ORIGINAL RESEARCH

Regulation of Angiopoietin-1/Tie-2 Receptor Signaling in Endothelial Cells by Dual-Specificity Phosphatases 1, 4, and 5

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***Background*—**Angiopoietin-1 (Ang-1) promotes survival and migration of endothelial cells, in part through the activation of mitogen-activated protein kinase (MAPK) pathways downstream of Tie-2 receptors. Dual-specificity phosphatases (DUSPs) dephosphorylate phosphotyrosine and phosphoserine/phosphothreonine residues on target MAPKs. The mechanisms by which DUSPs modulate MAPK activation in Ang-1/Tie-2 receptor signaling are unknown in endothelial cells.

***Methods and Results*—**Expression of various DUSPs in human umbilical vein endothelial cells exposed to Ang-1 was measured. The functional roles of DUSPs in Ang-1-induced regulation of MAPK activation, endothelial cell survival, migration, differentiation, and permeability were measured using selective siRNA oligos. Ang-1 differentially induces DUSP1, DUSP4, and DUSP5 in human umbilical vein endothelial cells through activation of the PI-3 kinase, ERK1/2, p38, and SAPK/JNK pathways. Lack-of-function siRNA screening revealed that DUSP1 preferentially dephosphorylates p38 protein and is involved in Ang-1-induced cell migration and differentiation. DUSP4 preferentially dephosphorylates ERK1/2, p38, and SAPK/JNK proteins and, under conditions of serum deprivation, is involved in Ang-1-induced cell migration, several antiapoptotic effects, and differentiation. DUSP5 preferentially dephosphorylates ERK1/2 proteins and is involved in cell survival and inhibition of permeability.

***Conclusions*—**DUSP1, DUSP4, and DUSP5 differentially modulate MAPK signaling pathways downstream of Tie-2 receptors, thus highlighting the importance of these phosphatases to Ang-1-induced angiogenesis. **(*J Am Heart Assoc*. 2013;2:e000571 doi: 10.1161/JAHA.113.000571)**

Key Words: angiogenesis • angiopoietin-1 • apoptosis • dual-specificity phosphatases • endothelial cells • mitogen-activated protein kinases

Angiopoietin-1 (Ang-1) is an agonist of Tie-2 receptors and promotes migration, proliferation, and differentiation of endothelial cells (ECs).[1] Mice lacking Ang-1 do not survive early development and exhibit major defects in vascular organization.[2] In adult tissues, Ang-1 exerts a dual role by stimulating angiogenesis at sites of active vascular remodeling and by promoting vascular quiescence in mature vessels through the inhibition of apoptosis and inflammation.[1]

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Ang-1 induces autophosphorylation of Tie-2 receptors and activates downstream signaling pathways such as the mitogen-activated protein kinase (MAPK) pathways, which include ERK1/2, p38, and SAPK/JNK.[3,4] MAPK activation is dependent on phosphorylation of threonine and tyrosine residues by dual-specificity MAPK kinases, which are activated through phosphorylation of serine/threonine residues by upstream MAPK kinase kinases.[5] In ECs, the ERK1/2 pathway mediates antiapoptotic properties of Ang-1, whereas the p38 pathway promotes apoptosis.[3] Ang-1-induced ERK1/2 and SAPK/JNK phosphorylation in combination with phosphatidylinositol 3-kinase activation induces IL-8 production, which is essential for EC migration and proliferation.[6] The transcription factors activating protein-1 and early growth response–1 (Egr-1) are activated downstream of Tie-2 receptors in cells exposed to Ang-1. They also play a role in migration and proliferation.[6]

Outcomes of MAPK signaling are determined by the magnitude and duration of MAPK phosphorylation, suggesting that mechanisms of signaling inactivation are just as important as the activation of cascades themselves.[5]

Specific dephosphorylation of MAPKs on phosphoserine/phosphothreonine and phosphotyrosine residues is mediated by dual-specificity phosphatases (DUSPs), a family of cysteine-dependent protein tyrosine phosphatases.[7] To date, 16 mammalian DUSPs that dephosphorylate MAPKs have been identified, of which 11 belong to a subfamily of CH2 (CDC25 homology)-motif-containing MAPK phosphatases (MKPs).[8] These MKPs contain a motif in their active site that shares high sequence similarity with the protein tyrosine phosphatase VH1 from the *Vaccinia* virus and an NH_2-terminal kinase interactive motif that contributes to substrate specificity.[8] They have been grouped into 3 major subfamilies based on their sequence similarity, substrate specificity, and subcellular localization.[7,8] The largest group includes 4 inducible nuclear phosphatases: DUSP1 (MKP-1), DUSP2, DUSP4 (MKP-2), and DUSP5.[9]

In ECs, recent studies have identified DUSP1 and DUSP5 as important negative modulators of those MAPK signaling pathways that are activated by angiogenesis factors like vascular endothelial growth factor (VEGF).[10] There is also evidence that DUSP4 regulates TNF-α-induced apoptosis in human umbilical vein endothelial cells (HUVECs).[11] However, despite their known importance to the regulation of MAPK signaling pathways downstream of angiogenic factors, no information is as yet available regarding the involvement of DUSPs in Ang-1/Tie-2 receptor signaling. The main focus of this study, therefore, is to characterize the ways in which DUSPs negatively regulate MAPK signaling and to investigate how they influence Ang-1-induced EC survival and migration.

Methods

Cell Culture and Adenoviral Infection

HUVECs were grown in MCDB131 medium supplemented with 20% fetal bovine serum (FBS), endothelial cell growth supplement, 2 mmol/L glutamine, heparin, and gentamicin. HUVECs stably transduced with control retroviruses (HUVEC-MSCV) and retroviruses expressing dominant-negative JNK1 (HUVEC-MSCV-JNK-APF) were prepared as previously described.[3] For some experiments, cells were transduced for 6 hours with 100 MOI of adenovirus-expressing GFP or Ad-TAM67[12] (Vector Biolabs, Philadelphia, PA) and allowed to recover for 48 hours prior to experimental treatment (online data supplement). To investigate the role of Tie-2 receptor activation in Ang-1-mediated regulation of DUSP expression, cells were infected for 6 hours with 100 MOI of Ad-GFP or Ad-Ex Tek. Tie-2 phosphorylation in HUVECs infected with Ad-GFP or Ad-Ex Tek was verified following 15-minute stimulation with Ang-1 using phospho-Tie-2 antibody (online data supplement).

Real-Time PCR

Total RNA was extracted using a GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, St. Louis, MO). Real-time PCR amplifications using specific primers for DUSP1, DUSP3, DUSP4, DUSP5, DUSP6, DUSP7, DUSP11, DUSP12, DUSP14, and DUSP22 were performed using SYBR green and a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA); see online data supplement. All experiments were performed in triplicate and relative mRNA expression was analyzed using the ΔC_T method and expressed as $2^{-\Delta\Delta CT}$.

Immunoblotting

Proteins from whole-cell lysates or from nuclear/cytosolic fractions obtained by centrifugation were separated using SDS-PAGE, transferred onto polyvinylidene difluoride membranes, blocked with 5% milk, and incubated overnight with specific primary antibodies. Proteins were detected with horseradish peroxidase–conjugated secondary antibodies and ECL reagents.

Phosphatase Activity Assay

Phosphatase activity was measured using immunoprecipitated DUSP proteins from HUVECs treated with vehicle (PBS) or Ang-1 (300 ng/mL). Immunoprecipitation was performed as previously described.[13] Phosphatase activity was measured using *p*-nitrophenyl phosphate (Sigma-Aldrich, St. Louis, MO) as a substrate.[13] Briefly, immunoprecipitate (20 μL) was added to 10 mmol/L *p*-nitrophenyl phosphate (130 μL) in phosphatase buffer and incubated at 30°C for 45 minutes. Reaction was stopped with 1 N NaOH (50 μL). Absorbance was measured at 405 nm.

mRNA Stability Assay

HUVECs were stimulated with Ang-1 (300 ng/mL) for 1 hour and maintained in serum-free medium containing actinomycin D (5 μg/mL) or actinomycin D plus Ang-1 (300 ng/mL). Total RNA was extracted at several times (0, 30, 60, and 90 minutes) and analyzed using real-time PCR.

Analysis of DUSP1 and DUSP4 Promoter Activities

HUVECs were cotransfected by electroporation with a *Renilla* luciferase plasmid (pRL-TK) and either DUSP1 (pGL3-erp7)[14] or DUSP4 reporter plasmids (MKP-2-Luc)[15] using an Amaxa Nucleofector System (Lonza, Walkersville, MD). Luciferase activity of cells maintained in medium with vehicle or Ang-1 (300 ng/mL) for 6 hours was quantified using a Dual-Luciferase Reporter Assay System (Promega, Madison, WI).

Transfection With siRNA Oligos

HUVECs were transfected with Egr-1-specific or scrambled negative control Dicer-substrate siRNA duplexes (Integrated DNA Technologies, Coralville, IA), siRNA (siGENOME SMARTpool) directed against DUSP1, DUSP4, and DUSP5, or a nontargeting siRNA pool (siCONTROL; Dharmacon, Lafayette, CO) using Lipofectamine RNAiMAX reagent (Life Technologies, Inc, Burlington, Ontario, Canada). All experiments were performed 48 hours posttransfection (online data supplement).

Cytotoxicity and Caspase 3/7 Activation Assays

HUVECs (24×10^3 cells per well) were plated in opaque-walled 96-well plates and maintained in 20% FBS medium, 0.2% FBS, or 0.2% FBS with Ang-1 (300 ng/mL). Cytotoxicity and caspase 3/7 activation in adherent cells were analyzed 48 hours later using an ApoTox-Glo Triplex Assay (Promega, Madison WI).

Cell Number Measurements

HUVECs (5×10^4 cells per well) were plated in 24-well plates 24 hours posttransfection with siGENOME SMARTpool siRNA specific for DUSP1, DUSP4, and DUSP5 or with siCONTROL scrambled siRNA. Cells were maintained in medium with 2% FBS with or without Ang-1 (300 ng/mL) for 48 hours posttransfection. Cell growth was assessed using an incubator-installed IncuCyte Live Cell Monitoring System (Essen Instruments, Ann Arbor, MI). Cells were photographed and counted 24 hours posttreatment.

Wound Healing Assay

HUVEC monolayers were wounded using a 200-μL pipette tip and maintained for 8 hours in 2% FBS media with or without Ang-1 (300 ng/mL). Wounded areas were visualized using an Olympus inverted microscope, quantified using Image-Pro Plus software (Media Cybernetics, Inc, Bethesda, MD), and reported as percent wound healing—$(1-[\text{wound area at } t_{8\ h}/\text{wound area at } t_0]\times100)$—where t_0 is the time immediately following wounding.

Capillary Tube Formation

HUVECs (2×10^5 cells per well) were seeded onto 24-well plates precoated with growth factor–reduced Matrigel in MCDB131 medium plus 1% FBS. Images from a total of 10 fields were captured 24 hours later using an Olympus inverted microscope (×40) and analyzed using Image-Pro Plus. Angiogenic tube formation activity was determined by counting branching points of formed tubes and the total tube length in each field, as previously described.[16]

Vascular Permeability Assay

HUVECs (1×10^5 cells per well) were plated in Costar transwell inserts coated with type I collagen (6.5-mm-diameter, 3.0-μm pore size polycarbonate filter; Corning Life Sciences, Tewkesbury, MA) and cultured for 3 to 4 days until a tight monolayer formed. Cells were serum-starved in MCDB131 medium with 1% bovine serum albumin for 1 hour prior to the addition of Ang-1 (300 ng/mL) and 1 mg/mL 40-kDa fluorescein isothiocyanate–labeled dextran (Life Technologies, Inc) to upper chambers. Permeability was assessed 30 minutes later by measuring the fluorescence of the lower chamber at 492 nm excitation/520 nm emission with a SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, CA).

Data Analysis

Data are expressed as means±standard errors. Differences between experimental groups were determined by the nonparameteric Mann–Whitney test. Differences in cytotoxicity and caspase 3/7 activity measured in cells maintained in media containing 20% FBS medium, 0.2% FBS, or 0.2% FBS plus Ang-1 in the presence of scrambled, DUSP1, DUSP4, or DUSP5 siRNA oligos were determined by 2-way analysis of variance followed by a Student–Newman–Keuls post hoc test. P values <0.05 were considered statistically significant.

Results

Ang-1 Induction of DUSP Expression

To identify DUSP induction in response to Ang-1 exposure, HUVECs were exposed for 1 hour to 300 ng/mL of Ang-1. DUSP1, DUSP3, DUSP4, DUSP5, DUSP6, DUSP7, DUSP11, DUSP12, DUSP14, and DUSP22 mRNA expression was measured using real-time PCR. Ang-1 elicited significant inductions of DUSP1, DUSP4, and DUSP5 but exerted no significant effects on any other DUSPs (Figure 1A). In comparison, VEGF elicited significant induction of DUSP1 and DUSP5 but had no effect on DUSP4 (Figure 2A). Exposure for 1 hour to a combination of Ang-1 and VEGF did not result in any additional increases in DUSP1 or DUSP5 mRNA levels, compared with those measured in response to Ang-1 alone (Figure 2B). Ang-1-induced expression of DUSP mRNA was dose dependent (Figure 1B) and time dependent, with peak levels detected 1 hour after Ang-1 exposure (Figure 1C). Exposure to Ang-2 (300 ng/mL) elicited time-dependent declines in DUSP1 and DUSP5 expression, whereas DUSP4 expression remained unchanged compared with control values (Figure 1D).

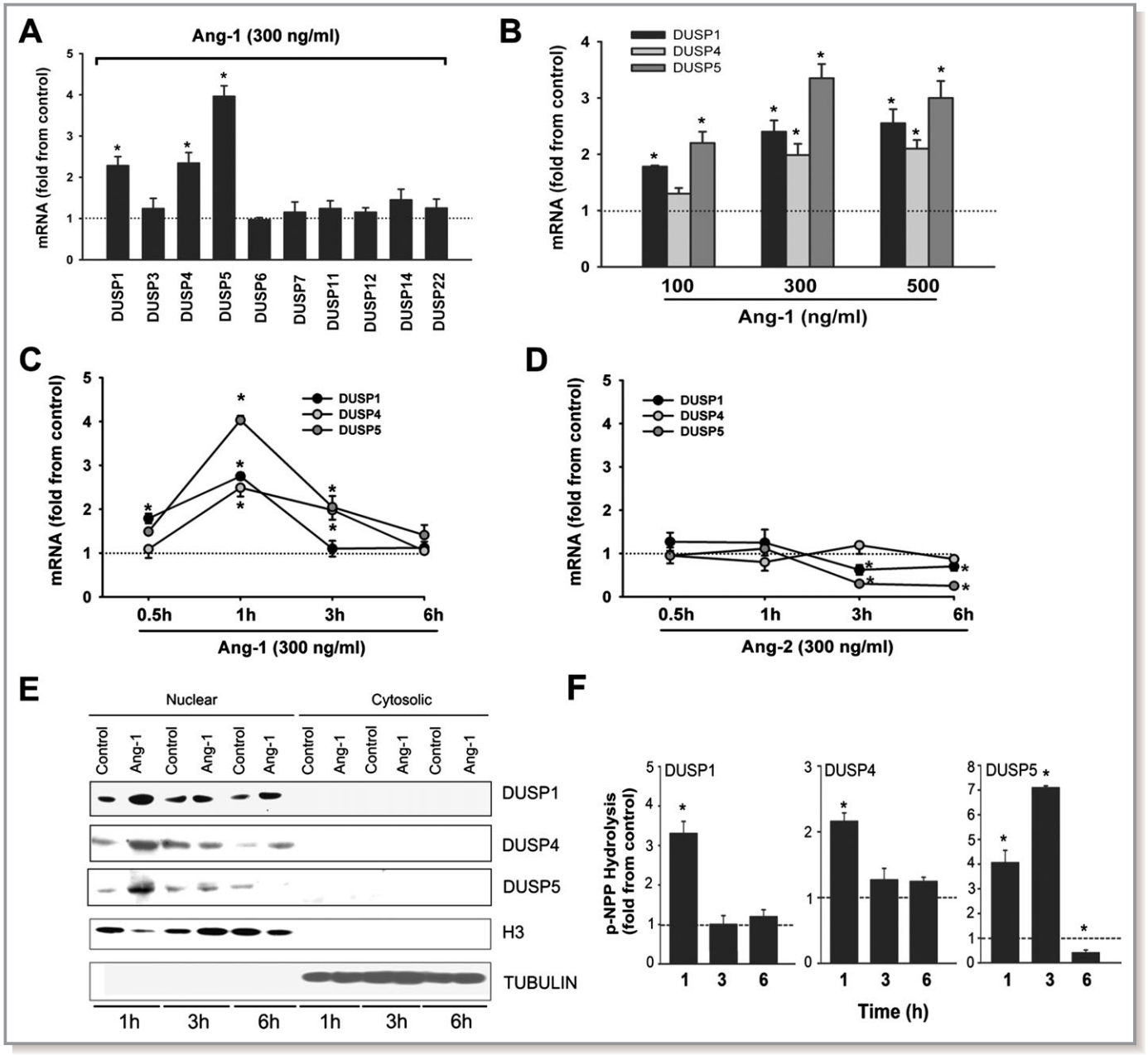

Figure 1. A, mRNA expression of DUSPs in HUVECs exposed to PBS (control) or Ang-1 (300 ng/mL) for 1 hour. Values expressed as mean±SE. **P*<0.05 compared with control; n=6 per group. B, mRNA expression of DUSP1, DUSP4, and DUSP5 in HUVECs exposed to PBS (control) or varying concentrations of Ang-1 for 1 hour. Values expressed as mean±SE. **P*<0.05 compared with control; n=6 per group. C, mRNA expression of DUSP1, DUSP4, and DUSP5 in HUVECs exposed to PBS (control) or Ang-1 (300 ng/mL) for varying durations. Values expressed as mean±SE. **P*<0.05 compared with control; n=6 per group. D, mRNA expression of DUSP1, DUSP4, and DUSP5 in HUVECs exposed to PBS (control) or Ang-2 (300 ng/mL) for varying durations. Values expressed as mean±SE. **P*<0.05 compared with control; n=6 per group. E, Representative immunoblots of DUSP1, DUSP4, and DUSP5 proteins in nuclear and cytosolic fractions of HUVECs exposed to PBS (control) for 1 hour or Ang-1 (300 ng/mL) for 1, 3, or 6 hours. H3 refers to histone 3, a marker of nuclear proteins. Tubulin was used as a marker for cytosolic proteins. F, Phosphatase activity of DUSP1, DUSP4, and DUSP5 in HUVECs exposed to PBS (control) for 1 hour or Ang-1 (300 ng/mL) for varying durations. Values expressed as mean±SE. **P*<0.05 compared with control; n=5 per group. Ang-1 indicates angiopoietin-1; DUSP, dual-specificity phosphatase; HUVEC, human umbilical vein endothelial cell; mRNA, messenger RNA; PBS, phosphate-buffered saline; *p*-NPP, *p*-nitrophenyl phosphate.

DUSP1, DUSP4, and DUSP5 protein levels and subcellular localization were evaluated by separating cell lysates into cytosolic and nuclear fractions. In PBS-treated cells (control), DUSP1, DUSP4, and DUSP5 were detected in the nuclear fraction (Figure 1E), and Ang-1 exposure significantly induced their levels within 1 to 3 hours (Figure 1E). Measurements of

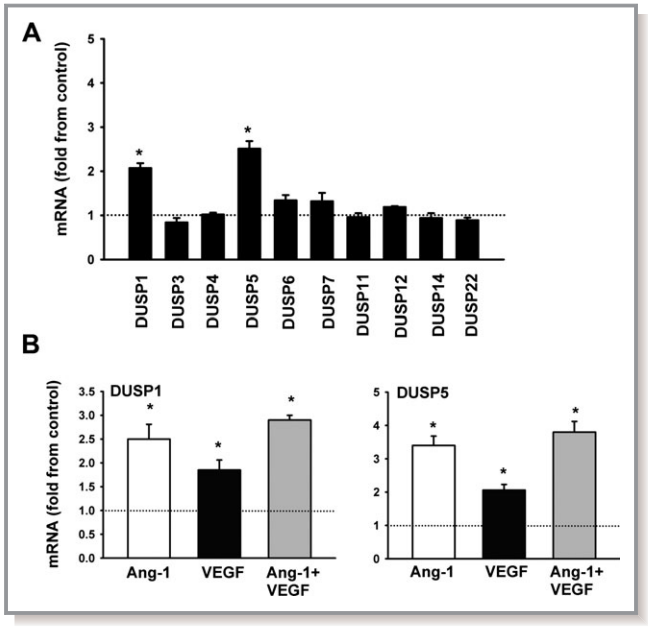

Figure 2. A, Mean±SE of mRNA expression of various DUSPs in HUVECs exposed to PBS (control) or VEGF (40 ng/mL) for 1 hour. **P*<0.05 compared with control; n=6 per group. B, Mean±SE of mRNA expression of DUSP1 and DUSP5 in HUVECs exposed for 1 hour to PBS (control), Ang-1 (300 ng/mL), VEGF (40 ng/mL), or combination of Ang-1 and VEGF. n=6. **P*<0.05 compared with control; n=5 per group. Ang-1 indicates angiopoietin-1; DUSP, dual-specificity phosphatase; HUVEC, human umbilical vein endothelial cell; mRNA, messenger RNA; PBS, phosphate-buffered saline; VEGF, vascular endothelial growth factor.

p-nitrophenyl phosphate hydrolysis revealed that phosphatase activity of DUSP1, DUSP4, and DUSP5 was significantly and transiently elevated within 1 to 3 hours of Ang-1 exposure (Figure 1F). Immunostaining confirmed that DUSP1, DUSP4, and DUSP5 were present in the cell nuclei of control and Ang-1-treated cells (Figure 3).

Ang-1 Regulation of DUSP Transcription and mRNA Stability

To identify mechanisms through which Ang-1 induces DUSP expression in ECs, luciferase reporter assays were used to measure DUSP1 and DUSP4 promoter activity in response to Ang-1 exposure. Ang-1 significantly induced DUSP1 and DUSP4 promoter activity, indicating that increased transcription is a mechanism of upregulation (Figure 4). To determine whether Ang-1 triggers increases in DUSP levels through alterations in mRNA stability, transcription inhibition by actinomycin D was performed, and the rate of decline of mRNA expression was measured. Ang-1 exerted no significant influences on the rate of decline in DUSP1, DUSP4, or DUSP5, indicating that Ang-1 does not affect mRNA stability (Figure 4).

Mechanisms of DUSP Induction

Ang-1 exposure activates the ERK1/2, p38, SAPK/JNK, and PI-3 kinase/AKT signaling pathways. Their roles in Ang-1 regulation of DUSP expression were assessed using pharmacological inhibitors of ERK1/2 (PD184352), p38 (BIRB0796), SAPK/JNK (SP600125), and PI-3 kinase (wortmannin). Ang-1 did not induce DUSP1 in the presence of p38 or PI-3 kinase inhibitors (Figure 5A). Ang-1 did not induce DUSP4 in the presence of p38, ERK1/2, SAPK/JNK, or PI-3 kinase inhibitors (Figure 5B). Ang-1 did not induce DUSP5 expression in the presence of ERK1/2 or PI-3 kinase inhibitors (Figure 5C). To further assess the roles of the SAPK/JNK pathway in Ang-1 regulation of DUSP mRNA expression, cells were transduced with empty retroviruses (MSCV) or retroviruses expressing a dominant-negative form of SAPK/JNK (MSCV-JNK-APF).[3] Ang-1 did not induce DUSP4 in MSCV-JNK-APF cells compared with in MSCV cells (Figure 5B). In contrast, Ang-1 did significantly induce DUSP1 (Figure 5A) and DUSP5 (Figure 5C) in these cells. These results indicate that Ang-1 induction of DUSP1 is mediated by the p38 and PI-3 kinase pathways, Ang-1 induction of DUSP4 induction is mediated by the ERK1/2, p38, SAPK/JNK, and PI-3 kinase pathways, and Ang-1 induction of DUSP5 is mediated by the ERK1/2 and PI-3 kinase pathways. To assess the importance of Tie-2 receptors to Ang-1-induced changes in DUSP1, DUSP4, and DUSP5 expression, cells were infected with Ad-GFP or Ad-Ex Tek. Ad-Ex Tek adenoviruses deliver a recombinant soluble Tie-2 receptor capable of blocking Tie-2 receptor activation in response to Ang-1 exposure[17] (online data supplement). Ang-1 significantly induced DUSP1, DUSP4, and DUSP5 expression in cells transduced with Ad-GFP (Figure 5). Ang-1 did not induce expression of these DUSPs in cells transduced with Ad-Ex Tek, indicating that Tie-2 receptor activation is necessary for Ang-1 to have a stimulatory effect on DUSP1, DUSP4, or DUSP5 expression (Figure 5).

Activating protein-1 (AP-1) and Egr-1 transcription factors are activated downstream of Tie-2 receptors in Ang-1-exposed cells and contribute to Ang-1-induced migration and proliferation.[6,18] The protein c-Jun, the main subunit of AP-1, is activated by Tie-2 receptors. To assess the role of AP-1 in Ang-1-induced DUSP expression, c-Jun was inhibited using adenoviruses expressing a dominant-negative form of the protein (TAM67).[12] In Ad-TAM67-transfected cells, Ang-1 did not induce DUSP4 or DUSP5 but significantly induced DUSP1 (Figure 6). This suggests that AP-1 is critically involved in Ang-1-induced DUSP4 and DUSP5 expression. To assess the role of Egr-1 in Ang-1-induced DUSP expression, cells transfected with Egr-1 siRNA oligos were used (Figure 6). In Egr-1-depleted cells, Ang-1 did not induce DUSP1, but significantly induced DUSP4 and DUSP5 (Figure 6), suggesting that Egr-1 is critically important to Ang-1-induced DUSP1 expression.

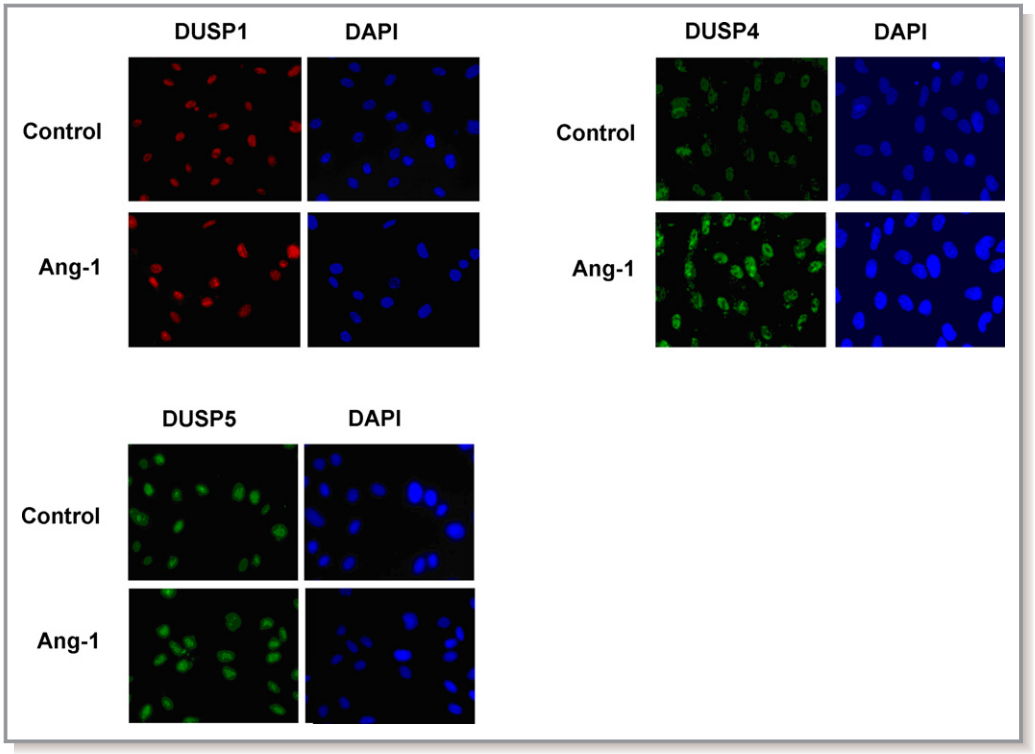

Figure 3. Immunostaining for DUSP1, DUSP4, and DUSP5 proteins in HUVECs exposed to PBS (control) or Ang-1 (300 ng/mL) for 1 hour. Ang-1 indicates angiopoietin-1; DUSP, dual-specificity phosphatase; HUVEC, human umbilical vein endothelial cell; mRNA, messenger RNA; PBS, phosphate-buffered saline.

Functional Roles of DUSPs

MAPK phosphorylation, cytotoxicity, caspase 3/7 activity, migration, capillary tube formation, and vascular permeability were evaluated in cells transfected with scrambled, DUSP1, DUSP4, or DUSP5 siRNA oligos. Initially, DUSP1, DUSP4, and DUSP5 levels were confirmed as being significantly attenuated using their corresponding siRNA oligos (online data supplement).

DUSP1

In cells transfected with scrambled siRNA oligos, Ang-1 induced significant increases in phosphorylation of ERK1/2, p38, and SAPK/JNK (Figure 7A). Knockdown of DUSP1 expression resulted in augmented Ang-1-induced p38 phosphorylation and attenuated Ang-1-induced SAPK/JNK phosphorylation, but had no influence on Ang-1-induced ERK1/2 phosphorylation (Figure 7A and 7B). These results suggest that DUSP1 plays an important role in Ang-1 regulation of p38 and SAPK/JNK. In response to serum deprivation of cells transfected with scrambled siRNA oligos, cytotoxicity and caspase 3/7 activity significantly increased, whereas cell numbers significantly decreased (Figure 7C and 7D). Knockdown of DUSP1 expression did not alter these results (Figure 7C), although the presence of Ang-1 significantly attenuated serum deprivation–induced cytotoxicity and caspase 3/7 activity and significantly increased cell numbers (Figure 7C and 7D). Moreover, these effects of Ang-1 did not change in the presence of DUSP1 knockdown, suggesting that DUSP1 does not play a major role in the antiapoptotic and prosurvival effects of Ang-1 (Figure 7C and 7D).

In cells transfected with scrambled siRNA oligos, Ang-1 significantly enhanced cell migration as measured by an in vitro wound healing assay (Figure 7E). Knockdown of DUSP1 expression resulted in complete elimination of this effect (Figure 7E). These results indicate that DUSP1 plays an important role in Ang-1 regulation of cell migration. Ang-1 significantly enhanced in vitro capillary tube formation in cells transfected with scrambled siRNA oligos, as indicated by significant increases in the number of branching points and capillary tube length (Figure 8A through 8C). Knockdown of DUSP1 resulted in complete elimination of these effects, indicating that DUSP1 is essential to Ang-1-induced endothelial cell differentiation (Figure 8A through 8C). Ang-1 significantly attenuated basal permeability in cells transfected with scrambled siRNA oligos (Figure 8D). This effect was not affected by DUSP1 knockdown, indicating that DUSP1 is not involved in Ang-1 effects on vascular permeability (Figure 8D).

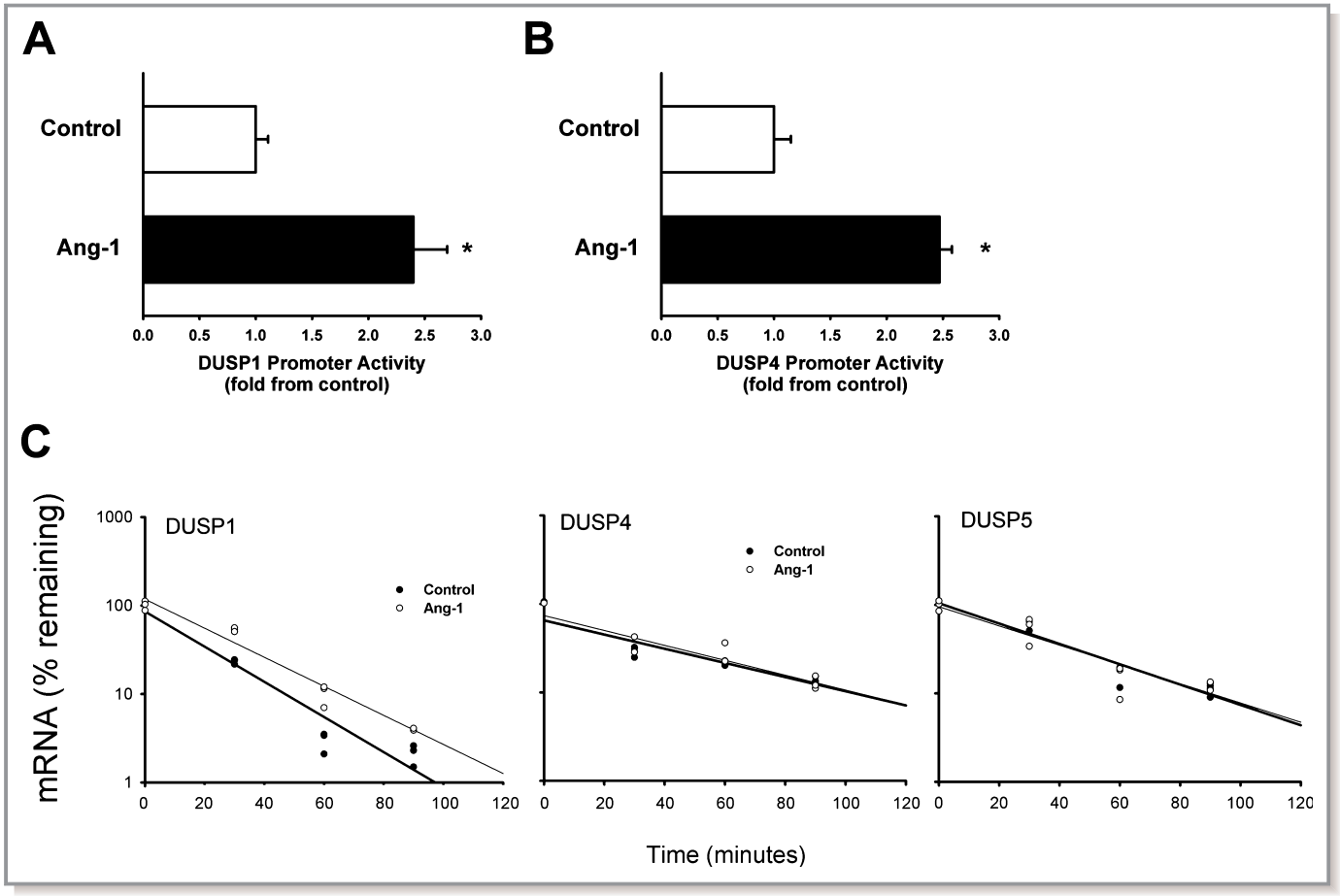

Figure 4. A, DUSP1 and DUSP4 promoter activities in HUVECs exposed to PBS (control) or Ang-1 (300 ng/mL) for 6 hours. Values expressed as meanSE. *$P<0.05$ compared with control; n=5 per group. B, Ang-1 regulation of DUSP1, DUSP4, and DUSP5 mRNA stability in HUVECs exposed to Ang-1 (300 ng/mL) for 1 hour. Cells were washed postexposure with PBS and maintained in serum-free medium containing actinomycin D (control) or medium containing actinomycin D plus Ang-1 (300 ng/mL). Total RNA was extracted after varying durations (0, 30, 60, and 90 minutes) of exposure. DUSP1, DUSP4, and DUSP5 transcripts were analyzed using real-time PCR. Ang-1 indicates angiopoietin-1; DUSP, dual-specificity phosphatase; HUVEC, human umbilical vein endothelial cell; mRNA, messenger RNA; PCR, polymerase chain reaction; PBS, phosphate-buffered saline.

DUSP4

Knockdown of DUSP4 expression resulted in augmentation and prolongation of Ang-1-induced ERK1/2, p38, and SAPK/JNK phosphorylation, suggesting that DUSP4 plays a major role in Ang-1 regulation of these pathways (Figure 9A and 9B). Knockdown of DUSP4 expression did not alter basal levels of cytotoxicity and caspase 3/7 activity but blunted serum deprivation-induced increases in both (Figure 9C). Inhibitory effects of Ang-1 on serum deprivation-induced cytotoxicity, caspase 3/7 activity, and decline in cell number were not observed in the presence of DUSP4 knockdown, suggesting that DUSP4 plays a major role in the antiapoptotic and prosurvival effects of Ang-1 (Figure 9C and 9D). Knockdown of DUSP4 also resulted in complete elimination of Ang-1-induced cell migration, indicating that DUSP4 also plays an important role in Ang-1 regulation of migration (Figure 9E). Furthermore, knockdown of DUSP4 resulted in complete elimination of Ang-1-induced capillary tube formation, indicating that DUSP4 is essential to Ang-1-induced endothelial cell differentiation (Figure 8A through 8C). The inhibitory effect of Ang-1 on basal vascular permeability was not affected by DUSP4 knockdown, indicating that DUSP4 does not play a major role in this effect (Figure 8D).

DUSP5

Knockdown of DUSP5 expression resulted in augmentation and prolongation of Ang-1-induced ERK1/2 phosphorylation but exerted no influence on Ang-1-induced p38 and SAPK/JNK phosphorylation, suggesting that DUSP5 plays a significant role in Ang-1 regulation of the ERK1/2 pathway (Figure 10A and 8B). Knockdown of DUSP5 significantly

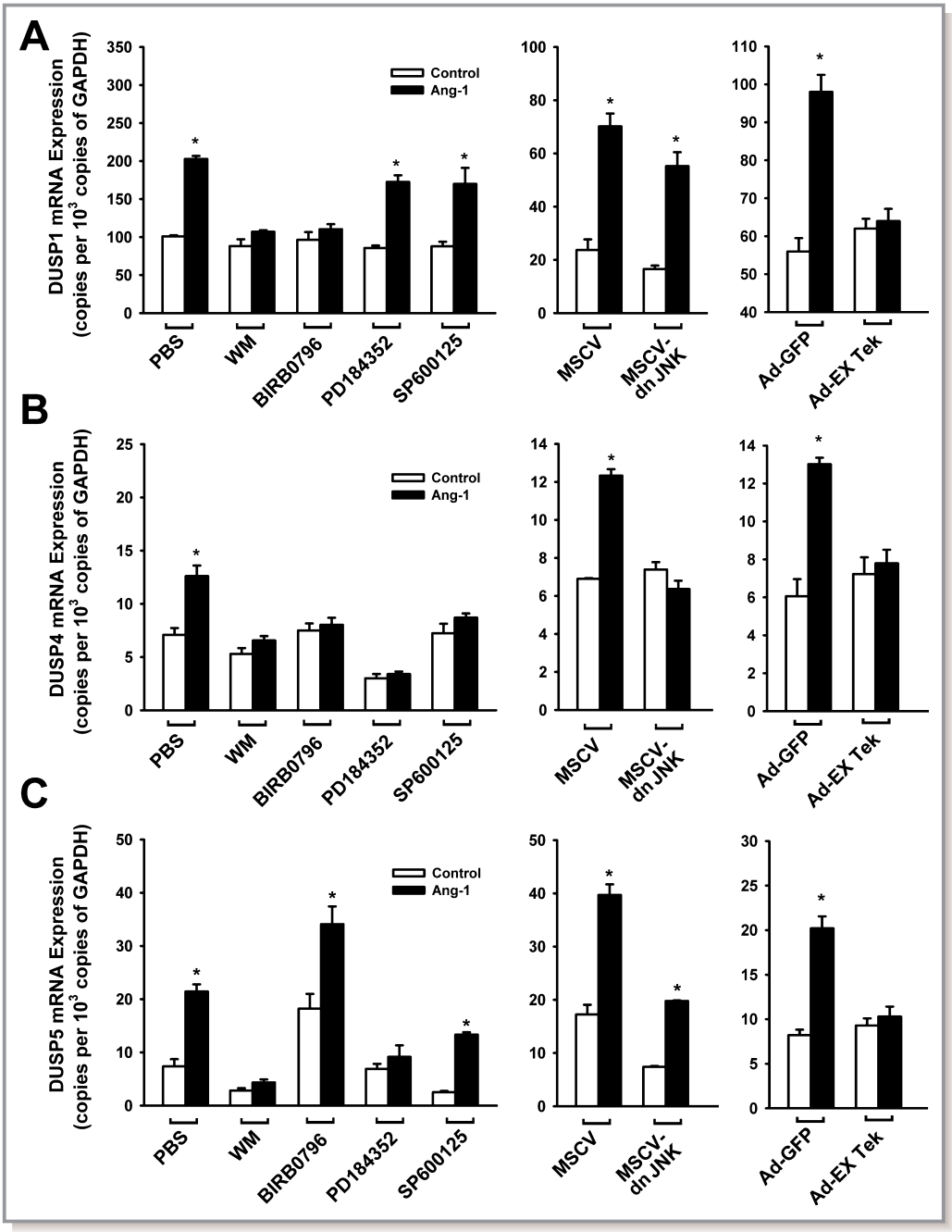

Figure 5. A through C, left, Effects of pretreatment with PBS, wortmannin (WM), PI-3 kinase inhibitor; BIRB0796 (p38 inhibitor); PD18435 (ERK1/2 inhibitor); or SP600125 (SAPK/JNK inhibitor) on mRNA expression of DUSP1, DUSP4, and DUSP5 in HUVECs exposed to PBS (control) or Ang-1. Values expressed as meanSE. *P<0.05 compared with own control; n=6 per group. A through C, middle, mRNA expression of DUSP1, DUSP4, and DUSP5 in HUVECs stably expressing empty vector (MSCV) or a dominant-negative form of JNK (MSCV-dn JNK) and exposed to PBS (control) or Ang-1. Values expressed as meanSE. *P<0.05 compared with own control; n=6 per group. A through C, right, mRNA expression of DUSP1, DUSP4, and DUSP5 in HUVECs infected with Ad-GFP or Ad-Ex Tek and exposed 48 hours postinfection to PBS (control) or Ang-1 (300 ng/mL). Values expressed as mean±SE. *P<0.05 compared with own control; n=6 per group. Ang-1 indicates angiopoietin-1; DUSP, dual-specificity phosphatase; GFP, green fluorescent protein; HUVEC, human umbilical vein endothelial cell; JNK, Jun N-terminal kinase; mRNA, messenger RNA; MSCV, murine stem cell virus; PBS, phosphate-buffered saline.

increased basal and serum deprivation–induced cytotoxicity and caspase 3/7 activity (Figure 10C and 10D). Inhibitory effects of Ang-1 on serum deprivation–induced cytotoxicity, caspase 3/7 activity, and decline in cell number were unaffected by DUSP5 knockdown (Figure 10C and 10D), suggesting that DUSP5 does not play a major role in the antiapoptotic and prosurvival effects of Ang-1. Knockdown of DUSP5 had no effect on Ang-1-induced cell migration

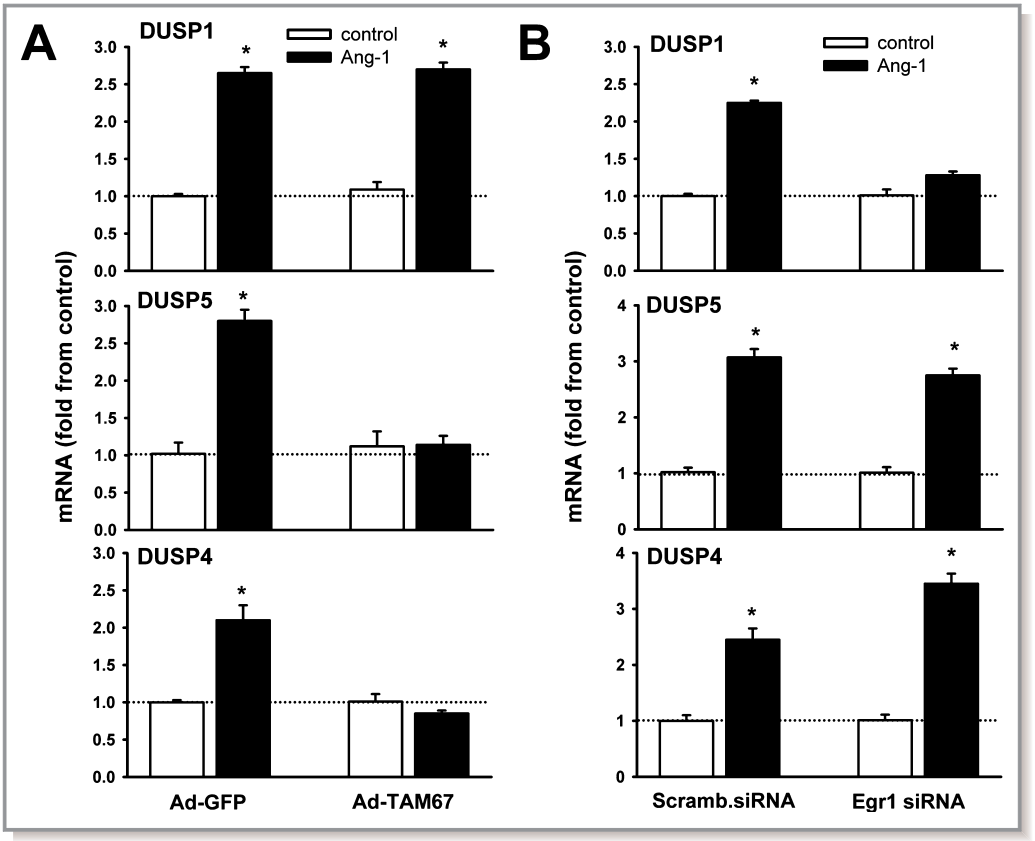

Figure 6. A, DUSP1, DUSP4, and DUSP5 mRNA expression in HUVECs infected with adenoviruses expressing GFP (control) or dominant-negative c-Jun (TAM67) and exposed 48 hours postinfection to PBS (control) or Ang-1 (300 ng/mL) for 1 hour. Values expressed as mean±SE. *P<0.05 compared with own control group; n=6 per group. B, DUSP1, DUSP4, and DUSP5 mRNA expression in HUVECs transfected with scrambled siRNA or Egr-1 siRNA oligos and exposed 48 hours posttransfection to PBS (control) or Ang-1 (300 ng/mL) for 1 hour. Values expressed as mean±SE. *P<0.05 compared with own control group; n=6 per group. Ang-1 indicates angiopoietin-1; DUSP, dual-specificity phosphatase; Egr-1, early growth response–1; HUVEC, human umbilical vein endothelial cell; mRNA, messenger RNA; PBS, phosphate-buffered saline; siRNA, small interfering RNA.

(Figure 10E) or capillary tube formation (Figure 8A through 8C). Knockdown of DUSP5 completely eliminated the inhibitory effect of Ang-1 on basal vascular permeability (Figure 8D).

Discussion

The principal findings of this study are: (1) in HUVECs, Ang-1 induces transient increases in the expression of DUSP1, DUSP4, and DUSP5 through activation of the PI-3 kinase, ERK1/2, p38, and SAPK/JNK signaling pathways; (2) Ang-1 induction of DUSP1, DUSP4, and DUSP5 expression is achieved through enhanced transcription, which is mediated by AP-1 and Egr-1 transcription factors; (3) DUSP1 and DUSP5 regulate Ang-1-induced p38 and ERK1/2 phosphorylation, respectively, whereas DUSP4 regulates Ang-1-induced ERK1/2, p38, and SAPK/JNK phosphorylation; (4) DUSP4 plays an important role in Ang-1-induced cell survival and inhibition of apoptosis; (5) DUSP1 and DUSP4 play important roles in Ang-1-induced cell migration and differentiation; and (6) DUSP5 is essential to Ang-1 inhibition of basal vascular permeability.

Regulation of DUSP1, DUSP4, and DUSP5

We report here that exposure of HUVECs to Ang-1 elicits significant induction of DUSP1, DUSP4, and DUSP5 expression and phosphatase activity. Previous studies in ECs have demonstrated that DUSP1 and DUSP5 are early response genes that are upregulated in response to angiogenic growth factors, including VEGF and thrombin.[10,19] In this study we have demonstrated that DUSP1 and DUSP5 mRNA and protein expression are transiently increased by Ang-1 in a fashion similar to that elicited by VEGF. Moreover, our results also indicate that DUSP4 is induced by Ang-1 but not by VEGF, suggesting that the regulatory functions of DUSP4 are unique to Ang-1 signaling and are unlikely to contribute to VEGF signaling.

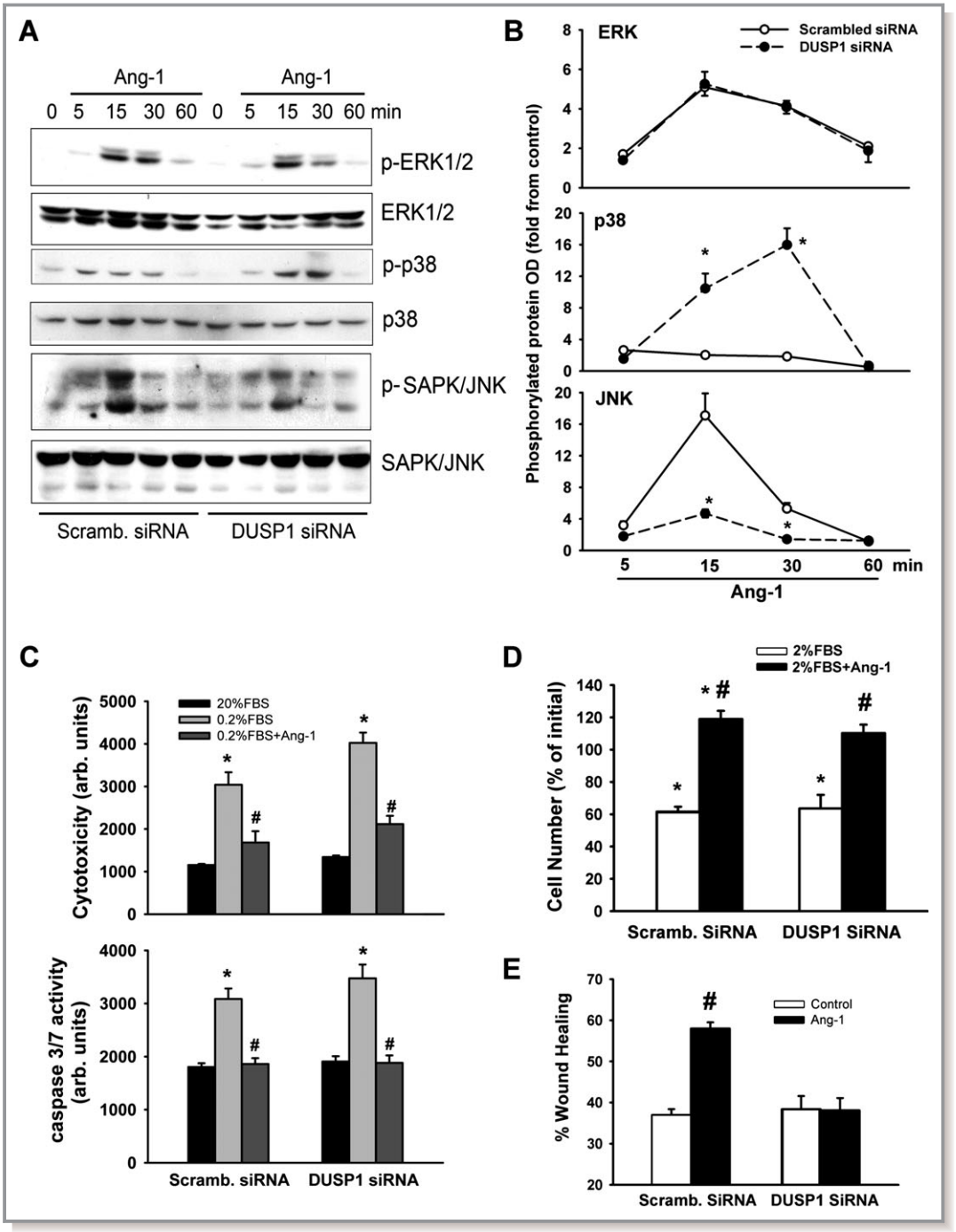

Figure 7. A, Representative immunoblots of ERK1/2, p38, and SAPK/JNK protein expression in HUVECs transfected with scrambled or DUSP1 siRNA and exposed 48 hours posttransfection to PBS (control) for 1 hour or Ang-1 (300 ng/mL) for varying durations. B, Relative protein optical densities of phosphorylated ERK1/2, p38, and SAPK/JNK in HUVECs transfected with scrambled or DUSP1 siRNA and exposed 48 hours posttransfection to PBS (control) for 1 hour or Ang-1 (300 ng/mL) for varying durations. Values expressed as mean±SE. **P*<0.05 compared with scrambled siRNA; n=5 per group. C, Cytotoxicity and caspase 3/7 activity in HUVECs transfected with scrambled or DUSP1 siRNA and maintained for 24 hours in medium containing 20% FBS, 0.2% FBS, or 0.2% FBS plus Ang-1. Values expressed as mean±SE. **P*<0.05 compared with 20% FBS; #*P*<0.05 compared with corresponding 0.2% FBS values; n=6 per group. D, Number of HUVECs transfected with scrambled or DUSP1 siRNA and maintained for 24 hours in medium containing 2% FBS (control) or 2% FBS plus Ang-1. Cell number expressed as percentage of initial cell count. **P*<0.05 compared with initial cell count; #*P*<0.05 compared with corresponding 2% FBS values; n=6 per group. E, Percent wound healing in HUVECs transfected with scrambled or DUSP1 siRNA and maintained for 8 hours in medium containing 2% FBS (control) or 2% FBS plus Ang-1. #*P*<0.05 compared with corresponding control values; n=6 per group. Ang-1 indicates angiopoietin-1; DUSP, dual-specificity phosphatase; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; HUVEC, human umbilical vein endothelial cell; PBS, phosphate-buffered saline; SAPK, stress activated protein kinase; siRNA, small interfering RNA.

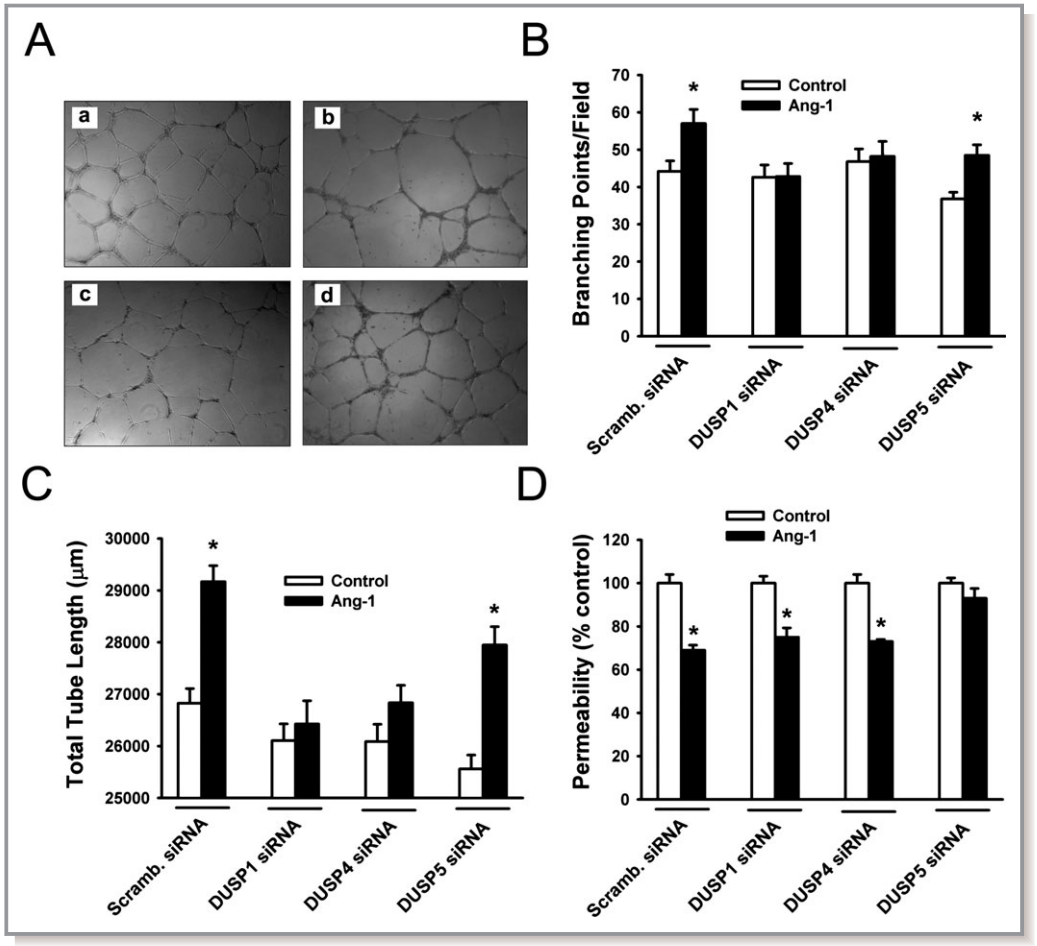

Figure 8. A, Representative micrograph of capillary tube formation in HUVECs transfected with (a) scrambled, (b) DUSP1, (c) DUSP4, or (d) DUSP5 siRNA and exposed 48 hours posttransfection for 24 hours to Ang-1. B and C, Branching points/field and total tube length in HUVECs transfected with scrambled, DUSP1, DUSP4, or DUSP5 siRNA and exposed 48 hours posttransfection for 24 hours to PBS (control) or Ang-1. Values expressed as mean±SE.*P<0.05 compared with corresponding control values; n=6 per group. D, Permeability changes in HUVECs transfected with scrambled, DUSP1, DUSP4, or DUSP5 siRNA and exposed 48 hours posttransfection for 30 minutes to PBS (control) or Ang-1. Values expressed as mean±SE.*P<0.05 compared with corresponding control values; n=6 per group. Ang-1 indicates angiopoietin-1; DUSP, dual-specificity phosphatase; HUVEC, human umbilical vein endothelial cell; PBS, phosphate-buffered saline; siRNA, small interfering RNA.

MAPK signaling pathways are well-known regulators of cell survival, migration, and proliferation. Our group and others have demonstrated that Ang-1 exposure triggers simultaneous and significant increases in ERK1/2, p38, and SAPK/JNK phosphorylation.[3,20] It has also been well established that MAPKs induce DUSPs as a transcriptional negative feedback mechanism to control their activities.[7,8] The results of this study indicate that the Ang-1/Tie-2 axis uses the ERK1/2, p38, and SAPK/JNK pathways to upregulate DUSP1, DUSP4, and DUSP5. Specifically, we found that although the ERK1/2 and p38 pathways separately regulate DUSP5 and DUSP1 expression, respectively, they, along with the SAPK/JNK pathway, also regulate DUSP4 expression (Figure 5).

We have previously found that in Ang-1-stimulated HUVECs the PI-3 kinase pathway activates transcription factors AP-1 and Egr-1 in a MAPK-independent fashion.[6,18] We suggest that these transcription factors also participate in PI-3 kinase pathway regulation of DUSPs. Specifically, we found that inhibition of the c-Jun subunit of AP-1 using a dominant-negative form of the protein resulted in elimination of Ang-1-induced DUSP4 and DUSP5 expression, but had no effect on Ang-1-induced DUSP1 expression. We have previously reported that Ang-1 induces transactivation of AP-1 in ECs by increasing c-Jun phosphorylation on Ser^{63} and Ser^{73} and that this effect is mediated through the ERK1/2, SAPK/JNK, and PI-3 kinase pathways.[6] These findings suggest that Ang-1 triggers activation of these pathways, which, in turn, induce transactivation of c-Jun, enhanced binding of c-Jun to DUSP4 and DUSP5 promoters, and transcription upregulation of both DUSPs. This scenario is supported by observations in cancer cells that implicate c-Jun activation in the regulation of DUSP5.[21]

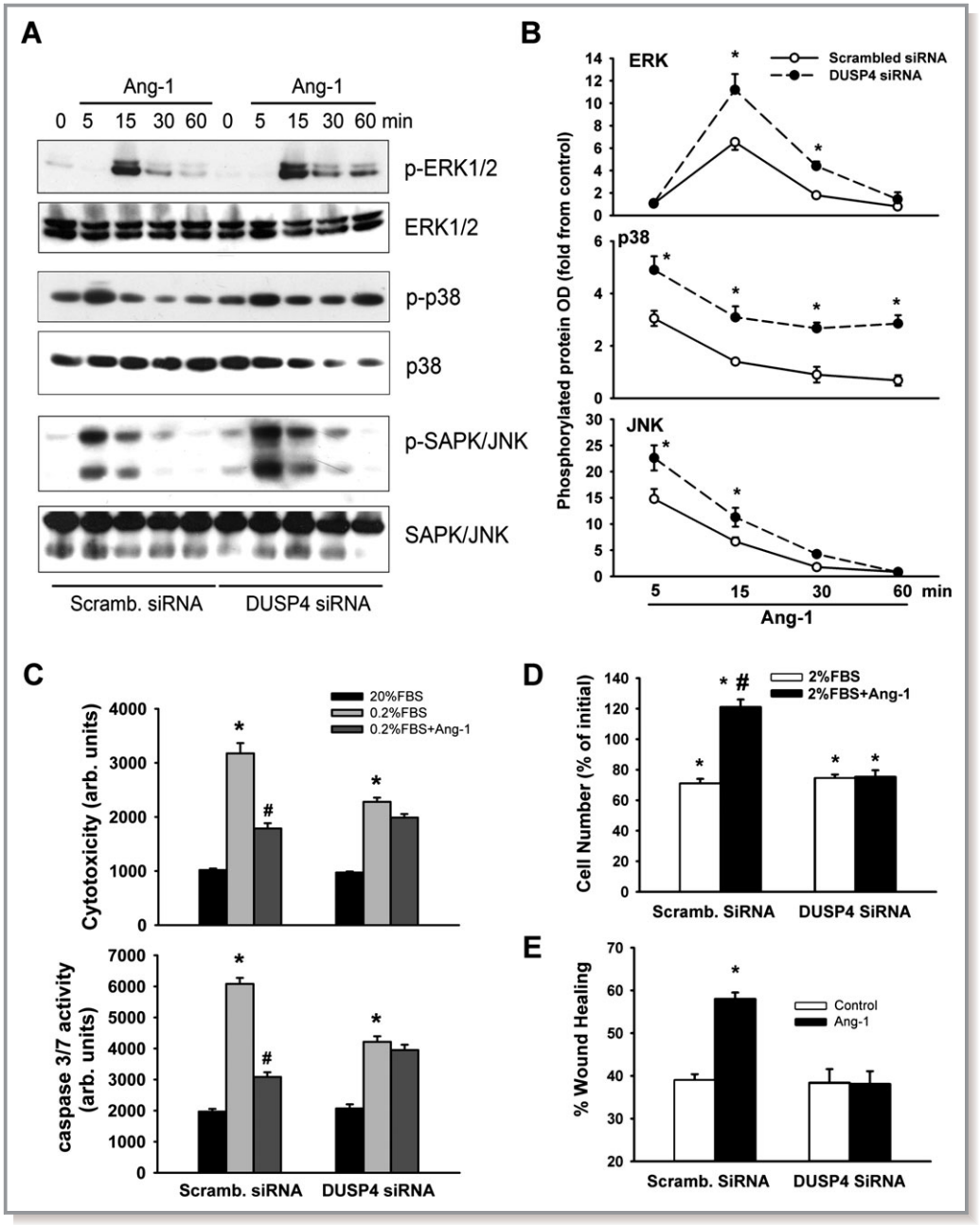

Figure 9. A, Representative immunoblots of ERK1/2, p38, and SAPK/JNK protein expression in HUVECs transfected with scrambled or DUSP4 siRNA and exposed 48 hours posttransfection to PBS (control) for 1 hour or Ang-1 (300 ng/mL) for varying durations. B, Relative protein optical density of phosphorylated ERK1/2, p38, and SAPK/JNK in HUVECs transfected with scrambled or DUSP4 siRNA and exposed 48 hours posttransfection to PBS (control) for 1 hour or Ang-1 for varying durations. Values expressed as mean±SE. *P<0.05 compared with scrambled siRNA; n=5 per group. C, Cytotoxicity and caspase 3/7 activity in HUVECs transfected with scrambled or DUSP4 siRNA and maintained for 24 hours in medium containing 20% FBS, 0.2% FBS, or 0.2% FBS plus Ang-1. *P<0.05 compared with 20% FBS. #P<0.05 compared with corresponding 0.2% FBS values; n=6 per group. D, Number of HUVECs transfected with scrambled or DUSP4 siRNA and maintained for 24 hours in medium containing 2% FBS (control) or 2% FBS plus Ang-1. Cell numbers expressed as percentage of initial cell count. *P<0.05 compared with initial cell count; #P<0.05 compared with corresponding 2% FBS values; n=6 per group. E, Percent wound healing in HUVECs transfected with scrambled or DUSP4 siRNA and maintained for 8 hours in medium containing 2% FBS (control) or 2% FBS plus Ang-1. #P<0.05 compared with corresponding control values; n=6 per group. Ang-1 indicates angiopoietin-1; DUSP, dual-specificity phosphatase; FBS, fetal bovine serum; HUVEC, human umbilical vein endothelial cell; PBS, phosphate-buffered saline; siRNA, small interfering RNA.

We also report here that attenuation of Egr-1 expression by selective siRNA oligos results in the elimination of Ang-1-induced DUSP1 expression. DUSP4 and DUSP5 expression remained unaffected. These findings suggest that Egr-1 is involved in the regulation of DUSP1 expression in HUVECs exposed to Ang-1.[22] Recently, we have observed that Egr-1

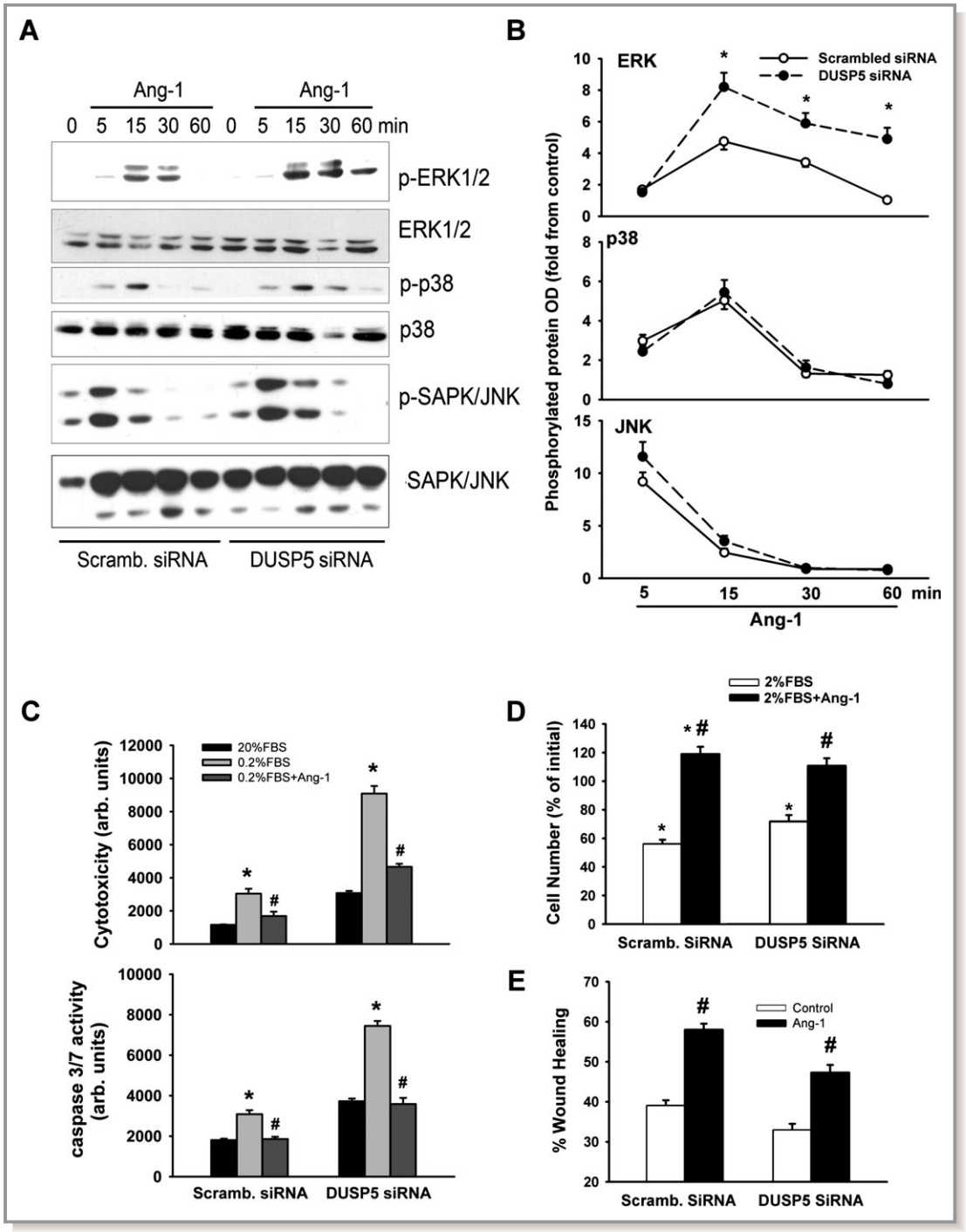

Figure 10. A, Immunoblots of ERK1/2, p38, and SAPK/JNK protein expression in HUVECs transfected with scrambled or DUSP5 siRNA and exposed to PBS (control) for 1 hour or Ang-1 for varying durations. B, Relative protein optical density of phosphorylated ERK1/2, p38, and SAPK/JNK in HUVECs transfected with scrambled or DUSP5 siRNA and exposed 48 hours posttransfection to PBS (control) for 1 hour or Ang-1 for varying durations. Values expressed as mean±SE. **P*<0.05 compared with scrambled siRNA; n=5 per group. C, Cytotoxicity and caspase 3/7 activity in HUVECs transfected with scrambled or DUSP5 siRNA and maintained for 24 hours in medium containing 20% FBS, 0.2% FBS, or 0.2% FBS plus Ang-1. Values expressed as mean±SE. **P*<0.05 compared with 20% FBS; #*P*<0.05 compared with corresponding 0.2% FBS values; n=6 per group. D, Number of HUVECs transfected with scrambled or DUSP5 siRNA and maintained for 24 hours in medium containing 2% FBS (control) or 2% FBS plus Ang-1. **P*<0.05 compared with initial cell count; #*P*<0.05 compared with corresponding 2% FBS values; n=6 per group. E, Percent wound healing in HUVECs transfected with scrambled or DUSP5 siRNA and maintained for 8 hours in medium containing 2% FBS (control) or 2% FBS plus Ang-1. #*P*<0.05 compared with corresponding control values; n=6 per group. Ang-1 indicates angiopoietin-1; DUSP, dual-specificity phosphatase; FBS, fetal bovine serum; HUVEC, human umbilical vein endothelial cell; PBS, phosphate-buffered saline; siRNA, small interfering RNA.

expression and activity are rapidly induced in HUVECs exposed to Ang-1 and that Egr-1 is essential to the proangiogenic effects of Ang-1 in ECs.[18] DUSP1 is a transcriptional target of several factors including p53, c-Jun, ATF2, CREB, E2F, and SAP-1.[23–26] To our knowledge, no information is as yet available regarding Egr-1 regulation of DUSP1 expression. One major weakness of the current study is that no ChIP assays were performed to document possible direct binding of Egr-1 to the DUSP1 promoter in HUVECs stimulated with Ang-1. In the absence of these assays, we speculate that Egr-1 may bind directly to the DUSP1 promoter, although DUSP1 promoter analyses have not revealed the presence of any distinct Egr-1 binding domains.[26] It is also possible that Egr-1 may indirectly act on DUSP1 transcription by interacting with other transcription factors, specificity factor 1, for example, which directly binds to several domains on the DUSP1 promoter.[26] This is plausible because the Egr-1 DNA-binding domain shares a high degree of homology with that of specificity factor 1.

It has been well established that the PI-3 kinase pathway is critical to the proangiogenic and antiapoptotic effects of Ang-1 in ECs.[27,28] Our finding that wortmannin markedly reduces Ang-1 inductions of DUSP1, DUSP4, and DUSP5 implies that the PI-3 kinase pathway plays a major role in the regulation of these DUSPs downstream of Tie-2 receptors. The PI-3 kinase pathway is known to regulate DUSP1 and DUSP4 expression in a variety of cells, including smooth muscles, macrophages, and cancer cells,[29–31] although little is known regarding DUSP5 regulation by this pathway. The mechanisms through which it regulates DUSP expression are likely to involve multiple factors, including those that are dependent on MAPK and MAPK-independent mechanisms. In the context of the Ang-1/Tie-2 receptor axis, we have reported that the PI-3 kinase pathway activates the ERK1/2 pathway in ECs exposed to Ang-1.[3] Thus, it is likely that the effects of the PI-3 kinase pathway on DUSP4 and DUSP5 that were observed in our experiments are also mediated through ERK1/2.

Regulation of MAPK Signaling by DUSPs

Despite their structural similarities, different DUSPs exhibit different substrate preferences, and their activities can be modulated on binding to these substrates.[32] We found that DUSP1 inactivates p38 downstream of the Ang-1/Tie-2 axis and that DUSP5 negatively regulates ERK1/2. These findings, that DUSP1 and DUSP5 are selective regulators of p38 and ERK1/2, respectively, are in accordance with those described in ECs exposed to VEGF.[10,19] Surprisingly though, we found that DUSP4 has the ability to inactivate ERK1/2, p38, and SAPK/JNK in response to Ang-1 exposure (Figure 9). Although some in vitro studies have demonstrated that this ability is more marked in relation to ERK1/2 and SAPK/JNK than it is in relation to p38,[33,34] others have identified an important role for DUSP4 in p38 inactivation. Indeed, DUSP4 is able to dephosphorylate all 3 MAPKs in CCL-38 cells[35] and can also function as a potent inhibitor of p38 activity in PC-12 cells in response to nerve growth factor.[36] These differences in DUSP4 substrate specificity can be explained in part by differential levels of phosphatase expression, because DUSP4 at very high concentrations loses its selectivity for determined MAPKs.[34] We should also emphasize that additional studies are required to assess the participation of DUSPs other than DUSP1, DUSP4, and DUSP5 in the regulation of MAPK signaling downstream of Tie-2 receptors.

DUSP Regulation of Ang-1-Induced Migration and Differentiation

It has been well established that Ang-1 stimulates cell migration and that Tie-2 receptors are essential to this response.[37–39] The signaling pathways through which Ang-1 stimulates migration involve PI-3 kinase and the adapter protein Dok-R, which is recruited to activated Tie-2 receptors and, in turn, creates binding sites for Nck and the serine kinase p21-activating kinase.[40] Enhanced Ang-1-induced migration also involves the GTPases RhoA and Rac1 and the adapter protein ShcA.[38,41] Moreover, Ang-1-induced migration requires the release of reactive oxygen species from NADPH oxidase. Reactive oxygen species modulate the activities of PI-3 kinase/AKT and the MAPKs, leading to enhanced cell migration.[42,43]

Selective contributions of various MAPK members to Ang-1-induced cell migration have yet to be fully explored, but indirect evidence suggests that ERK1/2 activation is involved.[6] Our study indicates that Ang-1-induced cell migration is strongly influenced by DUSP1 and DUSP4, because attenuation of their expression completely abrogates the promigratory effect of Ang-1. This observation, along with our findings that DUSP1 and DUSP4 regulate the intensity and kinetics of the ERK1/2, p38, and SAPK/JNK pathways in cells exposed to Ang-1, suggests that MAPKs are important mediators of the promigratory effects of Ang-1. To test this hypothesis, we measured Ang-1-induced EC migration in the presence of selective inhibitors of MAPKs. Our results indicate that inhibition of the ERK1/2 and p38 pathways, but not of the SAPK/JNK pathway, abrogated Ang-1-induced cell migration, thereby confirming the importance of ERK1/2 and p38 as mediators of migration. These results, which demonstrate the regulatory importance of DUSP1 and DUSP4 in Ang-1-induced migration, are similar to those that indicate that they also play important roles in VEGF-induced migration.[10,19]

We found that DUSP1 and DUSP4 knockdown results in significant reductions in Ang-1-induced tube formation, indicating that both have a strong effect on tube formation in HUVECs (Figure 8). We speculate that these effects may be mediated by the excessive p38 activation that is associated with their knockdown (Figures 7 and 9). This is based on observations that growth factor–induced angiogenesis is negatively affected by the p38 pathway.[44] It is also possible that attenuation of SAPK/JNK pathway activation in DUSP1-knockdown cells may contribute to poor tube formation because this pathway has been shown to play a central role in capillary tube formation in ECs.[45]

DUSP Contributions to Ang-1-Induced EC Survival

The Ang-1/Tie-2 receptor axis promotes cell survival through activation of the ERK1/2 and PI-3 kinase/AKT pathways, reduction in cytosolic levels of the mitochondrial caspase activator Smac, and upregulation of survivin.[27] In HUVECs, Ang-1 attenuates serum deprivation–induced cytotoxicity and caspase 3/7 activity while significantly increasing cell number (Figures 7, 9, and 10). Ang-1 does not attenuate serum deprivation–induced cytotoxicity and caspase 3/7 activity in DUSP4-knockdown cells, suggesting that DUSP4 serves as an antiapoptotic protein in cells exposed to Ang-1. However, DUSP4 knockdown reduced cytotoxicity and caspase 3/7 activity in HUVECs grown in medium containing 0.2% FBS alone, indicating that under serum-deprivation conditions, DUSP4 promotes apoptosis in the absence of Ang-1. These observations suggest that the degree and direction of DUSP4 regulation of EC survival and apoptosis are context dependent and are influenced by such factors as the presence or absence of nutrients or exposure to growth factors, Ang-1, for example. Additional experiments are clearly needed to identify the exact mechanisms through which DUSP4 regulates endothelial cell survival in response to serum deprivation and in the presence of Ang-1.

We speculate that the antiapoptotic effects of DUSP4 in ECs exposed to Ang-1 may be mediated through selective inhibition of DUSP4-induced SAPK/JNK activation. This is based on several reports indicating that that inactivation of SAPK/JNK by Ang-1 represents an important mechanism through which the Ang-1 pathway promotes EC survival[42,46] and the observation that overexpression of DUSP4 rescues HEK-239 cells from UV- and cisplatin-induced apoptosis, also a result of SAPK/JNK inhibition.[47] The proapoptotic role of DUSP4 in serum-deprived cells is more difficult to explain but might be the result of an imbalance between pro- and antiapoptotic MAPK pathways and the nature of interactions between MAPKs and other signaling pathways that are activated under conditions of serum deprivation.

Role of DUSPs in Vascular Permeability

Ang-1 is a strong inhibitor of vascular permeability, as shown in in vitro and in vivo studies.[48–50] Our observation that significant reduction in basal vascular permeability occurs in response to Ang-1 in HUVECs transfected with scrambled siRNA oligos (Figure 8) is in agreement with these studies. Ang-1 inhibited vascular permeability in DUSP1- and DUSP4-knockdown cells but not in DUSP5-knockdown cells, indicating that DUSP5 is essential to this Ang-1 effect (Figure 8).

Multiple signaling pathways modulate the effect of Ang-1 on vascular permeability. For instance, Ang-1 antagonizes VEGF-induced permeability through sequestration of Src by mammalian diaphanous and through the inhibition of nitric oxide release that results from phosphorylation of endothelial nitric oxide synthase by protein kinase C zeta.[48] Sphingosine kinase-1 has also been shown to mediate the inhibitory effect of Ang-1 on vascular permeability.[51] Furthermore, many reports have confirmed that MAPKs in general and ERK1/2 in particular are important regulators of vascular permeability. The ERK1/2 role is mediated in part through activation of myosin light-chain kinase, which promotes EC contractility and disruption of cell–cell junctions.[52–54] On the basis of these observations and that DUSP5 regulates ERK1/2 activation, we suggest that the role of DUSP5 in Ang-1 inhibition of vascular permeability is mediated primarily by regulation of the intensity and kinetics of ERK1/2 activation.

In summary, we report here that Ang-1 induces the expression and activation of DUSP1, DUSP4, and DUSP5 and that these effects are mediated through the PI-3 kinase, ERK1/2, p38, and SAPK/JNK pathways. We found that at least 2 transcription factors, AP-1 and Egr-1, play important roles in the regulation of DUSPs. We also report that DUSP1, DUSP4, and DUSP5 differentially contribute to Ang-1-induced EC migration, differentiation, vascular permeability, and survival.

Authorship Contributions

R. Echavarria performed experiments, analyzed results, and interpreted results. S. Hussain designed experiments, analyzed results, interpreted, and wrote the final manuscript.

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Disclosure

None.

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