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AGING IMPAIRS TRANSCRIPTIONAL REGULATION OF VASCULAR ENDOTHELIAL GROWTH FACTOR IN HUMAN MICROVASCULAR ENDOTHELIAL CELLS: IMPLICATIONS FOR ANGIOGENESIS AND CELL SURVIVAL

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In some tissues, aging impairs angiogenesis and reduces expression of vascular endothelial growth factor A (VEGF), a fundamental regulator of angiogenesis. We previously examined angiogenesis in aging and young gastric mucosa *in vivo* and *in vitro* and showed that an imbalance between expressions of VEGF (pro-angiogenic factor) and endostatin (anti-angiogenic protein) results in an aging-related impairment of angiogenesis in rats. However, the human relevance of these findings, and whether these mechanisms apply to endothelial cells derived from other tissues, is not clear. Since P-STAT3 and P-CREB are transcription factors that, in association with HIF-1α, can activate VEGF gene expression in some cells (*e.g.*, liver cancer cells, vascular smooth muscle cells), we examined the expression of these two proteins in human dermal microvascular endothelial cells (HMVECs) derived from aging and neonatal individuals. We examined and quantified *in vitro* angiogenesis, expression of VEGF, P-STAT3, P-CREB and importin-α in HMVECs isolated from neonates (neonatal) and a 66 year old subject (aging). We also examined the effects of treatment with exogenous VEGF and endostatin on *in vitro* angiogenesis in these cells. Endothelial cells isolated from aging individuals had impaired angiogenesis (vs. neonatal endothelial cells) and reduced expression of VEGF mRNA and protein. Aged HMVECs also had reduced importin-α expression, and reduced expression and nuclear translocation of P-STAT3 and P-CREB. Reduced VEGF gene expression in aged HMVECs strongly correlated with the decreased levels of P-STAT3, P-CREB and importin-α in these cells. Our study clearly demonstrates that endothelial cells from aging individuals have impaired angiogenesis and reduced expression of VEGF likely due to impaired nuclear transport of P-STAT3 and P-CREB transcription factors in these cells.

Key words: *aging, angiogenesis, vascular endothelial growth factor, endothelial cells, apoptosis, P-CREB, P-STAT3, importin-α*

INTRODUCTION

Aging has been demonstrated in several tissues to be associated with impaired angiogenesis - the formation of new blood vessels from pre-existing vessels (1, 2), and with reduced expression of vascular endothelial growth factor A (referred here to as VEGF) (3-9). VEGF is the fundamental regulator of angiogenesis (10, 11). It induces cell growth, migration, and tube formation in endothelial cells, which are the key targets and effectors of angiogenesis (4, 12). In some cells, VEGF gene expression involves transcriptional activation of the VEGF gene by proteins such as HIF1α, Signal transducer and activator of transcription (STAT) 3 and cAMP response element-binding protein (CREB) (13). STAT3 in some cells such as liver cells, glioma cells and human coronary artery smooth muscle cells directly upregulates VEGF, Ref-1, p21, and anti-apoptotic genes such as Bcl-xL (14-17). CREB was first described as a cAMP-responsive transcription factor regulating the somatostatin gene (18) and is known to activate VEGF gene expression in mouse macrophages and in human prostate cancer cells (19, 20). However, STAT3 and CREB are latent cytoplasmic transcription factors and require transport into the nuclei of cells in order to activate transcription of target genes. Importins -α and -β mediates nuclear transport of various proteins including STAT3 and CREB into cell nuclei (21-27). We previously identified importin-α as a critical factor regulating angiogenesis and VEGF expression in gastric mucosal endothelial cells (28). However, the human relevance of that study and the applicability of those findings to endothelial cells from other tissues have not been established.

In this study, we examined *in vitro* angiogenesis and the expression of VEGF, P-STAT3, P-CREB and importin-α in neonatal and aged human dermal microvascular endothelial cells (HMVECs). Here we present evidence that in aged HMVECs angiogenesis is impaired and VEGF expression is reduced vs. neonatal HMVECs due to the dysregulation of factors that regulate VEGF expression (importin-α, P-STAT3 and P-CREB). These novel findings together with our previous studies suggest that these defects associated with aging are common to the vasculature of different tissues. They may also have important

implications for angiogenesis, endothelial cell viability, and tissue injury healing.

MATERIALS AND METHODS

These studies were approved by the R&D Committee at the VA Healthcare System, Long Beach, CA.

Cell Culture

Human dermal microvascular endothelial cells (HMVECs) - aged HMVECs (C-12212, Lot # 5062103.1 isolated from a 66 year-old individual and cryopreserved) and neonatal HMVECs (CC-2505, Lot # 2F0391) were obtained from PromoCell (Heidelberg, Germany) and Lonza Walkersville Inc. (Walkersville, MD), respectively. Both cells types were free of mycoplasma and were negative for HIV, HBVDNA and HCVRNA. Cells were cultured in EGM-2-MV medium (Lonza Walkersville Inc., Walkersville, MD) in an atmosphere of 5% CO_2 and 95% air at 37°C in a humidified incubator and were used between passage numbers 4 and 7. Subcultures were made from confluent stock cultures by trypsinization in PBS containing 0.5 mM EDTA and 0.25% trypsin.

In vitro angiogenesis assay

Endothelial tube formation was examined by an *in vitro* angiogenesis assay as described in our previous studies (28-31). Aged and neonatal HMVECs were cultured in complete endothelial cell growth medium in 60-mm tissue culture dishes until they were about 80% confluent. The growth medium was replaced with basal medium supplemented with 1% FBS, 100 µg/ml heparin and antibiotics, and the cells were incubated for 24 more hours. Cells were then treated with endostatin (15 µg/ml, Sigma, St Louis, MO) or PBS (controls) for 2 hours. The cells were then trypsynized, counted, resuspended in basal medium supplemented with 1% FBS and seeded onto growth factor-reduced Matrigel (Becton Dickinson, Bedford, MA) in the presence of either PBS, endostatin (15 µg/ml, Sigma, St Louis, MO), VEGF (20 ng/ml, R&D Systems) or endostatin and VEGF for up to 24 hours. Six and 24 hours later, the cells were photographed using a Nikon inverted phase contrast photomicroscope (Nikon USA, Garden City, NY) and analyzed using a video image analysis system - MetaMorph 7.0 (Molecular Devices, Downington, PA). Endothelial tube formation was quantified by measuring the total length of capillary tubes in randomly selected fields under 200× magnification as described in our previous studies (28, 30).

Determination of vascular endothelial growth factor mRNA by reverse transcription real-time quantitative polymerase chain reaction (PCR)

Total cellular RNA was isolated from aged and neonatal HMVECs and VEGF mRNA levels were determined by real-time RT-PCR using pre-validated VEGF QuantiTect assays (Qiagen, Valencia, CA) and the iCycler real-time PCR detection system (Bio-Rad, Hercules, CA) as described in our previous study (29). Relative mRNA levels were calculated using the $2^{-\Delta\Delta Ct}$ method and glyceraldehyde-3-phosphate dehydrogenase was used as a reference.

Western blot analysis

Total cellular protein or cytoplasmic and nuclear proteins were isolated from aged and neonatal HMVECs using commercial buffers and kits (Pierce, Fisher Scientific, Pittsburg, PA). Expression of VEGF, importin-α, P-STAT3, P-CREB, STAT3 and CREB was determined in these samples by Western blotting with respective specific antibodies using methods described in our previous study (28). β actin was used as a reference. The primary antibodies used were against VEGF (1:250, sc-507, Santa Cruz Biotechnology, Santa Cruz, CA), importin-α (1:500, I1784, Sigma-Aldrich, St. Louis, MO), P-STAT3 (Tyr 705, 1:250, sc-8059, Santa Cruz Biotechnology, CA), P-CREB (Ser133, 1:250, #9191, Cell Signaling Technology, Danvers, MA), STAT3 (1:500, sc-8019, Santa Cruz Biotechnology, CA), CREB (1:250, #9197, Cell Signaling Technology, Danvers, MA), or β actin (1:1000, A5316, Sigma-Aldrich, St Louis, MO).

Immunohistochemical staining for P-STAT3 and P-CREB

To determine the mechanisms underlying reduced expression of VEGF, we examined the expression and localization of P-STAT3 and P-CREB (factors that can regulate VEGF gene expression) in aged and neonatal HMVECs by immunohistochemical staining using specific antibodies against P-STAT3 (1:100, Santa Cruz Biotechnology, CA) and P-CREB (1:100, Cell Signaling Technology, Danvers, MA). The staining intensity was quantified using MetaMorph 7.0 (Molecular Devices, Downington, PA).

Statistical analysis

Data are presented as mean ± S.D. Statistical significance was analyzed by either Student's t-test, analysis of variance or Pearson's correlation. A p value less than 0.05 was considered statistically significant.

RESULTS

Impaired angiogenesis and reduced VEGF expression in aged (vs. neonatal) HMVECs. We examined *in vitro* angiogenesis on Matrigel in neonatal and aged HMVECs, and quantified the expression of VEGF mRNA and protein in these cells. These studies showed that aged HMVECs had significantly reduced *in vitro* angiogenesis by 3.9-fold ($p<0.001$) (*Fig. 1A*); and, that aged HMVECs had significantly reduced expression levels of VEGF mRNA and VEGF protein by 2.2-fold ($p<0.05$) and 2-fold ($p<0.001$), respectively (*Fig. 1B*), which is similar to our *in vivo* findings in gastric mucosa of aging rats (9). These results further showed that reduced expression of VEGF protein closely correlated with reduced angiogenesis (correlation coefficient r=0.93; $p<0.05$).

Treatment with exogenous vascular endothelial growth factor and endostatin affects in vitro angiogenesis in human dermal microvascular endothelial cells

Since our previous *in vivo* study demonstrated that endostatin (anti-angiogenic protein) is increased in aging gastric mucosa (9), we examined the effects of exogenous VEGF and endostatin on *in vitro* angiogenesis in neonatal and aged HMVECs. Treatment of aged HMVECs with exogenous VEGF only partly restored impaired angiogenesis by 1.9-fold ($p<0.01$) (*Fig. 2A*). Treatment of neonatal HMVECs with exogenous endostatin significantly reduced *in vitro* angiogenesis by 2.5-fold ($p<0.001$) (*Fig. 2B*) to the levels present in aged-HMVECs and significantly reduced the angiogenic response of these cells to VEGF treatment. This finding further supports our previous *in vivo* study demonstrating that impairment of angiogenesis in

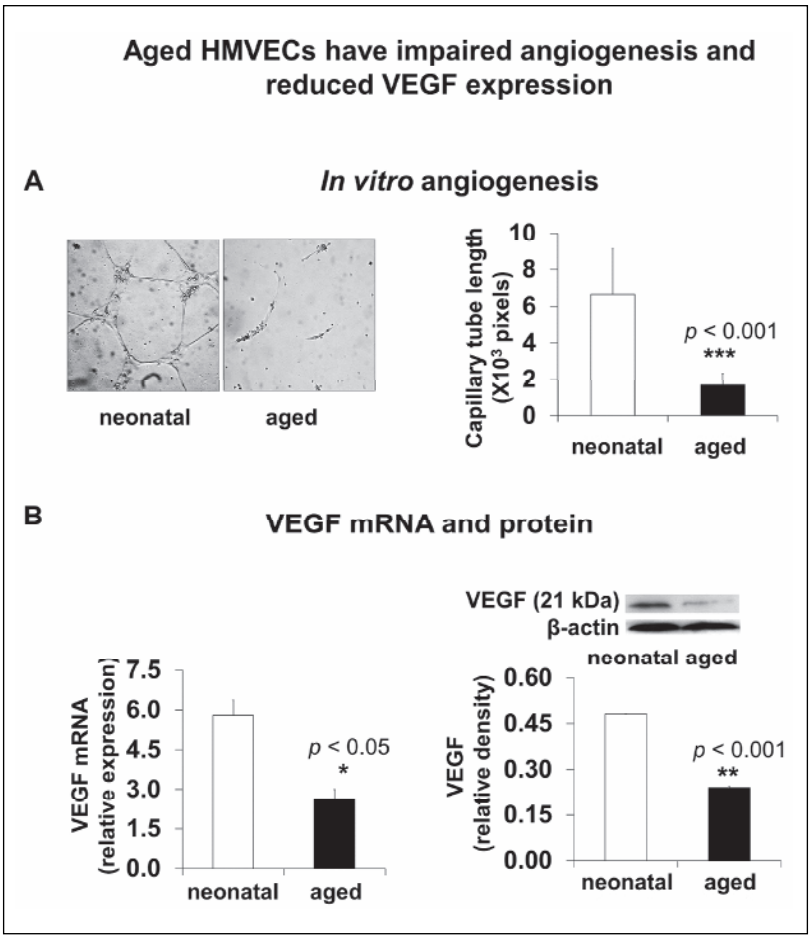

Fig. 1. Aged HMVECs exhibit impaired *in vitro* angiogenesis and reduced VEGF mRNA and protein levels. (*A*) Capillary tube formation reflecting *in vitro* angiogenesis in neonatal and aged HMVECs. Values are mean ± S.D. of 3 independent experiments. Aged HMVECs have significantly reduced (*** $p<0.001$) *in vitro* angiogenesis. (*B*) Determination of VEGF mRNA and protein expression by real time RT-PCR analysis and Western blotting, respectively. Values are mean ± S.D. of 3 independent experiments. Aged HMVECs have significantly reduced VEGF mRNA levels (* $p<0.05$) and VEGF (*** $p<0.001$) protein levels vs. neonatal HMVECs.

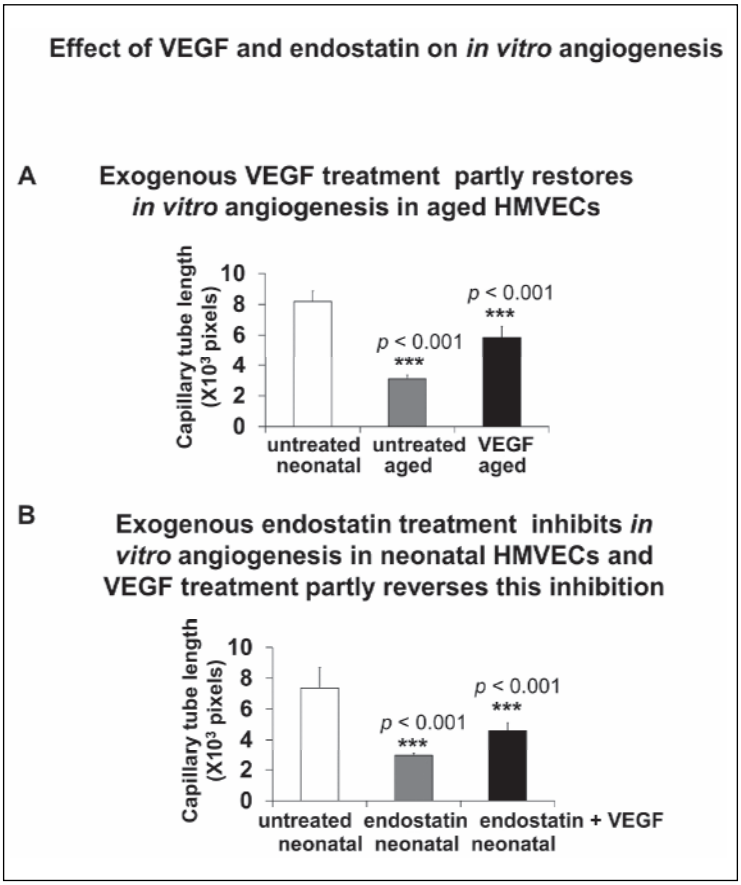

Fig. 2. Treatment with exogenous VEGF partly restores *in vitro* angiogenesis in aged HMVECs and endostatin treatment of neonatal HMVECs reduces *in vitro* angiogenesis. Capillary tube formation reflecting *in vitro* angiogenesis in neonatal and aged HMVECs was quantified as described in Materials and Methods. Values are mean ± S.D. of 3 independent experiments. (*A*) Treatment of aged HMVEC with exogenous VEGF increases *in vitro* angiogenesis (** $p<0.01$) but not to the extent seen in neonatal HMVEC. (*B*) Treatment of neonatal HMVEC with exogenous endostatin inhibits *in vitro* angiogenesis and significantly attenuates VEGF-induced *in vitro* angiogenesis (** $p<0.01$). Values are mean ± S.D. of 3 independent experiments.

aging gastric mucosa is due to an imbalance between VEGF and endostatin (9).

Reduced expression of P-STAT3 and P-CREB in aged (vs. neonatal) human dermal microvascular endothelial cells

To determine the mechanism of decreased VEGF mRNA and protein in aged vs. neonatal HMVECs, we examined the expression of P-STAT3 and P-CREB, transcription factors that are essential in addition to HIF1 for activation of the VEGF gene promoter (13). Aged HMVECs had significantly reduced expression of P-STAT3 protein by 2.7-fold (p<0.001) (*Fig. 3A*). The expression of P-STAT3 protein closely correlated with VEGF mRNA expression (correlation coefficient r=0.983; p<0.01). Immunostaining confirmed that nuclear levels of P-STAT3 protein was decreased in both nuclei and cytoplasm in aged (vs. neonatal) HMVECs (*Fig. 3B*). Aged HMVECs also had significantly reduced expression of P-CREB protein by 1.5-fold (p<0.001) (*Fig. 3C*), which was confirmed by immunostaining (*Fig. 3D*). The expression of P-CREB protein

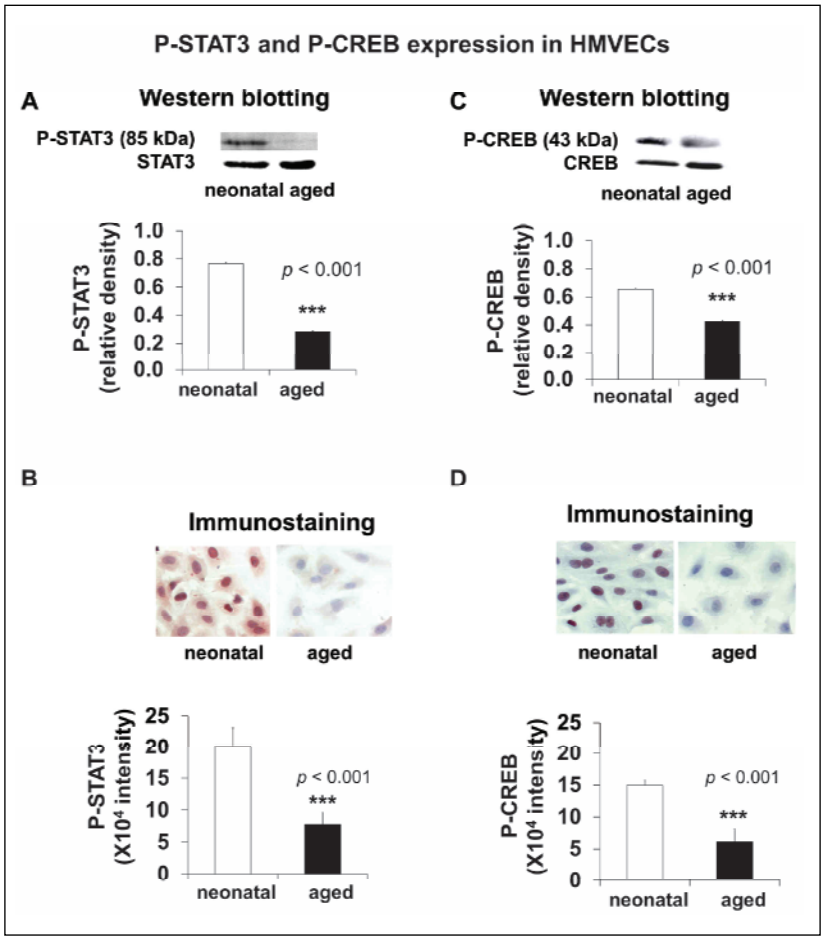

Fig. 3. Aged HMVECs exhibit reduced levels of P-STAT3 and P-CREB. (*A*) Western blotting determination of protein levels of P-STAT3. Values are mean ± S.D. of 3 independent experiments. Aged HMVECs had significantly reduced P-STAT3 (*** p<0.001). (*B*) Immunohistochemical staining confirmed significantly reduced P-STAT3 in cell nuclei of aged HMVECs (*** p<0.001). (*C*) Western blotting determination of protein levels of P-CREB. Values are mean ± S.D. of 3 independent experiments. Aged HMVECs had significantly reduced P-CREB (*** p<0.001). (*D*) Immunohistochemical staining confirmed significantly reduced P-CREB in cell nuclei of aged HMVECs (*** p<0.001).

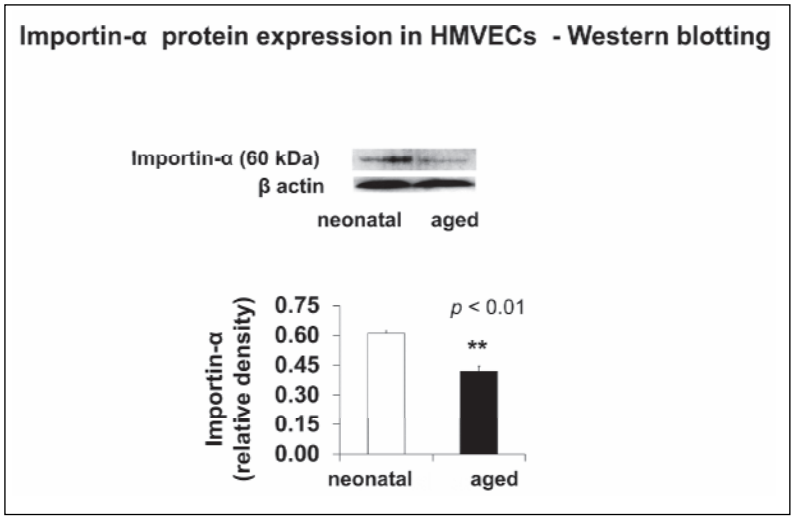

Fig. 4. Aged HMVECs exhibit reduced levels of importin-α. Western blotting determination of protein levels of importin-α. Values are mean ± S.D. of 3 independent experiments. Aged HMVECs have significantly reduced importin-α (*** p<0.01).

strongly correlated with VEGF mRNA expression (correlation coefficient r=0.986; $p<0.01$).

Reduced expression of importin-α protein in aged human dermal microvascular endothelial cells

To determine whether the levels of nuclear transport protein, importin-α, may play a role in the aging-associated impairment of angiogenesis and reduced VEGF gene expression, we next examined expression of importin-α protein in neonatal and aged HMVECs. Aged HMVECs had significantly reduced expression of importin- protein by1.4-fold ($p<0.01$) (*Fig. 4*). The expression of importin-α protein closely correlated with *in vitro* angiogenesis (correlation coefficient r=0.914; $p<0.01$) and with VEGF mRNA expression (correlation coefficient r=0.974; $p<0.01$).

DISCUSSION

This study demonstrates that impairment of angiogenesis in aging endothelial cells applies to human dermal microvascular endothelial cells and therefore indicates that it is a more general phenomenon occurring in endothelial cells derived from aging individuals and tissues other than the gastric mucosa. The impairment of angiogenesis in HMVECs has similar features to those found in rat gastric microvascular endothelial cells, *e.g.*, reduced expression of VEGF and importin-α. We also demonstrated impaired nuclear transport of P-STAT3 and P-CREB as the likely mechanism underlying reduced VEGF mRNA expression and decreased angiogenesis in aged vs. neonatal human dermal microvascular endothelial cells.

Our study showed significantly reduced expression of activated STAT-3 and CREB in aged HMVECs compared to neonatal HMVECs. The reduced expression of these transcription factors closely correlated with decreased VEGF gene expression in these cells. Activation of STAT3 and CREB by phosphorylation is ATP dependent. The levels of ATP decline in aging cells (31). Our finding that aged HMVECs have significantly reduced P-STAT3 and P-CREB levels, but that the total (phosphorylated and non-phosphorylated) levels of these transcription factors are similar between aged and neonatal HMVECs, indicate that reduced ATP levels and declined mitochondrial function during aging may contribute to reduced VEGF gene expression in endothelial cells. Furthermore, STAT3 can activate VEGF and anti-apoptotic genes such as Bcl-xL, and can likely promote cell survival and inhibit apoptosis in endothelial cells (32).

A recent study showed by immunofluorescence and chromatin immunoprecipitation that the activation of STAT3 results in its nuclear translocation and the transcriptional activation of the VEGF promoter (33). We demonstrated nuclear localization of phosphorylated STAT3 and CREB in neonatal HMVECs, and their significant reduction in aged HMVECs. Our findings suggest that activation and nuclear transport of STAT3 is impaired in aged HMVECs resulting in reduced VEGF gene expression in these cells. A previous study showed that hypoxia induces VEGF gene expression through a STAT3 signaling cascade in U251 glioma cells by the binding of activated STAT3 to the VEGF promoter (16). Hypoxia increases in HepG2 cells the recruitment of phospho-STAT3, HIF-1α, and CBP/p300 as a transcriptional complex within the VEGF promoter (17). Furthermore, STAT3 can stimulate VEGF expression by upregulating HIF-1α expression in human coronary artery smooth muscle cells (34). Over-expression of CREB in the RAW264.7 mouse macrophage cell line increased VEGF secretion by these cells (19). In human prostate cancer ARCaP cells, activation of CREB induced VEGF expression likely *via* a hypoxia-inducible factor-dependent mechanism (20). However, whether STAT3 and CREB can upregulate VEGF gene expression in endothelial cells has not been examined before.

A previous study examined expression of VEGF, angiostatin, and endostatin in two models of experimental UC (35). That study demonstrated for the first time increased expression of endostatin and increased VEGF levels in colonic mucosa at early stage of experimental UC, which likely explains why mucosal lesions heal slowly despite increased VEGF levels in UC (35). Therefore, inhibition of angiogenesis by endostatin may have significant implications for angiogenesis and tissue injury healing.

A recent study from Dr. Szabo's group demonstrated increased nuclear expression and nuclear transport of STAT3 and importin-α, and identified a critical role of STAT3-activated pathways in the protective mechanisms of duodenal mucosal cells in early duodenal ulceration (32). That study also demonstrated that STAT3 activates the expression of VEGF, bFGF, Ref-1, p21, and Bcl-xL genes, which likely enhance cell survival and is responsible for resistance to chemically induced duodenal ulcer mucosal injury (32).

Our previous study showed that the activation of metabolic sensor - AMP activated protein kinase (AMPK) - using the pharmacological stimulator AICAR reversed aging-related impairment of angiogenesis in aging myocardial microvascular endothelial cells by enhancing VEGF gene expression (36). A previous study showed that the simultaneous overexpression of FGF and VEGF in cultured murine myoblasts increased the proliferation of these cells (37). In another study, treatment with recombinant human VEGF was shown to increase capillary density and arteriolar density in hearts of 3 month of age (young) and 24 month old (aging) rats and to induce *in vitro* and *in vivo* angiogenesis during aging (38). These studies demonstrated the role of VEGF in endothelial cell function and reduction of VEGF expression during aging.

Our present study uncovered novel roles of STAT3, CREB and importin-α in VEGF gene expression in HMVECs and in aging associated impairment of angiogenesis. This has important implications for angiogenesis, endothelial cell survival and tissue injury healing.

Abbreviations: HMVECs, human dermal microvascular endothelial cells; mM, milli molar; mRNA, messenger ribonucleic acid; RT-PCR, reverse transcription and polymerase chain reaction; VEGF, vascular endothelial growth factor; STAT3, signal transducer and activator of transcription 3; CREB, cAMP response element-binding protein

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Conflict of interest: None declared.

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