

Original Article

New proangiogenic activity on vascular endothelial cells for C-terminal mechano growth factor

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Angiogenesis is crucial in wound healing. The administration of the C-terminal 24-a.a. peptide of mechano growth factor (MGF24E) has been previously demonstrated to induce more blood vessels in regenerating bone around defective areas compared with the control. Accordingly, this study aims to determine whether MGF24E promotes bone defect healing through MGF24E-increased angiogenesis and whether MGF24E has positive effects on angiogenesis *in vitro*. The roles of MGF24E on angiogenesis and the underlying mechanisms were investigated. The cell proliferation, migration, and tubulogenesis of the human vascular endothelial EA.hy926 cells co-treated with 2% serum and MGF24E were determined to assess angiogenesis in comparison with 100 ng/ml of vascular endothelial growth factor 165 (VEGF₁₆₅)-positive control or vehicle control (phosphate-buffered saline). MGF24E treatment (10 ng/ml) significantly promoted the biological processes of angiogenesis on EA.hy926 cells compared with the vehicle control. The suppression of vascular endothelial growth factor and angiopoietin-I expressions by 2% serum starvation was reversed by the addition of 10 ng/ml of MGF24E in 2% serum medium. This result suggests that MGF24E has a protective effect on angiogenesis. Moreover, the inhibition of ERK due to PD98050 pretreatment completely abolished and mostly blocked MGF24E-induced proliferation and migration, respectively, whereas the MGF24-induced tubulogenesis and the angiogenic factor expression were only partially inhibited. These new findings suggest that MGF24E promotes angiogenesis by enhancing the expression of angiogenic cytokines which involves the MAPK/ERK-signaling pathway.

Keywords MGF24E; vascular endothelial cell; angiogenesis; angiogenic cytokines; ERK1/2

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Introduction

Angiogenesis plays a paramount role on a wide range of physiologic and pathologic processes, such as fetal development and wound healing [1]. Many cases of wound healing failure, especially bone nonunion, are associated with insufficient blood vessel formation. Therefore, the issue of angiogenesis is crucial to be addressed for injury repair. Growth factors have gained a significant importance in the search of angiogenic therapies. Insulin-like growth factor-I (IGF-I) is one of the angiogenic bioactive molecules having angiogenesis as a major component of action. IGF-I can enhance the expression of vascular endothelial growth factor (VEGF) in several different cells [2–5]. Moreover, the *igf-I* gene can be alternatively spliced to generate different IGF-I isoforms [6]. Mechano growth factor (MGF), which has a unique C-terminal E domain (MGF24E), is the initial splice variant of IGF-I when tissue or cells suffer from injuries [7–12]. However, some different biological activities are observed between MGF and IGF-I. The MGF24E is not present in the gene structures or peptide sequences of other IGF-I isoforms and IGF-I. Therefore, MGF24E seems to be the key in distinguishing MGF from IGF-I or other IGF-I isoforms [7,11–15]. In recent years, increasing attention has been paid to MGF24E because of its regenerative effects on neurons [9,10], human mesenchymal stem cells [11], and osteoblasts [12]. Meanwhile, the tissue-protective actions of MGF24E have been demonstrated in acute myocardial infarction [8,9], neuron damage [10,11], and rabbit bone-defect models [12].

The previous bone-defect healing study showed that the administration of MGF24E induced more blood vessels in bone regeneration around defective areas [12]. This study is the first to determine whether MGF24E has positive effects on angiogenesis *in vitro*. Furthermore, previous studies have demonstrated that MGF24E acts via the MAPK/ERK

but not the Akt-signaling pathway on cardiomyocytes, dopamine neurons, or osteoblasts [9,12,14]. Thus, it appears attractive for the role of ERK signaling on vascular endothelial cell (EC) actions or angiogenic cytokine expression activated by MGF24E. The effects of MGF24E on cell proliferation, migration, and tubulogenesis were estimated in the present study. The expression of angiogenic cytokines in ECs incubated with MGF24E was investigated to explain the underlying molecular mechanisms. Meanwhile, the effects of the ERK inhibitor PD98059 on cell actions and angiogenic cytokine expressions induced by MGF24E were investigated to determine whether MGF24E acts on vascular ECs via the MAPK/ERK-signaling pathway similar to the other cells.

Materials and Methods

Cell culture

The human vascular EC line EA.hy926 (China Center for Type Culture Collection, Wuhan, China) was incubated at 37°C with 5% CO₂ in RPMI 1640 (Gibco, Carlsbad, USA) containing 10% fetal bovine serum (FBS; Gibco), 100 µg/ml of streptomycin, and 100 U/ml of penicillin. The cells were grown to 80% confluence for assays.

Cell counting kit-8 assay

The EA.hy926 cells were seeded at 3000 cells/well in 96-well plates, attached for 12 h, serum-starved overnight, and then treated by RPMI 1640 with 2% FBS containing 5–100 ng/ml MGF24E which was prepared as described previously [15]. Up to 100 ng/ml of VEGF₁₆₅ (Peprotech, Rocky Hill, USA) served as the positive control, and phosphate-buffered saline (PBS) served as the vehicle control. The cells were incubated for 1–5 days at various times, and cell viability was determined by using the cell counting kit-8 (CCK-8) assay kit (Jianchen, Nanjing, China). For ERK1/2 inhibition, the cells were pretreated with 100 µM of PD98059 (Sigma, St Louis, USA) for 30 min prior to 10 ng/ml of MGF24E administration.

Cell cycle distribution

The cells were seeded at 7×10^4 cells/well in 6-well plates, attached for 12 h, serum-starved overnight, and incubated with 10 ng/ml of MGF24E, 100 ng/ml of VEGF₁₆₅, or PBS for 2 days as described previously [16]. For ERK1/2 inhibition, the cells were pretreated with 100 µM of PD98059 for 30 min prior to 10 ng/ml of MGF24E administration. The cells were collected, fixed, and stained with propidium iodide solution containing ribonuclease according to the manufacturer's protocol of the Coulter DNA prep reagent kit (Beckman Coulter Inc., Carlsbad, USA). Flow cytometry analysis was performed on a flow cytometer (Coulter Epics XL; Beckman), and the

data were gated using pulse width to exclude doublets. The DNA content was measured by FL2-A, and the percentage of cells in each phase of the cell cycle was calculated using Expo32-ADC workstation (Beckman). For each sample, the data from 2×10^5 cells were acquired.

Migration assay

Cell migration was assessed by a Transwell Boyden Chamber (polycarbonate filters with 8.0 µm pore size; Costar, Cambridge, WA, USA). The serum-free cells suspensions at a concentration of 3×10^4 cells/well with MGF24E, VEGF₁₆₅, or PBS were added into the upper chamber. For the ERK1/2 inhibition, the cells were pretreated with 100 µM of PD98059 for 30 min prior to cell seeding. The chamber was incubated at 37°C for 10 h, fixed with 4% paraformaldehyde, and stained with 0.5% crystal violet in 20% methanol for 30 min. The cells on the upper surface were removed with a cotton swab. The cells migrating to the lower surface were photographed under the microscope. The results were expressed as percentage as compared with the migrated cells of the vehicle group, which was set to 100%.

Tubulogenesis assays

The formation of capillary-like structures was analyzed as described previously [17]. In brief, the cells were pretreated with serum-free RPMI 1640 with or without 100 µM of PD98059 for 30 min. The pretreated cells were seeded at 1×10^4 cells/well in matrigel-coated 48-well plates (BD Biosciences, Franklin Lakes, USA). After the addition of growth factors, the plates were incubated at 37°C in serum-free RPMI 1640 media for 48 h. Tube formation was quantified by tube branch points (area where a single point give rise to three divergent outgrowths) under a microscope at $\times 40$ magnification. The results were expressed as percentage as compared with the vehicle whose branch points were set to 100%.

Quantitative real-time polymerase chain reaction

The cells were seeded at 7×10^4 cells/well in 6-well plates, attached for 12 h, serum-starved overnight, and treated by RPMI 1640 with 2% FBS containing 10 ng/ml of MGF24E or PBS, or by RPMI 1640 with 10% FBS. For Erk1/2 inhibition, the cells were pretreated with 100 µM of PD98059 for 30 min prior to 10 ng/ml MGF24E administration and collected after 24 h. Total RNA from ECs was extracted using TRIzol reagent (Invitrogen, Foster, USA). Complementary DNA was synthesized from 2 µg of the total RNA using an RNA reverse transcriptase polymerase chain reaction (PCR) kit (TaKaRa, Dalian, China). The complementary DNA mixtures were diluted (1:20) in water, and 8 µl was subjected to quantitative real-time PCR amplification with SYBR Green (Applied Biosystems, Foster, USA) using the following primers. The VEGF

forward and backward primers were 5'-CGCCATT TTATTTTCTTGCTGC-3' and 5'-CCCCTCCCAACTCA AGTCCA-3', respectively (GenBank: NM_001171630, 260 bp). The primers for *Ang-I* were 5'-GGGACAGC AGGAAAACAGAGCAG-3' and 5'-GCCACAAGCATCA AACCACCATC-3' (GenBank: NM_001199859.1, 132 bp), and those for *GAPDH* were 5'-TGGGGTGATGCTG GTGCTGAGT-3' and 5'-AGGTTTCTCCAGGCGGCAT GTC-3' (GenBank: AF106860, 500 bp). These primers were used with amplification on each sample to control for RNA loading. PCRs were conducted on a PCR System 7500 (Applied Biosystems). The specificity of all individual amplification reactions was confirmed via the melting-curve analysis. The fold change of relative *VEGF* or *Ang-I* messenger RNA (mRNA) expression was calculated using the $2^{-\Delta\Delta C_t}$ method, and statistical analysis was based on the ratio of VEGF or Ang-I to GAPDH.

Western blot analysis

The cells were seeded at 7×10^4 cells/well in 6-well plate, attached for 12 h, serum-starved overnight, and treated by RPMI 1640 with 2% FBS containing 10 ng/ml of MGF24E or PBS, or by RPMI 1640 with 10% FBS. For ERK1/2 inhibition, the cells were pretreated with 100 μ M of PD98059 for 30 min prior to 10 ng/ml of MGF24E administration and collected after 3 days. Total cellular protein was extracted, separated through sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred onto polyvinylidene fluoride membranes (Millipore, Billerica, USA), and probed with human monoclonal antibody against VEGF or Ang-I (Santa Cruz Biotechnology, CA, USA) at 1 : 500 dilution overnight at 4°C. Immunoreactive protein bands were visualized using ECL chemiluminescence detection plus a western blot detection system (Bio-Rad, Foster, USA). The intensity ratio was the relative expression of VEGF and Ang-I normalized to GAPDH.

Statistical analysis

All experiments were performed with triplicate samples and repeated at least three times. The data were expressed as mean \pm SEM and analyzed through unpaired Student's *t*-test for comparisons between two groups or one-way analysis of variance for multiple comparisons; $P < 0.05$ was considered to indicate statistical significance.

Results

MGF24E promoted EC proliferation via an MAPK/ERK-dependent pathway

The proliferation of ECs is an absolute requirement for angiogenesis. The CCK-8 assay and cell cycle progression analysis were carried out to detect the efficacy of MGF24E on EC proliferation. The CCK-8 assay showed that

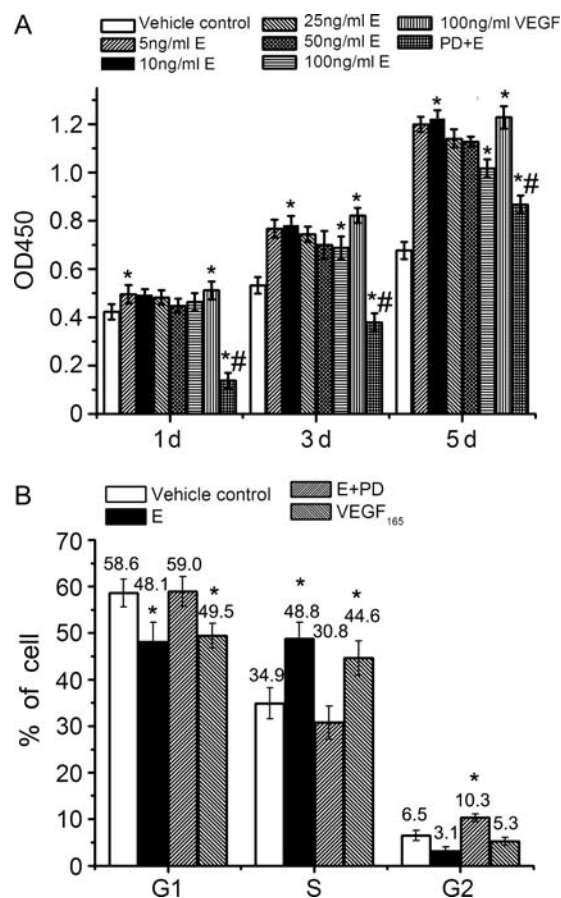


Figure 1 Effects of MGF24E on the cell proliferation ability of EA.hy926 vascular ECs. The cells were pretreated with or without 100 μ M of PD98059 for 30 min prior to 10 ng/ml of MGF24E administration, and incubated in various media. (A) MGF24E-induced cell viability was measured via the CCK-8 assay. (B) Cell cycle dynamics of EA.hy926 cell induced by different treatments measured by flow cytometry analysis. The cell proportions of the various treatments were presented on the top of the bars. PD, 100 μ M of PD98059 (ERK1/2 inhibitor); E, MGF24E. The vehicle control contains RPMI 1640 with 2% FBS and 0.05% (v/v) dimethylsulfoxide. # $P < 0.05$ versus 10 ng/ml of MGF24E; * $P < 0.05$ versus vehicle control; $n = 6$ per concentration.

MGF24E (5–100 ng/ml) induced higher cell proliferation compared with the vehicle control, especially at a low concentration of 5 or 10 ng/ml [Fig. 1, $P < 0.05$]. With 5 days of treatment, MGF24E promoted cell proliferation in a time-dependent manner. At the fifth day, 10 ng/ml of MGF24E increased the proliferation by 80.4% compared with the control [Fig. 1, $P < 0.05$]. MGF24E showed a great pro-proliferation activity. However, no significant difference was found between the 10 ng/ml of MGF24E-induced cell population and the 100 ng/ml of VEGF₁₆₅-induced cell population [Fig. 1, $P > 0.05$]. Moreover, the MGF24E-induced cell proliferation was completely abolished after 30 min of pretreatment with 100 μ M of PD98059 [Fig. 1(A), $P < 0.05$]. These data suggested that MAPK/ERK was completely responsible for the effect of MGF24E on vascular EC proliferation. This

finding is in accordance with the conclusion found in osteoblasts [12].

To determine whether the growth promotion of cells induced by MGF24E was due to the arrest of cell cycle at a certain phase(s), flow cytometry analysis was performed based on DNA content in nuclei stained by propidium iodide. Cell cycle progression analysis showed that 10 ng/ml of MGF24E induced more cells transition into the S phase, resulting in a marked accumulation of cells in the S phase [Fig. 1(B), 48.8% vs. 34.9% of control, $P < 0.05$], and a reduction in G2/M to 3.1% [Fig. 1(B)]. Meanwhile, 100 ng/ml of VEGF₁₆₅ also resulted in a marked accumulation of cells in the S phase [Fig. 1(B), 44.6% vs. 34.9% of control, $P < 0.05$]. However, no significant difference was found between VEGF₁₆₅ and MGF24E. The results also showed that 10 ng/ml of MGF24E, similar to 100 ng/ml of VEGF₁₆₅, promoted cell proliferation by inducing the cell cycle S-phase entry and transition to DNA replication. In accordance with the cell viability results, the inhibition of ERK signaling in the cell cycle analysis exhibited a similar decrease in cell amount in the S phase. This result indicated that the MGF24E-induced S-phase entry and DNA

replication were completely blocked. Taken together, these data revealed that MGF24E promoted cell viability and induced cell cycle S-phase transition via an MAPK/ERK-dependent signaling pathway.

MGF24E enhanced the motogenic capacity of EA.hy926 cells

A transwell assay was conducted to verify whether 10 ng/ml of MGF24E has a chemokinetic property. The cells treated with MGF24E spread into sprouts, but the cells of the vehicle control group curled up into groups [Fig. 2(A)]. In accordance with the observation in Fig. 2(A), the motogenic activity of 10 ng/ml MGF24E was 172.0% higher than that of the vehicle control, but still <100 ng/ml of VEGF₁₆₅ [Fig. 2(B), $P < 0.05$]. The result showing that MGF24E modified the mitogenic capacity of ECs was consistent with the observation in human myogenic precursor cells or human mesenchymal stem cells activated by MGF24E [8,11]. An inhibition of ERK significantly decreased the MGF24E-induced migration by 70.0% [Fig. 2(B), $P < 0.05$], which suggested that the ERK signaling was the main component in the MGF24E-mediated

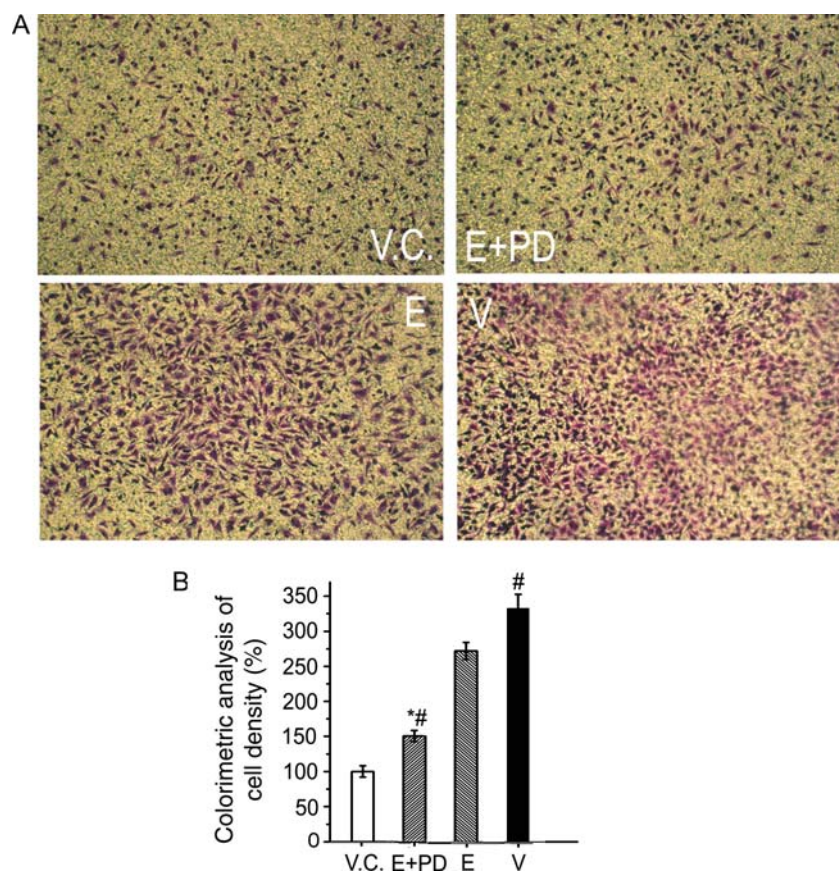


Figure 2 Effects of 10 ng/ml of MGF24E on cell migration The cells were pretreated with or without 100 μ M of PD98059 for 30 min prior to 10 ng/ml of MGF24E administration and incubated in various media for 10 h. (A) Representative light photomicrographs of different groups ($\times 40$). The migrated cells were stained into purple by crystal violet. The cells treated with MGF24E spread into sprouts, but the cells in the vehicle group curled up into groups. (B) Colorimetric analysis of migrated cell density in different groups. V.C., vehicle control containing 0.05% (v/v) dimethylsulfoxide; PD, 100 μ M of PD98059; E, MGF24E; V, 100 ng/ml of VEGF₁₆₅. [#] $P < 0.05$ versus 10 ng/ml MGF24E; ^{*} $P < 0.05$ versus vehicle control; $n > 3$ per group.

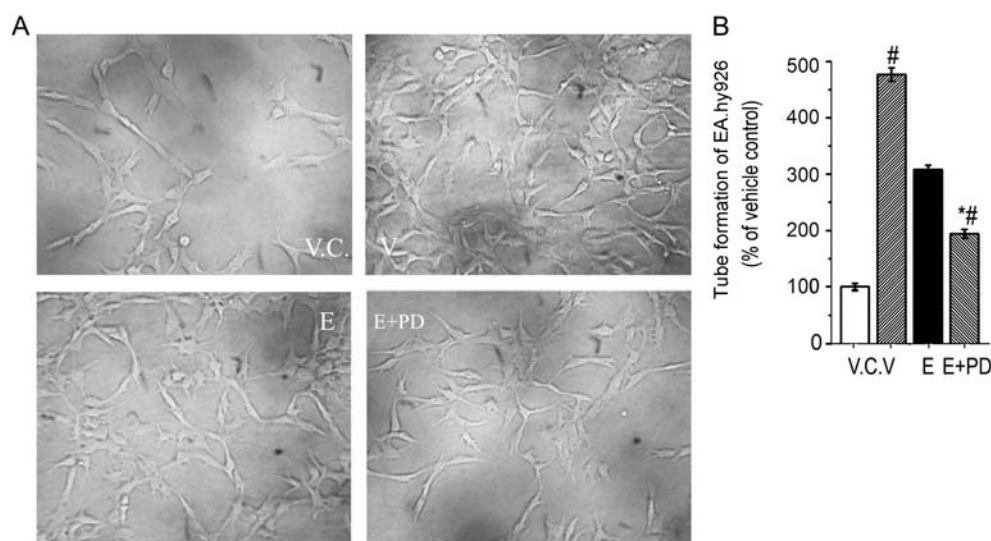


Figure 3 Effects of 10 ng/ml MGF24E on vascular endothelial tubule formation The cells were pretreated with or without 100 μ M of PD98059 for 30 min and incubated in different media for 48 h. (A) Representative light photomicrographs of different groups ($\times 40$). The MGF24E-induced capillary-like structures contained fewer branch points than the vehicle. (B) Summarized data from four separate experiments. V.C., vehicle control containing 0.05% (v/v) dimethylsulfoxide; PD, PD98059; E, MGF24E; V, 100 ng/ml of VEGF₁₆₅. # $P < 0.05$ versus MGF24E; * $P < 0.05$ versus vehicle control; $n > 3$ per group.

intracellular promigratory signal pathway in ECs. These results showed that MGF24E induced mainly a higher migratory activity via the MAPK/ERK pathway.

MGF24E-induced tubulogenesis partially involved ERK-signaling pathway

In vitro matrigel assay showed that 10 ng/ml of MGF24E promoted tube formation. The cells spreading on matrigel aligned with each other and formed branching anastomosing tubes, which produced a tubule-like structure. MGF24E led cell networks to contain more branch points and larger enclosed spaces than the vehicle control [Fig. 3(A)]. In addition, MGF24E increased tubulogenesis to approximately 308.0% of the vehicle control [Fig. 3(B), $P < 0.05$]. The inhibition of ERK1/2 decreased the MGF24E-induced tubulogenesis by 36.0% [Fig. 3(B), $P < 0.05$]. These data showed that the MAPK/ERK-signaling pathway was partially involved in the MGF24E-enhanced tubulogenesis.

MGF24E significantly up-regulated the expression of angiogenic cytokines

The process of angiogenesis is regulated by several growth factors. Among the many proangiogenic mediators, VEGF and Ang-I are two of the most impotent factors, especially for bone regeneration, and their gene and protein expression usually serve as the most important indices in elucidating the angiogenic mechanism [18–20].

Reverse transcriptase-PCR analysis revealed that 10 ng/ml of MGF24E remarkably enhanced the expression of VEGF and Ang-I mRNA [Fig. 4(A)]. Quantitative real-time PCR analysis quantified the expressions of VEGF and

Ang-I mRNA exposed to MGF24E treatment. The MGF24E treatment increased the total amount of VEGF and Ang-I mRNA by 309.0% and 315.0%, respectively, compared with that of the vehicle control [Fig. 4(B), $P < 0.05$]. The total amount of VEGF and Ang-I mRNA even increased by 133.0% and 107.0%, respectively, compared with that of 10% serum medium [Fig. 4(B), $P < 0.05$]. Furthermore, the ERK1/2 inhibitor effectively decreased the MGF24 up-regulated VEGF and Ang-I mRNA expression by 43.4% and 33.1%, respectively [Fig. 4(B), $P < 0.05$].

Western blot analysis further confirmed that MGF24E up-regulated the protein expression level of the angiogenic cytokines. The MGF24E treatment increased the total amount of VEGF and Ang-I protein by 114.0% and 144.0%, respectively, compared with that of the vehicle control [Fig. 4(C), $P < 0.05$]. The total amount of VEGF and Ang-I protein even increased by 56.0% and 88.0%, respectively, compared with that of 10% serum medium [Fig. 4(C), $P < 0.05$]. Moreover, the ERK inhibitor decreased the MGF24 up-regulated VEGF and Ang-I protein expression by 18.6% and 48.4%, respectively [Fig. 4(C), $P < 0.05$]. Taken together, the addition of 10 ng/ml of MGF24E reversed the angiogenic cytokine expressions inhibited by 2% serum starvation.

Discussion

Recent studies have shown that MGF24E plays a positive role on local nerve or muscle tissue repair through the promotion of cell growth, migration, and protection against

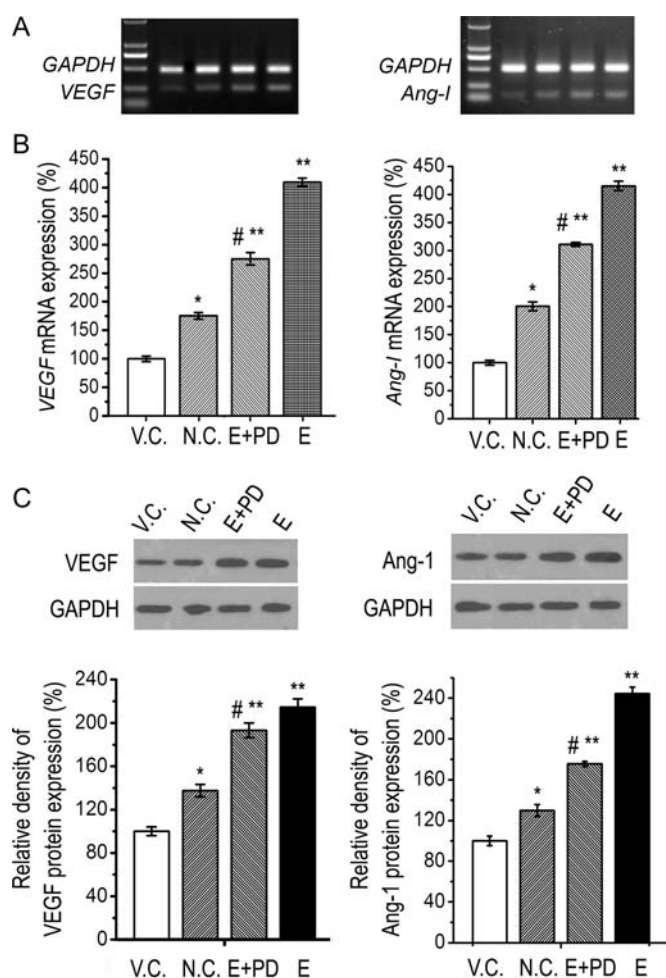


Figure 4 Effects of 10 ng/ml of MGF24E-induced angiogenic cytokines expressions (A) Reverse transcriptase-PCR of *VEGF* and *Ang-I* mRNA expressions. (B) The mRNA expression levels of *VEGF* and *Ang-I* were measured through real-time quantitative reverse transcriptase-PCR. The cells were pretreated with or without 100 μ M of PD98059 for 30 min and incubated in different media for 1 day. (C) MGF24E up-regulated VEGF and Ang-I protein expressions. The cells were incubated in different media for 3 days. V.C., vehicle control containing 0.05% (v/v) dimethylsulfoxide; N.C., normal cell culture medium with 10% FBS containing 0.05% (v/v) dimethylsulfoxide; PD, 100 μ M PD98059; E, MGF24E. # $P < 0.05$ versus MGF24E; * $P < 0.05$ versus vehicle control; ** $P < 0.05$ versus N.C.; $n > 3$ per group.

injury-induced cell apoptosis. The previous study demonstrated that MGF24E promoted bone-defect healing and induced more blood vessels in bone regeneration around the defective areas compared with the control [12], which showed that MGF24E promoted angiogenesis of bone tissue *in vivo*. However, angiogenesis commences by the migration of ECs from the parent vessel *in vivo*, which then proliferate in response to proangiogenic signals, resulting in the formation of nascent capillary sprouts and tubes [21]. This study aims to determine whether MGF24E promotes angiogenesis by enhancing the proliferation, migration, and tube formation of ECs. The results of this study *in vitro* supported the previous speculation and provided

the first evidence that MGF24E-induced angiogenesis in vascular ECs was mediated via the MAPK/ERK-signaling pathway.

In this study, we used the 2% serum-starvation model to mimic the bone-defect microenvironment without adequate nutrients *in vitro*. Preliminary studies show that serum starvation down-regulates some angiogenic cytokine expression in cultured cells, such as VEGF [22]. Angiogenic cytokines, such as VEGF or Ang-I, are key regulatory factors of angiogenesis during osteogenesis or fracture healing [19,20,23]. Therefore, the efficacy of MGF24E on the angiogenic cytokine expression of VEGF or Ang-I in ECs with 2% serum starvation was investigated. The assays benefited to identify the proangiogenic molecular mechanism of MGF24E during bone healing. The total amount of VEGF mRNA and protein in ECs decreased by approximately 43.0% and 27.0% in 2% serum starvation, respectively [Fig. 4(B,C), $P < 0.05$]. However, the reduction in VEGF expression was rectified by the addition of 10 ng/ml of MGF24E treatment. That is, the MGF24E-induced VEGF was higher than that of the cells without serum starvation. In good accordance with the enhanced VEGF expression induced by MGF24E, the expression of Ang-I exhibited similar increases. These results suggested that MGF24E had a protective effect on angiogenesis in ECs with 2% serum-starvation. Furthermore, an ERK inhibitor blocked partially the MGF24E-induced increase in the expressions of VEGF and Ang-I. These results indicated that the protective effects of MGF24E against low serum starvation-suppressed angiogenesis seemed to be relevant to the MAPK/ERK-signaling pathway.

MGF24E is different from IGF-I in terms of peptide sequence and some functions. However, the AKT or ERK signaling pathways of IGF-I usually served as the focus when the signaling transduction pathway of MGF24E, the splicing variant of IGF-I, was investigated. Previous studies have shown that the MGF C-terminal lacks the domain responsible for IGF-I receptor binding [24]. Therefore, MGF24E appears to signal through an IGF-I receptor-independent mechanism [15]. Recent studies have also demonstrated that MGF24E activates ERK signaling, but not Akt in some cell models [9,12,14]. In this study, ERK signaling was completely responsible for the MGF24E-induced proliferation and mostly involved in migration. At the same time, it partially participated in the MGF24E-induced tubulogenesis and angiogenic factor expression. This finding indicates that some actions of MGF24E on vascular EC may not be forecasted completely via the MAPK/ERK-signaling pathway. Some other MAPK pathways, such as the p38 pathway, may be involved in MGF24E-induced angiogenic events. The p38 pathway has been demonstrated to be involved in the migration of human umbilical vein endothelial cells

(HUVECs) [25]. The given hypothesis needs further investigation to explore the detailed signaling pathway mechanisms.

In summary, the results of this study suggested that a low concentration of MGF24E can promote angiogenesis by enhancing the angiogenic factor expression that involved the MAPK/ERK pathway. The new proangiogenic activity of MGF24E would contribute to some further studies as an angiogenetic therapy of injury healing.

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