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Celastrol inhibits vasculogenesis by suppressing the VEGF-induced functional activity of bone marrow-derived endothelial progenitor cells

Shuai Huang [a,1], Yubo Tang [b,1], Xingdong Cai [c], Xinsheng Peng [d], Xingmo Liu [a], Longjuan Zhang [e], Yucheng Xiang [a], Dechun Wang [a], Xi Wang [a], Tao Pan [a,*]

[a] Department of Orthopaedic Surgery, The Sixth Affiliated Hospital of Sun Yat-sen University, 510655 Guangzhou, China
[b] University Hospital Carl Gustav Carus at Technical University Dresden, Centre for Translational Bone, Joint and Soft Tissue Research, Germany
[c] Department of Respiratory Medicine, The First Affiliated Hospital of Sun Yat-sen University, 510080 Guangzhou, China
[d] Department of Orthopaedic Surgery/Orthopaedic Research Institute, The First Affiliated Hospital of Sun Yat-sen University, 510080 Guangzhou, China
[e] The Surgical Laboratory, The First Affiliated Hospital of Sun Yat-sen University, 510080 Guangzhou, China

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ABSTRACT

Bone marrow (BM)-derived endothelial progenitor cells (EPCs) play a critical role in tumor vasculogenesis because they provide both instructive (release of pro-angiogenic cytokines, such as VEGF) and structural (vessel incorporation and stabilization) functions. Celastrol, derived from *Trypterygium wilfordii* Hook F., a traditional Chinese medicine plant, has been studied for its antitumorigenic properties, but its mechanism of action is not well understood. The aims of this study are to investigate the effects of Celastrol on VEGF-induced functional activity of BM-EPCs and to identify any mechanisms associated with this process. Here, we show that Celastrol attenuates VEGF secretion in BM-EPCs in vitro. This attenuation, in turn, inhibits the in vitro VEGF-induced cell viability, cell–cell adhesion, cell–ECM adhesion, migration response and vascular tube formation of BM-EPCs. Additionally, Celastrol inhibits the phosphorylation of VEGFR2, endothelial nitric oxide synthase (eNOS), and Akt to attenuate cell functions. Taken together, the present study demonstrates that Celastrol decreases Akt/eNOS signaling in BM-EPCs in vitro. These findings identify novel mechanisms that regulate EPC function and may provide new insights for the medicinal use of Celastrol.

1. Introduction

Bone marrow (BM)-derived endothelial progenitor cells (EPCs) are not only vital for the maintenance of pre-existing vessels (angiogenesis) but also in vasculogenesis because they release pro-angiogenic cytokines (e.g., VEGF) and provide structural support through vessel incorporation and stabilization. Vasculogenesis, supported by BM-EPCs, is essential for organ/tissue growth and wound healing [1–5]. In disease conditions, abnormal neovascularization, such as that seen in cancer development also involves postnatal vasculogenesis [6,7]. Emerging evidence from preclinical models suggest that tumor-derived signals stimulate the quiescent bone marrow compartment. This compartment expands and mobilizes BM-derived VEGFR2$^+$ EPCs and, as a result, blocks EPC mobilization to inhibit vasculogenesis [8]. VEGF plays a central role in the cytokine-induced neovascularization of tumor-derived BM-EPCs by activating the VEGF signaling pathway. VEGF signaling induces EPCs proliferation, migration, adhesion and vascular tube formation. Some cancer patients were found to exhibit increased VEGF serum and tissue levels that correlates with the advanced stage, postoperative recurrence, metastasis, and prognosis of these patients [9,10]. It has also been shown that EPCs activate the "angiogenesis switch", a critical step in the transition of an avascular, dormant tumor to a vascularized, rapidly growing tumor [11]. Other studies have highlighted the important contribution of EPCs during the early phase of tumor neovascularization, a phase that has been associated with angiogenic status and patient prognosis [12].

Because access to the systemic blood flow is essential for neoplastic grow and metastasis, the inhibition of vessel formation by blocking angiogenesis and vasculogenesis through anti-angiogenic drugs has become an attractive target for cancer therapeutics [8,13,14].

Celastrol, also known as a tripterine, is a functional ingredient that was originally identified and extracted from a traditional Chinese medicinal plant, *Trypterygium wilfordii*. Celastrol has been found to exhibit anti-inflammatory and anticancer properties [15,16]. Although Celastrol has already shown to be promising in

* Corresponding author. Address: Department of Orthopaedic Surgery, the Sixth Affiliated Hospital of Sun Yat-sen University, No.19, Yuancuxin Street, Tianhe District, 510655 Guangzhou, Guangdong Province, China. Fax: +86 20 38254221.
E-mail address: pantao@126.com (T. Pan).
[1] These two authors contributed equally to this work.

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tumor prevention by inhibiting angiogenesis of endothelial cells [9], whether Celastrol could also modulate tumor vasculogenesis of BM-EPCs has not yet been investigated.

In this study, we isolated human BM-EPCs as previously described [17], detected VEGFR2 expression by Western blot analysis and examined whether Celastrol can inhibit VEGF-induced instructive (release of VEGF) and structural (vessel incorporation and stabilization) vasculogenesis. Specifically, we examined in vitro VEGF-induced cell viability, migration, cell–cell and cell–ECM adhesion and vascular tube formation activities. In addition, we further investigated the inhibitory effects of Celastrol on VEGF-induced cell signaling pathways. These findings provide important insights into the mechanism of the inhibition of VEGF-induced BM-EPCs vasculogenesis by Celastrol.

2. Materials and methods

2.1. Reagents

Purified Celastrol (>98%) was purchased from Sigma–Aldrich and used for the in vitro experiments. Celastrol was dissolved in DMSO (Sigma, St. Louis, MO) to generate a stock concentration of 50 mmol/L, and it was then aliquoted and stored at −20 °C. VEGF was purchased from Peprotech (Human VEGF165).

2.2. Isolation and cultivation of EPCs

Bone marrow was collected from the drill holes of the pedicle during internal spine fixation of patients with lumbar degenerative diseases (12 patients, age range 35–72 years, mean age 57.58 years). Informed consent for bone marrow collection was obtained from the patients, and all procedures were performed in accordance with the guidance and approval of a research ethics committee in the First Affiliated Hospital of Sun Yat-sen University (NO. 2008-55). The procedures for isolation, cultivation and identification of human EPC cultures followed previously published methods [17].

2.3. EPCs viability assay

Cell viability was determined by the CCK-8 (Dojindo, Japan) method, as described previously [17]. The CCK-8 was used to count living cells by combining WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] and 1-methoxy PMS. BM-EPCs were cultured with EGM-2 in 96-well culture plates at 1×10^4 cells/well per well for 24 h to 90% confluent. The medium was then replaced by fresh non-FBS EBM-2, with or without VEGF (10 ng/mL) and containing Celastrol at different concentrations (0, 0.1, 0.5, 1 and 2 μmol/L). After 24 h incubation, CCK-8 was used according to the manufacturer's instructions. Cell viability was assessed using a microplate reader at 450 nm.

2.4. Cell–matrix adhesion assay

The cell–matrix adhesion assay was performed as previously described [17]. Human BM-EPCs were cultured for 24 h at 90% confluency and treated with or without VEGF (10 ng/mL) and Celastrol for 24 h as above. Then, BM-EPCs at 1×10^4 cells/well were replated onto fibronectin-coated 96-well culture plates and incubated for 30 min at 37 °C. After incubation, non-adherent cells were removed by washing three times with PBS. The adherent cells were fixed with 4% paraformaldehyde for 20 min, washed with PBS, and stained with 100 μL 0.1% crystal violet for 30 min at room temperature. Adherent cells were counted using a phase contrast microscope by independent, blinded investigators.

2.5. Cell–cell adhesion assay

Cell–cell adhesion assays were performed in 96-well plates. BM-EPCs were seeded at a density of 1×10^4 cells/well in 96-well plate until confluency. Meanwhile, another group of BM-EPCs were cultured for 24 h at 90% confluency and treated with or without VEGF (10 ng/mL) and Celastrol for 24 h as above. The EPCs were then labeled (CellTracker™ Green CMFDA, Invitrogen) and seeded at a density of 1×10^4 cells/well in 96-well plate containing a 100% confluent EPC monolayer. The plate was incubated at 37 °C for 30 min. After incubation, non-adherent cells were removed by washing three times with PBS. Adherent cells were counted under the phase contrast fluorescence microscope (OLYPUMS IX81, JAPAN) (×100 objective lens) by independent blinded investigators.

2.6. EPCs migration assay

A transwell migration assay was performed as described previously [17]. Briefly, BM-EPCs (8×10^4 cell/well) along with the indicated concentrations of Celastrol were seeded into the upper chambers. The bottom chambers were filled with 600 μL EGM-2 supplemented with 10 ng/mL VEGF. This transwell system was then incubated for 6 h in 5% CO_2 at 37 °C. Non-migrating cells on the upper surface of the filter were removed using a cotton-tipped swab, and cells on the lower surface were colored with 1% crystal violet. Images were taken using an inverted microscope (×100 objective lens). The percent inhibition of migrated cells in the treated groups was recorded.

2.7. EPCs capillary-like tube formation assay

Tube formation mimicking new blood vessel development was assessed, as previously described [17]. Briefly, BM-EPCs were pretreated with various concentrations of Celastrol for 30 min and then seeded onto the Matrigel (BD Biosciences) layer in 96-well plates at a density of 1×10^4 cells/well. ECGM was used with or without 10 ng/mL VEGF. Capillary-like tubes were imaged after 24 h incubation using a 100× objective lens of an inverted microscope. All side branches were counted by three independent investigators in a blinded manner.

2.8. Enzyme-linked immunosorbent assay (ELISA)

To detect VEGF levels in the supernatant of BM-EPCs, we used an ELISA assay performed according to the manufacturer instructions (Excell, Shanghai, China). Human BM-EPCs were cultured for 24 h to 90% confluency and treated with different concentrations (0, 0.1, 0.5, 1 and 2 μmol/L) Celastrol for 24 h. The medium was then replaced with fresh non-FBS EBM-2 media and cultured for 24 h. At the end of treatment, the culture media was collected and centrifuged at 14,000 rpm for 5 min. The supernatants were stored at −70 °C and used for the ELISA assay.

2.9. Western blot analysis

For analysis of the changes in protein abundance, cell pellets treated with different concentrations were collected separately and washed three times with ice-cold phosphate-buffered saline. The cells were lysed with ice-cold lysing buffer (Novagen, Merck, Darmstadt, Germany). The lysates were centrifuged for 20 min at 4 °C at 12,000 × *g* to pellet any residual debris. The protein concentration was determined using a Bradford assay. For a control, protein (20 μg/lane) from total abnormal pulmonary venous drainage

was separated on a 10% SDS polyacrylamide gel. The protein expression was detected with the corresponding primary antibodies: phospho-VEGFR2, phospho-eNOS, phospho-Akt, VEGFR2, eNOS and Akt antibodies (1:1000, all from Cell Signaling Technology). Protein bands were visualized with Chemiluminescence Reagent Plus (Thermo, Waltham, MA, USA) and exposed on an X-ray film (Fujifilm, Tokyo).

2.10. Statistical analysis

All data are expressed as the mean ± SD. Statistical comparisons between treated group and untreated group were performed using Student's *t*-test. *P* values of $\leqslant 0.05$ were considered statistically significant.

3. Results

3.1. Celastrol inhibits VEGF-induced cell viability of BM-EPCs

To observe the possible cytotoxicity of Celastrol on the cells, we examined whether Celastrol modulates VEGF-induced cell viability of BM-EPCs by the CCK-8 assay. As shown in Fig. 1A, incubation of human BM-EPCs with Celastrol for 24 h exhibited a dose-dependent reduction in the number of BM-EPCs, with significant inhibition first occurring at 1 μmol/L and even greater inhibition at 2 μmol/L.

3.2. The adhesion ability of EPCs is inhibited by Celastrol

To investigate the possibility that Celastrol alters the adhesiveness of EPCs, cells were incubated with Celastrol for 24 h. After replating on fibronectin-coated dishes and a 100% confluent EPCs monolayer, EPCs pre-exposed to Celastrol exhibited a significant increase in the number of adhesive cells after a 30 min treatment. Celastrol reduced the number of adhesive cells of BM-EPCs in a dose-dependent manner, with significant inhibition first occurring at 1 μmol/L and greater inhibition at 2 μmol/L (Fig. 2B, E). Additionally, Celastrol could markedly inhibit cell–cell adhesion with the higher concentrations listed above (Fig. 2A, D).

3.3. Celastrol inhibits VEGF-induced chemotactic migration of BM-EPCs

It is known that cell mobility and maintenance of cell survival signaling are essential for capillary tube formation. To assess the anti-angiogenic properties of Celastrol in vitro, we examined the cellular response of Celastrol-treated BM-EPCs to VEGF-induced migration using a transwell assay. Celastrol reduced the number of migrating BM-EPCs in a dose-dependent manner, with significant inhibition first occurring at 1 μmol/L and greater inhibition at 2 μmol/L. Low Celastrol concentrations did not reduce BM-EPC migration (Fig. 2C, F).

3.4. Celastrol inhibits VEGF-induced capillary structure formation of BM-EPCs

To further determine the effect of Celastrol on vasculogenesis, we examined how Celastrol regulates capillary tubule formation of BM-EPCs. When EPCs were seeded on growth factor reduced, two-dimensional Matrigel, robust tube-like structures were formed in the presence of VEGF. However, treatment with 1 or 2 μmol/L Celastrol abolished the VEGF-induced tubule formation of BM-EPCs (Fig. 3A, B). These data suggest that Celastrol inhibits vasculogenesis.

3.5. Effects of Celastrol on VEGF secretion

Because VEGF can alter the marrow microenvironment from a quiescent state to a highly pro-angiogenic and pro-tumorigenic environment, it is important for the regulation of angiogenesis as well as cell function. Therefore, we evaluated the secretion level of VEGF in the supernatant of BM-EPCs. Over a period of incubation of 24 h after treated with Celastrol, the concentration of VEGF in the supernatant of BM-EPCs was significantly reduced with the treatment of Celastrol at 1 or 2 μmol/L as compared to untreated (control) EPCs (Fig. 1B).

3.6. Celastrol inhibits the phosphorylation of VEGFR2, eNOS and Akt

Interaction of VEGFR2 with VEGF leads to the activation of various downstream signaling molecules responsible for EPC migration, proliferation, and survival. To further delineate the mechanisms that contribute to the vasculogenesis inhibition effect of Celastrol, we examined the signaling molecules involved in the VEGF pathway using Western blot analysis. As shown in Fig. 4A, under the conditions used in the present experiments, the phosphorylation of VEGFR2 was suppressed by Celastrol in a dose-dependent manner (Fig. 4). Thus, the anti-angiogenic property of Celastrol may be at least partially due to VEGFR2 inhibition. Upon examination of the key pathway components that regulated the endothelial cell function in angiogenesis, we found that Celastrol can effectively suppress VEGF-triggered activation of the signaling cascade. Specifically, Celastrol affects Akt and eNOS kinase in

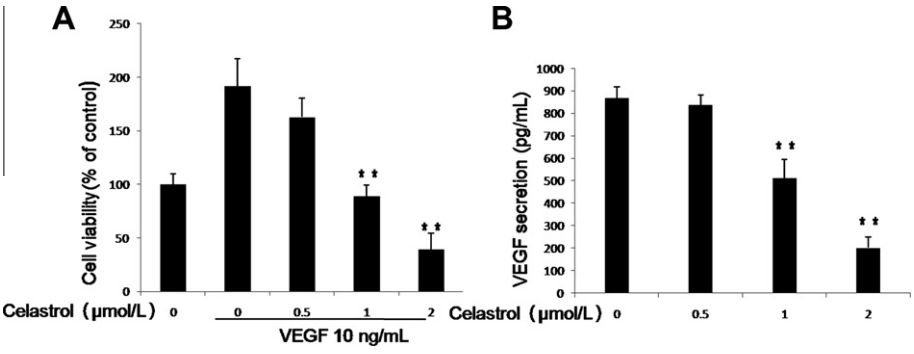

Fig. 1. Celastrol inhibited cell viability and VEGF secretion of BM-EPCs. (A) Cell viability was inhibited significantly by 1 and 2 μmol/L Celastrol for 24 h. (B) Compared with the control group, VEGF secretion was inhibited by the presence of Celastrol significantly at 1 and 2 μmol/L. (**) $P < 0.01$ versus VEGF (A) or control (B).The results are representative of at least three independent experiments.

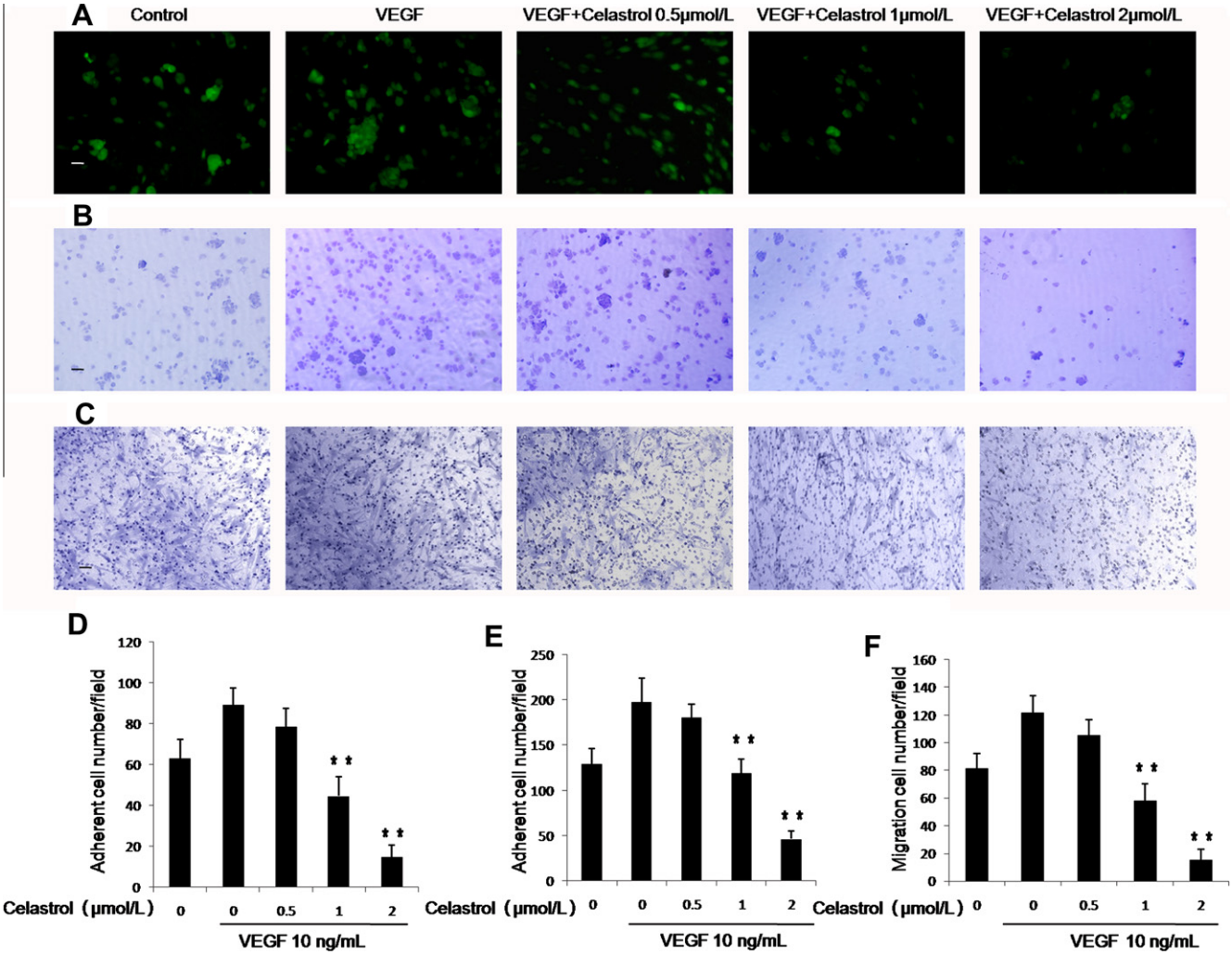

Fig. 2. Celastrol inhibited VEGF-induced cell–cell adhesion, cell–matrix adhesion, and chemotactic motility of BM-EPCs. (A, D) Celastrol inhibited VEGF-induced cell–cell adhesion significantly at 1 and 2 μmol/L. (B, E) Celastrol inhibited VEGF-induced cell–matrix adhesion significantly at 1 and 2 μmol/L. Adherent cells were photographed (magnification, ×100) and quantified by manual counting. (C, F) Celastrol inhibited VEGF-induced chemotactic motility of BM-EPCs significantly at 1 and 2 μmol/L. Migrated cells were photographed (magnification, ×100) and quantified by manual individual cell counting. (**) $P < 0.01$ versus VEGF alone. Bar = 100 μm. The results are representative of at least three individual experiments.

BM-EPCs (Fig. 4). This result suggests that Celastrol may inhibit tumor angiogenesis through blocking of these signaling pathways.

4. Discussion

Over the past 30 years, inhibition of vessel formation has been accepted as an effective strategy to treat human cancer. BM-derived cells contribute to tumor neovasculature and, when modified to express a vessel formation inhibitor, can restrict tumor neovascularization [7]. As such, the search for new antitumor drugs from natural sources, such as Celastrol, is one of the most important approaches for cancer prevention and therapy.

Extensive research has led to the identification and isolation of regulators of vasculogenesis, some of which represent therapeutic targets, such as VEGF. EPCs provide both instructive (release of pro-angiogenic cytokines, such as VEGF) and structural (vessel incorporation and stabilization) functions that contribute to the initiation of tumor neovasculogenesis. Thus, selective targeting of VEGF-induced BM-EPC-supported vasculogenesis has been heralded as a promising avenue for anti-angiogenic cancer therapy. We observed VEGF level dramatically decreased in the supernatant of BM-EPCs after inoculation with Celastrol at different concentrations. This finding indicates that a low dose of Celastrol at 1–2 μmol/L inhibited the VEGF secretion of BM-EPCs and, as a result, reduced the instructive functions of BM-EPCs that contribute to tumor neovasculogenesis.

Our rationale for investigating the potential inhibitory effects of Celastrol on VEGF-induced EPC structural contributions to vasculogenesis was based on its previous work, which found that Celastrol inhibited the angiogenesis of endothelial cells [9]. We investigated the role of Celastrol in these processes because, to date, no study has comprehensively described its inhibition effect on BM-EPC-supported vasculogenesis or characterized the underlying mechanisms.

In this paper, for the first time, we systematically evaluated the effects of Celastrol in vasculogenesis-related events involving BM-EPCs. Specifically, we examined its role in cell viability, cell–cell adhesion, cell–ECM adhesion, migration and capillary-like tube formation. Our results show that Celastrol can significantly inhibit VEGF-induced BM-EPC-supported vasculogenesis at a low dose (1–2 μmol/L through VEGFR2 signaling pathway. Therefore, understanding of the mechanisms that regulate EPC functions may provide new insights for the medicinal use of Celastrol.

Because several reports indicate that mobilization of EPCs from the BM to the peripheral circulation and its subsequent incorpora-

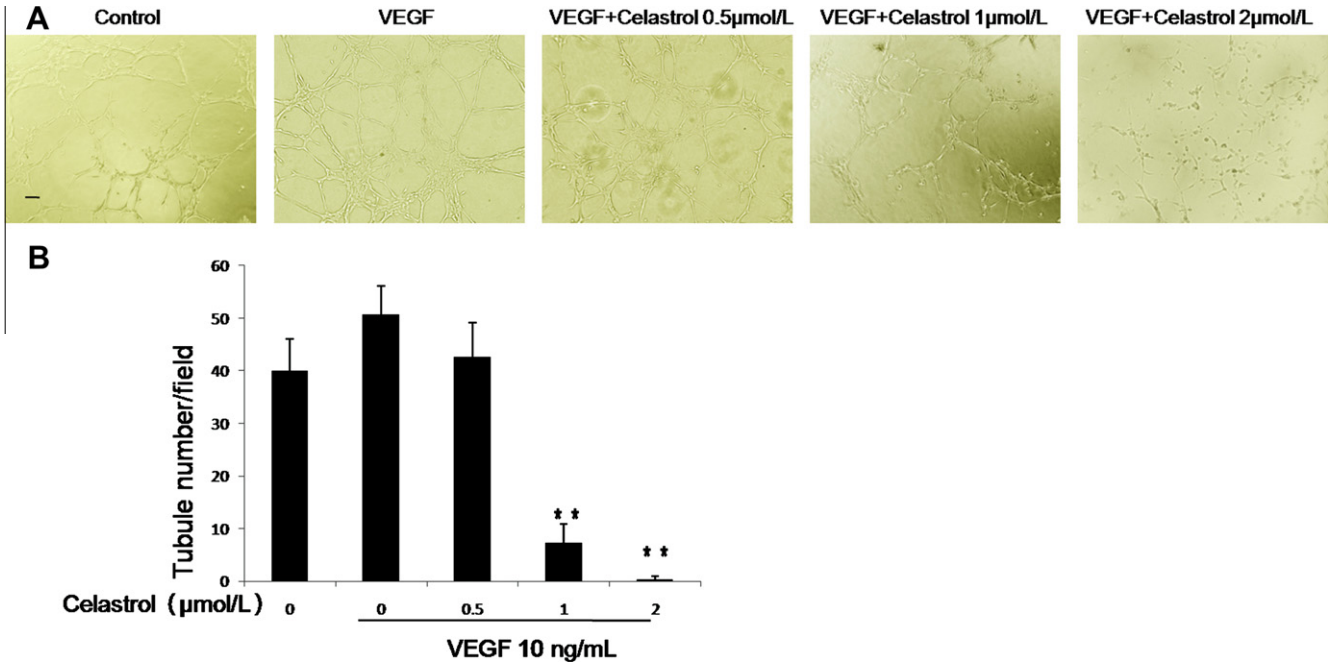

Fig. 3. Celastrol inhibited the VEGF-induced capillary structure formation on matrigel of BM-EPCs (A, B). Celastrol inhibited VEGF-induced capillary structure formation significantly at 1 and 2 μmol/L. After incubation, BM–EPCs were fixed, and tubular structures were photographed (magnification, ×100) quantified by manual counting. (**) $P < 0.01$ versus VEGF alone. Bar = 100 μm. The results are representative of at least three individual experiments.

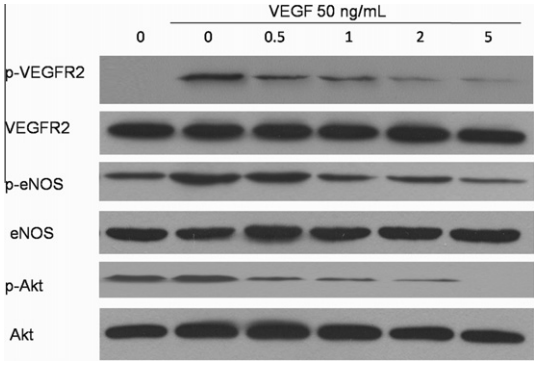

Fig. 4. Celastrol inhibited the VEGF-triggered activation of VEGFR2 and Akt pathways in BM–EPCs. Celastrol suppressed the phosphorylation of p-VEGFR2, p-eNOS and p-Akt triggered by VEGF in BM–EPCs. The relative density of immunoreactivity was measured. Similar experiments were performed in triplicate.

tion into neovessels are important for pathogenesis of many diseases, including cancer [8,2,18], targeting EPC mobilization may be an important therapeutic intervention for treatment of various pathological conditions. As shown in Fig. 2C, VEGF-induced migration was significantly decreased dose-dependently with Celastrol treatment.

VEGF can stimulate EPCs proliferation resulting in the expansion of BM-EPCs and, as a result, blocking EPC proliferation can inhibit vasculogenesis. In our results, as shown in Fig. 1A, VEGF-induced cell viability was significantly attenuated with a low dose of Celastrol (1–2 μmol/L). Moreover, Celastrol significantly inhibits cell–cell and cell–ECM adhesion of BM-EPCs and results in an attenuated level of EPCs located in a region of high VEGF expression and neovascularization via blocking VEGFR2 signaling pathways.

Here, we also found that the VEGF-induced activation of Akt and eNOS was dramatically attenuated. In other words, our data clearly demonstrate that Celastrol blocked recruitment of BM-EPCs and the effects of Celastrol on inhibition of EPC is due to a mechanism that inhibits VEGF/VEGFR2 signaling pathways, specifically targeting Akt and eNOS.

Celastrol might inhibit activation of VEGFR2 through direct regulation of VEGFR2 phosphorylation or by blocking the binding of VEGF to its receptor VEGFR2. eNOS is essential for the BM microenvironment, and increased BM NO levels results in the mobilization of EPCs from BM niches to the circulation, ultimately permitting their participation in vasculogenesis [19–21]. Neovascularization from EPCs is inhibited by treatment with Celastrol through inhibition of the Akt–eNOS signaling axis, which, in turn, attenuates cell proliferation and function.

In conclusion, we identified an inhibitory effect of Celastrol on VEGF-induced BM-EPC-supported vasculogenesis by targeting the signaling pathway VEGF/VEGFR2 and Akt–eNOS signaling axis. Taken together with previous studies, Celastrol has been identified as a candidate therapeutic agent to prevent cancer related vasculogenesis. Specifically, Celastrol may have therapeutic value in cancers where EPC mobilization is stimulated. Furthermore, Celastrol might be used for therapeutics in combination with anti-cancer agents and health foods to prevent and block progression of tumor development.

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