Homocysteine inhibits angiogenesis through cytoskeleton remodeling

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Running title: Hcy, an inhibitor of angiogenesis

Abstract

Homocysteine (Hcy) is an intermediate non-diet amino acid connecting methionine and folate cycles. Elevated total Hcy level in blood, denoted hyperhomocysteinemia, has emerged as a prevalent and strong risk factor for multiple diseases including atherosclerotic vascular disease in coronary, cerebral and peripheral vessels. Its detrimental effect on vascular system implies the potential application as an inhibitor of angiogenesis. However, the detailed mechanism is unveiled. Inhibitory effect of Hcy was assessed on vascular endothelial growth factor (VEGF)-induced cell proliferation and migration with endothelial cell culture system. Its effect on angiogenesis was further examined *in vitro* and *in vivo*. After Hcy treatment, key angiogenic factors were measured by RT-qPCR. Cellular skeletal structure was also evaluated by actin stress fiber staining. VEGF-induced human umbilical vein endothelial cell (HUVEC) proliferation and migration were dramatically down-regulated by Hcy in a dose responsive manner. Hcy treatment significantly inhibited the VEGF induced angiogenesis *in vitro* by tube formation assay and chick chorioallantoic membrane vessel formation *in vivo*. Key angiogenic factors like VEGFR1/2 and Ang1/2 were substantially reduced by Hcy in HUVEC and VEGF induced actin stress fiber cytoskeletal structure was abolished. We demonstrated that Hcy could inhibit angiogenesis by targeting key angiogenic factor and disruption of actin cytoskeleton which is crucial for cell migration.

KEYWORDS: homocysteine; angiogenesis; endothelial cells; actin stress fiber; HUVEC

Summary statement

Homocysteine was demonstrated as a potent anti-angiogenic factor in our study. It could inhibit VEGF-induced HUVEC cell growth and migration at dose responsive manner. The molecular mechanism of homocysteine mediated inhibition was partly accounted by its ability to down-regulate key angiogenic factors like VEGFR and angiopoietin, in addition to the well-established disruption of redox balance in cells. More importantly, we unveiled the unappreciated function of homocysteine to abolish VEGF-induced actin stress fiber formation and cytoskeletal remodeling in endothelial cells.

Abbreviations list

Homocysteine (Hcy); vascular endothelial growth factor (VEGF)

Introduction

Homocysteine (Hcy) is a sulfur-containing α amino acid. It is not found in protein and cannot be obtained from diet. In the cells it is biosynthesized from methionine via a cycle of chemical reactions. Firstly, methionine is converted into S-adenosylmethionine (SAM) by methionine adenosyltransferase (MAT) in ATP-dependent manner. SAM is a ubiquitous methyl group donor which is required for a large family of SAM-dependent methyltransferases for methylation of DNA, RNA, proteins and lipids. During the methylation reaction, SAM is then converted into S-adenosylhomocysteine (SAH) after the methyl group is transferred to acceptor molecules. SAH gives rise to Hcy via hydrolysis reaction to remove adenosine. Biochemically Hcy can be recycled to form methionine by methylation, or combined with serine to give rise to cysteine which is the precursor of an important anti-oxidant factor glutathione. Homocysteine transsulfuration pathway is critical for Hcy catabolism and is considered as a major source of glutathione in the liver (1-3).

The normal concentration of total homocysteine in plasma of adults is approximately 10 μM (4). Over 90% of total plasma homocysteine is bound to plasma proteins, and only traces of free homocysteine, approximately 0.1 μM, are present in plasma (5). Hyperhomocysteinemia, elevation of plasma homocysteine level, is caused by the deficiency of dietary intake of vitamins B12, B2, folate and choline (6). In this condition, increased level of homocysteine undergoes auto-oxidation of thiol group to generate hydrogen peroxide, and other reactive radical oxygen species which then leads to oxidative stress in cells (7, 8). Increased oxidative stress subsequently causes dysfunction of endothelial cells, swelling and vacuolization of endothelial cells, fibrin deposition and even clot formation in vascular vessels. Therefore homocysteine is considered a risk factor of cardiovascular disease (9, 10).

On the other hand, homocysteine is recently reported to play an important role in angiogenesis (11-15) which is a hall-mark of cancer development (16, 17). Angiogenesis by definition is the physiological process in which new blood vessels form from pre-existing ones. It is vital in growth and development, also it is essential for tumor growth which depends on the supply of nutrients, oxygen and waste disposal. Moreover, migration of tumor cells into distal regions also requires the route of blood vessel, and it has been the primary killing factor for tumor mortality.

Angiogenesis is under strict regulation of cellular microenvironment by the circulating positive and negative signals. The primary angiogenic signal is vascular endothelial growth factor (VEGF) which binds to VEGF receptor found on endothelial cell surface and promotes endothelial cell growth and migration toward the source of VEGF. Anti-angiogenesis factors have been continuously developed and widely used as anti-tumor agents, for instance avastin, endostatin and some VEGF inhibitors such as sorafenib, axitinib and pazopanib (18). Since homocysteine reduces endothelial cell proliferation, which plays a key role in angiogenesis, it has been proposed as inhibitor of angiogenesis. The homocysteine-dependent impairment of angiogenesis is largely caused by the decrease in glutathione peroxidase expression and consequent increase in oxidant stress, leading to endothelial progenitor cell dysfunction (19, 20); decreased bioactive

nitric oxide generation (7, 21); and dysregulation of matrix metalloproteinase (MMP) activity as well as tissue remodeling (22).

The actin cytoskeleton and associated proteins play a critical role in cell-cell adhesion (23). Through their cytoplasmic tails, junctional adhesion proteins may bind to cytoskeletal and signaling proteins, which allows the anchoring of the adhesion proteins to F-actin and the transfer of intracellular signals inside the cell (24, 25). Actin cytoskeleton is also implicated in angiogenesis (26, 27). In this study, we examined the function of homocysteine as inhibitor of angiogenesis in endothelial cell model and our results showed that homocysteine could counter-act the proliferative effect of VEGF on endothelial cell to suppress the cell migration and tube formation ability in vitro. At molecular level it reduced the mRNA levels of angiogenic factors such as VEGFR1/2, Ang1/2 and disrupted the actin stress fiber formation. The inhibitory role of homocysteine on angiogenesis was also confirmed in chicken chorioallantoic membrane assay which was more physiological relevant.

Materials and methods

Cell Culture

Human umbilical vein endothelial cells (HUVECs) were purchased from ScienCell (CA, USA), and the human hepatic epithelial cell line (WRL-68) and human fibroblast-like fetal lung cells (WI-38) were purchased from American Type Culture Collection (ATCC; VA, USA). HUVECs were cultured in Endothelial Cell Medium (ECM; ScienCell, USA) supplemented with 5% heat-inactivated fetal bovine serum (FBS; ScienCell, USA), 1% penicillin/streptomycin (ScienCell, USA) and 1% Endothelial Cell Growth Supplement (ECGS; ScienCell, USA). WI-38 and WRL-68 were maintained in Dulbecco's Modified Eagle Medium (DMEM; Gibco, CA, USA) and Roswell Park Memorial Institute medium 1640 (RPMI; Gibco, USA), respectively, supplemented with 10% heat inactivated FBS (Sigma-Aldrich, MO, USA) and 1% penicillin/streptomycin (Gibco, USA). All cells were incubated at 37°C in humidified 5% CO_2, 95% air.

In vitro migration and invasion assays

Migration assay was performed by the Boyden chamber method in 24-well plates with inserts of 6.5 mm diameter and 8-μm pore size (Transwell; Corning Inc., NY, USA). DMEM/F12 containing 10 % FBS as a chemoattractant was placed in the lower wells, respectively. In brief, 200 μl of the HUVEC cell culture was added to the upper compartments. The chamber was incubated at 37 °C under 5 % CO_2 for 16 h. After incubation, the non-migrating cells were removed from the upper surface of the filter using a cotton swab. The filters were fixed in methanol for 15 min and stained with 0.1 % crystal violet for 15 min. Migration was quantitated by counting the stained cells that migrated to the bottom side of the membrane using an optical microscope (100× magnification). All experiments were made in duplicate and replicated three times at least.

Scratch-Wound Directional Migration Assay

HUVECs were seeded at a cell density of $\times 10^5$ cells/well in a 96-well microtiter plate and allowed to grow into a confluent monolayer overnight. Then, the monolayer was scraped using a sterile 20–200 µl micropipette pipette tip to create a wound of ±1 mm width. The cells were washed twice with Hanks' Balanced Salt Solution (HBSS; Sigma-Aldrich, USA) and replaced with fresh medium containing indicated concentrations of NC. After 8 h, the cells were stained with Hoechst 33342 and Cellomics® whole cell stain green (Thermo Fisher Scientific, Waltham, MA, USA). Cell migration was estimated by measuring the number of endothelial cells that had migrated from the edge of the wounded monolayer. An area of 512×512 pixels of the wounded area was acquired using Cellomics Array Scan HCS Reader and the number of migrated cells was calculated by the HCS automated algorithm. Inhibition of migration was represented by a decrease in the number of cells in the image acquired relative to the untreated control. For each monolayer sample, three measurements were taken for three independent wounds.

Chick chorioallantoic membrane (CAM) assay

Fertilized white Leghorn eggs were incubated at 37°C in a humidified incubator and windowed. On day 7 of development, sterile filters soaked with either vehicle or VEGF (100 ng/disk) in the presence or absence of NC (9 µg/disk) were applied to relatively avascular regions of the CAM. CAMs were fixed (4% paraformaldehyde in PBS) *in ovo* on day 9 and photographed in the localized area of the filter. The newly capillarised area in the region of each filter was quantified using Leica QWin Lite software and neovascularisation is expressed as an angiogenic index (n=12–15 eggs per treatment). This study was approved by the ethics committee of The First Affiliated Hospital of Wenzhou Medical University

RNA extraction and Real-time PCR

HUVEC were treated with 7 mM NC for 16 h, PBS was used as control. Brief, RNA extraction from sub-confluent treated or non-treated cells was performed using 1.0 ml of Trizol (Invitrogen, Carlsbad, CA, USA) for each HUVECs 1×10^6 cells of sample according to manufacturer's recommendation. RNA integrity was assayed by agarose gel electrophoresis and treated with DNAse (RQ1 RNAse free DNAse – Promega, Madison, WI, USA). cDNA and PCR were performed using SuperScript III Platinum one-step qRT-PCR Systems (Invitrogen, USA). Gene expression was measured in 7500 Fast (Applied Biosystems, Waltham, MA, USA) using GAPDH (Hs99999905_m1) as endogenous gene. Taqman gene expression assay from Applied Biosystems were performed for VEGFR1 and Ang1/2 genes, respectively.

Immunocytofluorescence Microscopy

The effects of PA on the actin and tubulin cytoskeletal systems of HUVECs were investigated by immunofluorescence. Briefly, HUVECs at ~80% confluency were treated with PA for 16 h and stained with phalloidin for F-actin and anti-paxillin antibody for paxillin, respectively. Images were acquired on conventional fluorescence microscope and the effects on F-actin and paxillin were analyzed by Morphology BioApplication Algorithm (Thermo Fisher Scientific, USA).

Statistical analysis

All values are expressed as means ± SEM. The data were analyzed using Student's t-test for two-group comparisons or using two-way ANOVA followed by the Tukey post hoc tests. GraphPad Prism 7.0 statistical and graphing software was used for the statistical analyses. Differences were considered significant at $p < 0.05$.

Results

Homocysteine inhibits EGFP induced cell proliferation and migration

During the angiogenesis process, endothelial cells (EC) are stimulated to migrate, proliferate, and invade surrounding tissue to form capillary tubules capable of carrying blood. VEGF is a potent inducer of endothelial cell proliferation. Using an established cell model HUVEC, we first examined the effect of homocysteine on VEGF-driven cell proliferation assay, with dosage range based on literature and pilot study. As shown in Figure 1 A, HUVEC cells growth rate was promoted by VEGF (50 ng/ml) from 8 h to 24 h and maintained at steady state from 24 h to 48 h. Under different dosages of homocysteine, HUVEC growth was monitored. We noticed that 10 μM homocysteine did not make much difference, while 40 μM Hcy showed ~25% reduction of cell growth. Strikingly, 70 μM Hcy completely abolished the proliferative effect of VEGF at all time-points. It is worth noting that Hcy at higher doses than 70 μM could not induce further inhibition in our pilot study (data not shown). Next we investigated the migration capability of HUVEC cells under treatment of Hcy with modified Boyden chamber assay. Compared to control condition, VEGF could significantly enhance the migration of HUVEC to the extent of almost 2 folds. Similarly, although 10 μM homocysteine caused neglectable effect, 40 to 70 μM Hcy showed significant inhibitory effect at a dose responsive manner. As shown in Figure 1B, 40 μM Hcy completely reverted the VEGF effect to the level of control cells and 70 μM Hcy showed 40% suppressive effect further compared to 40 μM Hcy treatment condition. Collectively our results showed that high concentration Hcy treatment could robustly suppress HUVEC cell growth and migration.

Homocysteine suppresses the *in vitro* angiogenesis

Angiogenesis is normally activated in the wound healing process. We next performed the wound healing assay on HUVEC cell monolayer with different dosages of Hcy in the presence of VEGF. As shown in Figure 2A, VEGF could readily induce cell migration to fill the gap introduced by wound after 24 h, 10 μM Hcy treatment could reduce the cell migration by 25% and 70 μM Hcy completely inhibit VEGF induced cell migration, as quantified in Figure 2B. During the angiogenesis, endothelial cells need to protrude from old vessel in a tube format. The ability to form tubing is therefore important and is assessed as a standard method to quantify the potential of angiogenesis (28). We then examined the effect of Hcy with tube formation assay. As shown in Figure 2C, VEGF could stimulate the formation of capillary-like tubes on the surface of extracellular matrix, while Hcy treatment showed dose-dependent inhibition of tube formation as quantified in Figure 2D. Compared to VEGF control, 70 μM Hcy treatment reduced the length of tube by 70%. The above results demonstrated that Hcy could suppress the angiogenesis *in vitro*.

Homocysteine impairs angiogenesis *in vivo*

To validate the results in Figure 2, we further evaluated the Hcy effect on angiogenesis in a setting mimicking the *in vivo* situation. Chick embryo chorioallantoic membrane (CAM) assays have been widely used to study angiogenesis and tumor invasion as a robust and cost-effective *in vivo* model. From result in Figure 3A and 3B, Hcy treated CAMs clearly showed less capillary formed from main blood vessel, compared to the control. Quantitation of data from 12-15 eggs showed remarkable reduction of blood vessel numbers with dose responsive effect where 70 μM Hcy showed more than 70% suppression. This result confirmed the *in vitro* data that Hcy treatment could significantly inhibit angiogenesis *in vivo*.

Homocysteine represses the angiogenesis factors

To gain more molecular insight into the function of Hcy on angiogenesis, in addition to the reported oxidative stress, we investigated the key angiogenic factors such as VEGFR and angiopoietin (Ang) in the process. VEGFR1/2 are receptors for VEGF and play important role to convey the signal into cells and regulate gene expression. Angiopoietins form dimer or tetramer and bind to receptor Tie2 to activate downstream pathways involved in angiogenesis as well as vascular permeability regulation (29). HUVEC cells were treated with various doses of Hcy for 16 h and mRNA levels of VEGFR1/2, Ang1/2 were quantified by RT-qPCR method. As shown in Figure 4A and 4B, VEGFR1/2 mRNA level showed dose dependent decrease upon Hcy treatment ($p<0.05$) compared to untreated control. Ang1/2 mRNA showed 20-30% decrease with 10 and 40 μM Hcy ($p<0.05$) and even greater decrease with 70 μM Hcy treatment ($p<0.01$) (Figure 4C and 4D). These results supported the suppressive role of Hcy on multiple key factors for angiogenesis. This could partially account for the inhibitory effect of Hcy presented in previous figures (Figure 1-3).

Homocysteine disrupts the actin stress fiber formation

Stress fibers are contractile actin bundles found in non-muscle cells and are found to play an important role in cellular contractility by providing the mechanic force for multiple functions such as cell adhesion, migration and morphogenesis (30). The function of stress fiber in angiogenesis has been elusive. Here we tested Hcy effect on the formation of stress fiber in HUVEC cells as another mechanistic explanation for its suppressive role (Figure 5A-D). As shown in Figure 5, VEGF treatment could induce the robust formation of stress fiber inside cells. Interestingly, 10 μM Hcy could dramatically reduce the stress fiber length. 70 μM Hcy almost completely disrupted the stress fiber structure. Our result added a new layer of mechanism about homocysteine-mediated regulation of angiogenesis through perturbation of actin cytoskeleton dynamics and maintenance.

DISCUSSION

Homocysteine is an intermediate metabolite connecting the methionine cycle to cysteine biosynthesis. It has been suggested to play a role in oxidative stress, endothelial dysfunction and acute inflammatory response. However, the function of homocysteine in angiogenesis is still under debate. In this study, we demonstrate the inhibitory effect of

homocysteine on VEGF induced endothelial cell proliferation, migration. High dose of homocysteine could significantly impair angiogenesis in vitro and in vivo, possibly through down-regulation of key angiogenic factors such as VEGFR and angiopoietin, and disruption of actin stress fiber formation which is crucial for cell motility and morphology. Our results lend support to the potential application of homocysteine as anti-angiogenesis agent in cancer therapy.

One big concern for homocysteine as anti-angiogenesis drug is that high level of homocysteine would pose threat to the cardiovascular system as it causes the damage of endothelial cells and promotes atherosclerosis and thrombosis. Normal concentration of homocysteine in plasma is around 10 μM and more than 90% of total plasma homocysteine exists as conjugate with plasma proteins (31). When the concentration is raised to more than 15 μM in plasma, this will cause a medical condition called hyperhomocysteinemia which is frequently associated with deficiency of B6, B12 vitamins (32). There are two types of hyperhomocysteinemia: (1) the rare but severe forms are due to major genetic mutations of the enzymes implicated in homocysteine metabolism; (2) the more common forms cause moderately elevated homocysteine levels related to a pathogenesis such as genetic and environmental factors. When the level of homocysteine is between 16-30 μmol/L, it is classified as moderate, 31-100 μmol/L is considered intermediate and a value above 100 μmol/L is classified as severe hyperhomocysteinemia (33, 34). The concentration we tested to be effective to inhibit angiogenesis fell into the range of intermediate hyperhomocysteinemia. In fact, large scale meta-analysis concludes that elevated homocysteine is just a modest independent risk factor for ischemic heart disease and stroke incidence (35). Therefore, the value of Hcy as angiogenesis inhibitor could be explored more thoroughly in the clinical setting of tumor therapy since the inhibitory effect of Hcy is relatively rapid and the advantage for cancer patient will over-compensate the moderate risk.

In our study we first tested the effect of homocysteine on basic characteristics of endothelial cell growth and re-confirmed its inhibitory function on cell proliferation and migration (Figure 1). We used HUVEC as endothelial cell model which has been widely accepted in the field since its report by Rhim (36). Our result was consistent with previous studies of homocysteine with different cell line HMEC1(13), ECV304(37) , and tumor cell line (38). Endothelial cells play important role in forming new vessels in tube-like structure and sprouting out from old vessels during angiogenesis. Therefore we validated the effect of Hcy on angiogenesis in vitro by tube formation assay or in vivo by CAM assay. Both of these results demonstrated the dramatic inhibition of angiogenesis (Figure 2 and 3), which is also well supported by multiple studies (12-14).

The mechanisms of homocysteine induced endothelial cell dysfunction and inhibition of angiogenesis have been mainly attributed to the disruption of anti-oxidant glutathione production which subsequently leads to ROS formation and oxidative stress in the endothelial cells as well as other types of target cells (39-42). Hyperhomocysteinemia increases oxidative stress and is closely related to accumulation of asymmetric dimethylarginine (ADMA) , an endogenous nitric oxide (NO) synthase (NOS) inhibitor that inhibits the activity of endothelial NOS (eNOS) and inducible NOS (iNOS) which play crucial role in cardiovascular regulation (43-45). However, many of these studies used homocysteine concentrations far beyond physiological range (1 to 10

mmol/L). Under such conditions, it may lead to the generation of reactive oxygen species in the absence of in vivo antioxidant defense systems (46-48). Indeed, there was some discrepancy regarding the role of homocysteine in the promotion of oxidative stress(49), although it was still favored by the mainstream researchers that ROS plays an important role in homocysteine-induced endothelial dysfunction. In our study, we chose to tackle this problem from another aspect since there have been a plethora of studies supporting the oxidative stress theory. In addition to the impaired endothelial cell proliferation by ROS stress, endothelial cells were exposed to complex regulatory network of cytokine and growth factor such as VEGF and its receptor mediated signaling events, angiopoietin and Tie2 receptor signaling, among many others. Interestingly, our result suggested that homocysteine had suppressive function on those signaling factors in a dose dependent manner (Figure 4). Our finding about homocysteine and VEGF signaling pathway was supported by other researchers (15, 50). The connection between homocysteine and angiopoietin was first time reported in the field by us, which shed more light on the mechanism of homocysteine-mediated angiogenesis regulation. Nonetheless, we had to point out that the effect of homocysteine on those angiogenic signaling factors was not so dramatic as what we observed on the phenotypic effect of homocysteine on VEGF-induced angiogenesis and cell proliferation (Figure 1-3). This difference suggested that the regulation on cytokine signaling, particularly those mentioned ones, was not the primary player in the homocysteine effect on angiogenesis which was supposed to be multi-faceted mechanisms.

We continued to explore some new mechanism shown in Figure 5 that homocysteine disrupted the VEGF induced actin stress fiber formation. It has been reported that VEGF can promote actin remodeling and cell migration through Rho and ROCK signaling. However, it was relatively lack of attention for the role of homocysteine in angiogenesis, so far only Sen et al briefly mentioned that homocysteine and cyclic stretch combined together to regulate endothelial focal adhesion protein redistribution and cell remodeling (51). Our study pointed out that cytoskeletal dysregulation could be the new dimension of mechanism regarding to homocysteine induced angiogenic suppression. The detailed signaling pathway about how elevated homocysteine suppresses VEGF-induced cytoskeletal remodeling will be investigated in future.

Conclusions

In summary, homocysteine was demonstrated as a potent anti-angiogenic factor in our study. It could inhibit VEGF-induced HUVEC cell growth and migration at dose responsive manner. Further we confirmed moderately higher concentration of homocysteine (70 μM) could suppress angiogenesis process *in vitro* and *in vivo*. The molecular mechanism of homocysteine mediated inhibition was partly accounted by its ability to down-regulate key angiogenic factors like VEGFR and angiopoietin, in addition to the well-established disruption of redox balance in cells. More importantly, we unveiled the unappreciated function of homocysteine to abolish VEGF-induced actin stress fiber formation and cytoskeletal remodeling in endothelial cells.

Authors' contributions

Lemen Pan, Guanfeng Yu, Jingyong Huang, Xiangtao Zheng performed the experiments, analyzed and interpreted the data. Yinghua Xu wrote the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors have declared that no conflicts of interest exist.

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Figure legends

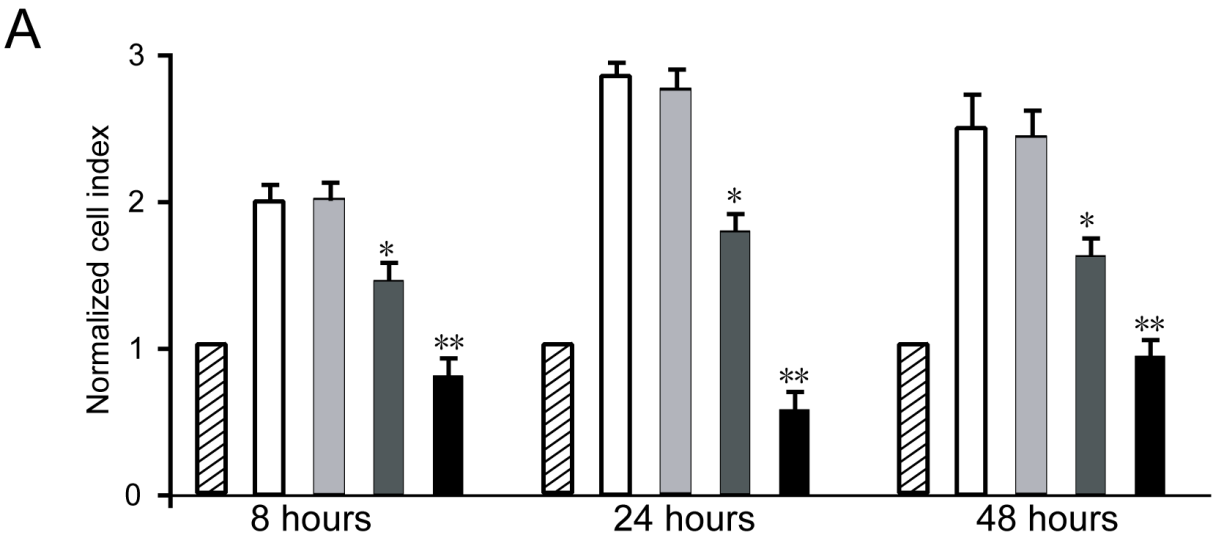
Figure 1. **Effects of homocysteine on HUVEC proliferation (A) and migration (B)**. Data are expressed as means ± SEM of three independent experiments. Migrated cells were observed using a modified Boyden chamber assay. The data are presented as percentages of inhibition. Statistical significance is expressed as **, $P<0.001$; *, $P<0.05$ versus VEGF control ($n=4$). Scale bar indicates 50 μm.

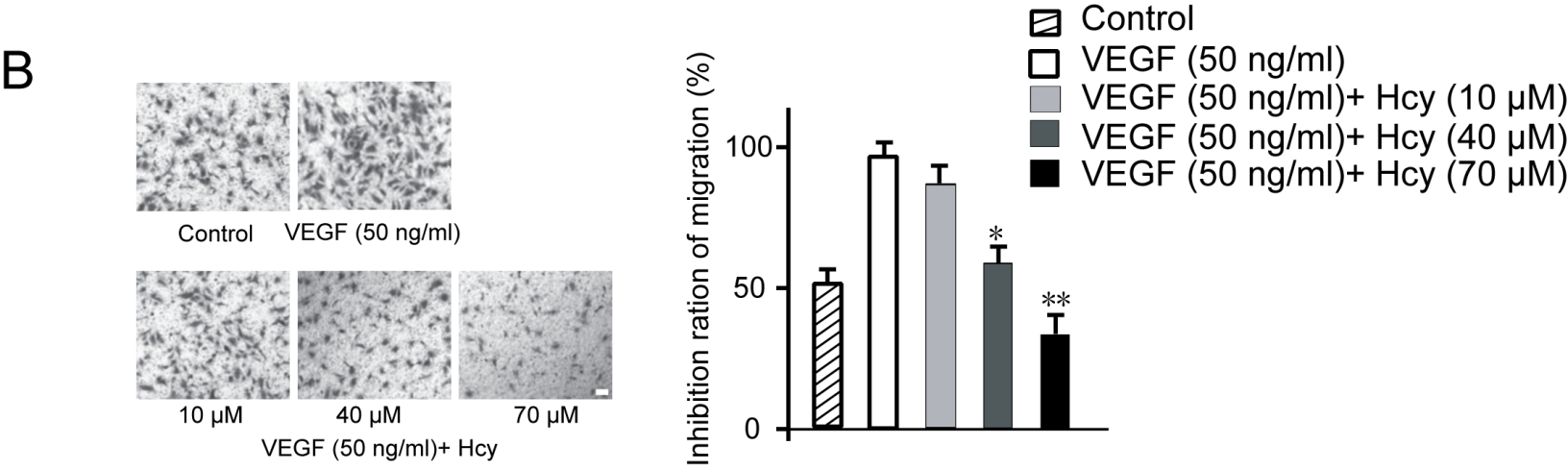
Figure 2. **Effects of homocysteine on HUVECs migratory ability and tube formation.** (A) Confluent monolayer of HUVECs was wounded and treated with either homocysteine (10, 40, 70 μM) or medium alone (untreated control) for 24 h following VEGF stimulation. The cells were then fixed and stained with Hoechst 33342 and Cellomics® whole cell stain green (B) Quantification of the number of migrated cells after 24 h exposure to indicated concentrations of homocysteine. For each monolayer sample, three measurements were taken in three independent wounds. Percentage of inhibition was expressed using untreated wells at 100%. (C) Effects of homocysteine on tube formation. (D) Quantitative data of tube formation after treated with homocysteine for 2 h following VEGF stimulation. Data are expressed as means ± SEM of three independent experiments. Statistical significance is expressed as **, $P<0.001$; *, $P<0.05$ versus VEGF. Scale bar indicates 50 μm (A)/20 μm (C).

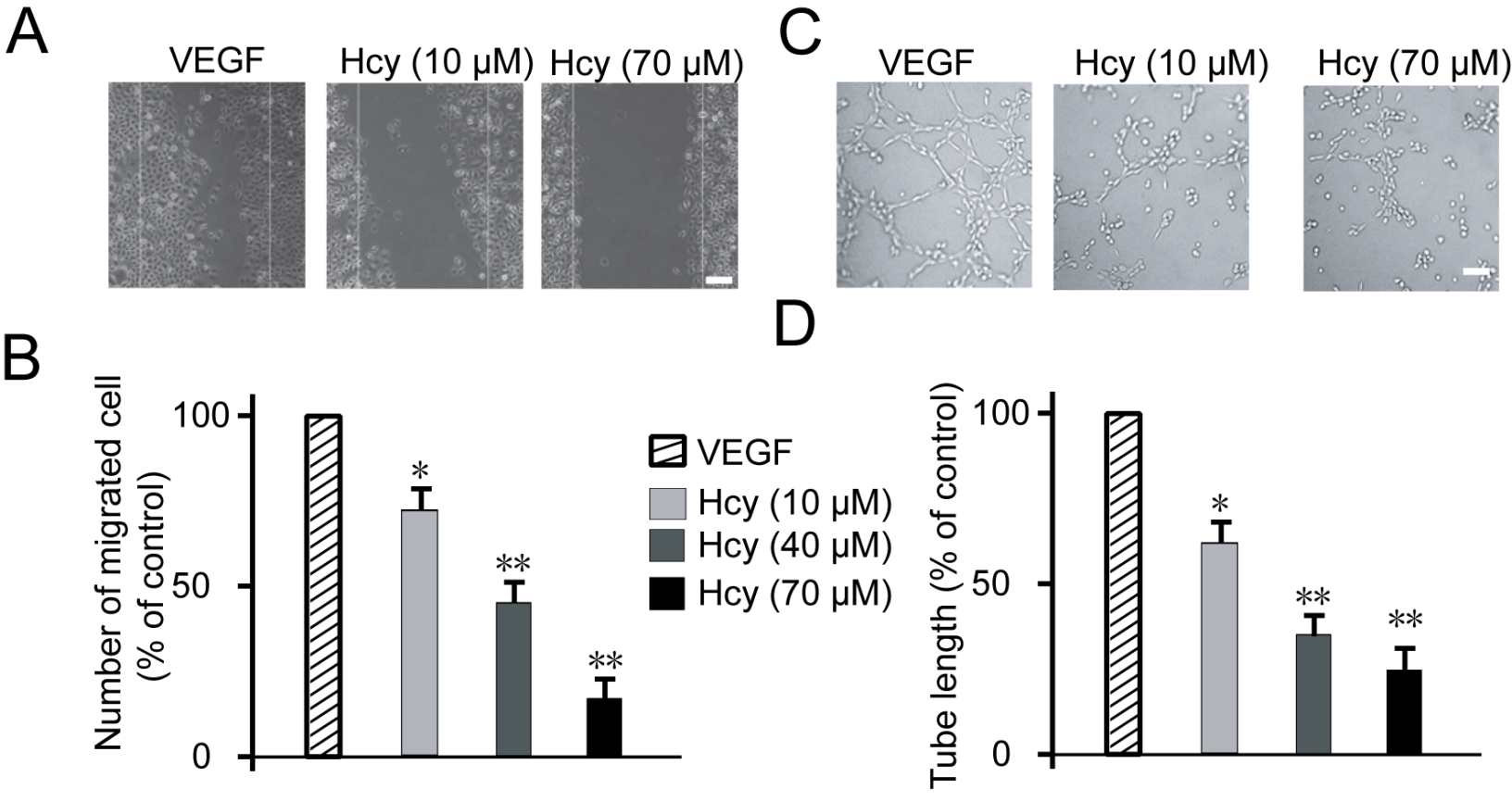
Figure 3. Homocysteine inhibited angiogenesis *in vivo*. Representative images of chick embryonic CAM after treated with homocysteine for 48 h (**A**). Quantitative data of chick embryonic CAM after treated with CA4 for 48 h (**B**). Pooled data from 12–15 eggs (mean ± SEM). Statistical significance is expressed as **, $P<0.001$; *, $P<0.05$ versus VEGF. Scale bar indicates 50 μm.

Figure 4. Homocysteine decreases VEGFR1/2 and angiopoietins (Ang1, Ang2) gene transcription. (A-B) RT-PCR analysis of VEGFR1 and VEGFR2 demonstrated that the treatment with different concentrations of homocysteine reduces VEGFR1/2 mRNA in HUVEC. Data are expressed as fold increase versus control cells treated with PBS only and are the mean ± SEM of three experiments. Homocysteine significantly decreased Ang1 (C) and Ang2 (D) gene expression in a dose-dependent way. The data are representative of three independent experiments performed in triplicate. Statistical significance is expressed as **, $P<0.001$; *, $P<0.05$ versus untreated control.

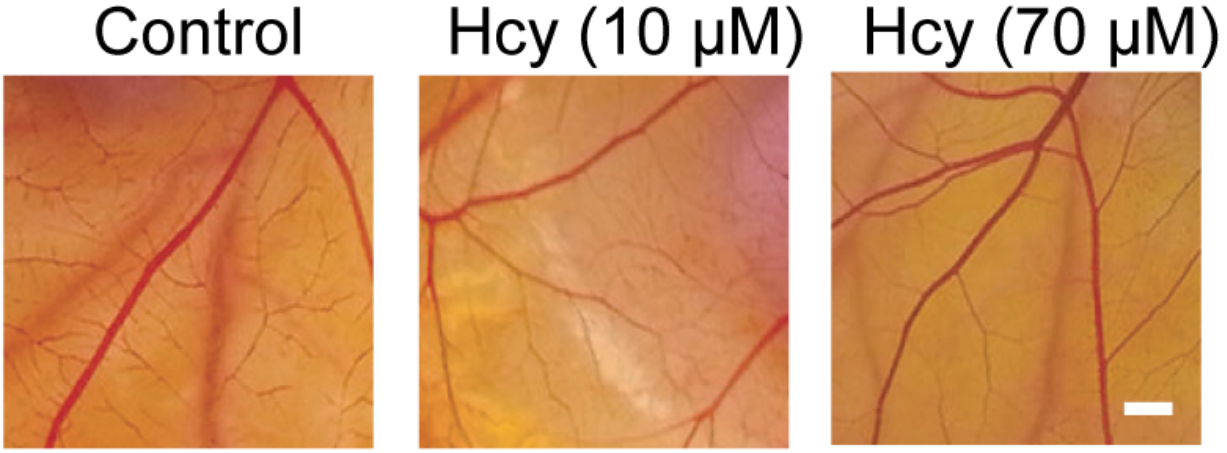
Figure 5. **Homocysteine inhibits VEGF induced actin-stress fibers in HUVECs.** (A) HUVECs were fixed and stained with phalloidin for F-actin (Red), and with Dil for membrane (Green), respectively. (B-D) HUVECs stimulated by 50 ng/ml VEGF were treated with different concentrations of homocysteine for 16 h. Scale bar indicates 20 μm.


A
Normalized cell index
3
2
1
0
*
**
8 hours
24 hours
48 hours

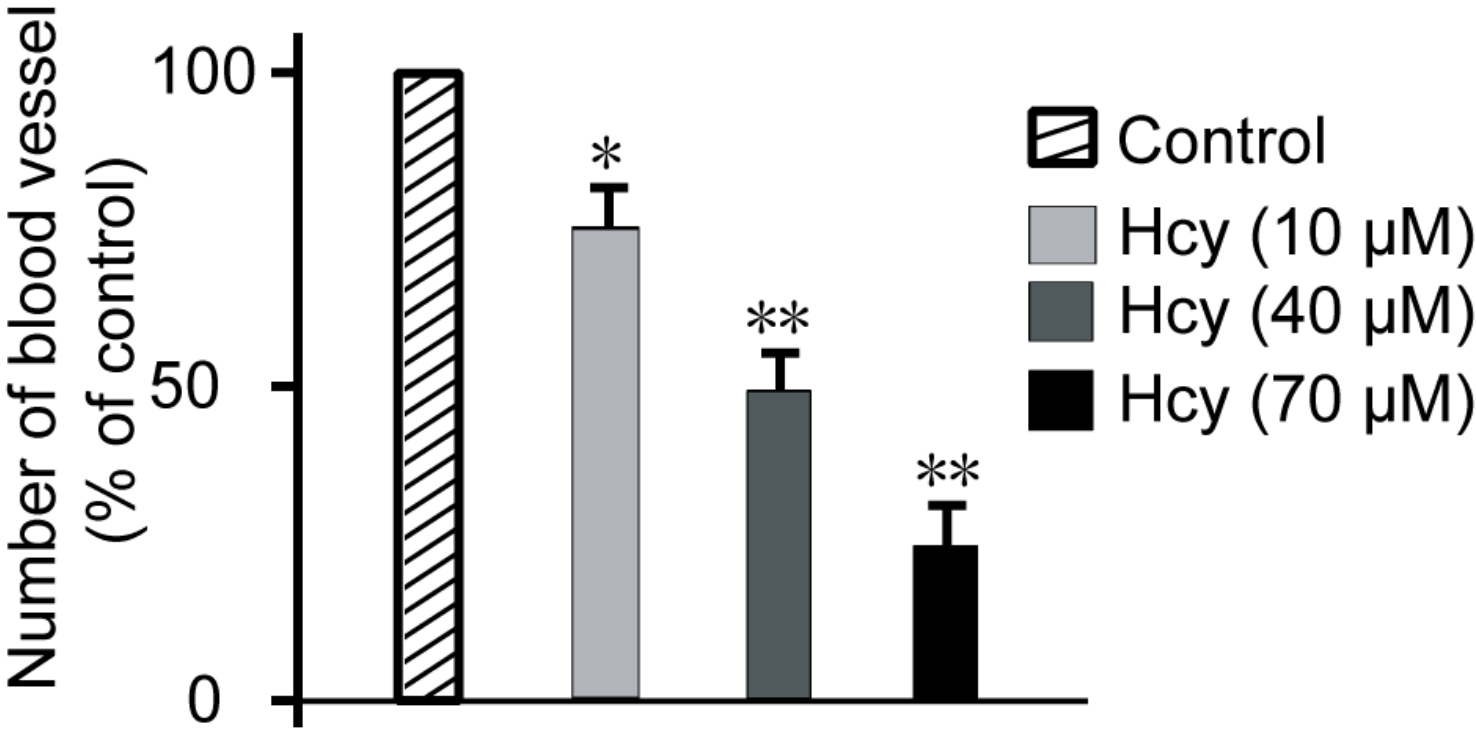

B
Control
VEGF (50 ng/ml)
10 μM
40 μM
70 μM
VEGF (50 ng/ml)+ Hcy
Control
VEGF (50 ng/ml)
VEGF (50 ng/ml)+ Hcy (10 μM)
VEGF (50 ng/ml)+ Hcy (40 μM)
VEGF (50 ng/ml)+ Hcy (70 μM)
Inhibition ration of migration (%)
100
50
0
*
**

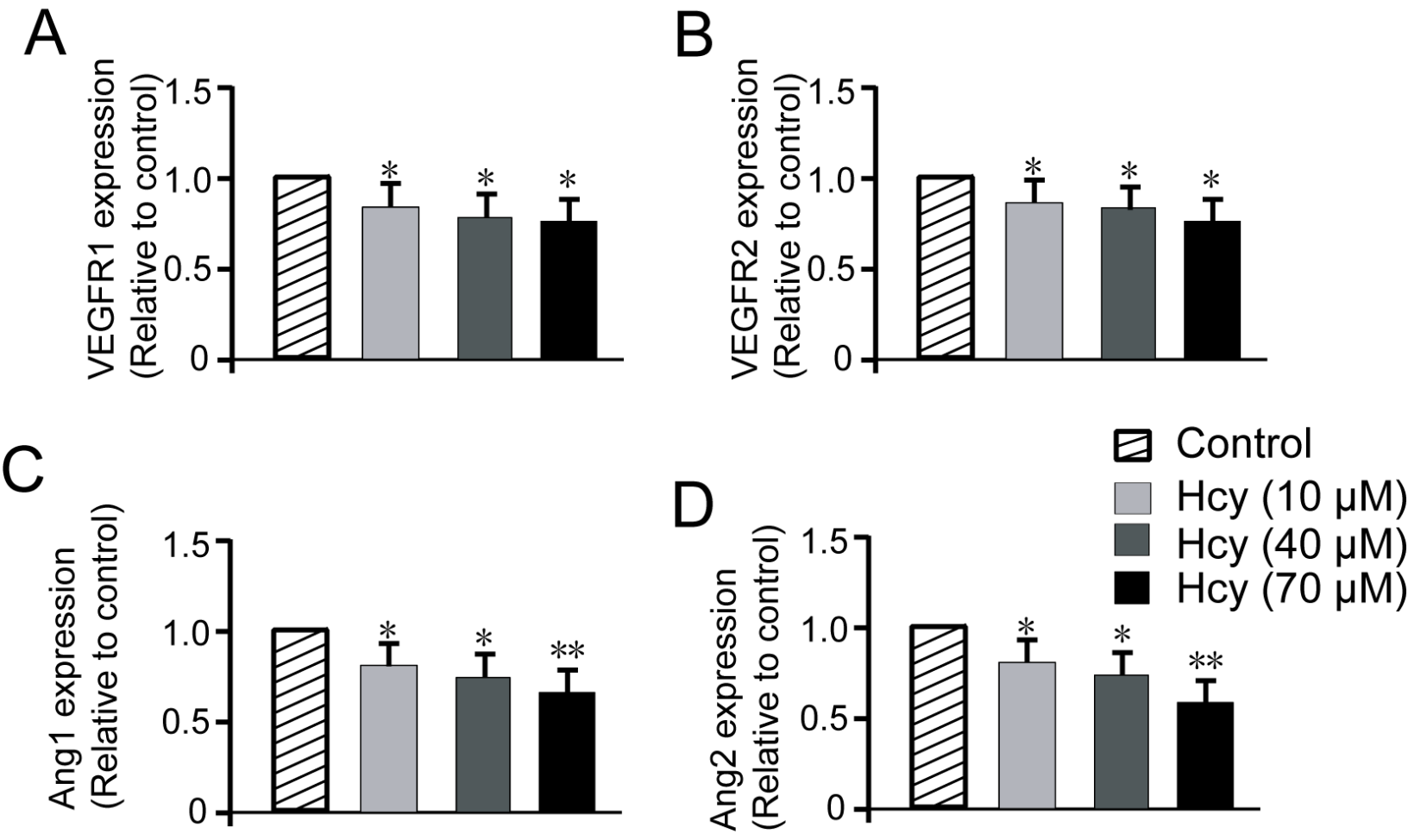

A
VEGF
Hcy (10 μM)
Hcy (70 μM)
C
VEGF
Hcy (10 μM)
Hcy (70 μM)
B
Number of migrated cell (% of control)
100
50
0
*
**
**
VEGF
Hcy (10 μM)
Hcy (40 μM)
Hcy (70 μM)
D
Tube length (% of control)
100
50
0
*
**
**

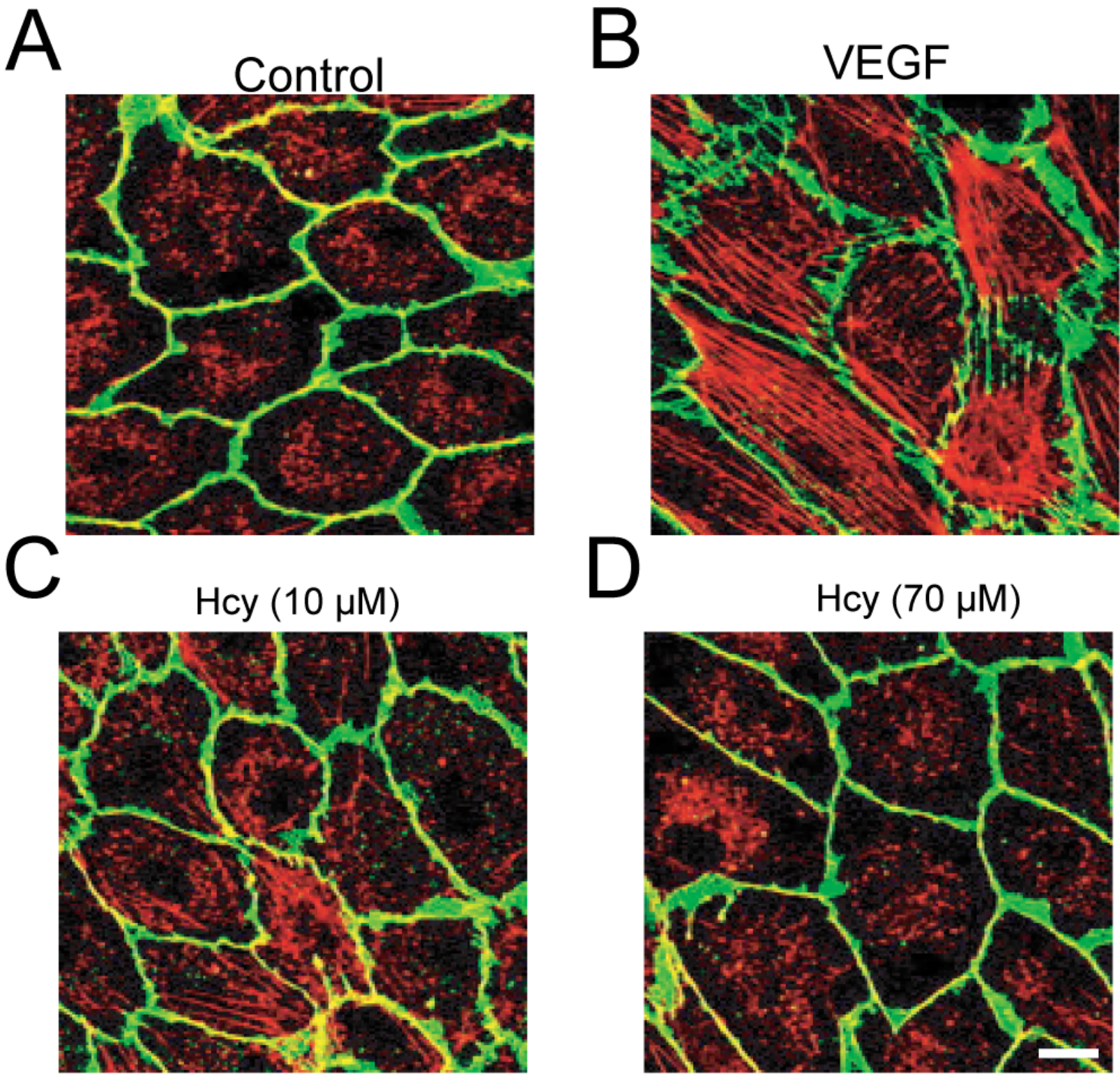
A


Control
Hcy (10 μM)
Hcy (70 μM)

B


Number of blood vessel
(% of control)
100
50
0
*
**
**
Control
Hcy (10 μM)
Hcy (40 μM)
Hcy (70 μM)


A
VEGFR1 expression
(Relative to control)
1.5
1.0
0.5
0
*
*
*
B
VEGFR2 expression
(Relative to control)
1.5
1.0
0.5
0
*
*
*
C
Ang1 expression
(Relative to control)
1.5
1.0
0.5
0
*
*
**
D
Ang2 expression
(Relative to control)
1.5
1.0
0.5
0
*
*
**
Control
Hcy (10 μM)
Hcy (40 μM)
Hcy (70 μM)


A
Control
B
VEGF
C
Hcy (10 μM)
D
Hcy (70 μM)