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Opposing roles of STAT-1 and STAT-3 in regulating vascular endothelial growth factor expression in vascular smooth muscle cells

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ABSTRACT

Increased microvessel density in atherosclerotic plaques plays a major role in promoting plaque destabilization resulting in increased risk of stroke and myocardial infarction. Previously we have shown that expression of the inflammatory cytokine, Oncostatin-M (OSM), in human atherosclerotic plaques correlated with increased microvessel density, indicating a role for OSM in promoting plaque angiogenesis. The purpose of this study was to determine the mechanism by which OSM regulates Vascular Endothelial Growth Factor (VEGF) expression in human coronary artery smooth muscle cells. Using shRNA and overexpression studies, we have shown that the transcription factor, STAT-1 inhibited VEGF expression, while STAT-3 promoted the expression of VEGF. We further show that the mechanism by which STAT-1 and STAT-3 regulates VEGF expression is through modulation of Hypoxia Inducible Factor-1α (HIF-1α). STAT-1 suppresses HIF-1α expression, whereas STAT-3 positively regulates HIF-1α expression. These results provide evidence that activated STAT-1 and STAT-3 regulate VEGF expression indirectly, by modulating HIF-1α activity.

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1. Introduction

The development of new capillary blood vessels by angiogenesis or vasculogenesis is essential to the development of a functional vascular system in the embryo, and in normal adult physiological settings such as wound repair [1]. However, uncontrolled blood vessel growth has also been implicated in diseases such as atherosclerosis, cancer and rheumatoid arthritis [2]. For example, in atherosclerosis, there is compelling evidence to suggest that angiogenesis plays a role in the development and destabilization of atherosclerotic plaques. Angiogenesis has been shown to increase atherosclerotic plaque size [3] and contribute to macrophage recruitment into the plaque which can destabilize the plaque by releasing proteases [4]. Also, increased neovascularization in atherosclerotic plaques is associated with adverse cardiovascular events such as stroke and myocardial infarction [5,6], likely due to immature microvessels within plaques that are prone to rupture [7,8]. These studies clearly demonstrate the importance of angiogenesis in the development of atherosclerosis.

We, and others, recently demonstrated that Oncostatin-M (OSM), a pro-inflammatory cytokine, is expressed in human atherosclerotic plaques [9,10]. Furthermore, we observed that the expression of OSM by SMC within atherosclerotic plaques correlated with increased plaque microvessel density, suggesting that OSM may be involved in promoting plaque angiogenesis. In support of this observation, it was shown that in smooth muscle cells (SMC), OSM promoted the expression of vascular endothelial growth factor (VEGF), a potent regulator of blood vessel development and growth [11]. OSM belongs to the interlukin (IL)-6 family of cytokines which includes, leukemia inhibitory factor, IL-11, IL-27, cardiotrophin 1 and ciliary neurotrophic factor [12,13]. Upon binding to their receptors, these cytokines exert their effects through activation of the Janus Kinase and Signal Transducer and Activator of Transcription signaling pathway (JAK/STAT) [14,15].

In this study, we have sought to determine the mechanism for the induction of VEGF expression by OSM in SMCs by focusing on the transcriptional factors STAT-1, STAT-3 and Hypoxia Inducible Factor-1α (HIF-1α). The findings from this study demonstrate that STAT-1 and STAT-3 have opposing roles in modulating HIF-1α activity and subsequently, VEGF expression.

2. Materials and methods

2.1. Cell culture

Human coronary artery SMCs (Cell Applications Inc.) were maintained at 37 °C in SMC growth medium (Cell Applications Inc.). Prior to experimentation, SMCs were incubated for 24 h in serum-free DMEM/F12 medium supplemented with insulin, trans-

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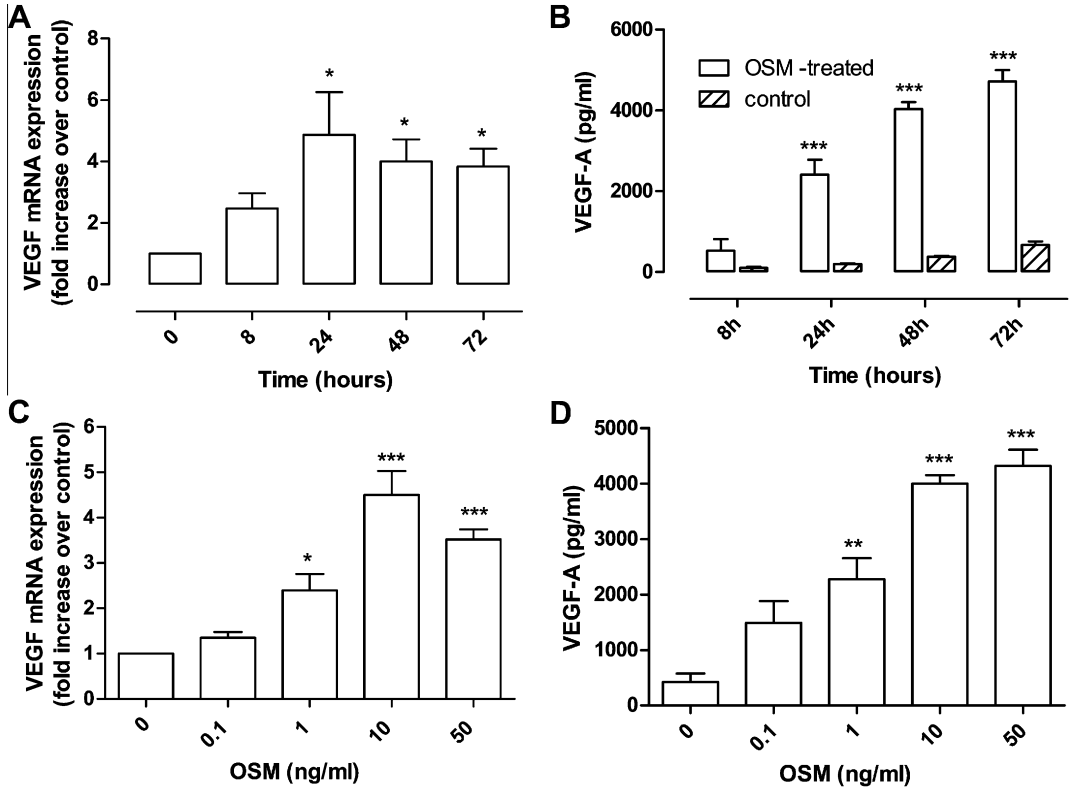

Fig. 1. OSM stimulates VEGF mRNA and protein synthesis. (A) and (B) Human coronary artery SMC were stimulated with OSM (10 ng/ml) for the indicated times or (C) and (D) stimulated for 24 h with the indicated doses. VEGF mRNA and protein from SMC cultures were assayed by qPCR and ELISA, as described in Materials and methods. Data are shown as the mean ± SEM of three independent experiments. Western-blot shown is representative of two separate experiments. $^{*}P \leqslant .05$, $^{**}P \leqslant .01$, $^{***}P \leqslant 0.005$ versus unstimulated controls.

ferrin and selenium. SMCs between passages 3 and 6 were used for all experiments.

2.2. Transfection of STAT-1 and STAT-3 cDNA

STAT-1 cDNA obtained from GeneCopoeia and STAT-3 cDNA obtained from Origene were subcloned into pcDNA 3.1 vector to give pcDNA 3.1/STAT-1 and pcDNA 3.1/STAT-3, respectively. Transfection of plasmids into SMCs was carried out as previously described [16]. Briefly, SMCs were transfected with 2 μg pcDNA 3.1/STAT-1 or pcDNA 3.1/STAT-3. SMCs transfected with empty pcDNA 3.1 served as controls. Transfection was carried out using the Transfast transfection reagent (Promega) as described by the manufacturer. SMC was maintained in growth medium for 72 h followed by incubation in serum-free DMEM-F12 medium supplemented with insulin, transferring and selenium (DMEM-F12/ITS) for a further 24 h prior to experimentation. Cultures were then stimulated with OSM (10 ng/ml) for the indicated times and RNA extracted as described below.

2.3. Quantitative real-time RT-PCR

Total RNA was prepared from cells using RNeasy Plus mini kit (Qiagen) according to the manufacturer's instructions. Prior to cDNA synthesis, genomic DNA was eliminated by treating RNA samples with DNase-1 (TURBO-DNA free kit, Ambion). RNA (100 ng) was converted to cDNA using the Go-Script kit (Promega) in a final volume of 20 μl and subsequently diluted to 100 μl with nuclease-free water. Both random hexamers and oligo dT were used for cDNA synthesis. Quantitative real-time PCR (qPCR) was performed using the Kapa sybr fast qPCR master mix (Kapa Biosystems). cDNA (5 μl) was used for the PCR reaction in a 20 μl final reaction volume. PCR reactions were run on an Eppendorf Realplex 2 system. The cycling conditions for PCR reactions comprised of a 3 min activation step at 95 °C followed by 40 cycles at 95 °C for 3 s and 60 °C for 30 s. The following primer sets were used: VEGF sense 5-TCCACCATGCCAAGTGGTCCCA-3′, antisense 5′-GCATCGCA TCAGGGGCACACA-3′, HIF-1α sense 5′-GCAGCCAGACGATCATGCA GCT-3′, antisense 5′-TCCATTGATTGCCCCAGCAGTCT-3′. Sequences for STAT-1, STAT-3 and the reference gene RPL13A primers sets are described in our recent publication [9]. Each sample was analyzed in duplicate. Relative gene expression was calculated by using the $\Delta\Delta C_T$ method and reported as fold change over controls [17].

2.4. Vegf elisa

VEGF from SMC culture supernatants was quantified using Quantikine ELISA kits (R&D Systems) and performed according to the manufacturer's instructions.

2.5. shRNA knockdown

SMC were infected with validated Mission shRNA pKLO.1 lentiviral particles (Sigma–Aldrich) at a multiplicity of infection of 10 in the presence of 8 μg/ml polybrene for 24 h in SMC growth medium followed by a further 24 h in fresh growth medium. Cultures were then incubated with serum-free DMEM/F12 supplemented with insulin, transferring and selenium (DMEM-F12/ITS) for a further 48 h prior to experimentation. The following shRNAs were used; shSTAT-1 (TRCN0000004265), shSTAT-3 (TRCN0000020842), HIF-1α (TRCN 0000003810) and non-targeting control shRNA (SHC002V). The knockdown efficacy of the STAT-1 and STAT-3 shR-

NAs was reported previously [9]. Knockdown of HIF-1α mRNA was estimated at 74% by qPCR (see Supplementary Fig. 1).

2.6. Western blot analysis

SMC cultures were lysed in cell lysis buffer (Cell Signaling Technology) containing protease inhibitors (Roche) and 0.1% SDS. Cell extracts (25 μg) were separated by electrophoresis on a gradient 4–12% Bis-Tris polyacrylamide gel (Invitrogen). Separated proteins were transferred to PVDF membranes and probed with antibodies to phospho-Stat1, Stat1, phospho-Stat3, Stat3, HIF1-α and β-tubulin. Blots were developed using the Western breeze kit (Invitrogen).

2.7. Statistical analysis

Statistical analyses were performed by Prism 5.0 software (GraphPad). Data are presented as mean ± SEM. Comparisons between groups were performed by one-way ANOVA. *P* values of <0.05 were considered significant.

3. Results

3.1. OSM promotes VEGF mRNA and protein synthesis in human coronary SMC

In our previous study, we showed a strong correlation between microvessel density and OSM antigen in human atherosclerotic plaques, suggesting a role for OSM in promoting plaque angiogenesis [9]. In this study, we sought to examine whether OSM stimulated VEGF expression, a potent angiogenic factor, in human coronary artery SMC and to study the mechanism of this induction. OSM (10 ng/ml) exposed to SMCs promoted a time-dependent increase in the expression of VEGF mRNA and protein (Fig. 1A and B). VEGF mRNA levels peaked at 24 h (5-fold) and remained elevated over the time period tested. VEGF protein increased significantly and remained elevated over the time period tested. OSM also induced a dose-dependent increase in the expression of VEGF mRNA and protein synthesis (Fig. 1C and D). A low dose of OSM (1 ng/ml) was sufficient enough to significantly stimulate VEGF synthesis.

3.2. Opposing roles of STAT-1 and STAT-3 in regulating VEGF expression

OSM signals primarily through activation of STAT-1 and STAT-3 [11,18]. Consistent with these studies, we observed that stimulation of SMCs with OSM (10 ng/ml) resulted in phosphorylation of both STAT-1 and STAT-3 (Fig. 2A). STAT-1 phosphorylation peaked at 30 min, returning to control levels by 60 min. However, STAT-3 phosphorylation was almost maximal by 2 min and remained phosphorylated throughout the time period tested. What is unclear is the precise role that activated STAT-1 and STAT-3 play in regulating VEGF expression. We tested the hypothesis that STAT-1 and STAT-3 may have opposing effects on VEGF expression. To accomplish this, we inhibited STAT-1 and STAT-3 protein expression by treating SMC with shRNA targeted to STAT-1 or STAT-3 respectively. We used the same shRNA that we previously demonstrated to effectively inhibit STAT-1 and STAT-3 expression [9]. For controls, SMCs were infected with a non-target (NT) shRNA. As shown in Fig. 2B and C, when SMCs expressing STAT-1 shRNA were stimulated with OSM (10 ng/ml), a significant increase in VEGF mRNA (2-fold) and protein expression was observed when compared to OSM-stimulated SMC cultures containing NT shRNA. In contrast, treatment of SMC cultures containing STAT-3 shRNA with OSM resulted in significant inhibition of VEGF expression. These

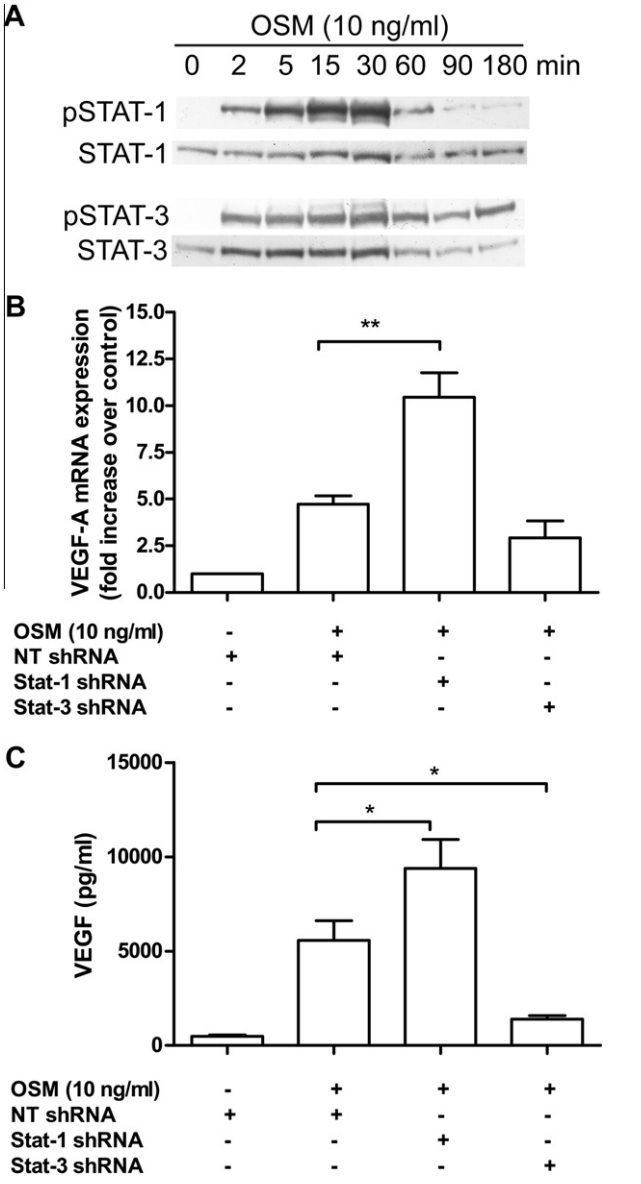

Fig. 2. Regulation of VEGF expression by STAT-1 and STAT-3. (A) Western analysis of the kinetics of phosphorylated STAT-1 and STAT-3 in SMC following treatment with 10 ng/ml OSM. Blots were stripped and reprobed with anti-STAT-1 and STAT-3. (B) and (C) SMCs were transduced with either control non-target (NT) shRNA, STAT-1 shRNA or STAT-3 shRNA for 24 h in growth medium. Fresh serum-free medium was added and cultures stimulated with 10 ng/ml OSM for an additional 24 h. RNA was extracted for VEGF mRNA analysis (B) and culture supernatants were assayed for VEGF protein (C). Values shown are the mean ± SEM of three separate experiments. $^{*}P \leqslant 0.05$, $^{**}P \leqslant 0.01$ versus OSM stimulated SMC transduced with NT shRNA.

results indicate that inhibiting STAT-1 activity allows STAT-3 to promote VEGF expression. In contrast, inhibiting STAT-3 allows STAT-1 to suppress VEGF expression.

3.3. Overexpression of STAT-1 inhibits VEGF expression

To directly address the contribution of STAT-1 and STAT-3 in regulating VEGF expression, we overexpressed STAT-1 and STAT-3 in SMC cultures by transfecting SMC cultures with plasmids encoding STAT-1 (pcDNA3.1/STAT-1) or STAT-3 (pcDNA3.1/STAT-3). SMC cultures transfected with empty pcDNA3.1 vector served as controls. Treatment of SMC cultures overexpressing STAT-1 with OSM (10 ng/ml) resulted in significantly lower levels of VEGF

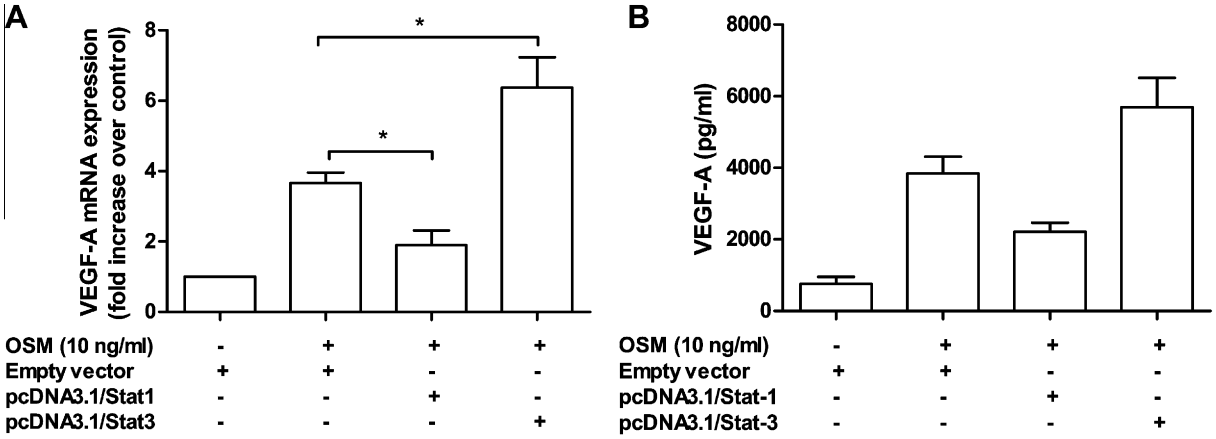

Fig. 3. Overexpression of STAT-1 SMC inhibits VEGF expression. pcDNA3.1-STAT-1, pcDNA3.1- STAT-3 or control vector without insert (pcDNA3.1) was transfected into SMC as described in Materials and methods. Cultures were treated with or without OSM (10 ng/ml) for 24 h, and VEGF mRNA (A) and VEGF protein (B) was analyzed by qPCR and ELISA, respectively. Data represents the mean ± SEM from three separate experiments. *$P < 0.05$ versus OSM stimulated cultures transfected with empty vector.

mRNA and protein when compared to OSM-stimulated SMC transfected with empty vector (Fig. 3A and B). In contrast, OSM-stimulated SMC cultures overexpressing STAT-3 had significantly higher levels of VEGF mRNA and protein levels. This result confirms our observation that STAT-1 inhibits VEGF expression, whereas STAT-3 promotes VEGF expression.

3.4. Regulation of HIF-1α by STAT-1 and STAT-3

Previous data have shown that the transcriptional factor HIF-1α plays a critical role in the transactivation of VEGF under hypoxic microenvironment and when stimulated by growth factors and cytokines under normal oxygen conditions [19–22]. In addition, STAT-3 has been shown to stimulate HIF-1α expression in tumor cells [20,23]. Therefore, we tested the hypothesis that STAT-1 and STAT-3 may play a role in regulating VEGF expression in SMCs indirectly by modulating HIF-1α expression. SMCs stimulated with OSM (10 ng/ml) promoted expression of HIF-1α mRNA (1.8-fold), with maximal activation at 8 h (Fig. 4A). Levels of HIF-1α mRNA remained elevated up to 72 h. Western blot analysis confirmed OSM promoted HIF-1α protein synthesis (Fig. 4B). To determine if HIF1-α is required for OSM-induced VEGF expression, we inhibited HIF-1α activity using shRNA. As shown in Fig. 4C and D, knockdown of HIF1-α significantly reduced OSM-induced VEGF mRNA and protein levels.

To determine if HIF-1α is regulated by STAT-1 and STAT-3, SMCs were incubated with shRNAs targeting STAT-1 and STAT-3. We observed that knockdown of STAT-1 promoted HIF-1α mRNA expression in OSM-stimulated SMC cultures whereas knockdown of STAT-3 resulted in reduced HIF-1α mRNA expression (Fig. 4E). To confirm that HIF-1α is modulated by STAT-1 and STAT-3, SMCs overexpressing STAT-1 and STAT-3 were stimulated with OSM (10 ng/ml) for 8 h. As shown in Fig. 4F, OSM-stimulated SMC cultures transfected with STAT-1 cDNA had significantly reduced levels of HIF-1α mRNA expression whereas SMC cultures transfected with STAT-3 cDNA expressed higher levels of HIF-1α mRNA when compared to control cultures. These data indicate that activated STAT-1 and STAT-3 regulate VEGF expression in SMCs indirectly by modulating HIF-1α activity.

4. Discussion

OSM and several members of the IL-6 cytokine family have been shown to be involved in the pathogenesis of vascular diseases such as atherosclerosis [10]. Typically, these cytokines activate the JAK-STAT pathway to elicit their biological effects. The major findings of the present study are: (1) activated STAT-1 and STAT-3 have opposing effects on the regulation of OSM-induced VEGF expression, and (2) STAT-1 and STAT-3 regulate VEGF expression by modulating HIF-1α activity. While several studies have documented that STAT-3 promotes VEGF expression in different cell types [11,24–26], the role of STAT-1 on VEGF expression remains unknown. Our results reveal that STAT-1 is a negative regulator of VEGF expression in SMCs. Furthermore, this study also identifies that the mechanism by which STAT-1 and STAT-3 control VEGF expression in SMCs is through modulation of HIF-1α expression.

Angiogenesis has been recognized as an important process in the progression of diseases such as atherosclerosis and cancer [2]. For example, in atherosclerosis, increased neovascularization in atherosclerotic plaques is believed to promote plaque instability and rupture [6–8]. Studies have shown that there is a correlation between increased plaque microvessel density and plaque rupture [5,27,28]. We, and others, recently observed a strong correlation between expression of OSM in SMCs of human carotid atherosclerotic plaques and increased microvessel density [9]. This suggests that OSM may promote plaque destabilization by stimulating microvessel growth. Indeed, previous studies have shown that multiple members of the IL-6 family, including OSM, can promote angiogenesis [29–31]. More recently, OSM was shown to stimulate VEGF expression in SMCs in a STAT-3 dependent manner [11]. In the current study, we confirm this observation. Our studies also demonstrate that the induction of HIF-1α by STAT-3 is critical for OSM-induced VEGF expression. Increase in HIF-1α expression in SMC under normal oxygen conditions has been shown to involve both increased transcription and translation [21]. More significantly, our studies also show, we believe for the first time, that in SMCs, STAT-1 negatively regulates VEGF expression by inhibiting HIF-1α transcription. STAT-1 has also been shown to suppress VEGF-induced endothelial cell growth and HIF-1α induced transcription of CXCR4 receptor [32,33]. Interestingly, in the study by Demyanets et al., they observed that interferon gamma (IFN-γ) inhibited OSM-induced VEGF expression in SMCs [11]. It is important to note that IFN-γ triggers persistent STAT-1 activation [34]. Therefore it is plausible that the inhibitory effects of IFN-γ on VEGF expression observed in the studies of Demyanets et al. are mediated by STAT-1, supporting our hypothesis that STAT-1 is a negative regulator of VEGF expression.

There is also now convincing evidence that the balance between activated STAT-1 and STAT-3 may be important in human cancers.

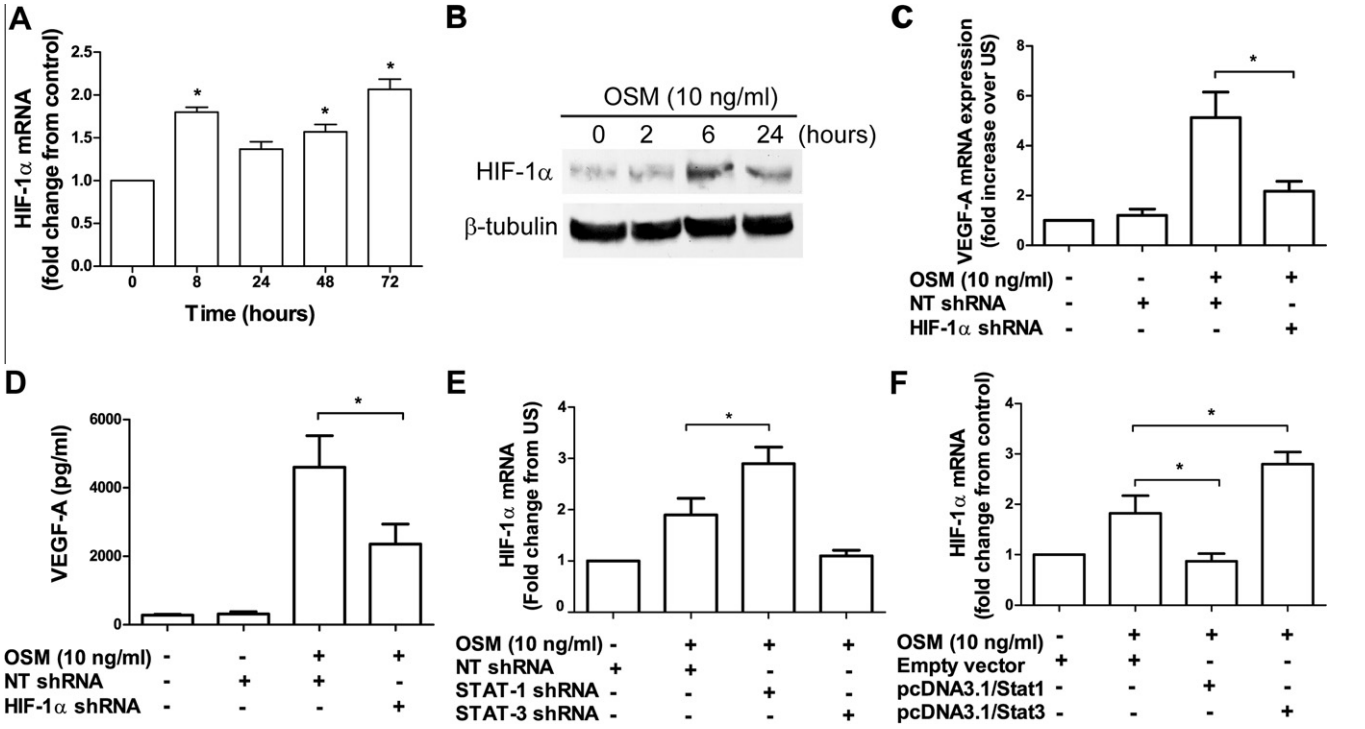

Fig. 4. Modulation of HIF-1α activity by STAT-1 and STAT-3. Human coronary artery SMC were stimulated with OSM (10 ng/ml) for the indicated times. HIF-1α mRNA (A) and protein (B) was determined by qPCR and western-blot analyses. (C) and (D) SMC cultures were transduced with HIF-1α or non-target (NT) shRNA. Cultures were treated with or without OSM (10 ng/ml) for 24 h. VEGFmRNA (C) and protein levels (D) were analyzed by qPCR and ELISA. (E) SMC cultures were transduced with STAT-1, STAT-3 or non-target (NT) shRNA. Cultures were stimulated with 10 ng/ml OSM for 24 h. RNA was extracted and HIF-1α mRNA was analyzed by qPCR. (F) SMC cultures were transfected with pcDNA3.1-STAT-1, pcDNA3.1- STAT-3 or control vector without insert (pcDNA3.1). Cultures were stimulated with 10 ng/ml OSM for 24 h. RNA was extracted and HIF-1α mRNA analyzed. Data for qPCR are expressed as mean ± SEM from three separate experiments. Western blot data is representative of two independent experiments. $^{*}P < 0.05$.

For example, constitutive activation of STAT proteins has been observed in a variety of human cancers and it has been suggested that aberrant STAT activation is involved in cancer development [35]. In particular, STAT-3 has been reported to be constitutively active in a number of tumors [20,36–38]. Overexpression of an active form of STAT-3 can transform a variety of non-malignant cells such as breast and prostate epithelial cells [39,40]. In contrast, STAT-1 can limit tumor growth by inhibiting cell proliferation and promoting apoptosis [41,42]. Unbalanced STAT-1 and STAT-3 activation can also promote tumor growth indirectly by promoting angiogenesis. STAT-3 has been shown to directly regulate VEGF synthesis by binding to STAT-3 responsive elements on the VEGF promoter [43,44]. STAT-3 can also regulate VEGF expression indirectly by promoting HIF-1α expression [20,45]. Vollmer et al., demonstrated in HepG2 cells that under normal oxygen conditions, OSM stimulated VEGF expression via STAT-3 activation of HIF-1α [26]. This observation is consistent with our present finding that STAT-3 mediated VEGF expression is regulated by HIF-1α in SMCs stimulated by OSM. Our study provides evidence that the control of HIF-1α by STAT-1 and STAT-3 is an important mechanism by which VEGF expression is regulated in SMCs.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.10.037.

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