Early Growth Response-1 Regulates Angiopoietin-1–Induced Endothelial Cell Proliferation, Migration, and Differentiation

Nelly A. Abdel-Malak, Mahroo Mofarrahi, Dominique Mayaki, Levon M. Khachigian, Sabah N.A. Hussain

Objective—Angiopoietin-1 (Ang-1) is an important regulator of angiogenesis in endothelial cells. It promotes migration, proliferation, and differentiation of cells, although the regulating factors involved in these processes remain unclear. In this study, we evaluated the contribution of the transcription factor early growth response-1 (Egr-1) to Ang-1–induced angiogenesis in human umbilical vein endothelial cells (HUVECs).

Methods and Results—Expression of Egr-1 was evaluated with real-time PCR and immunoblotting, whereas Egr-1 DNA binding activity was monitored with electrophoretic mobility shift assays. Cell migration was measured with wound healing and Boyden chamber assays, whereas cell proliferation and differentiation of cells into capillary-like tube structures were monitored with cell counting, BrdU incorporation and Matrigels. To selectively inhibit Egr-1 expression, we used both siRNA oligonucleotides and specific DNAzymes. Egr-1 mRNA expression rose approximately 9-fold within 2 hours of Ang-1 exposure and declined thereafter. Upregulation of Egr-1 expression was accompanied by an increase in nuclear mobilization and augmented DNA binding. These processes were mediated through the Erk1/2, PI-3 kinase/AKT, and mTOR pathways. Knockdown of Egr-1 expression completely abrogated Ang-1–induced endothelial migration and significantly reduced proliferation and capillary-like tube formation of HUVECs that overexpress Ang-1.

Conclusion—Ang-1 triggers significant and transient induction of Egr-1, and Egr-1 contributes to Ang-1–induced endothelial cell migration and proliferation. (Arterioscler Thromb Vasc Biol. 2009;29:209-216.)

Key Words: endothelial cells ■ angiopoietins ■ angiogenesis ■ transcription factors ■ cell migration

The receptor tyrosine kinase (Tie-2) and its associated ligands, the angiopoietins, have emerged as important regulators of angiogenesis both in adults and in embryos. In adult mice, Ang-1 stimulates in vivo vascular remodeling, vascular enlargement, wound healing, and lymphangiogenesis. In addition, Ang-1 inhibits endothelial cell (EC) apoptosis and stimulates migration, proliferation, and differentiation of these cells. Despite the importance of the Ang-1/Tie-2 receptor pathway to vascular homeostasis and angiogenesis, little is known about transcription factors that are activated downstream from it. Daly et al have reported that Ang-1 inhibits the transcriptional activity of FoxO1 downstream of the PI-3 kinase/AKT pathway, thereby reducing the expression of several proapoptotic proteins. Elk-1 is activated by Ang-1 downstream from the Erk1/2 pathway and forms a complex by binding serum response factor and serum response element in various promoters. However, neither of these studies addressed the importance of these transcription factors in mediating the biological functions of the Ang-1/Tie-2 pathway. Early growth response-1 (Egr-1) has recently been identified as a transcription factor that is significantly induced downstream from Tie-2 receptors. Egr-1 is an immediate-early gene that is rapidly and transiently induced by many stimuli, including hypoxia, shear stress, and injury. On activation it binds promoter regions of several growth factors, cytokines, receptors, and adhesion molecules. Two corepressors of Egr-1, Nab1 (constitutive) and Nab2 (inducible), negatively regulate the activity of Egr-1 through direct interaction. Moreover, Egr-1 knockdown, or overexpression of Nab2, attenuates the proangiogenic effects of fibroblast growth factor 2 (FGF-2) and vascular endothelial growth factor (VEGF) on proliferation and differentiation of ECs. However, although Egr-1 is induced downstream from Tie-2 receptors, it has yet to be definitively linked to Ang-1/Tie-2 signaling. The main aim of this study, therefore, is to test the hypothesis that Egr-1 induction downstream from the Ang-1/Tie-2 receptor pathway contributes significantly to the regulation of EC migration and proliferation.

Materials and Methods

Cell Culture

HUVECs were maintained in culture, as previously described. Cells were serum-starved overnight and were then exposed to either a
control vehicle (phosphate buffered saline, PBS) or 300 ng/mL of COMP–Ang-1 and standard Ang-1 proteins. COMP–Ang-1 is a soluble, stable, and potent recombinant chimera made by replacing the N-terminal of Ang-1 with the short coiled-coil domain of cartilage oligomeric matrix protein (COMP). Cells were collected after 30 minutes to 6 hours of exposure and examined for Egr-1 and Nab2 mRNA and protein expressions. Cells were also exposed to either VEGF-A (80 ng/mL), COMP–Ang-1 (300 ng/mL), or a combination of the two. Cells were collected after 1 hour of exposure and examined for Egr-1 mRNA and protein expression. In additional experiments, serum-starved HUVECs were preincubated for 1 hour with U0126 or PD98059 (30 μmol/L, inhibitor of Erk1/2 MAPK), SB203580 (10 μmol/L, inhibitor of p38 MAPK), SP60125 (15 μmol/L, inhibitor of SAPK/JNK pathway), wortmannin (50 mmol/L, inhibitor of PI-3 kinase), rapamycin (50 ng/mL, inhibitor of mammalian target of rapamycin, mTOR), and U0126+SB203580 or PD98059+SB203580. Cells were then exposed to a control vehicle (PBS) or COMP–Ang-1 (300 ng/mL) for 1 hour and were then collected and examined for Egr-1 mRNA and protein expression.

Transfection With siRNA Oligonucleotides
Serum-starved HUVECs were transfected with 10 nmol/L of either Egr-1-specific or scrambled negative control Dicer-substrate siRNA duplexes (Integrated DNA Technologies) using HiPerfect transfection reagent (Qiagen) according to the manufacturer’s instructions. Gene silencing was monitored 24 hours later by performing real-time PCR experiments and immunoblotting for Egr-1.

Transfection With DNAzymes
RNA-cleaving phosphodiester-linked DNA-based enzymes (DNAzymes) are cation-dependent enzymes made up entirely of DNA that can be designed to cleave target mRNA in a gene-specific and catalytically efficient manner. DzF is a DNAzyme engineered to target the A301U site of human Egr-1 mRNA. HUVECs were maintained in starvation medium for 6 hours and then transfected overnight with 0.05 μmol/L of either DzF (Egr-1 DNAzyme) or DzFSCR (scrambled DNAzyme) using HiPerfect transfection reagent. Gene silencing was monitored 24 hours later using real-time PCR and immunoblotting for Egr-1.

Generation of MSCV and MSCV-Ang1 HUVECs
HUVECs were transduced with retroviruses expressing an empty MSCV-pac (empty vector) or MSCV-pac containing murine Ang-1 cDNA, respectively. Transient transfections of the Amphi Phoenix packaging cell line were performed using Fugene 6 transfection reagent. Viral supernatants from transfected packaging cell lines were used to transduce HUVECs in multiple rounds of infection and nuclear extracts of HUVECs stimulated with control vehicle (PBS) or COMP–Ang-1 were performed according to the manufacturer’s instructions (Active Motif). Competition assays were performed with unlabeled wild-type and mutated Egr-1 oligo competitors (5′-GGA GCC GAG CGG CGG CGA-3′), and supershift analysis was performed with Egr-1 antibody (2 μg). Binding complexes were resolved by non-denaturing polyacrylamide gel electrophoresis. Gels were then fixed, dried, and exposed on autoradiography film.

Wound Healing Assay
Cells were maintained in complete medium to confluence and then wounded using a 200 μL-pipette tip. Control vehicle (PBS) or COMP–Ang-1 (300 ng/mL) was added and wound healing was quantified 12 hours later with inverted bright field microscopy, as previously described.

Boyden Chamber Assay
Migration of HUVECs was quantified using 24-well transwell polycarbonate inserts (8.0 μm pore size) of modified Boyden chambers. HUVECs transfected with either Egr-1-siRNA or scrambled-siRNA oligonucleotides were seeded in upper compartments at a density of 105 cells/100 μL of basal medium. PBS or COMP-Ang-1 (300 ng/mL) was added in lower compartments. The apparatus was then incubated at 37°C in a CO2 incubator for 5 hours. Migration was then quantified, as previously described.

Proliferation Assays
For cell counting, cells were cultured in basal medium plus 2% FBS. After 48 hours, cells were trypsinized and counted using a hemacytometer. For BrdU incorporation, cells were plated in 96-well plates. After 24 hours, cells were pulsed with 10 μmol/L of BrdU and incubated for an additional 24 hours. Cells were then fixed and labeled according to the manufacturer’s instructions (Roche Applied Science). The absorbance of samples was measured at 370 nm.

Capillary-Like Tube Formation Assays
MSCV and MSCV–Ang-1 HUVECs transfected with scrambled and Egr-1 siRNA oligos (2×107 cells per well) were seeded into growth factor-reduced Matrigel-coated 24-well plates in basal medium plus 1% FBS. Images of tube formation were captured 24 hours later using an inverted microscope using a 10× objective. Images from a total of 7 to 10 fields per well were analyzed by Image Pro software (Media Cybernetics). Angiogenic activity was determined by counting the branch points of formed tubes and the average number of branch points was calculated, as previously described. Each experiment was repeated 6 times.
Immunoblotting

Total cell lysates were boiled and then loaded onto tris-glycine SDS-polyacrylamide gels. Proteins were electrophoretically transferred onto polyvinylidene difluoride membranes, blocked with 5% dry milk, and subsequently incubated with specific polyclonal antibodies. Activation of Erk1/2 was assessed with a phosphospecific antibody (Thr202/Tyr204). Proteins were detected with HRP-conjugated secondary antibodies and ECL. For all immunoblots, equal protein loading was verified using anti-α-tubulin antibody.

Data Analysis

Data were expressed as means±SE. Differences between experimental groups were determined by 1-way ANOVA and were considered statistically significant at \( P<0.05 \). A similar analysis was used to compare cells transfected with siRNA oligos and DNAzymes and between MSCV and MSCV–Ang-1 cells.

Results

The time course of Egr-1 expression was assessed by treating HUVECs with COMP–Ang-1 (300 ng/mL) for 30 minutes, 1, 2, 3, and 6 hours. COMP–Ang-1 elicits significant induction of Egr-1 mRNA and protein expressions in a time-dependent fashion (Figure 1A through 1C). Standard Ang-1 protein (300 ng/mL) also elicits significant induction of Egr-1 protein, in a fashion similar to that elicited by COMP–Ang-1 (Figure 1D). Mobilization of Egr-1 protein to the nucleus was evaluated by separating HUVEC lysates collected after 1 hour of vehicle or COMP–Ang-1 treatment into cytosolic and nuclear fractions. COMP–Ang-1 enhances nuclear accumulation of Egr-1 protein (Figure 1E). In addition, COMP–Ang-1 significantly increases Egr-1 DNA binding activity as detected by electrophoretic mobility shift assays (Figure 1F).

To identify the mechanisms through which Ang-1 induces Egr-1 expression in HUVECs, we measured human Egr-1 promoter activity and Egr-1 mRNA stability. COMP–Ang-1 significantly induces Egr-1 promoter activity (Figure 2A) suggesting that Ang-1 does indeed enhance Egr-1 expression through increased transcription. However, COMP–Ang-1 has no influence on the rate of decline in Egr-1 mRNA expression after transcription inhibition by actinomycin D (Figure 2B), suggesting that Egr-1 mRNA stability is not influenced by COMP–Ang-1.
The involvement of the PI-3 kinase, Erk1/2, p38, SAPK/JNK, and mTOR pathways in Ang-1–induced Egr-1 expression was evaluated by using selective inhibitors of these pathways. When HUVECs are incubated with inhibitors alone, no significant induction of Egr-1 protein expression is observed (supplemental Figure I). However, inhibition of the Erk1/2, PI-3 kinase/AKT, and mTOR pathways results in significant attenuation of Ang-1–induced Egr-1 mRNA and protein expressions, with SAPK/JNK inhibition having no effect (Figure 2C and 2D). Inhibition of p38 MAPKs by SB203580 results in augmentation of COMP–Ang-1–induced Egr-1 expression; however, when SB203580 was present along with inhibitors of the Erk1/2 pathway (U0126 and PD98059), Egr-1 expression is attenuated to levels significantly lower than those observed with COMP–Ang-1 alone (Figure 2C and 2D). These results suggest that the Erk1/2 and PI-3 kinase/AKT pathways promote Egr-1 expression, whereas the p38 MAPK pathway inhibits Egr-1 expression. They also suggest that the inhibitory effects of p38 MAPK on Egr-1 expression are mediated through a negative influence on the Erk1/2 pathway.13 This was verified by observing that Erk1/2 activation in response to 15 minutes exposure to COMP–Ang-1 was augmented when p38 MAPKs were inhibited with SB203580 and PD169316 (supplemental Figure II).

To evaluate the nature of interactions between Ang-1 and VEGF pathways in regulating Egr-1 expression, we exposed HUVECs for 1 hour to VEGF, COMP–Ang-1, and a combination of the two. VEGF (80 ng/mL) significantly induces Egr-1 expression, and when combined with COMP–Ang-1 the augmentation of Egr-1 expression occurs in an additive fashion (Figure 3A and 3B). We also monitored the influence of Ang-1 on the expression of the inducible corepressor of Egr-1, namely, Nab2. COMP–Ang-1 elicits a transient, yet significant, inhibition of Nab2 mRNA expression, followed by a significant rise after 1 hour (Figure 3C). Inhibition of Nab2 protein expression occurs after 1 hour of COMP–Ang-1 treatment, followed by significant induction at 2 hours (Figure 3C).

To evaluate the functional importance of Egr-1 in the biological responses to Ang-1 in ECs, we used siRNA oligos and DNAzymes to attenuate Egr-1 expression. We first confirmed that Egr-1 mRNA and protein levels, in both control and COMP–Ang-1–treated HUVECs, are significantly attenuated after transfection with Egr-1 siRNA oligos and DNAzymes (DzF; supplemental Figure III). We then used wound healing and Boyden migration assays to evaluate the importance of Egr-1 in Ang-1–induced EC migration. In untransfected cells, and in those transfected with scrambled siRNA oligos but not in cells transfected with Egr-1 siRNA oligos (Figure 4B). To evaluate the involvement of Egr-1 in Ang-1–induced EC proliferation and capillary-like tube formation, HUVECs
that overexpress Ang-1 (MSCV–Ang-1 cells) were generated using a retroviral vector. These cells produce and secrete Ang-1 protein into the culture medium, which gets incorporated into the extracellular matrix (supplemental Figure IV). MSCV–Ang-1 HUVECs proliferate faster than MSCV cells (supplemental Figure IV). In addition, both MSCV and MSCV–Ang-1 HUVECs grown on growth factor-reduced Matrigel matrices show the appearance of a network of capillary-like tubes which peaks after 24 hours; however, these angiogenic changes are stronger and the tubes are more stable in MSCV–Ang-1 HUVECs as compared to MSCV cells (supplemental Figure IV). Finally, significantly greater levels of Egr-1 mRNA were detected in MSCV–Ang-1 cells, as compared to MSCV cells (supplemental Figure IV). These results are strongly supportive of the notion that elevated expressions of Egr-1 in MSCV–Ang-1 HUVECs are responsible, in part, for enhanced migration, proliferation, and capillary-like tube formation of these cells. This was confirmed by observing that enhanced proliferation and capillary-like tube formation of MSCV–Ang-1 cells are significantly attenuated when Egr-1 expression is knocked down (Figure 5A through 5C). Finally, we evaluated whether Egr-1 regulates the expression of proangiogenesis growth factors and receptors in HUVECs by transfecting these cells with scrambled and Egr-1 siRNA oligos. Transfection with Egr-1 siRNA oligos significantly reduces expressions of PDGF-B, FGF-2, IL-8, and Flt-1×58%, 48%, 37%, and 66%, respectively, but has no influence on VEGF-A, VEGF-C, HB-EGF, HGF, or CXCR4 expressions (supplemental material).

Discussion

The main findings of this study include: (1) in ECs, Ang-1 induces a significant increase in the expression and DNA binding of Egr-1, through the Erk1/2, PI-3 kinase/AKT, and mTOR pathways; (2) induction of Egr-1 by Ang-1 is accomplished through enhanced transcription, not through increased mRNA stability; (3) the effects of Ang-1 and VEGF on Egr-1 expression are additive; (4) Egr-1 plays an important role in Ang-1–induced migration, proliferation, and capillary-like tube formation of ECs; and (5) inhibition of Egr-1 expression in ECs significantly attenuates the expression of PDGF-B, FGF-2, Flt-1, and IL-8.

Regulation of Egr-1 Expression

In ECs, little is known about transcription factors that are involved in regulating gene expression downstream from Tie-2 receptors. We report here that exposure of ECs to COMP–Ang-1 or standard Ang-1 elicits significant induction
of Egr-1 expression and enhanced Egr-1 DNA binding activity. Previous reports have confirmed that Egr-1 is activated in ECs by many proangiogenesis growth factors, including FGF-2 and EGF, and, in the present study, the induction of Egr-1 expression by Ang-1 is qualitatively similar to those responses. We also report here that a combination of Ang-1 and VEGF has an additive effect on Egr-1 expression. This observation suggests that each of these growth factors activates similar signaling pathways. Indeed, we have recently reported that Ang-1 and VEGF exert an additive effect on Erk1/2 pathway activation. In addition, in HUVECs, many of the same genes that are upregulated after 4-hour exposure to VEGF are also upregulated by Ang-1 treatment.

The MAPK and the PI-3 kinase/AKT pathways have recently emerged as major signaling pathways through which Tie-2 receptors regulate EC functions such as survival, migration, adhesion, and proliferation. Moreover, reports have confirmed that the Erk1/2 pathway plays an important role in mediating Egr-1 responses to many mediators, including EGF, whereas the p38 MAPKs promote prostaglandin E2-induced Egr-1 expression. In turn, these signaling pathways activate various transcription factors, including serum response factor, c-Fos, c-Jun, ATF2, Elk-1, and SP-1, which

Figure 5. A and B, Effects of siRNA oligos on proliferation of MSCV and MSCV–Ang-1 HUVECs. \*P<0.05 compared with scrambled oligos-transfected MSCV cells. \#P<0.05 compared with scrambled oligos-transfected MSCV–Ang-1 cells (n=6 measurements per group). C, Effects of Egr-1 knockdown on capillary-like tube formation in MSCV–Ang-1 HUVECs. Tube formation measured 24 hours later. \#P<0.05 compared with scrambled oligos-transfected cells. D, Schematic depiction of the mechanisms through which Ang-1/ Tie-2 receptors regulate Egr-1 expression.
then bind selective binding sites on Egr-1 promoter. The present results indicate that Ang-1–mediated induction of Egr-1 transcription in HUVECs is dependent on the Erk1/2, PI-3 kinase/AKT, and the mTOR pathways. In a recent study, we reported that the Ang-1/Tie-2 receptor pathway stimulates the production in ECs of the chemokine IL-8 and that this response is mediated through the PI-3 kinase/AKT and Erk1/2 pathways and SAPK/JNK members of the MAPKs, whereas the p38 pathway inhibits IL-8 production through selective inhibition of Erk1/2 phosphorylation. The present results indicate that activated Tie-2 receptors use the Erk1/2 and PI-3 kinase/AKT pathways to upregulate Egr-1 expression in a fashion similar to that observed regarding IL-8 induction (Figure 5D). In addition to the Erk1/2 and PI-3 kinase/AKT pathways, we report here that Ang-1–induced Egr-1 expression is significantly attenuated by rapamycin, suggesting the involvement of the mTOR network (Figure 2). We have recently reported that mTOR and its downstream effector, p70S6 kinase, are activated downstream from Tie-2 receptors in HUVECs and that mTOR participates in Ang-1–induced alterations in the HUVEC transcriptome. Very little is known about interactions between Egr-1 and the mTOR network, although it has been demonstrated that induction of Egr-1 expression is mediated, in part, through the mTOR/P70S6 kinase pathway during skeletal myoblast differentiation.

We also confirm here that the p38 MAPK pathway inhibits Egr-1 transcription and that this effect is mediated through a negative influence on Erk1/2 pathway activation. It has been previously reported that this modulatory effect of the p38 MAPKs on Erk1/2 activity is mediated through the inhibitory effect of PP2A phosphatase on MEK1/2 phosphorylation. Previous and present results suggest that the Erk1/2 pathway contributes positively to Ang-1–induced survival, migration, and proliferation of ECs. The p38 MAPK pathway, in contrast, acts as an inhibitory biological switch through which other stimuli regulate the degree to which the Ang-1/Tie-2 axis modulates angiogenic processes.

**Regulation of EC Migration, Proliferation, and Capillary-Like Tube Formation by Egr-1**

The present study reveals that Ang-1 stimulates EC migration and capillary-like tube formation and that knocking down Egr-1 expression using siRNA oligos or DNAzymes attenuates these effects of Ang-1. Exogenous Ang-1 protein elicits a mild increase in cell proliferation. To enhance the proproliferative effects of Ang-1 and to reproduce conditions similar to the in vivo vascular environment, whereby ECs are exposed to sustained Ang-1 production derived from vascular smooth muscles, we generated a HUVEC line using retroviruses that stably produces Ang-1 (MSCV–Ang-1). In these cells, Ang-1 induces significant cell proliferation, which is associated with upregulation of Egr-1 expression. However, the mechanisms through which Egr-1 modulates EC proliferation, migration, and capillary-like tube formation are still under investigation. One possibility is that Egr-1 upregulates the production of proangiogenesis growth factors and that these factors act in an autocrine fashion to stimulate EC proliferation, differentiation, and migration. This hypothesis is supported by reports documenting Egr-1–mediated induction of FGF-2, PDGF-A, PDGF-B, and IGF-II in various models of in vitro and in vivo angiogenesis. The functional importance of Egr-1 in cellular proliferation that is induced by these factors, particularly by FGF-2, and G-CSF, was confirmed using siRNA oligos and selective Egr-1 DNAzymes.

The present study confirms that Egr-1 regulates the expression of selective proangiogenesis growth factors such as PDGF-B and FGF-2 but is not involved in regulating others, such as VEGF-A, VEGF-C, and HGF. This observation raises the possibility that Egr-1 contributes to Ang-1–induced proliferation and migration through the release of growth factors such as PDGF-B and FGF-2.

Another likely mechanism through which Egr-1 promotes Ang-1–induced proliferation, differentiation, and migration of HUVECs is the production of IL-8. IL-8 is endogenously produced by ECs and may directly promote EC proliferation, differentiation, and migration through activation of CXCR1 and CXCR2. We have recently reported that Ang-1 elicits significant upregulation of IL-8 production in HUVECs and that IL-8 contributes significantly to Ang-1–induced proliferation and migration of these cells. The present study suggests that Egr-1 is involved in the regulation of IL-8 production in ECs; however, little is known about the link between Egr-1 and IL-8 in these cells. Giri et al reported that attenuation of Egr-1 expression triggers a significant reduction of IL-8 expression in the monocytic THP-1 cells stimulated with amyloid peptides. Chromatin immunoprecipitation analysis has also revealed the presence of Egr-1 binding sites in IL-8 promoter. Additional studies are clearly needed to investigate the precise downstream pathways through which Egr-1 regulates Ang-1–induced proliferation and migration of ECs.

In summary, we report, as an original finding, that Ang-1 induces the expression and activation of the transcription factor Egr-1 and that this effect is mediated through the Erk1/2, PI-3 kinase/AKT, and mTOR pathways. Our results also indicate that Egr-1 contributes significantly to Ang-1–induced migration, proliferation, and capillary-like tube formation of ECs.

**Acknowledgments**

The authors are grateful to L. Franchi for his technical assistance.

**Sources of Funding**

This study was funded by the Canadian Institute of Health Research and the Heart and Stroke Foundation of Quebec.

**Disclosures**

None.

**References**

4. Abdel-Malak NA, Harfouche R, Hussain SN. Transcriptome of angio-


6. Fahmy RG, Dass CR, Sun LQ, Chesterman CN, Khachigian LM. Transcription factor Egr-1 supports FGF-dependent angiogenesis during neo-


11. Harfouche R, Malak NA, Brandes RP, Karsan A, Irani K, Hussain SN. Roles of reactive oxygen species in angiopoietin-1/1c-2 receptor sig-


19. Westerman J, Li SP, Kaluunki T, Han J, Kahari VM. p38 mitogen-ac-

20. Kanda S, Miyata Y, Machiiitaki Y, Matsuyama T, Kanetake H. Angio-


Early Growth Response-1 Regulates Angiopoietin-1-Induced Endothelial Cell Proliferation, Migration, and Differentiation

Nelly A. Abdel-Malak, Mahroo Mofarrah, Dominique Mayaki, Levon M. Khachigian and Sabah N.A. Hussain

Arterioscler Thromb Vasc Biol. 2009;29:209-216; originally published online December 26, 2008;
doi: 10.1161/ATVBAHA.108.181073

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2008 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/29/2/209

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2009/01/26/ATVBAHA.108.181073.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
**Supplementary Table:** Real-time PCR primers and fold changes in mRNA expression ±SEM of various genes in HUVECs transfected with Egr-1 siRNA oligos then exposed for 1h to basal culture medium containing 15% FBS, expressed as fold changes from values measured in HUVECs transfected with scrambled siRNA oligos. N=4 measurements per group.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5’-----3’)</th>
<th>Product size (bp)</th>
<th>Fold change</th>
</tr>
</thead>
</table>
| CXCR4  | Forward 5’-AGCATGACGGACAAGTACAGG-3’  
         Reverse 5’-GATGAAGTCGGGAATAGTCAGC-3’ | 309   | 0.80±0.12 |
| VEGF-C | Forward 5’-TGTACAAGTGTCAGCTAAGG-3’  
         Reverse 5’-CCACATCTATACACACCTCC-3’ | 183   | 1.10±0.15 |
| VEGF-A | Forward 5’-CTACCTCCACCATGCCAAGT-3’  
         Reverse 5’-CACACAGGATGGCTTGAAGA-3’ | 187   | 0.95±0.07 |
| HB-EGF | Forward 5’-GAGAGGCTAGCTGCTGGAA-3’  
         Reverse 5’-TGCTTGCTGGCTGGAGATA-3’ | 157   | 1.05±0.02 |
| HGF    | Forward 5’-GGACGCAGCTACAAGGGGAAC-3’  
         Reverse 5’-CCTTCTCCCCCTCGAGATT | 158   | 0.98±0.19 |
| PDGF-B | Forward 5’-CTCGTCCGTCTGCTCGATG-3’  
         Reverse 5’-GGAAGAAGATGGCGATGGAG-3’ | 167   | 0.42±0.06 |
| IL-8   | Forward 5’-CTCTTTGGCAGCCTCTTGAT-3’  
         Reverse 5’-ACAACCTCTGACCCAGTT-3’ | 242   | 0.63±0.11 |
| Flt-1  | Forward 5’-CACTGGCGACAGAAAATC-3’  
         Reverse 5’-TCACACCTTGCTCGGAATG-3’ | 109   | 0.34±0.04 |
| FGF-2  | Forward 5’-GAGACACCCCATCCGTGAACC-3’  
         Reverse 5’-GGCAGCGTGGTATGCTC-3’ | 285   | 0.52±0.08 |
| GAPDH  | Forward 5’-AAGAAGGTGGTGAAGCGAGGC-3’  
         Reverse 5’-ACCAGGAAATGAGCTTGACA-3’ | 166   |           |
Supplementary Figure I

Representative immunoblot showing Egr-1 protein expression in ECs pre-incubated with various inhibitors for 1h.
Supplementary Figure II

Representative immunoblot showing effect of p38 inhibition on Ang-1-induced Erk1/2 phosphorylation in ECs pre-incubated with PD169316 and SB203580 then exposed to COMP-Ang-1 for 15min. Note the increases in basal and Ang-1-induced Erk1/2 phosphorylation thereby confirming suppression of inhibitory action of p38 MAPK on Erk1/2 pathway.
Supplementary Figure III

A: Relative levels of Egr-1 mRNA (means±SEM) in cells transfected with scrambled and Egr-1 siRNA or DNAzymes. N=3 measurements per group. *P<0.05 compared with scrambled oligos and DNAzymes.

B: Representative immunoblot of Egr-1 protein in cells transfected with scrambled and Egr-1 siRNA and DNAzymes. Cells were treated with vehicle or COMP-Ang-1 (300 ng/ml) for 1 h.

C: Means± SEM of relative Egr-1 protein optical densities in ECs transfected with scrambled and Egr-1 siRNA oligos and DNAzymes and stimulated with COMP-Ang-1 for 1h. Values for vehicle-stimulated HUVECs are not shown because Egr-1 protein was not detected. N=6 measurements per group. *P<0.05 compared with cells transfected with scrambled oligos and DNAzymes.
**Supplementary Figure IV**

**A:** Detection of Ang-1 protein in medium and extracellular matrix (ECM) of MSCV-Ang-1 and MSCV HUVECs.

**B:** Proliferation of MSCV and MSCV-Ang-1 HUVECs measured after 2 days of growth. * P<0.05 compared with MSCV cells.

**C & D:** Representative capillary-like tube formation after 12h and 32h of growth on Matrigel™-coated plates and means± SEM of the number of branching points measured after 24h in MSCV and MSCV-Ang-1 HUVECs. Note that tube formation was evident within 12h and peaked after 24h. After 32h, intact tubes were still evident in MSCV-Ang-1 HUVECs whereas MSCV HUVECs had complete dissolution of tubes. *P<0.05 compared with MSCV HUVECs. N=6 measurements per group.

**E:** Egr-1 mRNA expression in MSCV and MSCV-Ang-1 HUVECs measured after 12h growth in basal medium containing 15% or 0% FBS, expressed as fold changes from those measured in MSCV cells. * p<0.05 compared with MSCV cells. N=6 measurements per group.