Hypoxia-inducible Transcription Factor-2α in Endothelial Cells Regulates Tumor Neovascularization through Activation of Ephrin A1

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The hypoxia-inducible transcription factors (HIF)-1α and -2α mediate responses to hypoxia, such as tumor neovascularization. To determine the function of HIF-2α in vascular endothelial cells (ECs), we examined vascular formation in HIF-2α knockdown (kd/kd) mice transplanted with tumors. We observed that both the tumor size and the number of large vessels growing within transplanted melanomas were significantly reduced in kd/kd recipients compared with wild-type (WT) mice. In contrast, we observed a similar extent of vascular formation within fibrosarcomas transplanted from either kd/kd or WT mice into WT recipients. Thus, HIF-2α expression in host animal ECs, but not in the tumor cells, is crucial for tumor neovascularization. HIF-2α may function through ephrin A1 as the expression of ephrin A1 and related genes was markedly reduced in kd/kd ECs, and HIF-2α specifically bound a hypoxia-response element sequence in the ephrin A1 promoter. Treatment of WT ECs with an ephrin A1 inhibitor (ephrin A1-Fc) also impaired neovascularization. We conclude that in ECs, HIF-2α plays an essential role in vascular remodeling during tumor neovascularization through activation of at least ephrin A1.

Angiogenesis is a multistep physiologic process involving the growth of new sprouts from pre-existing vessels during development and growth. In addition, angiogenesis can be activated under pathological conditions such as wound healing and tumor formation (1). Endothelial cells (ECs)3 line blood vessels and play essential roles in the formation of new blood vessels. During neovascularization, the first ECs translocate from their stable location by breaking through the basement membrane and migrating toward angiogenic stimuli, such as vascular endothelial growth factor (VEGF) and angiopoietins (Angs). Subsequently, after reaching their destination, these cells proliferate to provide sufficient additional cells for vessel formation.

The growth of tumors in vivo is completely dependent on vascularization (2). Most tumors grow so rapidly that they outstrip their existing blood supply, generate hypoxic conditions in surrounding normal tissues, and induce neovascularization. Inhibition of tumor neovascularization is considered a promising strategy for anticancer therapy. However, targeting tumor neovascularization in vivo is difficult. Numerous pro- and antiangiogenic factors contribute to tumor neovascularization (3). Therefore, characterizing the precise roles of individual molecules in this process is a critical first step toward developing anticancer therapies targeting neovascularization.

The hypoxic response is triggered by hypoxia-inducible factors (HIFs), a family of transcription factors activated by local hypoxic environments (4). Activated HIFs accumulate in the nucleus and heterodimerize with HIF-1β, also known as the aryl hydrocarbon receptor nuclear translocator (Arnt). These complexes bind to cis-acting hypoxia-response elements (HREs) present in the promoter sequences of hypoxia-responsive genes. More than 40 genes have been identified as targets of HIF-1α. These genes contribute to a diverse array of cellular processes, including cell survival, glycolysis, erythropoiesis, and vasculogenesis/angiogenesis (4). HIF-1α is ubiquitously expressed and may function as a key player in the transcriptional response to hypoxia. In contrast, HIF-2α, another member of the HIF family, is only expressed by ECs (5), kidney fibroblasts (6), hepatocytes (6), astrocytes (7), and certain tumor cells (8). The role of HIF-2α in neovascularization is not well understood (9).

Previously, we generated mice containing a “knockdown” allele of the HIF-2α gene (10). Homozygous mutant mice (kd/kd) display a 20–80% reduction in HIF-2α expression depend-

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3 The abbreviations used are: EC, endothelial cell; HIF, hypoxia-inducible transcription factor; WT, wild type; B(a)P, benzo(a)pyrene; WT, wild type; FBS, fetal bovine serum; PBS, phosphate-buffered saline; RT, reverse transcription; BM, bone marrow; VEGF, vascular endothelial growth factor; Ang, angiopoietin; ChIP, chromatin immunoprecipitation; Arnt, the aryl hydrocarbon receptor nuclear translocator; HRE, hypoxia-response element; Eph A4, and Eph receptor A4.
ing on the tissue but are born at expected Mendelian frequencies and are fertile. Neovascularization was markedly reduced in the retinas of kd/kd mice in response to local hypoxia. Intriguingly, hypoxic induction of erythropoietin, a known target of HIFs, was prominently lost in the retinas of kd/kd mice, whereas other angiogenic factors, such as VEGF, were induced normally. Therefore, one of the roles of HIF-2α in retinal vascular formation under hypoxic conditions is to activate expression of erythropoietin.

Here, we examined the role of HIF-2α during tumor neovascularization in mature animals. A variety of HIF target genes that promote angiogenesis are activated in tumors during neovascularization. Recent studies have suggested that HIF-1α and HIF-2α regulate distinct target genes in ECs, tumor cells, and embryonic stem cells (11–16). In immortalized mouse embryonic fibroblasts, only HIF-1α is activated in response to hypoxia, whereas endogenous HIF-2α remains transcriptionally inactive even under hypoxic conditions (17). Current models propose that HIF-1α and HIF-2α play different roles in cellular physiology and during tumor neovascularization. As both HIF-1α and HIF-2α are widely expressed in the cell types that mediate neovascularization, such as ECs, pericytes, and smooth muscle cells, discriminating the roles of these two related proteins within normal and tumor tissue is difficult. We addressed this problem by examining kd/kd mice that express reduced levels of HIF-2α, and we identified a specific role and mechanism of action for HIF-2α in ECs during tumor neovascularization. HIF-2α increases the expression of ephrin A1, a regulator of vascular tube formation, but not EC proliferation. Ephrin A1 was shown to be essential for vascular tube formation, and is essential for vascular tube formation and normal development (19, 20). Here, we clearly demonstrate that HIF-2α, but not HIF-1α, directly regulates the expression of ephrinA1 and promotes vascular tube formation.

**EXPERIMENTAL PROCEDURES**

**Tumorigenesis**—B16F10 melanoma and 3LLC lung carcinoma cells were grown in Dulbecco’s modified Eagle’s medium/high glucose (Sigma) supplemented with 10% FBS (Invitrogen). For transplantation studies, cells were harvested with 1 mM EDTA/PBS and resuspended in PBS at a concentration of 1 × 10⁷ cells/ml. Two hundred microliters of the cell suspension were injected into each WT or kd/kd mouse subcutaneously. Benzo[a]pyrene (B[a]P) (200 μl at 10 mg/ml) dissolved in corn oil (Wako Pure Chemical Industries) was injected subcutaneously into WT or kd/kd mice on the 1st and 5th day of the experiment (21). The resulting WT or kd/kd primary fibrosarcomas (20 mm³) induced by B[a]P were subcutaneously implanted into KSN nu/nu mice (SLC).

**Immunohistochemical Staining**—Tumors from host mice were harvested and fixed in 4% paraformaldehyde/PBS overnight at 4 °C. Samples were then embedded in polyester wax and sectioned for immunohistochemical staining. Sections were incubated overnight with anti-CD31 (PharMingen), anti-HIF-2α, or anti-HIF-1α antibodies at 4 °C (10), washed twice with PBS-MT (which contains 5% dried milk and 0.1% Tween 20) at 4 °C, and incubated with an horseradish peroxidase-conjugated secondary antibody (BIOSOURCE) for 1 h at room temperature and washed. Bound antibodies were detected by adding a color reaction buffer (250 μg/ml of 3,3’-diaminobenzidine with or without 0.05% NiCl₂) or by utilizing 3-amino-9-ethylcarbazole substrate kit (Vector Laboratories) to the samples. Sections were counterstained with nuclear fast red (Vector Laboratories) or hematoxylin solutions.

**Diffusion Chamber Assay**—The diffusion chamber contained a chamber ring and a 0.45-μm pore membrane (Millipore). Cultured B16F10 melanoma cells or 3LLC cells resuspended in PBS (5 × 10⁶ cells/ml) were added to the diffusion chamber. The chamber was then subcutaneously embedded into anesthetized mice. After 7 days, we examined newly formed vessels on the membrane of the diffusion chamber macroscopically. Images of the neovascularized area were obtained using NIH image.

**Microarray Analysis and Real Time PCR**—Forty eight hour post-implantation, total RNAs were prepared from newly formed vessels on the surface of the diffusion chamber using the RNeasy mini kit (Qiagen). RNA samples were analyzed by microarray analysis according to the manufacturer’s instructions (Agilent Technologies). cDNAs were synthesized by RT-PCR from 1 μg of total RNA using a cDNA synthesis kit (Invitrogen). The reaction mixtures for quantitative RT-PCR were prepared using POWER SYBR® Green PCR master mix (Applied Biosystems) and analyzed by a 7700 sequence detector (Applied Biosystems). Experiments were performed as triplicate, and the data were calculated by ΔΔCt method. Primers used for PCRs were as follows: ephrin B2 forward, 5’-TCTGTTGGAA-GTACTGTTGGGACTTT-3’; and reverse, 5’-TGTAGCCCTCTGGAGGATTT-3’; HIF-2α forward, 5’-CAGCC-TCAAGGTGAGAAGGAG-3’; and reverse, 5’-GTTGTAAGCTC-ACGGGCCCC-3’; HIF-1α forward, 5’-CAAGATCTCGGCGAAG-CAGAAGTCTGGA-3’; and reverse; 5’-GAAAGAATCCTCAG-GTGTGTGCGAAAGATCGTA-3’; and reverse; 5’-GAAAGAATCCTCAG-GTGTGTGCGAAAGATCGTA-3’. Primer sets used for quantitative PCR were as follows: ephrin B2 forward, 5’-TCTGTTGGAA-GTACTGTTGGGACTTT-3’; and reverse, 5’-TGTAGCCCTCTGGAGGATTT-3’; HIF-2α forward, 5’-CAGCC-TCAAGGTGAGAAGGAG-3’; and reverse, 5’-GTTGTAAGCTC-ACGGGCCCC-3’; HIF-1α forward, 5’-CAAGATCTCGGCGAAG-CAGAAGTCTGGA-3’; and reverse; 5’-GAAAGAATCCTCAG-GTGTGTGCGAAAGATCGTA-3’. Primer sets used for quantitative PCR were as follows: ephrin B2 forward, 5’-TCTGTTGGAA-GTACTGTTGGGACTTT-3’; and reverse, 5’-TGTAGCCCTCTGGAGGATTT-3’; HIF-2α forward, 5’-CAGCC-TCAAGGTGAGAAGGAG-3’; and reverse, 5’-GTTGTAAGCTC-ACGGGCCCC-3’; HIF-1α forward, 5’-CAAGATCTCGGCGAAG-CAGAAGTCTGGA-3’; and reverse; 5’-GAAAGAATCCTCAG-GTGTGTGCGAAAGATCGTA-3’.

**Plasmids**—Full-length mouse HIF-1α, HIF-2α, and Arnt cDNAs were inserted into a pEF-BOS vector driven by the human elongation factor-1α promoter. A 2.2 kb fragment of the ephrin A1 promoter was synthesized by using PCR and
inserted into a pGL3 promoter vector (Promega). The HRE sequence in this fragment of the ephrin A1 promoter was mutated with the QuickChange site-directed mutagenesis kit (Stratagene). The sequence of primer sets used for mutagenesis were as follows: primers for ephrin A1 -2.2-kb promoter, 5'-GGGACGGTTG(MluI)AGTGCCAGGTGGTTTACGCCCAC-3' (forward) and 5'-CCTAGATGCT(BglII)CTCCCATAGTCTGTGTGTCTC-3' (reverse); primers for the mutation of HRE, 5'-AGGAGGTGGGCGGATCTCCCTAGCTGAGCGGAG-3' (forward) and 5'-CTCCGCTCAGCTAGCGGAGATCCGCACCTCCTCT-3' (reverse).

A full-length mouse ephrin A1 cassette was ligated with internal ribosomal entry site-tRDE1, and they were inserted into mouse stem cell virus vector for retroviral transfection (Clontech). After the infection, vector alone- or ephrin A1-transfected kd/kd ECs were purified by using FACSVantage (BD Biosciences). The expression of ephrin A1 in ECs was examined by Western blot analysis.

**Cell Culture and Tube Formation Assay—**BM samples were harvested from the femurs of WT and kd/kd mice by flushing with PBS. Isolated cells were stained with APC-conjugated anti-CD45 antibody (PharMingen). The fraction of CD31-positive and CD45-negative was collected by flow cytometry with a FACSVantage (BD Biosciences). The expression of ephrin A1 in ECs was examined as an internal control.

**Luciferase Reporter Assay—**We transfected 293T cells in 12-well plates with pGL3 ephrin A1 -2.2 kb of luciferase (10 ng) or pGL3 ephrin A1 -2.2 kb of mut-Luc (10 ng) as a reporter gene using FuGENE 6 transfection reagent (Roche Applied Science). Reporter assays were performed following co-transfection of 293T cells with the total amount of 200 ng of DNA containing pEF1-Arnt (10 ng) adjusted by adding empty vector (pEF-BOS). The pEF1-Renilla-Luc plasmid (1 ng) was co-transfected as an internal control.

**Chromatin Immunoprecipitation (ChIP) Assay—**For each assay, 5 × 10^6 WT and kd/kd ECs cells were fixed with 1% formaldehyde for 10 min at room temperature, then washed with PBS containing 1 μg/ml protease inhibitor mixture (PIC) (Roche Applied Science), harvested, and treated with hypotonic solution (5 mM HEPES, 85 mM KCl, 0.5% Nonidet P-40, and 1 μM PIC). Subsequently, samples were centrifuged (5 min at 14,000 × g) to collect nuclei. Nuclei were lysed with lysis buffer (50 mM Tris-HCl (pH 8.1), 10 mM EDTA, 1% SDS, and 1 μM PIC). After fragmentation of DNA by sonication, immunoprecipitation reactions were performed using a rotating mixer at 4 °C with 1 μg/ml of anti-HIF-1α antibody (Novus Biologicals) or anti-HIF-2α antibody (10). Normal rabbit IgG was employed as a negative control to verify the specificity of the reaction. Following incubation with the antibody, reaction mixtures were incubated with pre-blocked protein A-agarose beads (Calbiochem) (4 °C, 1 h), and precipitated complexes were collected by centrifugation (5 min at 3000 × g). These complexes were washed three times with washing buffer (0.25 mM LiCl, 1% Nonidet P-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl (pH 8.1)) and eluted from the protein A-agarose beads by centrifugation (4 °C at 3000 × g) and denatured (65 °C, 4 h). DNA fragments were then extracted with phenol/chloroform and precipitated with ethanol. Subsequently, the recovered DNA sequences were resuspended in TE buffer (10 mM Tris-HCl (pH 7.5), 1 mM EDTA) and used as template for detection of the ephrin A1 HRE sequence by the PCR with Taq polymerase (Takara). The following primers were used for the PCR analysis: forward, 5'-GCTGACTGTCAGCGCCGAGG-3', and reverse, 5'-GCGACGCGT(MluI)AGTGCCCAGGTTTCAGCCAC-3'. Reporter assays were performed following transfection of 293T cells with the total amount of 200 ng of DNA containing pEF1-Arnt (10 ng) adjusted by adding empty vector (pEF-BOS). The pEF1-Renilla-Luc plasmid (1 ng) was co-transfected as an internal control.

**Western Blot Analysis—**Cells were harvested and homogenized with whole cell extract buffer (10 mM Tris-HCl (pH 7.5), 1% SDS, 7 M urea, 10% glycerol, 5 mM dithiothreitol, 1 μM PIC) at 4 °C. Twenty micrograms of whole cell extracts in each well were electrophoretically separated on 10% SDS-polyacrylamide gel and then electrotransferred to polyvinylidene difluoride membranes (Millipore). Membranes were blocked with 5% skim milk, 1% FBS in TBS-T (Tris-buffered saline containing 0.1% Tween 20) for 1 h at room temperature and then incubated with primary antibody (anti-ephrin A1 antibody at a dilution of 1:2,000, Santa Cruz Biotechnology) for 1 h at room temperature. After extensive washing with TBS-T, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (anti-rabbit IgG antibody at a dilution of 1:10,000, Zymed Laboratories Inc.) for 1 h at room temperature. Immunoreactive bands were detected with a chemiluminescence rea-
tumors were estimated by measuring the diameter. The maximal vessel density per field in each tumor section.

FIGURE 1. Analysis of tumor formation in WT and kd/kd mice. A–C, B16F10 melanoma cells, implanted subcutaneously into recipient WT and kd/kd mouse, were analyzed 14 days after transplantation. D–F, primary fibrosarcomas were analyzed at 15 weeks after subcutaneous injection of B[a]P into WT and kd/kd mice. G–I, primary fibrosarcomas induced by B[a]P treatment were harvested from WT and kd/kd mice. Pieces of fibrosarcomas (+/+ and kd/kd) were implanted subcutaneously into nude mouse recipients (+/+) and analyzed after 14 days. A, D, and G, tumor weights were measured for WT (panel a) and kd/kd (panel b) specimens; mean tumor weights are presented (panel c, filled column, WT; open column, kd/kd; n = 4–8, mean ± S.D., **, p < 0.01). Scale bar = 1 cm. B, E, and H, histological analyses following hematoxylin and eosin (H&E) staining (panels a and b) and anti-CD31 antibody immunostaining (panels c and d). EC stained positively in red (B, panels c and d) and brown (E and H, panels c and d). J, diameters of vessels (arrowheads) were categorized into three groups after anti-CD31 antibody immunostaining (less than 10 μm (panels a and d), between 10 and 100 μm (panels b and e), and greater than 100 μm (panels c and f) in diameter); the number of vessels was scored as the maximal vessel density per field in each tumor section. C, F, and I, sizes of newly formed vessels within tumors were estimated by measuring the diameter. t, tumor cell genotype; r, recipient mouse genotype. Scale bar = 100 μm. *, p < 0.05; **, p < 0.01.

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Statistical Analysis—Statistical evaluations of data were conducted using the Student’s t test for comparison analysis. Data are presented as means ± S.D.

RESULTS

Impaired Development of Tumor Vessels in HIF-2α kd/kd Mice—To investigate the function of HIF-2α in tumor growth and vessel formation, we first analyzed the in vivo growth of B16F10 melanomas following subcutaneous implantation into HIF-2α knockdown mice (kd/kd) (24). Fourteen days after implantation, tumors were harvested and weighed. Tumors harvested from kd/kd mice were significantly smaller than those isolated from the wild-type (WT) hosts (Fig. 1A; WT, 3.01 ± 1.12 g, versus kd/kd, 1.56 ± 0.99 g, n = 6, p = 0.038). To examine neovascular development in the tumors, we measured vessel diameters after staining with anti-CD31 antibodies (Fig. 1B). Vessels were categorized into three groups as follows: diameters less than 10 μm, diameters between 10 and 100 μm, and diameters greater than 100 μm (Fig. 1I). Fine vessels (those less than 10 μm in diameter) were more frequent in kd/kd mice than in WT mice (Fig. 1C; WT, 0.4 ± 0.5, versus kd/kd, 9.6 ± 2.7, n = 4, p = 0.0005), whereas intermediate vessels (10–100 μm) were markedly reduced in kd/kd animals in comparison with WT mice (WT, 9.2 ± 2.5, versus kd/kd, 4.2 ± 2.1, n = 4, p = 0.02). In contrast to WT mice, vessels over 100 μm in diameter were not detected in tumors from kd/kd hosts. Given that newly formed vessels originated from the recipient mice (WT or kd/kd), we conclude that the formation of vessels in tumors larger than 10 μm in diameter is impaired in kd/kd mice.

We next examined the effect of HIF-2α on tumor neovascularization. We generated primary tumors in kd/kd and WT mice by subcutaneous injection of B[a]P. At 15 weeks after the injection of B[a]P, tumors were harvested and analyzed. Tumors derived from kd/kd mice were significantly smaller than those from WT mice (Fig. 1D; WT, 3.35 ± 1.23 g,
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**FIGURE 2. Analysis of HIF-1α and HIF-2α expression in fibrosarcoma.** Primary fibrosarcomas induced by B[a]P treatment were harvested from WT (A–D) and kd/kd (E–H) mice and examined by immunohistochemical staining with hematoxylin and eosin (H.E.) (A and E) and anti-CD31 (B and F), anti-HIF-1α (C and G), or anti-HIF-2α (D and H) antibodies (Ab). Positively staining cells are brown. Sections are counterstained with hematoxylin. Asterisks represent vessels over 50 μm in diameter (B–D and G and H). Arrowheads represent HIF-2α positively stained cells (D). Scale bar = 100 μm (A, B, E, and F), 50 μm (C, D, G, and H).

| H.E. | WT | kd/kd |
|------|----|-------|
| αCD31 Ab | A | B |
| αHIF-1α Ab | C | D |
| αHIF-2α Ab | E | F |

versus kd/kd, 0.53 ± 0.31 g, n = 3, p = 0.018). Subsequently, we counted and categorized the vessels in these primary tumors (Fig. 1, E and F). Formation of vessels smaller than 10 μm in diameter was increased in kd/kd mice compared with WT mice (WT, 9.85 ± 3.18, versus kd/kd, 18.4 ± 3.01, p = 0.027). In contrast, formation of vessels larger than 10 μm in diameter was significantly reduced in kd/kd mice relative to WT mice (between 10 and 100 μm, WT, 39.6 ± 8.14, versus kd/kd, 18.9 ± 3.96, p = 0.017; larger than 100 μm, WT: 3.06 ± 1.29, versus kd/kd, 0.33 ± 0.34, p = 0.024).

The kd/kd- and WT-derived fibrosarcomas were also subcutaneously implanted into nude mice. Tumors were weighed 14 days after implantation (Fig. 1G). The tumors from mice of either HIF-2α genotype grew similarly in nude mice (WT, 2.17 ± 0.92 g, kd/kd, 2.27 ± 0.87 g, n = 4, p = 0.89). Consistent with the apparent tumor growth, vessels developed well in both two distinct tumor cell types, B16F10 melanoma cells (Fig. 3B) and 3LLC lung carcinoma cells (Fig. 3C), and control PBS (Fig. 3A) were placed in diffusion chambers for analysis of tumor neovascularization. These chambers were then embedded subcutaneously onto the backs of WT or kd/kd mice. Membranes of the chambers were examined 7 days after implantation for newly formed vessels.

On the membrane surfaces of the chambers implanted in WT mice, growing vessels were irregularly shaped, distorted, and dilated with a characteristic tumor vasculature (Fig. 3, B, panels a and c, and C, panels a and c). Bleeding was frequently observed, indicating that the vessels were leaky. These results were similar for chambers containing either B16F10 or 3LLC cells. In contrast, vessels developed poorly on the membranes of chambers embedded into kd/kd mice (Fig. 3, B, panels b and d, and C, panels b and d). Vessel formation in kd/kd mice was only

kd/kd- and WT-derived fibrosarcomas as determined by the distribution of vessel diameters (Fig. 1, H and I). We interpret from these results that HIF-2α expression in ECs, but not tumor cells, of host animals determines the rate of tumor growth by affecting tumor vessel growth and development.

**Distinct Expression Patterns of HIF-2α and HIF-1α in Tumor Vasculature**—We examined the expression of HIF-α in B[a]P-induced tumors obtained from WT and kd/kd mice (Fig. 2). To analyze the tissue structure and the location of endothelial cells, hematoxylin and eosin and anti-CD31 antibody staining (Fig. 2, A and F) and anti-CD31 staining (Fig. 2, B and F) were also performed. HIF-1α staining was observed in cells surrounding the vessels but was barely detectable in ECs of the tumor vessels in both WT and kd/kd hosts (Fig. B, C, F, and G). In contrast, HIF-2α was primarily detected in ECs of the vessels in WT, whereas the expression of HIF-2α was markedly reduced in kd/kd (Fig. 2, B, D, F, and H). We conclude that the reduced HIF-2α expression in ECs impairs the formation of vessels within tumors.

**Vessel Formation in kd/kd and WT Mice Induced by Tumor Cells**—We investigated the role of HIF-2α in tumor neovascularization with a diffusion chamber assay that permits direct macroscopic observation of newly formed vessels on the surface of a filter membrane of chambers containing tumor cells.
We investigated the target genes regulated by HIF-2α in the host animal is critical for vessel formation (24, 27). As the expression of genes, such as angiopoietin-1 and VEGF, which indicate vascular endothelial growth factor; PDGF indicates platelet-derived growth factor.

**TABLE 1**

| Genes          | WT     | kd/kd | Ratio |
|----------------|--------|-------|-------|
| Eph A4         | 2439.02| 420.78| 0.173 |
| Ephrin B2      | 7788.94| 2268.40| 0.291 |
| Ephrin A1      | 8256.14| 2722.74| 0.330 |
| Ephrin B1      | 8005.31| 2987.03| 0.373 |
| PDGF-α         | 8264.31| 3969.87| 0.480 |
| Eph B6         | 390.93 | 230.23| 0.589 |
| Eph A2         | 2212.52| 1701.22| 0.769 |
| Angiopoietin-1 | 93.18  | 68.36 | 0.734 |
| Angiopoietin-2 | 625.49 | 554.08| 0.886 |
| VEGF-C         | 176.89 | 264.22| 1.494 |
| VEGF-B         | 13,259.60| 11,650.18| 0.879 |
| VEGF           | 2054.87| 2086.13| 1.015 |
| Flt-1          | 4774.38| 5333.80| 1.122 |
| PDGF-C         | 1494.22| 1862.44| 1.246 |
| Flt-3          | 1719.89| 1827.83| 1.063 |
| Flt-4          | 76.83  | 49.44 | 0.643 |
| Ephrin A2      | 342.28 | 230.23| 0.685 |
| Ephrin B3      | 3610.42| 3425.14| 0.949 |
| Flk-1          | 1805.81| 1545.90| 0.856 |
| PDGF-β         | 372.30 | 490.43| 1.317 |
| EGF-1          | 1965.12| 2020.68| 1.028 |

from samples prepared from WT and kd/kd mice (Table 1) revealed that the expression levels of ephrins B2, A1, and B1 and Eph receptor A4 (Eph A4) were significantly decreased in kd/kd mice (p < 0.01) relative to WT mice. The expression levels of known vasculogenic factors, including Ang1, Ang2, VEGF, and VEGFR-2 (Flk-1), did not differ significantly between genotypes. We confirmed these differences in expression level by quantitative RT-PCR analysis (Fig. 4). Consistent with the results of the microarray screening, the expression levels of Eph A4 and ephrin A1 and B2 were reduced in vessels derived from kd/kd mice. Because it has been reported that erythropoietin affects vascularization (25) and is a target molecule for HIF-2α, we also examined the erythropoietin expression. There was no significant difference of erythropoietin expression between WT- and kd/kd-derived samples (data not shown).

**Gene Expression Profiles in ECs Derived from kd/kd Mice after Transfection with HIF-2α and Treatment with CoCl2**

Endothelial precursor cells contribute to vessel development in both physiologic and pathologic processes (26). Endothelial precursor cells translocate from the BM in response to various angiogenic stimuli and contribute to tumor vascular formation (24, 27). As the expression of genes, such as HIF-2α, by ECs is critical for neovascularization, we examined gene expression profiles of primary EC cultures from the WT and kd/kd BM.

To mimic hypoxia that induces inhibition of proline hydroxylases, ECs were treated with CoCl2. The expression of angiogenic factors was determined with quantitative RT-PCR (Fig. 5, A and B). In agreement with the results of the diffusion chamber experiments (Fig. 4), ephrin A1 expression was significantly reduced in ECs from the kd/kd BM. The expression of Eph A4,
however, did not differ between kd/kd and WT cells under normoxic conditions, and Eph A4 expression was similarly induced in both the kd/kd and WT cells by CoCl2 treatment. The

![Graphs showing mRNA expression levels of various angiogenic factors in WT and kd/kd ECs under different conditions.](image1)

**Figure 4.** Comparison of mRNA expression in ECs between WT and kd/kd. Nascent vessels (filled with PBS or B16F10 cells) formed in WT mice on chamber membranes were harvested and purified for mRNA isolation. mRNA expression levels of each angiogenic factor were analyzed by quantitative RT-PCR. Quantitative RT-PCR analysis was performed in two independent experiments.

![Graphs showing Eph A4/ephrin A1 expression after CoCl2 treatment in BM-derived ECs.](image2)

**Figure 5.** Eph A4/ephrin A1 expression after CoCl2 treatment in BM-derived ECs. A, expressions of Eph A4, ephrin A1, ephrin B2, CXCR4, VEGF, and Flk-1 in BM-derived ECs were analyzed by quantitative RT-PCR. ECs derived from WT (+) and kd/kd (−) mice were transfected with the pEF1-HIF-2α expression vector or mock-transfected and then cultured in the presence or absence of CoCl2. N, ECs cultured under normoxic conditions without CoCl2 treatment; CoCl2, ECs treated with CoCl2; HIF-2α, ECs transfected with exogenous HIF-2α; mock, ECs transfected with a control vector. RNAs prepared from these samples were subjected to quantitative RT-PCR. B, Western blot analysis of WT and kd/kd cells showing the expression of ephrin A1. Expression levels of ephrin A1 were measured by densitometry and indicated in histograms. Mean ± S.D. was obtained from three independent experiments.

* p < 0.05; ** p < 0.01.
expression level of ephrin B2 was greater in kd/kd cells than that in WT cells. From this expression analysis we conclude that whereas ephrin A1 is a direct target of HIF-2α in ECs, additional extracellular environmental cues may be required in vivo for the regulation of Eph A4 and ephrin B2 expression.

We transfected ECs with an expression plasmid encoding HIF-2α to determine whether kd/kd ECs can recover the gene expression of ephrin A1. Expression of ephrin A1 was rescued by transfection with the HIF-2α plasmid. The expression level was significantly elevated over that observed in WT ECs, indicating that HIF-2α regulates the expression of ephrin A1. Consistent with reports that the expression of VEGF and its receptor Flk-1 are reduced in HIF-1α null animals (28), the expression of these proteins was unaffected in kd/kd and WT ECs transfected with HIF-2α. To determine the ephrin A1 protein expression in ECs after the treatment with CoCl2, we performed Western blot analysis (Fig. 5C). Ephrin A1 expression was up-regulated in WT ECs (normoxia, 1.00 versus Co2+, 1.62 ± 0.46-fold, n = 3, p = 0.04), but not kd/kd ECs (normoxia, 0.60 ± 0.24 versus Co2+, 0.46 ± 0.15, n = 3, p = 0.14) (Fig. 5C). Thus, both HIF-1α and HIF-2α regulate the angiogenic molecules in ECs but control the expression of distinct genes and are not functionally redundant in the assays we employed.

Defective Vascular Tube Formation Is Associated with Reduced Ephrin A1 Expression in kd/kd ECs—An EC line lacking HIF-1α exhibits impaired cell proliferation under hypoxic but not normoxic conditions (28). To investigate the role of HIF-2α in EC proliferation, we examined the growth of WT and kd/kd ECs under both normoxic and hypoxic conditions (Fig. 6A). In contrast to HIF-1α-null ECs, HIF-2α kd/kd cells proliferated well under both normoxic and hypoxic conditions. However, in a tube formation assay, the number of tubes formed by kd/kd ECs was significantly reduced in comparison with WT ECs under normoxic conditions (Fig. 6B). WT, 64.8 ± 5.5 tubes/field, versus kd/kd, 28.5 ± 3.8 tubes/field, n = 3, p = 0.0007). Under hypoxic conditions, the number of tubes formed by both types of ECs was elevated, but again fewer were observed in cultures of kd/kd ECs (Fig. 6B; WT, 92.3 ± 4.0 tubes/field, versus kd/kd, 48.5 ± 8.81 tubes/field, n = 3, p = 0.0005).

As the expression of ephrin A1 is significantly reduced in kd/kd ECs, we examined the role of ephrin A1 in the vascular tube formation (Fig. 6C). Overexpression of ephrin A1 in kd/kd EC was prepared by using retrovirus system, and the tube formation assay was performed in Matrigel (Fig. 6C). An increased number of tube formation was observed in ephrin A1-overexpressed kd/kd EC (kd/kd EC (ephrin A1)) (kd/kd EC (vector); 56.3 ± 3.5 tubes/field versus kd/kd EC (ephrin A1); 141 ± 35 tubes/field, n = 3, p = 0.000007).

To investigate whether HIF-2α directly regulates tube formation via ephrin A1, HIF-2α-overexpressed WT EC was prepared for Matrigel assay (Fig. 6D). An increasing number of tube formations were observed in HIF-2α-overexpressed WT EC (vector, 43.3 ± 5.7 tubes/field, versus HIF-2α, 60 ± 4 tubes/field, n = 3, p = 0.014). Conversely, ephrin A1-Fc, a competitive inhibitor for receptors of ephrin A1, significantly inhibited tube formation in both vector control (control EC, 38 ± 6.9 tubes/field, versus ephrin A1-Fc, 24.6 ± 3 tubes/field, n = 3, p = 0.036) and HIF-2α-overexpressed (control EC, 61.3 ± 5 tubes/field, versus ephrin A1-Fc, 30 ± 5.2 tubes/field, n = 3, p = 0.001) WT EC. Consequently, ephrin A1 expression in EC is crucial for tube formation in Matrigel, suggesting that HIF-2α may associate with the expression of ephrin A1 in EC.

HIF-2α Regulates Ephrin A1 Expression by Binding to the HRE Site in Ephrin A1 Promoter—We investigated if HIF-2α can activate transcription of ephrin A1 with a reporter assay (Fig. 6E). The ephrin A1 promoter contains a putative HRE sequence 2.1 kb upstream of the predicted start codon. We constructed a reporter cassette for ephrin A1 gene expression by ligating the 2.2-kb region upstream of the ephrin A1 gene and the SV40 promoter to the sequence encoding luciferase (Fig. 6E). In cells transfected with HIF-2α and Arnt expression plasmids, reporter activity was enhanced by 4-fold over control levels (p < 0.01), whereas no induction was observed when the HRE sequence (Cggatcct) was mutated in the reporter plasmid. Cells transfected with the HIF-1α and Arnt expression plasmids exhibited only weak luciferase expression from both the intact and mutated ephrin A1 HRE reporter constructs (Fig. 6E).

To investigate if HIFs associate with the ephrin A1 promoter in WT and kd/kd ECs, we performed ChIP experiments (Fig. 6F). As shown in Fig. 6F (left panel), HIF-2α but not HIF-1α bound the HRE sequence of the ephrin A1 promoter under normoxic conditions. Similarly, after treatment with CoCl2, HIF-2α but not HIF-1α associated with the HRE of the ephrin A1 promoter in WT ECs. HIF-2α also bound the HRE sequence in kd/kd ECs under both normoxic and hypoxic conditions, albeit to a significantly lesser degree than WT ECs (normoxia, WT, 1.00 ± 0.05, versus kd/kd, 0.15 ± 0.07, n = 5, p = 0.00009; hypoxia, WT, 3.37 ± 0.93 versus kd/kd, 0.81 ± 0.1, n = 3, p = 0.009). This finding is consistent with the reduced expression of both HIF-2α and ephrin A1 in kd/kd ECs relative to WT ECs (Fig. 5C). Nonimmunized rabbit IgG did not precipitate detectable amounts of chromatin containing the ephrin A1 promoter sequence. Thus, HIF-2α but not HIF-1α binds the HRE sequence in the promoter of ephrin A1 gene to up-regulate expression of ephrin A1 in ECs.

In summary, we demonstrate with these experiments that HIF-2α expression in ECs plays a crucial role in neovascularization and that HIF-2α functions in part by up-regulating the expression of ephrin A1 to promote vascular tube formation.

**DISCUSSION**

HIF-1α and HIF-2α have been proposed to function as key factors in angiogenesis during tumor development. These two highly homologous proteins are co-expressed in ECs and bind identical HRE sequences. Therefore, HIF-1α and HIF-2α were considered to have overlapping or redundant functions in ECs during neovascularization. Recent studies, however, have demonstrated that these two factors have their own unique target genes and distinct roles in ECs and cancer cells (13, 14, 17, 29). Here we demonstrated that HIF-2α is an essential regulatory factor in ECs for vascular remodeling. This function cannot be substituted by HIF-1α as exemplified by the regulation of expression of ephrin A1 exclusively regulated by HIF-2α.

Functional differences between HIF-1α and HIF-2α during embryonic development have been identified by characterizing...
**FIGURE 6.** HIF-2α is involved in capillary tube formation through direct regulation of ephrin A1 expression in EC. A, growth rates of WT- and kd/kd-derived ECs from bone marrow. The average cell numbers present in cultures under normoxic (left panel) or hypoxic (5% O2, right panel) conditions were determined by counting three dishes at 24-h intervals. B, WT and kd/kd ECs were analyzed with a tube formation assay (photograph top, WT; bottom, kd/kd). The number of tubes formed on Matrigel was scored in three wells and averaged (mean ± S.D.; right panel, black bar, WT; white bar, kd/kd; N, normoxia; H, 5% O2). Scale bar = 50 μm. C, overexpression of ephrin A1 in kd/kd EC (kd/kd ephrin A1) was examined by Western blot, and tube formation assay was performed. kd/kd vector, control kd/kd EC transfected vector alone. **, *p < 0.01. D, effects of ephrin A1 on tube formation. Overexpression of HIF-2α in WT EC was examined for HIF-2α (left panel) and ephrin A1 (right panel) expression by Western blot. The ephrin A1-Fc inhibitor was added to the vector- or HIF-2α-transfected WT EC tube formation assay. This assay was performed in the presence of 200 ng/well of ephrin A1-Fc or CD28-Fc (control-Fc). The number of tubes was scored in a random field from each of three wells and averaged for each condition (mean ± S.D.; *, *p < 0.05; **, *p < 0.01). E, luciferase reporter assay was performed using the WT ephrin A1 − 2.2-kb promoter (left panel, top) and an HRE-mutated promoter (located at −2.1-kb position; left panel, bottom). The pEF1-HIF-2α, pEF1-HIF-1α, and pEF1-Arnt expression vectors were transfected. Luciferase activity was measured for the ephrin A1 (right panel, filled bar) and mutated ephrin A1 promoters (right panel, open bar). The values shown are the averages of three independent experiments (mean ± S.D.). Values were normalized to the luciferase activity measured for 293T cells transfected with each reporter plasmid and empty vector. *, *p < 0.05; **, *p < 0.01. F, ChIP assay was performed using anti-HIF-1α and anti-HIF-2α antibodies on WT and kd/kd ECs under normoxic (N) conditions or after treatment with CoCl2 (Co2⁺). Normal rabbit IgG was used as a negative control. The histogram represents ephrin A1 expression levels seen in WT and kd/kd ECs using anti-HIF-2α antibody after treatment with CoCl2. The expression levels observed in WT ECs were normalized to a value of 1. Mean ± S.D. was obtained from three independent experiments. **, *p < 0.01.
the phenotypes associated with targeted gene disruptions of these genes in mice. Mouse mutants for HIF-1α exhibit early embryonic lethality associated with severe defects of vascularization in the developing embryo and yolk sac (30). Cell type-specific disruption of HIF-1α in ECs, however, did not produce any lethal defects during embryonic vascular development, consistent with an essential role for HIF-1α expression in other cell types, potentially vascular pericytes, during embryonic vascular development (28). In contrast, targeted inactivation of HIF-2α by several groups has resulted in a variety of pathological phenotypes with these differences likely because of variation in genetic background. Peng et al. (31) detailed defects in vascular remodeling with abnormally fenestrated capillaries that resulted in local hemorrhage, whereas other groups reported defects in fetal catecholamine production (32), impaired homeostasis of reactive oxygen species (29), and defects in lung maturation because of deficient surfactant secretion from type 2 pneumocytes (33). Thus, HIF-1α and HIF-2α have unique functions that are not redundant during embryonic development. The different morphological features of these impaired vasculatures suggest that HIF-1α and HIF-2α exert differential functions in vascular formation at embryonic stages.

Vascular remodeling and EC proliferation have been proposed as the two major forms of neovascularization. In HIF-2α kd/kd mice, large vessels that require constant remodeling were rarely observed during tumor development (Fig. 1, A–C), although the total number of nascent vessels inaked/kd animals was comparable with that observed in WT mice. Indeed, our examination of cell growth in vivo did not detect any differences in proliferation of cultured ECs derived from kd/kd and WT mice under both normoxic and hypoxic conditions (Fig. 6A). From these results, we conclude that HIF-2α is essential for vascular remodeling. Although HIF-2α appears dispensable for EC proliferation, we cannot formally rule out the possibility that the low level of HIF-2α expression retained in kd/kd EC is sufficient to support EC proliferation. In contrast, loss of HIF-1α in ECs severely reduces EC proliferation in tumors. The EC line lacking HIF-1α exhibited impaired cell proliferation under hypoxic but not normoxic conditions (28). Therefore, HIF-1α and HIF-2α play independent roles in tumor angiogenesis in ECs.

Functional differences between HIF-1α and HIF-2α in tumor angiogenesis are supported by identification of unique target genes. In HIF-1α-null ECs, expression levels of VEGF are decreased, and the induction of VEGFR2/Flk-1 by hypoxia is lost, resulting in disruption of a VEGF-mediated autocrine loop induced by hypoxia that is critical for tumor angiogenesis (28). In contrast, expression of VEGF and Flk-1 was unchanged in tumor-derived vessels from HIF-2α kd/kd mice and in cultured kd/kd ECs (Table 1 and Fig. 4). Thus, HIF-1α but not HIF-2α specifically induces the VEGF/Flk-1 system in ECs to promote EC proliferation both in vivo and in vitro.

Reduced expression of ephrin-A1 was consistently observed in kd/kd vessels grown on diffusion chamber membranes and in ECs cultured from kd/kd mice. This decrease in ephrin-A1 levels accompanied defects in vascular remodeling (Table 1, Fig. 4, and Fig. 5). In addition, it is of note that HIF-2α overexpression increased tube formation in WT ECs, whereas soluble ephrin-A1-Fc clearly abrogated the effect of HIF-2α on tube formation by inhibiting ephrin-A1 function (Fig. 6D). Similarly, overexpression of ephrin-A1 in kd/kd ECs exhibited a significant increase in tube formation (Fig. 6C). Reciprocally, increasing HIF-2α expression activated not only an ephrin-A1 reporter construct in 293T cells (Fig. 6E) but also increased endogenous ephrin-A1 gene expression in kd/kd ECs to a level higher than that in controls (Fig. 5, A and B). ChIP analysis revealed that HIF-2α but not HIF-1α specifically bound the HRE sequence in the ephrin-A1 promoter (Fig. 6F). Based on these findings, we propose that HIF-2α influences tumor vascular remodeling by regulating the expression of ephrin-A1 in ECs.

The Eph family of receptor tyrosine kinases and their ligands were originally identified as critical factors for embryonic patterning and neuronal targeting (34). Mounting evidence supports the critical role of Eph/ephrin proteins in a variety of cellular responses during angiogenic remodeling (35, 36). Targeted disruptions of ephrin-B2, Eph-B2, Eph-B3, and Eph-B4 impair primary capillary network remodeling and formation of the embryonic vasculature, resulting in embryonic lethality (37–39). During tumor vasculogenesis, inhibition of ephrin-A1 or its receptor Eph-A2 causes defects in vascular tube formation and blocks tumor progression (40, 41). Interestingly, Cheng et al. (42) reported that inhibition of Eph-A2 receptor activity affects angiogenesis mediated by VEGF but not basic fibroblast growth factor, consistent with an essential role for ephrin-A1/Eph-A2 in mediating the induction of optimal neovascularization mediated by VEGF. Our findings help to discriminate the functions of HIF-2α. Together with HIF-1α, HIF-2α in ECs works specifically but cooperatively to control tumor angiogenesis by regulating the ephrin/Eph systems. It is important to note that reduced tumor growth in vivo has been observed in both HIF-1α EC null and HIF-2α kd/kd mice.

There are several questions to be answered concerning the complex mechanisms of HIFs in relation to the tumor neovascularization. To what extent do HIF-1α and HIF-2α play common and unique transcriptional roles? How do HIF-1α and HIF-2α discriminate their own target genes despite their seemingly identical binding sequence? Interestingly, HIF-2α but not HIF-1α binds the Oct-4 promoter and increases the expression of this transcription factor. This transcriptional cascade may contribute to the defects in embryonic development and hematopoietic stem cell differentiation observed in HIF-2α mutant mice (11).

In conclusion, HIF-2α expressed in ECs plays a unique and essential role in vascular remodeling during tumor development by inducing the expression of ephrin-A1, and probably its related factors, that cooperatively work with HIF-1α-specific target genes such as VEGF and Flk-1 to promote effective tumor angiogenesis. Thus, therapeutic approaches to restrict neovascularization in pathological conditions must target both the HIF-1α and HIF-2α pathways.

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