Identification of a New Way to Induce Differentiation of Dermal Fibroblasts Into Vascular Endothelial Cells

Xiaoling Cui
Shandong University
Jie Wen
Shandong University
Xiao Li
Shandong University
Nan Li
Shandong University
Xuxiao Hao
Shandong University
BaoXiang Zhao
Shandong University
Xunwei Wu
Shandong University
Junying Miao (miaojy@sdu.edu.cn)
Shandong Provincial Key Laboratory of Animal Cells and Developmental Biology, School of Life Science, Shandong University, Qingdao 266237, P.R. China https://orcid.org/0000-0002-7006-1357

Research Article

Keywords: Small chemical molecule, Human dermal fibroblasts, Vascular endothelial cells, Differentiation

Posted Date: February 12th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-216763/v1

Abstract

Background: Human dermal fibroblasts (HDFs) have the potential to differentiate into vascular endothelial cells (VECs), but their differentiation rate is low and the mechanism involved is unclear. The small molecule pathway controls the phenotype of fibroblasts by activating cellular signaling pathways, which is a more convenient method in the differentiation strategy of dermal fibroblasts into vascular endothelial cells.

Methods: In this study, dermal fibroblasts were treated with the different doses of CPP, and the mRNA level and protein level were detected by qPCR, Western blot and immunofluorescent staining. Matrigel assays also were used to teste the angiogenic ability of vascular endothelial cells derived from dermal fibroblasts.

Results: Here, we report that a small chemical molecule, CPP ((E)-4-(4-(4-(7-(diethylamino)-2-oxo-2H-chromene-3-carbonyl) piperazin-1-yl) styryl)-1-methylpyridin-1-ium iodide), efficiently induces the differentiation of dermal fibroblasts into Vascular endothelial cells. First, we observed that the morphology of CPP-treated dermal fibroblasts elongated, curved and formed circular patterns. Western blot and qRT-PCR analyses revealed that CPP effectively reduced the level of the dermal fibroblasts-marker Vimentin and increased levels of the vascular endothelial cells -markers CD31 and CD133. Detection of the percentage of CD31-positive cells from immunofluorescent staining confirmed that CPP efficiently induces dermal fibroblasts to differentiate into vascular endothelial cells. Matrigel assays showed that CPP-treated dermal fibroblasts have the functions of vascular endothelial cells. Western blot and qRT-PCR analyses of pro-angiogenic factors (VEGF, FGF-2 and PDGF-BB) showed that CPP induces dermal fibroblasts to vascular endothelial cells by promoting the expression of pro-angiogenic factors (VEGF, FGF-2 and PDGF-BB).

Conclusions: Our results indicate that the small chemical molecule CPP efficiently induces the differentiation of dermal fibroblasts into vascular endothelial cells. Simultaneously, this new inducer provides a potential to develop new approaches to restore vascular function for the treatment of ischemic vascular diseases.

Background

The main reason for the damage of the vascular repair mechanism is vascular endothelial cells (VECs), that the endothelial progenitor cells in the body are damaged due to lack, poor mobilization or dysfunction[1, 2]. Therefore, the use of generate ECs for the treatment of vascular injury-related diseases has become a current research hotspot. However, the differentiation rate of using adult stem cells and progenitor cells to form endothelial cells is relatively low[3, 4].

Human dermal fibroblasts (HDFs) in the skin are derived from mesenchymal stem cells (MSCs) during embryonic development [5, 6]. HDFs are abundant in the human body and have a multi-directional differentiation potential. Previous studies have reported that HDFs can differentiate into endothelial-like

cells, fat-like cells, cartilage-like cells, bone-like cells and spinal motor neurons [7–9]. At present, the main strategy to induce the differentiation of HDFs is reprogramming. However, reprogramming has the disadvantages of a low differentiation rate and a high cost, so it is urgent to develop more efficient differentiation induction strategies[10–12].

The small molecule pathway can control the phenotype of fibroblasts by activating cellular stress-related signaling pathways, which is a more convenient application method for the differentiation strategy of fibroblasts into endothelial cells. Simultaneously, small chemical molecules have been reported to possess great advantages in inducing cell differentiation, which can produce faster biological effects and facilitate in-depth studies of signaling pathways [13, 14]. In our research, we are committed to using small chemical molecules as tools to discover new factors and new pathways [15–17]. Therefore, our aim is to identify new chemical molecules that can efficiently induce HDFs to differentiate into endothelial cells. Recently, we synthesized and identified a new water-soluble fluorescence probe for the detection of hypochlorous acid (HOCl) ((E)-4-(4-(4-(7-(diethylamino)-2-oxo-2H-chromene-3-carbonyl) piperazin-1-yl)styryl)-1-methylpyridin-1-ium iodide)(CPP) [18].

Methods And Materials

Antibodies

Antibodies against CD31 (sc-1506), PDGF-BB (sc-7878), VEGF (sc-7269), FGF-2 (sc-271847) and CD133 (sc-30219) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against Vimentin (10366-1-AP) was from Proteintech group (Wuhan, China). The antibody against β-actin was from Sigma-Aldrich (St. Louis, MO, USA). Horseradish peroxidase-conjugated secondary antibodies were from Jackson Immunoresearch (West Grove, PA, USA). The secondary antibody used for immunofluorescence was donkey anti-rabbit IgG Alexa Fluor-546 (A-11037; Invitrogen, Carlsbad, CA, USA).

Cell culture

Human primary HDFs were derived from adult foreskins, and were isolated according to our previous publication [19]. HDFs were cultured in DMEM Basic medium (C11995500BT, Gibco, Grand Island, NY, USA) supplemented with 10% (v/v) bovine calf serum. HDFs were cultured in a humidified incubator at 37°C in a 5% CO_2 atmosphere. Cells were seeded in appropriate dishes (35,000 cells/ml), and all cell lines were authenticated by DNA short tandem repeat (STR) profiling and were confirmed to be mycoplasma negative.

Cell morphology

Morphological changes of HDFs were examined using an inverted phase contrast microscope (Eclipse TS-100; Nikon, Tokyo, Japan) after 10 days of treatment with CPP at the indicated concentrations.

Cell viability assay

HDFs were seeded in 96-well plates and were then treated with 0.1% DMSO (as a control) or with CPP at the indicated concentrations for 48 h. Cell viability was determined using a sulforhodamine B (SRB) assay (L109288, Aladdin, Shanghai, China) according to the manufacturer's instructions.

Western blot analysis

Cell lysates (30 μg protein per lane) were separated by SDS-PAGE, after which the proteins were transferred to polyvinylidene difluoride membranes. At room temperature, the membranes were blocked with 5% non-fat milk in TBST (TBS containing 0.05% Tween-20) for 1 h. After that, the membranes were incubated with the primary antibody overnight at 4°C, then were washed three times with TBST for 5 min each. Each membrane was incubated with the secondary antibody for 1 h at room temperature, and was then washed 3 times with TBST, each time for 5 min. Antibodies bound to proteins were detected using an enhanced chemiluminesence detection kit (34080, Thermo Fisher, Waltham, MA, USA). Relative quantities of specific bands were analyzed by Image J software and were normalized to loading controls.

Quantitative real-time PCR

RNA was extracted from the whole-cell fraction using the Trizol reagent method (Takara, Tokyo, Japan), and extracted total RNAs were reverse transcribed using the primer sequences of the target genes. The reverse transcription step used the PrimeScript RT reagent kit with gDNA Eraser (Takara). PCR reactions involved the use of SYBR Premix Ex Taq (Tli RNaseH Plus, Takara) and levels of expressed genes were measured by the 2 − ΔΔCt method with MxPro 4.00 (Stratagene, La Jolla, CA, USA). The following primers were used: VEGF: 5'-ATCGAGTACATCTTCAAGCCAT-3' (forward) and 5'-GTGAGGTTTGATCCGCATAATC-3' (reverse); FGF-2: 5'- CATCAAGCTACAACTTCAAGCA-3' (forward) and 5'-CCGTAACACATTTAGAAGCCAG-3' (reverse); PDGF-BB: 5'-ACCGCACCAACGCCAACTTC-3' (forward) and 5'-TCTTCCGCACAATCTCGATCTTTCTC-3' (reverse); CD31: 5'-TCAGACGTGCAGTACACGGA-3' (forward) and 5'-CTTTCCACGGCATCAGGGAC-3' (reverse); CD133: 5'-GTGGCGTGTGCGGCTATGAC-3' (forward) and 5'-CCAACTCCAACCATGAGGAAGACG-3' (reverse); Vimentin: 5'-GGTGGACCAGCTAACCAACG-3' (forward) and 5'-TTGCAGGGTGTTTTCGGCTT-3' (reverse); Actin: 5'- CCTGGCACCCAGCACAAT-3' (forward) and 5'-GCCGATCCACACGGAGTACT-3 (reverse).

Angiogenesis assays

Aliquots of Matrigel were stored at -80°C and were melted in ice overnight immediately prior to use. After mixing the culture medium and Matrigel (3:1), 300 μl Matrigel was added to each well in 24-well plates. The 24-well plates were cultured in a humidified incubator at 37°C in a 5% CO_2 atmosphere for 30 min. Cells were digested with trypsin and were then resuspended in culture medium and seeded at a concentration of 4 × 10,000 cells/ml in the 24-well plates. Morphological changes of HDFs were examined using an inverted phase contrast microscope (Eclipse TS-100; Nikon). Tubular lengths were analyzed by Image J software and were normalized to the control group.

Statistical analysis

Data are reported as means ± SE from at least three separate experiments and were analyzed by t-test with SPSS 17.0 (SPSS Inc., Chicago, IL, USA). Differences with a $p < 0.05$ are recognized as statistically significant.

Results

CPP altered the morphology of HDFs.

In order to test whether the hypochlorous acid (HOCl) probe CPP could potentially promote the differentiation of HDFs into endothelial cells, we first investigated whether CPP affects the cell viability of HDFs. Using the sulforhodamine B (SRB) assay, we observed that CPP did not significantly affect the viability of HDFs (Fig. 1A-1B). Secondly, we investigated whether treatment with CPP affects the morphology of HDFs. We observed that HDFs treated with CPP for 6 days and 10 days were elongated, curved and formed circular patterns (Fig. 1C), which resembles the function of endothelial cells.

From the above results we draw a conclusion that CPP, as a hypochlorous acid probe, can affect the morphology of HDFs. In order to identify whether other hypochlorous acid probes can also promote HDFs to form circular patterns, we treated HDFs with other HOCl probes for 10days. Interestingly, these HOCl probes failed HDFs to elongated, curved and formed circular patterns (Fig. 1D). Taken together, these data indicate that we may have found a new inducer that can induce HDFs to change their morphology and possess endothelial cell functions.

CPP promotes the expression of endothelial cell marker CD133.

In order to prove that HDFs indeed differentiated into VECs after treatment with CPP, we treated HDFs with different doses of CPP for 10 days. Cells treated with CPP had significantly decreased protein levels of the endothelial cell marker CD133 (Fig. 2A, 2B). Next, we further text the expression of CD133 in CPP-treated HDFs by qPCR. Consistent with western blot results, CPP can significantly increase the mRNA level of CD133 (Fig. 2C).

Furthermore, we used immunofluorescence staining to detect the level of CD133 in HDFs and found that CPP can promote the increase of CD133 levels. Collectively, CD133, as an important regulator for the maintenance of endothelial progenitor cell stemness, plays an important role in the process of endothelial cell differentiation[20]. From the above data, we found that CPP can significantly promote the expression of CD133.

CPP promotes the expression of endothelial cell marker CD31.

CD31, as the main marker of VECs, is the main factor to identify whether there is vascular endothelial cell production. In order to prove that CPP induce HDFs to differentiate into VECs. We treated HDFs with CPP at 1, 10 and 20 μM for 10 days, the protein level of CD31 was measured by western blot. As expected, the protein level of CD31 was significantly increased (Fig. 3A, 3B). Next, we tested the mRNA level of CD31 by qPCR, we also proved that the mRNA level of CD31 was increased (Fig. 3C). These results were verified by

immunofluorescence staining (Fig. 3D). Together, these data showed that CPP promoted the expression of CD31.

Moreover, we quantified the percentage of CD31-positive cells using immunofluorescence staining, and found that nearly 80% of cells after 10 days of treatment with 10 or 20 μM CPP expressed CD31, and less than 10% of CD31-positive cells was observed in the control group (Fig. 4A). Taken together, these data demonstrate that CPP efficiently induced the differentiation of HDFs into VECs in vitro.

CPP reduced the level of HDFs marker Vimentin.

In the process of transforming one type of cell into another, the level of the marker protein of the source cell will decrease, and the level of the marker protein of the target cell will increase. Therefore, in order to further prove that CPP induces the differentiation of HDFs into VECs, we detected Vimentin, a marker protein of HDFs. After CPP treated HDFs at 1, 10 and 20 μM for 10 days, western blot was used to detect the protein level of Vimentin. We found that CPP significantly reduced the protein level of Vimentin at 10μM (Fig. 5A, 5B). Interestingly, these results were verified by qRT-PCR analysis and by immunofluorescence staining (Fig. 5C, 5D).

CPP promotes the angiogenesis of VECs derived from HDFs in vitro.

Next, we tested the angiogenic ability of HDFs treated with CPP using an in vitro Matrigel tube formation assay. HDFs treated with CPP at concentrations of 10 or 20 μM for 12 days, we observed the formation of HDFs into tubules in vitro on 4 days, 8 days, 10 days, and 12 days. We found that CPP-treated HDFs began to form tubules on day 10, and the number of tubes has increased significantly on day 12. In contrast, tube-like structures didn't appear in the control group without treatment of CPP (Fig. 6).

CPP induces HDFs to differentiate into VECs by promoting the expression of pro-angiogenic factors.

As a secretory cell, VECs can secrete a variety of cytokines. Studies have shown that Vascular endothelial growth factor (VEGF), fibroblast growth factor 2 (FGF-2) and Platelet derived growth factor (PDGF-BB) are closely related to the maturation of VECs. In order to clarify the mechanism of CPP-induced differentiation of HDFs into VECs, we analyzed whether CPP(0,1,10,20 μM) treatment enhanced the expression levels of vascular endothelial function related factors in HDFs, and qRT-PCR analysis revealed that mRNA levels of VEGF, FGF-2 and PDGF-BB were strongly increased in cells treated with CPP for 10 days (Fig. 7A-C). Next, in order to further prove that CPP promoted the secretion of VEGF, FGF-2 and PDGF-BB, western blot was further verified. As expected, CPP significantly promoted the increase of VEGF, FGF-2 and PDGF-BB protein levels (Fig. 7D-I). Through the above results, we preliminarily proved that VECs derived from CPP-induced HDFs have their typical secretory function.

Discussion

HDFs have the potential to differentiate into VECs, but at present, there are few effective strategies to induce HDFs to differentiate into VECs. Lee et al. showed the direct reprogramming of human HDFs into

endothelial cells using ER71/ETV2 [21]. Recently, analyses of induced neuron production by single cell RNA-Seq revealed that silencing of reprogramming factors, death from an epigenetically unstable state and reprogramming toward alternative fates, limits the number of cells that successfully reprogram [22, 23]. Therefore, there are some safety issues in the genetic delivery of exogenous genes, such as gene mutations or insertions, etc[24–26]. Here, based on small chemical molecules that provide a simple, efficient and cost-effective induction method, we found that the small chemical molecule CPP induced HDFs to differentiate into VECs with a high rate of differentiation. At the same time, we discovered that treatment of HDFs with CPP for 10 days will cause them to elongate, curved and form circular patterns. In addition, we speculate that these changes in cell morphology indicate that the cells already have the function of VECs, rather than being simple endothelial-like cells.

More and more evidence has shown that small chemical molecules can regulate cell phenotypes by targeting signaling pathways, epigenetic modifications and metabolic processes [27, 28]. In research studies of signaling pathways, it was found that the Wnt signaling pathway, the TGF-β signaling pathway and the MAPK/ERK signaling pathway play important roles in the maintenance of cell pluripotency [29–31]. Small molecules maintain cell pluripotency by affecting those signaling pathways[32]. However, in previous studies, researchers used a variety of small molecules to treat cells together to achieve a high induction rate[27, 33, 34]. In this article, we use a small molecule to induce HDFs with an induction rate of up to 80%. Secondly, because different small chemical molecules regulate cells in different ways, the use of different small molecules to treat cells is not conducive to detailed mechanism research on cell differentiation. In our reports, we treated HDFs with CPP, and proved that CPP induced HDFs to differentiate into VECs.

VEGF regulates the function of endothelial cells through its three receptors VEGFR1, VEGFR2, and VEGFR3[35]. At the same time, the VEGF signaling pathway has a regulatory role in the differentiation of endothelial progenitor cells into VECs[36, 37]. In this study, we found that the expression of VEGF in CPP-treated HDFs was significantly increased, which further proved that VEGF played a key role in the differentiation of HDFs into VECs induced by CPP. PDGF signaling pathway also plays an important regulatory role in the differentiation of endothelial progenitor cells into mature endothelial cells[38, 39]. PDGF-BB can induce the production of mature endothelial cells under serum-free conditions. In previous reports, it was found that VEGF and FGF-2 synergistically activate the endogenous PDGF-B-PDGFRβ signaling pathway[40]. Therefore, in this study, compared with the control group, HDFs treated with CPP Significantly increased the level of VEGF, FGF-2 and PDGF-BB.

Conclusions

In conclusion, we have found a new induction method that can induce HDFs to differentiate into VECs with a high differentiation rate. these results provide new ideas for the study of HDF differentiation, and also provide a new compound to potentially develop effective new drugs against ischemic dermopathy.

Abbreviations

mesenchymal stem cells	MSCs
hypochlorous acid	HOCl
Human dermal fibroblasts	HDFs
vascular endothelial cells	VECs
Vascular endothelial growth factor	VEGF
fibroblast growth factor 2	FGF-2
Platelet derived growth factor	PDGF

Declarations

Ethics approval and consent to participate

All experimental procedures in this study were performed in accordance with the ARRIVE guidelines 39 and were approved by the Ethics Committee in Shandong University.

Authors' contributions

Junying Miao and Baoxiang Zhao designed experiments. Xiaoling Cui, Jie Wen, Xiao Li, Nan Li, and Xuxiao Hao performed experiments. Xiaoling Cui and Xunwei Wu analyzed the results. Xiaoling Cui, Junying Miao and Xunwei Wu wrote the manuscript. The authors read and approved the final manuscript.

Availability of data and materials

The data generated or analyzed during this study are included in this article, or if absent are available from the corresponding author upon reasonable request.

Consent for publication

Written informed consent was obtained from all patients.

Funding

This study was supported by the National Key Research and Development Program of China (2017YFA0104604), the Natural Science Foundation of Shandong Province (ZR2019ZD36, ZR2018MB042), and the National Natural Science Foundation of China (No. 31871407, 31741083, 31870831, 81772093).

Acknowledgments

We thank Public technology platform for large scale instruments of Shandong University for Laser scanning confocal microscope. We thank Professor Wu Xunwei’s laboratory for providing Human dermal fibroblasts.

Conflict of interest

The authors declare that they have no conflict of interest.

References

1. Coultas L, Chawengsaksophak K, Rossant J. Endothelial cells and VEGF in vascular development. Nature. 2005;438(7070):937-45.
2. Godo S, Shimokawa H. Endothelial Functions. Arterioscler Thromb Vasc Biol. 2017;37(9):e108-e14.
3. O'Neill TJt, Wamhoff BR, Owens GK, Skalak TC. Mobilization of bone marrow-derived cells enhances the angiogenic response to hypoxia without transdifferentiation into endothelial cells. Circ Res. 2005;97(10):1027-35.
4. Lin Y, Gil CH, Yoder MC. Differentiation, Evaluation, and Application of Human Induced Pluripotent Stem Cell-Derived Endothelial Cells. Arterioscler Thromb Vasc Biol. 2017;37(11):2014-25.
5. Thulabandu V, Chen D, Atit RP. Dermal fibroblast in cutaneous development and healing. Wiley interdisciplinary reviews Developmental biology. 2018;7(2).
6. Stunova A, Vistejnova L. Dermal fibroblasts-A heterogeneous population with regulatory function in wound healing. Cytokine Growth Factor Rev. 2018;39:137-50.
7. Karlsson LK, Junker JP, Grenegard M, Kratz G. Human dermal fibroblasts: a potential cell source for endothelialization of vascular grafts. Ann Vasc Surg. 2009;23(5):663-74.
8. Junker JP, Sommar P, Skog M, Johnson H, Kratz G. Adipogenic, chondrogenic and osteogenic differentiation of clonally derived human dermal fibroblasts. Cells Tissues Organs. 2010;191(2):105-18.
9. Son EY, Ichida JK, Wainger BJ, Toma JS, Rafuse VF, Woolf CJ, et al. Conversion of mouse and human fibroblasts into functional spinal motor neurons. Cell Stem Cell. 2011;9(3):205-18.
10. Margariti A, Winkler B, Karamariti E, Zampetaki A, Tsai TN, Baban D, et al. Direct reprogramming of fibroblasts into endothelial cells capable of angiogenesis and reendothelialization in tissue-engineered vessels. Proc Natl Acad Sci U S A. 2012;109(34):13793-8.
11. Smith ZD, Sindhu C, Meissner A. Molecular features of cellular reprogramming and development. Nat Rev Mol Cell Biol. 2016;17(3):139-54.
12. Vierbuchen T, Ostermeier A, Pang ZP, Kokubu Y, Sudhof TC, Wernig M. Direct conversion of fibroblasts to functional neurons by defined factors. Nature. 2010;463(7284):1035-41.
13. Qin H, Zhao A, Fu X. Small molecules for reprogramming and transdifferentiation. Cell Mol Life Sci. 2017;74(19):3553-75.

14. Xie X, Fu Y, Liu J. Chemical reprogramming and transdifferentiation. Curr Opin Genet Dev. 2017;46:104-13.
15. Ge D, Han L, Huang S, Peng N, Wang P, Jiang Z, et al. Identification of a novel MTOR activator and discovery of a competing endogenous RNA regulating autophagy in vascular endothelial cells. Autophagy. 2014;10(6):957-71.
16. Liu S, Li X, Lin Z, Su L, Yan S, Zhao B, et al. SEC-induced activation of ANXA7 GTPase suppresses prostate cancer metastasis. Cancer Lett. 2018;416:11-23.
17. Ning J, Lin Z, Zhao X, Zhao B, Miao J. Inhibiting lysine 353 oxidation of GRP78 by a hypochlorous probe targeting endoplasmic reticulum promotes autophagy in cancer cells. Cell Death Dis. 2019;10(11):858.
18. Zhang L-J, Zhao X, Yang D, Jia Z-Z, Han X, Sun L-Q, et al. A new water-soluble and mitochondria-targeted fluorescence probe for ratiometric detection of hypochlorous acid in living cells. Sensors Actuators B: Chem. 2018;276:8-12.
19. Wu X, Scott L, Jr., Washenik K, Stenn K. Full-thickness skin with mature hair follicles generated from tissue culture expanded human cells. Tissue Eng Part A. 2014;20(23-24):3314-21.
20. Duan Y, Yu S, Xu P, Wang X, Feng X, Mao Z, et al. Co-immobilization of CD133 antibodies, vascular endothelial growth factors, and REDV peptide promotes capture, proliferation, and differentiation of endothelial progenitor cells. Acta Biomater. 2019;96:137-48.
21. Lee S, Park C, Han JW, Kim JY, Cho K, Kim EJ, et al. Direct Reprogramming of Human Dermal Fibroblasts Into Endothelial Cells Using ER71/ETV2. Circ Res. 2017;120(5):848-61.
22. Srivastava D, DeWitt N. In Vivo Cellular Reprogramming: The Next Generation. Cell. 2016;166(6):1386-96.
23. Treutlein B, Lee QY, Camp JG, Mall M, Koh W, Shariati SA, et al. Dissecting direct reprogramming from fibroblast to neuron using single-cell RNA-seq. Nature. 2016;534(7607):391-5.
24. Cohen DE, Melton D. Turning straw into gold: directing cell fate for regenerative medicine. Nat Rev Genet. 2011;12(4):243-52.
25. Knoepfler PS. Deconstructing stem cell tumorigenicity: a roadmap to safe regenerative medicine. Stem Cells. 2009;27(5):1050-6.
26. Yamanaka S. A fresh look at iPS cells. Cell. 2009;137(1):13-7.
27. Li W, Ding S. Small molecules that modulate embryonic stem cell fate and somatic cell reprogramming. Trends Pharmacol Sci. 2010;31(1):36-45.
28. Federation AJ, Bradner JE, Meissner A. The use of small molecules in somatic-cell reprogramming. Trends Cell Biol. 2014;24(3):179-87.
29. Cole MF, Johnstone SE, Newman JJ, Kagey MH, Young RA. Tcf3 is an integral component of the core regulatory circuitry of embryonic stem cells. Genes Dev. 2008;22(6):746-55.
30. Yang J, van Oosten AL, Theunissen TW, Guo G, Silva JC, Smith A. Stat3 activation is limiting for reprogramming to ground state pluripotency. Cell Stem Cell. 2010;7(3):319-28.

31. Shi Y, Do JT, Desponts C, Hahm HS, Scholer HR, Ding S. A combined chemical and genetic approach for the generation of induced pluripotent stem cells. Cell Stem Cell. 2008;2(6):525-8.
32. Wang Y, Zhu G, Li N, Song J, Wang L, Shi X. Small molecules and their controlled release that induce the osteogenic/chondrogenic commitment of stem cells. Biotechnol Adv. 2015;33(8):1626-40.
33. Qi Y, Zhang XJ, Renier N, Wu Z, Atkin T, Sun Z, et al. Combined small-molecule inhibition accelerates the derivation of functional cortical neurons from human pluripotent stem cells. Nat Biotechnol. 2017;35(2):154-63.
34. Shan J, Schwartz RE, Ross NT, Logan DJ, Thomas D, Duncan SA, et al. Identification of small molecules for human hepatocyte expansion and iPS differentiation. Nat Chem Biol. 2013;9(8):514-20.
35. Ballermann BJ. Glomerular endothelial cell differentiation. Kidney Int. 2005;67(5):1668-71.
36. Li L, Liu H, Xu C, Deng M, Song M, Yu X, et al. VEGF promotes endothelial progenitor cell differentiation and vascular repair through connexin 43. Stem Cell Res Ther. 2017;8(1):237.
37. Nourse MB, Halpin DE, Scatena M, Mortisen DJ, Tulloch NL, Hauch KD, et al. VEGF induces differentiation of functional endothelium from human embryonic stem cells: implications for tissue engineering. Arterioscler Thromb Vasc Biol. 2010;30(1):80-9.
38. Kemp SS, Aguera KN, Cha B, Davis GE. Defining Endothelial Cell-Derived Factors That Promote Pericyte Recruitment and Capillary Network Assembly. Arterioscler Thromb Vasc Biol. 2020;40(11):2632-48.
39. Bowers SLK, Kemp SS, Aguera KN, Koller GM, Forgy JC, Davis GE. Defining an Upstream VEGF (Vascular Endothelial Growth Factor) Priming Signature for Downstream Factor-Induced Endothelial Cell-Pericyte Tube Network Coassembly. Arterioscler Thromb Vasc Biol. 2020;40(12):2891-909.
40. Kano MR, Morishita Y, Iwata C, Iwasaka S, Watabe T, Ouchi Y, et al. VEGF-A and FGF-2 synergistically promote neoangiogenesis through enhancement of endogenous PDGF-B-PDGFRbeta signaling. J Cell Sci. 2005;118(Pt 16):3759-68.

Figures

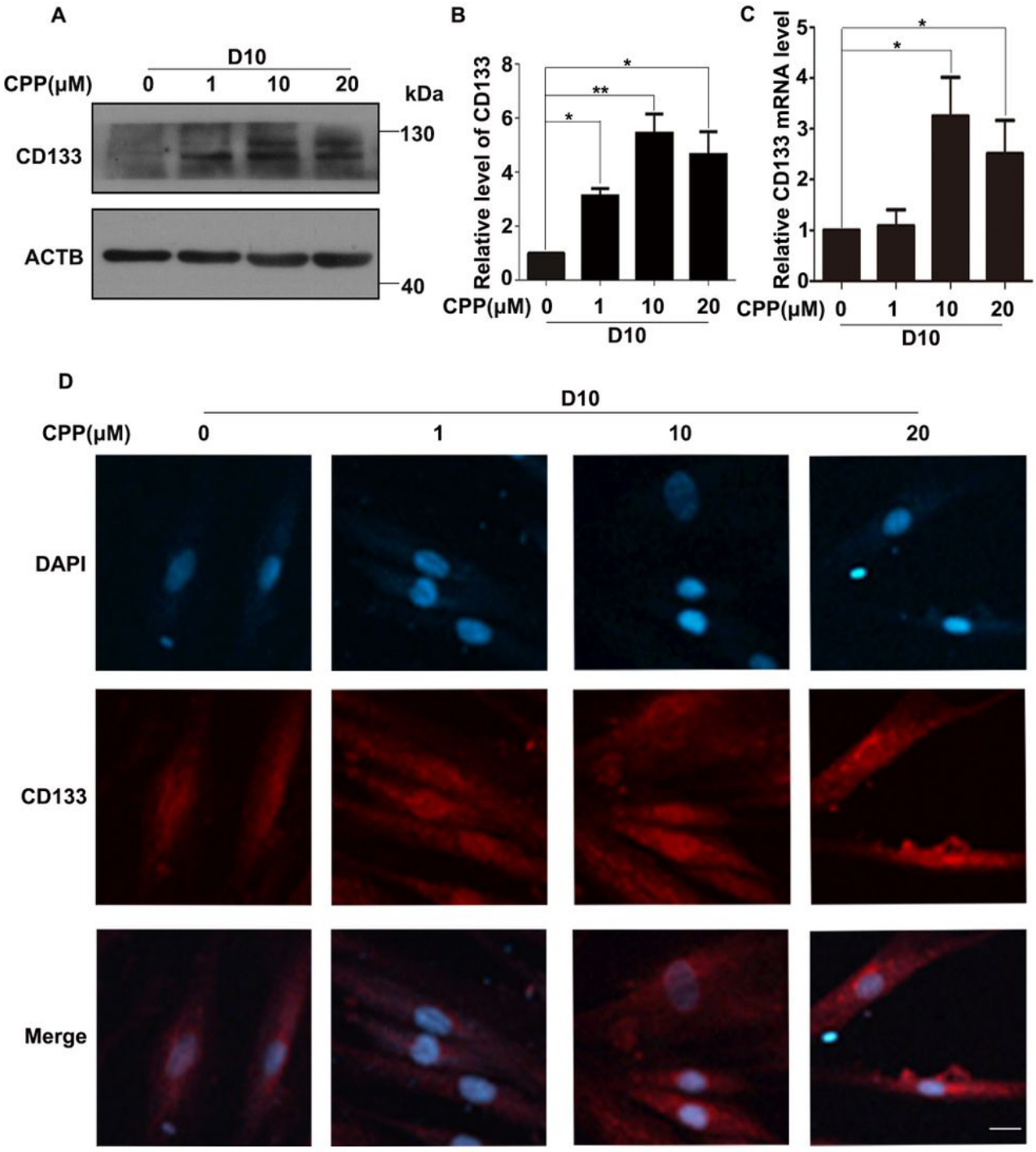

Figure 1

CPP induces morphological changes of HDFs. (A) The chemical structure of CPP. (B) Cell viability was determined using the sulforhodamine B (SRB) assay according to the manufacturer's instructions. HDFs were seeded in 96-well plates, and were then treated with 0.1% DMSO (as a control) or with CPP at the indicated concentration for 48 h, after which the SRB assay was used to determine cell viability. (C) HDFs were treated with the small chemical molecule CPP (0, 1, 10 or 20 μM) for 6 days (D6) or 10 days(D10),

after which morphological changes of HDFs were examined using an inverted phase contrast microscope. (D) HDFs were treated¬ with other HOCl probes for 10 days, and morphological changes of HDFs were examined by inverted phase contrast microscope (Eclipse TS-100; Nikon, Tokyo). Scale bar: 20 μm.

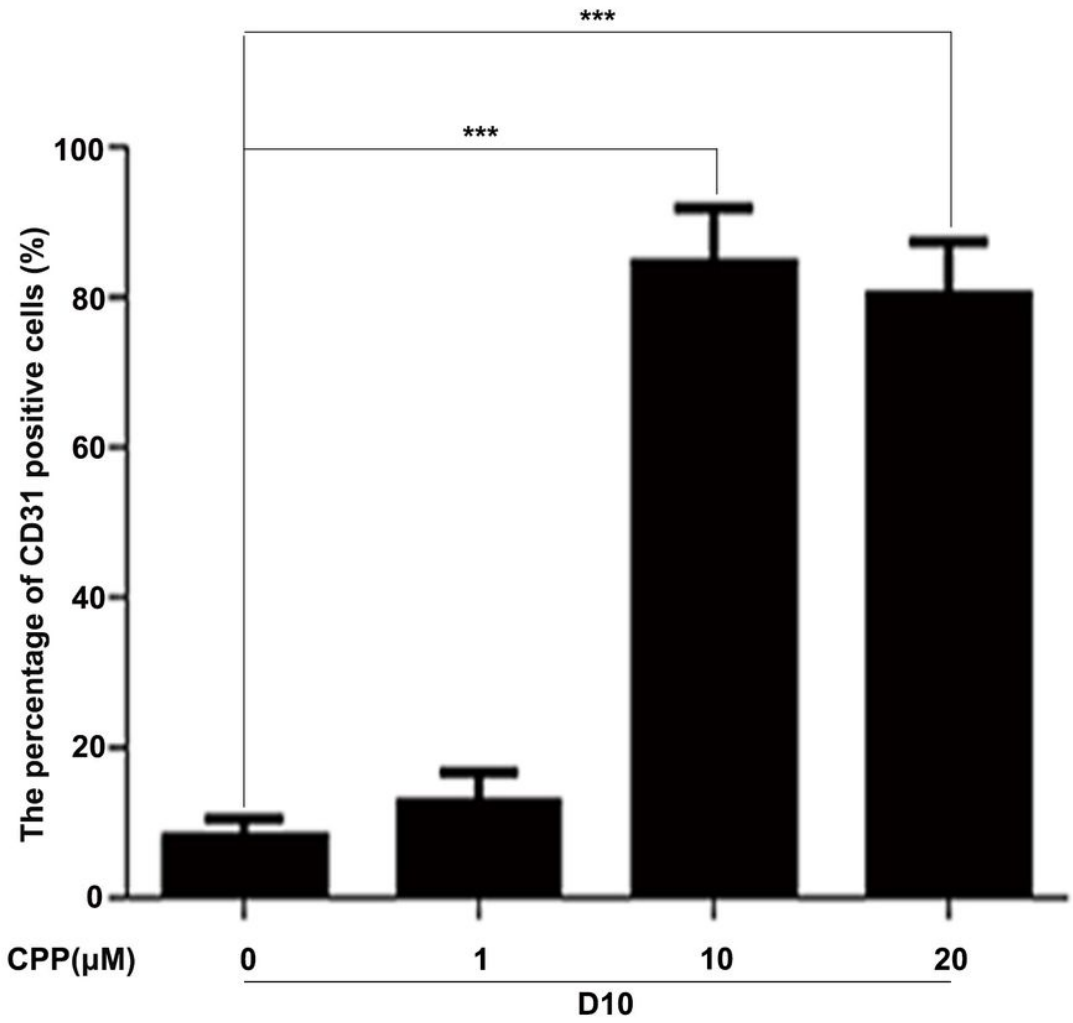

Figure 2

CPP promoted the expression of CD133. (A)-(B) HDFs were treated with 0, 1, 10 or 20 μM CPP for 10 days (D10), after which the protein level of CD133 was determined by Western Blot. β-actin (ACTB) was used as a loading control. Quantitation of bands in the Western blots (A) is shown in (B). (C) Different doses of CPP (0, 1, 10 or 20 μM) were used to treat HDFs for 10 days, after which mRNA levels of CD133 were

detected by qPCR. (D) Different doses of CPP (0, 1, 10 or 20 μM) were used to treat HDFs for 10 days (D10), after which protein levels of CD133 was detected by immunofluorescence. Scale bar: 20 μm. Data are presented as means ± SEM, *p < 0.05, **p < 0.01, n = 3.

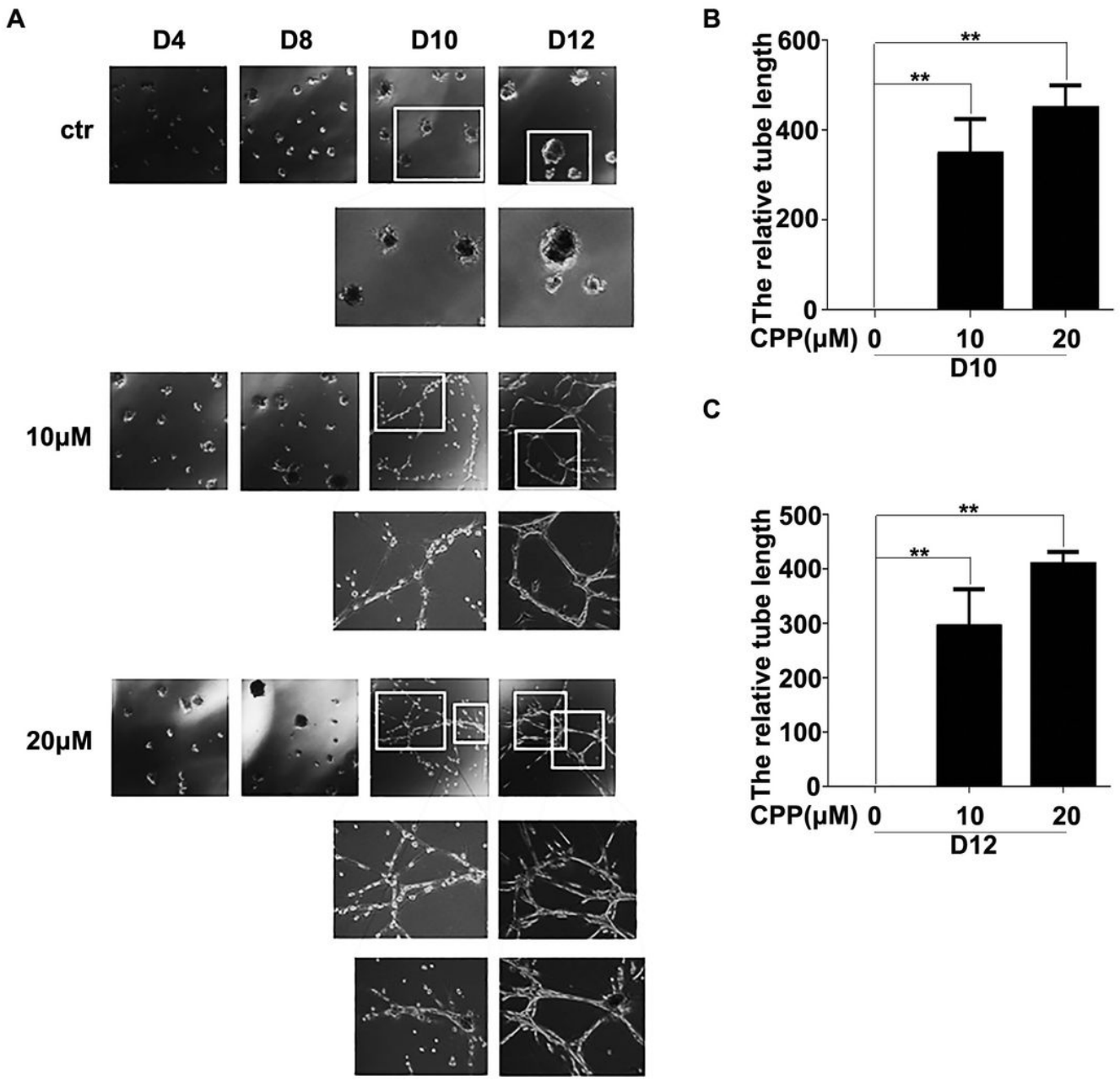

Figure 3

CPP promoted the expression of CD31. (A)-(B) HDFs were treated with 0, 1, 10 or 20 μM CPP for 10 days (D10), and the protein level of CD31 was determined by Western Blot. β-actin (ACTB) was used as a loading control. Quantitation of bands in the Western blots (A) is shown in (B). (C) Different doses of CPP

(0, 1, 10 or 20 μM) were used to treat HDFs for 10 days, and the mRNA levels of CD31 were detected by qPCR. (D) Different doses of CPP (0, 1, 10 or 20 μM) were used to treat HDFs for 10 days (D10), after which protein level of CD31 was detected by immunofluorescence. Scale bar: 20μm. Data are presented as means ± SEM, *p < 0.05, **p < 0.01, n = 3.

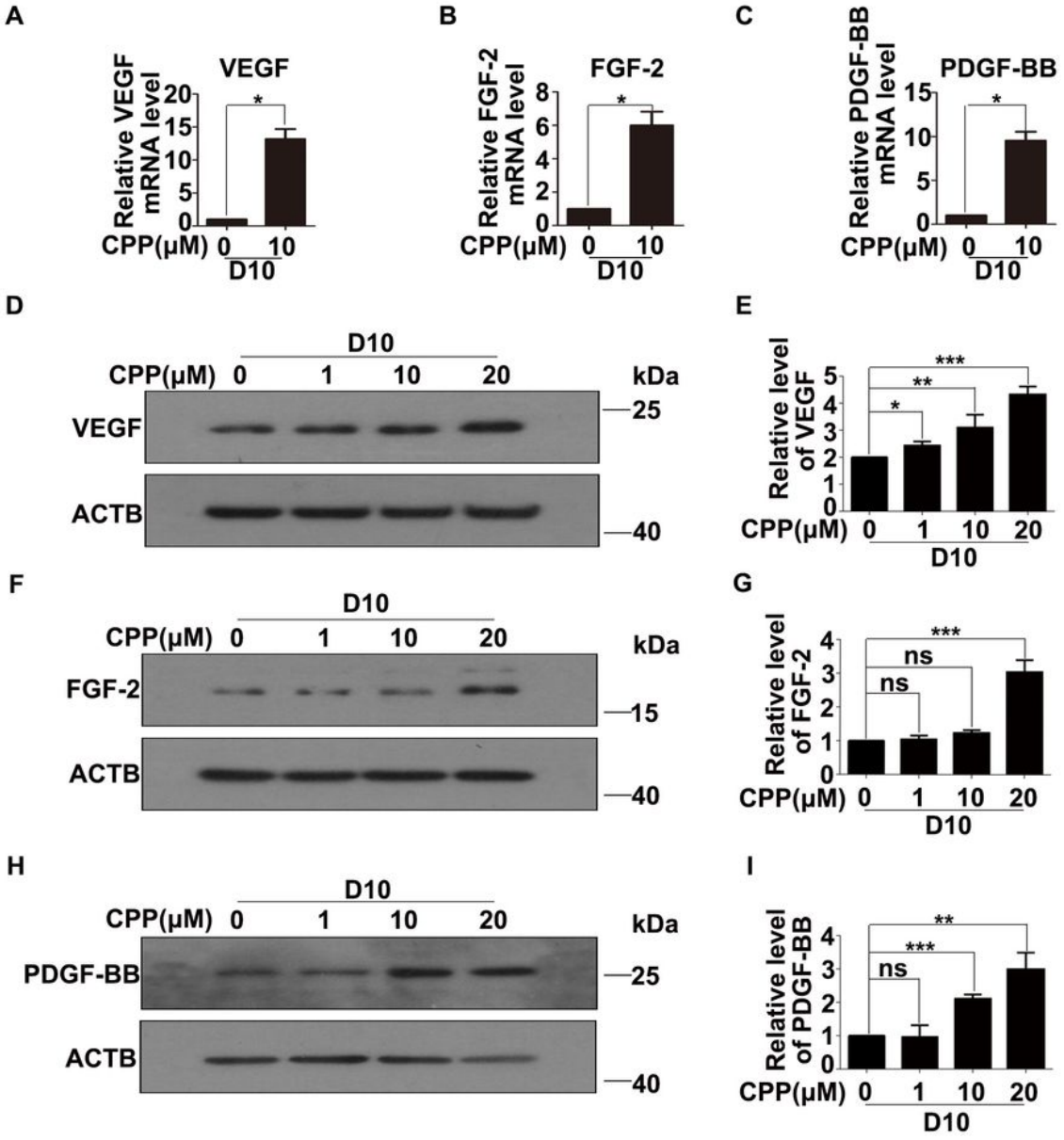

Figure 4

Differentiation rate of HDFs into vascular endothelial cells. Different doses of CPP (0, 1, 10 or 20 μM) were used to treat HDFs for 10 days, and then the ratio of the number of CD31-positive cells to the total number of cells was counted. Data are presented as means ± SEM, ***p < 0.001, n = 3.

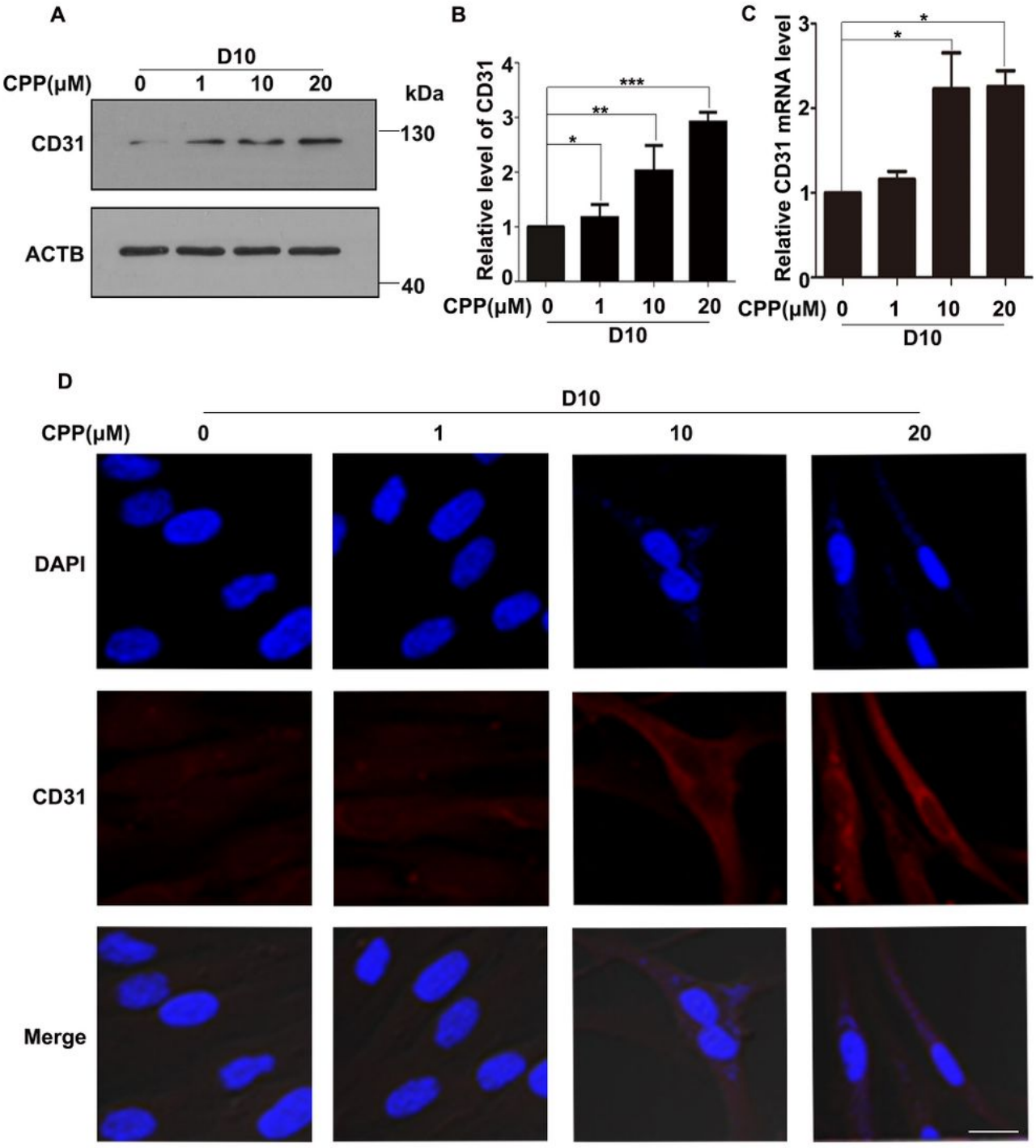

Figure 5

CPP reduced the level of Vimentin. (A)-(B) HDFs were treated with 0, 1, 10 or 20 μM CPP for 10 days (D10), after which the protein level of Vimentin was determined by Western Blot. β-actin (ACTB) was used

as a loading control. Quantitation of bands in the Western blots (A) is shown in (B). (C) Different doses of CPP (0, 1, 10 or 20 μM) were used to treat HDFs for 10 days, after which mRNA levels of Vimentin were detected by qPCR. (D) Different doses of CPP (0, 1, 10 or 20 μM) were used to treat HDFs for 10 days (D10), after which protein levels of Vimentin was detected by immunofluorescence. Scale bar: 20 μm. Data are presented as means ± SEM, *p < 0.05, **p < 0.01, n = 3.

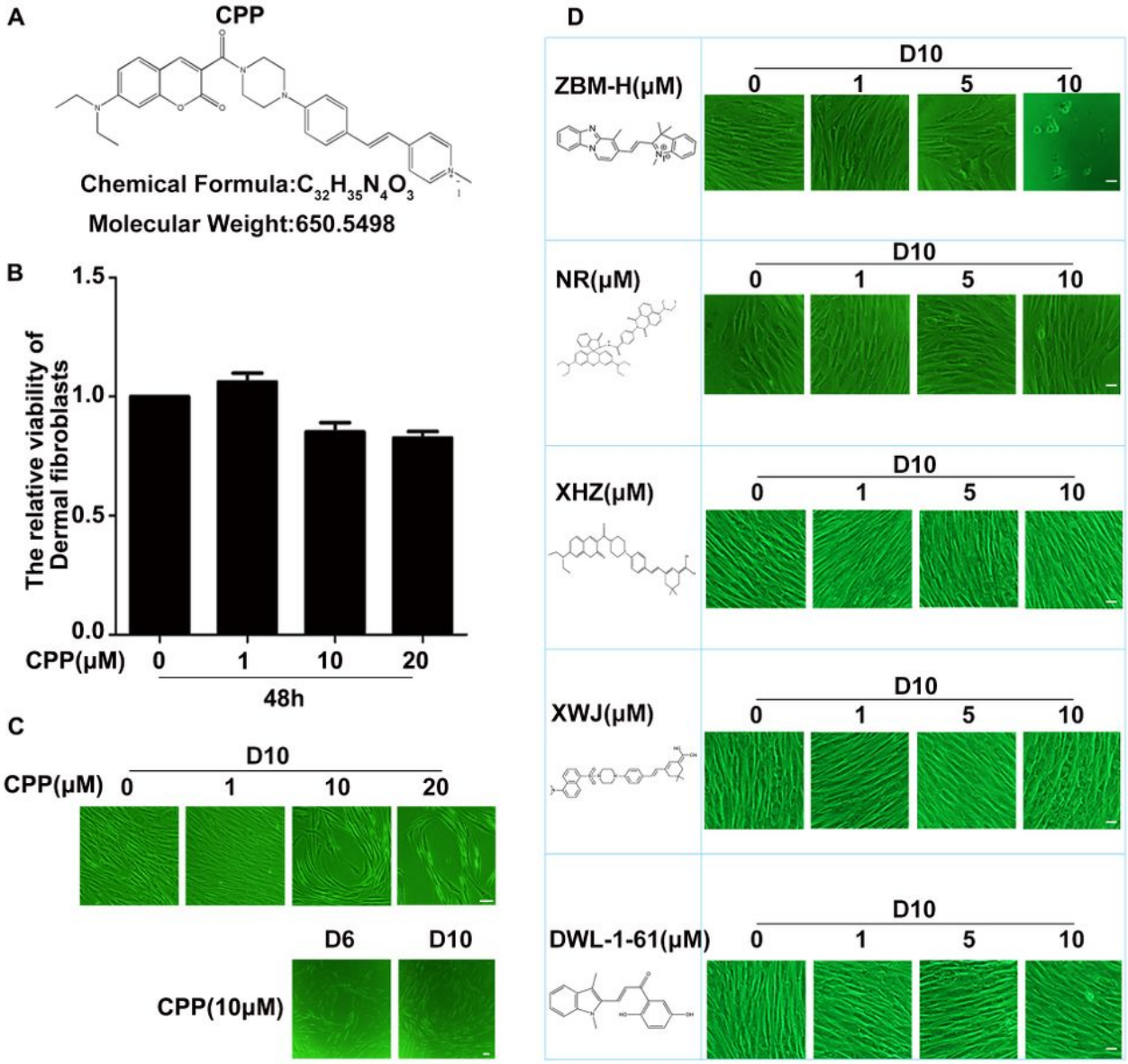

Figure 6

CPP promotes the angiogenesis of VECs derived from HDFs in vitro. (A)-(C) In vitro capillary-like tube formation by HDFs treated with different concentrations of CPP (0, 10, 20 μM) for 4, 8, 10 and 12 days. Representative images of capillary morphogenesis are shown at different points in time (A), and tubular lengths at day 10 (D10) and 12 (D12) were analyzed by Image J software and normalized to the control group (B) and (C). Data are presented as means ± SEM, **p < 0.01, n = 3.

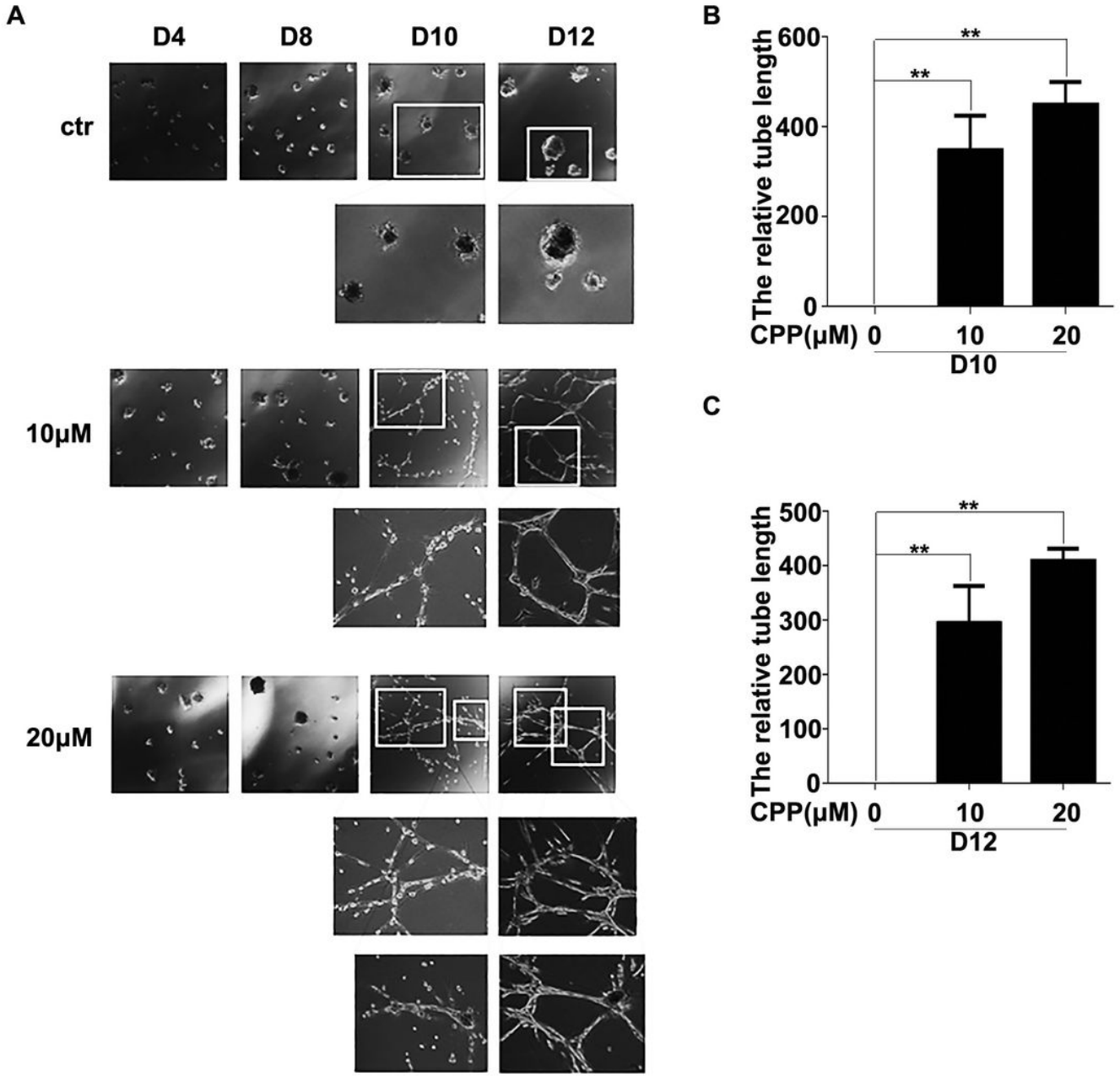

Figure 7

HDFs treated with CPP secrete angiogenesis-related factors. (A)-(C) mRNA expression levels of VEGF, FGF-2 and PDGF-BB in HDFs treated with or without CPP (10 μM) for 10 days (D10) were analyzed by qPCR. (D)-(I) HDFs were treated with or without CPP for 10 days (D10), and protein levels of VEGF, FGF-2 and PDGF-BB were determined by Western Blot (D), (F) and (H). β-actin (ACTB) was used as a loading control. Quantitation of bands in the Western blot bands (D), (F) and (H) are shown in (E), (G) and (I).