Activation of the VEGFR1 Chromatin Domain

AN ANGIOGENIC SIGNAL-ETS1/HIF-2α REGULATORY AXIS*

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Angiogenesis is induced by multiple growth factors including vascular endothelial growth factor (VEGF) and fibroblast growth factor 2 (FGF2). In vascular endothelium VEGF signals through two receptor-tyrosine kinases, VEGFR1 and VEGFR2. The VEGFR1 gene encodes both a receptor-tyrosine kinase and a secreted splice variant, soluble VEGFR1. Whereas VEGFR1 is essential for vascular development, mechanisms that regulate VEGFR1 expression in endothelial cells are poorly understood. We demonstrate here that in endothelial cells, FGF2 and epidermal growth factor (EGF) signaling induce VEGF1 mRNA expression in a combinatorial fashion. EGF/FGF2-mediated VEGFR1 induction is mediated via functional interaction of transcription factors ETS1 and HIF-2α. Mechanistic analyses revealed that in endothelial cells EGF/FGF2 signaling induces ETS1 expression, increases HIF-2α protein level in absence of hypoxia, and recruits both ETS1 and HIF-2α to the VEGFR1 chromatin domain. Knockdown of ETS1 and HIF-2α by RNA interference inhibits EGF/FGF2-induced VEGFR1 expression, and loss of expression is associated with impaired DNA-polymerase II recruitment and histone modifications at the VEGFR1 promoter region. In addition, using a mouse embryonic stem cell in vitro differentiation system, we found that induction of VEGFR1 in embryoid bodies is also associated with ETS1 and HIF-2α recruitment to the VEGFR1 locus. These results establish an angiogenic signal-ETS1/HIF-2α axis that regulates the VEGFR1 chromatin domain to induce VEGFR1 transcription in endothelial cells and in differentiating embryonic stem cells.

Angiogenesis involves endothelial cell differentiation, proliferation, migration, and cell adhesion, which lead to tubulogenesis to form vessels. VEGFR2 family of receptor-tyrosine kinases is crucial for vascular development during embryogenesis as well as physiological and pathological angiogenesis (1). VEGFR1 and VEGFR2 are closely related receptor-tyrosine kinases and have both shared and specific ligands. Despite their differential kinase activation potentials, both VEGFR1 and VEGFR2 are required for normal development and angiogenesis (2, 3). Gene targeting studies demonstrate that VEGFR1−/− mice die in utero between days 8.5 and 9.0. Endothelial cells develop but do not form an organized vasculature. Three different gene products including placental growth factor, VEGFA, and VEGFB are known to bind VEGFR1 (4). VEGFR1 is also present in a soluble form (5), resulting from alternative splicing, which lacks the tyrosine kinase domain but retains the ability to bind ligands.

Several lines of evidence suggest that VEGFR1 plays both negative and positive roles in angiogenesis. Deletion of the VEGFR1 tyrosine-kinase domain is compatible with normal vascular development (6). Therefore, VEGFR1 seems to function as a “decoy receptor” for VEGFA during embryogenesis by regulating its accessibility for VEGFR2 on developing blood vessels. However, recent evidence suggests that the ability of VEGFR to modulate angiogenesis is not limited to mechanism by which it acts as a VEGF-trapping receptor. Activation of VEGFR1 by VEGF induces migration of endothelial cells lacking VEGFR2 (7). Recent studies showed that VEGFR1 loss is associated with decreased vascular sprout formation, migration, and vascular branching (8, 9). This phenotype was also observed in vivo, as VEGFR1−/− embryos had defective sprouting from the dorsal aorta (9). Consequently, signaling through VEGFR1 positively modulates endothelial cell migration and vascular branching. The biological outcome of VEGFR1 function might also be influenced through type of ligands it binds and “cross-talk” with VEGFR2. Several studies have reported that VEGFR1 both negatively and positively regulates VEGFR2 signals (10). It has been shown that, under pathological conditions, activation of VEGFR1 by placental growth factor, a VEGFR1-specific ligand, contributes to angiogenesis (11), and under certain circumstances, upon stimulation with placental growth factor, VEGFR1 may heterodimerize with VEGFR2, leading to transactivation of VEGFR-2 and angiogenesis (12). VEGFR1 plays multiple roles in tumor metastasis and angiogenesis (13). The growth and metastasis of the majority of tumors depend on the formation of new blood vessels (14).

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; shRNA, short hairpin RNA; ChIP, chromatin immunoprecipitation; kb, kilobase(s); EB, embryoid body.
Angiogenic factors, including VEGF, are released by tumor cells and promote activation, proliferation, and migration of endothelial cells to the tumor tissue, allowing rapid formation of functional neo-vascular structures. Emerging evidence indicates that bone marrow-derived VEGFR1 expressing circulating endothelial progenitor cells (CEPs) can contribute to the angiogenesis and growth of certain tumors (15, 16), and co-mobilization of VEGFR1+ hematopoietic stem and progenitor cells facilitates the incorporation of CEPs into functional tumor neo-vascular structures (16). VEGFR1+ hematopoietic progenitors have also been implicated in the regulation of metastasis due to their ability to initiate the pre-metastatic niche (17).

Whereas multiple studies have been performed to determine the biological functions and signaling mechanisms of VEGFR1, mechanisms that regulate VEGFR1 transcription are poorly understood. Analysis with reporter genes have implicated multiple transcription factors including ETS1 and HIF-2α in VEGFR1 transcriptional regulation (18–21). Transient transfection analysis (18, 19) using VEGFR1 promoter constructs provide evidence that ETS1 and HIF-2α regulate VEGFR1 promoter activity. However, their role in the regulation of the endogenous VEGFR1 chromatin domain is poorly understood.

Earlier, we showed that an endothelial cell growth supplement containing FGF2, EGF, induces VEGFR1 transcription in mouse yolk sac endothelial cells (YSECs) and human umbilical vein endothelial cells (HUVECs) (23). Although both FGF2 and EGF-signaling induce angiogenesis and VEGF production in certain cell types (24, 25), their role in regulation of the endogenous VEGFR1 gene expression in endothelial cells is unknown.

We here report that EGF/FGF2 signaling induces VEGFR1 in endothelial cells by recruiting ETS1 and HIF-2α transcription factors to the VEGFR1 chromatin domain. ETS1 and HIF-2α occupancy is associated with Pol II recruitment and transcriptionally favorable histone modifications at the VEGFR1 locus. Our work indicates an angiogenic signal-ETS1/HIF-2α axis that regulates gene expression in endothelial cells.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Reagents**—YSEC, HUVEC, and human lung microvascular endothelial cells (HLMECs) were cultured in medium 200 (M200; Invitrogen) containing EGF (10 ng/ml), FGF2 (3 ng/ml), hydrocortisone (1 μg/ml), and heparin (10 μg/ml) along with fetal bovine serum (2% final). “Supplemented endothelial cell culture condition” refers to the incubation of cells in the presence of the growth supplement, whereas its absence indicates the starved culture condition. For experiments with EGF and FGF2, 10 ng/ml recombinant human EGF (R&D Systems, Minneapolis, MN) and/or 3 ng/ml recombinant mouse FGF2 (R&D Systems) were added to the supplement-free M200 medium.

HEK-293T cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (Atlas Biologicals, Fort Collins, CO). LY294002 (2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one, phosphatidylinositol 3-kinase inhibitor), G66983 (2-[1-(3-dimethylamino propyl)-5-methoxyindol-3-yl]-3(1H-indol-3-yl) maleimide, broad spectrum protein kinase C (PKC) inhibitor), and Gö6976 (12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo(2,3-a)pyrrolo(3,4-c)-carbazole, PKCα and PKCβ inhibitor) were obtained from Sigma Aldrich. Mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (MEK) inhibitor U0126 (1,4-diamino-2,3-dicyano-1,4-bis(2-amino-phenylthio)butadiene) was obtained from Calbiochem. BAPTA-AM was purchased from Calbiochem. All other chemicals were from Sigma-Aldrich.

**Quantitative Reverse Transcription (RT)-PCR**—RNA was extracted from different cell samples with Trizol reagent (Invitrogen). cDNA was prepared by annealing RNA (1 μg) with 250 ng of a 5:1 mixture of random and oligo(dT) primers heated at 68 °C for 10 min. This was followed by incubation with Moloney murine leukemia virus reverse transcriptase (50 units) (Invitrogen) combined with 10 mM dithiothreitol, RNasin (Promega, Madison, WI) and 0.5 mM dNTPs at 42 °C for 1 h. Reactions were diluted to a final volume of 100 μl and heat-inactivated at 97 °C for 5 min. 20–μl PCR reactions contained 2 μl of cDNA, 10 μl of SYBR Green Master Mix (Applied Biosystems, Foster City, CA), and corresponding primer sets. Control reactions lacking RT yielded very low signals. Relative expression levels were determined from a standard curve of serial dilutions of untreated YSEC and HUVEC/HLMVEC cDNA samples and were normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and hypoxanthine-guanine phosphoribosyltransferase (HPRT), respectively. Forward and reverse primers for quantitive RT-PCR (5’- 3’) were: mouse GAPDH, TGCCCCCATGTTTGTGATG and TTGGGTATGTCAGTGTGCATCT; mouse VEGFR1, CGGAAGAAAGAAACGGGAATC and TGGGTATGTCAGTGTGCATCTA; mouse ETS1, GTTTCACAAAAAGACACGAGCAGG and TTTCTGTCACACTGGCGGC; mouse HIF-2α, CTCCTCGGACATAAGCTCTG and AGCTTGAGATCTTACGAGA; mouse VEGFR2 (exon2/3), TTGGCGCAATACACCCCTTCAG and CTTTTCCTCAGATCTCAAGT; human VEGFR1, CTCGCACCCTTAAATTTTGTGTAAGA and AAA CGATGACACGGCGCTT; human HIF-2α, AATTTGGTGGATGATGCTCTCAACTT and GCCGAGCTCAAATTATCCTTCACAA.

**Western Blot Analysis**—Cell lysates were prepared in SDS-gel loading buffer and resolved by 8 or 10% PAGE. Monoclonal anti-VEGFR1 was obtained from Abcam, Cambridge, MA. Polyclonal anti-ETS1 antibody was obtained from Santa Cruz Biotechnology, Santa Cruz, CA. Polyclonal HIF-2α antibody was obtained from Novus Biologicals, Littleton, CO, and monoclonal anti-α-tubulin was obtained from Calbiochem. Anti-VEGFR2 antibody was obtained from Cell Signaling Technology, Danvers, MA. Horseradish peroxidase-conjugated goat-anti-rabbit and anti-mouse from Santa Cruz Biotechnology were used as secondary antibodies.

**RNA Interference**—Lentiviral vectors containing short hairpin RNAs (shRNAs) targeting mouse ETS1 and HIF-2α mRNAs were cloned in pLKO1 (Open Biosystems, Huntsville, AL). Lentiviral supernatants were produced in HEK-293T cells by transient transfection with calcium phosphate. Briefly, HEK-293T
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FIGURE 1. EGF/FGF2 signaling combinatorially induces VEGFR1 expression in endothelial cells. A, YSECs were starved for 12 h and then treated with supplemented M200 medium (Sup) for different time intervals. VEGFR1 mRNA was quantitated by real-time RT-PCR and normalized to GAPDH mRNA (means ± S.E. of three independent experiments). B, a representative Western blot of VEGFR1 expression in samples analyzed in A. C, YSECs were starved for 12 h and then treated with or without EGF (10 ng/ml), FGF-2 (3 ng/ml), or hydrocortisone (1 μg/ml) for 3 h. VEGFR1 mRNA was quantitated by real-time RT-PCR and normalized to GAPDH mRNA (means ± S.E. of three independent experiments). C, HUVECs and HLMECs were starved for 12 h and then treated with or without EGF (10 ng/ml) and FGF-2 (3 ng/ml) in M200 for 3 h. VEGFR1 mRNA was quantitated by real-time RT-PCR and normalized to HPRT mRNA (means ± S.E. of the mean for three independent experiments). D, Western blot analysis of VEGFR1 expression of samples analyzed in C.

cells were grown to 80% confluence. pMDLg/pRRE (HIV-1 Gag/Pol), pMD2.G (VSV G), pRSV-Rev, and shRNA-containing vectors were mixed with 0.25M CaCl2 to a final volume of 2 μl. An equal volume of 2X Hepes-buffered saline buffer was added dropwise and mixed with gentle vortexing. The resulting mixture was added on the HEK-293T cells. The infection medium was replaced with fresh medium after 15 h. Lentiviral supernatants were collected after 24 and 48 h of incubation. YSECs grown to 70% confluence were incubated with 8 μg/ml of Polybrene (Sigma Aldrich) for 1 h followed by transfection with lentiviral soups. Transfected YSECs were selected by the addition of 3 μg/ml of puromycin (Sigma Aldrich) after 48 h of infection. After 3 days, selected cells were starved overnight in supplement-free M200 medium containing puromycin and treated with EGF/FGF2-containing medium at 37 °C. After 3 h samples were prepared for RNA and protein analysis. The knockdown was confirmed by quantitative RT-PCR and Western blotting. Constructs with five different target sequences each for ETS1 and HIF-2α were analyzed. The ETS1 target sequences 5'-GCCAGACGACTCTTTGGCATT-3' (clone 1) and 5'-GACAGCTTTGACTAG-AGGAT-3' (clone 2) and HIF-2α target sequence 5'-GACAGAATCTTGGAACTGATT-3' (clone ID TRCN0000082307, Open Biosystem) successfully knocked down the corresponding genes.

Quantitative ChIP Assay—Real-time PCR-based quantitative ChIP analysis was performed according to an earlier described protocol (26). Subconfluent YSECs were starved overnight, and then EGF/FGF2-containing M200 medium was added for 3 h. Cells were trypsinized and protein-DNA cross-linking was conducted by treating cells with formaldehyde at a final concentration of 1% for 10 min at room temperature with gentle agitation. Glycine (0.125 M) was added to quench the reaction. Antibodies against ETS1, HIF-2α, di-acetylated histone 3 (acH3; Millipore, Billerica, MA), tetra-acetylated histone 4 (acH4; Millipore), histone 3 di-methylated at lysine 4 (H3MeK4; Abcam), histone 3 di-methylated at lysine 79 (H3K79; Abcam), and Pol II (Santa Cruz) were used to immunoprecipitate protein-DNA cross-linked fragments. Primers were designed to amplify 60- to 100-bp amplicons and were based on sequences in Ensembl data base for mouse VEGFR1 locus. Samples from three more immunoprecipitations were analyzed. Products were measured by SYBR Green fluorescence (Power SYBR Green Master mix, Applied Biosystems) in 25-μl reactions. The amount of product was determined relative to a standard curve of input chromatin. Dissociation curves showed that PCRs yielded single products.

Forward and reverse primers for ChIP assays (5'-3') were: VEGFR1(-) 1.5 kb, GAGGCGCCAGGATCCAGCTG and GAGTGGCCCGCAGCTAGTTA; VEGFR1(-) 1 kb (used for HIF-2α recruitment at ~980-bp motif), ATTTACAGGAC-AAGACTGGGC and GAAGGCTGACTCTCACC; VEGFR1(-) 750 bp (used for HIF-2α recruitment at ~795-bp motif), ATTTACAGGAC-AAGACTGGGC and GAAGGCTGACTCTCACC; VEGFR1(-) 400 bp, TGACCT-AGACGGTTCCCTC and TTAATAGCTCTGAGTACC; VEGFR1(-) 250 bp (used for HIF-2α recruitment at...
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RESULTS

EGF and FGF2 Induce VEGFR1 in Endothelial Cells through a Protein Kinase C-dependent Pathway—Despite the importance of VEGFR1 in multiple biological functions of endothelial cells and other cell types, VEGFR1 transcriptional regulation is poorly understood. Earlier, we showed that VEGFR1 expression is induced when YSECs are starved from growth factors and treated with endothelial growth supplement (23). To understand the mechanism of supplement-mediated VEGFR1 induction, we performed time course analysis in YSECs. Time course analysis showed that, in the presence of the supplement, VEGFR1 mRNA is rapidly induced in starved YSECs. The VEGFR1 mRNA induction is maximal between 3 and 7 h after supplement addition showing nearly a 12-fold induction in VEGFR1 mRNA level compared with that in the starved cells (Fig. 1A). However, the mRNA level was reduced with time, and after 20 h of supplement addition only a 5-fold increase in VEGFR1 mRNA was observed. Western blot analysis validated the mRNA expression pattern (Fig. 1B).

The endothelial supplement contains EGF, FGF2, and hydrocortisone along with fetal bovine serum. We tested which of these components induces VEGFR1 transcription in YSECs. We found that both EGF and FGF2 partially induce VEGFR1 mRNA (Fig. 1C). However, a combination of EGF and FGF2 is capable of maximal transcriptional induction. To determine whether EGF/FGF2 signaling regulates VEGFR1 in primary human cells, we tested VEGFR1 induction in HUVECs and HLMECs. Similar to YSECs, EGF/FGF2 rapidly induced VEGFR1 in starved HUVECs and HLMECs (Fig. 1, D and E). Thus, EGF/FGF2 signaling regulates VEGFR1 induction in diverse endothelial cell types.

As multiple signaling pathways are implicated downstream of the receptors of EGF and FGF2 (27, 28), we tested which signaling pathway is involved in EGF/FGF2-mediated VEGFR1 transcriptional induction. We found that pretreatment of YSECs with the broad spectrum PKC inhibitor Go6983 (29) inhibited EGF/FGF2-induced VEGFR1 mRNA expression by ~65% (Fig. 2A). However, pretreatment with U0126 (MEK1/2 inhibitor) (30) or LY294002 (phosphatidylinositol 3-kinase inhibitor) (31) did not have any effect on the VEGFR1 induction, indicating that EGF/FGF2-induced VEGFR1 induction is not mediated via phosphatidylinositol 3-kinase- or MEK1/2-dependent pathway. A, YSECs were starved for 12 h, pretreated with or without U0126 (10 μM), LY294002 (10 μM), Go6983 (10 μM), and Go6976 (1 μM) for 30 min, and treated with supplemented medium with or without chemical inhibitors for 3 h. VEGFR1 mRNA was measured by quantitative RT-PCR (means ± S.E.; four independent experiments). B, YSECs were loaded with Fluo-4/AM in supplement-free M200 medium. Intracellular Ca2+ was monitored every 10 s. The graphs depict change in intracellular Ca2+ levels, plotted as a percentage of fluorescence intensity at time 0 (F0), of a representative cell. The arrow indicates the time of addition of EGF/FGF2-containing medium (three independent experiments showed that intracellular Ca2+ levels are increased in more than 60% cells in response to EGF/FGF2). C, fluorescence images at two different time points showing intracellular Ca2+ levels of the cell analyzed in B (objective, 40×; eyepiece, 10×; objective NA, 0.60). D, starved YSECs were treated with or without 4 μM BAPTA-AM for 1 h in supplement-free medium, treated with EGF/FGF2 containing medium with or without BAPTA-AM for 3 h, and VEGFR1 mRNA expression was measured (means ± S.E.; three independent experiments).
FIGURE 3. EGF/FGF2-signaling induces ETS1 expression and recruits ETS1 to the VEGFR1 locus. A, starved YSECs were treated with or without cycloheximide (20 μg/ml) for 15 min in supplement-free medium or treated with supplemented medium with or without cycloheximide for 3 h, and VEGFR1 mRNA expression was measured by quantitative RT-PCR (means ± S.E.; four independent experiments). B and C, RT-PCR and Western blot analysis, respectively, of ETS1 expression in YSECs. Starved YSECs were treated with supplemented (Sup) or EGF/FGF2-containing medium for 3 h, and ETS1 expression was measured. D, rVISTA alignment plot of ∼4-kb regions of mouse and human VEGFR1 loci. The red bars indicate conserved ETS motifs. UTR, untranslated region. E, quantitative ChIP analysis of ETS1 recruitment at the VEGFR1 locus in YSECs. Starved YSECs were treated with EGF/FGF2 containing medium for 3 h, and analysis was performed with both starved and EGF/FGF2-treated cells. The dotted line shows the average signal obtained when nonspecific preimmune serum was used as a control antibody (means ± S.E.; four independent experiments).

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ETS1 and HIF-2α Interact Functionally to Induce VEGFR1 in Endothelial Cells—To test the functional role of ETS1 in EGF/FGF2-mediated VEGFR1 induction, we knocked-down ETS1 by RNA-mediated interference. We found that when ETS1 mRNA was knocked down by ∼90% in YSECs (Fig. 4A), VEGFR1 mRNA induction was inhibited by ∼60% (Fig. 4B). Western blot analysis also validated the loss of ETS1 and VEGFR1 expression (Fig. 4D). ETS1-specific small interfering RNA did not inhibit the mRNA expression of the related transcription factor, ETS2 (Fig. 4C), indicating its specificity for ETS1. However, both mRNA and protein analysis showed that almost complete (90%) loss of ETS1 mRNA and protein levels results in only a partial loss of VEGFR1 induction. Therefore, we hypothesized that another factor(s) cooperates with ETS1 in EGF/FGF2-mediated VEGFR1 expression.

Transcription factor HIF-2α has been implicated in the regulation of receptor-tyrosine kinases including VEGFR1 in endothelial cells (19, 33). In addition, cooperative function of ETS1 and HIF-2α has been demonstrated in the regulation of VEGFR2 (34) and VE-cadherin promoters (35) in endothelial
cells. Therefore, we tested whether HIF-2α is induced/stabilized in response to EGF/FGF2 signaling in endothelial cells under our experimental conditions and whether they functionally interact in EGF/FGF2-mediated VEGFR1 induction.

RT–PCR analysis showed that HIF-2α mRNA is expressed in YSECs but is not induced in response to EGF/FGF2 (Fig. 5A). Surprisingly, Western blot analysis detected HIF-2α protein in YSECs without hypoxia treatment. We also found that compared with starved cells, HIF-2α protein levels in EGF/FGF2 treated (3 h) cells increases significantly (Fig. 5B), and the protein level is similar to that in cells which are simultaneously treated with EGF/FGF2 and 150 μM CoCl₂, a chemical inducer of the hypoxic response. Western blot analysis in HUVECs also detected HIF-2α protein in normoxic conditions. Furthermore, similar to YSECs, EGF/FGF2 significantly increased HIF-2α protein levels in HUVECs (Fig. 5C). These results indicate that HIF-2α protein is present at a detectable concentration in endothelial cells under our experimental conditions, and EGF/FGF2 signaling further increases HIF-2α proteins in endothelial cells.

Because HIF-2α protein levels increase in response to EGF/FGF2-signaling, we wanted to test whether HIF-2α is also recruited to the VEGFR1 locus in response to EGF/FGF2 signaling. Sequence analysis of the ~4-kb mouse and human VEGFR1 loci, which we analyzed for ETS1 binding (Fig. 3D), indicated the presence of three conserved R(A/G)CGTGT motifs (putative HIF motifs) (Fig. 5, D and E). One of those putative HIF motifs is located at −980 bp corresponding to the transcriptional start site. This motif (mentioned as −959-bp HIF motif) has been implicated in VEGFR1 promoter regulation by an earlier study (36). Two other conserved RCGTGT motifs are located at −207 bp and at −795 bp corresponding to the transcriptional start site. Therefore, we tested whether HIF-2α is also recruited to the VEGFR1 locus in response to EGF/FGF2 treatment.

Quantitative ChIP analysis in YSECs detected EGF/FGF2-dependent HIF-2α recruitment at the −207-bp region as well as at the −980-bp region (Fig. 5E). However, significantly less occupancy was detected using primers spanning the −792-bp RCGTGT motif, indicating that this motif may not be occupied by HIF-2α. Given the size of the chromatin fragments (300–600 bp), the occupancy signal at −795-bp region may come from the immunoprecipitated chromatin fragments, in which the −980-bp but not the −795-bp motif is occupied by HIF-2α. From these results we hypothesized that like ETS1, HIF-2α also directly regulates VEGFR1 transcription in response to EGF/FGF2-signaling, and functional interaction of these two factors is necessary for the maximal transcriptional induction of VEGFR1.

We next instituted the RNA-mediated interference approach to test this hypothesis. We found that knockdown of HIF-2α (Fig. 6, A and B) also reduced EGF/FGF2-induced VEGFR1 expression in YSECs (Fig. 6, C and D). Furthermore, combinatorial knockdown of both ETS1 and HIF-2α in YSECs resulted in near complete loss of VEGFR1 induction (Fig. 6, C and D). These results indicate that EGF/FGF2-induced VEGFR1 expression is mediated by ETS1 and HIF-2α in a combinatorial fashion.

ETS1- and HIF-2α-dependent Remodeling of the Histone Modification Pattern and Pol II Recruitment at the VEGFR1 Chromatin Domain—Tissue-specific transcriptional activation often involves the selective targeting of histone modification enzymes and recruitment of Pol II to specific regulatory regions of the gene (37). Both ETS1 and HIF-2α interact with the transcriptional coactivators; that is, cAMP-responsive element binding protein and the related protein p300 (CBP/p300) (38, 39). ETS1-CBP/p300 complex possesses histone acetyl transferase activity (38) and has been implicated in transcriptional regulation of multiple genes (40). Because supplemental-mediated induction of VEGFR1 is associated with the recruitment of ETS1 and HIF-2α at the VEGFR1 chromatin domain and small interfering RNA-mediated knockdown of those factors resulted in loss of transcriptional induction, we wanted to test their function of post-chromatin occupancy. To that end we tested whether the histone modification patterns are altered and Pol II is recruited to the VEGFR1 promoter region in response to EGF/FGF2-signaling and whether the histone modification and Pol II recruitment are dependent on ETS1 and HIF-2α. Quantitative ChIP analysis showed that EGF/FGF2-mediated transcriptional induction of VEGFR1 in YSECs is associated with a 3-fold increase in acH3 levels and H3MeK4 levels and a 2-fold increase in acH4 levels (Fig. 7, A–C) at the VEGFR1 promoter region. The H3MeK79 levels were very high at VEGFR1 pro-
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In this study we have shown that an EGF/FGF2-Ca^2+-PKC-ETS1/HIF-2α regulatory axis mediates VEGFR1 transcriptional induction in endothelial cells. We found that combinatorial function of ETS1 and HIF-2α, in which these two factors directly modulate the nucleoprotein structure at the VEGFR1 chromatin domain, is important for maximal induction of VEGFR1. We have also shown that the induction of VEGFR1 in ES cell-derived EBs is also associated with ETS1 and HIF-2α recruitment at the VEGFR1 locus, indicating that these factors directly regulate VEGFR1 during ES cell differentiation to endothelial and other cell lineages.

ETS1 is expressed in mesoderm lineage cells including endothelial cells during embryonic development (41). ETS1 expression is detected in all developing vascular structures, including the heart, arteries, capillaries, and meninges (42). Moreover, increased expression of ETS1 is observed in endothelial cells of neo-vessels during tumor angiogenesis in the adult (43) and

moter region in the starved cells, and the levels did not change significantly in the presence of EGF/FGF2 (Fig. 7D). However, in the presence of EGF/FGF2, Pol II occupancy was increased by 4-fold at the VEGFR1 promoter (Fig. 7E), indicating that transcriptional induction is associated with Pol II recruitment at the promoter region. Interestingly, knockdown of ETS1 and HIF-2α by RNA-mediated interference impaired histone modifications as well as Pol II recruitment at the VEGFR1 promoter. Thus, in response to EGF/FGF2 signaling an ETS1/HIF-2α-dependent altered nucleoprotein structure assembles at the VEGFR1 promoter region, leading to Pol II recruitment and transcriptional induction.

**Induction of VEGFR1 in Differentiating ES Cells Is Associated with ETS1 Recruitment to the VEGFR1 Chromatin Domain**—Our studies in endothelial cells indicate that an angiogenic signal-ETS1/HIF-2α axis regulates VEGFR1 transcription in endothelial cells. To test whether the same mechanism is involved in the regulation of VEGFR1 transcription during endothelial differentiation, we differentiated ES cells to generate EBs and asked whether induction of VEGFR1 during ES cell differentiation is associated with ETS1 and HIF-2α recruitment to the VEGFR1 locus.

Time course analysis during ES cell differentiation showed that both VEGFR1 and ETS1 mRNAs are induced by an ~4-fold in day 3 EBs (Fig. 8A), and this induction is maintained in day-4.5 EBs. VEGFR2, an early marker of endothelial progenitors, is also highly induced in day 4.5 EBs (Fig. 8, A and B). However, HIF-2α mRNA levels did not change significantly. Western blot analysis validated the mRNA analyses (Fig. 8B).

To determine whether ETS1 and HIF-2α are recruited to the VEGFR1 locus in undifferentiated and differentiating ES cells (day 3 EBs), we performed a ChIP analysis. Interestingly, ETS1 occupancy at the VEGFR1 locus was only detected in day 3 EBs (Fig. 8C). However, HIF-2α occupancy was detected both in undifferentiated ES cells and in day 3 EBs (Fig. 8D). Thus, similar to endothelial cells, a HIF-2α- and ETS1-containing nucleoprotein complex assembles at the transcriptionally active VEGFR1 locus during ES cell differentiation.

**DISCUSSION**

In this study we have shown that...
also in endothelial cells during angiogenesis and after denudation (44). Interestingly, ETS1-deficient mice develop normally despite an increase in perinatal mortality (45). Whether this is because other factors can compensate for its loss of function remains an open question. However, direct involvement of ETS1 in angiogenesis was shown through a variety of in vitro and in vivo approaches (46).

The hypoxia-inducible factors mediate the cellular adaptive response to low O2 levels. HIF-1α and HIF-2α are the critical mediators of the hypoxia response, and they regulate both overlapping and unique transcriptional targets (47). HIF-2α is expressed mainly in vascular endothelial cells. In addition to endothelial cells, HIF-2α transcripts are found in neural crest cell derivatives, lung type II pneumocytes, liver parenchyma, and interstitial cells of the kidney (39). Inactivation of HIF-2α by three independent groups resulted in embryonic lethality or death shortly after birth due to insufficient catecholamine synthesis (48), insufficient surfactant production from type II alveolar cells (49), or defects in vascular remodeling (50), depending on the genetic background. These results indicate that multiple developmental pathways including vascular development are regulated by HIF-2α. Interestingly, expression of HIF-2α cDNA in the vascular endothelium of HIF-2α−/− embryos by an ES cell-mediated transgenic approach rescued vascular development in HIF-2α−/− embryos, indicating an intrinsic requirement for HIF-2α by endothelial cells (51).

Surprisingly, in this study we found that HIF-2α protein is detectable, albeit at low levels, in endothelial cells and in undifferentiated ES cells under normal tissue culture conditions without hypoxia treatment. EGF/FGF2 signaling further increases HIF-2α protein levels in endothelial cells. We performed this study in a 3-h window after the addition of EGF/FGF2-containing medium. Therefore, it is possible that increases in HIF-2α protein levels are a transient event in response to growth factor signaling. Whether this increase in HIF-2α protein levels is due to protein stability or protein synthesis is an interesting question for further analysis. Because endothelial cells experience reduced oxygen tension during embryonic development (47) as well as pathological conditions (during tumor growth) along with canonical hypoxia-dependent stabilization, this growth factor signaling-mediated increase in HIF-2α protein level may contribute to an additional level of control for HIF-2α function in vascular endothelium.

Interestingly, we have discovered another conserved HIF motif at the VEGF1 promoter region (~207-bp HIF motif). This motif contains the consensus HIF binding sequence B(C/G)T(G/T)R (A/G)CGTGS(C/G)K(G/T) (36). We found that HIF-2α occupies this motif at the transcriptionally active endogenous

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FIGURE 6. ETS1 and HIF-2α functionally interact to induce VEGFR1 in response to EGF/FGF2-signaling. A and B, quantitative RT-PCR and Western blot analysis of HIF-2α expression in YSECs showing knockdown of HIF-2α expression. YSECs were infected with lentiviral vectors expressing HIF-2α shRNA as mentioned under “Experimental Procedures” and analyzed for mRNA (means ± S.E.; three independent experiments) and protein expression. C, quantitative RT-PCR analysis of VEGFR1 mRNA expression in YSECs. ETS1 and HIF-2α were knocked down individually or in combination in YSECs, cells were starved and treated with EGF/FGF2 containing medium for 3 h, and VEGFR1 mRNA expression was measured (means ± S.E.; three independent experiments). D, representative Western blot for determining VEGFR1 protein expression in samples analyzed in C.

FIGURE 7. EGF/FGF2-mediated histone modifications and Pol II recruitment at the VEGF1 promoter is dependent upon ETS1 and HIF-2α. Quantitative ChIP analysis of acH3 (A), acH4 (B), H3MeK4 (C), H3MeK79 (D), and Pol II recruitment (E) at the VEGF1 promoter region in YSECs (means ± S.E. of the mean for three independent experiments). YSECs, control or infected with lentiviral vectors expressing ETS1 and HIF-2α shRNAs, were starved, treated with EGF/FGF2 for 3 h, and analyzed for histone modifications. PI, preimmune.
VEGFR1 chromatin in endothelial cells and in EBs, indicating its functional importance in VEGFR1 regulation.

ChIP analysis showed a peak of ETS1 occupancy near the transcription start site at the VEGFR1 locus, whereas HIF-2α occupancy are detected at the −207 and −980-bp HIF motifs. However, their functional interaction indicates that probably these regions are in close proximity in higher order chromatin conformation. Earlier, it has been shown that ETS1 and HIF-2α can interact physically (34). Thus, occupancy of one factor at the VEGFR1 locus may facilitate occupancy of the other factor.

Evidence is accumulating regarding the cooperative functions of transcriptional factors in endothelial cell-specific gene regulation (34, 35, 52, 53). Several studies have shown that multiple ETS family transcription factors including ETS1 can cooperate with HIF-2α for target gene regulation (39). These interactions are predicted to specify HIF-2α target genes (54). Reporter gene analysis showed that HIF-2α and ETS1 function cooperatively to regulate VEGFR2 promoter activity (34).

Another recent study showed that in endothelial cells, ETS1 and HIF-2α cooperatively regulates VE-cadherin promoter in the absence of hypoxia (35). We found that in response to EGF/FGF2-signaling, ETS1 and HIF-2α interact functionally to activate endogenous VEGFR1 chromatin domain to induce VEGFR1 expression in endothelial cells.

The angiogenic signal-ETS1/HIF-2α axis of VEGFR1 regulation might be important both in the context of vascular development as well as during pathological angiogenesis. Multiple angiogenic growth factors including EGF, FGF2, and VEGF have been shown to induce ETS1 mRNA in endothelial cells (43, 44, 55). We have shown that both FGF2 and EGF induce VEGFR1 in endothelial cells (Fig. 1C), and another study showed that VEGF also induces VEGFR1 expression in endothelial cells (55). VEGF is crucial for vascular development (56), and FGF2 have also been implicated in early endothelial differentiation (57). Because both ETS1 and HIF-2α are expressed in endothelial cells during embryonic development (33, 41, 42), it is conceivable that, during vascular development, growth factors like FGF2 and VEGF might regulate VEGFR1 expression via ETS1 and HIF-2α.

Studies with multiple human astroglial tumors showed that ETS1 expression in human astrocytomas is associated with VEGFR1 synthesis and neoangiogenesis (22). Given the fact that glioma cells produce growth factors including EGF, FGF2, and VEGF (58, 59), the angiogenic signal-ETS1/HIF-2α axis might regulate VEGFR1 expression during neoangiogenesis in astrocytomas.

Collectively, ETS1 and HIF-2α may comprise an important regulatory duo controlling endothelial gene regulation during vascular development and pathological angiogenesis. Further analysis in other model systems including knock-out mice models will provide additional information regarding the combinatorial function of ETS1 and HIF-2α.

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