSKM.

Among the five lines, 253G1, 201B7, and #25 successfully generated VECs. Although cell morphologies slightly differed from one another (Fig. 1A), we could effectively expand the VECs generated from all the three lines: the hiPSC-derived VECs were subcultured by 2:1 ~ 3:1 dilutions up to 10 passages, in the case of 201B7, or even higher, in the cases of 253G1 and #25 (Fig. 1B). Both 253G1-derived and #25-derived VECs expanded with comparable growth rates to hESCs-derived VECs, whereas the growth speed of 201B7-derived VECs was slightly lower. After 10 ~ 20 passages, the hiPSC-derived VECs underwent senescence as demonstrated by SA-β-galactosidase assays as in the cases of hESC-derived VECs and HUVEC (Fig. 1C). In agreement with this, the expressions of senescence-associated gene products of $p16^{INK4A}$ and/or $p21^{CIP1}$ were induced in the senesced cells (Fig. 1D). Using the cells at exponentially growing phases, we evaluated the functions and maker expressions. All the hiPSC-derived VECs showed high Ac-LDL-uptaking capacities (Fig. 2A) and cord-forming activities (Fig. 2B). Although the expressions of VE-cadherin and PECAM1 messages were hardly detectable in #25-derived VECs (Fig. 2C), these cells showed comparable protein expressions of eNOS (Fig. 2D), Tie-2 (Fig. 2E), and VEGFR1 (Fig. 2F) to the other hiPSCs-derived VECs and HUVEC.

Thus, hiPSCs can generate VECs with equivalent expansion potentials to hESCs, although maturation levels of hiPSCs-derived VECs vary depending on the lines of hiPSCs.

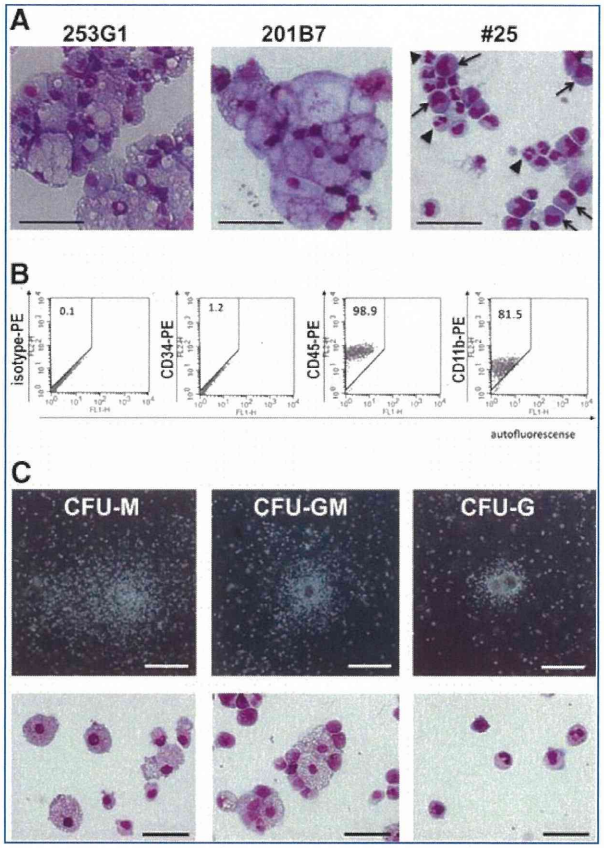

FIG. 4. Evaluation of hematopoietic potentials of hiPSC-derived cells (**A**) Cytological observation. Hematopoietic cells generated from hiPSCc (253G1, 201B7, and #25 as indicated) were stained by Wright-Giemsa solution. In #25-derived cells (right), azure granule-positive myeloid precursor cells (arrows) and segmented neutrocytes (arrow heads) were observed, whereas only macrophages were detected in 253G1-derived (left) and 201B7-derived (middle) cells. Scale bars indicate 50 μm. (**B**) Flow cytometry. The #25-derived hematopoietic cells were subjected to flow cytometric analyses using antihuman CD34, CD45, and CD11b antibodies as indicated. Number on each figure indicates the percentage of the corresponding marker-positive cells. (**C**) Colony assays. The #25-derived hematopoietic cells were subjected to colony assays. CFU-M, CFU-GM, and CFU-G were determined by phase contrast microscopic observation (upper panels; scale bars indicate 500 μm), which were further confirmed by Wright-Giemsa staining studies (lower panels; scale bars indicate 50 μm).

hiPSCs can generate reproducible HPCs with comparable colony-forming activities to hESCs

We previously established a feeder-free method for the neutrophil differentiation of hESCs (Saeki et al., 2009). By our system, HPCs are generated within a unique construction named the "sac-like structure." Within this structure, HPCs are repeatedly generated: they are reproduced within a few days after manually puncturing the sac walls and releasing the inner HPCs into the culture supernatant (Saeki et al., 2009). The reproduction process can be repeated three or four times (Saeki et al., 2009).

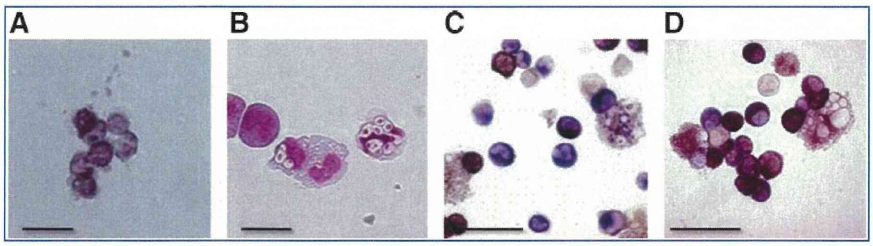

FIG. 5. Functional assessments and special staining. (**A, B**) The #25-derived hematopoietic cells were subjected to an NBT-reducing assay (**A**) or phagocytosis assay (**B**). Almost all the cells showed NBT-reducing activities (**A**) and zymosan-phagocytizing myeloid cells were often observed (**B**). Scale bars indicate 20 μm. (**C, D**) The #25-derived hematopoietic cells (**C**) or hESC (khES-3)-derived hematopoietic cells (**D**) were subjected to double esterase staining assay. Note that typical neutrophil-specific dark blue staining patterns were clearly observed in #25-derived cells, whereas hESC-derived hematopoietic cells showed rather mixed (brownish blue) staining patterns. Scale bars indicate 50 μm.

By applying this method, we performed neutrophil differentiation of 251G3, 201B7, and #25. All three lines successfully generated sac-like structures that were filled with abundant spheroid cells (Fig. 3A–C). However, the walls of 201B7-derived sacs seemed rather fragile because the inner spheroid cells spontaneously permeated the sac-like structures (Fig. 3B). Furthermore, we failed in reproducing spheroid cells after manually puncturing the sac walls and releasing inner spheroid cells into the culture supernatant (data not shown). In the case of 253G1, the sac walls seemed solid; however, spheroid cells were scarcely reproduced after sac wall puncturing (Fig. 3D and E). In contrast, spheroid cells were actively reproduced in the case of #25 (Fig. 3F and G). Eventually, the reproduction process persisted up to 60 days after the start of differentiation (data not shown), which was longer than that of hESCs (up to 40 days) (Saeki et al., 2009). Thus, the #25 line bears an equivalent, or even higher, spheroid cell-reproducing potential to hESCs.

We further evaluated the qualities of hematopoietic differentiation of 253G1, 201B7, and #25. First, cytological examinations were performed around day 30 of differentiation, when abundant neutrophil production was observed in the case of hESCs (Saeki et al., 2009). However, vast majorities of the products of 253G1 and 201B7 were macrophages (Fig. 4A, left and middle). On the other hand, various stages of granulocyte-lineage cells, from azurophilc granule-positive myeloid progenitors to segmented granulocytes, were observed in the case of #25 (Fig. 4A, right). Flow cytometric analyses demonstrated that #25-derived spheroid cells were highly positive for CD45, a pan-hematopoietic cell marker, and the majority of cells were positive for CD11b, a granulomonocytic marker (Fig. 4B). To confirm their hematopoietic activities, colony assays were performed (Fig, 4C). The # 25-derived spheroid cells demonstrated equivalent, or even higher, colony-forming unit-granulocyte (CFU-G) ($8.0 \pm 5.3/10^4$ cells; $n = 3$), colony-forming unit-granulocyte/macrophage (CFU-GM) ($12.3 \pm 5.5/10^4$ cells; $n = 3$), colony-forming unit-macrophage (CFU-M) ($21.0 \pm 3.5/10^4$ cells; $n = 3$) to those of hESCs, whose average CFU-G, CFU-GM, and CFU-M per 10^4 cells were 2.3, 7.9, and 3.1, respectively ($n = 2$). Thus, hiPSCs can produce HPCs with equivalent colony-forming activities to hESCs.

We also confirmed the functional maturation of #25-derived neutrophils by performing a superoxide production study (Fig. 5A), a phagocytosis assay (Fig. 5B), and double esterase-staining test (Fig. 5C). Interestingly, the double-esterase staining test, in which neutrophil-specific esterase is stained blue while that of nonspecific monocyte/macrophage esterase is stained brown, demonstrated that #25-derived cells showed even clearer neutrophil-specific blue staining patterns than hESC-derived ones, the majority of which showed brownish blue or bluish brown staining (Fig. 5D). Thus, hiPSCs can generate neutrophil with equivalent, or even higher, maturation levels than hESCs.

For further assessment of possible hematopoietic potentials of 253G1-derived and 201B7-derived spheroid cells, we checked the morphologies of spheroid cells over time. Surprisingly, small round cells with high nucleus/cytoplasm

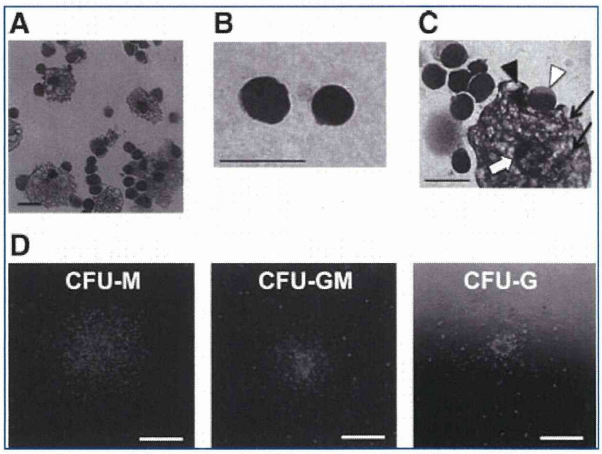

FIG. 6. The hiPCS-derived differentiated cells at early phases. (**A–C**) The 253G1-derived nonadherent cells at day 21 of differentiation were collected and stained by Wright-Giemsa solution. Abundant small cells with particularly high nuclear cytoplasmic ratios were detected (**A, B**). Note that the macrophage contained condensed nucleus-like substances (closed arrow head) and/or chromatin-like substances (arrows) in addition to its own nucleus (open arrow). Small cells were occasionally encompassed by the cytoplasm of macrophages (open arrow head). Scale bars indicate 20 μm. (**D**) The 253G1-derived nonadherent cells at day 23 of differentiation were subjected to colony assays. CFU-M, CFU-GM, and CFU-G were determined by phase contrast microscopic observation. Scale bars indicate 500 μm.

ratios were detected in 253G1-derived samples around day 20 of differentiation (Fig. 6A and B), whereas no such cells were detected in 201B7-derived samples (data not shown). Interestingly, hemophagocytosis-like scenes, where the small cells were phagocytized by macrophages, were often observed (Fig. 6C). Because the morphologies of the small cells resembled to hematopoietic stem/progenitor cells, we checked their colony-forming activities (Fig. 6D). Colony assays performed around day 20 indicated that 253G1-derived cells had comparable CFU-G ($3.3 \pm 2.3/10^4$ cells; $n=3$), CFU-GM ($3.3 \pm 1.2/10^4$ cells; $n=3$), CFU-M ($15.0 \pm 1.0/10^4$ cells; $n=3$) to those of hESCs. On the other hand, few hematopoietic colonies were observed in the case of 201B7 at any time points (data not shown).

Thus, hiPSCs can generate reproducible HPCs with equivalent colony-forming activities to hESC-derived HPCs, although some lines of hiPSCs suffer from defective hematopoietic differentiation.

Discussion

In this article, we have provided the counterexamples to a previously reported finding that hiPSC-derived hemangioblast, the common progenitor of hematopoietic and endothelial cells, suffered from early senescence. In that report, hiPSC-derived HPCs was shown to have substantially decreased colony-forming activities and the majority of hiPSC-derived endothelial cells senesced after one passage (Feng et al., 2010). However, our data have clearly shown that the issue of early senescence can be overcome by selecting appropriate lines of hiPSCs and applying proper differentiation methods to them. Moreover, our results proved that retroviral insertion of reprogramming transgenes was not the cause of early senescence contrary to the discussion by the authors (Feng et al., 2010). We have also shown that, after sequential passages, hiPSC-derived VECs enter senescence as in the cases of hESC-derived VECs and primary human VECs, guaranteeing that hiPSC-derived VECs bear very low tumorigeneity, if any.

The key to our success in producing hiPSC-derived VECs that bear as high growth potentials as hESC-derived counterparts may reside, at least in part, in our usage of multiple hematopoietic cytokines in addition to VEGF. As we have shown previously, the six cytokines, SCF, IL6, IL3, BMP4, Flt3-L, and VEGF, as a whole work for the stable and high-purity production of subculturable VECs (Saeki et al, 2008). Interestingly, we are also observing that, under serum-free conditions, the presence of hematopoietic cytokine cocktail is crucial for the formation of spheres and their subsequent growth on gelatin-coated plates (M.N., unpublished finding). Thus, the usage of hematopoietic cytokine cocktail is advantageous not only for an achievement of high-efficiency differentiation but also survival and proliferation of the differentiated cells. Alternatively, the differentiation process per se, which is often followed by apoptosis, might include antiapoptotic processes as far as the differentiated cells keep surviving. In any event, stressful conditions should be avoided as much as possible from the differentiation procedures of hESCs/hiPSCs as in the case of their maintenance culture, where chromosomal aberrations are reportedly induced via stressful handling of the cells (Draper et al., 2004, Mitalipova et al., 2005).

As we mentioned, two lines of hiPSCs, 253G4 and 201B2, failed in directed differentiation into VECs. The 253G4- and 201B2-derived cells showed poor cord-forming activities and lacked VEC marker expressions, although they possessed Ac-LDL-uptaking capacities and were subculturable over 10 passages (data not shown). Their disadvantageous natures concerning VEC differentiation may be resulted from the possible line dependency in differentiation propensity among hiPSCs as reported in the case of hESCs (Osafune et al., 2008). Indeed, 253G4 and 201B2 showed very poor or no hemaotopoietic differentiation (data not shown). The finding that hiPSC lines with poor VEC-differentiating potentials bear little hemaotocyte-producing capacities seems very reasonable, because hematopoietic cells are derived from a specific population of vascular endothelial cells (Eilken et al., 2009).

Our findings together indicate that, although hiPSCs may be imposed line-dependent limitations in their differentiation capacities, they are not put inevitable fates of differentiation-dependent early senescence.

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Author Disclosure Statement

The authors declare that no conflicting financial interests exist.

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