A Novel Class of Vascular Endothelial Growth Factor-responsive Genes That Require Forkhead Activity for Expression\*\*\*1

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Recently, we have shown that transient phosphorylation and inhibition of the pro-apoptotic transcription factor, forkhead, by vascular endothelial growth factor (VEGF) is essential for endothelial cell (EC) survival and proliferation. The goal of the present study was to determine whether forkhead (FKHR) also plays a positive role in agonist-mediated gene induction.

Human coronary artery ECs were transduced with adenovirus overexpressing constitutively active phosphorylation-resistant triple mutant FKHR or transfected with small interference RNA (siRNA) against FKHR. The cells were then treated in the absence or presence of VEGF and assayed for gene expression using quantitative real-time PCR and Northern blots analyses.

The data revealed a novel set of VEGF-responsive genes that require FKHR activity for optimal expression in ECs, including bone morphogenetic protein 2, cbp/p300-interacting transactivator 2, decay accelerating factor (DAF), vascular cell adhesion molecule-1 (VCAM-1), manganese superoxide dismutase, endothelial-specific molecule-1, RING1 and YY1-binding protein, and matrix metalloproteinase-10. Consistent with a positive role for FKHR in mediating VEGF induction of DAF and VCAM-1 mRNA, siRNA against FKHR attenuated the effect of VEGF on complement-mediated EC lysis and monococyte adhesion, respectively. VEGF induction of the forkhead-dependent genes was down-regulated by the NF-κB inhibitor, constitutively active Ad-1κB, and in some cases by the nuclear factor of activated T-cells (NF-AT) inhibitor, cyclosporin. Together, these findings suggest that the VEGF-forkhead signaling axis plays an important functional role in ECs beyond the regulation of cell survival/apoptosis and cell cycle.

Vascular endothelial growth factor (VEGF)\# plays a critical role in endothelial cell (EC) survival, proliferation, migration, and is involved in wound repair, angiogenesis of ischemic tissue, tumor growth, microvascular permeability, vascular protection, and hemostasis (1–8). VEGF has been shown to activate a number of different intracellular signaling pathways, including protein kinase C, PI3K and Akt, MEK1/2, p38 mitogen-activated protein kinase, and phospholipase Cγ (9–13).

VEGF-mediated activation of intracellular intermediates results in altered gene expression and EC phenotype. Among the transcription factors that have been shown to be activated by VEGF are NF-κB, Egr-1, NFAT-1, Ets-1, and Stat-3/5 (14–18). In contrast, forkhead transcription factors have been shown to be negatively regulated by VEGF (19).

The mammalian members of the winged helix, or forkhead, transcription factors include FKHR (Foxo1), FKHRL1 (Foxo3a), and AFX (Foxo4). Forkhead transcriptional activity is coupled to three principal functions: growth inhibition, apoptosis, and regulation of metabolism. In non-endothelial cells, forkhead-mediated effects on cell growth and survival have been shown to be governed by cell type-specific induction of cell-cycle arrest and/or pro-apoptotic genes, including kinase inhibitor protein p27 (p27Kip1), growth arrest and DNA damage 45A (GADD45A), B-cell translocation gene 1 (BTG1), Fas ligand, and pro-apoptotic Bcl-2 interacting member (20–23). Forkhead effects on metabolism are mediated by a distinct set of target genes involved in energy metabolism and stress resistance, including glucose-6-phosphatase, phosphoenolpyruvate carboxykinase, insulin-like growth factor-1-binding protein-1, and others.

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3 The abbreviations used are: VEGF, vascular endothelial growth factor; EC, endothelial cell; P38, phosphatidylinositol 3-kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; Stat, signal transducers and activators of transcription; FKHR, forkhead; BTG1, B-cell translocation gene 1; SOD, superoxide dismutase; TM, triple mutant; siRNA, small interference RNA; HCAEC, human coronary artery endothelial cell; WT, wild type; Adv, adenovirus encoding the CDNA of β-galactosidase; Ad, adenovirus; BSA, bovine serum albumin; m.o.i., multiplicity of infection; HA, hemagglutinin; BMP2, bone morphogenetic protein 2; CITED2, cbp/p300-interacting transactivator 2; DAF, decay accelerating factor; VCAM-1, vascular cell adhesion molecule-1; ESM-1, endothelial specific molecule-1; MMP, matrix metalloproteinase; siFKHR, siRNA against FKHR; GADD45A, growth arrest and DNA damage 45A; NF-AT, nuclear factor of activated T-cells.
manganese superoxide dismutase (Mn-SOD), and catalase (24–29). Studies in non-ECs suggest that agonists involved in promoting cell growth, survival, or metabolism, including growth factors, cytokines, and hormones, result in PI3K-Akt-dependent phosphorylation, nuclear exclusion, and inactivation of forkhead (30–36). According to this model, growth factor-mediated inactivation of forkhead and secondary inhibition of the forkhead-responsive genes is a prerequisite for cell proliferation and metabolism.

Consistent with this paradigm, we have recently demonstrated that VEGF signaling in ECs results in PI3K-Akt-dependent phosphorylation and nuclear exclusion of forkhead transcription factors, with subsequent down-regulation of the forkhead target gene, p27kip1, increased cell proliferation, and decreased apoptosis (19).

In contrast to non-ECs, where insulin suppresses Mn-SOD levels via a PI3K-Akt-forkhead-dependent signaling pathway (27), VEGF was shown to induce expression of Mn-SOD in ECs (37). A positive role for forkhead in this pathway was evidenced by the observation that VEGF-mediated induction of Mn-SOD was enhanced by inhibition of PI3K or Akt or by overexpression of TM-FKHR1 (38).

According to the classic view of forkhead as a pro-apoptotic and cell-cycle arrest gene, its deletion in mice might predict for increased cell survival and growth. However, mice that are null for FKHR demonstrate impaired vascular development and embryonic lethality (39, 40). Moreover, ECs derived from FKHR−/− embryonic stem cells demonstrated an abnormal morphological response to VEGF (40). Finally, siRNA-mediated inhibition of FKHR in serum-treated human umbilical vein endothelial cells resulted in altered expression of angio-genesis-related genes (41). Together with our findings that forkhead proteins are involved in mediating VEGF induction of the antioxidant Mn-SOD (38), these data suggest that the VEGF-forkhead signaling axis plays an important role in vascular biology, over and above its ability to inhibit pro-apoptotic/cell-cycle arrest genes. Here, we provide evidence for the existence of a subset of VEGF-responsive genes whose expression is both dependent on and modulated by forkhead activity in ECs.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Reagents**—Human coronary artery ECs (HCAECs) were from Clonetics (San Diego, CA) and grown in endothelial growth medium-2-MV (EGM-2) BulletKit (Clonetics) at 37 °C and 5% CO2. ECs from passage 3 to 6 were used for all experiments. Cells were serum-starved in 0.5% fetal bovine serum prior to treatment with 50 ng/ml human VEGF165 (Pepro Tech Inc., Rocky Hill, NJ). Where indicated, cells were preincubated for 30 min with 50 μg/ml LY294002 (Biomol, Plymouth Meeting, PA). The anti-endoglin (CD105) monoclonal antibody (IgG2a) was from Covance (Berkley, CA). Cyclosporin A was from Calbiochem.

**Adenoviruses**—HCAECs were transduced with replication-deficient adenoviruses encoding the cDNAs of β-galactosidase (Adv), wild-type (WT)-FKHR, and triple mutant (TM)-FKHR as previously described (42). The triple mutant version of FKHR contains T24A, S256A, and S319A and is resistant to agonist-induced phosphorylation. Adenovirus expressing constitutively active IkB was a generous gift from Chritiane Ferran of Beth Israel Deaconess Medical Center.

**siRNA-mediated Inhibition of Endogenous FKHR**—HCAECs were grown to 70–80% confluency in 6-cm plates and transfected with siRNA against one of the following FKHR target sequences: siFKHR1, ATG GAG GTA TGA GTC AGT ATA, and siFKHR2, CAG CGC CGA CTT CAT GAG CAA (Qiagen, Valencia, CA) in Opti-MEM containing Lipofectin (10 μg/ml) for 4 h. The cells were then incubated in EGM-2 medium for 24 h and serum-starved in 0.5% serum for 12–16 h before VEGF treatment for the times indicated.

**Quantitative Real-time PCR**—Total RNA was prepared using the RNeasy RNA extraction kit with DNaseI treatment following the manufacturer’s protocol (Qiagen). To generate cDNA, total RNA (100 ng) from each of triplicate samples was mixed and converted into cDNA using random primers and Superscript III reverse transcriptase (Invitrogen). All cDNA samples were aliquoted and stored at −80 °C. Primers were designed using the Primer Express oligonucleotide design software (Applied Biosystems, Foster City, CA) and synthesized by Integrated DNA Technologies (Coralville, IA). All primer sets were subjected to rigorous data base searches to identify potential conflicting transcript matches to pseudogenes or homologous domains within related genes. Amplicons generated from the primer set were analyzed for melting point temperatures using the first derivative primer melting curve software supplied by Applied Biosystems. The sequences of the real-time PCR primers used in this study are listed in Table 1. The SYBR Green I assay and the ABI Prism 7700 Sequence Detection System were used for detecting real-time PCR products from the reverse-transcribed cDNA samples, as previously described (43). 18 S rRNA, which exhibits a constant expression level across all the HCAECs samples, was used as the normalizer. PCR reactions for each sample were performed in duplicate and copy numbers were measured as described previously (43). The level of target gene expression was normalized against the 18 S rRNA expression in each sample, and the data presented as mRNA copies per 106 18 S rRNA copies.

**Western and Northern Blot Analyses**—ECs were harvested for total protein, and Western blots were carried out as previously described (19). The phosphospecific antibodies against Ser-256 FKHR and Ser-473 Akt were purchased from Cell Signaling (Beverly, MA), and anti-β-actin was from Sigma. Anti-Akt and anti-FKHR antibodies were obtained from Cell Signaling. Anti-β-actin antibody was from BD Biosciences. RNA extraction and Northern blot assays were performed as described previously (37).

**Immunolocalization Studies**—These studies were carried out in HCAECs grown onto four-well chamber slides (Lab-Tek, Christchurch, New Zealand) and treated with or without VEGF for the times indicated. Subcellular localization of FKHR was determined using a primary anti-FKHR antibody and a Cy3-conjugated secondary antibody as described previously (19). Quantitative analyses were carried out by counting 200 cells per time point.

**Complement-mediated Cell Lysis Assay**—HCAECs were plated in 24-well plates and incubated at 37 °C for 3 h prior to adenoviral transduction or 24 h prior to siRNA transfection.
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The cells were serum-starved overnight and incubated with or without VEGF for 24 h. Calcein acetoxymethyl ester (7 μl, Molecular Probes, Leiden, The Netherlands) were added to HCAECs monolayer for 30 min. Following washing with Hanks’ balanced salt solution plus 1% BSA, 250 μl of monoclonal anti-endoglin (CD105) antibody (IgG2a) was added to HCAECs for 30 min. HCAECs were washed with Hanks’ balanced salt solution plus 1% BSA, and the supernatant from each well was transferred to a 96-well microtiter plate. The remaining HCAECs in the 24-well plate were assayed for phosphorylated Ser-256-FKHR (pFKHR) by Western blot. The same membrane was stripped and reprobed with anti-HA antibody to detect the total level of expression of exogenous FKHR and anti-β-actin antibody to control for loading. B, HCAECs were transfected with 100 nm siRNA (siFKHR1 or siFKHR2) as described under “Experimental Procedures.” After 48 h, cell lysates were prepared and Western blots were performed using antibodies to total FKHR and ERK1/2. The blots shown are representative of three independent experiments.

FIGURE 1. Overexpression of WT- and TM-FKHR by adenoviruses and knockdown of FKHR by siRNA in HCAECs. A, HCAECs were transduced with adenoviruses encoding the cDNAs of β-galactosidase (Adv), wild-type (WT)-FKHR, or triple mutant (TM)-FKHR at multiplicity of infection (MOI) as indicated. Proteins were harvested as cell lysates after 24 h and 48 h and assayed for phosphorylated Ser-256-FKHR (pFKHR) by Western blot. The same membrane was stripped and reprobed with anti-HA antibody to detect the total level of expression of exogenous FKHR and anti-β-actin antibody to control for loading. B, HCAECs were transfected with 100 nm siRNA (siFKHR1 or siFKHR2) as described under “Experimental Procedures.” After 48 h, cell lysates were prepared and Western blots were performed using antibodies to total FKHR and ERK1/2. The blots shown are representative of three independent experiments.

RESULTS

Modulation of Intracellular Levels of FKHR in Primary ECs by Adenovirus and siRNA—To determine the role of forhead of adenoviral transduction. On the contrary, FKHR siRNA resulted in significant reduction of FKHR protein expression in HCAECs, with siFKHR2 demonstrating the greatest effect (>85% reduction) (Fig. 1B). Thus, Ad and siRNA are effective in increasing and decreasing intracellular FKHR levels in ECs, respectively.

Forhead Differentially Modulates VEGF-responsive Genes in Primary ECs—We initially employed DNA microarrays to assay for the effect of PI3K inhibition and/or TM-FKHR1 on VEGF-mediated gene expression in HCAECs. We used these data as a guide to identify two classes of genes: 1) classic VEGF-repressible, forhead-responsive transcripts, and 2) novel VEGF-inducible, forhead-responsive genes. Recognizing the primary importance of FKHR in ECs (39, 40), and assuming some degree of redundancy between FKHRL1 and FKHR isoforms, we chose to confirm the existence of these distinct classes downstream of the VEGF-FKHR signaling axis. HCAECs were transduced with Adv- or TM-FKHR, or transfected with FKHR siRNA, treated in the absence or presence of VEGF and then assayed for candidate genes by reverse transcription-PCR. Table 1 shows the list of the primers used for the genes that were tested by reverse transcription-PCR.

In keeping with the results of our DNA microarray studies, two distinct patterns of response were noted (Table 2). One group of genes, including BTG-1, GADD45A, and p27Kip1, demonstrated the usual forhead response pattern observed in non-ECs, namely agonist (VEGF)-mediated inhibition of expression, TM-FKHR-mediated induction of mRNA, and FKHR siRNA-mediated repression of basal mRNA levels.

A second group of genes was induced both by VEGF and TM-FKHR and super-induced by VEGF plus TM-FKHR (Table 2). VEGF-mediated induction of this novel group was blocked by FKHR siRNA, suggesting that VEGF requires FKHR for their optimal expression. These genes include bone morphogenic protein 2 (BMP2), cbp/p300-interacting transactivator 2 (CITED2), decay accelerating factor (DAF), vascular cell adhesion molecule (VCAM-1), manganese superoxide dismutase (Mn-SOD), endothelial specific molecule-1 (ESM-1, also known as endocan), RING1 and YY1 binding protein, matrix metalloproteinase-10 (MMP-10), and MGC5618. For purposes
of discussion, we will refer to the first (class) group as Class I, and the second (novel) group as Class II.

**VEGF Inhibits Expression of Class I Genes in Primary ECs via an FKHR-dependent Mechanism**—To further characterize the Class I genes, we focused on the expression patterns of GADD45A and BTG1 in ECs. HCAECs were pretreated in the absence or presence of the PI3K inhibitor, LY294002, or transduced with adenovirus overexpressing β-galactosidase (Adv), constitutively active phosphorylation-resistant triple mutant FKHR (TM-FKHR) or wild-type FKHR, and then treated with VEGF or vehicle for varying time points. Cells were harvested for RNA and assayed for gene expression using Northern blot analyses. VEGF resulted in a decrease in the expression of GADD45A and BTG1 (Fig. 2, A and B). We have previously shown that the PI3K inhibitor, LY294002, inhibits VEGF-mediated phosphorylation, nuclear exclusion, and inactivation of forkhead proteins in ECs (19). Preincubation with LY294002 resulted in increased basal levels of GADD45A and BTG1 and blocked the inhibitory effect of VEGF on these genes (Fig. 2, A and B). TM-FKHR increased GADD45A and BTG1 mRNA levels (Fig. 2, C and D), suggesting that VEGF-mediated down-regulation of Class I genes occurs through phosphorylation and nuclear exclusion of FKHR. These data are consistent with those previously reported for p27kip1 (19) and suggest that one role for the VEGF-FKHR signaling axis is to reduce the expression of pro-apoptotic and/or cell-cycle inhibitory genes.

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### TABLE 1

Primers used in the RT-PCR analyses

| Gene name | Forward primer | Reverse primer |
|-----------|----------------|----------------|
| BTG1      | GCCACCTGAGTATACGGAAGAT | CTTTGTGCGGAGAACACTTG |
| GADD45A   | TGGATCAAGTGGCACATTGCA   | GCAGATCGCAGAACCAGCCTAG |
| p27kip1   | GAAGGAGGCGCAGCACCTCTTGC | GCCACCTGAGTATACGGAAGAT |
| BMP2      | GAGACCGCAACACTGATGGTC  | AACATCTCCTGTGGAGACACCTTG |
| DAF       | AATGTTCAGCAACACGATCCGAT | GCTACATCACTGACCATCTTCTOTA |
| CITED2    | CACTCAGGCTCCGATTGGCA   | TCTCGAGCTCTCCTCTTGGCA |
| ESM1      | TGTGACTGCTCAACACACTTG  | TCGACACTGCTCTCCTCTGCC |
| MinSOD    | GGTGCTGTCATCATCATTCACTAGC | GCTTCGAGACATCTCTTCTT |
| MGC5618   | CCAAGAAGGGTTTTGCTGAGTT | TCCAACACTAATCATGCTGAG |
| MMP10     | TTCTCTGCGATGACCAACAGAAGGGTGAA | CATTGTTCCTGCACTTGGAGGTT |
| VCAN-1    | TCTCTGAGCTGCCCCTGACAT | TCTGCTGTCGTCCTCCTTG |
| RYBP      | AAGACCTGCTCCTCATACGACAT | TCTGCTGTCGTCCTCCTTG |

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### TABLE 2

Induction (-fold) or reduction of the genes in endothelial cells by RT-PCR analyses

The basal levels of expression for each gene in unstimulated, serum-starved HCAEC were arbitrarily considered as 1 (-fold) per 10^6 18 S mRNA copies. Numbers are expressed as -fold induction (or reduction if less than 1) over the basal levels.

| VEGF | TM-FK | TM-FK+VE | siFK | siFK+VE | FKHRRE* | Function         |
|------|-------|----------|------|---------|---------|------------------|
| Class I |      |          |      |         |         |                  |
| BTG1 | 0.7 ± 0.1 | 1.3 ± 0.2 | 3.2 ± 0.2 | 0.65 ± 0.1 | 0.73 ± 0.08 | 4 | Cell cycle |
| GADD45A | 0.6 ± 0.06 | 2 ± 0.03 | 2.6 ± 0.05 | 0.5 ± 0.09 | 0.6 ± 0.07 | 1 | Cell cycle |
| p27kip1 | 0.7 ± 0.08 | 2 ± 0.06 | 2.7 ± 0.1 | 0.7 ± 0.3 | 0.8 ± 0.1 | 2 | Cell cycle |
| Class II |      |          |      |         |         |                  |
| BMP2 | 3.2 ± 0.2 | 2.5 ± 0.2 | 4.1 ± 0.6 | 0.6 ± 0.01 | 0.8 ± 0.05 | 1 | Angiogenesis |
| DAF | 2 ± 0.1 | 4.6 ± 0.3 | 5.9 ± 0.9 | 0.6 ± 0.2 | 1 ± 0.2 | 1 | Complement inhibition |
| CITED2 | 2.4 ± 0.3 | 2.8 ± 0.4 | 5.2 ± 0.05 | 0.8 ± 0.02 | 1.2 ± 0.1 | 1 | Cardiac development |
| ESM1 | 2.2 ± 0.2 | 1.8 ± 0.2 | 4 ± 0.4 | 0.15 ± 0.06 | 0.4 ± 0.09 | 2 | Angiogenesis |
| MnSOD | 2.1 ± 0.6 | 2.4 ± 0.67 | 4.2 ± 0.9 | 0.7 ± 0.2 | 1 ± 0.6 | 1 | Redox regulation |
| SMC5618 | 2 ± 0.2 | 2.5 ± 0.6 | 4.1 ± 0.7 | 0.6 ± 0.08 | 0.8 ± 0.9 | 1 | Unknown |
| MMP10 | 2.8 ± 0.04 | 17.3 ± 0.9 | 22.9 ± 15 | 1.2 ± 0.1 | 1.4 ± 0.2 | 1 | Matrix remodeling |
| VCAN-1 | 16.3 ± 0.2 | 6.7 ± 0.3 | 19 ± 0.8 | 1.3 ± 0.1 | 4.5 ± 0.2 | 2 | Cell adhesion |
| RYBP | 2 ± 0.04 | 1.2 ± 0.03 | 2.8 ± 0.09 | 1 ± 0.1 | 1 ± 0.05 | 1 | Neural development |

*Number of FKHR consensus elements (FKHREs) in upstream promoter.

<sup>a</sup> p < 0.01 relative to TM-FK.

<sup>b</sup> p < 0.001 relative to basal level.

<sup>c</sup> p < 0.05 relative to TM-FK.

<sup>d</sup> p < 0.05 relative to uninduced/basal level.

<sup>e</sup> p < 0.05 relative to siFK.

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**FIGURE 2.** VEGF inhibits expression of GADD45A and BTG1 in HCAECs through a PI3K/FKHR-dependent pathway. A and B, HCAECs were serum-starved overnight and then treated with or without VEGF (50 ng/ml) for 1 h. Where indicated, HCAECs were pretreated with or without the PI3K inhibitor, LY294002 (50 μM), for 30 min. Total RNA was extracted and processed for Northern blot analyses for GADD45A (C) and BTG1 (D). The lower panels show ethidium bromide-stained 28 S RNA as a loading control. Blots shown are representative of three independent experiments.
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VEGF-mediated Induction of Class II Genes in Primary ECs Depends on FKHR—To further characterize the Class II genes, we focused on the expression patterns of Mn-SOD, VCAM-1, ESM-1, MMP-10, BMP2, and CITED2 in HCAECs. In Northern blot analyses, VEGF increased the mRNA levels for each of these genes (Fig. 3). TM-FKHR alone increased expression of Mn-SOD, ESM-1, MMP10, and BMP2, and CITED2 (Fig. 3, A and B). VEGF-mediated induction of all the above genes, Mn-SOD, VCAM-1, ESM-1, MMP10, BMP2, and CITED2, was significantly attenuated by FKHR siRNA (Fig. 3, C and D). These findings support a role for FKHR as a positively acting transcription factor required for transduction of the VEGF signal.

VEGF-mediated Protection against Complement-mediated Lysis of ECs Is Dependent on FKHR—Expression of DAF, a membrane-bound complement regulatory protein, is induced by VEGF in ECs and provides protection from complement-mediated injury/lysis of ECs (44). As shown in Table 2, VEGF-mediated increase in DAF expression is dependent on FKHR (Table 2). We next wished to determine the functional relevance of these findings. In control or VEGF-treated ECs, overexpression of TM-FKHR resulted in decreased complement-mediated cell lysis (Fig. 4). In contrast, siRNA against FKHR blocked the protective effect of VEGF against cell lysis (Fig. 4). Together, these findings support an important role for FKHR in VEGF-mediated, DAF-dependent protection of ECs against complement.

VEGF-mediated Monocyte Adhesion to ECs Is Dependent on FKHR—VCAM-1, a member of the immunoglobulin superfamily of genes, promotes the adhesion of monocyte to ECs. As shown in Fig. 5, VEGF resulted in more than 3-fold induction of U937 adhesion on endothelial monolayer in vitro. As shown for VCAM-1 expression (Table 2 and Fig. 3), VEGF-induced monocyte adhesion to HCAECs was further enhanced by overexpression of a constitutively active TM-FKHR (Fig. 5A). In contrast, a reduction in the level of FKHR by siFKHR in HCAECs resulted in a decrease in the VEGF-induced monocyte adhesion (Fig. 5B), suggesting an important role for FKHR in this process.

VEGF-mediated Induction of Class II Genes Also Depends on NF-AT and/or NF-kB Activity—Our findings indicate that Class II genes are forkhead-responsive. For example, strategies that increase nuclear localization of FKHR, e.g. inhibition of PI3K-Akt or overexpression of TM-FKHR, result in increased expression of Class II genes. Moreover, VEGF-mediated induction of Class II genes requires the presence of FKHR. However, the fact that VEGF signaling promotes the nuclear exclusion, hence transcriptional inactivation, of FKHR and the observation that VEGF super-induces Class II gene expression in the presence of TM-FKHR, strongly suggest that additional positive acting transcription factors are involved.

Several transcription factors have been implicated in VEGF signaling, including NF-kB and NF-AT (38, 45–47). To deter-
FIGURE 5. VEGF-mediated monocyte adhesion to EC is dependent on FKHR. HCAECs were transduced with either Adv or TM-FKHR (3 m.o.i.) (A), or transfected with either control siRNA (100 nM) (NS) or siFKHR (B), as described under “Experimental Procedures.” The cells were serum-starved for 12–16 h before VEGF stimulation. U937 monocyte adhesion on HCAECs monolayer was determined as described under “Experimental Procedures.” The bar graph results shown are mean ± S.D.* p < 0.05 control versus VEGF-treated; †, p < 0.05 VEGF-treated versus TM-FKHR (or siFKHR) plus VEGF-treated.
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TABLE 3
Induction (-fold) or reduction of the Class II genes in endothelial cells by RT-PCR analyses

The basal levels of expression for each gene in unstimulated, serum-starved HCAECs, were arbitrarily considered as 1 (−fold) per 10^4 18 S mRNA copies.

| Gene       | VEGF | IκB | IκB+VE |
|------------|------|-----|-------|
| BMP2       | 2.4  | 0.8 | 1.0   |
| DAF        | 2.6  | 0.6 | 1.2   |
| CITFED2    | 1.4  | 0.8 | 1.1   |
| ESM-1      | 1.9  | 1   | 1.2   |
| MnSOD      | 2.0  | 0.6 | 0.6   |
| MGC5618    | 2.2  | 0.5 | 0.6   |
| MMP10      | 1.8  | 0.6 | 0.6   |
| VCAM-1     | 15.2 | 0.5 | 3.9   |
| RYBP       | 1.6  | 0.9 | 0.9   |

*p < 0.05 relative to basal level.
*p < 0.001 relative to basal level.
*p < 0.001 relative to IκB.

FIGURE 6. VEGF-mediated induction of Class II genes Mn-SOD, VCAM-1, ESM-1, and DAF is dependent on NF-κB. HCAECs were transduced with adenoviruses encoding the cDNAs of β-galactosidase (Adv) or constitutively active IκB (Adv-IκB) at the m.o.i. indicated (1 and 10 m.o.i.). The cells were serum-starved overnight, treated with or without VEGF (50 ng/ml) for 4 h, and then harvested for total RNA. Northern blot analyses of the genes were carried out using the same membrane. The lower panel shows ethidium bromide-stained 28 S RNA as a loading control. The blots shown are representative of at least three independent experiments. Two transcripts for Mn-SOD are indicated.

FIGURE 7. Overexpression of TM-FKHR reverses IκB-mediated inhibition of the Class II genes Mn-SOD, VCAM-1, and ESM-1. HCAECs were transduced with adenoviruses encoding the cDNAs of β-galactosidase (Adv) or constitutively active IκB (Adv-IκB) and/or TM-FKHR (3 m.o.i.) as indicated. The cells were serum-starved overnight, treated without or with VEGF (50 ng/ml) for 4 h, and then harvested for total RNA. Northern blot analyses of the genes were carried out using the same membrane. The lower panel shows ethidium bromide-stained 28 S RNA as a loading control. The blots shown are representative of two independent experiments. Two transcripts for Mn-SOD are indicated.
VEGF-mediated Subcellular Localization (Nuclear Export) of FKHR in Primary EC Is Transient in Nature—VEGF inhibition of Class I genes occurred early (at 1 h), whereas VEGF induction of Class II genes occurred only after 1 h (data not shown). We were interested in determining whether these differences correlated with the temporal pattern of FKHR nuclear exclusion in VEGF-treated ECs. VEGF-mediated phosphorylation of FKHR in HCAECs reached maximal levels between 15 and 30 min and returned to basal levels after 60 min of VEGF addition (Fig. 9A). In immunofluorescent studies of HCAECs, VEGF-mediated exclusion of FKHR from the nucleus was similarly transient in nature (Fig. 9B). In untreated serum-starved cells, FKHR was localized primarily in the nucleus (65 ± 5.6% in the nucleus versus 35 ± 3.1% in the cytoplasm). 30 min after addition of VEGF, this ratio was reversed, with 32 ± 4.3% nuclear and 68 ± 4.4% cytoplasmic. The nuclear to cytoplasmic ratio of FKHR returned to basal levels after 60 min (Fig. 9B). These data demonstrate that VEGF-mediated phosphorylation and nuclear export of FKHR in HCAECs are transient in nature.

DISCUSSION

Previous studies in non-endothelial cells have demonstrated that growth factors exert their proliferative, pro-survival, and metabolic effects in part by PI3K-Akt-dependent phosphorylation and exclusion of forkhead proteins from the nucleus, with secondary reduction in target gene expression (19, 20, 38, 46, 48, 49). Among these target genes are p27kip1 and BTG1, both inhibitors of G1 to S cell-cycle progression (21, 23), and GADD45A, a DNA-damage repair protein that regulates the G2/M cell-cycle checkpoint (22, 50).

More recently, we have demonstrated that VEGF promotes PI3K-Akt-dependent phosphorylation and nuclear exclusion of FKHR in ECs (19). As a consequence, VEGF signaling resulted in decreased expression of p27kip1 (19). Here, we have extended these findings by demonstrating a similar mechanism for BTG1 and GADD45A, a DNA-damage repair protein that regulates the G2/M cell-cycle checkpoint (22, 50).

Importantly, the present study provides evidence for the existence of a previously unrecognized group of FKHR-dependent genes (Class II genes) whose expression is normally
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induced by VEGF. These genes, which include BMP2, MMP-10, CITED2, VCAM-1, Mn-SOD, DAF, ESM-1, and RING1 and YY1 binding protein, encode for proteins known to be involved in metabolism, cell signaling, cell adhesion, stress response, matrix reorganization, differentiation, and other cellular functions. The functional relevance of these findings is evidenced by our studies of monocyte adhesion and complement-mediated EC lysis.

Recently, FKHR was shown to be essential for embryonic vascular development in mice (39, 40). In two independent studies, deletion of FKHR resulted in embryonic lethality (at embryonic day 11) and abnormalities in angiogenesis. Furuyama et al. (40) extended their findings by showing that the ECs differentiated in vitro from isolated embryonic stem cells of FKHR-null mice had an abnormal morphological response to exogenous VEGF. These data raised an interesting question. If FKHR is a death-promoting gene that requires exclusion from the nucleus for cell viability and growth, then why did these mice not demonstrate uncontrolled cell proliferation and overgrowth? Based on the data presented in this study, we propose that FKHR is not simply a death-promoting, cell-cycle-arrest factor, but also a positive-acting transcriptional protein necessary for VEGF-mediated EC homeostasis. In other words, FKHR is an essential transacting factor that is involved in the coordinated regulation of a distinct set of genes in ECs involved in cell maintenance/health.

There are three possible explanations for the finding that VEGF induces the expression of FKHR-responsive genes (Class II), while promoting transient nuclear export of this transcription factor. One is that FKHR exerts an unusual dose response whereby too much nuclear FKHR is inhibitory and too little is insufficient to transduce the VEGF signal. However, the observation that LY294002 or TM-FKHR result in super-induction of Class II gene expression argues against this hypothesis.

A more likely explanation is that VEGF induces the activity of other positive acting factors which, together with residual FKHR in the nucleus, transactivates downstream target genes. In support of this model is the finding that VEGF-mediated induction of Class II genes is dependent on NF-κB and in some cases NF-AT. Further studies are required to determine the relative contribution of these and other non-FKHR transcription factors in mediating the effect of VEGF on Class II gene expression.

A final possibility relates to the time course of FKHR subcellular localization. Our data suggest that VEGF-mediated phosphorylation and nuclear exclusion of FKHR is transient, returning to baseline by

FIGURE 10.
Model for VEGF-responsive genes that require FKHR for expression. In the classic pathway (left panel), VEGF-mediated activation of PI3K-Akt leads to phosphorylation and nuclear exclusion of forkhead (FKHR) with secondary reduction of forkhead-dependent transcription of Class I genes (OFF). In the novel pathway (right panel), VEGF normally induces expression of Class II genes (ON) via a pathway that requires FKHR and one or more non-forkhead transcription factors (e.g. NF-κB or NFAT; indicated by the oval) for optimal expression. The non-forkhead proteins may interact with residual nuclear FKHR (at an earlier time point) (1) and/or interact with FKHR that has returned to the nucleus (at a later time point) (2) to induce target gene expression.

REFERENCES

1. Dvorak, H. F. (2000) Semin. Perinatol. 24, 75–78
2. Zachary, I., Mathur, A., Yla-Herttuala, S., and Martin, J. (2000) Arterioscler. Thromb. Vasc. Biol. 20, 1512–1520
3. Matsumoto, T., and Claesson-Welsh, L. (2001) Sci. STKE 2001, RE21
4. Ferrara, N., Gerber, H. P., and LeCouter, J. (2003) Nat. Med. 9, 669–676
5. Mukhopadhyay, D., Zeng, H., and Bhattacharya, R. (2004) Mol. Cell Biochem. 264, 51–61
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6. Zelzer, E., and Olsen, B. R. (2005) *Curr. Top. Dev. Biol.* 65, 169–187
7. Folkman, J. (2004) *APMIS* 112, 496–507
8. Stupack, D. G., and Cheresh, D. A. (2004) *Curr. Top. Dev. Biol.* 64, 207–238
9. Xia, P., Aiello, L. P., Ishii, H., Jiang, Z. Y., Park, D. I., Robinson, G. S., Takagi, H., Newsome, W. P., Jirousek, M. R., and King, G. L. (1996) *J. Clin. Invest.* 98, 2018–2026
10. Guo, D., Jia, Q., Song, H. Y., Warren, R. S., and Donner, D. B. (1995) *J. Biol. Chem.* 270, 6729–6733
11. Kroll, J., and Waltenberger, J. (1997) *J. Biol. Chem.* 272, 32521–32527
12. D’Angelo, G., Struman, I., Martial, J., and Weiner, R. I. (1995) *Proc. Natl. Acad. Sci. U. S. A.* 92, 6374–6378
13. Rousseau, S., Houle, F., Landry, J., and Huot, J. (1997) *Oncogene* 15, 2169–2177
14. Chen, Z., Fisher, R. J., Riggs, C. W., Rhim, J. S., and Lautenberger, J. A. (1999) *Science* 285, 530–534
15. Skurk, C., Maatz, H., Kim, H. S., Yang, J., Abid, M. R., Aird, W. C. (2001) *FASEB J.* 15, 2548–2550
16. Johnson, E. N., Lee, Y. M., Sander, T. L., Rabkin, E., Schoen, F. J., Kaushal, S., and Bischoff, J. (2003) *Proc. Natl. Acad. Sci. U. S. A.* 101, 2975–2980
17. Folkman, J. (2004) *Arterioscler. Thromb. Vasc. Biol.* 24, 3002–3007
18. Zelzer, E., and Olsen, B. R. (2005) *Science* 310, 169–187
19. Aicher, A., Kollipara, R., Depinho, R. A., Zeiher, A. M., and Dimmeler, S. (2005) *J. Clin. Invest.* 115, 2382–2392
20. Bilbao, G., Contreras, J. L., Zhang, H. G., Pike, M. J., Overturf, K., Mikhailova, G., Krasnykh, V., and Curiel, D. T. (1999) *J. Virol.* 73, 6992–7000
21. Shih, S. C., Robinson, G. S., Perruzi, C. A., Calvo, A., Desai, K., Green, J. E., Ali, I. U., Smith, L. E., and Senger, D. R. (2002) *Am. J. Pathol.* 161, 35–41
22. Mason, J. C., Steinberg, R., Lidentification, E. A., Kinderluer, A. R., Ohba, M., and Haskard, D. O. (2004) *J. Biol. Chem.* 279, 41611–41618
23. Minami, T., Horiuchi, K., Miura, M., Abid, M. R., Takabe, W., Suguchi, N., Kohro, T., Ge, X., Aburatani, H., Hamakubo, T., Kodama, T., and Aird, W. C. (2004) *J. Biol. Chem.* 279, 50537–50554
24. Skurk, C., Maatz, H., Kim, H. S., Yang, J., Abid, M. R., Aird, W. C., and Walsh, K. (2004) *J. Biol. Chem.* 279, 1513–1525
25. Hesser, B. A., Liang, X. H., Camenisch, G., Yang, S., Lewin, D. A., Scheller, R., Ferrara, N., and Gerber, H. P. (2004) *Blood* 104, 149–158
26. Bilbao, G., and Cheresh, D. A. (2004) *J. Biol. Chem.* 279, 41611–41618
27. Minami, T., Horiuchi, K., Miura, M., Abid, M. R., Takabe, W., Suguchi, N., Kohro, T., Ge, X., Aburatani, H., Hamakubo, T., Kodama, T., and Aird, W. C. (2004) *J. Biol. Chem.* 279, 50537–50554
28. Shih, S. C., Robinson, G. S., Perruzi, C. A., Calvo, A., Desai, K., Green, J. E., Ali, I. U., Smith, L. E., and Senger, D. R. (2002) *Am. J. Pathol.* 161, 35–41
29. Mason, J. C., Steinberg, R., Lidentification, E. A., Kinderluer, A. R., Ohba, M., and Haskard, D. O. (2004) *J. Biol. Chem.* 279, 41611–41618
30. Minami, T., Horiuchi, K., Miura, M., Abid, M. R., Takabe, W., Suguchi, N., Kohro, T., Ge, X., Aburatani, H., Hamakubo, T., Kodama, T., and Aird, W. C. (2004) *J. Biol. Chem.* 279, 50537–50554