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Original Article

MEK inhibitor PD0325901 upregulates CD34 expression in endothelial cells via inhibition of ERK phosphorylation

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ABSTRACT

Introduction: CD34-positive endothelial progenitor cells (EPCs) promote angiogenesis and are a promising tool for regenerative cell therapy of ischemic diseases. However, the number and quality of CD34-positive cells decrease owing to various external and internal factors; thus, an efficient method is needed to establish CD34-positive EPCs. The generation of functional cells by reprogramming, that is, manipulating cell fate via gene transfer and/or treatment with chemical compounds, has recently been reported. Therefore, we aimed to generate CD34-positive cells by the reprogramming of endothelial cells (ECs).
Methods: Based on previous reports, seven candidate chemical compounds were selected to reprogram human umbilical vein ECs (HUVECs) to CD34-positive cells. Following stimulation with the chemical compounds, the expression of CD34 was evaluated using quantitative PCR, flow cytometry, and immunocytochemistry.
Results: HUVECs treated with the compounds exhibited increased CD34 expression. We cultured cells in alternate media lacking one of the seven compounds and found no CD34 expression in cells treated with PD0325901-free media, suggesting that PD0325901—a MEK inhibitor—mainly contributed to the increase in CD34 expression. We found that 98% of cells were CD34-positive after PD0325901 treatment alone for 7 d. Western blotting revealed that the phosphorylation of ERK was suppressed in PD0325901-treated cells. No upregulation of CD34 was observed in fibroblast cell lines, even after PD0325901 treatment. These results suggested that PD0325901 induces CD34-positive cells by inhibiting ERK phosphorylation in ECs.
Conclusions: CD34 expression was strongly induced in ECs by treatment with the MEK inhibitor PD0325901 in vitro. Our study provides a useful reference for the establishment of CD34-positive EPCs and will contribute to the development of regenerative therapies, especially for ischemic diseases.

1. Introduction

Circulating CD34-positive endothelial progenitor cells (EPCs) promote angiogenesis in vivo and are a promising cell source for regenerative therapy [1]. EPCs promote angiogenesis by secreting cytokines such as VEGF and HGF [2] and some studies indicates that they are directly incorporated into new blood vessels [3,4]. Over the last two decades, the clinical efficacy of cell therapy using autologous CD34-positive cells has been demonstrated in ischemic diseases, such as severe limb ischemia, refractory angina, and myocardial infarction[5–9]. Iwasaki et al. [10] reported that transplantation of human CD34-positive cells protected cardiac

Abbreviations: ALK, anaplastic lymphoma kinase; BMP, bone morphogenetic protein; EC, endothelial cell; EPC, endothelial progenitor cell; ERK, extracellular signal-regulated kinase; HUVEC, human umbilical vein endothelial cell; MACS, magnetic-activated cell sorting; MAPK, mitogen -activated protein kinase; MEK, MAPK/ERK kinase; PPIA, peptidylprolyl isomerase A; ROCK, Rho-associated kinase; TGF-β, transforming growth factor β; VPA, valproic acid; cAMP, cyclic adenosine monophosphate.

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function after myocardial infarction in a dose-dependent manner in a nude rat model. However, the number and quality of circulating EPCs may vary based on donor characteristics, such as smoking, aging, hypertension, diabetes, dyslipidemia, and other factors [11–13]. Therefore, to promote the applicability of EPCs in clinical cell therapies, new methods are needed for establishing stable cells in sufficient numbers and with retained proliferation and angiogenesis capacities.

Reprogramming refers to the artificial manipulation of cell fate by regulating transcription factors and epigenetic status. There are two approaches to reprogram cells: genetic and chemical. Pluripotent stem cells, somatic stem cells, progenitor cells, and other cell lineages can be generated from somatic cells [14,15]. Genetic approaches can efficiently reprogram cells by forcibly expressing certain genes, but they require complex manipulation processes and expertise in controlling gene transcription and translation. In contrast, chemical reprogramming, in which functional proteins are stimulated or inhibited using chemical compounds, allows for easier control of the experimental process, with predictable biological effects and fine-tuning capabilities. Indeed, liver progenitor cells have been generated from mature hepatocytes through chemical reprogramming alone [16]. Functional cardiomyocytes have also been generated from fibroblasts using a chemical approach [17].

In this study, we investigated the applicability of chemically reprogramming endothelial cells (ECs) to EPCs. Accordingly, we analyzed the effects of various chemical compounds and their combinations. We found that PD0325901, an inhibitor of the MEK/ERK pathway, substantially increased the expression of CD34, a representative marker of EPCs, in ECs via the inhibition of ERK phosphorylation.

2. Methods

2.1. Cell lines and cultures

Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza (Basel, Switzerland) and used in the experiments at passage 4–7. HUVECs were cultured in endothelial growth medium 2 (EGM2, Lonza). Human fetal lung fibroblasts (MRC-5) were purchased from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan) and cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum and 1% penicillin– streptomycin solution (FUJIFILM Wako Pure Chemical, Osaka, Japan).

2.2. Chemical screening

HUVECs and MRC-5 cells were seeded onto fibronectin-coated culture dishes (Wako or Sigma-Aldrich). With the seeding of the HUVECs, the following chemical compounds were added to EGM2: 3 μM CHIR-99021 (Cayman Chemical Company, Ann Arbor, MI, USA), 1 μM A83-01 (Wako), 1 μM LDN193189 (Sigma-Aldrich), 10 μM Y-27632 (Wako), 500 μM valproic acid (VPA) (Wako), 20 μM Forskolin (Tokyo Chemical Industry, Tokyo, Japan), and 1 μM PD0325901 (Wako). For the further analysis of PD0325901, HUVECs or MRC-5 cells were cultured in basal media containing 0.5, 1, or 5 μM PD0325901. The culture medium was changed every 2 d, and confluent cells were passaged using TrypLE™ Express Enzyme (Thermo Fisher Scientific, Waltham, MA, USA). Cells were harvested for evaluation at 1, 4, 7, and 10 d after treatment.

2.3. Realtime PCR

Samples were lysed in TRIzol (Thermo Fisher Scientific). Total RNA was extracted using the phenol chloroform method and reverse-transcribed into single-strand cDNA using a High-Capacity RNA-to-cDNA™ Kit (Thermo Fisher Scientific). Real-time PCR was performed using Fast SYBR™ Green Master Mix (Thermo Fisher Scientific) and a StepOnePlus real-time PCR system (Thermo Fisher Scientific). The primers for the PCR are listed in Supplemental Table 1. The target mRNA expression was normalized to that of the housekeeping gene peptidylprolyl isomerase A (*PPIA*), and calculated using the $2^{-\Delta\Delta Ct}$ method.

2.4. Flow cytometry

Cultured cells were detached after incubation with TrypLE™ Express Enzyme (Thermo Fisher Scientific) for 5–13 min at 37 °C. Cells (adjusted to 5.0×10^4–1.0×10^5 per sample) were treated with primary antibodies for 20 min at 4 °C. The following primary antibodies and isotype control antibodies were used: mouse FITC anti-human CD34 antibody, 1 μg/100 μL/sample (BioLegend, San Diego, CA, USA, #343604); APC anti-human CD31 antibody, 0.2 μg/100 μL/sample (BioLegend, #303116); mouse FITC mouse IgG2a, κ-isotype control antibody, 1 μg/100 μL/sample (BioLegend, #400207); and APC mouse IgG1, κ-isotype control antibody, 0.2 μg/100 μL/sample (BioLegend, #400120). Secondary antibody reactions were not performed because all primary antibodies were fluorescently labeled. The reacted samples were centrifuged at 3000 *g* for 5 min and washed twice with 2% bovine serum albumin (BSA, Wako) in phosphate-buffered saline (PBS). After filtering the samples using a cell strainer (Corning, Corning, NY, USA), flow cytometry was performed using a Spectral Analyzer SA3800 (SONY, Tokyo, Japan). The obtained data were analyzed using FlowJo™ software (Becton, Dickinson & Company, Franklin Lakes, NJ, USA, version 10.8.0).

2.5. Immunocytochemistry

Adherent cells were fixed with 4% paraformaldehyde in PBS (Wako) for 10 min at room temperature (RT) and permeabilized with 0.1% *v/v* Tween-20 (Wako) in PBS (PBST) for 5 min at RT. Blocking was performed for 30 min at RT in PBST. Samples were incubated at 4 °C overnight with the following primary antibodies in PBST containing 2% *w/v* BSA: 1:200 anti-CD31 mouse antibody (Abcam, Cambridge, UK, #ab9498) and 1:200 anti-CD34 rabbit antibody (Abcam, #ab81289). Secondary antibody reactions were conducted at RT for 1 h using the following secondary antibodies in PBST containing 2% *w/v* BSA: 1:300 goat anti-mouse IgG (Alexa Fluor® 647; Abcam, #ab150115) and 1:300 donkey anti-rabbit IgG (Alexa Fluor® 488; Abcam, #ab150073). Nuclei were stained with 4′,6-diamidino-2-phenylindole (AAT Bioquest, Pleasanton, CA, USA; 1:3500 in PBS containing 2% *w/v* BSA). Cells were imaged using a BZ-X700 fluorescence microscope (KEYENCE, Osaka, Japan).

2.6. Western blotting

After 24 h of exposure to the chemical compounds, cells were collected and lysed on ice for 30 min using RIPA buffer (Wako) with 1:100 protease inhibitor cocktail (Wako), 1:100 phosphatase inhibitor cocktail (Wako), and 5 mM DTT (Wako). Protein concentrations were measured using a BCA Protein Assay Kit (Takara Bio, Kusatsu, Japan) and SpectraMax M2 (Molecular Devices, San Jose, CA, USA). The protein lysates were mixed with equal volumes of sample buffer containing 2-mercaptoethanol (Nacalai Tesque, Kyoto, Japan) and Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA, USA) and incubated at 95 °C for 7 min. Samples were electrophoresed using Mini-PROTEAN TGX Gels (Bio-Rad) at 200 V for 25–40 min with Precision Plus Protein Dual Color Standards (Bio-Rad), and 15 μg protein was applied per well. Proteins were

blotted on membranes using a Trans-Blot Turbo Transfer Pack (Bio-Rad) on a Trans-Blot Turbo system (Bio-Rad). The membranes were washed with Tris-buffered saline (TBS, Bio-Rad) for 5 min and blocked with 5% *w/v* nonfat dry milk in TBS (Bio-Rad) containing 0.1% *v/v* Tween-20 (Wako) (TBST) for 1 h at RT. Primary antibody reactions were performed at 4 °C overnight with the following antibodies: GAPDH (D4C6R) mouse mAb [Cell Signaling Technology (CST), Danvers, USA, #97166], 1:2000 in 5% *w/v* nonfat dry milk in TBST; Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) XP® rabbit mAb (CST, #4370), 1:2000 in 5% *w/v* BSA in TBST; and p44/42 MAPK (Erk1/2) rabbit mAb (CST, #4695), 1:1000 in 5% *w/v* BSA in TBST. Secondary antibody reactions were performed at RT for 1 h with the following antibodies: HRP-linked anti-mouse IgG (CST, #7076), 1:3000 in 5% *w/v* nonfat dry milk in TBST, and HRP-linked anti-rabbit IgG (CST, #7074), 1:3000 in 5% *w/v* nonfat dry milk in TBST. Target proteins on the membranes were detected using Clarity Western ECL Substrate (Bio-Rad) on a Fusion Solo 7s Edge (VILBER, Marne-la-Vallée, France).

2.7. Magnetic activated cell sorting

Adherent cells were incubated with TrypLE™ Express Enzyme (Thermo Fisher Scientific) for 5–13 min at 37 °C. Detached cells were filtered with a pluriStrainer (30 μm; pluriSelect, Leipzig, Germany) and incubated with CD34 antibody-linked magnetic beads (Miltenyi Biotech, Bergisch Gladbach, Germany) at 4 °C for 30 min. Cells were washed with PBS containing 0.5% *w/v* BSA and 2 mM ethylenediaminetetraacetic acid (Wako) and separated by magnetic-activated cell sorting (MACS) using a miniMACS separator and MS columns (Miltenyi Biotech). Unlabeled cells were collected from the flow-through (negative fraction). Obtained cells were immediately used for each application.

2.8. Statistical analysis

Statistical analyses were performed with GraphPad Prism (version 5.01, GraphPad Software, San Diego, CA, USA). Values were presented as means plus/minus standard deviation (SD) or standard error of the mean (SEM). The averaged variables were compared using Student's two tailed *t*-test (Two-group comparison) or one-way analysis of variance (ANOVAs) (Multiple-group comparison). *P*-values less than 0.05 were considered statistically significant and the notations of *p*-values are as follows: $p < 0.05$ as single asterisk (*): $p < 0.01$ as double asterisks (**): $p < 0.001$ as triple asterisks (***).

3. Results

3.1. Screening of chemical compounds to reprogram ECs into CD34-positive EPCs

We selected candidate chemical compounds for EC reprogramming from previous reports regarding reprogramming of somatic cells into progenitor-like, somatic stem-like, or other lineage cells using chemical compounds [16–27]. The most common chemical compounds activated Wnt, inhibited transforming growth factor β (TGF-β), inhibited bone morphogenetic protein (BMP), inhibited Rho-associated kinase (ROCK), inhibited histone deacetylase, induced cyclic adenosine monophosphate (cAMP), or inhibited MEK activity [16–27]. Based on their activities and potential to reprogram endothelial cells into EPCs, we selected the following seven chemical compounds: CHIR99021 (glycogen synthase kinase-3 inhibitor) as the Wnt signaling activator, A83-01 [anaplastic lymphoma kinase (ALK) 4, ALK5, and ALK7 inhibitor] as the TGF-β signaling inhibitor, LDN193189 (ALK1, ALK2, ALK3, and ALK6 inhibitor) as the BMP signaling inhibitor, Y-27632 as the ROCK1 inhibitor, VPA as the histone deacetylase inhibitor, forskolin (adenylate cyclase activator) as the cAMP inducer, and PD0325901 as the MEK inhibitor. HUVECs were cultured in EGM2 containing the seven chemical compounds for 7 d (Fig. 1A). We assessed the expression of *PECAM1*, a marker of ECs, and *CD34*, a representative marker of EPCs, by qPCR [1,28]. Both *PECAM1* and *CD34* expressions were upregulated in HUVECs treated with seven chemical compounds compared to those in untreated or vehicle-treated HUVECs. Especially, the level of *CD34* was 98-fold increased by treatment of seven chemical compounds (Fig. 1B). To investigate the protein expression of CD31 (*PECAM1*) and CD34, we performed flow cytometry and revealed that the $CD31^{+}CD34^{+}$ cell population increased after chemical exposure (95.6% ± 0.36%) compared with that in untreated HUVECs (5.2% ± 0.77%) (Fig. 1C).

To identify the specific chemical compound associated with the increase in CD34 expression, we prepared EGM2 culture media lacking each of the seven compounds and cultured HUVECs in the separate media for 7 d (Fig. 1D). The expression of *CD34* was not detected in cells treated with PD0325901-free media, while there was no significant reduction in expression in the other groups (Fig. 1E). In contrast, cells treated with A83-01- or forskolin-free media showed significantly higher levels of *CD34* than the all-compound-treated cells (Fig. 1E). These findings suggested that PD0325901 promotes CD34 expression in HUVECs.

3.2. PD0325901 upregulated CD34 expression via inhibition of ERK phosphorylation

We investigated the detailed effects of PD0325901 on CD34 expression in HUVECs. Cells were exposed to 1 μM PD0325901 for 7 d (Fig. 2A). qPCR analysis showed that *CD34* expression was 1095-fold higher in PD0325901-treated than in untreated HUVECs (Fig. 2B), indicating a more pronounced increase than that observed under exposure to all compounds (Supplemental Fig. 1A). Flow cytometric analysis showed that the frequency of $CD31^{+}CD34^{+}$ cells was 98.2% ± 0.36% after PD0325901 exposure (Fig. 2C). Immunocytochemical analysis revealed that PD0325901-treated cells expressed CD34 in the cytosol and cell membrane (Fig. 2D). Additionally, these cells showed reduced cell proliferation compared to vehicle-treated cells (Supplemental Fig. 2A), and the number of viable PD0325901-exposed cells remained almost unchanged for 7 d (Supplemental Fig. 2B and C). We assessed the effects of treatment duration and concentration on HUVECs cultured in EGM2 containing 0.5, 1, or 5 μM PD0325901 for 4, 7, or 10 d (Supplemental Fig. 3A). *CD34* expression increased with the increasing duration and concentration of PD0325901 treatment, but there were no significant differences between 0.5 μM for 10 d, 1 μM for 7 d, 1 μM for 10 d, 5 μM for 7 d, and 5 μM for 10 d (Supplemental Fig. 3B). In addition, cell proliferation was reduced in cells treated with 5 μM PD0325901 compared with those treated with 0.5 μM and 1 μM PD0325901 at all time points (data not shown). We found that 1 μM PD0325901 increased CD34 expression more quickly than 0.5 μM PD0325901 and retained cell proliferation compared with 5 μM PD0325901. We examined the time-dependent changes in CD34 protein expression in HUVECs cultured in EGM2 containing 1 μM PD0325901. Flow cytometry analysis indicated that the $CD31^{+}CD34^{+}$ cell population increased from day 1–7 (Supplementary Fig. 3C).

To confirm the inhibitory effects of PD0325901 on MEK activity in HUVECs, we investigated the phosphorylation of extracellular signal-regulated kinase (ERK) 1/2—the downstream signaling protein of the MEK pathway—by western blotting using samples treated with 1 μM PD0325901 for 24 h (Fig. 3A). Western

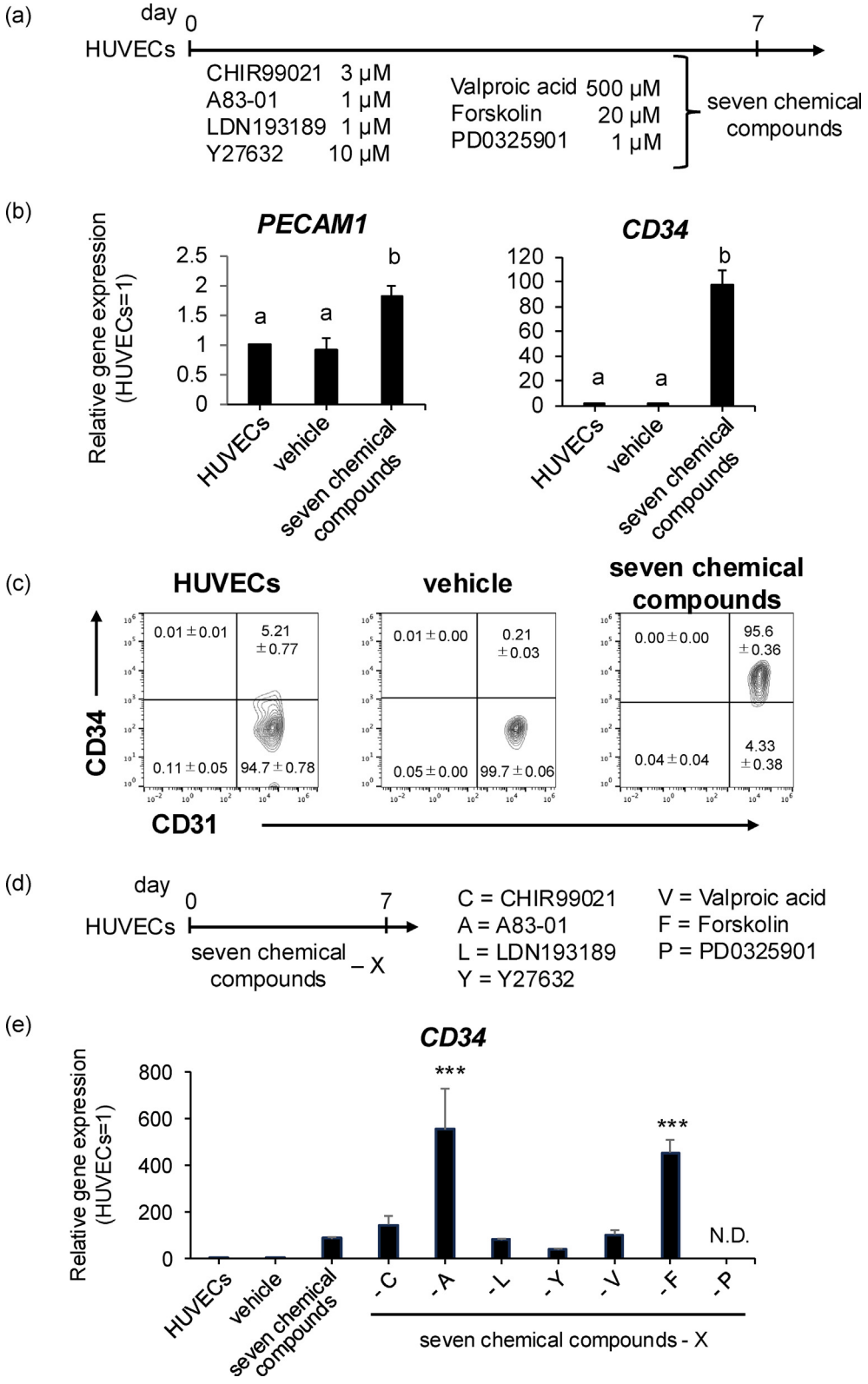

Fig. 1. Chemical screening of compounds affecting CD34 expression. (a) Experimental protocol. (b) Expression of endothelial marker *PECAM1* and endothelial progenitor cell (EPC) marker *CD34* in HUVECs following treatment with vehicle and seven chemical compounds based on RT-qPCR. Different letters above the columns indicate significant differences ($p < 0.05$, one-way ANOVA followed by Tukey's multiple comparison test). (c) Expression of CD31 and CD34 based on flow cytometry. (d) Experimental protocol. (e) RT-qPCR analysis of *CD34* in cells exposed to six compounds (seven compounds - X). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; one-way ANOVA followed by Dunnett's multiple comparison test compared with seven chemical compounds as control. Data are presented as the mean ± SD, $n = 3$.

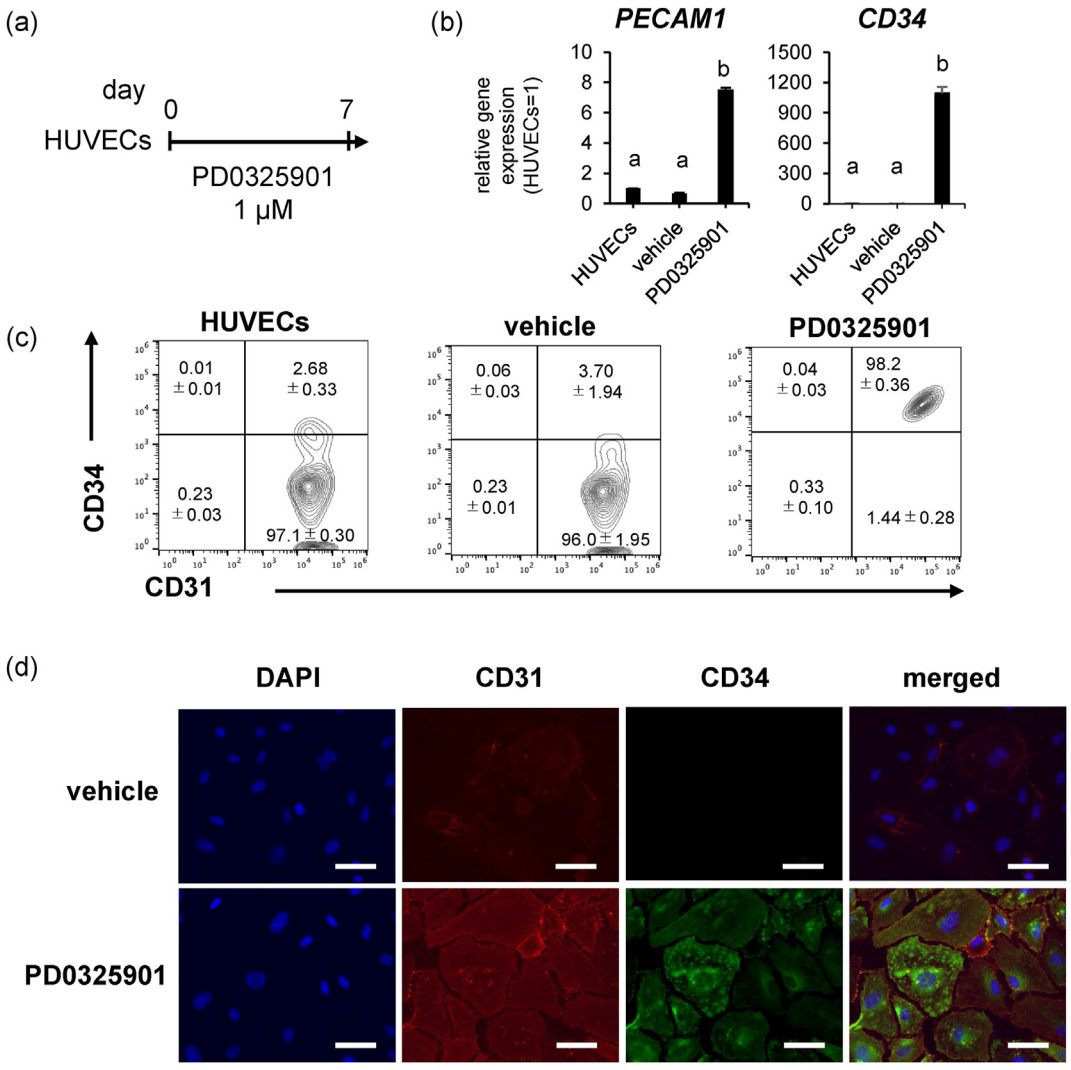

Fig. 2. PD0325901 upregulates CD34 expression. (a) Experimental protocol. (b) RT-qPCR analysis of *PECAM1* and *CD34* in HUVECs treated with vehicle and PD0325901. Data are presented as the mean ± SEM of four independent trials, $n = 3$. Different letters above the columns indicate significant differences ($p < 0.05$, one-way ANOVA followed by Tukey's multiple comparison test). (c) Flow cytometric analysis of CD31 and CD34 in HUVECs treated with vehicle and PD0325901. Data are presented as the mean ± SD, $n = 3$. (d) Immunofluorescence of CD31 and CD34 in vehicle- and PD0325901-treated samples. Scale bar = 50 μm.

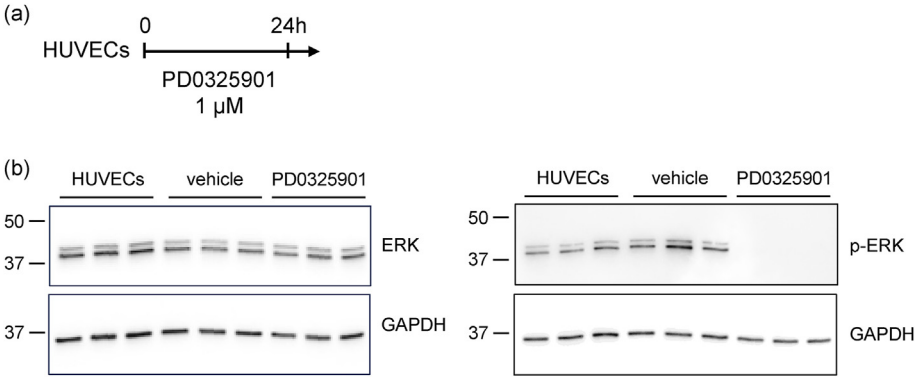

Fig. 3. Western blotting indicates PD0325901 inhibits ERK phosphorylation in HUVECs. (a) Experimental protocols. (b) Western blot analysis of extracellular signal-regulated kinase (ERK) phosphorylation. All data are presented as the mean ± SD, $n = 3$.

blotting revealed that ERK1/2 was present in all samples, but phosphorylated ERK1/2 levels were substantially reduced in PD0325901-treated samples compared to those in untreated and vehicle-treated HUVECs (Fig. 3B). These results indicated that PD0325901 upregulates the expression CD34 by inhibiting ERK1/2 phosphorylation.

3.3. Isolation of CD34-negative HUVEC fraction and PD0325901 exposure

As demonstrated in Fig. 1C and 2C, and Supplemental Fig. 3C, we measured the original percentage of $CD34^+$ HUVECs. To determine whether the increased expression of CD34 was due to cell selection (elimination of the CD34-negative fraction) or cell induction (transforming of the CD34-negative to -positive fraction), we purified the CD34-negative fraction from untreated HUVECs using MACS and cultured it in medium supplemented with PD0325901 (Fig. 4A). Flow cytometric analysis immediately after MACS showed that the population of $CD34^+$ cells in the negative fraction was significantly lower than that in the untreated HUVECs (Fig. 4B). In contrast, after exposure to PD0325901, no significant differences were observed in the $CD34^+$ population between the negative fraction and total HUVECs (Fig. 4C). Almost no CD34-positive cells were detected immediately after MACS, whereas they dominated after PD0325901 treatment in both cell groups (Supplemental Fig. 4A). Cell proliferation on day 7 was almost equal and non-confluent in both the negative and total HUVECs (no passage for 7 d) (Supplemental Fig. 4B and 4C). These findings suggested that PD0325901 induces the expression of CD34 in HUVECs.

3.4. Effects of PD0325901 on other types of cells

We investigated the specificity of PD0325901 in upregulating CD34 expression in cell types other than ECs, namely fetal lung fibroblasts (MRC-5), which showed very low levels of CD31 and CD34. We cultured MRC-5 cells in medium with 1 μM PD0325901 on fibronectin-coated dishes for 7 d (Fig. 5A). Interestingly, regardless of treatment with PD0325901, there were no significant changes in *CD34* expression, whereas *PECAM1* expression was significantly decreased in PD0325901-treated MRC-5 cells (Fig. 5B). Flow cytometric analysis revealed that the population of $CD31^+CD34^+$ cells did not increase in MRC-5 cells treated with PD0325901 (Fig. 5C). These findings indicated that PD0325901 upregulates CD34 expression in specific cell types.

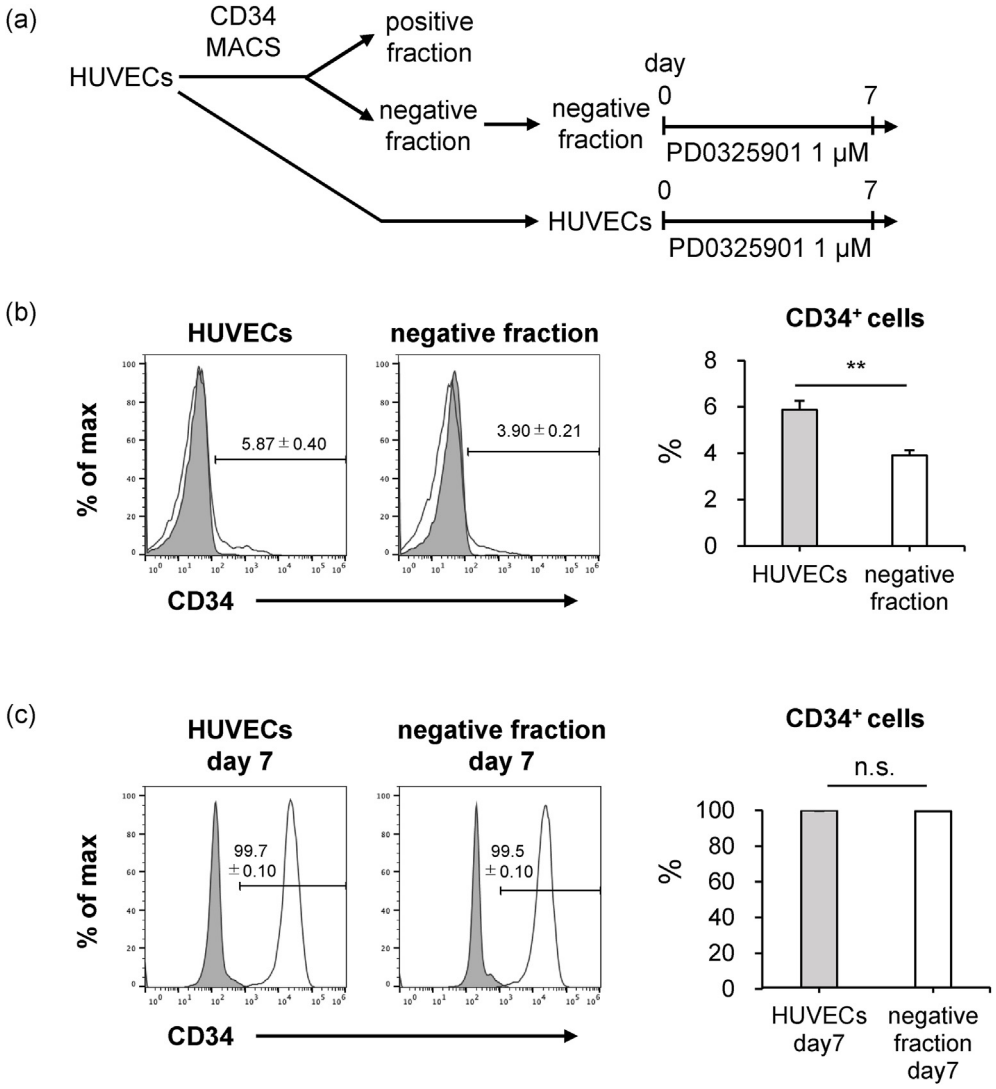

Fig. 4. Isolation of CD34-negative fraction and exposure to PD0325901. (a) Experimental protocol. (b) Flow cytometric analysis of CD34 in HUVECs and negative fraction isolated by magnetic-activated cell sorting (MACS). $^{**}p < 0.01$ (unpaired Student's *t*-test). (c) Flow cytometry analysis of CD34 in HUVECs and negative fraction after 7 d of PD0325901 exposure. n.s., non-significant (unpaired Student's *t*-test).

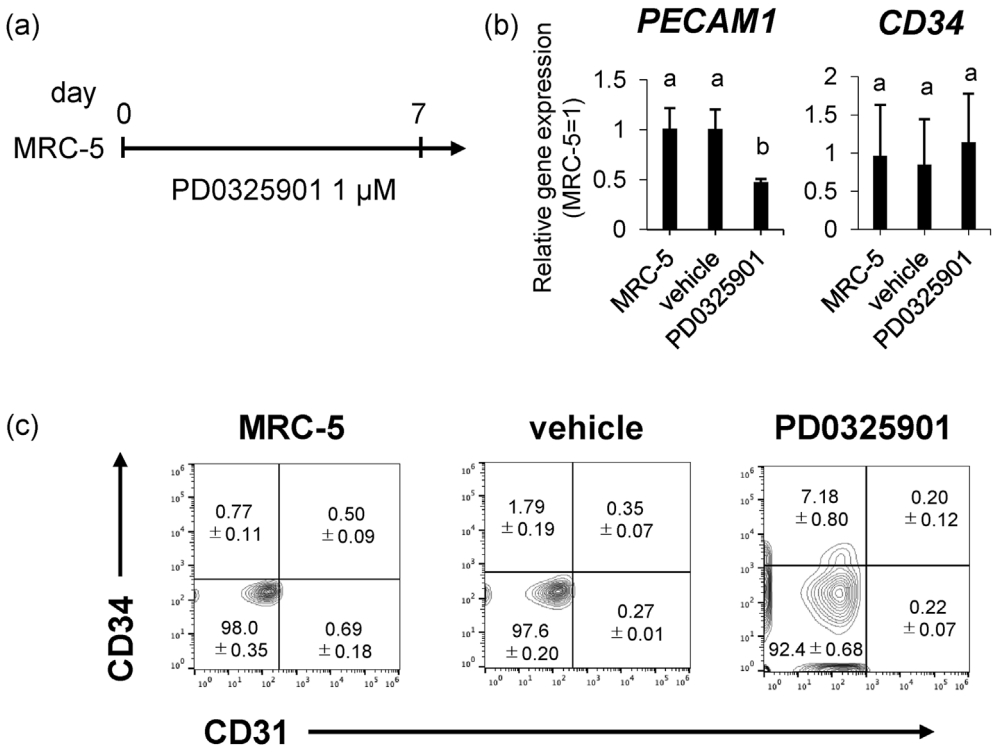

Fig. 5. Effects of PD0325901 on fetal lung fibroblast MRC-5 cells. (a) Experimental protocol. (b) RT-qPCR analysis of *PECAM1* and *CD34* expression in MRC-5 cells treated with vehicle and PD0325901-exposed samples. Different letters above the columns indicate significant differences ($p < 0.05$, one-way ANOVA followed by Tukey's multiple comparison test). (c) Flow cytometry analysis of CD31 and CD34 in MRC-5 cells treated with vehicle and PD0325901. Data are presented as the mean ± SD, $n = 3$.

4. Discussion

In this study, we screened candidate chemical compounds that can reprogram ECs into EPCs based on the upregulated expression of CD34. We showed that the MEK inhibitor PD0325901 upregulated the expression of CD34 in HUVECs by inhibiting ERK phosphorylation. PD0325901 potently induced CD34 expression in 98% of ECs after treatment with 1 μM for 7 d. Approximately 80% of HUVECs are CD34-positive immediately after isolation from the umbilical cord; however, this percentage rapidly decreases with cell proliferation after the start of in vitro cell culture [29]. However, the mechanism underlying the changes in CD34 expression in ECs remains unclear. We showed that PD0325901 directly induced CD34 expression in CD34-negative cells (Fig. 4B and C). In contrast, CD34 expression was not upregulated in lung fibroblast MRC-5 cells after PD0325901 treatment (Fig. 5B and C). These results indicated that certain signaling pathways may regulate CD34 expression in ECs. We observed that CD34 expression was significantly increased in cells treated with A83-01- and forskolin-free media (Fig. 1E), suggesting that A83-01 and forskolin may suppress CD34 expression in ECs and that unknown signaling pathways other than the MEK pathway may participate in the regulation of CD34 expression. A previous study reported that $CD34^+$ neuroblastoma cancer stem cells (NB-CSCs) and $CD34^-$ NB-CSCs demonstrated significant differences in gene expression related to stem cell pluripotency and Wnt signaling [30]. In hematopoietic stem cells, the thrombopoietin pathway was shown to be upregulated in lineage marker-positive (Lin^+) $CD34^+$ cells compared to Lin^-CD34^- cells [31]. Collectively, these results suggest that CD34 expression in ECs is induced through the inhibition of the MEK signaling pathway, but other types of pathways may also be involved. Future transcriptome analyses are needed to elucidate these complicated regulatory mechanisms.

PD0325901 suppressed the phosphorylation of ERK1/2 in ECs (Fig. 3B). Ras–Raf–MEK–ERK signaling participates in various cellular activities, such as proliferation, migration, and differentiation. The ERK1/2 signaling pathway is activated by growth factors [32,33]; therefore, the reduction in $CD34^+$-cell proliferation in this study may have been caused by the suppression of proliferation signals via MEK inhibition.

Regarding the relationship between cell proliferation and CD34 expression in ECs, Delia et al. [29] showed that CD34 expression decreased with repeated cell division in vitro and increased under suppressed cell proliferation, such as in high-density or growth factor-free medium cultures. CD34-positive HUVECs also showed lower proliferative activity than CD34-negative HUVECs [34]. These studies suggested that cell proliferation and CD34 expression may be inversely correlated in HUVECs. In other cell types, Avolio et al. [35] recently demonstrated that cardiac pericytes can be reprogrammed to vascular smooth muscle cell (VSMC)-like cells using PD0325901 and suggested that the resulting cell proliferation arrest may be independent of the reprogramming effect. In the same study, the expression of *CCND1* (cyclin D1-encoded gene), which is regulated by MEK–ERK signaling and required for cell cycle progression from G1 to S, decreased in PD0325901-treated cells. However, they also showed that inhibition of cyclin-dependent kinase 4/6 (CDK4/6), which is activated by cyclin D1, did not reprogram cardiac pericytes into VSMC-like cells. These results suggested that cell proliferation arrest and the reprogramming effects of PD0325901 occur simultaneously but independently.

In the present study, we observed simultaneous cell proliferation arrest (Supplemental Fig. 2A, B, and C) and $CD34^+$ cell induction by PD0325901 (Fig. 2B, C, D), which seems to be consistent with the inverse relationship between proliferation and CD34 expression reported in previous studies. To determine whether there is a direct relationship between cell proliferation arrest and CD34 upregulation by PD0325901, future studies should consider the effects of cell cycle-associated protein inhibitors.

The viability and safety of autologous CD34$^+$ cell transplantation has been reported in various ischemic diseases [5–9]. Several recent studies reported the potential of an in vivo reprogramming approach using PD0325901. For instance, PD0325901 effectively reprogrammed cardiac pericytes into VSMCs in mice, thereby promoting angiogenesis and inhibiting cardiac dysfunction [35]. Ma et al. [36] transdifferentiated cochlear supporting cells into hair cells (HCs) using PD0325901 and showed the induction of HCs regeneration in vivo. These findings support the potential of PD0325901 for reprogramming ECs into CD34$^+$ EPCs for the treatment of ischemic diseases, though further in vivo studies are needed.

Our study had one obvious limitation in that we did not assess the angiogenic potential of CD34-positive cells generated by PD0325901. Future studies should consider the activities of angiogenesis-related cytokines and evaluate their angiogenic ability under treatment with PD0325901. In addition, we used HUVEC at passage 4–7 for the experiments in this study. Comparisons of the obtained CD34-positive cells with freshly isolated HUVEC should be also needed for the precise assessments of angiogenic potential of cells.

5. Conclusions

CD34 expression was strongly induced in ECs by treatment with the MEK inhibitor PD0325901 in vitro. Our findings may inform the establishment of a generalizable method for preparing CD34-positive EPCs and contribute to the development of regenerative therapies, especially for ischemic diseases.

Author contribution

Conceptualization: S.M. and C.H.; Investigation: C.H., S.M., S.K., Y.O. and Y.T.; Formal analysis: C.H. and S.M.; Data curation: C.H. and S.M.; Writing – Original Draft: C.H.; Writing – review and editing: C.H., S.M., A.S., M.S., and K.T.; funding acquisition: M.S. and K.T.

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Declaration of competing interest

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.reth.2024.08.009.

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