DARC Regulates Angiogenesis by Mediating Vascular Endothelial Cell Migration

Xiaolin Wang
Second Affiliated Hospital，Air Force Medical University
Yongqian Bian
Second Affiliated Hospital, Air Force Medical University
Yuejun Li
Second Affiliated Hospital, Air Force Medical University
Jing Li
Second Affiliated Hospital, Air Force Medical University
Congying Zhao
Second Affiliated Hospital, Air Force Medical University
Jinqing Li (lijqfmmu@hotmail.com)
Second Affiliated Hospital，Air Force Medical University
Xueyong Li
Second Affiliated Hospital, Air Force Medical University

Research

Keywords: Angiogenesis, DARC, Endothelial cell, RhoA

Posted Date: December 1st, 2020

DOI: https://doi.org/10.21203/rs.3.rs-115340/v1

Abstract

Background: DARC (The Duffy antigen receptor for chemokines) is a kind of glycosylated membrane protein that binds to members of the CXC chemokine family associated with angiogenesis and has recently been reported to be implicated in diverse normal physiologic processes. This study aimed to investigate the involvement of DARC in angiogenesis, which is known to generate new capillary blood vessels from preexisting ones.

Methods: HDMECs (Human dermal microvascular endothelial cells) were divided into two groups (DARC overexpression group, and control group). We used Brdu staining to detect cell proliferation, and wound healing assay to detect cell migration. Then tube formation assay were observed. Also, western blot and immunofluorescent staining were used to estimate the relationship between DARC and RhoA (Ras homolog gene family, member A).

Results: HDMECs proliferation, migration, and tube formation were inhibited significantly when DARC was overexpressed intracellular. DARC impaired microfilament dynamics and intercellular connection in migrating cells, and RhoA activation underlay the effect of DARC on endothelial cell. Furthermore, DARC inhibited the formation of new capillaries in vitro.

Conclusion: Our findings revealed the role of DARC in the angiogenic process and provided a novel mechanism for RhoA activation during endothelial cell migration and angiogenesis.

Backgroud

It is accepted that angiogenesis is the formation of new capillary blood vessels from preexisting ones, and it is a balanced process regulated by many angiogenic factors and anti-angiogenesis factors[1, 2]. The process of generating new blood vessels is closely related to physiological and pathological processes such as tissue development, wound repair, and tumor growth[3–5]. Among them, the growth and development of tissues require the new blood vessels to provide nutrients, and the preparation of the wound bed also requires the new blood vessels to provide nutrients and various growth factors[6, 7]. This process is highly controlled by the intricate balance of both proangiogenic and antiangiogenic factors, and involves a cascade of events of which migration of capillary endothelial cells is an essential component[8, 9].

The Duffy antigen receptor for chemokines (DARC) is a glycosylated membrane protein that belongs to the subfamily of silent chemokine receptors, which is expressed mainly on erythrocytes and endothelial cells[10]. However, the function of DARC expressed on endothelial cells remains unclear. Previous studies have indicated that DARC is a negative regulator of breast carcinoma and inhibits tumor growth by inhibiting tumorigenic chemokine, and DARC overexpression in breast cancer patients is associated with a better prognosis, lower metastatic potential, and less lymphocytic infiltrate[11–13]. Similar experimental results were reported in the lung[14] and laryngeal squamous cell carcinomas[15]. Another

study showed that tumors from DARC-deficient mice had higher intra-tumor concentrations of angiogenic chemokines, larger tumor vessel density, and they had greatly augmented prostate tumor growth[16].

The negative involvements of DARC in the development of prostate cancer[16] and breast cancer[13] suggested that this protein might play a negative regulatory role in angiogenesis, of which vascular endothelial cell proliferation and migration are essential components. Our study was undertaken to test this hypothesis directly. There has been a growing body of evidence that coordinated action of microtubules and actin filaments is critical for cell migration[17, 18]. Microtubule dynamics are precisely regulated by various microtubule-binding proteins and the Rho family[18–20]. Microfilaments can also regulate the activity of Rho GTPases, and the interaction between cytoskeleton and Rho GTPases is required for efficient cell migration and intercellular connection[20]. We hypothesized that the negative regulation of DARC may be related to its effect on the cytoskeleton and thus inhibiting cell migration.

However, the precise role of DARC in angiogenesis and the exact molecular mechanisms of DARC in the endothelial cells are still not clear. Therefore, the current study was to explore the involvement of the DARC in angiogenesis in vitro and also in vivo respectively.

Material And Methods

Cell culture and transfection

Human dermal microvascular endothelial cells (HDMECs, ScienCell Research Laboratories, catalog number #2000) were cultured according to the manufacturer's protocol. Briefly, HDMECs were cultured in Endothelial Cell Medium (ECM, ScienCell Research Laboratories, catalog number #1001) supplemented with 5% fetal bovine serum (FBS, ScienCell Research Laboratories), 4 mmol/l L-glutamine, 100 U/ml penicillin-G, 100 g/ml streptomycin, and 1% endothelial cell growth supplement (v/v; ScienCell Research Laboratories). HDMECs were used from the passage 4 to the passage 5.

Adenoviruses that over-express DARC (Ad-DARC, experimental group) and empty vector of adenovirus without target gene (Ad-eGFP, control group) were made by the Cyagen Biotechnology Co., Ltd. All the adenoviruses were labeled with green fluorescent tags. HDMECs were infected with Ad-DARC or Ad-eGFP at 1×10^6 PFU, in a minimal volume of serum-free medium, and the infection efficiency of HDMECs was confirmed to be above 80% by preliminary experiments.

Cell proliferation and viability assay

Cells were grown on glass coverslips and cultured with anti- bromodeoxyuridine (BrdU) antibody (1:500, ThermoFisher #B35128) at 4°C through the night. After the coverslips were washed with phosphate buffer saline (PBS, Gibco, catalog number 20012027), the cells were fixed with 4% paraformaldehyde for 15 minutes at room temperature. Then the coverslips were placed in 2N HCl for 30 minutes and washed with PBS. After that, the cells were incubated with antibodies against BrdU (Abcam, ab6326), and Cy3-conjugated secondary antibodies, and then unspecific bindings were blocked with PBS containing 5%

goat serum. Finally, the cells were stained with 4',6-diamidino-2-phenylindole (DAPI) for 10 minutes, and then examined by fluorescence microscopy.

CellTiter-Glo Luminescent Cell Viability Assay Kit (Cat# G7570, Promega) was used according to the manufacturer's protocol. Briefly, cells were cultured in 96-well plates and infected with Ad-DARC as well as Ad-eGFP in the following day. Equal volumes of medium and CellTiter-Glo Reagent were added at 1st day, 3rd day, and 5th day respectively, and the results were analyzed. Luminescence was detected by a multiwell scanning spectrophotometer.

Cell migration assay

Oris™ Cell Migration Assay Kit (PLATYPUS Technologies, Product No: CMA1.101) was used for cell migration detection according to the manufacturer's protocol. Briefly, cells were infected with Ad-DARC as well as Ad-eGFP and then seeded in each test well through one of the side ports of the Oris™ Cell Seeding Stopper. When cells had grown to be a confluent monolayer, we removed all the stoppers. Cells were imaged kinetically for up to 24 hours in a Cytation^{TM}3(BioTek Instruments, Inc.) microplate imager with incubation at 37℃ and a gas control module set to 5% CO_2 using the settings outlined. Then photos were taken at 6-hour intervals with 3 random locations to examine the extent of wound closure. The gap area was calculated with Image-Pro Plus version 6.0 (Media Cybernetics, Inc., Warrendale, PA). The analysis relied on the determination of the area of the detection zone of post-migration wells in comparison with pre-migration wells in which no migration occurred to calculate post-migration percent closure using imaging data.

Tube formation assay

Matrigel (BD Biosciences) was added to 96-well plates with a volume of 50 μl per well, and HDMECs (2×10^4 cells/100 μl) were cultured after the matrigel had solidified at 37℃. To observe tube formation, photographs were taken 24 hours later with the fluorescence microscope, and the degree of tube formation was quantified by measuring the branch points with the use of Image J software.

Cytoskeleton staining

Cells were grown on glass coverslips and fixed with 4% paraformaldehyde for 15 minutes at room temperature. Then they were incubated with 10 μg/ml phalloidine for 30 minutes. After that, the cells were washed with PBS and stained with DAPI for 10 minutes. Finally, the cells were observed under the Olympus IX71 fluorescent microscope.

Immunofluorescence staining

Cells on glass coverslips were fixed with 4% paraformaldehyde for 15 minutes at room temperature and then permeabilized using 0.1% Triton X-100 for 5 minutes. After blocking in 5% goat serum for 60 minutes, the samples were immunostained with primary antibodies against connexin 43(1:5000, ab11369, Abcam,), RhoA(10 μg/ml, ab86297, Abcam), followed by incubation with the goat anti-mouse

IgG Alexa Fluor-cy3-conjugated and the goat anti-rabbit IgG Alexa Fluor-488-conjugated secondary antibodies(1:200, Invitrogen). A confocal laser scanning microscopy with an Olympus FV 1000 device was used to observe cellular fluorescence associated with the cell connection.

Western blot analysis

After being washed twice with PBS, the cells were lysed for 30 minutes in ice-cold lysis buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1 mM sodium orthovanadate, 5 mM sodium fluoride, 1 mM ethylene diamine tetraacetic acid (EDTA), and the Protease Inhibitors Cocktail (Sigma). Then the samples were boiled at 100℃ for 10 minutes and were placed in ice immediately. Cell lysates were subjected to western blot analysis using rabbit antibodies against RhoA (1 μg/ml, ab86297, Abcam) and mouse antibodies against GAPDH (1 μg/ml, ab9484, Abcam) as the primary antibodies. The significance of the protein expression differences was calculated at last.

In vivo matrigel angiogenesis model

Eight-week-old nude mice were purchased from the Animal Center of the Air Forth Medical University. All animal procedures were performed in accordance with the Animal Care and Use Committee at the University of Air Forth Medical. Matrigel (BD Bioscience, catalog number 354248) was diluted with serum-free Dulbecco's modified Eagle's medium(DMEM, Gibco, catalog number 11965092) to 10 mg/ml. All the nude mice were weighed and anesthetized by 60 μl/20 g bodyweight injection of 1% sodium pentobarbital. The dorsal skin was disinfected with 70% ethanol, then tissue engineering chambers were filled with matrigel and then implanted subcutaneously into the dorsal of athymic nude mice. Sixteen nude mice were implanted with matrigel and randomly divided into four groups: the positive control group (n = 4), tissue engineering chambers that were filled with matrigel containing angiogenic factors (heparin and vascular endothelial growth factor); the negative control group (n = 4), tissue engineering chambers that were filled with matrigel without angiogenic factors; the DARC group (n = 4), tissue engineering chambers that were filled with matrigel containing angiogenic factors and Ad-DARC adenovirus; the control group (n = 4), tissue engineering chambers that were filled with matrigel containing angiogenic factors and Ad-eGFP adenovirus. The tissue engineering chambers were removed 6 days after implanted and Dextran was injected into nude mice via the tail vein before removed to mark the blood vessels. New blood capillaries in the matrigel were observed by fluorescence microscopy. To quantify the neovascularization in vivo, the cells were recovered from the matrigel by dispase (Corning, Cat. No. 354235) digestion and cell recovery solution (Corning, Cat. No. 354253). Then the recovered cells were stained with FITC-lectin for statistical analysis .

Results

Overexpression of DARC impairs angiogenesis in vitro

To investigate the potential role of DARC in angiogenesis, we first examined the effect of DARC overexpression on proliferation, viability, migration, and apoptosis in vitro. Two adenoviruses were used to infect HDMECs. Ad-DARC was able to overexpress DARC expression effectively in HDMECs (Fig. 1A). The cells were stained with BrdU to investigate the proliferation of DARC overexpression in the cells. The result showed that DARC overexpression inhibited HDMECs proliferation and also inhibited the proliferation caused by 20 ng/ml VEGF (Fig. 1B). To evaluate the effects of DARC on the cell viability, cell viability assay was performed on HDMECs. Overexpression of DARC resulted in significant inhibition of the cell viability at the 3rd day and the 5th day respectively (Fig. 1C). Similarly, cell migration was also inhibited in the DARC overexpression group observed by the Oris™ cell migration assay (Fig. 1D).

DARC regulates angiogenesis by inhibiting tube formation

To further investigate the function of DARC in angiogenesis, we examined the effect of DARC overexpression on vascular endothelial tube formation in vitro. We observed the branch point structures at 24 hours after plating cells onto matrigel. The results showed that overexpressed DARC remarkably impaired tube formation compared with the control group, and also impaired the tube formation caused by VEGF (Fig. 2). By counting the branch points, we found that overexpressed DARC in HDMECs inhibited HDMECs tube formation by 67.16% and 60.92%, respectively, normal cultured group and VEGF added group.

DARC modulates microfilament dynamics and intercellular connection in migrating cells

We conducted the cytoskeleton staining. According to the Phalloidine staining result, the cytoskeleton protein degraded and reconstructed in the DARC overexpression group, also the intercellular connection decreased significantly (Fig. 3A), and cell spreading was inhibited. On the basis of the finding that cells gap junctions were decreased, we then detected connexin 43. The result obviously showed that DARC overexpression inhibited the expression level of connexin 43 (Fig. 3B). These data suggested that DARC might regulate the migration of vascular endothelial cells through modulation of microfilament dynamics.

RhoA activation impairs the effects of DARC on endothelial cell migration

To gain more mechanistic insight into how DARC mediates endothelial cell migration, we detected the activities of RhoA, which is known mainly involved in cell polarization and migration primarily through interplay with the cytoskeleton[20, 21]. According to the immunofluorescence staining result, the RhoA expression decreased significantly in the DARC overexpression group (Fig. 4A). The western-blot result showed that DARC overexpression significantly decreased the intracellular RhoA expression (Fig. 4B). To examine whether RhoA activation contributes to the action of DARC in endothelial cell migration, endothelial cells were transfected with pSUPER-RhoA and then wound healing assay was performed. We found that overexpression of RhoA could remarkably rescue DARC overexpression-induced migration defects (Fig. 4C), indicating that RhoA activation underlies the effect of DARC on endothelial cell migration.

DARC inhibits angiogenesis in vivo

To further confirm the in vitro data indicating the importance of DARC in angiogenesis, we studied the effect of DARC on the angiogenic response in nude mice. Tissue engineering chambers were filled with matrigel containing heparin and vascular endothelial growth factor and were implanted into the dorsal area of athymic nude mice (Fig. 5A).

We found apparent vascular structures in the tissue engineering chambers 6 days after implantation (Fig. 5B Positive control), and no vascular structures were observed in tissue engineering chambers that were filled with matrigel without heparin and vascular endothelial growth factor (Fig. 5B Negative control). It is very important that the tissue engineering chamber with Ad-DARC addition obviously inhibited vascular growth which was induced by the angiogenic factors. On the contrary, the control group did not affect vascular growth into the tissue engineering chamber (Fig. 5B). By laser confocal microscope, obvious vascular structures were found in the control group as in the DARC group, and this was consistent with the results of the positive and negative controls (Fig. 5C).

In order to quantify the neovascularization in vivo, cells were recovered from the matrigel by dispase digestion and stained with FITC-lectin. As shown in Fig. 5D, the fluorescence intensity of cells recovered from the DARC group was dramatically decreased compared with the control group. These results confirmed the important role of DARC in angiogenesis in vivo.

Discussion

In the past few decades, significant progress has been achieved in identifying angiogenic factors and inhibitors. Although lots of new inhibiting angiogenesis molecules have been discovered, the precise molecular mechanisms remain to be elucidated. In our study, we have found that DARC had the negative effects not only for endothelial cell migration and for intercellular connection in vitro but also for angiogenesis in vivo. Our results were partly consistent with certain previous studies that DARC was involved in reducing angiogenesis in tumorigenesis[16, 22, 23], and also DARC was highly expressed on endothelial cells post-capillary venules and endothelial cell membrane[24, 25]. In these studies, Xu et al. have further investigated the mechanism, and they have shown that DARC on endothelial cells attenuated the angiogenic activity by causing senescence[26]. However, our study provides the evidence showing a novel possibility in DARC-induce angiogenesis inhibition, and we propose that DARC inhibited the migration of endothelial cells by inhibiting the expression of RhoA, resulting in the inhibition of angiogenesis.

In the current studies, we show that HDMECs proliferation was inhibited significantly when DARC was overexpressed intracellular. Meanwhile, the proliferation of HDMECs induced by VEGF was also inhibited obviously (Fig. 1B). Our data are also supported by the cell viability assay showing that the ATP concentration was inhibited by DARC both normal cultured cells and VEGF induced cells (Fig. 1C). As shown by overexpressing DARC and performing Oris™ cell migration assay, we revealed the effects of DARC on cell migration, that is, the inhibitory effect of DARC on cell migration became more significant

with the increase of time (Fig. 1D). These results suggest that DARC has the effect of reducing the angiogenic potential.

Cell migration plays a central role during tube formation and cell movement in angiogenesis, and cellular movement is a complex, tightly regulated multistep process[27]. Through tube formation assay and cytoskeleton staining, we have found that DARC inhibited vascular endothelial cell migration by impairing intercellular connections that were related to microfilament dynamics.

The microfilament cytoskeleton is known to undergo dramatic rearrangement in response to signals that stimulate cell migration, and such property of microfilaments relies on precise control of microfilament dynamics in cells. Microfilaments can also regulate the activity of Rho GTPases, and the interaction between cytoskeleton and Rho GTPases is required for efficient cell migration and intercellular connection[21]. Interestingly, we clearly showed that the inhibitory effect of DARC on the intercellular connection was induced by RhoA, which is well known to play an important role in cell movement, cytoskeleton recombination, and cell proliferation[28–30]. Our data supported that RhoA expression decreased with the overexpression of DARC by showing the results of fluorescence staining and protein expression (Fig. 4A, 4B). Through the results of the wound healing experiment after the overexpression of DARC and RhoA simultaneously, we found that overexpression of RhoA could remarkably rescued DARC overexpression-induced migration defects (Fig. 4C), indicating that RhoA activation underlies the effect of DARC on endothelial cell migration.

In vitro studies have demonstrated that DARC significantly inhibited endothelial cell growth and tube formation. Similarly, by the matrigel angiogenesis model in vivo, we found that the control group showed a large number of new capillary vessels, but only a very few new capillary vessels could be found in the DARC group. These data suggested that DARC inhibited the formation of new capillaries which induced by angiogenic factors in vivo. The in vivo results were consistent with our previous finding that DARC inhibited angiogenesis in vitro.

In conclusion, our data showing dramatic decreases in new capillary vessels formation provided strong evidence that the DARC had an important regulatory function in this process, and microfilament was involved in the regulation of this process. Our finding that RhoA inhibition was involved in DARC's effects on endothelial cell migration and angiogenesis is very interesting. Considering the interplay between RhoA activity and microfilaments in migrating cells, it is conceivable that the action of DARC in Rac1 down-regulation might be an event downstream of the modulation of microtubule dynamics by DARC. However, it is still unclear that how DARC regulates microfilament through RhoA exactly and what the specific signal transduction pathways it affects. This will be important to investigate in the future.

Abbreviations

DARC
Duffy Antigen Receptor for Chemokines;
RhoA

Ras homolog gene family, member A;
HDMECs
Human Dermal Microvascular Endothelial Cells;
ECM
Endothelial Cell Medium;
FBS
Fetal Bovine Serum;
Ad-DARC
Adenoviruses that over-express DARC;
Ad-eGFP
Empty vector of adenovirus without target gene;
BrdU
bromodeoxyuridine;
PBS
Phosphate Buffer Saline;
DAPI
4’,6-diamidino-2-phenylindole;
EDTA
Ethylene Diamine Tetraacetic Acid;
DMEM
Dulbecco’s Modified Eagle’s Medium;
VEGF
Vascular Endothelial Growth Factor.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

Funding

This work was supported by grants from the National Natural Science Foundation of China (Grant No: 81701909)

Authors' contributions

X.W., J.L., and X.L. designed the research; X.W. and Y.B. wrote the paper; and X.W., Y.L., J.L., and C.Z. performed the research and analyzed the data.

Acknowledgments

We thank the Experimental Center of the Third Affiliated Hospital of the Air Force Medical University for their assistance with the laser confocal microscopy.

References

1. S S, FT Z, MS L, NA G, CM M. Mechanisms of angiogenesis in microbe-regulated inflammatory and neoplastic conditions. ANGIOGENESIS. 2018;21(1):1-14.https://doi.org/10.1007/s10456-017-9583-4 PMID: 29110215
2. A B. History and conceptual developments in vascular biology and angiogenesis research: a personal view. ANGIOGENESIS. 2017; 20(4): 463-78. https://doi.org/10.1007/s10456-017-9569-2 PMID: 28741165
3. Mikkelsen VE, Stensjoen AL, Granli US, Berntsen EM, Salvesen O, Solheim O et al. Angiogenesis and radiological tumor growth in patients with glioblastoma. BMC CANCER. 2018;18(1):862. https://doi.org/10.1186/s12885-018-4768-9 PMID: 30176826
4. Korntner S, Lehner C, Gehwolf R, Wagner A, Grutz M, Kunkel N et al. Limiting angiogenesis to modulate scar formation. Adv Drug Deliv Rev. 2018; https://doi.org/10.1016/j.addr.2018.02.010. PMID: 29501628
5. Maruotti N, Annese T, Cantatore FP, Ribatti D. Macrophages and angiogenesis in rheumatic diseases. Vasc Cell. 2013; 5(1):11. https://doi.org/10.1186/2045-824X-5-11 PMID: 23725043
6. OC V. Angiogenesis and vasculogenesis: inducing the growth of new blood vessels and wound healing by stimulation of bone marrow-derived progenitor cell mobilization and homing. J VASC SURG. 2007;45(6):39-47. https://doi.org/10.1016/j.jvs.2007.02.068 PMID: 17544023
7. Greaves NS, Ashcroft KJ, Baguneid M, Bayat A. Current understanding of molecular and cellular mechanisms in fibroplasia and angiogenesis during acute wound healing. J DERMATOL SCI. 2013;72(3):206-17. https://doi.org/10.1016/j.jdermsci.2013.07.008 PMID: 23958517
8. Lamalice L, Le Boeuf F, Huot J. Endothelial Cell Migration During Angiogenesis. CIRC RES. 2007;100(6):782-94. https://doi.org/10.1161/01.RES.0000259593. 07661.1e PMID: 17395884
9. Daub JT, Merks RMH. A Cell-Based Model of Extracellular-Matrix-Guided Endothelial Cell Migration During Angiogenesis. B MATH BIOL. 2013;75(8):1377-99.https://doi.org/ 10.1007/s11538-013-9826-5 PMID: 23494144

10. SC P, ZX W, K N, AW M, HJ S, MJ C et al. The Duffy antigen/receptor for chemokines (DARC) is expressed in endothelial cells of Duffy negative individuals who lack the erythrocyte receptor. The Journal of experimental medicine. 1995;181(4):1311-7. https://doi.org/10.1084/jem.181.4.1311 PMID: 7699323
11. Zeng XH, Ou ZL, Yu KD, Feng LY, Yin WJ, Li J et al. Coexpression of atypical chemokine binders (ACBs) in breast cancer predicts better outcomes. Breast Cancer Res Treat. 2011;125(3):715-27. https://doi.org/10.1007/s10549-010-0875-2 PMID: 20369284
12. Wang J, Ou ZL, Hou YF, Luo JM, Chen Y, Zhou J et al. [Duffy antigen receptor for chemokines attenuates breast cancer growth and metastasis: an experiment with nude mice]. Zhonghua Yi Xue Za Zhi. 2005;85(29):2033-7. PMID: 16313795
13. Wang J, Ou ZL, Hou YF, Luo JM, Shen ZZ, Ding J et al. Enhanced expression of Duffy antigen receptor for chemokines by breast cancer cells attenuates growth and metastasis potential. ONCOGENE. 2006;25(54):7201-11. https://doi.org/10.1038/sj.onc.1209703 PMID: 16785997
14. Addison CL, Belperio JA, Burdick MD, Strieter RM. Overexpression of the duffy antigen receptor for chemokines (DARC) by NSCLC tumor cells results in increased tumor necrosis. BMC CANCER. 2004;4(1):28. https://doi.org/10.1186/1471-2407-4-28 PMID: 15214968
15. Sun G, Wang Y, Zhu Y, Huang C, Ji Q. Duffy antigen receptor for chemokines in laryngeal squamous cell carcinoma as a negative regulator. Acta Otolaryngol. 2011;131(2):197-203. https://doi.org/10.3109/00016489.2010.516012 PMID: 21105847
16. Shen H, Schuster R, Stringer KF, Waltz SE, Lentsch AB. The Duffy antigen/receptor for chemokines (DARC) regulates prostate tumor growth. The FASEB Journal. 2006;20(1):59-64. https://doi.org/10.1096/fj.05-4764com PMID: 16394268
17. DD T, BD G. The roles and regulation of the actin cytoskeleton, intermediate filaments and microtubules in smooth muscle cell migration. RESP RES. 2017;18(1):54. https://doi.org/PMID:
18. S E. Microtubules in cell migration. ANNU REV CELL DEV BI. 2013;29(1):471-99. https://doi.org/10.1186/s12931-017-0544-7 PMID: 28390425
19. A H, LX T, KH G, KJ L, E M, L T. Rho GTPases and regulation of cell migration and polarization in human corneal epithelial cells. PLOS ONE. 2013;8(10):e77107. https://doi.org/10.1371/journal.pone.0077107 PMID: 24130842
20. CD L, AJ R. Rho GTPase signaling complexes in cell migration and invasion. Journal of cell biology. 2018;217(2):447-57. https://doi.org/10.1083/jcb.201612069 PMID: 29233866
21. AJ R. Rho GTPase signalling in cell migration. CURR OPIN CELL BIOL. 2015;36:103-12.https://doi.org/10.1016/j.ceb.2015.08.005 PMID: 26363959
22. LW H, Y Y, S Z, RM S, A R. Opposing roles of murine duffy antigen receptor for chemokine and murine CXC chemokine receptor-2 receptors in murine melanoma tumor growth. CANCER RES. 2007;67(20):9791-9. https://doi.org/10.1158/0008-5472.CAN-07-0246 PMID: 17942909
23. Maeda S, Kuboki S, Nojima H, Shimizu H, Yoshitomi H, Furukawa K et al. Duffy antigen receptor for chemokines (DARC) expressing in cancer cells inhibits tumor progression by suppressing CXCR2

signaling in human pancreatic ductal adenocarcinoma. CYTOKINE. 2017;95:12-21. https://doi.org/10.1016/j.cyto.2017.02. 007 PMID: 28214673

24. Hadley TJ, Lu ZH, Wasniowska K, Martin AW, Peiper SC, Hesselgesser J et al. Postcapillary venule endothelial cells in kidney express a multispecific chemokine receptor that is structurally and functionally identical to the erythroid isoform, which is the Duffy blood group antigen. J CLIN INVEST. 1994;94(3):985. https://doi.org/10.1172/JCI117465PMID: 8083383
25. Pruenster, Mudde M, Bombosi L, Dimitrova P, Zsak S, Middleton M et al. The Duffy antigen receptor for chemokines transports chemokines and supports their promigratory activity. NAT IMMUNOL. 2009;10(1):101. https://doi.org/10.1038/ni.1675 PMID: 19060902
26. Xu L, Ashkenazi A, Chaudhuri A. Duffy antigen/receptor for chemokines (DARC) attenuates angiogenesis by causing senescence in endothelial cells. ANGIOGENESIS. 2007;10(4):307-18. https://doi.org/10.1007/s10456-007-9084-y PMID: 17955335
27. Michaelis, Ruth U. Mechanisms of endothelial cell migration. Cellular & Molecular Life Sciences. 2014;71(21):4131-48. https://doi.org/10.1007/s00018-014- 1678-0 PMID:25038776
28. Raftopoulou M, Hall A. Cell migration: Rho GTPases lead the way. DEV BIOL. 2004;265(1):23-32.https://doi.org/10.1016/j.ydbio.2003.06.003 PMID:14697350
29. Ridley AJ. RhoA, RhoB and RhoC have different roles in cancer cell migration. J MICROSC-OXFORD. 2013;251(3):242-9. https://doi.org/10.1111/jmi.12025 PMID:23488932
30. Pertz O, Hodgson L, Klemke RL, Hahn KM. Spatiotemporal dynamics of RhoA activity in migrating cells. NATURE. 2006;440(7087):1069-72. https://doi.org/10.1038/nature04665 PMID:16547516

Figures

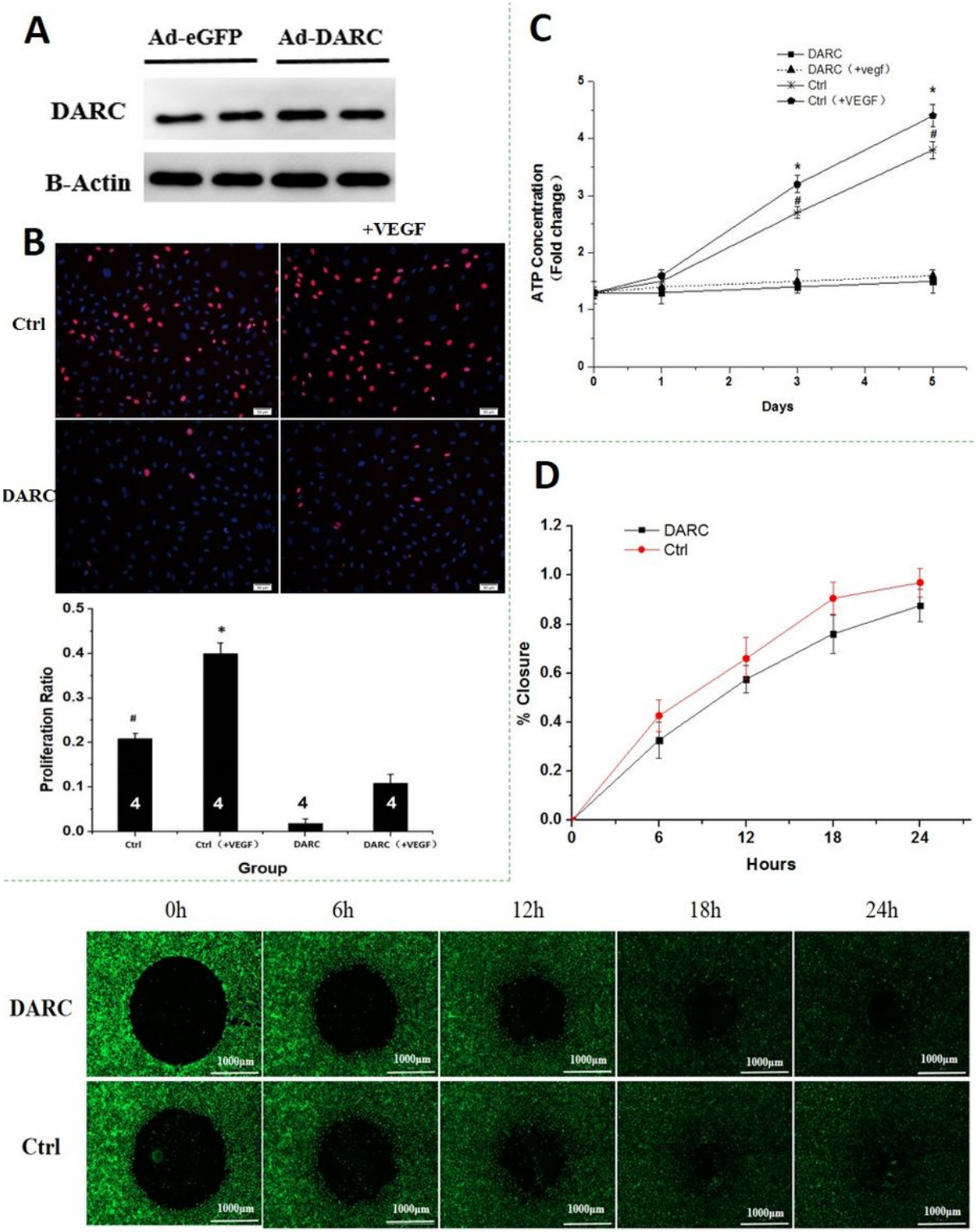

Figure 1

Overexpression of DARC in the endothelial cells impairs cell proliferation, cell migration. (A) Immunoblot analysis of DARC and β-actin expression in HDMECs infected with Ad-DARC or Ad-eGFP. (B) Cells were stained with BrdU. The red ones were proliferative cell nuclei, and the blue ones were the normal nuclei(#indicates $p<0.05$ vs DARC, and *indicates $p<0.05$ vs DARC+VEGF). (C) Cells were processed for proliferation assay using CellTiter-Glo Luminescent Cell Viability Assay Kit (Cat# G7570, Promega)

(*indicates p<0.05 vs DARC+VEGF, and #indicates p<0.05 vs DARC). (D) Cells were treated according to the in-structions, and cell migration was determined by the area of the detection zone of post-migration wells.

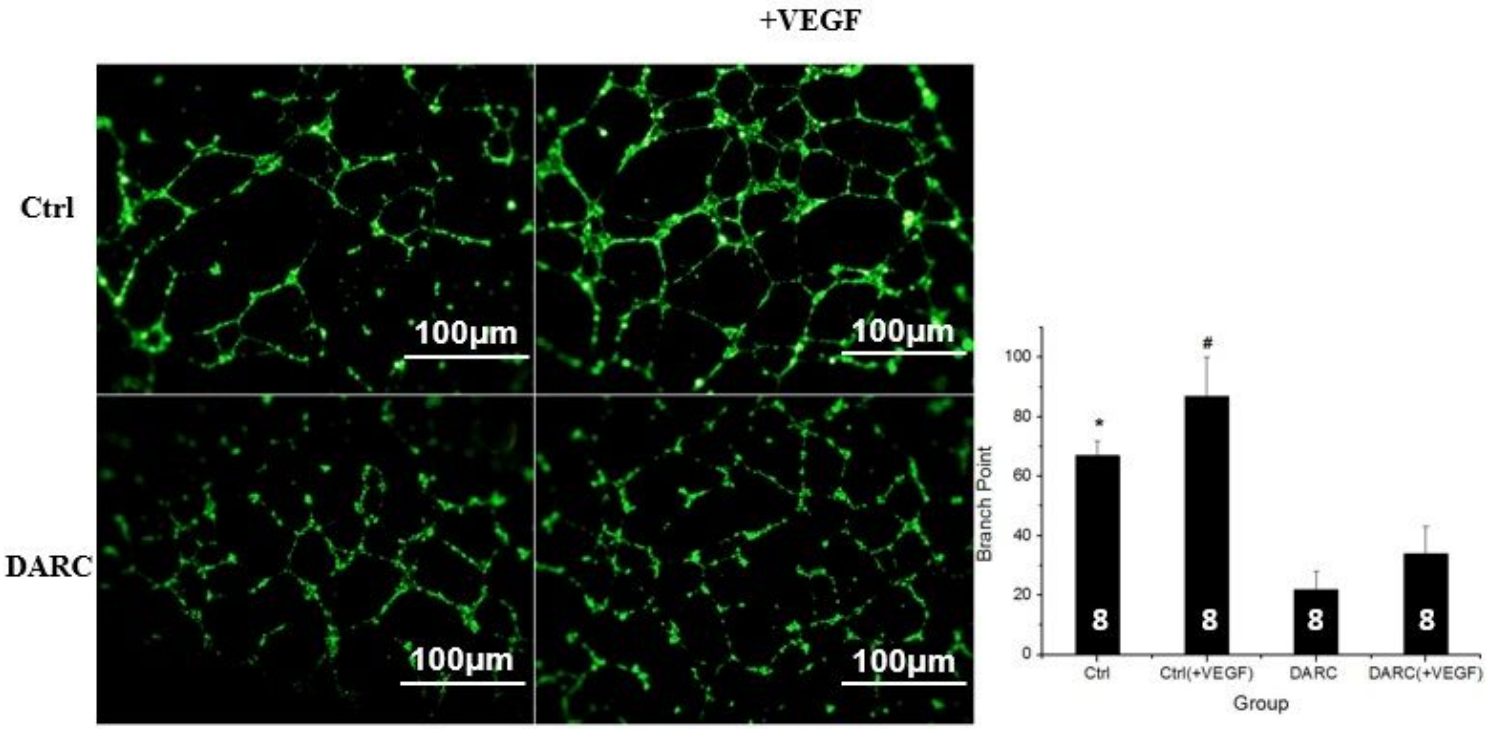

Figure 2

Representative image of tubes formed on the matrigel after HDMEC after incubating for 24 hours. The cells were cultured on the solid matrigel to observe the tube formaiton. The histogram on the right showed the number of branch points of each group. (*indicates p<0.05 vs DARC, and #indicates p<0.05 vs DARC+VEGF)

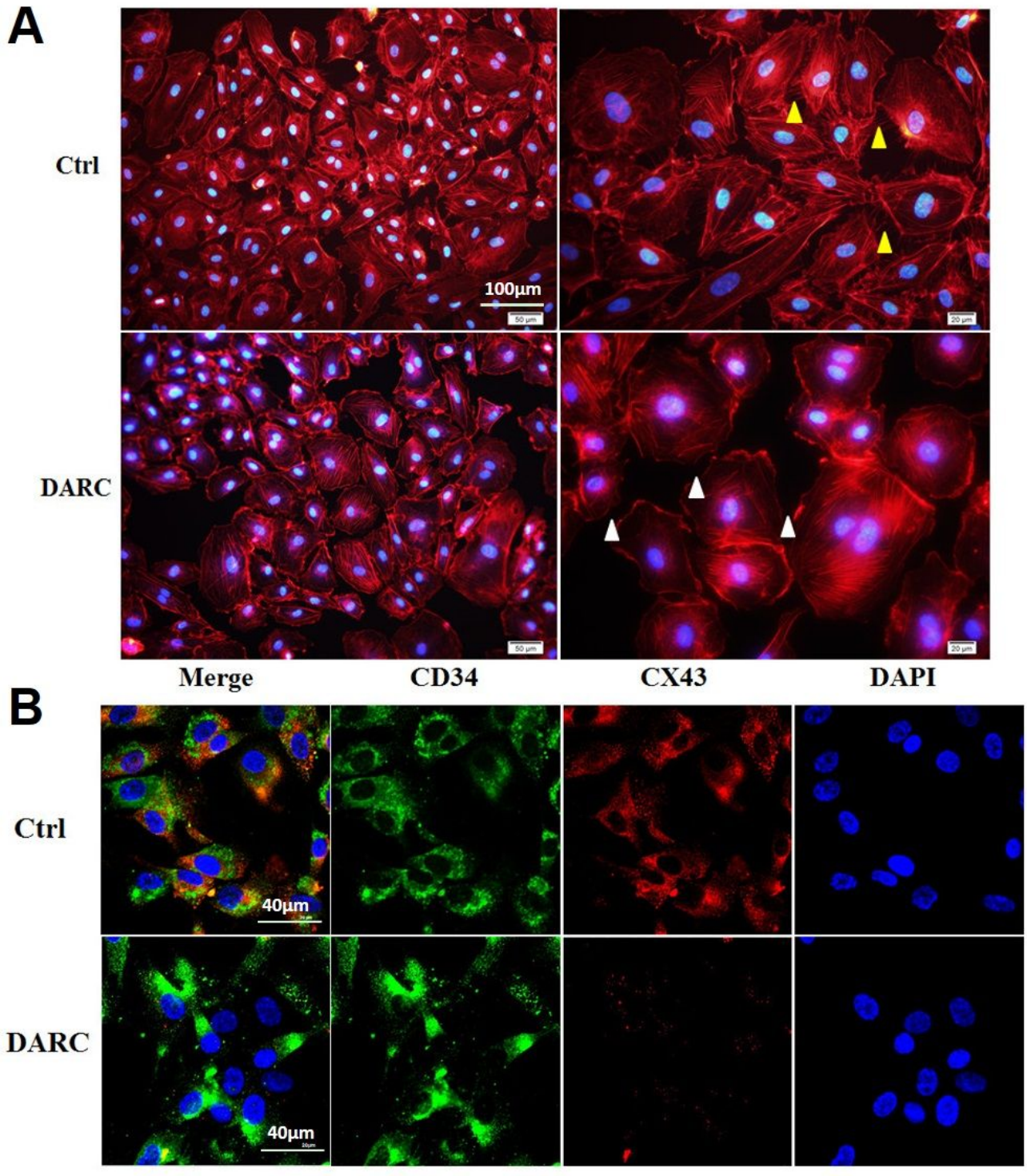

Figure 3

DARC modulates microfilament dynamics and intercellular connection in migrating cells. (A) Phalloidine was used to mark cytoskeleton protein (The red fluorescence). DAPI was used to mark the nuclei (The blue fluorescence). Yellow arrowheads indicate cell junctions, and white arrowheads indicate the gaps between cells. (B) CX43 was used to mark gap junctions (Red), and CD 34 was used to mark HDMECs (Green). DAPI was used to mark the nuclei.

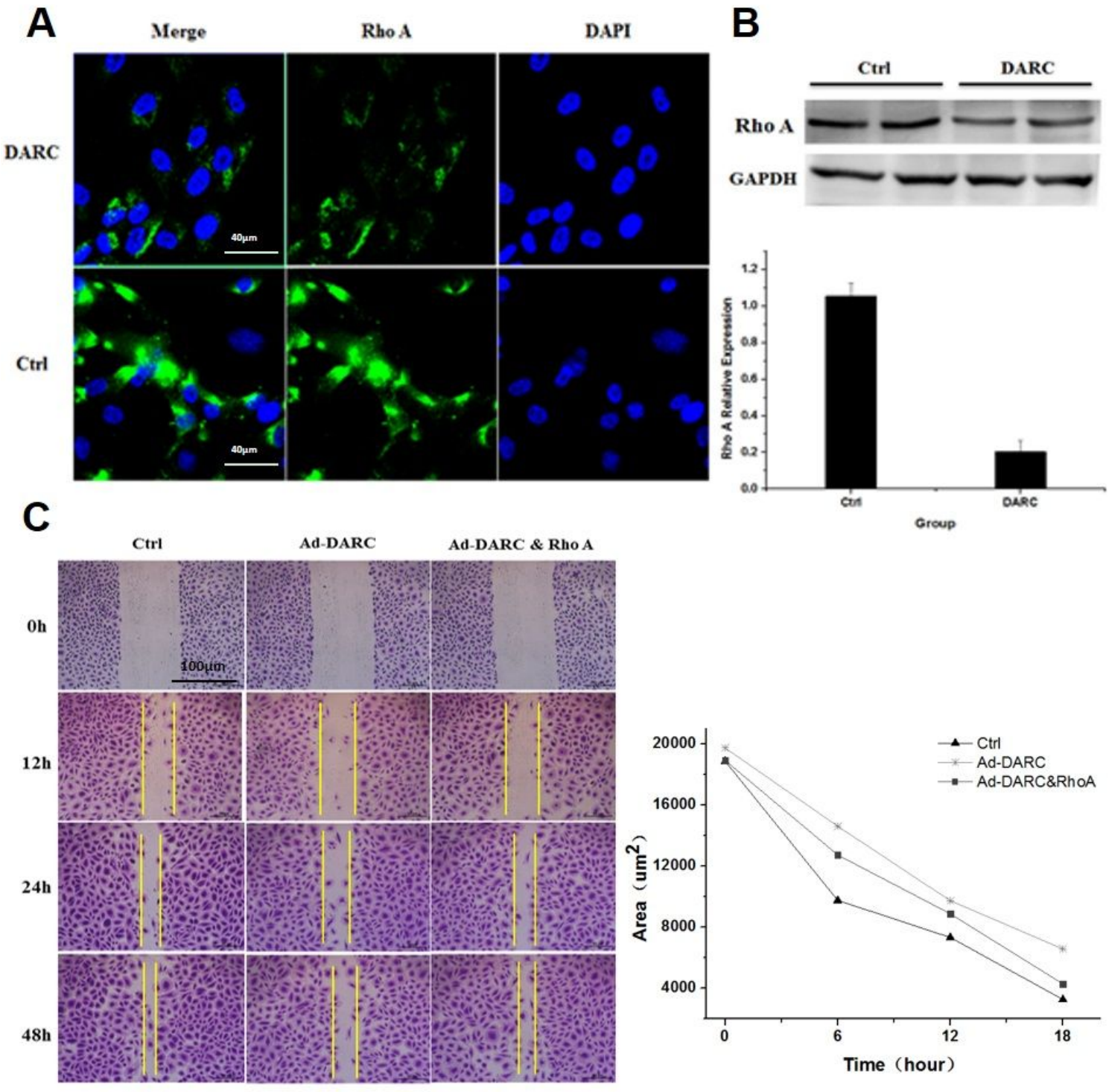

Figure 4

RhoA activation impairs the effects of DARC on endothelial cell migration. (A) Immunofluorescence staining for RhoA in endothelial cells. RhoA was marked green, and DAPI was used to mark nuclei. (B) Western-bolt assay(*indicates $p<0.05$ vs control group). (C) En-dothelial cells infected with adenovirus DARC and transfected with pSUPER-RhoA were scratched, and wound margins were stained with crystal violet and imaged at the beginning, the 12th hour, the 24th hour, and the 48th hour. Yellow lines indicate wound margins.

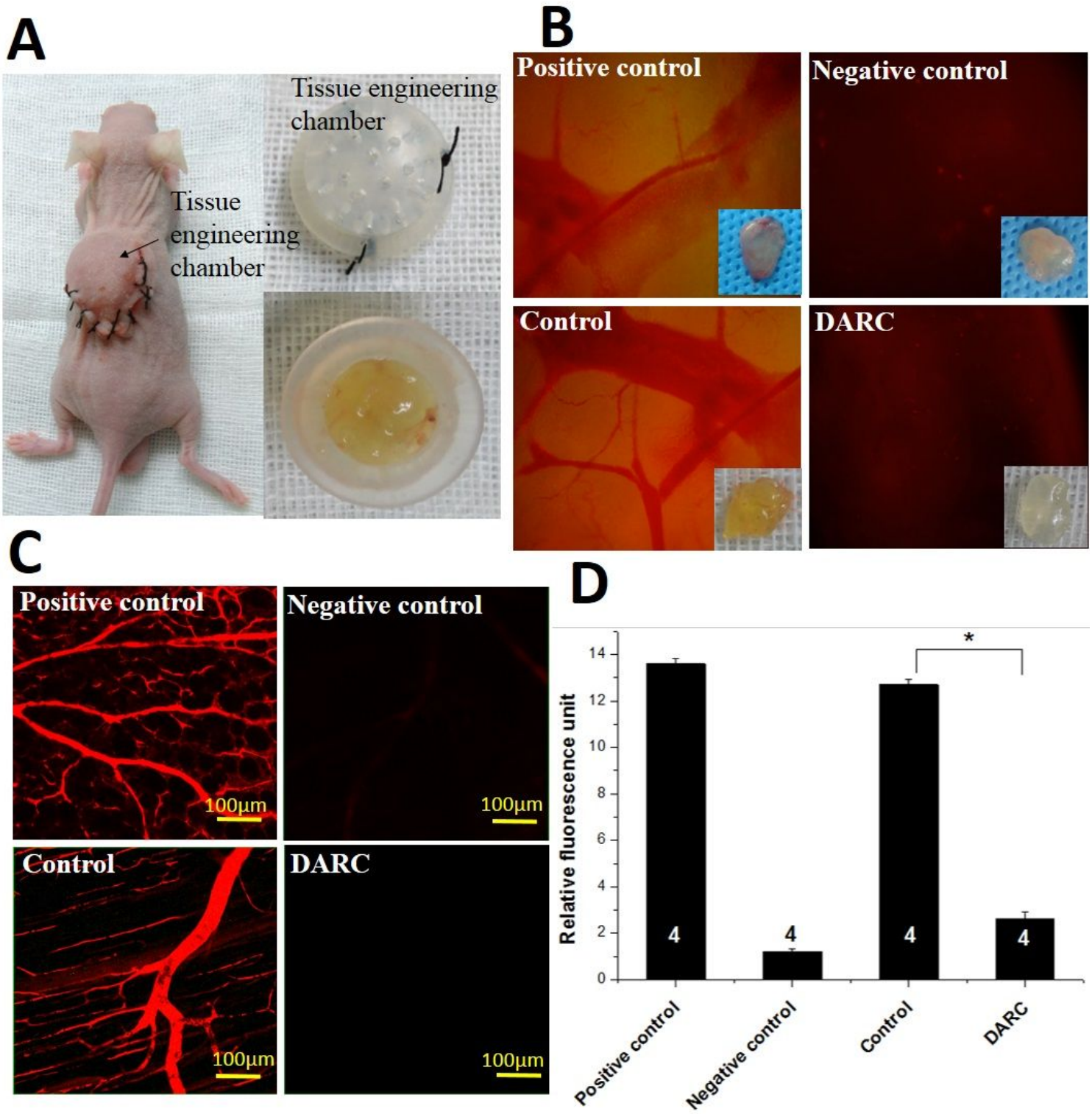

Figure 5

DARC inhibited the formation of new capillaries in vivo. (A)A nude mouse with a tissue engineering chamber implanted, and the arrowhead indicates the surgical implantation site. On the right is the tissue engineering chamber before implantation and after removal respectively. (B)Angiogenesis in the matrigel observed by an optical microscope. Tissue engineering chambers filled with matrigel containing angiogenic factors (VEGF, Heperhin) serve as the positive control, and matrigel not containing angiogenic

factors serve as the negative control. In our experiments, tissue engineering chambers were filled with matrigel containing angiogenic factors and Ad-DARC adenovirus or control adenovirus. (C) Before removed the tissue engineering chambers, Dextran Texas RedTM, 70000MV(Cat# D1830) were injected into the nude mice tail via tail vein, then observed by confocal laser. (D) Statistical analysis was performed as in panel B, and angi-ogenesis was quantified by FITC-lectin staining of recovered cells from the matrigels (*indicates $p<0.05$ between the DARC group and the control group).