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α-Melanocyte-stimulating hormone inhibits angiogenesis through attenuation of VEGF/VEGFR2 signaling pathway

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ABSTRACT

Background: Gene therapy of proopiomelanocortin, the precursor of α-melanocyte-stimulating hormone (α-MSH), suppresses the neovascularization in tumors. However, the roles of α-MSH in angiogenesis remain unclear.
Methods: The influence of α-MSH on angiogenesis was evaluated by ex vivo rat aorta and in vivo, including transgenic zebrafish and chicken chorioallantoic membrane (CAM) assays. The effect of α-MSH on proliferation, matrix metalloproteinase (MMP) secretion, migration and tube formation was examined using human umbilical vein endothelial cells (HUVECs). The expression of vascular endothelial growth factor (VEGF) and VEGF receptor 2 (VEGFR2) was investigated by quantitative RT-PCR, immunoblot and immunofluorescent analysis. Antibodies' neutralization was employed to dissect the receptor(s) transmitting α-MSH signaling.
Results: Application of α-MSH potently suppressed the microvessels sprouting in organotypic aortic rings. Besides, α-MSH perturbed the vessels development in zebrafish and chicken embryos. α-MSH (0.01–10 nM) inhibited the MMP-2 secretion, migration and tube formation of HUVECs without affecting proliferation. Mechanistic studies unveiled α-MSH decreased the VEGF expression and release in HUVECs. Besides, α-MSH downregulated the VEGFR2 expression at transcriptional and translational levels. Importantly, α-MSH attenuated the Akt phosphorylation, but enhanced the expression of PTEN, endogenous antagonist of PI3K/Akt signaling. Expression analysis and antibody neutralization revealed that MC1-R and MC2-R participated in α-MSH-induced blockage of migration and VEGF/VEGFR2/Akt signaling. However, VEGF supply failed to reverse the anti-angiogenic function of α-MSH.
Conclusions: α-MSH inhibits the physiological angiogenesis by attenuating VEGF/VEGFR2/Akt signaling in endothelial cells.
General significance: α-MSH is a potent angiogenesis inhibitor targeting at endothelial VEGF/VEGFR2 signaling, which may have potential for therapeutic application.

Abbreviations: α-MSH, α-melanocyte-stimulating hormone; MC-Rs, melanocortin receptors; HUVECs, human umbilical vein endothelial cells; VEGF, vascular endothelial growth factor; VEGFR2, VEGF receptor 2

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1. Introduction

α-Melanocyte-stimulating hormone (α-MSH) is a tridecapeptide from the precursor proopiomelanocortin (POMC) and belongs to melanocortin family [1]. The amino-acid sequence of α-MSH is highly conserved across animal species, extending into invertebrates [2]. The production of α-MSH is widespread and mainly generated by the pituitary, skin tissue and immune cells [2]. The biological activities of α-MSH are mediated through the G-protein coupled melanocortin receptors (MC-Rs) which increase intracellular cAMP to exert different biological functions. α-MSH mainly binds to activates MC1-, MC3-, MC4- and MC5-R to mediate diverse function [3]. α-MSH is well-known for its role in the skin where α-MSH influences human melanocytes to stimulate melanogenesis formation by MC1-R and it can affect pigmentation [4]. In addition to pigment regulation, the anti-inflammatory and immunomodulatory properties of α-MSH have been paid attention in the last few years [1,3]. Given in pharmacological concentration, α-MSH is extremely effective in preclinical treatment of local and systemic inflammatory disorders including sepsis syndrome, acute respiratory distress syndrome, rheumatoid arthritis, inflammatory bowel disease, and encephalitis [2,5]. Recently, α-MSH has been proposed to be the important role in food intake, energy balance and protective role in hypoxia conditions acting upon MC4-R in the brain [6–8]. Despite the numerous studies available on the classical roles of α-MSH, it is still unclear whether α-MSH could modulate endothelial functions, especially angiogenesis.

Angiogenesis, the formation of new capillaries from the existing vasculature, is a key event in physiological processes (such as embryonic development and wound healing) and pathological states (such as tumor growth and metastasis) [9,10]. In recent years, angiogenesis blockage has become a therapeutic strategy for human diseases due to aberrant angiogenesis including cancer, inflammation, cardiac hypertrophy [9], and peripheral artery disease [10] and ischemic heart diseases [11]. Angiogenesis is a multiple cellular process consisting of endothelial cell proliferation, migration and morphological differentiation [12] and is closely regulated by growth factors and intracellular signaling pathways. One of the most specific and potent inducers associated with angiogenesis is vascular endothelial growth factor (VEGF), which modulates endothelial proliferation, permeability, and survival [13,14]. VEGF, generally referred to as VEGF-A, is an apparently endothelial cell-specific mitogen derived from arteries, veins, and lymphatics [15]. VEGF not only has the potent angiogenic ability to stimulate capillary formation in vivo but also has direct mitogenic actions on endothelial cells [15]. VEGF exerts its biological functions via activation of the protein tyrosine kinase receptors, VEGF receptor 1(VEGFR1) and VEGFR2 [16]. These receptors regulate physiological as well as pathological angiogenesis. VEGFR2 is predominantly located on the surfaces of endothelial cells and is thought to initiate intracellular signal transduction regulating endothelial cell proliferation, migration, and in vivo angiogenesis [16].

Our previous studies indicated systematic POMC gene delivery reduced tumor angiogenesis in melanoma models and also inhibited vascularization in rat model of osteoarthritis [17,18]. We further delineated that POMC-derived melanocortins such as α-MSH may participate in POMC-mediated melanoma suppression [19]. However, the influence of α-MSH on physiological angiogenesis and function of endothelial cells have never been explored. In this study, we evaluated the effects of α-MSH on endothelial cell functions in vitro and in vivo. Subsequently, we delineated the role of VEGF/VEGFR2 signaling in α-MSH-mediated angiogenesis regulation.

2. Materials and methods

2.1. Peptides and antibodies

α-MSH was purchased from Bachem Bioscience Inc. (King of Prussia, PA). VEGF was from Sigma Chemical (St. Louis, MO). All the drugs were dissolved in normal saline. Antibodies against MC-Rs (MC1-5R), VEGF, VEGFR2, Akt, pAkt (Ser473), PTEN and β-actin were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

2.2. Aortic ring assay

This ex vivo angiogenesis assay was performed as previously described [20]. Thoracic aortas were removed from Sprague–Dawley rats (male; 8-week-old) and immediately transferred to a culture dish containing ice-cold serum-free MCDB131 media (Life technologies Ltd., Paisley, Scotland). The peri-aortic fibroadipose tissue was removed with micro-dissecting forceps and carefully not to damage the aortic wall. Each aortic ring was sectioned and extensively rinsed in five subsequent washes of MCDB131 media. Ring-shaped explants of aorta were then embedded in the 1 ml mixtures of Matrigel and MCDB131 (1:1). Then, the aortic rings were polymerized and kept in triplicate at 37 °C in the 24 well culture plates. After polymerization, each well was added with 1 ml of MCDB131 (Life Technologies Ltd., Paisley, Scotland) supplemented with 25 mM $NaHCO_3$, 2.5% rat serum, 1% glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and PBS or α-MSH (10 nM) or/combined with VEGF (10 ng/ml) to the upper on Matrigel-based embedded aortic ring. The rings were kept at 37 °C in a humidified environment for 7 days and the vascular sprouting was examined by microscope equipped with digital images system (Olympus; Tokyo, Japan). The greatest distance from the aortic ring body to the end of the vascular sprouts (sprout length) was measured by NIH Image program at three distinct points per ring.

2.3. Zebrafish angiogenesis model

Transgenic *Tg*(*fli-1*:*EGFP*) zebrafish embryos, in which Enhanced Green Fluorescent Proteins (EGFP) is expressed in all endothelial cells of the vasculature, were used to monitor the effects of α-MSH on embryonic angiogenesis [21]. Zebrafish embryos were generated by natural pair-wise mating and raised at 28 °C in embryo water (0.2 g/l of Instant Ocean Salt in distilled water). Approximately 20 healthy embryos were placed in 6 cm dishes and various concentrations of α-MSH were separately added into embryo water at 6 h post fertilization (hpf). The embryo water containing α-MSH was replaced daily. At 72 hpf, the embryos were anesthetized using 0.05% 2-phenoxyethanol in embryo water. The embryos were further observed for blood vessel development, especially in the intersegmental vessels (ISV) and subintestinal vessel plexus (SIV), using a microscope with digital images system (Olympus; Tokyo, Japan).

2.4. Chorioallantoic membrane assay (CAM assay)

Based on the previously described protocol [22], fertilized chicken eggs were incubated in a MultiQuip Incubator at 37 °C and constant humidity. After 3 day of incubation, a square window (1 cm^2) was cut into the shell with small dissecting scissors to reveal the embryo and CAM vessels under aseptic conditions. The window was resealed with adhesive tape, and the eggs returned to the incubator for a further 5 days. At day 8 of incubation, 1-mm diameter gelatin sponges (Spongostan; Ethicon) soaked in either PBS, or α-MSH (10 nM) were implanted onto the CAM between branches of blood vessels. Samples were observed until 4 day after placement wherein they were photographed with a stereomicroscope equipped with a Camera System (Leica MZ FL111) to quantify the blood vessels surrounding the implants ($n = 6$).

2.5. Endothelial cells cultures

Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical veins and cultured in M199 medium (Life Technologies, Gaithersburg, MD) as previously described [23]. HUVECs were used for

all the experiments at passages 2 to 5. EA.hy926 (transformed human endothelial cells), ECV 304 and BMEC (human brain microvessel endothelial cells) were cultured as previously reported [18,24,25].

2.6. Cell proliferation assay

Cell viability was measured by a quantitative colorimetric assay with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay. HUVECs were cultured in a 24-well plate at a density of 4×10^4 cells/ml overnight. Cells were incubated in M199 medium containing 0.5 mg/ml of MTT for 2 h at 37 °C. The formazan in viable cells were dissolved with dimethylsulfoxide and determined by reading optical densities in micro-plate reader (DYNEX Technologies Inc., Chantilly, VA) at an absorption wavelength of 570 nm.

2.7. Gelatin zymography

The matrix metalloproteinase (MMP) secretion of endothelial cells treated by α-MSH were measured by gelatin zymography. Briefly, HUVECs at near 80% confluence were supplemented with serum-free media and treated with α-MSH (0.01–10 nM) for 24 h. Aliquots of conditioned media were subjected to separation with 10% SDS-PAGE containing 0.1% type-A gelatin (Sigma Chemical Co., St. Louis, MO). After electrophoresis, the gel was washed twice with 2.5% Triton X-100, incubated in buffer containing 40 mM Tris–HCl, pH 8.0; 10 mM $CaCl_2$; and 0.01% sodium azide at 37 °C for 18–24 h, stained with 0.25% Coomassie blue R-250 in 50% methanol and 10% acetic acid for 1 h, and was destained with 10% acetic acid and 20% methanol. The gelatinolytic regions by MMPs were visualized as white bands in a blue background and quantified by densitometer.

2.8. Scratch wound healing assay

The migration of endothelial cells was assessed using a scratch migration assay as described previously [26]. Briefly, a gap of approximately 1 mm was created in the adherent layer of confluent endothelial cells (in six-well plates) by using a sterile 0.1-ml pipette tip (Gilson, Inc., Middleton, WI). After treatment with PBS or α-MSH (0.01–10 nM), the closure extent of the cell-free gap was performed by microscope with digital images system (Olympus; Tokyo, Japan) at different time intervals and measured by NIH Image program.

2.9. Migration assay

The cell migration assay was performed as previously described [27]. HUVECs were seeded in triplicate in the upper compartment of the chamber (2.5×10^4 cells/50 μl per well) and supplemented with M199 serum free media. The lower compartment was filled with 30 μl of M199 media containing 10% FBS serum media. A polycarbonate filter (8-μm pore size Nucleopore; Costar, Cambridge, MA) which was coated with 0.1% gelatin to allow cell adhesion was separated the compartments. After incubation for 4 h in a humidified 5% CO_2 atmosphere chamber at 37 °C, cells on the upper side of the filter were removed to lower side. Migrated cells were fixed in absolute methanol and stained with 10% Giemsa solution (Merck, Germany). Finally, the fixed cells were photographed by microscope with digital images system (Olympus; Tokyo, Japan), and counted as mean ± S.D. per filter under five different high-power fields.

2.10. Tube formation assay

The tube formation assay was performed as previously described [27]. Matrigel (Becton Dickinson, Bedford, MA) was diluted with cold M199 serum-free media to 10 mg/ml. The diluted Matrigel solution was added to 96-well plates (70 μl per well) and allowed to form a gel at 37 °C for 1 h. Cell suspensions (3×10^4 cells/70 μl per well) in M199 media containing 10% FBS were plated on Matrigel-coated wells and incubated for 6–8 h at 37 °C in 5% CO_2. After incubation, the endothelial tubes were observed and photographed by microscope with digital images system (Olympus; Tokyo, Japan).

2.11. Immunofluorescence assay

In order to observe the expression of MC-R subtypes, VEGF and VEGFR2 on α-MSH-treated endothelial cells, immunofluorescence staining was performed as described previously [23]. After treatment with PBS or α-MSH (10 nM) for 24 h, the fixed HUVECs were permeabilized using buffer containing 0.1% normal goat serum and 0.1% Triton X-100 in PBS, and incubated with MC-Rs or VEGF or VEGFR2 antibody 1:100 dilution) at 4 °C overnight. The cells were then washed three times with PBS and incubated with the corresponding Alexa-488-conjugated (or Alexa-546-conjugated) secondary antibody (1:1000 dilution; Molecular Probes) for 1 h at room temperature. Finally, the cells were rinsed twice with PBS and incubated with DAPI for 5 min. After mounting in anti-Fade media (Invitrogen), the fluorescent images of cells were captured by a ZEISS LSM PASCAL multiphoton confocal microscope image system (Carl Zeiss, Germany).

2.12. Flow cytometry analysis

The surface VEGFR2 expression in HUVECs was determined by flow cytometry analysis. After treatment with PBS or α-MSH (10 nM) for 24 h, HUVECs were trypsinized and incubated with VEGFR2 antibody (1:200 dilution) in PBS at 4 °C for 2 h. After wash with PBS twice, cells were incubated with Alexa-488-conjugated secondary antibody (1:100 dilution; Molecular Probes) at 4 °C for 1 h. Finally, the cells were washed with PBS twice, and resuspended in PBS for analysis in a flow cytometer (BD Biosciences; San Jose, CA).

2.13. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

HUVECs were homogenized with TRIzol reagent (TEL-TEST, Inc., Friendswoods, TX, USA) to collect total RNA. Then, 5 μg of the total RNA was used for the reverse transcription with Superscriptase III (Invitrogen; Carlsbad, CA) using oligo-dT and random primers. The cDNA used for real-time PCR was performed in Lightcycler (Roche) using a SYBR green assay. PCR reaction was performed in SYBR Green PCR Master Mix (Roche) following protocols provided by the manufacturer. The primer sequences for VEGF: forward 5′-CCCTGATGAGATCG AGTACA-3′, reverse 5′-AGGAAGCTCATCTCTCCTAT-3′. The primer sequences for VEGFR2: forward 5′-TCATTATTCTAGTAGGCACGGCG-3′, reverse 5′- GACAAGTAGCCTGTCTTCAGTT-3′. Expression was normalized to β-actin: forward 5′-TCACCCACACTGTGCCCATCTACGA-3′, primer 5′- CAGCGGAACCGCTC ATTGCCAATGG-3′.

2.14. Western blot analysis

HUVEC lysates were prepared using RIPA lysis buffer (50 mM Tris–HCl pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM PMSF and protease inhibitors). An aliquot of proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and transferred onto the polyvinylidene difluoride membranes (PVDF) (Immobilon-P membrane; Millipore, Bedford, MA). After blocking for 30 min, the membrane was incubated with primary antibodies for 2 h at room temperature, and then conjugated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Vector Laboratories, Burlingame CA, USA) (1:5000 dilution) for 1 h. Immunoreactivity was detected by ECL plus luminal solution

(Amersham Biosciences, Piscataway, NJ, USA). The immunoband intensities were quantified by densitometric scanning. The primary antibodies used in this study were antibodies against MC1-5R, VEGF, VEGFR2, Akt, pAkt (Ser473), PTEN (1:1000 dilution) and β-actin (1:5000 dilution).

2.15. Enzyme-linked immunosorbent assay (ELISA)

To explore VEGF release from α-MSH-incubated HUVECs, we analyzed the VEGF concentrations of supernatant by VEGF ELISA kit (R&D Systems Inc.). After treatment with α-MSH (0.01–10 nM) for 24 h, the cultured media of HUVECs were collected following protocols provided by manufacturer.

2.16. Measurement of cAMP

After treatment with α-MSH (0.01–10 nM) for 24 h, HUVECs were washed and lysed with 0.01 N HCl. The cellular cAMP expression was measured by cAMP EIA kit (Cayman, Chemical Inc., USA) following protocols provided by the manufacturer. The amount of cAMP in the cell samples was standardized to the amount of protein in each sample as determined by the BCA protein assay kit.

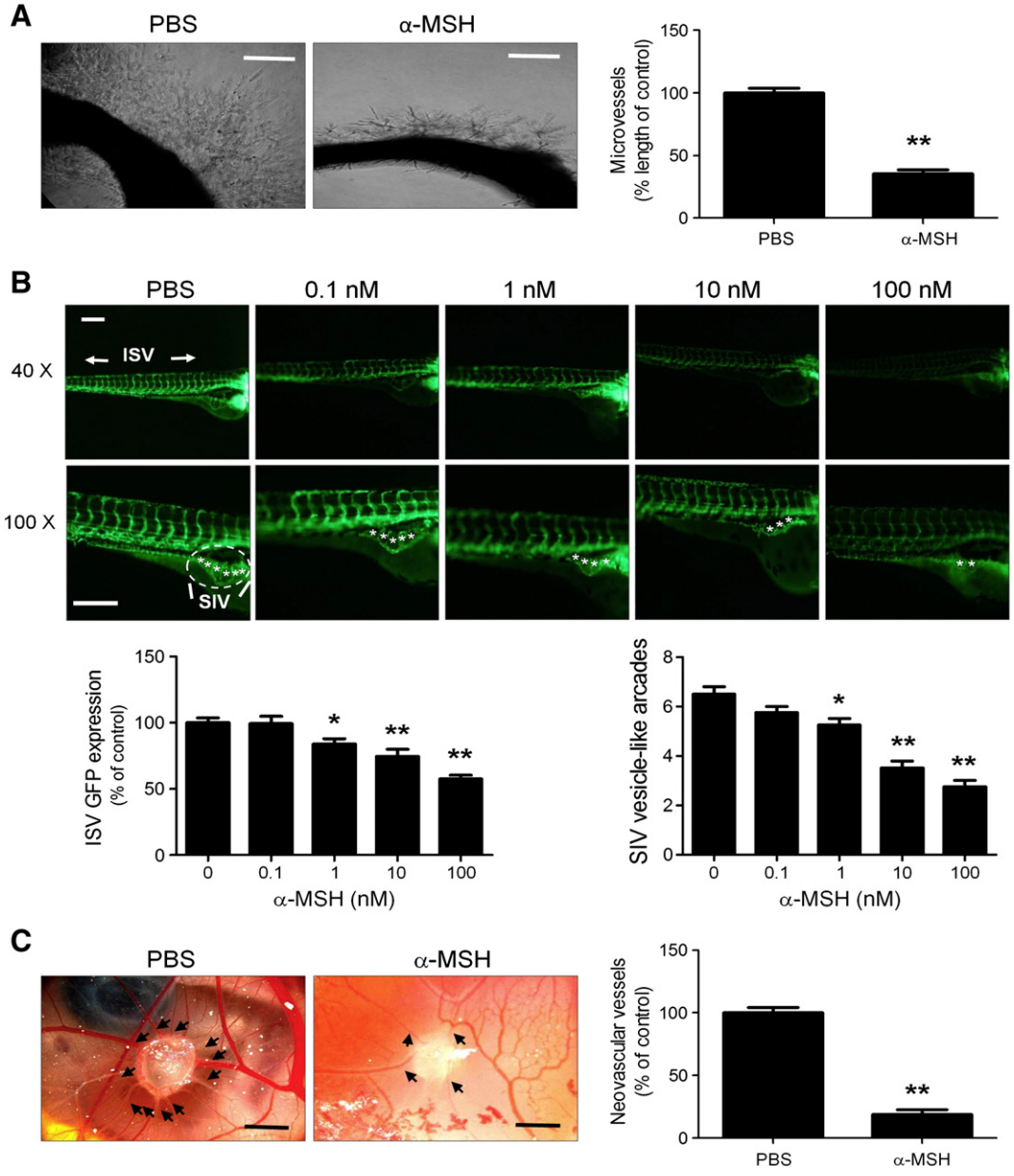

Fig. 1. Inhibition of angiogenesis by α-MSH ex vivo and in vivo. (A) Effect of α-MSH on the microvessel sprouting in aorta rings. Rat aortic rings placed in Matrigel were treated with PBS or α-MSH (10 nM) and vessel sprout from various aorta samples was observed on day 7. n = 6; Scale bar = 2 mm. (B) Effect of α-MSH on angiogenesis in transgenic *Tg(fli-1:EGFP)* zebrafish embryos. Embryos were treated with α-MSH (0.01–10 nM) at 6 hpf (n = 12 per group) then monitored for imaging recording at various time intervals. At 48 hpf, the representative photographs of intersegmental vessels (ISV) fluorescence in *Tg(fli-1:EGFP)* zebrafish treated with α-MSH (0.1–10 nM) were shown (40× magnification; Scale bars, 100 μm). The ISV fluorescence was quantified and expressed as mean ± SD percentages of control (n = 12; bottom left panel). At 72 hpf, the representative photographs of subintestinal vessel plexus (SIV) fluorescence in *Tg(fli-1:EGFP)* zebrafish treated with α-MSH (0.1–10 nM) were analyzed (100× magnification; Scale bars, 50 μm). White asterisks indicated arcades in the vesicle-like structure. The SIV arcades were quantified and expressed as mean ± SD (n = 12; bottom right panel). (C) Effect of α-MSH on angiogenesis in CAM assay. The representative pictures of PBS- or α-MSH-treated CAM were shown (left panel). Quantification analysis of the new blood vessel growth (black arrows) in a defined area was performed mean ± SD (right panel). Bars = 3 mm. n = 6 per group. *, $p < 0.05$ and **, $p < 0.01$.

2.17. Statistical analysis

All values were expressed as mean ± standard deviation (SD). A paired *t* test was used to assess the statistical differences between the groups. The differences were considered statistically significant when *p* was less than 0.05.

3. Results

3.1. α-MSH perturbs angiogenesis ex vivo and in vivo

We first employed the organotypic aortic rings to evaluate the function of α-MSH on angiogenesis in physiological conditions. It was found that application of α-MSH (10 nM) significantly perturbed the vessels outgrowth in organotypic aorta cultures by more than 60% of control (Fig. 1A). We then investigated the influence of α-MSH on vascular development in transgenic *Tg*(*fli-1:EGFP*) zebrafish. It was found that α-MSH supply significantly elicited a dose-dependent reduction in fluorescent intensities of intersegmental vessels (ISV) in zebrafish (Fig. 1B). Moreover, prolonged α-MSH exposure disrupted the formation of subintestinal vessel plexus (SIV) in zebrafish (Fig. 1B). By using CAM assay, it was observed α-MSH application potently suppressed the blood vessel formation in chicken embryos (Fig. 1C). Together, these results indicated that α-MSH inhibited the physiological angiogenesis in vivo.

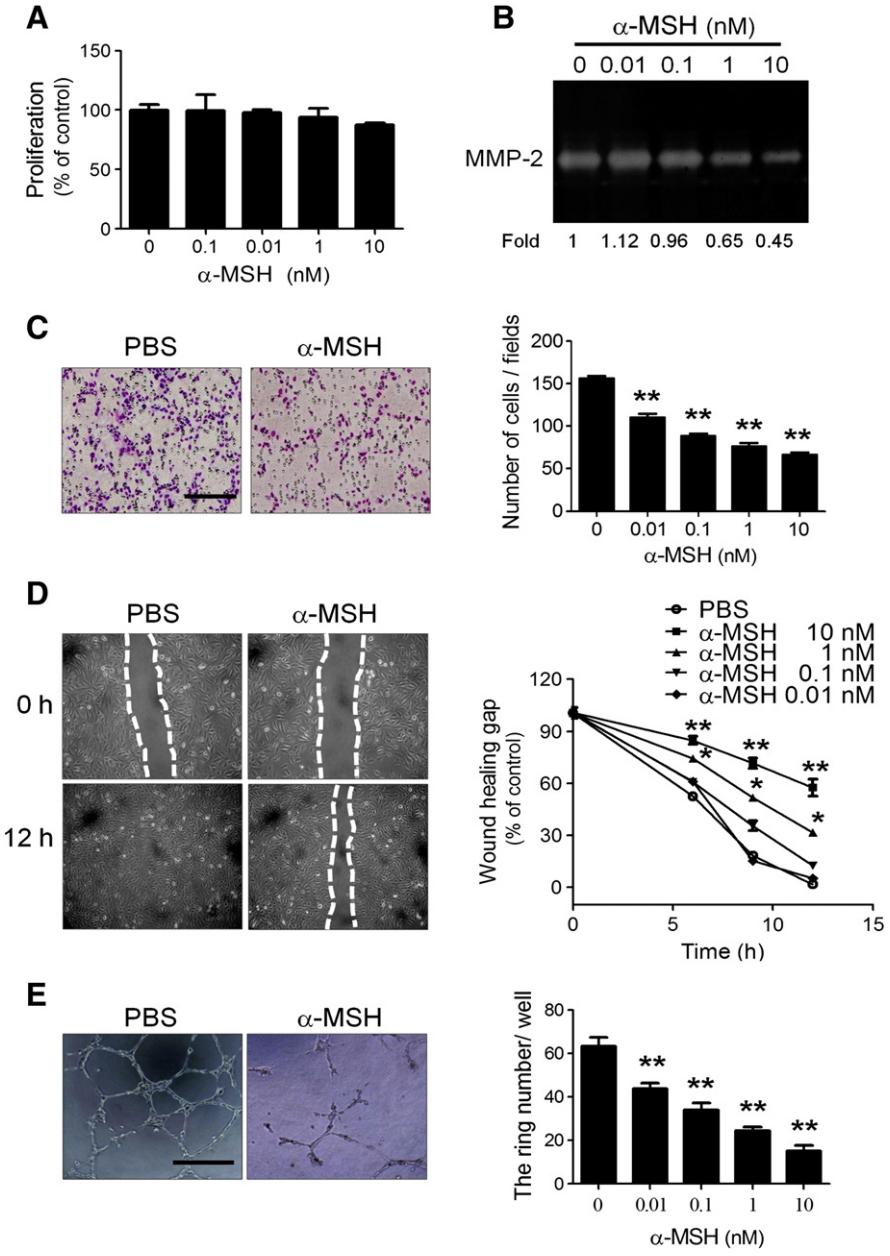

Fig. 2. Effects of α-MSH on angiogenic processes of endothelial cells. (A) Effect of α-MSH on proliferation. HUVECs were treated with various dosages of α-MSH (0.01–10 nM) in serum free medium for 24 h. The proliferation of HUVECs was measured with MTT assay and expressed as mean ± SD percentages of control in triplicate. (B) Effect of α-MSH on MMP secretion. After α-MSH (0.01–10 nM) incubation for 24 h, culture media of HUVECs were analyzed for MMP activities by gelatin zymography. Data were average fold over control from three independent experiments. After simultaneous incubation with α-MSH (0.01–10 nM), the effects of α-MSH on angiogenic process, including migration (C), scratch wounded assay (D) and tube formation (E) in vitro, were displayed in HUVEC. The representative photograph of HUVECs treated by α-MSH (10 nM) was shown (left panel). Quantification analysis was performed as mean ± SD in high-power fields from triplicate experiments (right panel). Scale bars = 100 μm. Asterisks indicated statistical significance vs. control groups (*$P < 0.05$ and **$P < 0.01$).

3.2. α-MSH suppresses multiple angiogenic processes in endothelial cells

We subsequently evaluated the effects of α-MSH on the distinct angiogenic steps, including proliferation, MMPs secretion, migration and tube formation, in cultured endothelial cells. By MTT assay, it was observed that α-MSH (in physiological concentrations; 0.01–10 nM) had no significant effect on proliferation of endothelial cells (Fig. 2A). Despite lack of effect on endothelial proliferation, α-MSH potently inhibited the MMP-2 secretion as revealed by gelatin zymography analysis (Fig. 2B). In Boyden chamber assay, α-MSH dose-dependently attenuated the migration of endothelial cells with a half maximal inhibitory concentration (IC_{50}) around 0.1–1 nM (Fig. 2C). Consistently, α-MSH significantly perturbed the healing of scratch wound in endothelial cells (Fig. 2D). Finally, α-MSH treatment dose-dependently abolished the formation of tube-like structure of HUVECs in Matrigel with an IC_{50} around 1 nM (Fig. 2E). Together, these results indicate that α-MSH suppresses MMPs secretion, migration and tube formation of endothelial cells without affecting the cell growth.

3.3. α-MSH reduces VEGF expression in endothelial cells at transcriptional level

Because VEGF plays a pivotal role in angiogenesis, we investigated the effect of α-MSH on VEGF expression in endothelial cells. By qRT-PCR analysis, it was shown that α-MSH dose-dependently decreased the VEGF mRNA level in HUVEC (Fig. 3A). Immunofluorescence analysis revealed that α-MSH (10 nM) treatment prominently depleted the VEGF expression in endothelial cells (Fig. 3B). Western blot analysis further confirmed showed that α-MSH inhibited the VEGF protein level in a dose-dependent manner (Fig. 3C). Moreover, α-MSH also significantly reduced the VEGF secretion in cultured media of HUVEC (Fig. 3D). Thus, α-MSH potently suppresses VEGF expression in endothelial cells.

3.4. α-MSH attenuated the VEGFR2 expression and Akt phosphorylation but elevates PTEN protein level in endothelial cells

VEGFR2 is the main receptor mediating the angiogenic function of VEGF in endothelial cells. To evaluate the influence of α-MSH on VEGFR2 expression, qRT-PCR analysis showed that α-MSH significantly decreased the VEGFR2 mRNA level in endothelial cells (Fig. 4A). Immunofluorescence and western blot analysis further revealed that α-MSH reduced the VEGFR2 expression in endothelial cells (Fig. 4B and C). Moreover, flow cytometry analysis confirmed that α-MSH (10 nM) treatment depleted the surface VEGFR2 expression in endothelial cells (16.9 ± 2.6% versus 41.7 ± 2.6% in control cells; $P < 0.01$; Fig. 4D).

Since Akt is a downstream effector of VEGFR2 signaling [28,29], we investigated the effect of α-MSH on Akt activities and found that α-MSH dose-dependently perturbed Akt phosphorylation in endothelial cells (Fig. 4E). Because PTEN/Akt signaling plays an important role in vascular remodeling, we examined PTEN expression in α-MSH-treated HUVEC by western blot analysis. Our study confirmed that α-MSH enhanced PTEN protein level in α-MSH-treated HUVEC in a

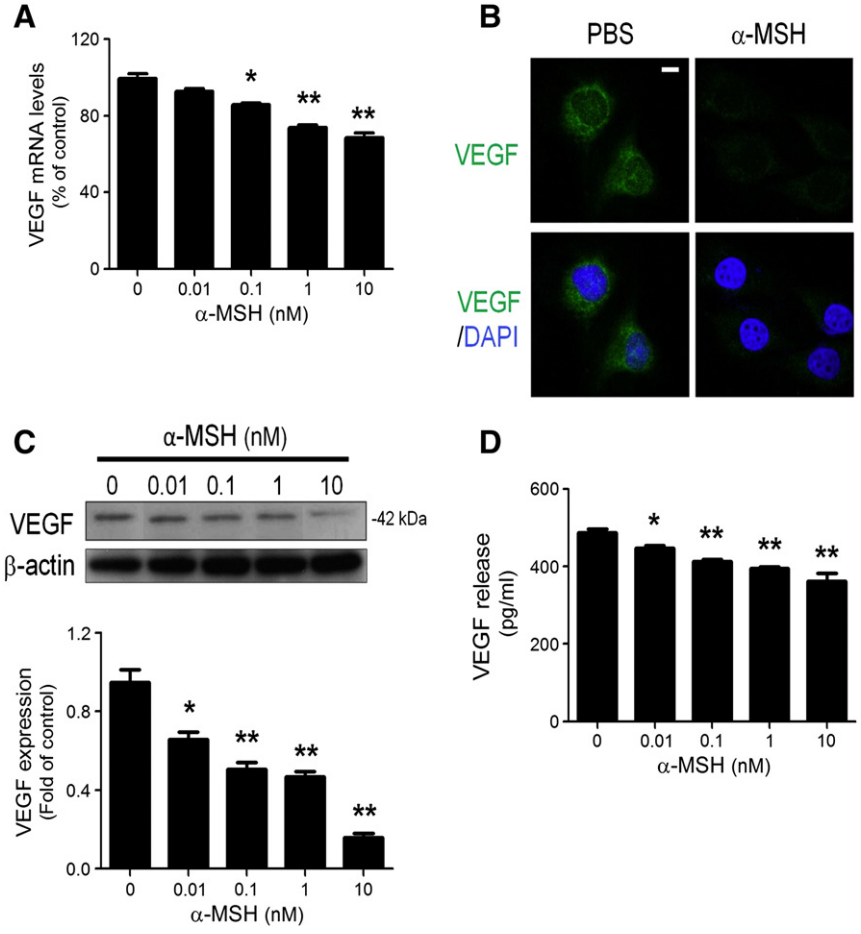

Fig. 3. Effect of α-MSH on VEGF expression in endothelial cells. HUVEC were treated with α-MSH (0.01–10 nM) for 24 h and separately subjected to VEGF mRNA and protein expression assay. (A) VEGF mRNA level was determined by quantitative RT-PCR analysis. VEGF protein expression was respectively shown by immunofluorescence (B) and western blot (C) analysis. The cell nuclei was stained with DAPI; bar = 20 μm; (D) Dose-dependent effect of α-MSH on VEGF secretion level. After treatment with α-MSH for 24 h, the condition media of HUVEC were collected to measuring the VEGF release by ELISA. Data were mean ± SD of triplicates. *: $P < 0.05$; **$P < 0.01$, versus control groups.

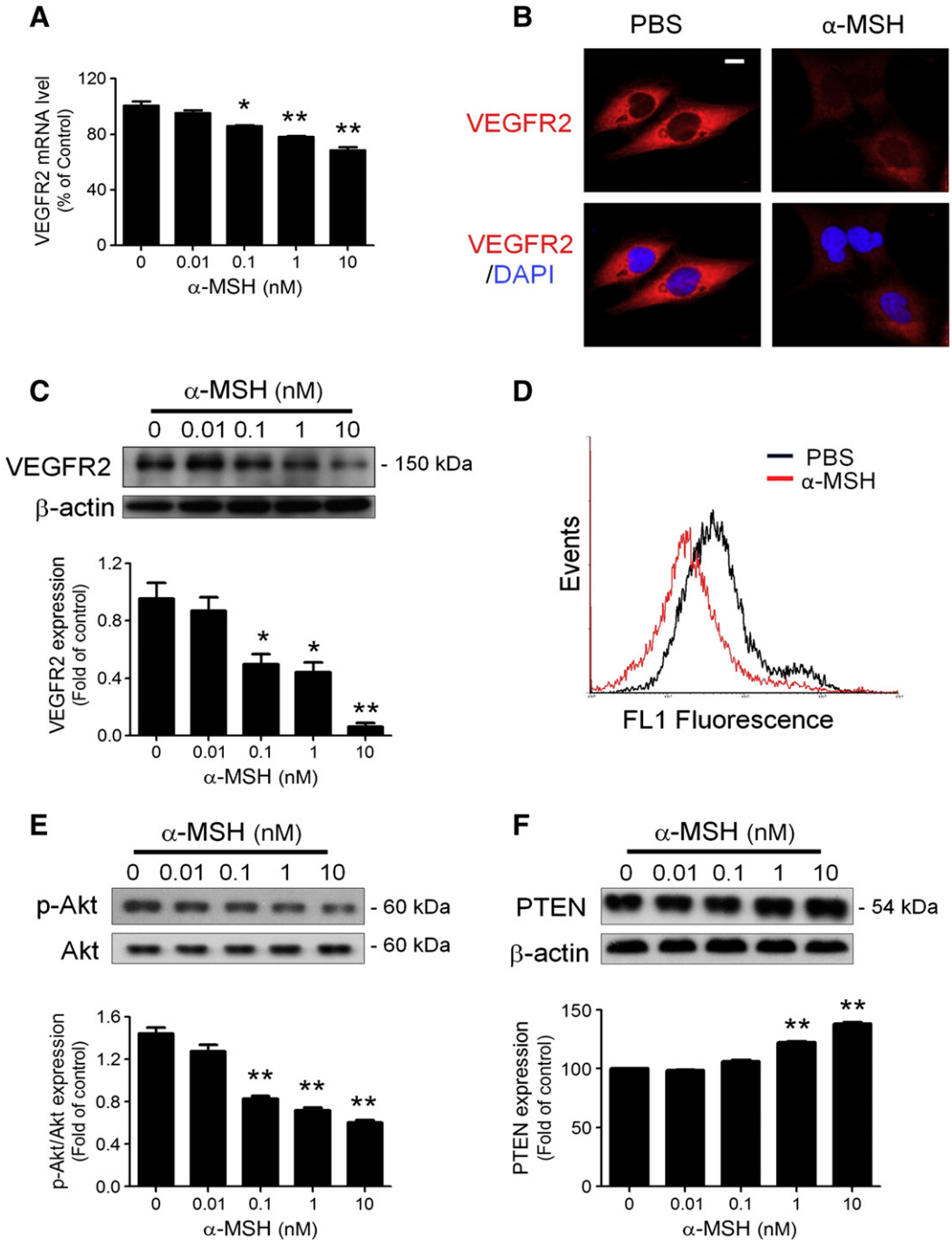

Fig. 4. Effect of α-MSH on VEGFR2 expression and Akt/PTEN signaling in HUVEC. (A) Effect of α-MSH on VEGFR2 mRNA expression. After treatment with α-MSH (0.01–10 nM) for 24 h in HUVECs, VEGFR2 mRNA levels were determined by quantitative RT-PCR analysis. Moreover, VEGFR2 protein levels were shown by immunofluorescence (B) and western blot (C) analysis, respectively. The cell nuclei were visualized using DAPI staining (blue). Scale bar = 20 μm. (D) Flow cytometry analysis of surface VEGFR2 expression after α-MSH treatment. After α-MSH (10 nM) treatment for 24 h, the cell surface VEGFR2 expression in endothelial cells was analyzed by FACScan. (E) Effect of α-MSH on Akt activation by western blot analysis. Quantification indicated mean fold change compared with the control (n = 3). (F) Effect of α-MSH on PTEN expression by western blot analysis. Quantification indicated mean fold change compared with the control (n = 3). All data were mean ± SD of triplicates. *: $P < 0.05$; **$P < 0.01$, versus control groups.

dose-dependent manner (Fig. 4F). These data demonstrate that α-MSH induces VEGFR2 down-regulation and a unique profile of Akt inactivation and PTEN upregulation in α-MSH-treated HUVEC.

3.5. MC1-R and MC2-R are involved in α-MSH-induced angiogenesis inhibition in endothelial cells

α-MSH exerts its biological functions through the activation of MC-Rs. We first analyzed the expression of MC-R subtypes in endothelial cells by semi-quantitative RT-PCR and detected the transcripts of MC1-R, MC2-R, MC4-R and MC5-R in endothelial cells (data not shown). Immunofluorescent analysis revealed the presence of MC1-R, MC2-R, MC4-R and MC5-R, but not MC3-R, in HUVEC (Fig. 5A). Moreover, western blot analysis of a panel of human endothelial cells (including EA.hy926, ECV304 and BMEC) showed similar results (Fig. 5B). Because cAMP mediates the MC-R signaling, we determined the effect of α-MSH on cAMP concentration in HUVEC and found α-MSH dose-dependently stimulated the cAMP level in HUVEC (Fig. 5C). Thus, MC-R/cAMP signaling pathway was involved in α-MSH-mediated effects in endothelial cells.

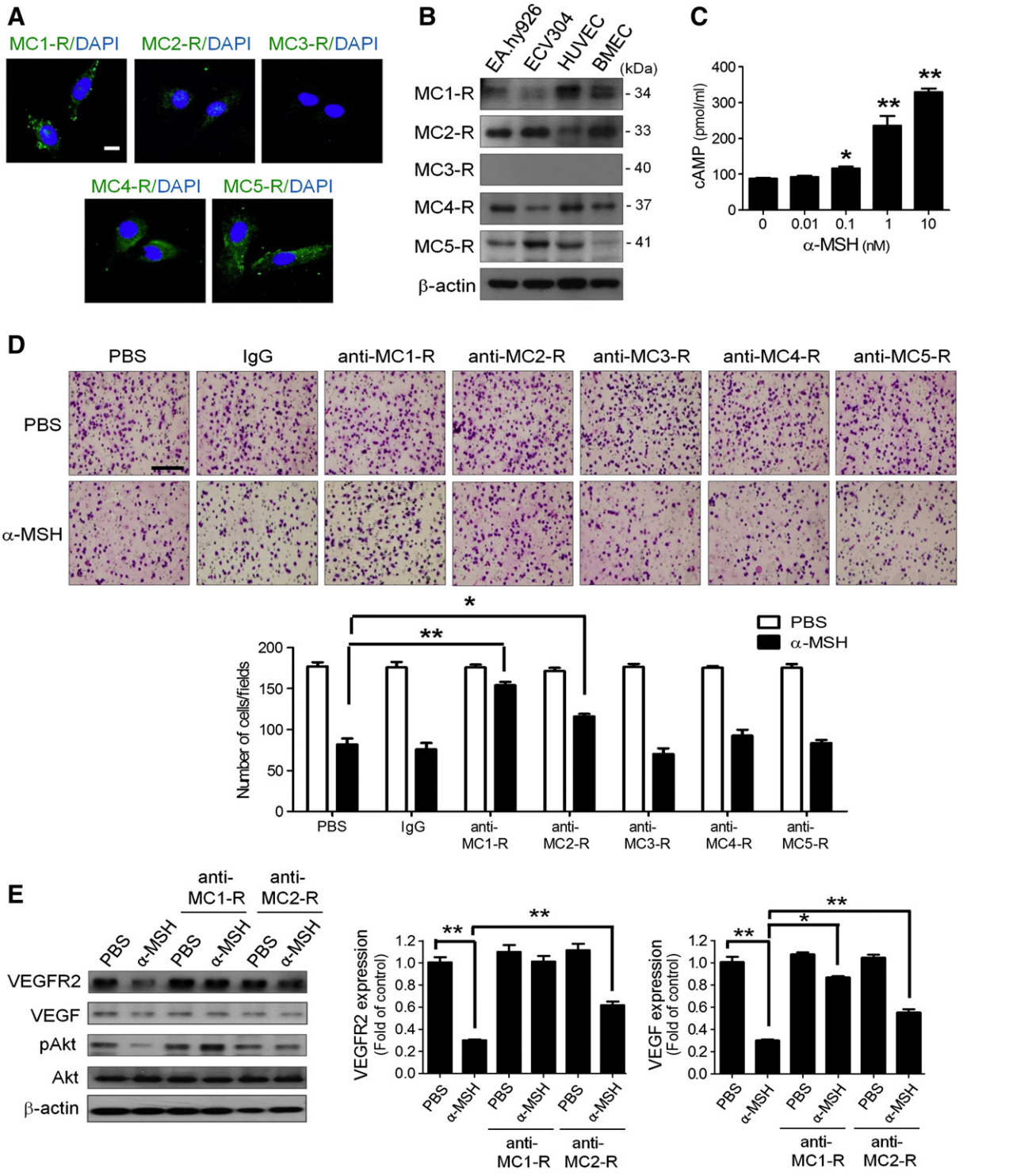

Fig. 5. The profiles of melanocortin receptors expression and signaling of α-MSH-regulated angiogenesis inhibition in endothelial cells. The protein expression of MC-Rs in cultured endothelial cells was shown in immunofluorescence (A) and western blot (B) analysis, respectively. The cell nuclei were visualized using DAPI staining (blue). Scale bar = 10 μm. (C) Enzyme-linked immunosorbent assay of cAMP levels in α-MSH-incubated HUVECs. After α-MSH (0.01–10 nM) treatment for 24 h, cAMP expression in HUVECs was analyzed by cAMP EIA kit. All data were expressed as the mean ± SD of triplicates. (D) Effect of MC-Rs neutralization on α-MSH-inhibited migration. After treatment with α-MSH (10 nM) or/combined with MC-Rs antibody (10 μg/ml), the migratory abilities were assessed by Boyden chamber assay; Data were mean ± SD in high-power fields from triplicate experiments. Scale bars = 100 μm. (D) Effect of MC-1R and MC2-R neutralization on α-MSH-induced inhibition of VEGF/VEGFR2/Akt signaling pathway. After pretreatment with MC1-R or MC2-R antibody for 30 min, the Akt, pAkt, VEGF and VEGFR2 protein expression were examined by western blot analysis. Quantification indicated mean fold change compared with the control (n = 3). *: $P < 0.05$; **$P < 0.01$, versus control groups.

To delineate which MC-Rs were contributed to the anti-angiogenic signaling of α-MSH, we utilized various MC-Rs' antibodies to investigate the effect of antibody neutralization on α-MSH-induced inhibition of endothelial migration. It was found that pretreatment with anti-MC1-R and anti-MC2-R antibody significantly reversed the α-MSH-induced inhibition of endothelial migration by about 90% and 60% of control, respectively ($P < 0.05$; Fig. 5D). In contrast, application of anti-MC4-R or ani-MC5-R had no such effect. Furthermore, western

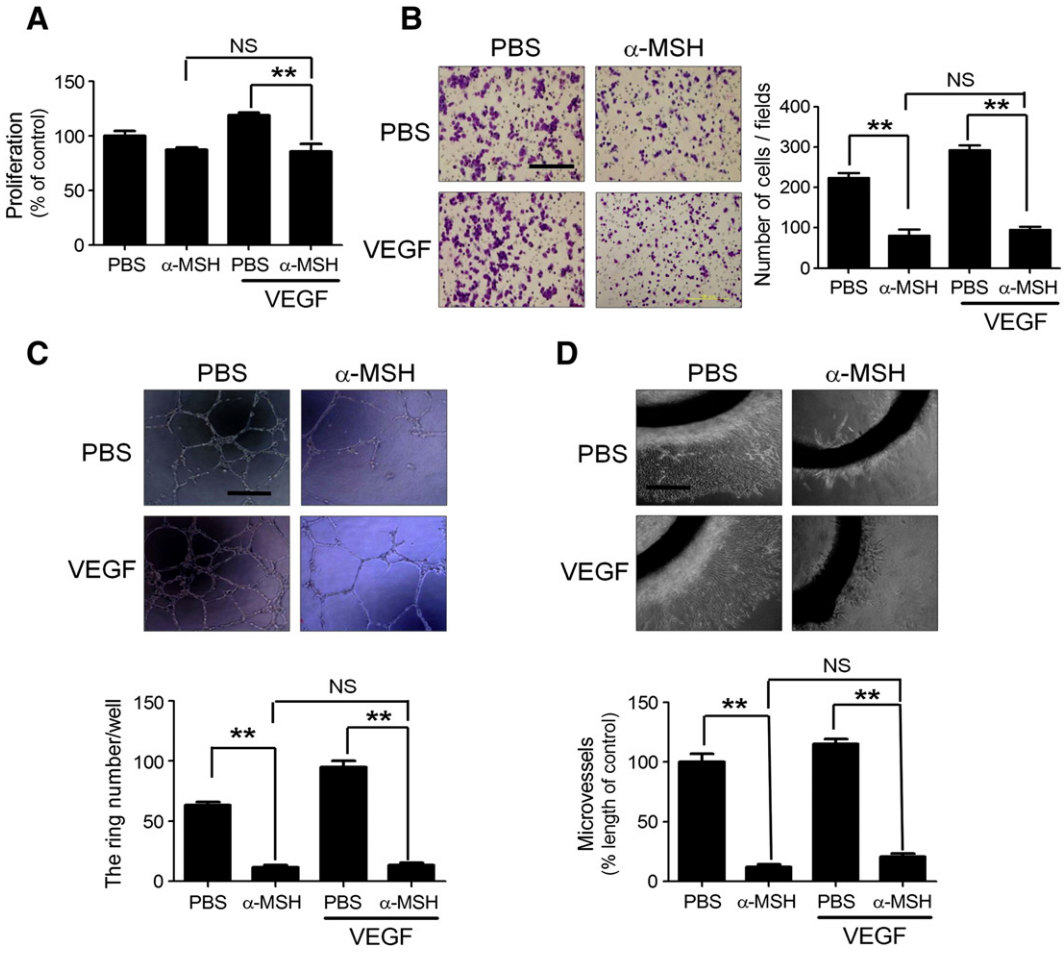

Fig. 6. Effects of VEGF supply on α-MSH-induced angiogenesis in vitro and ex vivo. After simultaneous incubation with PBS or α-MSH (10 nM) or/combined with VEGF (10 ng/ml), the effects of α-MSH on VEGF-induced proliferation (A), migration (B), tube formation (C) in vitro were observed in HUVEC. (D) The effects of α-MSH (10 nM) on the VEGF-induced microvessel sprouting in aorta rings assay ex vivo. Data were mean ± SD of quadruplicate experiments. (B),(C) Scale bars = 100 μm; (D) Scale bars = 2 mm. Asterisks indicate statistical significance vs. control group (*P < 0.05 and **P < 0.01). NS indicates no statistical significance.

blot analysis revealed that MC1-R and MC2-R neutralization significantly attenuated the α-MSH-induced VEGF/VEGFR2 down-regulation and Akt de-phosphorylation in endothelial cells (Fig. 5E). Interestingly, blocking MC1-R was more efficient in counteracting the function of α-MSH than MC2-R blockade. These results suggest that both MC1-R and MC2-R participate in α-MSH-induced angiogenesis inhibition.

3.6. Excessive VEGF supply fails to restore the α-MSH-induced angiogenesis inhibition

Because α-MSH treatment depleted the cellular VEGF level in endothelial cells, we elucidated the influence of exogenous VEGF supply on angiogenic processes in α-MSH-treated endothelial cells and rat aorta rings. It was found that, despite of its potent efficacy in stimulating the angiogenic processes in vitro, VEGF supply exerted marginal effect in alleviating the α-MSH-induced inhibition of proliferation (Fig. 6A), migration (Fig. 6B) and tube formation (Fig. 6C) in endothelial cells. Likewise, VEGF supply had no effect on α-MSH-induced inhibition of vessel sprouting in aorta rings (Fig. 6D). Thus, α-MSH potently suppresses the angiogenesis even in the presence of excessive VEGF.

4. Discussion

It has been recently established that gene delivery of POMC, the precursor of α-MSH, suppressed the primary and metastatic melanoma and Lewis lung carcinoma through blockade of tumor angiogenesis [19,30,31]. Besides, prophylactic POMC gene transfer attenuated the plasma leakage in lung during capsaicin-induced neurogenic inflammation [32], and significantly suppressed angiogenesis in a rat model of osteoarthritis [17]. The anti-neoplastic function of POMC therapy for multiple cancers seems to arise from blockade of tumor vasculature by POMC-derived melanocortins, particularly α-MSH. The present study first demonstrated that α-MSH at physiological concentration (0.01–10 nM) potently suppressed the vessels sprouting in organotypic aorta rings. Moreover, application of α-MSH dose-dependently disrupted the vessels development in zebrafish. It may seem paradoxical how a human hormone exerts function in fish at such a low concentration. However, previous studies showed that α-MSH at the concentration around nM was sufficient to stimulate melanosome translocation and pigmentation in zebrafish [33,34], suggesting the dose range (0.01–10 nM) of α-MSH used in our study was adequate and functional. Besides, given that melanocortin system is highly evolutionarily conserved in vertebrates [35], human α-MSH may likely exert its anti-angiogenic signaling through zebrafish MC-Rs. Together with previous studies, the present study indicates that α-MSH is an important endogenous regulator for either the physiological or pathological angiogenesis in vertebrates.

Our study brings important insights on how α-MSH regulates the angiogenesis at distinct angiogenic steps in vitro. Angiogenesis can be divided at least into the following steps: MMP secretion, breakdown of endothelial barrier, endothelial migration, endothelial proliferation, and interaction with extracellular matrix/mural cells [12]. Unlike many anti-angiogenic agents, α-MSH was not cytotoxic and exerted little influence on proliferation of stimulated endothelial cells at

physiological concentrations. Although lacking of cytotoxicity, α-MSH potently repressed various angiogenic processes, including MMP-2 release, migration and tube formation, with an IC_{50} in the range of 0.1–1 nM. Thus, the high anti-angiogenic efficacy of α-MSH plays a pivotal role in the anti-tumor and anti-metastatic function of POMC therapy.

To our knowledge, the present study provided the first evidence for the co-expression of MC1-R, MC2-R, MC4-R and MC5-R in human endothelial cells since previous studies revealed only MC1-R in human endothelial cells [36,37]. Moreover, we further delineated the involvement of MC1-R and MC2-R, but not MC4-R and MC5-R, in the anti-angiogenic mechanism of α-MSH in endothelial cells. Given that α-MSH preferentially binds to MC1-R over MC4-R or MC5-R [38], it seemed reasonable to exclude MC4-R and MC5-R from α-MSH-mediating signaling in endothelial cells. Nevertheless, the role of MC2-R in transmitting α-MSH signaling is intriguing because MC2-R binds only ACTH and has no affinity for the other melanocortin peptides [39]. To address the role of MC2-R, pilot study using MC2-R antibody failed to augment the efficacy of MC1-R antibody in counteracting α-MSH. Therefore, one likely mechanism is that MC2-R is not directly involved in, yet required for fully activation of MC1-R signaling by α-MSH. Further studies are warranted to elucidate the detailed mechanism.

The signaling mechanism underlying α-MSH-mediated angiogenesis inhibition remains unclear. It has been known that melanocortins exert their effects by via coupling to heterotrimeric G proteins, and further result in adenylase cyclase-dependent cAMP synthesis, protein kinase A (PKA) activation. α-MSH has elucidated to act as a potent inhibitor of LPS- or TNF-α-activated MC1-R-mediated NF-κB activation by maintaining the cytosolic IκBα in HDMECs [40]. This may imply that cAMP/PKA-dependent signaling transduction participate in the NF-κB regulation of α-MSH in endothelial cells. In the present study, we found α-MSH significantly reduced Akt activation to affect VEGF/VEGFR2 expression in a dose-dependent manner. Recently, the regulation of NF-κB activation through PKA/Akt-dependent pathway has been explored in HUVEC [41]. Therefore, we speculate the insights that α-MSH inhibits VEGF/VEGFR2 gene expression by PKA/Akt/NF-κB through MC-Rs' activations.

The present study unveils that α-MSH inhibits angiogenesis by downregulating VEGF/VEGFR2 expression in endothelial cells. However, exogenous VEGF supply failed to reverse the anti-angiogenic effects of α-MSH in HUVEC. It may implicate the involvement of other mechanisms contributing to the α-MSH-mediated anti-angiogenesis in HUVEC. It has been known that α-MSH reduces the LPS- or TNF-α-induced adhesion molecule expression, in human microvascular endothelial cells [40,42]. And the adhesion molecules may regulate the spreading, migration, and cell–cell and cell–matrix adhesion, which belong to the angiogenesis processes. Therefore, inhibition of cell adhesion may be one possible mechanism of α-MSH-mediated anti-angiogenesis in endothelial cells. Additionally, altered endothelin-1 (ET-1) homeostasis may be also involved in the anti-angiogenic function of α-MSH in endothelial cells. ET-1 is the most potent angiogenic factor, and a reduction in ET-1 secretion might directly contribute to the POMC-mediated angiogenesis inhibition in endothelial cells [18]. Thus, we speculate that altered ET-1 homeostasis may be other possibility mechanisms. Another possibility is the nitric oxide (NO) homeostasis. Endothelium-derived NO is required for the pro-angiogenic activity of growth factors such as VEGF. Autocrine NO production following activation of eNOS in endothelial cells promotes cell migration and organization in tubes, a phenomenon participating in the first steps of angiogenesis. Future studies are warranted to elucidate the mechanism underlying α-MSH-induced angiogenesis inhibition.

In conclusion, the present study has demonstrated that α-MSH inhibits angiogenesis in vitro and in vivo by attenuating VEGF/VEGFR2/Akt signaling through MC1-R and MC2-R activation in endothelial cells. Our findings support the novel function of α-MSH as an endogenous angiogenesis inhibitor, which could be of therapeutic potential for pathological conditions due to VEGF/VEGFR2 activation.

Acknowledgements

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References

[1] T. Brzoska, T.A. Luger, C. Maaser, C. Abels, M. Bohm, Alpha-melanocyte-stimulating hormone and related tripeptides: biochemistry, antiinflammatory and protective effects in vitro and in vivo, and future perspectives for the treatment of immune-mediated inflammatory diseases, Endocr. Rev. 29 (2008) 581–602.
[2] S. Gatti, G. Colombo, R. Buffa, F. Turcatti, L. Garofalo, N. Carboni, L. Ferla, L.R. Fassati, J.M. Lipton, A. Catania, alpha-Melanocyte-stimulating hormone protects the allograft in experimental heart transplantation, Transplantation 74 (2002) 1678–1684.
[3] A. Catania, S. Gatti, G. Colombo, J.M. Lipton, Targeting melanocortin receptors as a novel strategy to control inflammation, Pharmacol. Rev. 56 (2004) 1–29.
[4] J.L. Rees, The melanocortin 1 receptor (MC1R): more than just red hair, Pigment Cell Res. 13 (2000) 135–140.
[5] J.M. Lipton, A. Catania, Anti-inflammatory actions of the neuroimmunomodulator alpha-MSH, Immunol. Today 18 (1997) 140–145.
[6] A.V. Savos, J.M. Gee, D. Zierath, K.J. Becker, alpha-MSH: a potential neuroprotective and immunomodulatory agent for the treatment of stroke, J. Cereb. Blood Flow Metab. 31 (2011) 606–613.
[7] Y.K. Yang, C.M. Harmon, Recent developments in our understanding of melanocortin system in the regulation of food intake, Obes. Rev. 4 (2003) 239–248.
[8] D. Giuliani, L. Minutoli, A. Ottani, L. Spaccapelo, A. Bitto, M. Galantucci, D. Altavilla, F. Squadrito, S. Guarini, Melanocortins as potential therapeutic agents in severe hypoxic conditions, Front. Neuroendocrinol. 33 (2012) 179–193.
[9] J. Folkman, Tumor angiogenesis: therapeutic implications, N. Engl. J. Med. 285 (1971) 1182–1186.
[10] J. Folkman, Angiogenesis in cancer, vascular, rheumatoid and other disease, Nat. Med. 1 (1995) 27–31.
[11] P.A. D'Amore, Y.S. Ng, Won't you be my neighbor? Local induction of arteriogenesis, Cell 110 (2002) 289–292.
[12] F. Bussolino, A. Mantovani, G. Persico, Molecular mechanisms of blood vessel formation, Trends Biochem. Sci. 22 (1997) 251–256.
[13] D.O. Bates, F.E. Curry, Vascular endothelial growth factor increases hydraulic conductivity of isolated perfused microvessels, Am. J. Physiol. 271 (1996) H2520–H2528.
[14] N. Ferrara, Vascular endothelial growth factor: basic science and clinical progress, Endocr. Rev. 25 (2004) 581–611.
[15] N.M. Pandya, N.S. Dhalla, D.D. Santani, Angiogenesis—a new target for future therapy, Vascul. Pharmacol. 44 (2006) 265–274.
[16] J. Dai, A.B. Rabie, VEGF: an essential mediator of both angiogenesis and endochondral ossification, J. Dent. Res. 86 (2007) 937–950.
[17] P.C. Shen, A.L. Shiau, I.M. Jou, C.H. Lee, M.H. Tai, H.Y. Juan, P.R. Lin, G.S. Liu, C.L. Wu, J.L. Hsieh, Inhibition of cartilage damage by pro-opiomelanocortin prohormone overexpression in a rat model of osteoarthritis, Exp. Biol. Med. (Maywood) 236 (2011) 334–340.
[18] H.C. Lam, S.M. Kuo, M.J. Chuang, H.M. Keng, P.R. Lin, G.S. Liu, C.M. Hsu, S.L. Howng, M.H. Tai, Blockade of endothelin-1 release contributes to the anti-angiogenic effect by pro-opiomelanocortin overexpression in endothelial cells, Exp. Biol. Med. (Maywood) 231 (2006) 782–788.
[19] G.S. Liu, H.E. Tsai, W.T. Weng, L.F. Liu, C.H. Weng, M.R. Chuang, H.C. Lam, C.S. Wu, R. Tee, Z.H. Wen, S.L. Howng, M.H. Tai, Systemic pro-opiomelanocortin expression induces melanogenic differentiation and inhibits tumor angiogenesis in established mouse melanoma, Hum. Gene Ther. 22 (2011) 325–335.
[20] Y.S. Bee, S.J. Sheu, Y.L. Ma, H.C. Lin, W.T. Weng, H.M. Kuo, H.C. Hsu, C.H. Tang, J.C. Liou, M.H. Tai, Topical application of recombinant calreticulin peptide, vasostatin 48, alleviates laser-induced choroidal neovascularization in rats, Mol. Vis. 16 (2010) 756–767.
[21] N.D. Lawson, B.M. Weinstein, In vivo imaging of embryonic vascular development using transgenic zebrafish, Dev. Biol. 248 (2002) 307–318.
[22] D. Ribatti, B. Nico, A. Vacca, M. Presta, The gelatin sponge-chorioallantoic membrane assay, Nat. Protoc. 1 (2006) 85–91.
[23] H.M. Kuo, C.Y. Lin, H.C. Lam, P.R. Lin, H.H. Chan, J.C. Tseng, C.K. Sun, T.F. Hsu, C.C. Wu, C.Y. Yang, C.M. Hsu, M.H. Tai, PTEN overexpression attenuates angiogenic processes of endothelial cells by blockade of endothelin-1/endothelin B receptor signaling, Atherosclerosis 221 (2010) 341–349.
[24] T. Kumagai, T. Ishino, Y. Nakagawa, Acidic sphingomyelinase induced by electrophiles promotes proinflammatory cytokine production in human bladder carcinoma ECV-304 cells, Arch. Biochem. Biophys. 519 (2012) 8–16.
[25] P.M. Rood, J. Calafat, A.E. von dem Borne, W.R. Gerritsen, C.E. van der Schoot, Immortalisation of human bone marrow endothelial cells: characterisation of new cell lines, Eur. J. Clin. Invest. 30 (2000) 618–629.
[26] N. Zhu, R. Lalla, P. Eves, T.L. Brown, A. King, E.H. Kemp, J.W. Haycock, S. MacNeil, Melanoma cell migration is upregulated by tumour necrosis factor-alpha and suppressed by alpha-melanocyte-stimulating hormone, Br. J. Cancer 90 (2004) 1457–1463.

[27] M.H. Tai, S.M. Kuo, H.T. Liang, K.R. Chiou, H.C. Lam, C.M. Hsu, H.J. Pownall, H.H. Chen, M.T. Huang, C.Y. Yang, Modulation of angiogenic processes in cultured endothelial cells by low density lipoproteins subfractions from patients with familial hypercholesterolemia, Atherosclerosis 186 (2006) 448–457.
[28] B.H. Jiang, L.Z. Liu, AKT signaling in regulating angiogenesis, Curr. Cancer Drug Targets 8 (2008) 19–26.
[29] I. Shiojima, K. Walsh, Role of Akt signaling in vascular homeostasis and angiogenesis, Circ. Res. 90 (2002) 1243–1250.
[30] H.E. Tsai, L.F. Liu, G.J. Dusting, W.T. Weng, S.C. Chen, M.L. Kung, R. Tee, G.S. Liu, M.H. Tai, Pro-opiomelanocortin gene delivery suppresses the growth of established Lewis lung carcinoma through a melanocortin-1 receptor-independent pathway, J. Gene Med. 14 (2012) 44–53.
[31] G.S. Liu, L.F. Liu, C.J. Lin, J.C. Tseng, M.J. Chuang, H.C. Lam, J.K. Lee, L.C. Yang, J.H. Chan, S.L. Howng, M.H. Tai, Gene transfer of pro-opiomelanocortin prohormone suppressed the growth and metastasis of melanoma: involvement of alpha-melanocyte-stimulating hormone-mediated inhibition of the nuclear factor kappaB/cyclooxygenase-2 pathway, Mol. Pharmacol. 69 (2006) 440–451.
[32] G.S. Liu, H.T. Huang, C.J. Lin, J.Y. Shi, L.F. Liu, R.T. Tseng, W.T. Weng, H.C. Lam, Z.H. Wen, T.L. Cheng, K.S. Hsu, M.H. Tai, Prophylactic proopiomelanocortin expression alleviates capsaicin-induced neurogenic inflammation in rat trachea, Shock 32 (2009) 645–650.
[33] T.Y. Choi, J.H. Kim, D.H. Ko, C.H. Kim, J.S. Hwang, S. Ahn, S.Y. Kim, C.D. Kim, J.H. Lee, T.J. Yoon, Zebrafish as a new model for phenotype-based screening of melanogenic regulatory compounds, Pigment Cell Res. 20 (2007) 120–127.
[34] D.W. Logan, S.F. Burn, I.J. Jackson, Regulation of pigmentation in zebrafish melanophores, Pigment Cell Res. 19 (2006) 206–213.
[35] J.R. Metz, J.J. Peters, G. Flik, Molecular biology and physiology of the melanocortin system in fish: a review, Gen. Comp. Endocrinol. 148 (2006) 150–162.
[36] T.E. Scholzen, T. Brzoska, D.H. Kalden, M. Hartmeyer, M. Fastrich, T.A. Luger, C.A. Armstrong, J.C. Ansel, Expression of functional melanocortin receptors and proopiomelanocortin peptides by human dermal microvascular endothelial cells, Ann. N. Y. Acad. Sci. 885 (1999) 239–253.
[37] T.E. Scholzen, D.H. Kalden, T. Brzoska, T. Fisbeck, M. Fastrich, M. Schiller, M. Bohm, T. Schwarz, C.A. Armstrong, J.C. Ansel, T.A. Luger, Expression of proopiomelanocortin peptides in human dermal microvascular endothelial cells: evidence for a regulation by ultraviolet light and interleukin-1, J. Invest. Dermatol. 115 (2000) 1021–1028.
[38] P.M. Holloway, H.K. Smith, D. Renshaw, R.J. Flower, S.J. Getting, F.N. Gavins, Targeting the melanocortin receptor system for anti-stroke therapy, Trends Pharmacol. Sci. 32 (2011) 90–98.
[39] M.P. Corander, M. Fenech, A.P. Coll, Science of self-preservation: how melanocortin action in the brain modulates body weight, blood pressure, and ischemic damage, Circulation 120 (2009) 2260–2268.
[40] T.E. Scholzen, C. Sunderkotter, D.H. Kalden, T. Brzoska, M. Fastrich, T. Fisbeck, C.A. Armstrong, J.C. Ansel, T.A. Luger, Alpha-melanocyte stimulating hormone prevents lipopolysaccharide-induced vasculitis by down-regulating endothelial cell adhesion molecule expression, Endocrinology 144 (2003) 360–370.
[41] S. Balwani, R. Chaudhuri, D. Nandi, P. Jaisankar, A. Agrawal, B. Ghosh, Regulation of NF-kappaB activation through a novel PI-3K-independent and PKA/Akt-dependent pathway in human umbilical vein endothelial cells, PLoS One 7 (2012) e46528.
[42] D.H. Kalden, T. Scholzen, T. Brzoska, T.A. Luger, Mechanisms of the antiinflammatory effects of alpha-MSH. Role of transcription factor NF-kappa B and adhesion molecule expression, Ann. N. Y. Acad. Sci. 885 (1999) 254–261.