### Title
Reduced ghrelin in endothelial cells plays important mechanistic role in aging-related impairment of angiogenesis.




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# REDUCED GHRELIN IN ENDOTHELIAL CELLS PLAYS IMPORTANT MECHANISTIC ROLE IN AGING-RELATED IMPAIRMENT OF ANGIOGENESIS

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Ghrelin, a hormone produced mainly by gastric mucosal cells stimulates growth hormone (GH) release. Ghrelin is also expressed in the endothelial cells of blood vessels suggesting its physiological role and a function in these cells. We recently demonstrated that ghrelin induces angiogenesis - new capillary blood vessel formation- in neonatal human microvascular endothelial cells (HMVECs). Angiogenesis is impaired in aging individuals both *in vitro* and *in vivo*, but the precise mechanism(s) of this phenomenon is unknown. We examined whether HMVECs derived from aging individuals (66 years and 90 years old), 66-HMVECs and 90-HMVECs have reduced ghrelin levels *vs*. neonatal (Neo) HMVECs and whether treatment with exogenous ghrelin can restore impaired *in vitro* angiogenesis on matrigel in aged HMVECs. Ghrelin levels were reduced in the aged HMVECs by 3.2-fold ($p<0.05$) compared to Neo-HMVECs. Angiogenesis was significantly decreased in the aged 66- and 90-HMVECs by 39.7% ($p = 0.003$) and 62.4% ($p = 0.003$), respectively compared to Neo-HMVECs. Treatment with exogenous ghrelin significantly reversed impaired angiogenesis in aged HMVECs with the $EC_{50}$ 0.05 nM. Ghrelin induced angiogenesis in Neo-HMVECs mainly through ERK2 activation. This study is the first demonstration that reduced ghrelin is one of the factors responsible for aging-related impairment of angiogenesis.

Key words: *ghrelin, human microvascular endothelial cells, aging, angiogenesis*

## INTRODUCTION

Ghrelin is a peptide hormone secreted by gastric mucosal cells that stimulates release of human growth hormone at the hypothalamic and pituitary levels (1-4). Ghrelin has been recently implicated in regulation of cardiovascular functions including vasodilation, increased contractility and cardioprotection (5-8). Moreover, exogenous ghrelin inhibits proinflammatory response in human umbilical vein endothelial cells *in vitro* and reverses endothelial dysfunction in patients with metabolic syndrome (9, 10). Recent studies have demonstrated that ghrelin exerts a potent protective action on the gastric mucosa and accelerates the healing of ischemia/reperfusion-induced gastric lesions (11, 12). Since healing of gastric lesions requires angiogenesis - sprouting new capillary blood vessels (13), this indirectly indicates that ghrelin may stimulate angiogenesis. This contention was directly proven by our recent studies, which demonstrated that ghrelin is constitutively expressed in neonatal human microvascular endothelial cells (HMVECs) and exogenous ghrelin induces *in vitro* angiogenesis in these cells *via* ERK2 signaling pathway (14). The role of angiogenesis and growth factors such as VEGF and transforming growth factor-beta (TGF-β) in healing of gastric ulceration has been investigated (15, 16). Aging is associated with impaired angiogenesis (17-23). However, the cellular and molecular mechanisms of impaired angiogenesis in aging are not well defined. The aims of this study were to determine the expression of ghrelin in aging and neonatal HMVECs and to determine whether exogenous ghrelin can reverse impaired angiogenesis in aged HMVECs.

## MATERIAL AND METHODS

### *Cell lines and reagents*

Neonatal HMVECs (Neo-HMVECs) were obtained from Cambrex Biosciences (Walkersville, MD). Aged HMVECs from 66 and 90 years old donors (66-HMVECs and 90-HMVECs) were obtained from PromoCell (Heidelberg, Germany) and Cambrex Biosciences (Walkersville, MD), respectively. Cells were maintained in culture as an adherent monolayer in EGM-2 MV endothelial cell growth media (Cambrex Biosciences). Recombinant human ghrelin was purchased from Phoenix Pharmaceutical (Belmont, CA). Anti-phospho ERK (SC-7383) and anti-ERK (SC-154) were obtained from Santa Cruz (Santa Cruz, CA) and used at a dilution of 1:500. MEK inhibitor PD98059 was purchased from BIOMOL (Plymouth Meeting, PA). VEGFR2 inhibitor 1 (Z)-3-((2,4-Dimethyl-3-(ethoxycarbonyl)pyrrol-5-yl)methylidenyl)indolin-2-one) was purchased from Calbiochem/EMD Biosciences (#676480, San Diego, CA).

*These authors contributed equally to the manuscript

*ELISA measurement of ghrelin levels*

We examined ghrelin levels in cell culture supernatants and in cell lysates of HMVECs using the human Bioassay ELISA kit (US Biological, Swampscott, MA) according to the manufacturer's protocol. Cells were grown in 6-well plate for 48 hrs and the cell culture supernatant and cell lysate were collected. Fifty µl of either: culture supernataunt, cell lysates or recombinant human ghrelin protein at different concentrations, and 25 µl of ghrelin polyclonal antibody and 25 µl biotinylated ghrelin standard peptide were added to each well. After 2 h incubation, the plates were washed, and the immunoreactivity was determined using the avidin-HRP-TMB detection system. The reactions were stopped by adding 100 µl of 0.18 N $H_2SO_4$ and absorbance was determined using an ELISA microtiter plate reader (DYNEX Technologies, Inc. Chantilly, Virginia) at 450 nm. A standard curve of the absorbance and ghrelin concentration was plotted and the ghrelin levels in the samples determined from this standard curve.

*Immunocytochemical (ICC) staining for ghrelin receptor (GHSR1)*

Ghrelin receptor (GHSR1) expression and localization in HMVECs were analyzed by ICC staining as described in our previous study (14). Briefly, endothelial cells were plated on 24-well coverslips coated with rat type I collagen gel, cultured for 48 h and used for immunostaining. Samples were washed twice with PBS and fixed in 4% paraformaldehyde (Sigma-Aldrich) in PBS for 10 min and permeabilized with acetone for 5 min. After washing twice with PBS, cells were incubated with a blocking solution (DAKO, Carpentaria, CA) for 7 min at room temperature. Excess blocking solution was drained and samples were incubated with anti-GHSR1 antibodies for 2 h at room temperature. The samples were then rinsed with PBS and incubated with Alexa-conjugated secondary antibody (Molecular Probes, Eugene, CA). Cells were then washed, mounted using anti-fade mounting media (Molecular Probes, Eugene, CA) and examined under a Nikon epifluorescence microscope. As a negative control we performed staining using all reagents except the primary antibody.

*In vitro angiogenesis on matrigel*

First, we examined angiogenic ability of neo- and aged-HMVECs using *in vitro* capillary tube formation assay performed as described in our previous studies (14, 24). Briefly, 2 x $10^4$ HMVECs were plated on a Matrigel (BD Biosciences, Mountain View, CA) coated 48-well plate with either medium alone or medium containing ghrelin 0.1, 1 and 10 nM. To determine the mechanism of ghrelin-induced angiogenesis, cells were treated with PD98059 (ERK inhibitor, 10 µM) for 30 min prior to treatment with ghrelin. After 6 hours, the plates were examined for capillary tube formation under an inverted Nikon microscope and photographed. Each assay was done in triplicate and each experiment was repeated three times. The total length of capillary tube in control and each of treatment groups was measured in 5 random fields on coded samples by two independent observers. The tube formation was examined and quantified using the image analytical software - Metamorph version 7.0 (Universal Imaging Corporation, West Chester, PA)

*Effect of exogenous ghrelin on endothelial cell proliferation*

We examined endothelial cell proliferation using bromodeoxyuridine (BrdU) ELISA assay (Chemicon, Temecula, CA) and BrdU immunostaining. Briefly, endothelial cells were seeded into 96-well culture plates in triplicate and allowed to adhere overnight. The cultures were then washed and cultured with medium alone (control) or medium containing different concentrations of ghrelin. Following 24 hr incubation, cell proliferation was determined by BrdU ELISA following the manufacturer's instruction.

*Western blotting*

Cell lysates were subjected to SDS-PAGE and transferred onto a nitrocellulose membrane. The membranes were incubated with primary antibody against p-ERKs or total ERKs followed by peroxidase-conjugated secondary antibody. Immunoreactive proteins were visualized using ECL detection system (Amersham, Arlington Heights, IL), similarly as in our previous study (24).

*Statistical analysis*

Values are expressed as means ± SD. Student's t test was used to compare data between two groups. One-way ANOVA followed by Bonferroni correction was used to compare data between three or more groups. A P value < 0.05 was considered statistically significant.

## RESULTS

*Aged HMVECs exhibit decreased ghrelin expression*

Our previous study using neonatal HMVECs was the first to demonstrate the expression of ghrelin and its receptor - GHSR1 (14) in human endothelial cells. In the present study, we examined the levels of ghrelin and its receptor (GHSR1) in neonatal and aged HMVECs. Using ELISA we measured the levels of ghrelin in HMVECs. Compared to Neo-HMVECs, the aged 90- HMVECs had significantly 3.2-fold ($p<0.05$) reduced ghrelin level (*Fig. 1*). In a previous study we found GSHR1 mRNA and protein expression in neonatal HMVECs. In the present study immunofluorescence staining demonstrated no significant difference in expression of ghrelin receptor (GSHR1) between Neo-HMVECs and aged 90- HMVECs (data not shown).

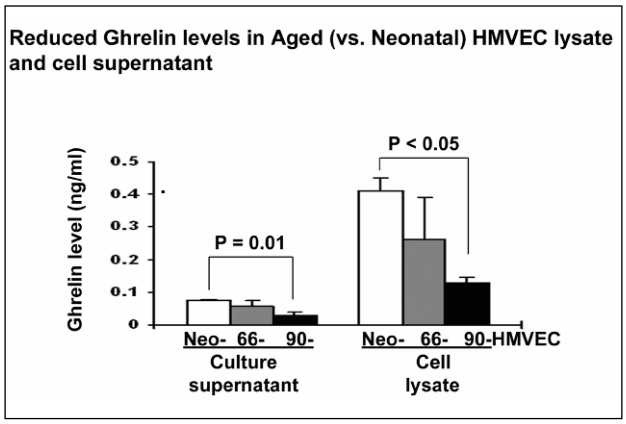


*Fig. 1.* Reduced ghrelin levels in aged HMVECs. Ghrelin levels were determined in cell culture supernatants and cell lysates of Neo- and aged HMVECs by ELISA. The values are mean ghrelin concentration ± S.D. This is representative of two experiments done in duplicate (n=4).

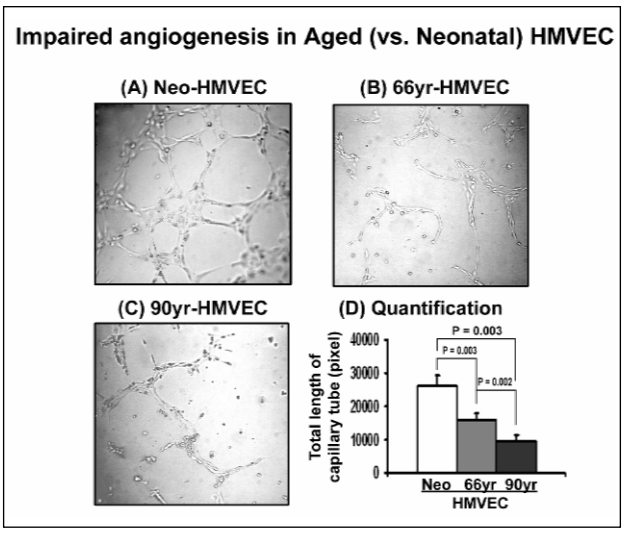


*Fig. 2.* Impaired angiogenesis in aged HMVECs. Cells were grown on matrigel for 6 hrs under normal growth condition (a) Neo-HMVECs, (b) 66-HMVECs, (c) 90-HMVECs, (d) quantification of capillary tube formation in Neo- and aged HMVECs. The values are mean capillary tube length ± S.D. This is representative of three experiments done in triplicate (n=9).

*Aged HMVECs exhibit decreased in vitro angiogenesis*

Using an *in vitro* angiogenesis assay on matrigel, we compared the angiogenic ability of Neo- and aged HMVECs. In aged 66- and 90-HMVECs, *in vitro* angiogenesis was significantly decreased by 39.7% (p=0.003) and 62.4%-fold (p=0.003) respectively, compared to Neo-HMVECs (*Fig. 2*).

*Ghrelin increases in vitro angiogenesis in aged HMVECs*

We next examined if exogenous ghrelin could reverse impaired angiogenesis in aged HMVECs using an *in vitro* angiogenesis assay on matrigel. Ghrelin at 0.1 nM significantly increased *in vitro* angiogenesis in Neo-HMVECs by 36.5%-fold (p<0.01) (*Fig. 3*). In aged HMVECs, 10 nM ghrelin significantly increased by 36.7% (p<0.05) *in vitro* angiogenesis to levels similar seen in untreated Neo-HMVECs. These data demonstrate that ghrelin can partly reverse impaired angiogenesis in aged HMVECs (*Fig. 3*).

*Ghrelin stimulates proliferation of HMVECs*

One of the essential components of angiogenesis is endothelial cell proliferation. Since aged HMVECs exhibit decreased ghrelin expression, next we determined if exogenous ghrelin can increase cell proliferation in HMVECs. Ghrelin added to culture medium at 0.1 nM significantly increased endothelial cell proliferation in Neo-HMVECs by 2.3-fold (p<0.05) compared to untreated cells (*Fig. 4*). However in aged HMVECs, a substantially higher doses of ghrelin, 1 nM was required to increase cell proliferation by a significant 2.2-fold (p<0.05) compared to untreated cells (*Fig. 4*). These data indicate that aged HMVECs have reduced ghrelin levels are also less responsive to ghrelin compared to Neo-HMVECs.

*Ghrelin induces phosphorylation of ERKs*

Since MAPK/ERK2 pathway is the mitogenic signaling pathway, the most likely mechanism for ghrelin mediated cell proliferation and angiogenesis is through the MAPK/ERK2. To examine whether the mechanism of ghrelin action involves MAPK/ERK2, we next examined using Western blotting the phosphorylation of ERKs in Neo- and aged HMVECs in response to ghrelin treatment. Ghrelin stimulates the phosphorylation of ERKs in Neo- and aged HMVECs by 2.4-fold or 139% (p<0.05) and 57.4% (p<0.05) respectively (*Fig. 5*).

*ERK phosphorylation is required for angiogenesis*

To further determine whether ghrelin-induced angiogenesis is mediated through ERK2 activation, Neo-HMVECs were treated with the MEK/ERK2 inhibitor PD98059 prior to ghrelin treatment and then subjected to *in vitro* angiogenesis assay on matrigel. Inhibition of ERK2 phosphorylation by PD98059 abolished ghrelin-induced *in vitro* angiogenesis (*Fig. 6*). These data demonstrate that ERK phosphorylation is required for angiogenesis in HMVECs.

## DISCUSSION

Aging is associated with impaired angiogenesis - formation of new capillary blood vessels. Healing of tissue injury including gastric lesions and ulcers requires angiogenesis (13). Recent studies have demonstrated that ghrelin stimulates gastric growth, exerts a potent protective action on the gastric mucosa and accelerates the healing of alcohol- induced and

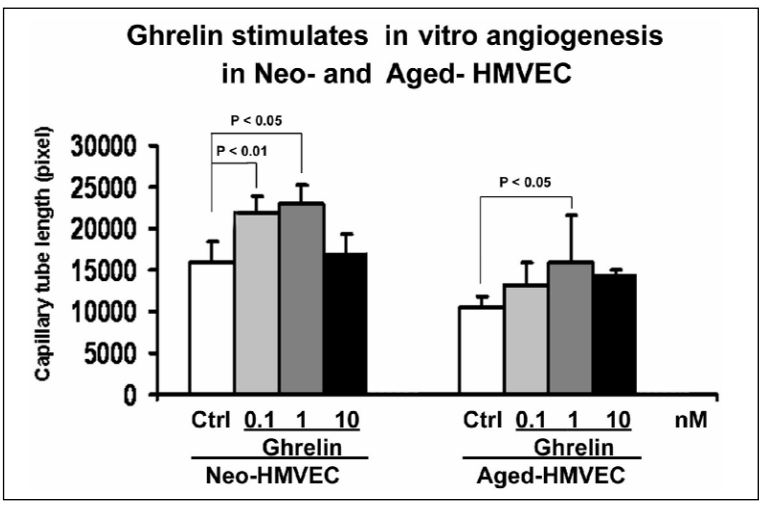


*Fig. 3.* Ghrelin increases *in vitro* angiogenesis in aged HMVECs. Cells were grown on matrigel for 6 hrs under normal growth condition in the presence or absence of ghrelin. The values are mean capillary tube length ± S.D. This is representative of three experiments done in triplicate (n=9).

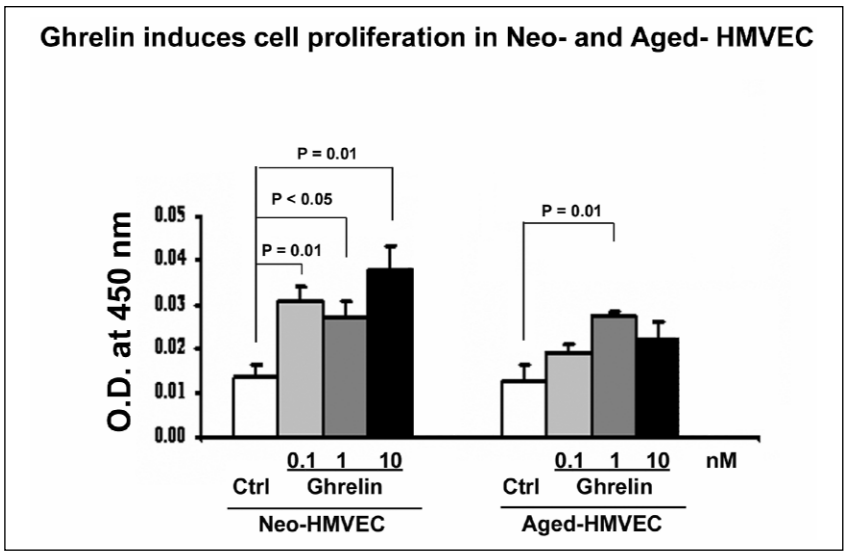


*Fig. 4.* Ghrelin stimulates proliferation of HMVECs. Cell proliferation was assessed by BrdU labeling in Neo- and aged HMVECs. The values are mean viable cell O.D. ± S.D. of triplicate culture. This is representative of three experiments done in triplicate (n=9).

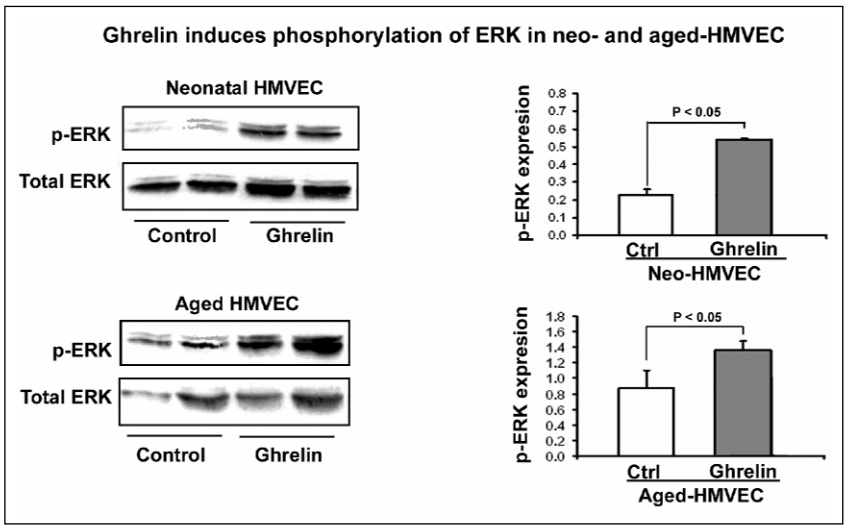


*Fig. 5.* Ghrelin induces phosphorylation of ERKs. Effect of 1 nM ghrelin on phosphorylation of ERKs in Neo- and aged HMVECs was examined using Western blotting. The values shown are mean intensity of bands ± S.D. This is representative of two experiments done in duplicate (n=4).

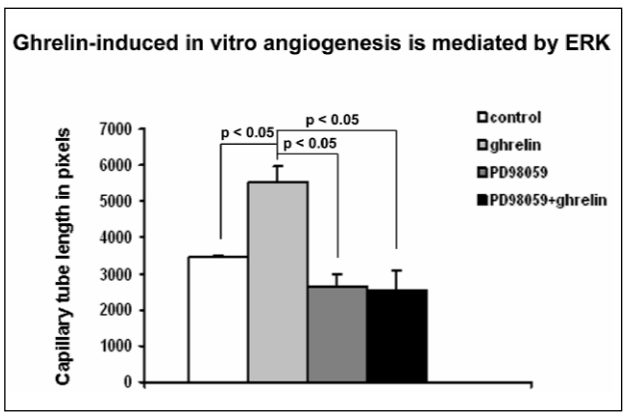


*Fig. 6.* ERK phosphorylation is required for angiogenesis. Capillary tube formation in ghrelin treated cells in the presence or absence of the Erk inhibitor PD98059 was measured on matrigel. Photograph was taken after 6 hrs. The values are mean capillary tube length ± S.D. This is representative of three experiments done in triplicate (n=9).

ischemia/reperfusion-induced gastric lesions (11, 12, 25, 26). Ghrelin has also been implicated in regulation of cardiovascular functions including vasodilation, increased contractility and cardioprotection (5-8), inhibiting proinflammatory response in human umbilical vein endothelial cells (9) and reversing endothelial dysfunction in patients with metabolic syndrome (10). These studies taken together suggest a pro-angiogenic role for ghrelin. However, there have been no studies exploring the role and mechanism of ghrelin in promoting angiogenesis and also its regulation during aging. Also, the cellular and molecular mechanisms of impaired angiogenesis in aging remain not fully explained. The aims of this study were to determine the expression of ghrelin during aging, the role of ghrelin in angiogenesis and the mechanism of ghrelin-induced angiogenesis in aged HMVECs.

Our previous study using neonatal HMVECs was the first to demonstrate the expression of ghrelin and its receptor (GHSR1) and that exogenous ghrelin promotes angiogenesis in neonatal HMVECs (14). Our present study reveals the presence of ghrelin and ghrelin receptor in both neonatal and aged HMVECs and further demonstrates reduced ghrelin in aged HMVECs. Reduced ghrelin is responsible, at least in part for impaired angiogenesis in aged HMVECs since exogenous ghrelin can significantly restore angiogenesis in aged HMVECs. We also demonstrated that aged HMVECs have reduced phosphorylation of ERKs.

This is the first demonstration that reduced angiogenesis in aging HMVECs is due to reduced ghrelin. Previous studies on angiogenesis using pathological models such as ischemia in the rabbit hind limb have demonstrated impaired angiogenesis in

aging tissues and have implicated reduced angiogenesis in delayed wound healing (21). Recent studies have suggested use of ghrelin and its receptor (GHS-R1A) antagonists as novel therapeutic approaches for age-related metabolic and physiological changes (27, 28). However, the role and regulation of angiogenesis by ghrelin during aging has not been examined in endothelial cells. Our study showed for the first time that ghrelin levels are significantly reduced in aged HMVECs. Moreover, treatment with ghrelin partly restores angiogenesis in aged HMVECs demonstrating that the reduction in ghrelin levels accounts in part for the significantly reduced angiogenic capability of aged HMVECs. Regarding the relevance of *in vitro* angiogenesis to *in vivo* condition, a recent study demonstrated that during *in vivo* angiogenesis, endothelial cells form capillary like endothelial tubes and develop lumina, very similar to those seen during *in vitro* angiogenesis models (29). Therefore, *in vitro* angiogenesis process closely resembles *in vivo* angiogenesis and likely represents a relevant model (29).

Cell proliferation significantly contributed to the process of *in vitro* angiogenesis, but this process in addition also requires, as demonstrated in previous publication, G-actin polymerization, cell migration and assembly of capillary tubes (30).

We have demonstrated that the mechanism of ghrelin action involves phosphorylation and thus activation of ERKs and that this process is reduced in aged HMVECs. Activation of MAPK pathway and phosphorylation of ERKs by ghrelin was reported previously in hepatoma cells and neonatal HMVECs (31, 14). However, these studies did not examine ghrelin's action on ERK activation in endothelial cells during aging.

In summary, our present study has identified reduced ghrelin levels in aged endothelial cells as one of the mechanisms that mediate the impairment of angiogenesis. Ghrelin plays a key role in angiogenesis by promoting endothelial cell proliferation through activation of the MAPK/ERK2 mitogenic signaling pathway. Our findings provide a rationale for use of ghrelin as a novel therapy for improving aging related impairment of angiogenesis.

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