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Authors

Li, Tao

Wu, Haopeng

Wang, Pingping

et al.

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HDACs Regulate the Differentiation of Endothelial Cells from Human IPSCs

Tao Li[1,2], **Haopeng Wu**[1], **Pingping Wang**[1], **Amy M Kim**[2], **Junjing Jia**[2], **Jan A. Nolta**[2,3,4], **Ping Zhou**[2,3,4]

[1]School of Medicine, Hunan Normal University, Changsha, Hunan, 410081, China

[2]Stem Cell Program, University of California Davis Medical Center, Sacramento, CA, USA, California, USA

[3]Department of Internal Medicine, University of California Davis Medical Center, Sacramento, CA, USA, California, USA

[4]University of California Davis Gene Therapy Center, Sacramento California, USA

Abstract

Human induced pluripotent stem cells (hiPSCs) possess the potential to differentiate toward vascular cells including endothelial cells (ECs), pericytes, and smooth muscle cells (SMCs). Epigenetic mechanisms including DNA methylation and histone modification play a crucial role in regulating lineage differentiation and specification. Herein, we utilized a three-stage protocol to induce differentiation of mesoderm, vascular progenitors and ECs from hiPSCs and investigated the regulatory effects of histone acetylation on the differentiation processes. We found that the expression of several histone deacetylases (HDACs) including HDAC1, HDAC5, and HDAC7 were greatly upregulated at the second stage and downregulated at the third stage. Interestingly, while HDAC1 remained in the nucleus during the EC differentiation. HDAC 5 and HDAC7 displayed cytosol/nuclear translocation during the differentiation process. Inhibition of HDACs with sodium butyrate (NaBt) or BML210 could hinder the differentiation of vascular progenitors at the second stage and facilitate EC induction at the third stage. Further investigation revealed that HDAC may modulate the stepwise EC differentiation via regulating the expression of endothelial transcription factors *ERG*, *ETS1*, and *MEF2C*. Opposite to the expression of EC markers, the smooth muscle/pericyte marker *ACTA2* was upregulated at the second stage and downregulated at the third stage by NaBt. The stage specific regulation of *ACTA2* by HDAC inhibition was likely through regulating the expression of *TGFβ2* and *PDGFB*. This study suggests that HDACs play different roles at different stages of EC induction by promoting the commitment of vascular progenitors and impeding the later stage differentiation of ECs.

Correspondence: Ping Zhou, Ph.D., Stem Cell Program, Department of Internal Medicine, University of California Davis, 2921 Stockton Blvd, Sacramento, CA 95817, Phone: 916 703 9371; Fax: 916 703 9310; pizhou@ucdavis.edu.

Author contributions

T.L. operated the experiments; P.P.W. wrote the manuscript; H.P.W. analyzed the data; A.M.K. and J. J. collected data, J.A.N. and P.Z. designed the experiments, edited the manuscript and provided financial support.

Competing interests

The authors declare there are no competing interests

Keywords

endothelial cells; induced pluripotent stem cells; histone deacetylase; differentiation; HDAC inhibitor

Introduction

Human induced pluripotent stem cells (hiPSCs) are generated by reprogramming somatic cells with defined transcription factors (c-MYC, OCT4, SOX2, KLF4). HiPSCs share high similarity with human embryonic stem cells (hESCs) in morphological characteristics and pluripotency, the capacity to differentiate toward any cell type in a body under proper stimuli. Different from hESCs, hiPSCs can be generated from patients as immune-matched autologous cells to avoid immunogenicity concerns[1] and thus present an opportunity for generation of functional autologous cells for transplantation.

Endothelial cells (ECs) derived from pluripotent stem cells emerge as a potential cell source for regenerative medicine. Several serious diseases such as limb amputation, strokes, myocardial infarction, and heart failure, frequently attribute to endothelial dysfunction. Transplantation of ECs is a promising therapeutic approach for repairing injured vessels and restoring perfusion of ischemic tissues. Meanwhile, vascularization of implanted tissues has been an utmost demand to repair or replace disabled organs. ECs can collaborate with stromal cells, pericytes, and smooth muscle cells (SMCs) to self-assemble a microvasculature. Therefore, incorporation of ECs into engineered tissues prior to implantation, is a promising strategy to achieve rapid vascularization of the transplanted tissues for access to nutrients and oxygen [1].

Differentiation of ECs from iPSCs has been extensively studied not only for generating ECs for transplantation but also for understanding molecular mechanisms underlying EC differentiation. Three general approaches have been developed for differentiation of hiPSCs into ECs: three-dimensional (3D) embryoid body (EB)-mediated differentiation, two-dimensional (2D) monolayer-directed differentiation, and coculture with feeder cells[2,3]. A stepwise differentiation of hiPSCs into ECs with timely administration of different chemical molecules or growth factors in a two-dimensional culture system is an efficient strategy. Substantial evidence overwhelmingly demonstrates that EC differentiation from ESCs or iPSCs *in vitro* imitates vascular morphogenic events *in vivo*. The progressive differentiation program firstly drives ESCs or iPSCs to differentiate into mesoderm cells. Subsequently, mesoderm intermediates will be induced into vascular progenitors which can give rise to ECs, pericytes, or SMCs. Eventually, vascular progenitors are coerced to adopt a fate toward mature and functional ECs. Recently, many efforts have been made to improve the differentiation efficiency, clinical applicability of ECs, biomaterial-mediated EC delivery, and therapeutic vascularization with ECs [2,3].

The differentiation of iPSCs toward ECs is tightly governed by extrinsic signals and intrinsic regulatory factors. Several growth factors have been found to promote mesoderm and endothelial lineage specification, such as bone morphogenetic protein 4 (BMP4), Activin A, basic fibroblast growth factor (bFGF), insulin-like growth factor-1 (IGF-1), and

vascular endothelial growth factor (VEGF)[4–6]. Wnt, Notch, MAPK, and PI3K pathways also contribute to mesoderm induction, the commitment to vascular lineages, and/or the differentiation of vascular progenitors to ECs[5,7]. Manipulating Wnt signaling by GSK3β inhibitors, such as CHIR99021 and BIO, stimulates the differentiation of hiPSCs toward mesoderm cells. Inhibition the Notch pathway by addition of the γ-secretase inhibitor DAPT during the late phase of EC induction, effectively increases the efficiency of EC production[8]. Moreover, a set of specific transcriptional factors such as ERG, GATA2, ETS1, ETV2, COUP-TFII, and FOXO1, are critical in the elaborate process of EC differentiation[9,10].

Additionally, epigenetic modification also temporally and spatially exerts crucial effects on vasculogenesis. DNA methylation and histone modification as epigenetic mechanisms participate in cell fate determination and specification via regulating dynamic activation or repression of genes. Histone acetylation mediated by histone acetylases (HATs) is a reversible event marked by transferring the acetyl moiety from acetyl co-enzyme A to lysine residues, thereby liberating chromatin structure and favoring the access of transcription factors to DNA. In contrast, histone deacetylases (HDACs) remove acetyl groups from the N-acetylated lysine residues on histones and enforce histone-DNA binding to mediate gene silence. The HDAC superfamily consists of 18 members and is classified into four classes. Class I includes HDAC1, 2, 3, and 8; Class II is represented by HDAC4, 5, 6, 7, 9, and 10; Class III includes SIRT1–7; and Class IV comprises HDAC11. Previous studies have shown that general inhibitors of class I and II HDACs (HDACi), such as sodium butyrate (NaBt) and trichostatin A (TSA), can manipulate stem cell differentiation into different cell lineages[11].

In this study, we investigated the expression profile of HDACs during EC differentiation from hiPSCs. HDAC5 expression underwent a significant fluctuation along the entire process of EC differentiation. Furthermore, we found that HDAC inhibition exerted distinct effects on EC differentiation at different phases. HDAC inhibition could impair EC differentiation and conversely enhance the expression of smooth muscle genes at the second differentiation phase. However, HDAC inhibition improved the expression of EC-specific genes at the third differentiation phase. HDAC inhibition affected EC differentiation via regulating the expression of endothelial transcription factors and cytokines. Therefore, our finding indicated that HDACs play different roles at different differentiation stages during EC induction of hiPSCs.

Materials and Methods

Reagents

CHIR99012 was obtained from Tocris Bioscience (Avonmouth, UK). BMP4, FGF2, and VEGF were purchased from R&D Systems (Minneapolis, MN). HDAC inhibitors sodium butyrate (NaBt) and BML210 were purchased from Selleck Chemicals (Houston, TX, USA). The inhibitors of ERK1/2 and PI3K, U0126 and LY294002, were also obtained from EMD4Bioscinens (Darmstadt, Germany). Antibodies that were used in the present study included CD31 (Bethyl, Montgomery, TX) and α-smooth muscle actin (α-SMA, Sigma-Aldrich, St. Louis, MO). Antibodies against HDAC1(#110831) and HDAC7 (#34589) were

purchased from Cell Signaling Technology (Boston, MA), Anti-HDAC5 antibody (ab55403) was obtained from Abcam (Cambridge, MA).

Cell Cultures for hiPSCs

Human iPSC line Dura6.9 was obtained from the Stem Cell Core at University of California Davis and previously characterized[12]. Dura6.9 cells were cultivated on Matrigel (Corning, Corning, NY)-coated culture plates in StemFlex medium (Thermo Fisher, Grand Island, NY). Cell medium was refreshed as manufacturer's instructions every other day. When reach 70–80% confluence, the cells were passaged with the ratio 1:9 using the ReLeSR cell dissociation solution (STEMCELL Technologies, Cambridge, MA).

Cell Differentiation

EC differentiation from hiPSCs was progressively induced in according to a three-stage protocol as described previously[7]. Briefly, one day after passaging hiPSCs onto hESC-qualified Matrigel-coated culture plates in StemFlex medium, cells were cultured in StemDiff APEL medium (STEMCELL Technologies, Cambridge, MA) with 6 μM of CHIR99012 for 2 days. The next 2 days, the culture medium was altered to StemDiff APEL medium (STEMCELL Technologies) supplemented with 25 ng/ml BMP4, 10 ng/ml FGF2, and 50 ng/ml VEGF. The cells at day 4 were lifted with Accutase (Innovative Life Technologies, San Diego, CA) and plated onto cell culture plates in ECGM-MV2 (PromoCell) medium with an additional 50 ng/ml VEGF. The medium was changed every 2 days for 4 days to generate mature ECs.

Quantitative RT-PCR (qRT-PCR)

RNA was isolated using QIAshredder and RNeasy Mini Kit (Qiagen, Germantown, MD) according to the manufacturer's instructions. cDNA was generated from 500 ng RNA using the High-Density cDNA Conversion Kit (Applied Biosystems) according to the manufacture's protocol. To quantitate the gene expression of interest, qPCR was conducted using 500 ng cDNA from each sample in the presence of specific primers and SYBR Green PCR Master Mix (Applied Biosystems) in the ABI-7300-Real Time-PCR System (Applied Biosystems). Primer sequences for tested genes are listed in Supporting Information Table 1. Ct value was first normalized to the internal GAPDH level and presented as fold change compared to the average of the control group. For each condition, 3–4 independent samples were tested in one hiPSC line first and repeated in another hiPSC line.

Immunofluorescence Staining

Cultured cells were fixed in 4% paraformaldehyde (Affymetrix, Cleveland, Ohio) for 20 minutes. Immunostaining was performed as previously described[13]. Antibodies against human CD31 and α-SMA were used at a 1:200 and 1:400 dilutions, respectively. Images were taken with Nikon Eclipse Ti microscope (Nikon Instruments Inc., Melville, NY).

Tube Formation

For *in vitro* tube formation, hiPSC-ECs were cultured on a solid layer of growth factor reduced Matrigel (Corning) in a 96-well plate (1.5×10^4 cells per well) in EC Growth Medium MV2 (PromoCell). Images were taken at 24 hours after plating.

Statistics

Each group had at least 3 samples. Data is presented as average with standard deviation. One-way analysis of variance (ANOVA) was performed to detect whether a significant difference existed between groups with different treatments, and Tukey's multiple comparisons test was used for post-analysis. Probability (p) less than 0.05 was considered statistically significant between samples in comparison.

Results

Stepwise differentiation of ECs from hiPSCs

We induced the generation of ECs from hiPSCs with high efficiency and reproducibility according to a stepwise procedure[7] (Fig.1A). Firstly, hiPSCs were exposed to GSK3β inhibitor (GSKi) CHIR99021 at 6 μM for 2 days, thus mesoderm induction was triggered due to the activation of WNT signaling pathway. Subsequently, a combined treatment of VEGF, FGF2, and BMP4 for 2 days further drove the transformation of mesodermal cells into vascular progenitors. Finally, vascular progenitors were cultured in endothelial medium ECGM-MV2 supplemented with extra 50 ng/ml of VEGF for 4 days to achieve EC specification and maturation. The hiPSC-derived ECs exhibited homogeneous cobblestone morphology, characteristic of ECs (Fig.1B). Immunofluorescence assay confirmed the expression of endothelial lineage marker CD31/PECAM1 in hiPSC-derived ECs (Fig.1C). Only a small fraction of cells were positively stained for human alpha-smooth muscle actin (ACTA2), a marker for smooth muscle cell lineage (Fig.1C). The time-course analysis of EC markers *CD31* and *CD144* (*VE-cadherin*) also validated the stepwise differentiation of ECs from hiPSCs (Fig.1D). Vascular progenitors at the second differentiation stage initiated the expression of *CD31* and *CD144*. Mature ECs expressed higher levels of *CD31* and *CD144*. Moreover, when seeded on matrigel, hiPSC-derived ECs could assemble into tube-like structures *in vitro*, indicating that these cells displayed the competence of ECs in function (Fig.1E). Taken together, we successfully induced EC differentiation from pluripotent stem cells via a multiple-stage induction protocol.

Expression profile of HDAC genes during EC differentiation

Subsequently, we assessed the expression profile of HDAC family genes throughout the differentiation process. As shown in Fig.2A, vascular progenitors at the second differentiation stage (day 4) exhibited 2-fold increase in the expression of Class I HDAC, HDAC1. Interestingly, the expression level of HDAC1 continued to undergo a decrease at day 6, and a slight increase at day 8. The other tested Class I HDACs (HDAC2, 3 and 8) did not change expression significantly during the EC differentiation. In addition, three Class II HDACs, HDAC5, 6, and 7, also exhibited a marked enhancement of mRNA levels in vascular progenitors (day 4) (Fig.2B). Specially, *HDAC5* displayed a significant expression

fluctuation and was slightly downregulated at day 2, greatly upregulated at day 4, greatly downregulated again at day 6 and then moderately upregulated at day 8. Similarly, the expression *HDAC6 and 7* were greatly enhanced at day 4, declined at day 6 and increased moderately again at day 8. HDAC7 showed the highest increase in vascular progenitors and ECs among the tested HDACs.

To further investigate the expression of HDACs during EC differentiation, the iPSCs (Day 0), iPSC-derived vascular progenitors (Day 4) and ECs (day8) were immunostained with HDAC1, 5 and 7, respectively. HDAC1 were detected in the nucleus of all three stage cells, while HDAC5 was detected mostly in the cytosol of all three stage cells. HDAC7, on the other hand, was primarily located in the cytosol of iPSCs and vascular progenitors but was present mainly in the nucleus of the ECs. Furthermore, the ECs showed decreased expression of HDAC5 and HDAC7 compared to the vascular progenitors, which is consistent with the qRT-PCR analysis of the cells at these stages (Fig. 2B). Taken together, these results suggest that vascular progenitors at the second differentiation stage have an increased expression of multiple Class I and Class II HDACs. The expression of these HDACs is downregulated at the third stage of the differentiation. It suggests that HDACs may exert different effects during the stepwise induction.

Expression regulation of HDACs at the second differentiation stage

Since the expression of several HDACs was significantly enhanced in vascular progenitors (day 4), we subsequently investigated the underlying mechanism. The occurrence of vascular progenitors attributed to the combined induction of VEGF, FGF2, and BMP4. Therefore, we focused on the effects of these growth factors on HDAC expression. BMP4 and VEGF played a role in mediating the upregulation of *HDAC7* (Fig.3A). Combined stimulation of BMP, FGF and VEGF not only further enhanced the expression of HDAC7 but also enhanced the expression of HDAC5 (Fig. 3A). Furthermore, inhibition of ERK1/2 by the specific inhibitor U0126 attenuated the expression of *HDAC5* but not *HDAC1* and *HDAC7* (Fig.3B), while inhibition of PI3K by the inhibitor LY294002 had no effect on the expression of these HDACs (Fig.3B). Seemingly, the synergic treatment of VEGF, FGF2, and BMP4 partially accounts for the upregulation of HDAC5 and HDAC7 at the second differentiation stage.

The localization alteration of HDACs during EC differentiation

Subsequently, we investigated the expression and localization of HDAC1, HDAC5, and HDAC7 during EC differentiation by immunostaining the iPSCs, the differentiated cells at day 4 and day 8. Interestingly, HDAC1 always remained in the nucleus during the process of EC differentiation (Fig.4). In contrast, HDAC5 and HDAC7 were localized in both the nucleus and cytoplasm in hiPSCs. The majority of HDAC5 and HDAC7 displayed cytoplasmic localization in vascular progenitors at day 4. After the accomplishment of EC differentiation, HDAC5 restored to both of nuclear and cytoplasmic localization at day 8. The nuclear distribution of HDAC7 was much more prominent at day 8 (Fig.4). These results further imply the involvement of HDACs in the regulation of EC differentiation.

HDAC inhibition represses endothelial differentiation at the second stage

Since several HDACs were upregulated at the second differentiation stage and were conversely regulated at the third stage, we speculated that HDAC inhibition at different induction stages might yield completely different effects on EC differentiation. Thus, we added a pan HDAC inhibitor NaBt (1 or 2 mM) into the culture medium at the second differentiation stage (Fig.5A). After NaBt exposure for 2 days, vascular progenitors at day 4 expressed higher levels of smooth muscle markers *ACTA2* and *CNN1* (Fig.5B). Simultaneously, the expression of EC markers *CD31* and *CD144* was greatly inhibited by NaBt treatment. Administration of another HDAC inhibitor BML210 at the second differentiation stage also decreased the expression of EC markers *CD31* and *CD144* (Fig.5C) and significantly enhanced in the expression of smooth muscle markers *ACTA2*. Cells treated by NaBt for 2 days at the second stage were allowed to further culture and differentiation. At day 8, a sharp decrease in the efficiency of EC differentiation was observed in these NaBt-treated cells, as evidenced by reduced CD31 staining (Fig.5D). After NaBt treatment at the second stage, the number of ACTA2-positive cells was greatly enhanced (Fig.5D). Thus, we conclude that HDACs are required for EC differentiation at the second stage and HDAC inhibition at the second stage blocks EC differentiation.

HDAC inhibition promotes EC differentiation at the third stage

We assessed the effect of HDAC inhibition at the third stage on EC differentiation. NaBt (1 or 2 mM) treatment for 4 days at the third stage remarkably decreased the expression of *ACTA2* and *CNN1*, and enhanced the expression of *CD31* and *CD144* at the same time (Fig.6A, B). Consistently, BML210 at the third differentiation stage strongly reduced the expression of *ACTA2* and *CNN1*, and enhanced *CD144* expression (Fig.6C). Collectively, these findings suggest that HDAC inhibition at different stages could block EC induction at the second differentiation stage but promote EC differentiation at the third differentiation stage.

HDAC inhibition affects the expression of endothelial transcription factors

Next, we investigated the mechanism underlying the stage-specific effects of HDAC inhibition. At the second differentiation stage (day 4), vascular progenitors exhibited enhanced expression of endothelial transcription factors, such as *GATA2*, *GATA4*, *ERG*, and *ETS1* (Fig.7A). As shown in Fig.7B, NaBt exposure at the second differentiation stage greatly decreased the expression of endothelial transcription factors *ERG* and *MEF2C*, slightly enhanced or unaffected the expression of *GATA2* and *ETS1*. On the other hand, NaBt treatment at the third differentiation stage upregulated the expression of *ERG*, *ETS1*, and *MEF2C*, while downregulated the expression of *GATA2* (Fig.7C). Thus, NaBt may modulate the stepwise EC differentiation via regulating endothelial transcription factors *ERG*, *ETS1*, and *MEF2C*.

HDAC inhibition affects the expression of TGFβ and PDGFB signaling

Since TGFβ and PDGF families are important cytokine signals regulating the differentiation of pericytes and SMCs. Next, we evaluated the expression of *TGFβ1*, *TGFβ2*, and *PDGFB*. As shown in Fig.8A, NaBt exposure at the second differentiation stage enhanced the

expression of *TGFβ2* but not *TGFβ1*. Simultaneously, NaBt greatly deceased *PDGFB* expression at the second stage. Furthermore, NaBt treatment at the third differentiation stage also remarkably enhanced *TGFβ2* but not *TGFβ1*expression (Fig.8B). Interestingly, NaBt treatment at the third differentiation stage robustly enhanced *PDGFB* expression. Therefore, HDAC inhibition differently affects the expression of *TGFβ2* and *PDGFB* expression.

Subsequently, we evaluated the effect of PDGF-BB at differentiation stages. As shown in Fig.8C, PDGF-BB exerted a repressive effect on *ACTA2* expression at the second stage. Moreover, PDGF-BB treatment at the third stage also decreased *ACTA2* expression (Fig.8D). Thus, HDAC inhibition might affect EC differentiation via regulating TGFβ and PDGFB signaling.

Discussion

iPSC-derived ECs are a promising cell source for transplantation therapies, such as tissue-engineered vascular grafts and revascularization of ischemic tissues. To date, a variety of induction protocols have been developed to differentiate pluripotent stem cells into functional ECs. Herein, our study shows that HDAC expression was regulated during EC differentiation from iPSCs and manipulation of HDAC activity can impact EC differentiation in a temporal fashion.

Our results showed that the expression of HDACs underwent a significant fluctuation along the entire process of EC differentiation. Several HDACs including *HDAC1*, *HDAC5*, and *HDAC7* underwent an increase in mRNA levels at the second stage (day 4). Intriguingly, the expression of these HDACs was significantly repressed at the third stage. These observations strongly implicate that HDACs may exert different effects during the stepwise induction of ECs. The treatment of VEGF, FGF2, and BMP4 partially accounts for the upregulation of certain HDACs during the generation of vascular progenitors from mesodermal cells. On the other hand, we could not exclude the possibility that the removal of Wnt agonist contributed to the upregulation of several *HDACs* at the second stage. Previous study has shown that Wnt activation led to the downregulation of several *HDACs* including *HDAC1* during cardiac differentiation[14]. Furthermore, our study validated that inhibition of HDAC with NaBt or BML210 hindered EC induction at the second differentiation stage but promoted EC differentiation at the third stage. Meanwhile, we observed that HDAC1 always remained in the nucleus during the whole process of EC differentiation. In contrast, HDAC5 and HDAC7 exhibited the localization alteration during EC differentiation. Seemingly, the nuclear distribution of HDAC5 and 7 was associated with EC maturation. Thus, HDACs exert different effects during the stepwise induction of ECs from hiPSCs.

Epigenetic mechanisms such as DNA methylation and histone modification undoubtedly play a critical role in establishing tempo-spatial expression patterns of tissue-specific genes during embryonic development. Interestingly, several studies have shown that histone modification can repress or promote cell differentiation at different phases. For instance, H3K9ac gradually declined at the early stage of *in vitro* neural differentiation of human ESCs (hESCs) and then increased at the late stage. Several HDAC inhibitors favored hESC pluripotency and reduced its neural differentiation at the early stage of

neural commitment. In contrast, HDACi treatment at the late stage evoked an augment of H3K9 acetylation, therefore facilitating neural differentiation and improving multiple neurodevelopmental genes[15]. Another study showed that histone acetylation exerted positive effects on mesoderm induction and the formation of cardiac progenitors at the early stage of cardiac differentiation. However, HDACi at the late stage hindered cell exit from pluripotency, thus mitigated the amount of cardiac functional proteins[16]. Meanwhile, HDACs also play a pleiotropic role in the regulation of erythropoiesis. HDACi specifically promoted the generation of immature erythroid cells, while minimized the generation of relatively mature erythroid cells[17]. Our study provides additional evidence on temporal-dependent effects of HDACs for lineage commitment and maturation using a different type of cells.

Previous studies have shown conflicting data on the relationship between HDACs and angiogenesis. A study showed that downregulation of HDAC impaired angiogenesis by decreasing endothelial VEGFR-2 protein half-life partially via a VE-cadherin-dependent manner[18]. Another study showed that Class I HDAC inhibitors could repress vasculogenic mimicry by increasing the expression of anti-angiogenesis genes in triple-negative breast cancer cells[19]. In contrast, other studies showed that HDAC5 and HDAC7 are repressors of angiogenesis. HDAC5 exhibited an anti-angiogenic effect in ECs through repressing the angiogenic genes *FGF2* and *Slit2*, which are involved in migration and sprouting of ECs[20]. Another study indicated that VEGF elicited HDAC5 phosphorylation and nuclear export in ECs through a VEGFR2-PKD-dependent pathway. HDAC5 export alleviated its inhibition on MEF2 transcription factor, and thus triggered the expression of angiogenic genes in response to VEGF[21]. Furthermore, HDAC5 expression was shown to be significantly increased in the ECs from patients with systemic sclerosis and caused poor angiogenesis owing to the repression of proangiogenic factors in ECs[22]. Silencing of HDAC5 in systemic sclerosis restored normal angiogenesis of ECs[22]. Interestingly, VEGF also stimulated HDAC7 phosphorylation and cytoplasmic accumulation. Thus, inactivation of HDAC7 plays a role in VEGF-stimulated endothelial cell migration, tube formation, and microvessel sprouting[23]. Moreover, flow-induced mechanical stimuli induced HDAC1 phosphorylation and nuclear export in angiogenic sprouting[24]. On the other hand, HDAC7 expression and splicing exerted a positive effect on SMC differentiation from ES cells through modulation of the SRF-myocardin complex[25,26].

Our finding showed that HDACi treatment at the third stage could promote the expression of EC genes and simultaneously inhibit the expression of SMC genes. Seemingly, these findings are consistent with previous reports demonstrating that HDAC5 and HDAC7 are negative regulators for angiogenesis. However, HDACi treatment at the second stage inhibited the differentiation of vascular progenitor, suggesting a positive role of HDACs in EC lineage commitment. The exact mechanisms responsible for the different impact of HDACs on ECs remain to be determined. Other factors in specific stages or types of cells may account for the different outcomes of HDACs.

Our study suggests that HDACs may modulate the stepwise EC differentiation via regulating endothelial transcription factors *ERG*, *ETS1*, and *MEF2C*. The HDAC inhibitor NaBt downregulated *ERG*, *ETS1*, and *MEF2C* at the second stage but upregulated these

transcription factors at the third stage. The ETS family member ETS1 and ERG are expressed in ECs and their progenitors, and proven to participate in vasculogenesis and angiogenesis[4]. Previous study also implicated that VEGF acts as a critical regulator of MEF2C. Thus, MEF2 may be an important mediator of VEGF action in endothelial cells to affect vascular development[27].

The heterogeneity of EC differentiation from iPSCs remains as a major obstacle of iPSC-EC application. During EC induction, a fraction of iPSCs may be differentiated into stromal cells, pericytes, or SMCs. It is well known that PDGF-BB and TGFβ1 were identified as inducing factors for SMCs/pericytes[1,28]. Our results show that HDACs also regulated TGFβ and PDGF signals. Interestingly, inhibition of HDACs with NaBt at both the second and third stages enhanced the expression of *TGFβ2*. In addition, NaBt enhanced the expression of *PDGFB* at the second stage but reduced *PDGFB* expression at the third stage. Furthermore, our data indicated that PDGF-BB exerted a repressive effect on *ACTA2* expression at both stages, which is consistent with a report that PDGF-BB suppressed the expression of ACTA2(α-SMA) and SM22-α in bone marrow or smooth muscle progenitor cells[29]. It is reasonable to speculate that the rising and falling levels of HDACs at different stages during EC differentiation not only facilitate the differentiation of ECs through regulation of endothelial transcription factors but also limit the differentiation SMAs/pericytes through regulation of TGFβ and PDGF signals.

Taken together, our results suggest that HDACs play different roles at different stages of EC induction: HDACs promote the generation of vascular progenitors but impede the later stage of EC differentiation. The precise mechanisms remain to be determined.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgement

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Data availability statement

The data sets used or analyzed in this study are available from the corresponding author on reasonable request.

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Significance of the study

Histone modification play a crucial role in regulating endothelial differentiation. In this study, we found the expression of several HDACs fluctuated significantly during endothelial differentiation. Inhibition of HDACs with sodium butyrate or BML210 could hinder or facilitate EC induction at different differentiation stages.

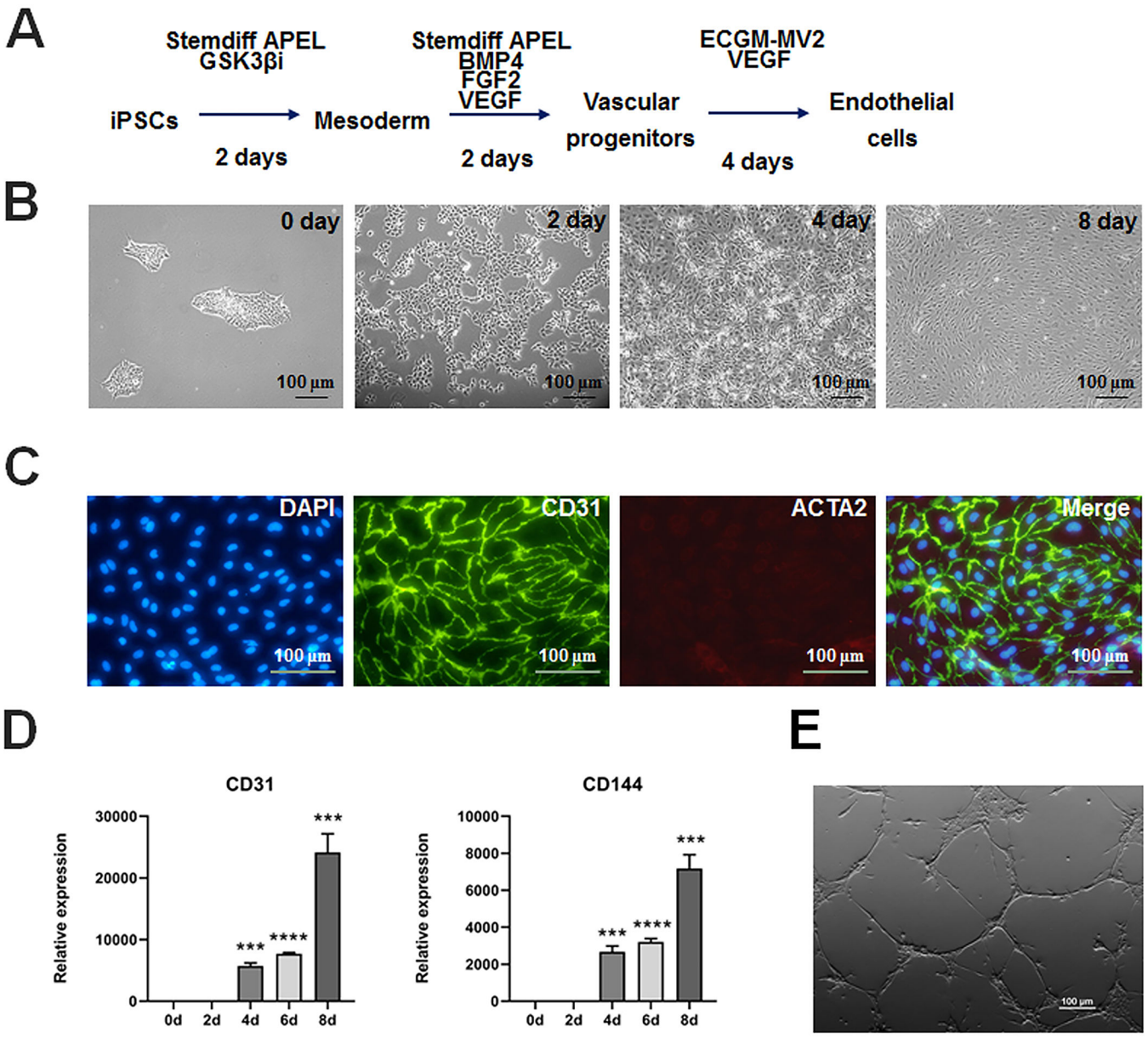

Figure 1. Differentiation of hiPSCs toward ECs.

(A) Schematic protocol for the induction of ECs from hiPSCs. (B) Typical microscope images of hiPSCs and differentiating cells at different stages. (C) Fluorescent images of hiPSC-ECs immunostained for CD31 and ACTA2. (D) The expression of *CD31* and *CD144* in hiPSCs and the differentiating cells was assessed by qRT-PCR. **P*<0.05; ***P*<0.01; ****P*<0.001; *****P*<0.0001 versus hiPSCs. (E) Tubes formed by hiPSC-ECs on matrigel *in vitro*.

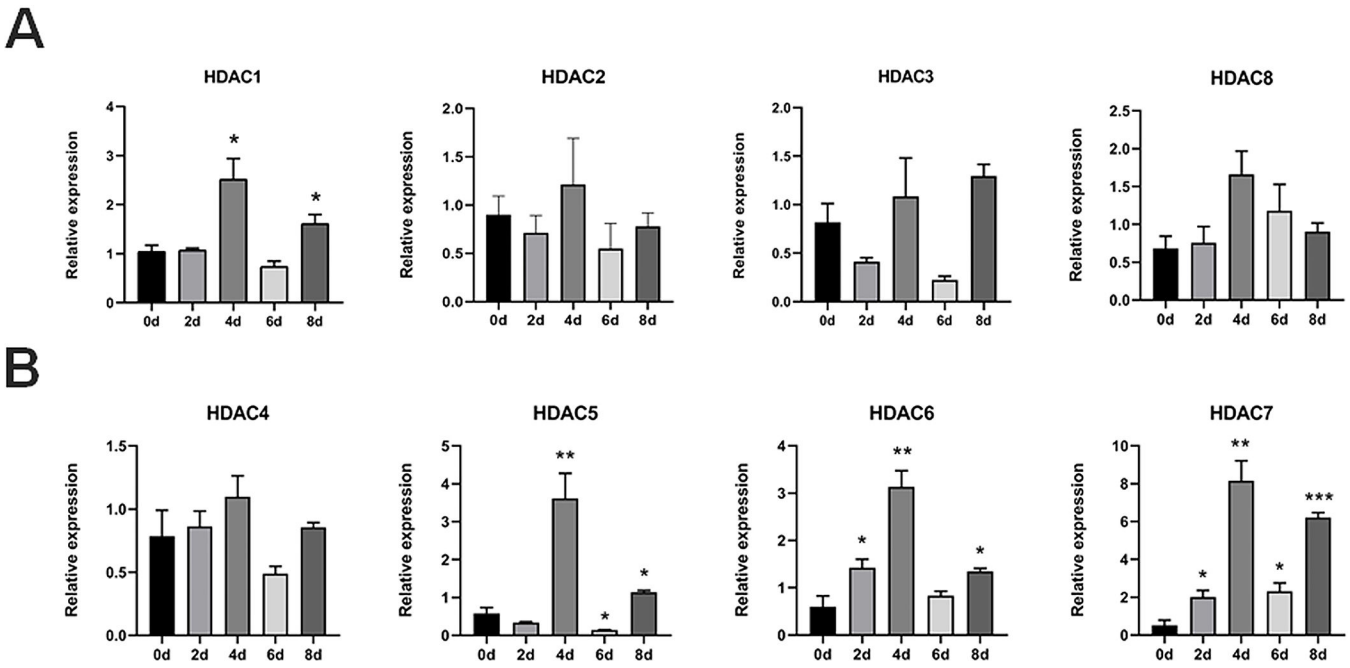

Figure 2. The expression of HDAC genes during EC differentiation.

(A) The expression of Class I HDACs (HDAC1, 2, 3, and 8) in hiPSCs and differentiating cells was assessed by qRT-PCR. (B) The expression of Class II HDACs (HDAC4, 5, 6, and 7) in hiPSCs and differentiating cells was assessed by qRT-PCR.

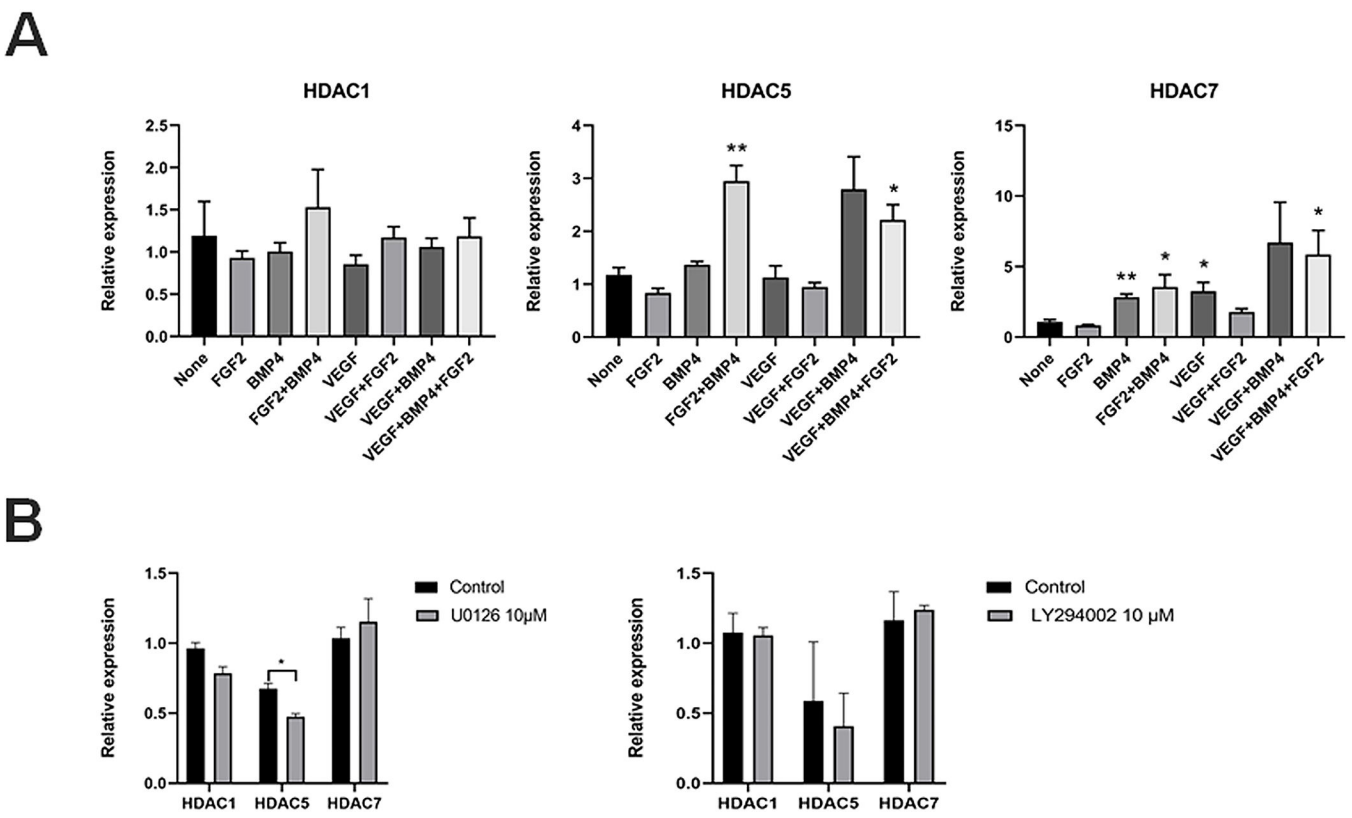

Figure 3. Expression regulation of HDAC1, 5, and 7 at the second differentiation stage.

(A) qRT-PCR analysis of the expression of *HDAC1*, *5*, and *7* in differentiating cells cultured with the indicated growth factors from day 2 to day 4. (B) Differentiating cells were cultured with either ERK1/2 or PI3K inhibitor, U0126 (10 μM) and LY294002 (10 μM) from day 2 to day 4. The expression of *HDAC1*, *5*, and *7* was assessed by qRT-PCR.

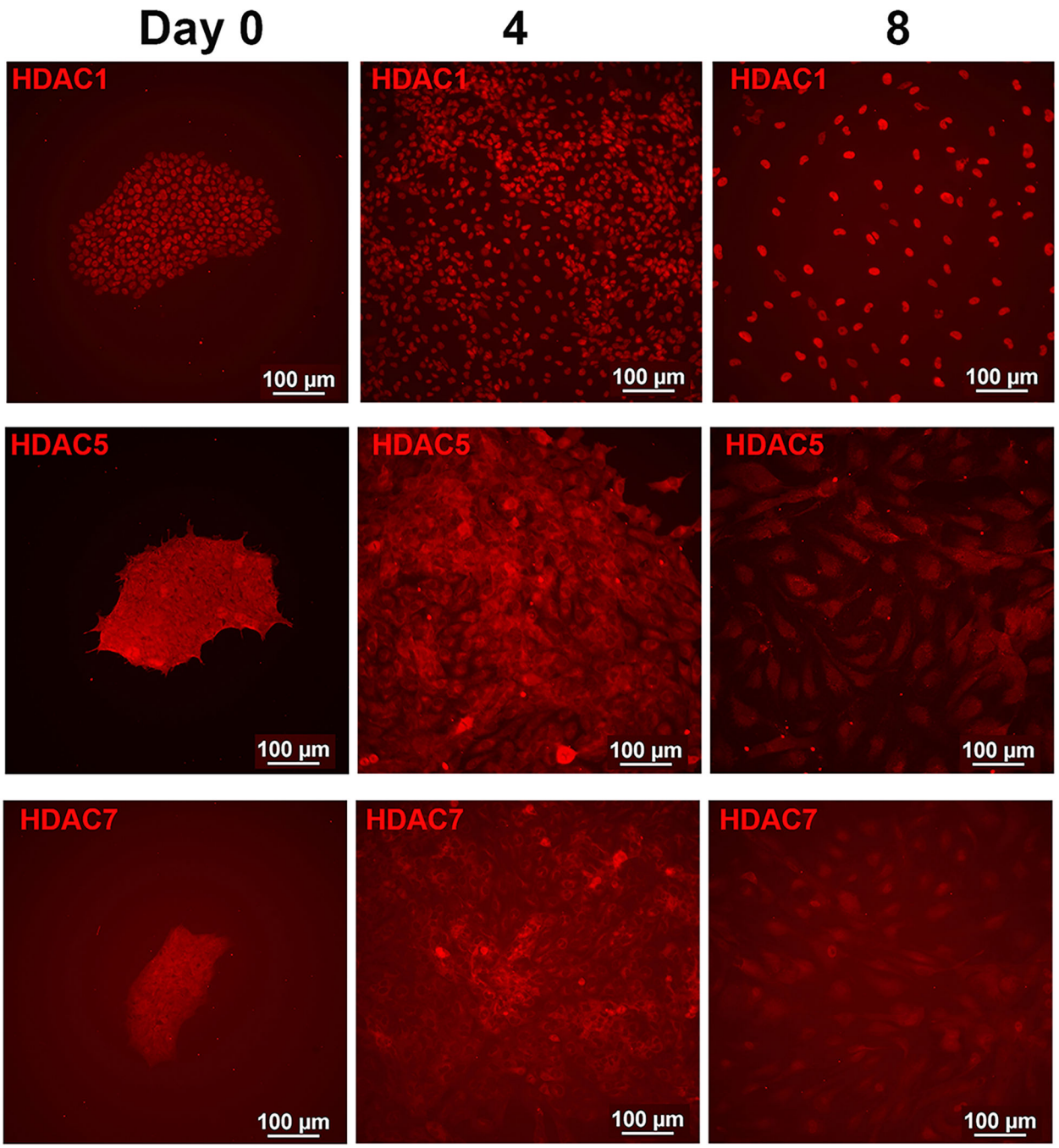

Figure 4. The localization of HDAC1, 5, and 7 during EC differentiation from iPSCs.
Immunofluorescent staining for HDAC1, 5, and 7 in iPSCs (Day 0), the vascular progenitors (day 4) and ECs (day 8). Scale bar=100μm.

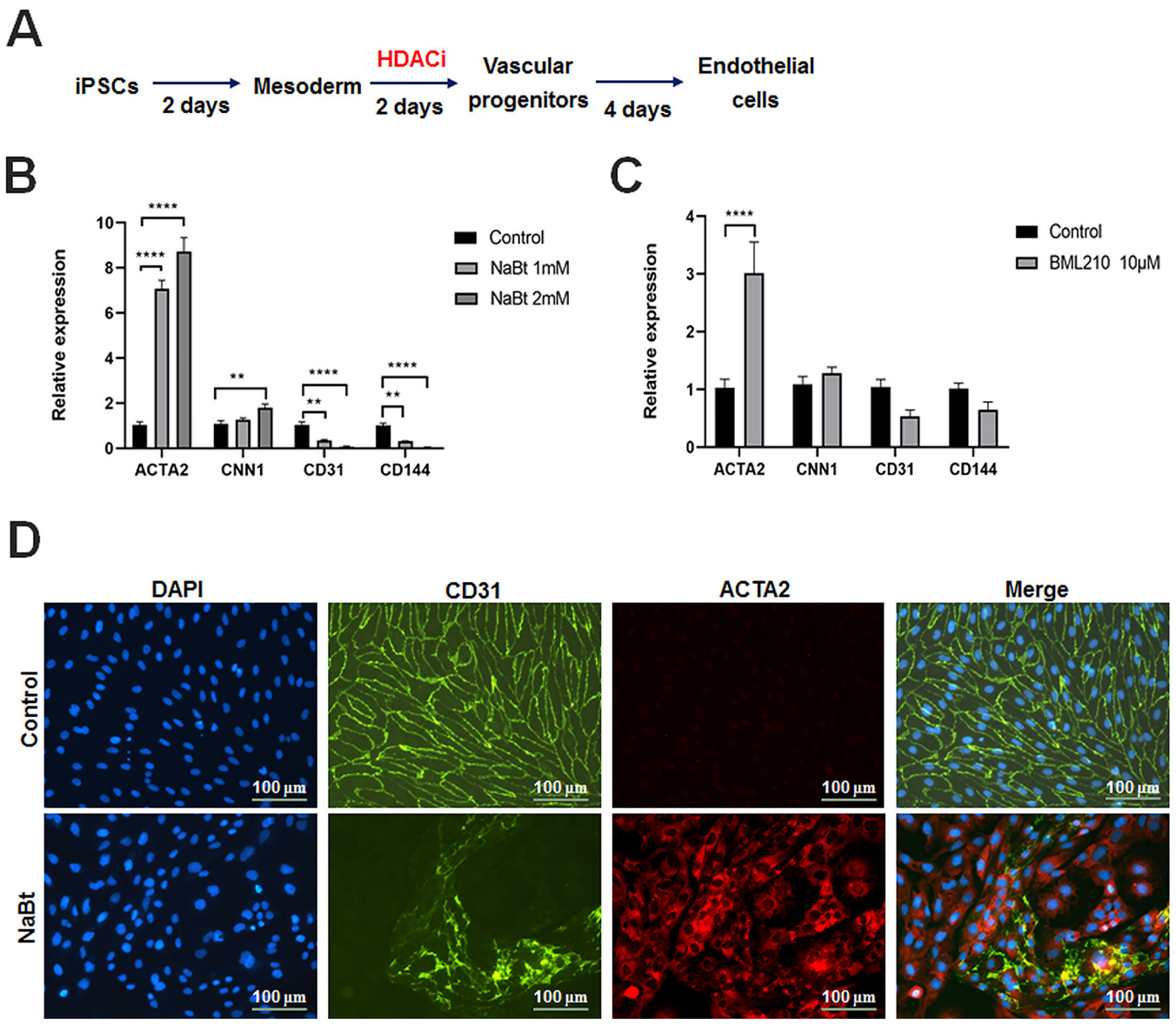

Figure 5. Effects of HDAC inhibition on EC differentiation at the second stage.

(A) Schematic representation of the protocol for HDAC inhibition. (B) After treatment with NaBt (1 or 2 mM) from day 2 to day 4, the cells were assessed for their expression of *ACTA2*, *CNN1*, *CD31*, and *CD144* by qRT-PCR. (C) After treatment with BML210 (10 μM) from day 2 to day 4, , the cells were assessed for their expression of *ACTA2*, *CNN1*, *CD31*, and *CD144* by qRT-PCR. (D) After treatment of NaBt (1 or 2 mM) from day 2 to day 4, the cells were allowed to further culture and differentiation. The cells at day 8 were immunostained with antibodies against CD31 (green) and ACTA2 (red). The nuclei were stained with DAPI (blue).

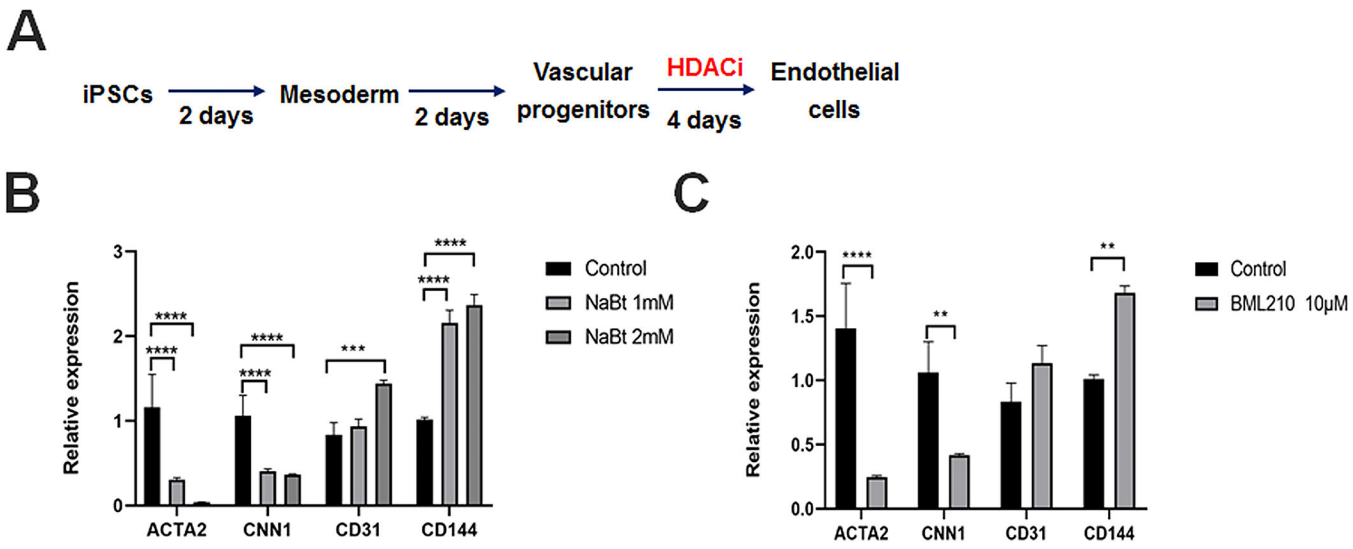

Figure 6. Effects of HDAC inhibition on EC differentiation at the third stage.

(A) Schematic representation of the protocol for HDAC inhibition. (B) After treatment with NaBt (1 or 2 mM) from day 5 to day 8, the cells were assessed for their expression of *ACTA2*, *CNN1*, *CD31*, and *CD144* by qRT-PCR. (C) After treatment with BML210 (10 μM) from day 5 to day 8, the cells were assessed for their expression of *ACTA2*, *CNN1*, *CD31*, and *CD144* by qRT-PCR.

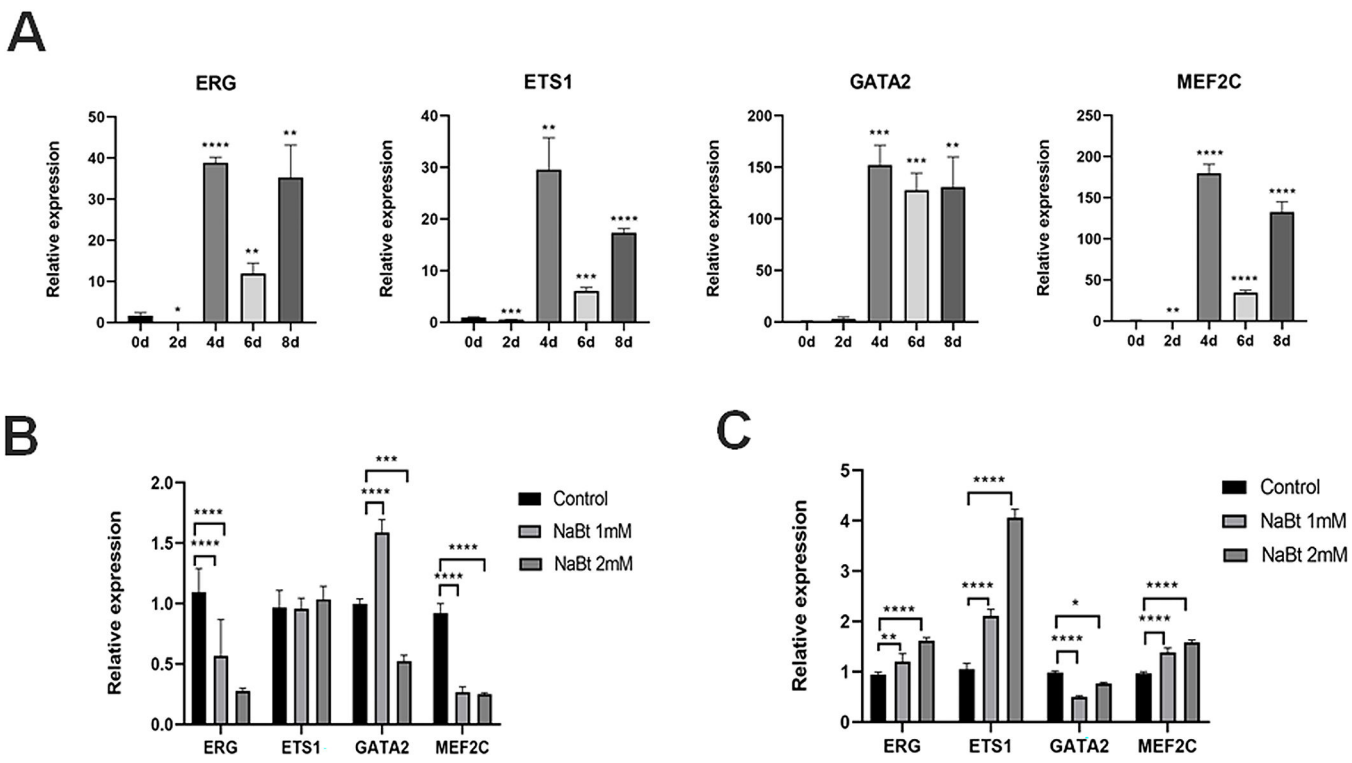

Figure 7. Effects of NaBt on the expression of endothelial transcription factors.

(A) The expression of endothelial transcription factors such as *ERG*, *ETS1*, *GATA2*, and *MEF2C*, was assessed by qRT-PCR. (B) After treatment with NaBt from day 2 to day 4, the cells were assessed for their expression of endothelial transcription factors by qRT-PCR. (C) After treatment with NaBt from day 5 to day 8, the cells were assessed for their expression of endothelial transcription factors by qRT-PCR.

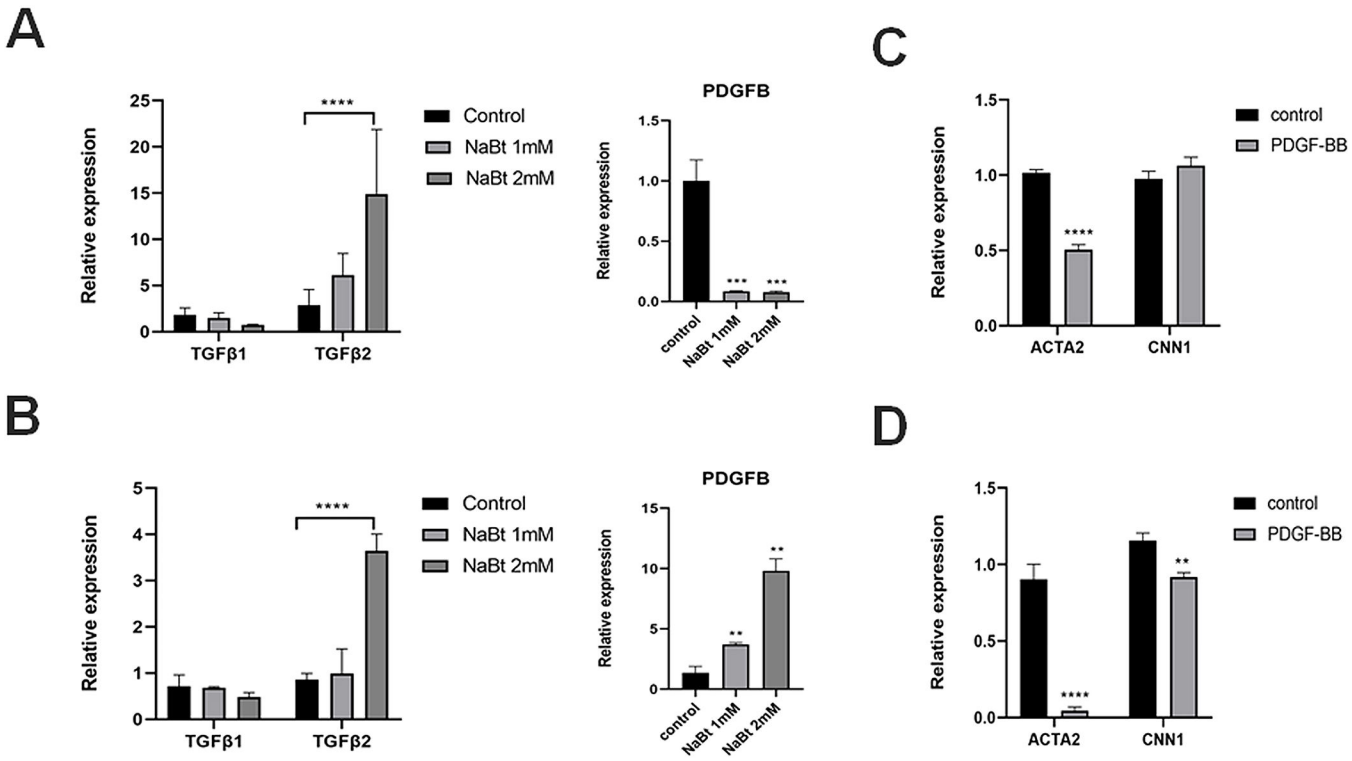

Figure 8. Effects of NaBt on the expression of the *TGFβ* and the *PDGFB* genes.

(A) After treatment with NaBt from day 2 to day 4, the cells were assessed for their expression of *TGFβ1*, *TGFβ2*, and *PDGFB* by qRT-PCR. (B) After treatment with NaBt from day 5 to day 8, the cells were assessed for their expression of *TGFβ1*, *TGFβ2*, and *PDGFB* by qRT-PCR. (C) After treatment with PDGF-BB (100ug/ml) from day 2 to day 4, the cells were assessed for their expression of *ACTA2* and *CNN1* by qRT-PCR. (D) After treatment with PDGF-BB (100ug/ml) from day 5 to day 8, the cells were assessed for their expression of *ACTA2* and *CNN1* by qRT-PCR.