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TRAF6 inhibits proangiogenic signals in endothelial cells and regulates the expression of vascular endothelial growth factor ☆

Sarah Bruneau, Dipak Datta, Jesse A. Flaxenburg, Soumitro Pal, David M. Briscoe *

Transplantation Research Center, Division of Nephrology, Department of Medicine, Children's Hospital Boston, Boston, MA, United States
Department of Pediatrics, Harvard Medical School, Boston, MA, United States

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ABSTRACT

TNF-family molecules induce the expression *Vascular Endothelial Growth Factor* (VEGF) in endothelial cells (EC) and elicit signaling responses that result in angiogenesis. However, the role of TNF-receptor associated factors (TRAFs) as upstream regulators of VEGF expression or as mediators of angiogenesis is not known. In this study, HUVEC were cotransfected with a full-length VEGF promoter-luciferase construct and siRNAs to TRAF 1, -2, -3, -5, -6, and promoter activity was measured. Paradoxically, rather than inhibiting VEGF expression, we found that knockdown of TRAF6 resulted in a 4–6-fold increase in basal VEGF promoter activity compared to control siRNA-transfected EC ($P < 0.0001$). In addition, knockdown of TRAF 1, -2, -3 or -5 resulted in a slight increase or no change in VEGF promoter activation. Using [^{3}H]thymidine incorporation assays as well as the *in vitro* wound healing assay, we also found that basal rates of EC proliferation and migration were increased following TRAF6 knockdown; and this response was inhibited by the addition of a blocking anti-VEGF antibody into cell cultures. Using a limited protein array to gain insight into TRAF6-dependent intermediary signaling responses, we observed that TRAF6 knockdown resulted in an increase in the activity of Src family kinases. In addition, we found that treatment with AZD-0530, a pharmacological Src inhibitor, reduced the regulatory effect of TRAF6 knockdown on VEGF promoter activity. Collectively, these findings define a novel pro-angiogenic signaling response in EC that is regulated by TRAF6.

1. Introduction

Angiogenesis, the generation of new blood vessels from pre-existing ones, is characteristically associated with cell-mediated immune responses and is a well-established pathological component of many chronic inflammatory diseases [1]. However, surprisingly little is known about mechanism(s) whereby the immune response results in the induced expression of angiogenesis factors and/or endothelial cell (EC) proliferation and migration. Previous studies, including ours, have shown that proinflammatory stimuli such as TNFα and CD40-induced signaling mediate the expression of the potent angiogenesis factor *Vascular Endothelial Growth Factor* (VEGF) in EC and other cell types [2–7]. In addition, CD40-induced pro-angiogenic responses in EC function in cell-mediated immunity [2,8] and are critical for EC proliferation in association with tumor angiogenesis *in vivo* [4,5].

TNF family receptors recruit members of the TNF-receptor associated factor (TRAF) family in order to elicit signaling events [9–13]. The six TRAF family members (called TRAF1 through -6), are characterized by a conserved C-terminal domain that mediates association with the TNF receptor family [14,15], and all TRAFs, except TRAF1, contain Ring finger and Zinc finger domains that function to elicit downstream signaling events. The cytoplasmic domain of CD40 contains a proximal TRAF6-binding domain and a distal TRAF2/3/5-binding domain [9]. The TNFα receptor TNFR1 binds TRAF2 indirectly, through the recruitment of TNFR1-associated death domain protein (TRADD) [16], while TNFR2 binds TRAF2 directly [17]; it is suggested that TRAF5 can substitute for TRAF2 in TNFα-induced signals [18]. TRAF1 has been shown to bind the same sites than TRAF2 on TNFR1, TNFR2 and CD40, and is thought to act as a regulator of TNFα and CD40-induced signals [13,19]. TRAF4, while structurally related to the other TRAFs, localizes to the cell nucleus and is unable to bind to the TNFα receptors and CD40 [20]. Therefore, CD40- and TNFα-induced responses are likely associated either with a TRAF2/5 and/or a TRAF6–mediated signaling response. However, no study to date has identified roles or functions for TRAFs in VEGF expression or in the angiogenesis response.

In these studies, we have used a knockdown approach to identify whether TRAF-mediated signals in EC function for VEGF

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* Corresponding author at: Children's Hospital Boston, Division of Nephrology, 300 Longwood Ave., Boston, MA 02115, United States. Fax: +1 617 730 0130.
E-mail address: david.briscoe@childrens.harvard.edu (D.M. Briscoe).

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expression and/or EC migration and proliferation. While we find some redundancy in TRAF1-, 2-, 3- and 5- dependent signaling responses, paradoxically, we observe that TRAF6 is a potent regulator of basal as well as the inducible expression of VEGF in EC. In addition, we find that TRAF6 serves as an endogenous inhibitor of EC proliferation and EC migration, and its ability to regulate angiogenesis is in part associated with interaction(s) with Src family kinases. Collectively, these studies identity TRAF6 as a critical factor in the regulation of pro-angiogenesis signaling in EC.

2. Materials and methods

2.1. Antibodies and reagents

For Western blotting, anti–phospho-Akt (Ser^{473}) and anti-phospho-Src (Tyr^{416}) were purchased from Cell Signaling Technology (Danvers, MA), and anti-TRAF1, -TRAF2, -TRAF3, -TRAF5, -TRAF6 and anti-VEGF were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). β-actin and GAPDH antibodies were obtained from Sigma–Aldrich (St. Louis, MO). Soluble CD40L was purchased from Ancell (Bayport, MN) and AZD-0530 was purchased from LC Laboratories (Woburn, MA). Neutralizing anti-human VEGF antibody used in cell culture was gifted by Genentech (South San Francisco, CA).

2.2. Cell culture

Single donor human umbilical vein endothelial cells (HUVEC) purchased from Clonetics (Walkersville, MD) were cultured in complete endothelial growth medium (EGM-2 BulletKit; Clonetics), according to the manufacturer's instructions.

2.3. siRNA knockdown

Validated small interfering RNAs (siRNA) for TRAF1, TRAF2, TRAF3, TRAF5, TRAF6 and control siRNA were purchased from Qiagen (Valencia, CA). Transfection of HUVEC with each siRNA (50nM) was performed using RNAimax lipofectamine (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. Knockdown efficiency was tested by Western Blot analysis for each experiment.

2.4. Promoter-reporter assay

A VEGF promoter-luciferase construct containing the 2.6 kb full-length VEGF promoter (gifted by Debabrata Mukhopadhyay, Mayo Clinic, Minneapolis, MN) was used as described [8,21].

2.5. Real-time PCR

Total mRNA was isolated from HUVEC using the RNeasy isolation kit (Qiagen) and used as a template to generate cDNA using random primers (Invitrogen). Quantitative real-time PCR analysis of human VEGFA and GAPDH was performed using the 7300 real-time PCR system and specific TaqMan® Gene Expression Assays (Applied Biosystems, Foster City, CA). Relative expression was calculated according to the $2^{-\Delta\Delta Ct}$ method, as described [22].

2.6. Western blot analysis

HUVEC were lysed with ice-cold RIPA buffer (Boston Bioproducts), run on SDS–polyacrylamide gels, and transferred onto polyvinylidene difluoride membranes (Millipore Corporation, Billerica, MA). Membranes were blocked with 5% BSA for 1 h and incubated overnight with primary antibody. After three washes, membranes were incubated with a species-specific secondary peroxidase-conjugated antibody for 1 h, and reactive bands were developed by chemiluminescence (Thermo Fisher Scientific-Pierce, Rockford, IL).

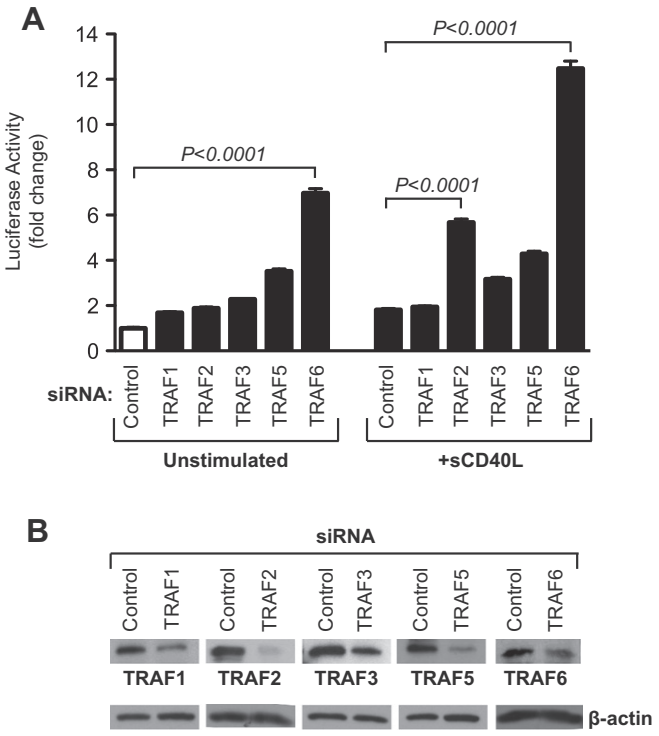


Fig. 1. Effect of TRAF knockdown on basal and CD40-inducible expression of VEGF. (A) HUVEC were transfected with a control siRNA or with siRNAs to TRAF 1/2/3/5/6 as illustrated. After 24 h, the cells were co-transfected with a full-length 2.6-kb VEGF promoter-luciferase construct and were cultured in the absence or presence of sCD40L (3 μg/ml) for an additional 24 h. The cells were lysed and promoter luciferase activity was evaluated. Induced promoter activity was calculated as the fold change in luciferase counts from each group of cells, compared to control siRNA-transfected cells. Illustrated are the mean results from four independent experiments (± 1SEM). (B) For each experiment, knockdown efficiency was evaluated 48 h after siRNA transfection by Western blot analysis. A representative blot is illustrated.

2.7. Cell proliferation assay

HUVEC were seeded onto 96-well cell culture plates (5000 cells per well), transfected with siRNAs, and cultured for 72 h. Proliferation was assessed by [^{3}H] thymidine incorporation (0.5 μCi/well) in the final 16 h of cell culture, using a Tomtec automated cell harvester (Hamden, CT).

2.8. In vitro migration

Migration of EC was evaluated using the *in vitro* wound-healing assay. Briefly, HUVEC were transfected either with control or TRAF6 siRNA, and cultured for 48 h. At this time, a linear wound was created in the cell monolayer using a pipette tip. Groups of cells (in triplicate) were cultured for an additional 18 h in the absence or presence of a neutralizing anti-VEGF antibody (10 μg/ml), and migration of cells into the wound was monitored by microscopy.

2.9. Phospho-kinase array

Phospho-kinase arrays were performed using the Human Phospho-Kinase Array Kit (Proteome Profiler™ Array) from R&D Systems (Minneapolis, MN), according to the manufacturer's instructions.

2.10. Statistical analyses

Statistical analysis was performed using the student *t* test for two groups of data or by one-way ANOVA for three or more groups. *P* values < 0.05 were considered statistically significant.

3. Results

3.1. The TRAF family of adaptor proteins regulate the endogenous expression of VEGF

It is well established that most cell types, including EC [23] express TRAF family molecules. Furthermore, the expression of individual TRAFs, notably TRAF1 in EC can be regulated by different cytokines, especially following ligation of CD40 by sCD40L [23]. This observation indicates that individual TRAFs have potential to function as adaptors to initiate signals that result in EC proliferation. We initially wished to determine the function of individual TRAF adaptor molecules in the expression of VEGF. EC were transfected either with siRNAs to TRAF1, -2, -3, -5 or -6 or with a control siRNA, and were co-transfected with a full-length VEGF promoter-luciferase reporter construct. Following co-transfection, EC were cultured for 48 h in the absence or presence of sCD40L. Paradoxically, rather than inhibiting VEGF expression, we found a generalized increase in VEGF transcriptional activation following transfection of EC with each TRAF siRNA as compared to control (Fig. 1A). Moreover, while the effect of TRAF1, -2, -3 and -5 was modest, TRAF6 knockdown resulted in a marked induction of basal levels of VEGF expression ($P < 0.0001$). Also, and consistent with our previously published studies [2,8,21,24], we found that the treatment of EC with sCD40L resulted in a 2–3-fold increase in VEGF transactivation. However, following activation, there was a greater increase in VEGF promoter activity in EC transfected with siRNAs to TRAF2 (approx. 6-fold induction, $P < 0.0001$) and TRAF6 (approx.12-fold induction, $P < 0.0001$) compared to control siRNA-transfected EC. Following activation, VEGF promoter activity was also increased in cells transfected with siRNAs to TRAF3 and TRAF5, but levels of expression were more modest. Thus, TRAF6 is a negative regulator of both endogenous as well as inducible expression of VEGF in EC, while other TRAFs, notably TRAF2, function more selectively as regulators following activation.

3.2. Role for TRAF6 in the regulation of EC proliferation and migration

To evaluate the physiological relevance of these observations we also analyzed the function of TRAF2 and TRAF6 in EC proliferative responses. As above, EC were transfected either with siRNAs to TRAF2 or TRAF6 and after 72 h, EC proliferation was assessed by [^{3}H] Thymidine incorporation. As illustrated in Fig. 2A, we found a greater than five fold increase in basal rates of EC proliferation

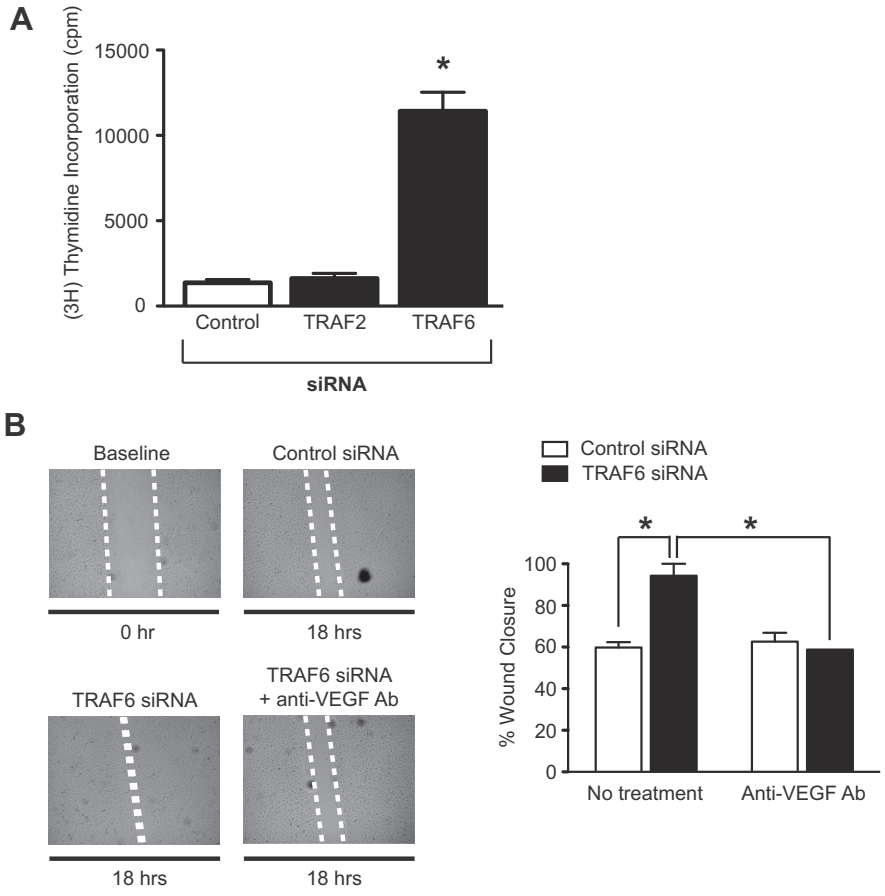
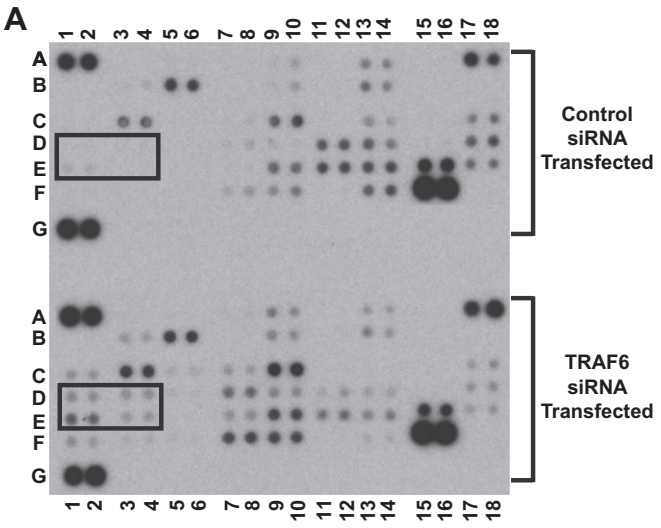


Fig. 2. Effect of TRAF6 knockdown on EC proliferation and migration *in vitro*. (A) HUVEC were transfected either with a control siRNA, or with siRNAs to TRAF2 or TRAF6. After 72 h, proliferation was evaluated using the [^{3}H] thymidine incorporation assay (0.5 μCi/well). The mean proliferative responses (± 1SEM) of three independent experiments are shown. $^*P < 0.0005$ vs. control siRNA. (B) HUVEC were transfected with a control siRNA or with the TRAF6 siRNA. After 48 h, a linear wound was created in confluent cell culture monolayers using a pipet tip. The cells were subsequently cultured in the absence or presence of a neutralizing anti-VEGF antibody (10 μg/ml), and the migration of cells into the wound was monitored by microscopy every 6 h for up to 24 h. Representative photomicrographs of wound closure in each group of cells are shown. The dotted white line highlights the wound (representative of $n = 3$). The bar graph illustrates the mean percentage wound closure (± 1SEM) in three experiments. $^*P < 0.05$. As illustrated, wound closure occurred after ~18 h in the TRAF6 siRNA transfected cells, earlier than control siRNA-transfected cells and untransfected cells.



Coordinate	Target/Control	Coordinate	Target/Control
A1, A2	Positive control	D9, D10	STAT5a (Y699)
A3, A4	p38α (T180/Y182)	D11, D12	p70 S6 Kinase (T421/S424)
A5, A6	ERK1/2 (T202/Y204, T185/Y187)	D13, D14	RSK1/2/3 (S380)
A7, A8	JNK pan (T183/Y185, T221/Y223)	D15, D16	p27 (T157)
A9, A10	GSK-3α/β (S21/S9)	D17, D18	PLCγ-1 (Y783)
A13, A14	p53 (S392)	E1, E2	Fyn (Y420)
A17, A18	Positive control	E3, E4	Yes (Y426)
B3, B4	MEK1/2 (S218/S222, S222/S226)	E5, E6	Fgr (Y412)
B5, B6	MSK1/2 (S376/S360)	E7, E8	STAT3 (Y705)
B7, B8	AMPKα1 (T174)	E9, E10	STAT5b (Y699)
B9, B10	Akt (S473)	E11, E12	p70 S6 Kinase (T229)
B11, B12	Akt (T308)	E13, E14	RSK1/2 (S221)
B13, B14	p53 (S46)	E15, E16	c-Jun (S63)
C1, C2	TOR (S2448)	E17, E18	Pyk2 (Y402)
C3, C4	CREB (S133)	F1, F2	Hck (Y411)
C5, C6	HSP27 (S78/S82)	F3, F4	Chk-2 (T68)
C7, C8	AMPKα2 (T172)	F5, F6	FAK (Y397)
C9, C10	β-Catenin	F7, F8	STAT6 (Y641)
C11, C12	p70 S6 Kinase (T389)	F9, F10	STAT5a/b (Y699)
C13, C14	p53 (S15)	F11, F12	STAT1 (Y701)
C15, C16	p27 (T198)	F13, F14	STAT4 (Y693)
C17, C18	Paxillin (Y118)	F15, F16	eNOS (S1177)
D1, D2	Src (Y419)	F17, F18	Negative control
D3, D4	Lyn (Y397)	G1, G2	Positive control
D5, D6	Lck (Y394)	G5, G6	Negative control
D7, D8	STAT2 (Y689)		

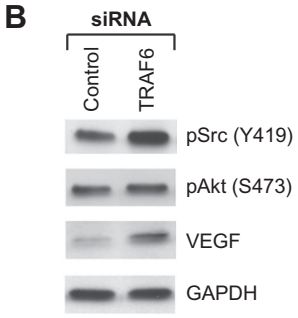


Fig. 3. Effect of TRAF6 on proangiogenic signaling pathways in EC. HUVEC were transfected with control siRNA or with TRAF6 siRNA, and after 48 h, a phosphokinase protein array was performed on cell lysates (in duplicate). (A) Illustrated is a representative blot showing the relative expression of individual phosphokinases in control and in TRAF6 siRNA transfected EC. The table on the right of the blot indicates the location of proteins tested in this array. (B) Lysates from 48 h siRNA-transfected EC were subjected to Western blot analysis using anti-phospho-Src (Tyr419), anti-phospho-Akt (Ser473) and anti-VEGF antibodies. The expression of GAPDH served as an internal control. The illustrated blot is representative of three with similar results.

following knockdown of TRAF6 as compared to control siRNA-transfected cells ($P < 0.0005$). However, TRAF2 knockdown did not increase EC proliferation.

To further evaluate the role of TRAF6 as an endogenous regulator of angiogenesis, we transfected EC with control siRNA or TRAF6 siRNA, and we examined EC migration using the well-established wound-healing assay [25]. After 18 h, we found that wound healing/EC migration was significantly increased in EC transfected with TRAF6 siRNA as compared to control siRNA-transfected EC ($P < 0.05$, Fig. 2B). To test if TRAF6 mediates this effect via the regulation of VEGF expression, we performed the identical assay using EC cultured in the presence of a neutralizing anti-VEGF antibody. Illustrated in Fig. 2B, we found that anti-VEGF completely blocked the TRAF6-induced migratory response to baseline, indicating that TRAF6 regulates basal migratory responses via augmentation of VEGF production.

3.3. TRAF6. regulates pro-angiogenic signaling pathways in EC

To gain insight into intermediary signaling pathways regulated by TRAF6, we compared the activity of 46 kinases and transactivators in control siRNA- and TRAF6 siRNA-transfected EC using a commercially available phosphokinase array. As illustrated in Fig. 3A and as anticipated [8], we found differences in the expression of kinases associated with the Akt/mTOR signaling pathway in TRAF6 siRNA-transfected cells vs. controls (Fig. 3A, lanes D and E 11–12). In addition, we found marked differences in the phosphorylation/activation of Src family kinases (including Lyn, Fyn, and Yes) (Fig. 3A, lanes D and E 1–4) following TRAF6 knockdown, and there were notable effects of TRAF6 on the activity of the STAT family of transcription factors (Fig. 3A, lanes F7–10).

We confirmed the effect of TRAF6 on PI-3K/Akt and Src activation by Western blot analysis (Fig. 3B). Although we had anticipated that TRAFs knockdown would result in an inhibition of Akt activity [8], we found minimal effects of either TRAF1, -2, -3 and -5 and -6 knockdown on the expression of phospho-Akt as compared to control siRNA-transfected EC (Fig. 3B and data not shown). We interpret these findings to suggest redundancy in the function of individual TRAFs for activation of PI-3K/Akt signaling. Nevertheless, and consistent with the array data, by Western blot analysis, we found a modest increase in phospho-Src expression in TRAF6 siRNA-transfected EC vs. controls (Fig. 3B). In addition, and consistent with our findings illustrated in Fig. 1, we also observed that knockdown of TRAF6 resulted in a marked induction in VEGF protein expression (Fig. 3B). Collectively these observations indicate that TRAF6-mediated regulation of Src may in part account for its effect on the regulation of endogenous VEGF expression in EC.

3.4. Src activation mediates the expression of VEGF in EC

To evaluate the effect of Src on VEGF overexpression, we next treated EC with the pharmacological Src kinase inhibitor AZD-0530 (1 μM or 6 μM) and we determined its effect on VEGF at the mRNA level. As illustrated in Fig. 4A, we found that pharmacological inhibition of Src resulted in a significant reduction in VEGF

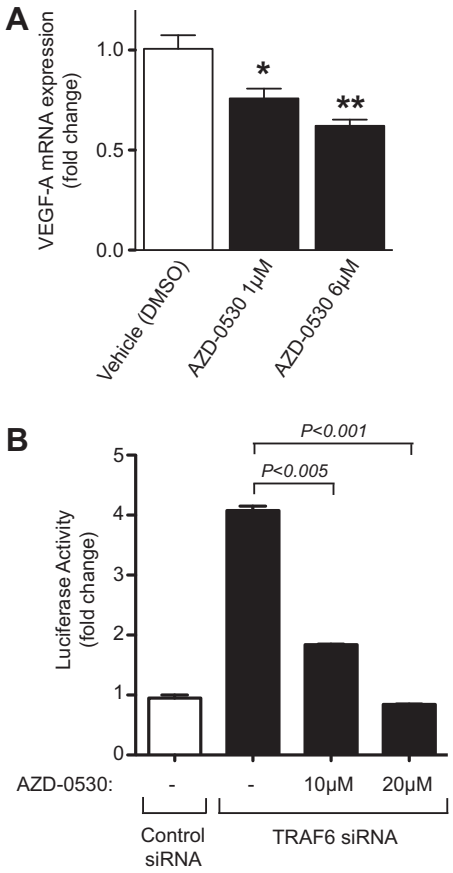


Fig. 4. Effect of TRAF6-Src crosstalk for VEGF expression in EC. (A) Confluent monolayers of HUVEC were cultured in the absence or presence of increasing concentrations of the Src kinase inhibitor AZD-0530. The cells were harvested as a time course from 18–36 h and VEGF mRNA expression was evaluated by real-time qPCR. Illustrated are the mean results of four experiments performed after 18 h of treatment. The data is expressed as mean fold change in expression over baseline (± 1SEM). (B) HUVEC were transfected with a control siRNA or with TRAF6 siRNA and, after 24 h, were co-transfected with a full-length 2.6-kb VEGF promoter-luciferase construct and were cultured in the absence or presence of increasing concentrations of AZD-0530 for an additional 24 h. The cells were lysed and promoter luciferase activity was calculated as the fold change in luciferase counts from each group of cells, compared to control siRNA-transfected cells. $^{*}P < 0.05$, $^{**}P < 0.005$.

mRNA expression as compared to cells treated with vehicle alone. Since TRAF6 knockdown increases Src activity as well as VEGF expression, we co-transfected EC with our VEGF promoter luciferase construct and TRAF6 siRNA, and we evaluated transactivation in the absence or presence of AZD-0530. As illustrated in Fig. 4B, we found that TRAF6 knockdown again resulted in an increase in VEGF promoter activity. Moreover, we found an inhibitory effect of AZD-0530 on VEGF promoter activation; inhibition was variable at low concentrations while higher concentrations (>10 mM) had a notable effect (Fig. 4B). In some experiments using the highest concentrations of AZD-0530, there were toxic effects on siRNA and promoter co-transfected EC (data not shown). This finding is most suggestive that TRAF6 inhibits basal VEGF expression in part via its ability to regulate Src activity in EC.

4. Discussion

In these studies, we establish a new function for the TRAF family of adaptor molecules in the maintenance of both basal as well as inducible expression of VEGF by EC. While we find some redundancy in the function of TRAF2, TRAF3 and TRAF5, our studies indicate that TRAF6 is a potent endogenous regulator of angiogenesis in part via its ability to inhibit Src family kinase activity. Collectively, these observations define a model whereby TRAF6 serves as a cell-intrinsic regulator of the angiogenesis response.

TRAF adaptor molecules are well established to function in TNF family signaling cascades [15,26–29]. However, only a few studies have evaluated their expression and function in EC [23,30–32], and their role in angiogenesis has not been previously explored. CD40-induced responses in EC are potent to induce VEGF expression, VEGF-dependent EC proliferation *in vitro* and angiogenesis *in vivo* [2,3,8,21,24]. Furthermore, CD40-induced angiogenesis is associated with breast cancer growth and progression [4,5]. TNFα also induces VEGF expression in different cell types including EC [6,7] and has been shown to induce angiogenesis *in vivo* at low concentrations [33–35]. Thus, it seems likely that TRAFs, especially TRAF2, TRAF5 and TRAF6, will serve to augment endogenous VEGF expression in EC and the angiogenesis response. However, we paradoxically found that all TRAFs, and notably TRAF6, serve to regulate basal as well as inducible levels of VEGF expression as well as EC proliferation and migration. Perhaps therefore, the relative activation of TRAF6 within EC will be a determinant of VEGF-dependent EC proliferation and angiogenesis. Indeed, consistent with this possibility, we find that induced EC proliferation and migration following knockdown of TRAF6 is inhibited by a blocking anti-VEGF antibody.

Using a phosphokinase array and Western blot analysis, we also found that the potent effect of TRAF6 on VEGF overexpression was associated with its ability to regulate the Src family kinases, JNK family kinases, and the activation of the STAT family of transcription factors. All of these kinases and transcription factors have been found to be important in EC survival, proliferation as well as in VEGF expression [36–38]. Thus, the relative activation of different members of the TRAF family in EC may suppress cell intrinsic signaling responses associated with the initiation, amplification as well as the suppression of angiogenesis.

Although not the subject of this study, the observed regulatory effect of TRAF6 on Src kinase activation and its ability to mediate crosstalk with other signaling pathways might also have important implications for proinflammation. Indeed, and consistent with this possibility we find that TRAF6 regulates families of STATs, which are well established to function in cytokine and chemokine-dependent responses. Also consistent with this possibility, a previous study demonstrated that TRAFs may function in EC to regulate chemokine expression [23]. TRAF6 was found to suppress MCP-1 expression in EC following CD40-dependent activation, and TRAF6 knockout mice were more prone to the development of atherosclerosis [23,32], which is an angiogenesis dependent process [1,39,40]. In addition, and consistent with our findings in this report, it was also observed that the function of TRAF2, TRAF3 and TRAF5 were different than those of TRAF6 on the regulation of chemokine/inflammatory responses [23]. Collectively, all these data indicate that TRAF6 has intrinsic regulatory functions in EC, and our observations in this report have broad implications for its role in angiogenesis-dependent diseases.

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