maintained as described previously [4]. Briefly, every 5–6 days, undifferentiated cells of both cell lines were detached with dissecting pipettes and transferred to dishes with mitomycin C-treated mouse feeder cells. All endothelial cells used in this study were not passaged more than five times.

2.2. Induction of differentiation

Undifferentiated human ES or iPS cells were harvested and transferred to a collagen I-coated dish after adjusting the colonies to an appropriate size. On the second day of incubation, the culture medium was replaced with human ES/iPS cell maintenance medium without basic FGF, supplemented with N2 supplement (Invitrogen)/B27 supplement (Invitrogen) and BIO (SIGMA). Thereafter, the cells were incubated for another 3 days, at which time the culture medium was replaced with StemPro-34 SFM (Invitrogen) supplemented with VEGF (50 ng/ml; PeproTech EC Ltd). After another 3–5 days of incubation, Flk1/VE-cadherin+/+ cells were sorted using FACSAria flow cytometer and used for the following experiments. Sorted cells were confirmed to remain VE-cadherin positive during the following cell culture and analyses.

2.3. Immunohistochemistry

Cultured cells were stained with an anti-VE-cadherin antibody (BV-9, Abcam) [9], or the indicated monoclonal antibodies as described [4].

2.4. MTT assay

Cell proliferation was assessed in colorimetric 3,4,5-dimethylthiazol-2-yl-2,5-diphenyl tetrazolium bromide (MTT) assays carried out as described previously [10]. Briefly, cells were incubated with MTT (Nakarai Tesque, Kyoto, Japan) solution for 4 h, after which the medium was discarded. The remaining dye was then dissolved in dimethyl sulfoxide, and the absorbance was measured at 570 nm.

2.5. In vitro wound healing assay

Wound healing assays were carried out as we described previously [11]. Briely, ECs were grown to overconfluence in six-well plates, after which a wound approximately 2 mm in width was made with a cell scraper. The wound was then allowed to heal (re-endothelialize) for 24 h in the same medium. The wounded monolayer was photographed before and after the incubation period, and the area of re-endothelialization was evaluated.

2.6. Annexin V assay

Confluent monolayers (80–90%) of ECs grown in 6-well plates were treated with or without 300 μmol/l H_2O_2. After 8 h of exposure, annexin V-FITC in combination with Via-Probe was used to quantitatively determine the percentage of cells undergoing apoptosis, as described previously [12]. Briefly, after treating the cells with the indicated reagents, the monolayer was detached by a brief incubation with trypsin–EDTA solution. Aliquots of cells (10^5) were then resuspended in 1× binding buffer (BD Pharmingen, San Diego, CA) and incubated with annexin V-FITC for 15 min at room temperature in the dark, stained with Via-Probe, and analyzed within 1 h in a FACSAria flow cytometer. FACS Diva software (Becton Dickinson) was used to analyze the data. Early apoptotic cells were stained with annexin V only, while late apoptotic or necrotic cells were stained with both annexin V and Via-Probe.

2.7. Quantitative real-time PCR

Total RNA was isolated using an RNeasy® Mini Kit and treated with an RNase-free DNase set (QIAGEN, Germany) to remove any contaminating genomic DNA. Quantitative real-time PCR was then performed using Premix ExTaqTM(Takara Bio Inc., Shiga, Japan). The PCR primers used were as follows: for Sirt1, GCCTCACATGCAAGCTCTAGTGAC(forward) and TTCGAGGATCTGTGCCAATCATAA(reverse); for Delta-like 4 (DLL4), GTGGACTGTGGCCTGGACAA(forward) and AGCATATCGCTGATATCCGACACACTC(reverse); for CXCR4, GCCAACGTCAGTCAGTGAGGCAGA(forward) and GCCAACCATGATGTGCTGAAAC(reverse) and for β-actin, CATCCGTAAAGACCTCTATGCCAC(forward) and ATGGAGCCACCATCCACA(reverse). All primers were produced by Takara Bio. Levels of Sirt1, DLL4 and CXCR4 mRNA are presented after normalization to the level of β-actin mRNA.

2.8. Western blot analysis

Western blotting was carried out using a standard protocol described previously [13]. Anti-Sirt1 antibody was purchased from Santa Cruz Biotechnology, Inc.

2.9. siRNA transfection

Small interference RNA (siRNA) against Sirt1 and negative control were provided by Qiagen. The target sequence for the Sirt1 siRNA was 5′CAA GCG ATG TTT GAT ATT GAA3′. ECs were trypsinized, washed with Hank's balanced salt solution, resuspended (5×10^5 cells) in human umbilical vein endothelial cell solution (Amaxa Biosystems) containing 3 μg of siRNA duplex, and then transfected using a Nucleofector (Amaxa Biosystems) following the manufacturer's instructions. After transfection, the cells were immediately plated in dishes.

2.10. Endothelial tube formation assay

Endothelial tube formation was assayed as described previously [14]. ECs (20,000 cells/well) were seeded into matrigel-coated 24-well plates. The cells were then incubated for 12 h at 37 °C, after which the formed tubes were digitally imaged and analyzed using MetaMorph software (Universal Imaging Corp.).

2.11. Statistical analysis

Results are presented as means ± SEM. Differences between groups were analyzed using ANOVA followed by Fisher's analysis for comparisons between two means. Values of $P < 0.05$ were considered significant.

3. Results

3.1. Morphological comparison of HAECs, ESECs and iPSECs

HAECs, ESECs and iPSECs were morphologically similar in that they all exhibited a cobblestone-like appearance on culture dishes, were positive for eNOS, and showed a marginal staining pattern when stained for CD31 and VE-cadherin (Fig. 1). All of these features are characteristic of vascular endothelial cells. Furthermore, HVECs, an example of venous endothelial cells, were analyzed together with HAECs, ESECs and iPSECs by real-time PCR for the expression of arterial endothelial marker genes, DLL4 and CXCR4. The expression levels of DLL4 and CXCR4 in both ESECs and iPSECs were comparable with those in HAECs and higher than those in HVECs

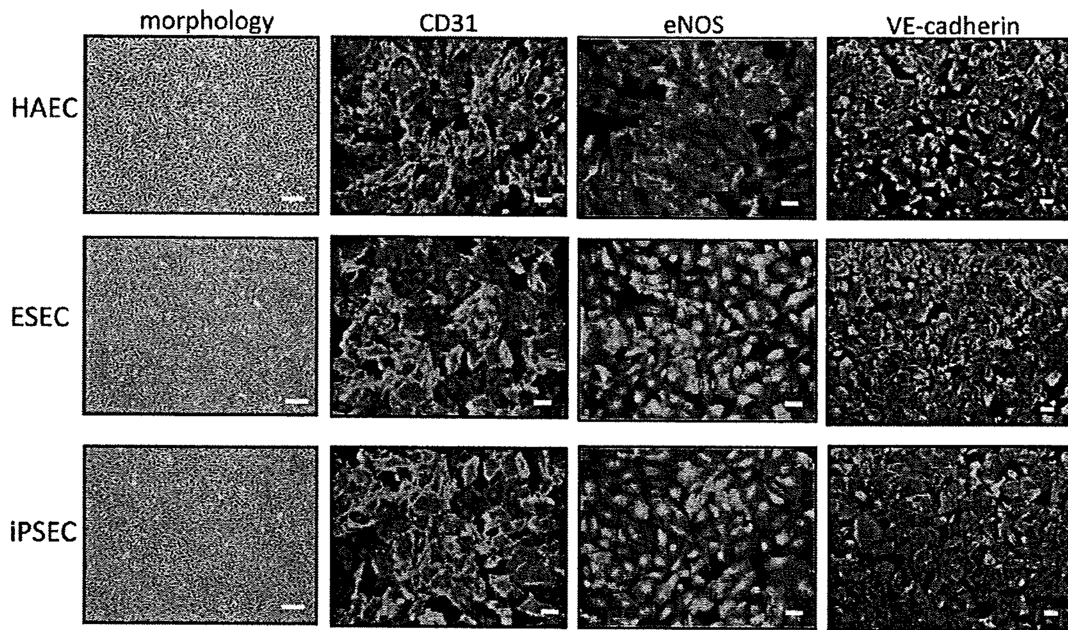

Fig. 1. Phenotypes of HAECs, ESECs and iPSECs. Typical morphology of an EC monolayer and immunofluorescence staining of the monolayer for CD31, eNOS and VE-cadherin; cell nuclei are stained with DAPI. Scale bar, 100 μm.

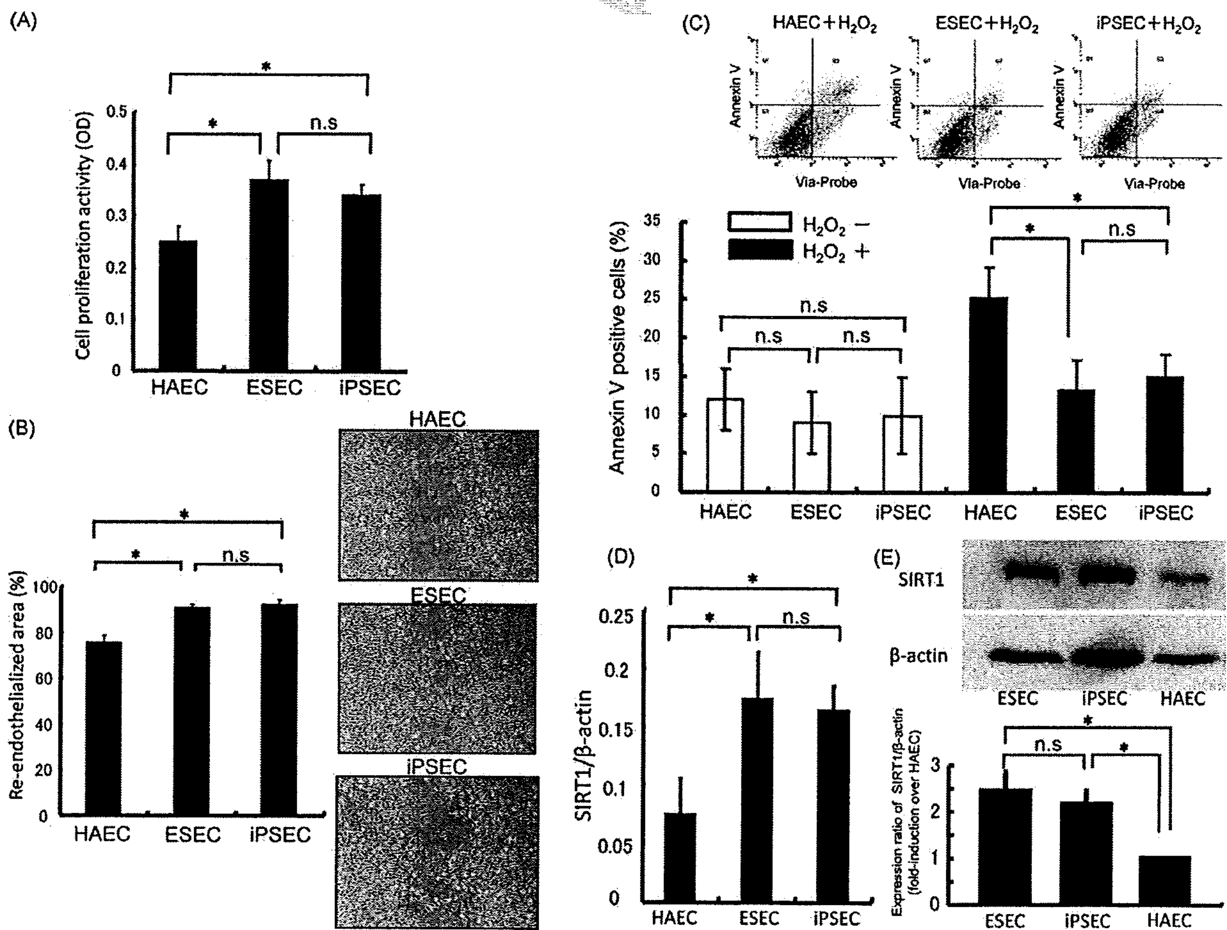

Fig. 2. Comparison of the potential for proliferation and migration, tolerance for oxidative stress, and Sirt1 expression in HAECs, ESECs and iPSECs. (A) MTT assay ($n=8$; $^*P<0.05$), (B) wound healing assay ($n=4$; $^*P<0.05$), (C) annexin V assay ($n=4$–6; $^*P<0.05$), (D) Quantitative real-time PCR ($n=8$; $^*P<0.05$) and (E) Western blot analysis ($n=4$; $^*P<0.05$).

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(Supplementary Fig. I), suggesting that both ESECs and iPSECs are near artery-lineage.

3.2. Comparison of the potentials for proliferation and migration and tolerance for oxidative stress of HAECs, ESECs and iPSECs

MTT assays carried out to evaluate their proliferative potential revealed ESECs and iPSECs to have a significantly greater potential for proliferation than HAECs, but there was no significant difference between ESECs and iPSECs (Fig. 2A). Similarly, an in vitro wound healing assay revealed that ESECs and iPSECs have a significantly higher potential for migration after EC loss than HAECs, and again there was no significant difference between ESECs and iPSECs (Fig. 2B). Following induction of oxidative stress by exposure to H_2O_2, moreover, there was a significantly lower percentage of annexin V-positive apoptotic cells among ESECs and iPSECs than among HAECs, with no significant difference between ESECs and iPSECs (Fig. 2C).

3.3. Differences in the expression of Sirt1 in HAECs, ESECs and iPSECs

To identify factors responsible for the functional differences between HAECs, ESECs and iPSECs, we next carried out a gene expression analysis using gene chip technology (Supplementary method, Supplementary Fig. II). We found that the expression level of Sirt1, an aging-related gene that encodes a NAD-dependent histone deacetylase, was higher in both ESECs and iPSECs than in HAECs. We then confirmed that finding by using real-time PCR to quantitatively compare the levels of Sirt1 mRNA expression in the three cell types. Sirt1 mRNA was expressed at significantly higher levels in both ESECs and iPSECs than in HAECs (Fig. 2D), but there was no significant difference in expression between ESECs and iPSECs. Subsequent Western blot analysis of Sirt1 protein expression yielded analogous results (Fig. 2E).

3.4. Contribution of Sirt1 to the cellular functionality of HAECs, ESECs and iPSECs

In view of the possibility that differences in Sirt1 expression contributes to the observed differences in the cellular functionality of HAECs, ESECs and iPSECs, we examined the effects of knocking down Sirt1 expression using siRNA or inhibiting Sirt1 protein using sirtinol (Calbiochem, San Diego, CA), a specific Sirt1 antagonist [15,16]. Using real-time PCR, we found that the targeted siRNA reduced the level of Sirt1 mRNA by about 70%, as compared to the nontargeted siRNA (data not shown). Although under control conditions ESECs and iPSECs showed a significantly greater proliferative potential than HAECs, knocking down Sirt1 expression diminished proliferation of all three cell types and abolished all differences in proliferative potential (Fig. 3A). Likewise, the difference in the cells' potential for migration also disappeared when Sirt1 was knocked down (Fig. 3B). Similar results were obtained when 50 μM sirtinol was applied to the cells (Fig. 3A upper, B upper). The vehicle control was 0.025% DMSO because sirtinol was dissolved Q2 in DMSO. On the other hand, differences in tolerance for oxidative stress became nonsignificant, but the trend remained upon Sirt1 knock down (Fig. 3C).

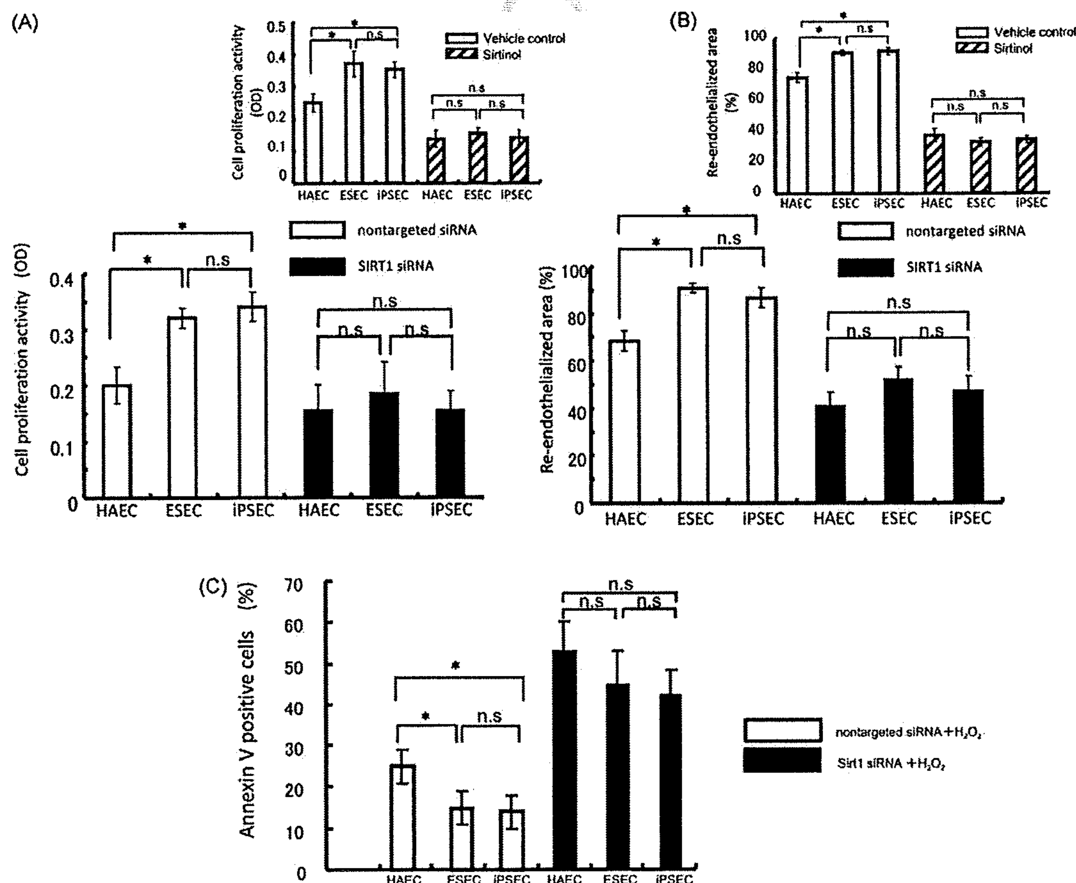

Fig. 3. Effects of Sirt1 siRNA or a Sirt1 inhibitor on the potential for proliferation and migration, and tolerance for oxidative stress in HAECs, ESECs and iPSECs. (A) MTT assay (n = 6–8; $^*P$ < 0.05). (B) Wound healing assay (n = 4; $^*P$ < 0.05). (C) annexin V assay (n = 4–6; $^*P$ < 0.05).

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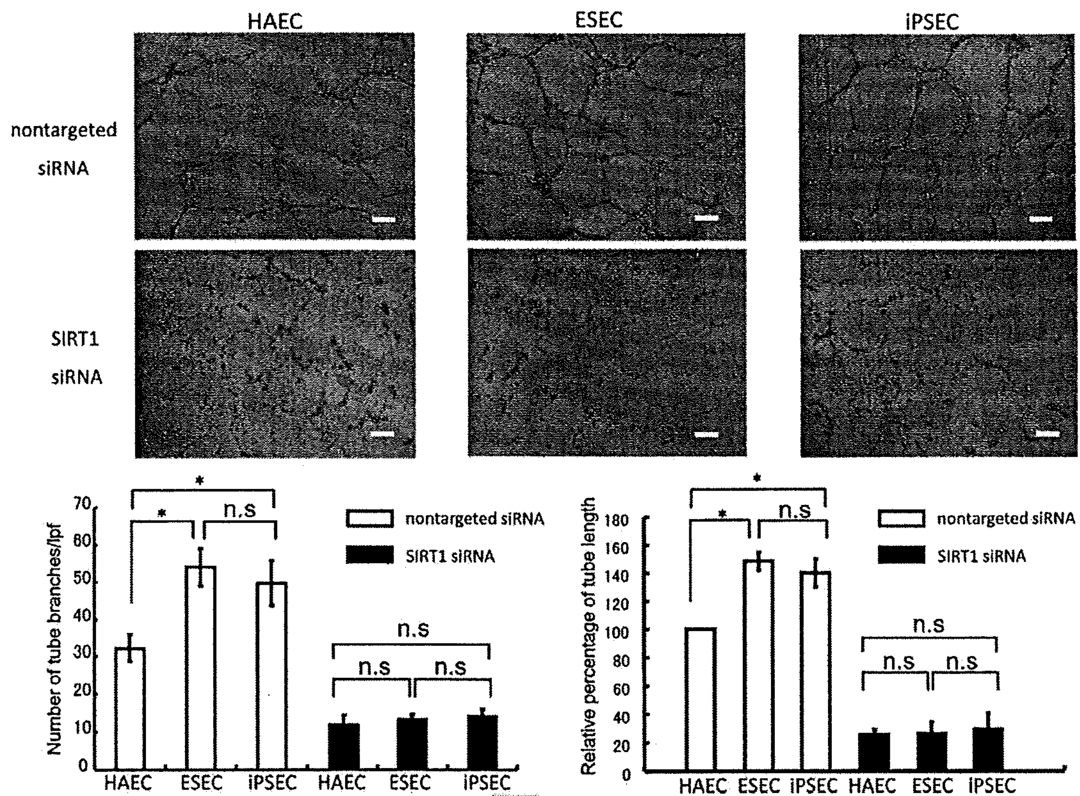

Fig. 4. Tube formation by HAECs, ESECs and iPSECs after 12 h of culture on Matrigel. Representative photomicrographs; scale bar, 100 μm. Quantitative analysis of tube formation ($n = 4$; $^{*}P < 0.05$).

3.5. *Changes in Sirt1 expression level during differentiation towards endothelial cells, and effects of Sirt1 on endothelial cell differentiation*

To investigate the involvement of Sirt1 in the differentiation process towards endothelial cells, Sirt1 gene expression level was determined by real-time PCR before and after VEGF stimulation. The level of Sirt1 expression in differentiated mesoblastic cells before VEGF addition was not significantly different from that in the VEGF-stimulated cells immediately before cell sorting (Supplementary Fig. IIIA). Furthermore, inhibition of Sirt1 by the addition of 25 mM or 50 mM sirtinol during the induction period did not alter endothelial cell differentiation efficiency (Supplementary Fig. IIIB).

3.6. *Contribution of Sirt1 to the angiogenic activities of HAECs, ESECs and iPSECs*

Finally, we used Matrigel assays to determine whether the differences in the functionality of the three cell types are reflected in their angiogenic activities. Consistent with the results summarized above, ESECs and iPSECs showed greater tube formation than HAECs, but that difference was abolished by knocking down Sirt1 expression (Fig. 4, Supplementary videos 1 and 2). Similar results were obtained when 50 μM sirtinol was applied to the cells (data not shown).

4. Discussion

The results of the present study demonstrate that human ESECs have a greater potential for proliferation and migration and a greater tolerance for oxidative stress than HAECs. They also demonstrate that the functionality of iPSECs is similar to that of ESECs.

Gene chip analysis and quantitative comparison using real-time PCR revealed that levels of Sirt1 expression differed between ESECs and HAECs. Sirt1, a mammalian homologue of Sir2, has recently been drawing attention due to its relationship to endothelial function [14–17] and eNOS expression in ECs [18]. It has also been reported that Sirt1 is important for the normal function of not only mature endothelial cells but also endothelial progenitor cells [19]. All of these previous studies demonstrated a protective role of Sirt1 in endothelial regeneration. In the present study, we found that when Sirt1 activity was knocked down by siRNA or inhibited by a specific Sirt1 antagonist, the aforementioned differences in the cellular functionality of ESECs and HAECs were abolished, which suggests that differences in Sirt1 activity contribute significantly to the observed differences in the cellular functionality of the two cell types. Moreover, this implies that changes in Sirt1 expression play a key role in mediating the effects of aging on human EC function, and that intervention to regulate Sirt1 expression may represent a useful approach to slowing the aging of ECs and improving their functionality.

Cell proliferation and migration are important steps in the process of angiogenesis. Sirt1 was previously shown to promote cell proliferation by suppressing p53 [20] and to promote cell migration through deacetylation of cortactin [21]. The differences in their potentials for proliferation and migration are also likely reflected in the results of the in vitro tube formation assays performed to assess the cells' angiogenic potential. Furthermore, the Matrigel assay showed strongly impaired tube formation due to the suppression of Sirt1 in all the three cell types. This suggests that Sirt1 may play an essential role in endothelial sprouting.

The post-transplant engraftment rate is another important indicator of a cell's utility for transplantation therapy. Several studies

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have shown that the engraftment rate is closely related to the incidence of apoptosis induced by inflammatory cytokines produced in inflamed or injured tissues [22]. Thus the observed anti-apoptotic effect in ESECs and iPSECs may be indicative of their potential for use in cell transplantation therapy. Nonsignificant but residual differences in tolerance for oxidative stress remained after suppressing Sirt1, suggesting the possible involvement of other factors. The protective effect of Sirt1 against oxidative stress has also been observed by several other studies [15,16,23]. Balestrieri et al. reported that metabolic intervention enhances therapeutic effects achieved by the administration of bone marrow cells or hemangioblasts alone in a mouse model of hindlimb ischemia [24]. In vivo experiments investigating the actual efficacy of a therapy using Sirt1 as a target are anticipated in the future.

The present and previous studies clearly demonstrated that Sirt1 plays an important role in endothelial cell function; however, whether it is involved in endothelial cell differentiation remains unclear. In this study, we showed that Sirt1 expression level was unchanged during induction, and that Sirt1 inhibition during induction did not alter the endothelial cell differentiation efficiency. Therefore, at least in the in vitro induction protocol used in the present study, Sirt1 appeared to have no effect on endothelial cell differentiation.

We found that iPSECs are very similar to ESECs, as judged from their expression of aging-related genes and their cellular functionality. iPS cells originate from somatic cells and are dedifferentiated through reprogramming. This is noteworthy, as it suggests that cells differentiated from iPS cells (e.g., iPSECs) are relieved of the aging they experienced as somatic cells so that they are functionally as young as cells differentiated from ES cells. This highlights the importance of reprogramming technology and the elucidation of the underlying mechanisms. In addition, in contrast to human ES cells, iPS cells can be established from every human being irrespective of their genetic backgrounds. The establishment of iPS cell lines from patients with inherited diseases presenting vascular abnormality should enable clarification of their pathogenesis. The establishment of iPS cell lines from patients with various genetic backgrounds should make it possible to dissect out cellular mechanisms in human vascular development, aging and diseased states such as arteriosclerosis.

In conclusion, we have shown that ESECs and iPSECs are younger and more viable than HAECs and are thus potentially useful cellular materials for vascular regeneration. We also showed that the functional differences between ESECs/iPSECs and HAECs is mediated by Sirt1 expression, suggesting Sirt1 plays a pivotal role in aging-associated functional impairment of human ECs.

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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.atherosclerosis.2010.04.021.

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Therapeutic Advances in Cardiovascular Disease Review

Potential use of endothelial progenitor cells for regeneration of the vasculature

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Kenichi Yamahara and Hiroshi Itoh

Abstract: Over the past decade, interest has been generated in the study of endothelial progenitor cells (EPCs). EPCs have been studied for their role in endogenous maintenance and for their therapeutic potential in vascular regenerative medicine. Despite their obvious potential in clinical practice, there still remain many controversies regarding how EPCs actually enhance endothelial repair and neovascularization. In addition, because of the limited expansion ability of EPCs, expansion of sufficient EPC populations for therapeutic angiogenesis remains a major task. On the other hand, embryonic stem (ES) cells have an extended self-renewal activity and can be expanded without limit, thus ES-cell-derived endothelial cells could be feasible as a novel cell source for therapeutic angiogenesis. In this review, we discuss recent experimental and clinical findings of EPCs and human ES-cell-derived endothelial cells for the treatment of ischemic cardiovascular diseases.

Keywords: endothelium, angiogenesis, cardiovascular diseases, smooth muscle

PROOF ONLY

Introduction

Neovascularization represents the formation of new blood vessels, either from the preexisting vascular network (angiogenesis) or through a *de novo* process from circulating primitive endothelial precursors (vasculogenesis) [Asahara *et al.* 1999; Takahashi *et al.* 1999; Shi *et al.* 1998; Folkman, 1995]. Many researchers have reported the identification and isolation of endothelial progenitor cells (EPCs) participating in vasculogenesis [Asahara *et al.* 1997]. EPCs were originally identified in the peripheral blood of bone marrow (BM) origin [Asahara *et al.* 1997], and later studies indicated that cord blood is a rich source of EPCs [Murohara *et al.* 2000]. These EPCs are now being studied for their role in the endogenous maintenance and repair of damaged endothelium, as well as for their regenerative potential. In particular, cell therapies using EPCs have proven beneficial in patients with coronary artery disease [Assmus *et al.* 2002]. The Transplantation of Progenitor Cells and Regeneration Enhancement in Acute Myocardial Infarction (TOPCARE-AMI) trial showed that intracoronary infusion of EPCs was safe and feasible in patients with AMI who successfully revascularized via stent implantation [Schachinger *et al.* 2004; Assmus *et al.* 2002]. However, in spite of the angiogenic potential of EPCs for treatment of ischemic diseases, these cell sources have limitations for clinical application. To isolate EPCs in sufficient numbers requires a large amount of blood or BM [Kalka *et al.* 2000] This may cause complications in some patients with severe cardiovascular disease including myocardial infarction.

Recently, embryonic stem (ES) cells have been highlighted as a promising cell source for therapeutic angiogenesis. ES cells are known to exhibit extensive regeneration potential and functional multilineage differentiation capacity, and they are ideal resources for regenerative medicine, which requires large numbers of transplant cells including endothelial cells (ECs) [Yamashita *et al.* 2000]. After the *in vitro* induction of differentiation in mouse and human ES cells, ECs can be obtained from vascular endothelial growth factor receptor (VEGFR)-2 positive cell fractions [Sone *et al.* 2007, 2003; Yamashita *et al.* 2000]. ES-cell-derived ECs express endothelial-specific markers and have been found to form capillary structures *in vitro* and *in vivo* [Yamahara *et al.* 2008; Sone *et al.* 2007; Yurugi-Kobayashi

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et al. 2003]. In addition, previous studies reported that transplantation of human ES-cell-derived ECs accelerated vascular regeneration in mouse hindlimb ischemia [Yamahara *et al.* 2008; Cho *et al.* 2007]. Thus, ES cells could be a promising source of ECs for the treatment of ischemia. In this review, recent experimental and clinical findings of these cell sources will be discussed along with our recent studies using mouse and human ES cells.

Definition of EPCs

Circulating EPCs were first described by Asahara *et al.* [1997] who showed that human peripheral blood cells enriched in both $CD34^+$ and VEGFR-2^+ cells could differentiate into mature ECs and form new vessels *in vivo.* Both CD34 and VEGFR-2 are expressed on primary hemangioblast islets in the yolk sac mesoderm during early embryonic vasculogenesis [Pelosi *et al.* 2002]. Therefore, $CD34^+$VEGFR-2^+ can be considered immature cells and represent putative EPCs. However, CD34 is expressed on hematopoietic stem cells and on the activated endothelium of certain microvasculature, but not on large vessels [Fina *et al.* 1990]. VEGFR-2 is also expressed on hematopoietic stem cells and mature endothelial cells [Ziegler *et al.* 1999]. In addition, recent evidence suggests that since different subsets of BM or circulating cells can differentiate into endothelial-like cells, the original definition of EPCs as $CD34^+$/VEGFR-2^+ might not represent all EC progenitors. The various markers used so far to identify EPCs indicate the uncertainty of the precise angiogenic phenotype of EPCs. Some studies have identified CD133 as an additional marker [Gehling *et al.* 2000] which is never expressed on mature endothelial cells. However this marker is expressed on more immature cells than CD34 is and so occurrence of $CD133^+$VEGFR-2^+ cells is lower than that of $CD34^+$VEGFR-2^+ cells in circulation. Despite these limitations, several studies chose CD34/VEGFR-2 or CD34/CD133 combinations to count circulating EPCs in several clinical conditions [Ghani *et al.* 2005; Heiss *et al.* 2005; George *et al.* 2003; Heeschen *et al.* 2003; Cho *et al.* 2003; Gill *et al.* 2001].

Furthermore, the definition of EPCs is complicated since they seemingly are comprised of two distinct cell populations [Gulati *et al.* 2003; Gehling *et al.* 2000; Lin *et al.* 2000]. Early outgrowth cells from isolated EPCs, which are used for transplantation in most studies possess the phenotype of monocytes, do not proliferate and begin to gradually die after a few weeks in culture [Fernandez Pujol *et al.* 2000, Eggermann *et al.* 2003, Rehman *et al.* 2003, Hur *et al.* 2004]. In contrast, late outgrowth cells, which arise from culture colonies more than 2 weeks after isolation, proliferate very rapidly and resemble microvascular ECs with a cobblestone morphology [Delorme *et al.* 2005; Yoon *et al.* 2005; Hur *et al.* 2004; Gulati *et al.* 2003a, 2003b] Because of this replicative potential, these late outgrowth cells are considered to be true stem/progenitor cells. The distinction of these two cell types is confirmed by their expression of different markers. Late outgrowth cells do not express the monocyte marker CD14 and have a low expression of the pan-leukocyte marker CD45 [Gehling *et al.* 2000; Lin *et al.* 2000]. Despite the obvious *in vitro* distinction between early and late outgrowth cells, their *in vivo* or therapeutic significance remains unclear. Most early studies of cell therapy utilized early outgrowth cells and these transplanted cells were thought to replace damaged endothelium and/or contribute to neovascularization [Murayama *et al.* 2002; Jackson *et al.* 2001; Llevadot *et al.* 2001; Lyden *et al.* 2001; Crosby *et al.* 2000]. However, recent studies reported that early outgrowth cells might enhance neovascularization in an indirect paracine manner [Yoder *et al.* 2007; Yoon *et al.* 2005; Rehman *et al.* 2003]. In contrast, late outgrowth cells have not been well studied *in vivo* [Yoon *et al.* 2005; Hur *et al.* 2004; Gulati *et al.* 2003]. Therefore, clarification of the character of EPCs is critical for determining the optimal cell for EPC therapy.

How does EPC enhance neovascularization?

In small animal models of hindlimb ischemia, direct injection of human circulating EPCs improved neovascularization and limb blood flow [Kalka *et al.* 2000]. Similarly, studies using various myocardial ischemia models have shown that systemically administered or directly injected EPCs stimulate angiogenesis, with subsequent improvement in myocardial perfusion and left ventricular function [Kawamoto *et al.* 2003, 2001; Kocher *et al.* 2001]. Although it is difficult to collectively understand the data from studies since they employ various cell types and doses, the preclinical evidence for the ability of EPCs to stimulate neovascularization seems to be obvious. Mature ECs have been shown to lack the ability

to induce neovascularization [Hur *et al.* 2004; Kocher *et al.* 2001; Kalka *et al.* 2000], suggesting that the undifferentiated nature of EPCs is crucial to their therapeutic function.

The mechanism by which EPCs stimulate neovascularization was initially thought to involve EPC differentiation into ECs and incorporation into neovessels. However, studies have shown that actual EPC incorporation into the vasculature is often low [Yamahara *et al.* 2008; Lyden *et al.* 2001]. Recently, their ability to stimulate neovascularization via paracrine effects has gained greater attention [Urbich *et al.* 2005]. This is supported by evidence for their ability to secrete various growth factors including VEGF, stromal cell-derived factor-1 (SDF-1), insulin-like growth factor-1 (IGF-1), and hepatocyte growth factor (HGF), which could stimulate angiogenesis [Lyden *et al.* 2001]. Therefore, the overall neovascularization induced by EPCs might be dependent not only on their ability to incorporate into the vasculature, but also on their ability to secrete factors that stimulate adjacent ECs. Although the question of how EPCs actually enhance neovascularization has not been satisfactorily answered, results from experiments have supported the use of EPCs for various therapeutic purposes, some of which have already been initiated clinically.

Cell therapy for myocardial infarction using EPCs

Preclinical experiments

There are many explanations for the mechanism by which EPCs improve myocardial function in post-myocardial infarction (MI) after cell therapy. Previous studies showed that EPCs could transdifferentiate into cardiomyocytes, ECs and smooth muscle cells (SMCs) [Badorff *et al.* 2003; Yeh *et al.* 2003]. Now much controversy exists regarding the ability of EPCs to differentiate into cells other than ECs, but still the ability of EPCs to improve cardiac function post-MI is supported by a number of preclinical studies [Wu *et al.* 2006; Kawamoto *et al.* 2003, 2001; Kocher *et al.* 2001; Asahara *et al.* 1999]. Many theories exist explaining the benefit to MI patients following EPC transplantation. First, transplanted EPCs were reported to stimulate neovascularization in the peri-infarct zone through direct endothelial incorporation or paracrine effect [Urbich *et al.* 2005], rescuing ischemia and increasing myocardial perfusion [Kocher *et al.* 2001]. The increased perfusion would improve myocardial regional contractility, thereby increasing myocardial function. Second, transplanted EPCs may attenuate deleterious ventricular remodeling, as was suggested by several groups [Kocher *et al.* 2001; Orlic *et al.* 2001, 2001]. Regenerating lost myocardial tissue would also lead to increased contractility and improved cardiac function. Recent experimental reports of cell therapy using EPCs in post-MI patients suggest that these processes occur synergistically. If the myocardial perfusion is augmented, myocyte apoptosis is prevented, and damaged cells have a better chance of surviving and regenerating lost myocardium [Kocher *et al.* 2001]. Also, EPCs were reported to stimulate mature ECs and cardiac resident progenitor cells via the secretion of VEGF, SDF-1 and IGF-1, which would enhance angiogenesis and myogenesis [Urbich *et al.* 2005]. Because elucidating such mechanisms is difficult in animals, and even more so in humans, there is little consensus for EPC-mediated myocardial regeneration post-MI.

An alternative strategy for cell therapy using EPCs is the mobilization of these cells from BM, which can avoid the need for cell isolation and delivery. Hypoxia-inducible factor 1 (HIF-1) produced in ischemic tissue stimulates secretion of VEGF and SDF-1, which can act synergistically to mobilize EPCs from BM via a matrix metalloproteinase (MMP-9) dependent mechanism [De Falco *et al.* 2004; Heissig *et al.* 2002; Takahashi *et al.* 1999], VEGF [Asahara *et al.* 1999] granulocyte-colony stimulating factor (G-CSF) [Ohtsuka *et al.* 2004; Cho *et al.* 2003], SDF-1 [De Falco *et al.* 2004] and erythropoietin [Bahlmann *et al.* 2004, 2003] have been reported to increase EPC mobilization in preclinical models, with subsequent myocardial neovascularization and improved cardiac function after acute MI [Ohtsuka *et al.* 2004; Orlic *et al.* 2001; Takahashi *et al.* 1999].

In addition, recent reports suggest that non-BM-derived EPCs might be more important than BM-derived EPCs in the setting of postnatal neovascularization [Urayama *et al.* 2008; Aicher *et al.* 2007; Smart *et al.* 2007]. Aicher *et al.* reported that circulating progenitor cells are mobilized from organs such as liver and intestine and contribute to neovascularization following tissue ischemia. EPCs are also mobilized from tissue-resident sources including epicardium in

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response to paracrine factors. These findings will provide the basis for a new physiological concept of EPCs and for possible therapeutic applications for ischemic disorders.

Clinical trials

Several clinical trials have been performed to test the regenerative capacity of BM or peripheral blood-derived cells for the treatment of cardiovascular diseases. Most trials using BM cells have utilized a density gradient to separate mononuclear cells consisting of a mixture of primitive hematopoietic, endothelial and mesenchymal stem/progenitor cell populations as well as mature monocytes and lymphcytes. These trials were not designed to determine the appropriate cell type for transplantation. Because we focused on the role of EPCs for regeneration of the vasculature, we only described clinical trials designed to use EPCs.

The TOPCARE-AMI clinical study was the first trial to show the efficacy of EPCs derived from peripheral blood [Assmus *et al.* 2002, Schachinger *et al.* 2004]. This trial allocated 59 patients who had AMI and successful percutaneous intervention (PCI) within 6 days to receive either autologous BM-derived mononuclear cells ($n=29$) or peripheral blood-derived EPCs ($n=30$) via intracoronary catheter delivery. Improved ejection fraction, coronary flow reserve and myocardial viability in the infarct zone were observed in the cell therapy groups compared with patients receiving PCI and medical treatment. As measured by quantitative left ventricular (LV) angiography at four months, LV ejection fraction (EF) significantly increased ($50\pm10\%$ to $58\pm10\%$), and end-systolic volumes significantly decreased (54 ± 19 ml to 44 ± 20 ml). There were no significant differences between the two cell groups. Contrast-enhanced magnetic resonance imaging after 1 year revealed an increased EF and reduced infarct size. Stamm *et al.* [2003] attempted to isolate EPCs using surface marker CD133 from BM and injected along the infarct border zone at the time of coronary artery bypass in six patients with recent MI. After surgery, all patients were alive and well, global LV function was enhanced in four patients, and infarct tissue perfusion had improved in five patients. Recently, Erbs and colleagues [2005] reported a randomized study in patients after recanalization of chronic coronary total occlusion (CTO) who received intracoronary EPCs or placebo. They mobilized BM cells using G-CSF, harvested EPCs from peripheral blood and infused EPCs via the coronary artery. After recanalization of CTO, 26 patients were randomly assigned to the treatment and control groups. At 3 months there was an increase in coronary flow reserve of 43%, and the number of hibernating segments in the target region had declined in the treatment group, whereas no significant changes were observed in the control group. MRI revealed a reduction in infarct size of 16% and an increase in LV ejection fraction of 14% in the treatment group (from 51.7 ± 3.7 to $58.9\pm3.2\%$) because of an augmented wall motion in the target region. Although the precise mechanisms of functional improvement remain to be elucidated, based on the observations of these trials, regenerative therapy using EPCs appears to interfere with the development of postinfarction heart failure.

Clinical trials studying the ability of G-CSF to mobilize EPCs in patients with coronary artery disease did not reach a conclusion on efficacy. Results from recently randomized controlled trials have shown no improvement in myocardial function following G-CSF administration, despite an increase in circulating EPC levels [Zohlnhofer *et al.* 2007; Ellis *et al.* 2006; Nienaber *et al.* 2006; Ripa *et al.* 2006; Zohlnhofer *et al.* 2006; Ince *et al.* 2005]. Because G-CSF mobilizes inflammatory cells as well as EPCs, it is possible that mobilization could accelerate arterial restenosis, plaque destabilization or cause other complications [Erbs *et al.* 2005, Hill *et al.* 2005].

It is reported that an inverse correlation exists between the number of coronary artery disease risk factors and the number and migratory ability of EPCs [Hill *et al.* 2003; Tepper *et al.* 2002]. Reports also suggest that advanced age [Heiss *et al.* 2005], smoking [Michaud *et al.* 2006], and diabetes [Loomans *et al.* 2004; Tepper *et al.* 2002] lead to impaired survival, proliferation, migration and reduced incorporation into vascular structures of EPCs. Since cell therapy using EPCs is done in an autologous manner, EPC dysfunction may reduce the therapeutic efficacy of cell therapy. Recent reports demonstrate that HMG-CoA reductase inhibitors (statins) [Dimmeler *et al.* 2001], estrogens [Iwakura *et al.* 2003], exercise [Laufs *et al.* 2004], and cessation of smoking [Kondo *et al.* 2004] enhance the number of circulating EPCs in patients, which is more practical than growth

factor or chemokine administration to improve EPC function.

ES cell-derived ECs

Recent reports suggest that the number of EPCs in bone marrow or peripheral blood is limited [Kalka *et al.* 2000]. In addition, somatic stem/progenitor cells usually have a limited proliferative capacity, thus expansion of sufficient EPC populations for therapeutic angiogenesis remains a major task. In contrast, ES cells have an excellent self-renewal capacity and can be expanded without limit. Therefore, ES cells could be feasible as a novel cell source for therapeutic angiogenesis.

Previously, our group established a novel ES cell differentiation system for vascular cells including ECs and mural cells (MCs: vascular SMCs and pericytes) [Yurugi-Kobayashi *et al.* 2006; Yamashita *et al.* 2000]. VEGFR-2 is the earliest functional differentiation marker for blood and ECs, and an indicator for the lateral plate mesoderm. VEGFR-2$^+$ cells are induced from undifferentiated mouse ES cells and purified by fluorescent-activated cell sorting. When VEGFR2$^+$ cells are recultured on type-IV collagen-coated dishes with serum and VEGF, CD31$^+$/vascular endothelial (VE)-cadherin$^+$ ECs, and α-smooth muscle actin (αSMA),$^+$ MCs are selectively induced. Induced vascular cells can form mature vascular-like structures with endothelial tubes and surrounding MCs *in vitro* and can contribute to newly formed vessels as ECs and MCs *in vivo* [Yurugi-Kobayashi *et al.* 2003; Yamashita *et al.* 2000]. Therefore, we termed these VEGFR-2$^+$ cells 'vascular progenitor cells (VPCs)'.

Recently, we were able to clarify the differentiation process from human ES cells to mature vascular cells (Figure 1) [Sone *et al.* 2007; 2003]. Unlike mouse ES cells, undifferentiated human ES cells already express VEGFR-2, but after differentiation, a VEGFR-2-positive but tumor rejection antigen 1-60 (TRA1-60; undifferentiated marker)-negative population emerged. These VEGF-R2-positive TRA1-60-negative cells were also positive for platelet-derived growth factor (PDGF) receptor α and β chains and could be effectively differentiated into both VE-cadherin$^+$ ECs and αSMA$^+$ MCs. VE-cadherin$^+$ cells, which were also CD34$^+$ and VEGFR-2$^+$ were considered to be ECs in the early differentiation stage, and could be easily

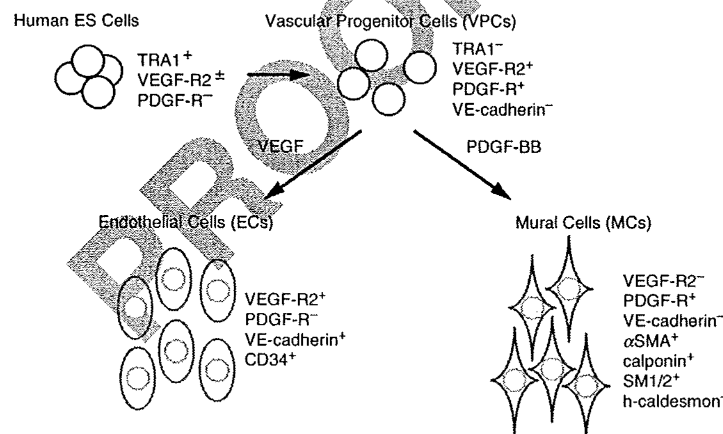

Figure 1. Differentiation pathway of vascular cells from human ES cells via vascular progenitor cells (VPCs). In contrast to mouse ES cells, undifferentiated (=TRA1-60 positive) human ES cells expressed VEGFR-2. By culturing undifferentiated human ES cells on OP9 feeder cells, VEGFR-2 expression first disappeared and then reappeared after differentiation. After the isolation of TRA1-60-negative, VEGFR-2-positive and VE-cadherin-negative-cells by a cell sorter, additional culturing of these cells with VEGF resulted in the appearance of VE-cadherin$^+$ ECs. In the presence of PDGF-BB, these VEGFR-2$^+$VE-cadherin$^-$ cells can also differentiate into αSMA$^+$ MCs. Therefore, human ES cell-derived TRA1-60$^-$VEGFR-2$^+$VE-cadherin$^-$ cells can act as VPCs.

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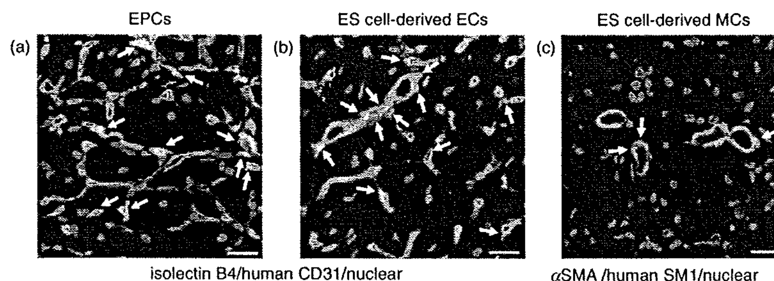

Figure 2. Incorporated human VPC-derived vascular cells at the sites of vascular regeneration. In a mouse model of experimentally induced hindlimb ischemia, intra-arterially-administered human ES cell-derived ECs or MCs were incorporated into the host vascular vessels (Figure 2(b) and (c), arrow). However, many of the transplanted EPCs were located within the lumen of host capillaries (Figure 2(a), arrow; scale bar: 500 µm, a, b). Fluorescence staining of endothelial marker GSL I-isolectin B4 (green) and human specific endothelial/monocyte marker CD31 (blue = transplanted human-derived endothelial/monocyte cells) in (a) human peripheral blood-derived EPCs; or (b) human VPC-derived EC transplanted mice. (c) Immunostaining of αSMA (green)/human specific smooth muscle myosin heavy chain (SM) 1 (blue = transplanted human-derived MCs) in human VPC-derived MC transplanted mice.

expanded while maintaining their maturity and form a network structure on Matrigel-coated dishes.

Based upon these findings, we transplanted human ES cell-derived vascular cells in a murine hindlimb ischemia model [Yamahara *et al.* 2008]. After the expansion of human VPC-derived ECs and MCs, these cells were intra-arterially administered, resulting in significantly augmented blood flow in nude mice with experimentally induced ischemic hindlimb, compared with human peripheral blood or umbilical cord-derived EPCs. Transplanted human VPC-derived vascular cells were incorporated into the host circulation as both ECs and MCs, whereas a considerable number of transplanted EPCs were localized inside the capillary lumen, not in the vessel wall (Figure 2). Recently, Ferreira *et al.* [2007] reported that transplantation of human ES-cell-derived ECs into nude mice using Matrigel as a scaffold contributed to the formation of blood vessels. Yang *et al.* [2008] demonstrated that human ES-cell-derived cardiovascular progenitor cells transplanted into the heart of NOD/SCID mice could differentiate into the cardiac, endothelial and vascular smooth muscle lineages. However, they did not show the direct integration of transplanted human ES-cell-derived ECs and MCs into host blood vessels. Judging from the double staining, using intravenously injected isolectin B_4 and anti-human specific CD31 antibody we found that the transplanted human VPC-derived EC incorporated into host circulating vessels. These transplanted ECs could on their own form *de novo* capillaries, as reported by other groups [Cho *et al.* 2007]. In addition, by the double immunostaining of human-specific smooth muscle myosin heavy chain 1 and α-smooth muscle actin (αSMA), we confirmed that transplanted human VPC-derived MCs were also incorporated into host vessel walls. We demonstrated for the first time the structural contribution of transplanted human VPC-derived ECs and MCs to form new vessels in the process of vascular regeneration.

We also confirmed that the combined transplantation of human VPC-derived ECs and MCs could markedly induce vascular regeneration, compared with the single fraction transplantation of VPC-derived ECs or MCs (Figure 3). Interaction between ECs and MCs is essential for vascular development and maintenance of vascular stability [Chan-Ling *et al.* 2004]. Compared with EC- or MC-transplanted mice only, the mice transplanted with the combined transplantation showed significant improvement after the induction of ischemic hindlimb. At day 42 after cell transplantation, the blood flow of ischemic hindlimb in the EC + MC group was significantly higher compared with the EC- or MC-transplanted groups only. Mouse and/or human CD31 and also αSMA positive capillary density at day 42 significantly increased in the

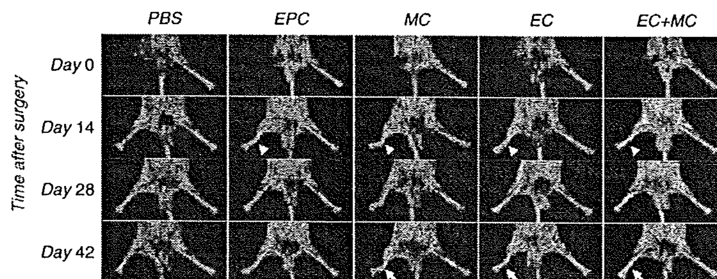

Figure 3. Augmented vascular regeneration by intra-arterial transplantation of human VPC-derived vascular cells in a murine hindlimb ischemia model. Serial laser Doppler perfusion image analysis of hindlimb ischemia in the transplanted mice. At day 14, the blood flow of the ischemic limb in all cell transplanted groups increased significantly compared to the control group (white arrowhead). After 42 days, significant blood flow recovery was observed in and human VPC-derived EC and/or MC-transplanted groups (white arrow), but not in EPC.

EC + MC group. We also found that the density of αSMA-positive arterioles also significantly increased in the EC + MC group. Therefore, combined transplantation of human VPC-derived ECs and MCs could synergistically contribute to vascular regeneration.

These results indicate that human ES-cell-derived ECs and MCs can be used as a new promising cell source for therapeutic vascular regeneration in patients with tissue ischemia. However, to enable their therapeutic potential to be realized, effective strategies need to be developed to produce sufficient quantities of human ES-cell-derived ECs and MCs. In addition, because human ES cell-derived differentiated cells will express transplant antigens including human leukocyte antigens (HLAs), effective prevention of graft rejection is also needed. Recently, Taylor *et al.* [2005] proposed the creation of a bank of HLA-typed human ES cells from which a best match could be selected, helping to reduce the likelihood of graft rejection [Nakajima *et al.* 2007; Taylor *et al.* 2005]. However, use of human embryos faces ethical controversies that hinder the application of human ES cells. One way to circumvent this issue is to induce pluripotent status in somatic cells by direct reprogramming. Takahashi *et al.* [2007] demonstrated the generation of induced pluripotent stem (iPS) cells from human dermal fibroblasts by transduction of four defined transcription factors: Oct3/4, Sox2, Klf4, and c-Myc. In theory, reprogramming a patient's somatic cells into pluripotent stem cells can facilitate differentiation into the required cell type for retransplantation therapy. Once the safety issue is overcome, human iPS cells should be applicable in regenerative medicine.

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Conclusion

Although cell therapy using EPCs is now being performed clinically for cardiac disease, the mechanism by which EPCs enhance endothelial repair and neovascularization has not been determined. In addition, because of the limited proliferative capacity of EPCs, human ES-cell-derived ECs could potentially be useful as an alternative cell source for vascular regenerative therapy. However, human ES cell-derived vascular cell therapy should be further tested in preclinical trials to confirm its therapeutic effect in the treatment of ischemic diseases.

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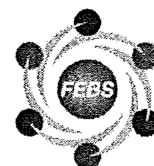

Adipogenic differentiation of human induced pluripotent stem cells: Comparison with that of human embryonic stem cells

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ABSTRACT

Induced pluripotent stem (iPS) cells were recently established from human fibroblasts. In the present study we investigated the adipogenic differentiation properties of four human iPS cell lines and compared them with those of two human embryonic stem (ES) cell lines. After 12 days of embryoid body formation and an additional 10 days of differentiation on Poly-L-ornithine and fibronectin-coated dishes with adipogenic differentiation medium, human iPS cells exhibited lipid accumulation and transcription of adipogenesis-related molecules such as C/EBPα, PPARγ2, leptin and aP2. These results demonstrate that human iPS cells have an adipogenic potential comparable to human ES cells.
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1. Introduction

Pluripotent embryonic stem (ES) cells have been considered potent candidates for regenerative medicine as an unlimited source of cells for the transplantation therapy and a useful tool for the investigation of cell development/differentiation, especially after establishment of human ES cells [1]. We previously clarified the differentiation process of mouse, monkey and human ES cells into vascular cells [2–4] and demonstrated that transplantation of vascular cells derived from human ES cells may constitute a novel strategy for vascular regeneration [4,5]. A number of immunological and ethical problems remain to be overcome before clinical application of the ES cells, however. Recently, novel ES cell-like pluripotent cells, termed induced pluripotent stem (iPS) cells, were generated by introducing four transcription factors (Oct3/4, Sox2, Klf4 and c-Myc) into mouse skin fibroblasts [6], and soon thereafter iPS cells were also generated from human skin fibroblasts [7,8]. Since then, a new generation of human iPS cells has been generated by introducing into fibroblasts just three of the aforementioned transcription factors (c-Myc was omitted) [9]. By overcoming the immunological and ethical problems associated with ES cells, iPS cells open a new avenue for cell transplantation-based regenerative medicine and provide a powerful new tool with which to investigate organ development/differentiation in specific disease states, especially in inherited diseases.

Generalized lipodystrophy consists of congenital and acquired types characterized by the lack of the whole adipose tissue, which leads to severe insulin-resistant diabetes, hypertriglyceridemia and fatty liver. We previously analyzed genes and phenotypes of congenital generalized lipodystrophic Japanese [10] and also demonstrated the long-lasting efficacy and safety of the leptin-replacement therapy in these patients [11–13]. Since metabolic abnormality in the mouse model is known to be cured by mature

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adipocytes transplantation, the regeneration therapy of the adipose tissue with human iPS cells-derived adipocytes is the ideal goal for lipodystrophic patients. Moreover, in vitro adipogenic differentiation system of human iPS cells will contribute to elucidate the pathogenesis of congenital generalized lipodystrophy when iPS cell lines are established from patients with lipodystrophy. In the present study we have investigated the adipogenic differentiation of human iPS cells and compared with that of human ES cells.

2. Materials and methods

2.1. Cells and culture

Four human iPS cell lines (201B6, 201B7, 253G1 and 253G4) were investigated. The 201B6 (B6) and 201B7 (B7) lines were generated by introducing four transcription factors (Oct3/4, Sox2, Klf4 and c-Myc) into human skin fibroblasts while the 253G1 (G1) and 253G4 (G4) lines were generated using only three factors (c-Myc was omitted) [9]. These iPS cell lines were maintained as previously described [7]. Two human ES cell lines (H9 and KhES-1) were used and maintained as previously described [1,14].

2.2. Adipogenic differentiation

For embryoid body (EB) formation, iPS and ES colonies were digested with 1 mg/ml collagenase type IV (GIBCO, CA, USA) and plated onto non-adherent bacterial culture dishes, where they were allowed to aggregate in maintenance medium without bFGF. Retinoic acid (Sigma–Aldrich, Japan) was added to the medium to a concentration of 100 nM from day 2 to day 5. After 12 days, EBs were transferred to 6-well plates coated with a combination of 30 μg/ml Poly-L-ornithine (Sigma–Aldrich) and 2 μg/ml fibronectin (Sigma–Aldrich). To induce adipocyte differentiation from iPS and ES cells, we applied a modification of a procedure described previously for use with mouse and human ES cells (Fig. 1) [15–19]. Differentiation was induced for 10 days using medium consisting of DMEM-F12, 10% KSR, and an adipogenic cocktail (0.5 mM IBMX, 0.25 μM dexamethasone, 1 μg/ml insulin, 0.2 mM indomethacin and 1 μM pioglitazone).

2.3. Immunocytochemistry

Immunocytochemistry was carried out as previously described [7]. The anti-human primary antibodies included Nanog (R&D Systems, MN, USA) and Alexa 488-conjugated SSEA-4 (Santa Cruz Biotechnology Inc., CA, USA) and TRA1-60 (CHEMICON, LA, USA). The TRA1-60 antibody was labeled using an Alexa Fluor 488 Monoclonal Antibody Labeling Kit (Molecular Probes, OR, USA). Alexa 546-conjugated donkey anti-sheep IgG (Molecular Probes, OR, USA) served as the secondary antibody. Alkaline phosphatase activity was detected using a BCIP/NBT substrate system (Dakocytomation, CA, USA).

2.4. Oil Red O staining and microscopic analysis of adipocytes

Cells were washed with phosphate-buffered saline (PBS) twice, fixed in 3.7% formaldehyde for 1 h and then stained with 0.6% (w/v) Oil Red O (Nacalai Tesque, Japan) solution (60% isopropanol, 40% water) for 2 h at room temperature. The cells were then washed with water to remove unbound dye. Subsequently, the bound Oil Red O was eluted with isopropanol.

Table 1
Primers for reverse-transcription polymerase chain reaction.

Gene		Sequence
Nanog	Sense	CAGCCCCGATTCTTCCACCAGTCCC
	Antisense	CGGAAGATTCCCAGTCGGGTTCACC
PPARγ2	Sense	ATTGACCCAGAAAGCGATTC
	Antisense	CAAAGGAGTGGGAGTGGTCT
C/EBPα	Sense	GCAAACTCACCGCTCCAATG
	Antisense	TTAGGTTCCAAGCCCAAGTC
aP2	Sense	AACCTTAGATGGGGGTGTCCTG
	Antisense	TCGTGGAAGTGACGCCTTTC
Leptin	Sense	GAACCCTGTGCGGATTCTTGTG
	Antisense	CGTTTCTFFAAGGCATACTGGTGAG
GAPDH	Sense	ACCACAGTCCATGCCATCAC
	Antisense	TCCACCACCCTGTTGCTGT
PPARγ2 (real-time RT-PCR)	Sense	GATACACTGTCTGCAAACATATCACAA
	Antisense	CCACGGAGCTGATCCCAA
	Probe	AGAGATGCCATTCTGGCCCACCAACTT

After staining with Oil Red O, each EB was examined microscopically for the presence of adipocyte colonies, and the percentage of EBs with outgrowths showing adipocyte positivity was determined as previously described [15]. EBs in which adipocytes accounted for more than half of their circumference were considered adipocyte-positive. The percent area of Oil Red O staining (+) was determined at 20× magnification by counting the number of pixels exhibiting Oil Red O positivity in selected microscope fields (449 × 338 pixels). Four randomly selected fields were examined in each well of a 6-well plate, and the percent area was calculated as the average for the four fields. Six independent experiments were performed for each cell line.

2.5. Reverse-transcription polymerase chain reaction (RT-PCR) and quantitative real-time PCR

Total RNA was extracted using TRizol Reagent (Invitrogen, CA, USA) and treated with RNase-Free DNase Set (QIAGEN, Germany) to remove any contaminating genomic DNA. For RT-PCR, cDNA was synthesized using a PrimeScript RT reagent Kit (Takara Bio Inc., Japan), after which RT-PCR was run using ExTaq (Takara Bio Inc.). For quantitative real-time PCR, TaqMan PCR was carried out using a Step One Plus Real-Time PCR System as instructed by the manufacturer (Applied Biosystems, CA, USA). Levels of mRNA were normalized to those of 18S mRNA. The primers used are listed in Table 1.

2.6. Statistical analysis

Data are expressed as means ± S.E.M. Statistical significance was evaluated using ANOVA for comparison among six groups. Values of $P < 0.05$ were considered significant.

3. Results

3.1. Adipogenic differentiation of human iPS and ES cells

Morphological phenotypes, immunoreactivities of Nanog, SSEA-4 and TRA-1-60, and ALP activity of human iPS cells did not differ

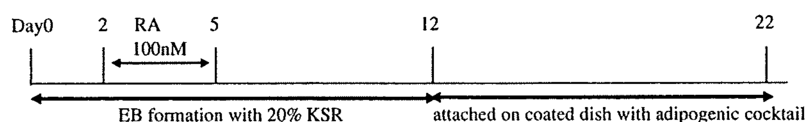

Fig. 1. Schematic diagram of the experimental protocol used for adipocyte differentiation from human ES and human iPS cells. EB: embryoid body. Adipogenic cocktail: 0.5 mM IBMX, 0.25 μM dexamethasone, 1 μg/ml insulin, 0.2 mM indomethacin and 1 μM pioglitazone.

from those of human ES cells (Fig. 2). In order to assess their potential for adipogenic differentiation, the human iPS cells were subjected to adipogenic induction culture. After 12 days of EB formation, EBs derived from human iPS cells were attached to coated dishes to induce differentiation. Several kinds of coating for the dishes, including gelatin, collagen IV and fibronectin were compared, and the efficiency of EB attachment and adipogenic differentiation were the best on dishes coated with a combination of Poly-L-ornithine and fibronectin. On day 15, after 3 days of adipogenic differentiation following the EB formation, differentiated cells containing small cytoplasmic lipid droplets were observed spreading outward from the attached EBs. On day 22, the lipid accumulation was evaluated by staining the cells with Oil Red O.

To evaluate the adipogenic potential of individual iPS cell lines, the percentage of EB outgrowths having adipocyte colonies and the percent area of Oil Red O staining (+) were determined. For each of iPS and ES cell lines tested, 40–60% of EBs formed adipocyte colonies (Table 2). In all of the iPS cell lines, lipid accumulation was similar to that seen in human ES cell lines (Fig. 3), though the B7 line showed stronger lipid accumulation than the other cell lines.

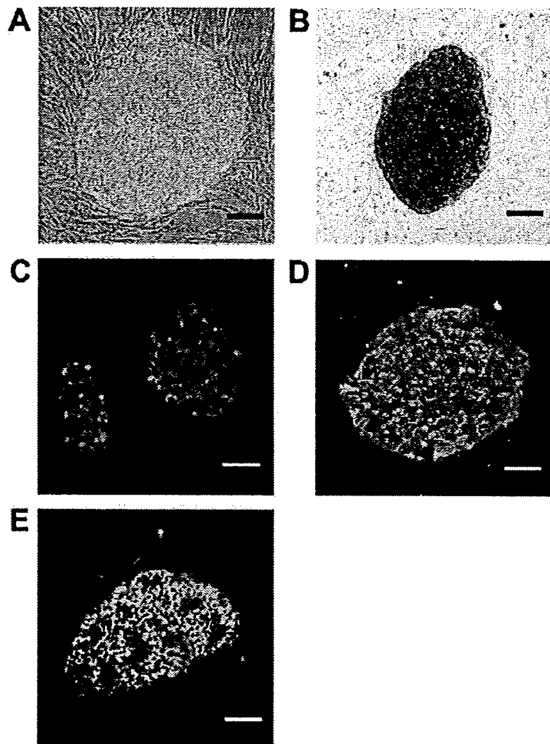

Fig. 2. Morphology of undifferentiated human iPS cells (G4). (A) Phase-contrast photomicrograph of an undifferentiated colony. (B) Alkaline phosphatase activity. (C) Immunofluorescent staining with Nanog. (D) Immunofluorescent staining with SSEA-4. (E) Immunofluorescent staining with TRA1-60. Scale bar = 100 μM.

Table 2
% of EBs with adipocyte colonies.

Cell line	[Number of EBs with adipocytecolonies/total number of EBs]
201B6	54.1% [40/74]
201B7	59.7% [46/77]
253G1	50.0% [35/70]
253G4	56.4% [44/78]
H9	48.8% [39/80]
KhES-1	45.5% [35/77]

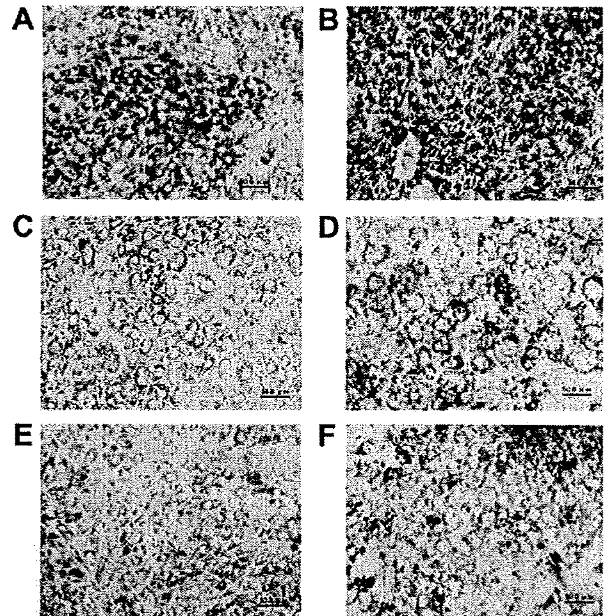

Fig. 3. Oil Red O staining of adipocytes derived from human iPS cells (A–D) and ES cells (E, F) on day 22. B6 (B) B7 (C) G1 (D) G4 (E) H9 (F) KhES-1. Scale bar = 50 μM.

Statistical analysis of the percent area of Oil Red O staining (+) showed no significant differences among the cell lines (Fig. 4).

3.2. Expression of adipogenesis-related molecules

Using RT-PCR, transcription of adipogenic markers was investigated on days 0 and 22 of differentiation (Fig. 5A). Though not detected at day 0, mRNAs encoding the adipogenic transcription factors C/EBPα (CCAAT/enhancer binding protein α) and PPARγ2 (peroxisome proliferator-activated receptor γ2) were detected on day 22. In contrast, expression of Nanog mRNA was strongly suppressed on day 22, as compared with its expression on day 0. Expression of the mature adipocyte markers leptin and aP2 (adipocyte fatty acid binding protein) was also clearly detected on day 22. All of the human iPS cell lines expressed mRNAs encoding adipogenesis-related molecules at levels that were comparable to the levels seen in human ES cell lines (Fig. 5A). In Quantitative real-time PCR analysis, expression of PPARγ2 mRNA differed somewhat among the iPS and ES cell lines. The differences between the B7 line and the two ES cell lines were significant, but other differences were not significant (Fig. 5B).

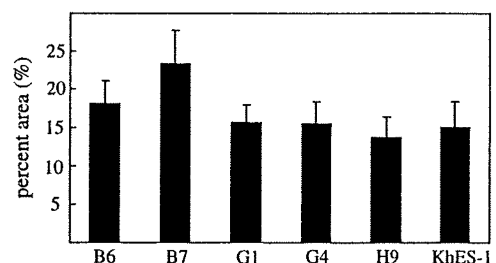

Fig. 4. Percent area of Oil Red O staining. Results are means of six independent experiments. No significant differences were observed among the iPS and ES cell lines.

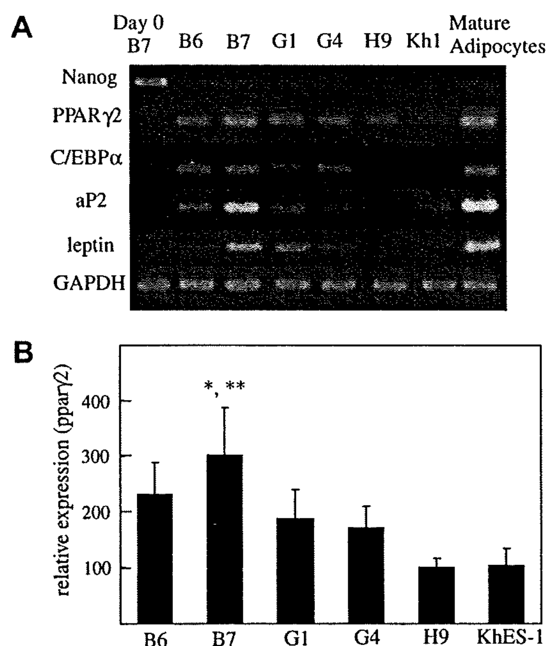

Fig. 5. (A) Transcription of the adipocyte-specific markers PPARγ2, CEBP/α, aP2 and leptin. RNA samples from undifferentiated human iPS cells (B7, day 0) and differentiated stage iPS cells (B6, B7, G1, G4) and human ES cells (H9, KhES-1), as well as mature human adipocytes differentiated from human adipose-derived mesenchymal stem cells (positive control), were analyzed by RT-PCR. Nanog is an undifferentiated human ES cell marker. GAPDH served as an internal standard for RT-PCR. Kh1: KhES-1. Adipose: human mature adipocytes differentiated from human adipose-derived mesenchymal stem cells. (B) Relative levels of PPARγ2 mRNA expression are shown as means ± S.E.M. of 4–6 independent experiments and normalized to those of 18S. The levels are expressed as percentages of the expression in the H9 cell line. $^{*}P < 0.05$ vs. H9. $^{**}P < 0.05$ vs. KhES-1.

4. Discussion

The present study demonstrates that human iPS cells have adipogenic potential comparable to human ES cells. Four human iPS cell lines of two generations were investigated. The B6 and B7 were generated by introducing four transcription factors (Oct3/4, Sox2, Klf4 and c-Myc) into human skin fibroblasts while the G1 and G4 were generated using only three factors (c-Myc was omitted) [9]. After 12 days of embryoid body formation and an additional 10 days of differentiation on Poly-L-ornithine and fibronectin-coated dishes with adipogenic differentiation medium, all human iPS cell lines of both generations exhibited lipid accumulation and transcription of such adipogenesis-related molecules as C/EBPα, PPARγ2, leptin and aP2. We also compared differentiation efficiency between human iPS and ES cells using two lines of human ES cells and found no apparent difference between human iPS and ES cells in properties of adipogenic differentiation including the time course and potential. In terms of lipid accumulation and transcription of adipogenesis-related molecules, human iPS-derived adipocytes appear to reach at least the same level of maturity as those derived from human ES cells. The B7 line tended to show stronger adipogenic potential than the other five iPS lines and the ES cell lines, but the difference in terms of percent area of Oil Red O staining (+) was not significant. The B7 line also showed significantly stronger expression of PPARγ2 than the two ES cell lines tested, but PPARγ2 expression varied among the different iPS cell lines, despite their having the same genetic background. We conclude that the adipogenic potential of iPS cells did not essentially differ from ES cells, though their adipogenic potentials were rather varied in each line.

Despite the prevalence of obesity, systems for research into human adipocyte biology remain underdeveloped, in part because of a lack of available human adipocyte cell lines. There are significant differences between adipocyte development in humans and mice [20]. The established in vitro adipocyte differentiation system using human iPS cells in the present study should make it possible to dissect out the cellular mechanisms underlying human adipocyte differentiation. It should also contribute to the better understanding of adipocyte biology and serve as a basis for advances in research into obesity and adipotoxicity, which has been proposed as the sum of the negative effects associated with obesity [21].

Adipogenesis is largely divided into two phases: the early phase consisting of the lineage commitment of adipocytes from pluripotent stem cells and the late phase consisting of the terminal differentiation of preadipocytes into adipocytes [22]. The molecular mechanism underlying the terminal adipocyte differentiation has been identified through analysis of the differentiation process in immortalized mouse preadipocyte cell lines (e.g., 3T3-L1 and 3T3-F442A cells) [22–24], but the differentiation from pluripotent stem cells during the early stage of adipogenesis must await further clarification. The establishment of adipocyte differentiation system with human iPS cells should facilitate that line of research.

In contrast to human ES cells, iPS cells can be induced from any human being irrespective of their genetic make-up. Consequently, the study of iPS cells should contribute to the identification of new susceptibility genes associated with obesity and metabolic syndrome, and to the clarification of the functions of those genes. The establishment of iPS cell lines from patients with inherited diseases presenting adipocyte abnormality should enable clarification of their pathogenesis. And because they overcome the immunological and ethical problems associated with human ES cells, iPS cell systems should also contribute to the development of novel regenerative therapies for reconstruction of soft tissue defects after tumor resections, extensive deep burns and lipodystrophy. The induced cells obtained with our protocol are not a homogeneous population. Consequently, at this stage human iPS cells may not yet have as much adipogenic potential as adipose-derived stem cells (ADSCs), which are derived from the stromal vascular fraction of human adipose tissue and are thought to be a safe and useful tool in adipose regenerative medicine [25]. About 80% of ADSCs differentiate into adipocytes under suitable conditions [26]. The next issue we plan to address will be the establishment of an improved differentiation protocol that includes a purification process such as cell sorting.

In conclusion, the present study demonstrates that human iPS cells have adipogenic potential that is generally equal to that of human ES cells. The use of iPS cells will contribute to the development of regenerative therapies of adipose tissue for lipodystrophy. This work should also contribute to our understanding of human adipogenesis and to the clarification of the pathogenesis and pathophysiology of obesity and metabolic syndrome, potentially leading to the development of new drug therapies.

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