Orphan nuclear receptor TR3/Nur77 regulates VEGF-A–induced angiogenesis through its transcriptional activity

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Vascular endothelial growth factor (VEGF)-A has essential roles in vasculogenesis and angiogenesis, but the downstream steps and mechanisms by which human VEGF-A acts are incompletely understood. We report here that human VEGF-A exerts much of its angiogenic activity by up-regulating the expression of TR3 (mouse homologue Nur77), an immediate-early response gene and orphan nuclear receptor transcription factor previously implicated in tumor cell, lymphocyte, and neuronal growth and apoptosis. Overexpression of TR3 in human umbilical vein endothelial cells (HUVECs) resulted in VEGF-A–independent proliferation, survival, and induction of several cell cycle genes, whereas expression of antisense TR3 abrogated the response to VEGF-A in these assays and also inhibited tube formation. Nur77 was highly expressed in several types of VEGF-A–dependent pathological angiogenesis in vivo. Also, using a novel endothelial cell-selective retroviral targeting system, overexpression of Nur77 DNA potently induced angiogenesis in the absence of exogenous VEGF-A, whereas Nur77 antisense strongly inhibited VEGF-A–induced angiogenesis. B16F1 melanoma growth and angiogenesis were greatly inhibited in Nur77$^{-/-}$ mice. Mechanistic studies with TR3/Nur77 mutants revealed that TR3/Nur77 exerted most of its effects on cultured HUVECs and its pro-angiogenic effects in vivo, through its transactivation and DNA binding domains (i.e., through transcriptional activity).

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Abbreviations used: bFGF, basic fibroblast growth factor; HUVEC, human umbilical vein endothelial cell; LBD, ligand binding domain; MOT, mouse ovarian tumor; VEGF, vascular endothelial growth factor; VPF, vascular permeability factor.

To grow beyond minimal size, tumors must induce the formation of new blood vessels (1, 2). They do so by secreting angiogenic factors, including basic fibroblast growth factor (bFGF), platelet-derived growth factor B, and members of the vascular permeability factor (VPF)/vascular endothelial growth factor (VEGF) family (3). Among these, VPF/VEGF (VEGF-A) is thought to be the most important for several reasons. It is expressed abundantly by most human and animal tumors and is able by itself to induce typical tumor blood vessels. In addition, when neutralized, the growth of VEGF-A–expressing tumors is strikingly inhibited (3, 4). VEGF-A is a multifunctional cytokine that acts through receptors that are expressed on vascular endothelium as well as on some other cell types. After interaction with VEGF-A, endothelial cells undergo extensive reprogramming of protease, integrin, and glucose transporter expression; are stimulated to migrate and divide; and are protected from apoptosis and senescence (4, 5). In addition, VEGF-A and other VPF/VEGF family members are the only angiogenic cytokines identified thus far that render microvessels hyperpermeable to circulating macromolecules, a characteristic property of tumor and other angiogenic blood vessels (3).

Although extensive efforts have been made to delineate the mechanisms by which VEGF induces angiogenesis, little is known about the transcriptional events that regulate this important process. We therefore set out to identify endothelial cell immediate-early response genes whose expression was induced by VEGF-A with the thought that such genes might play key downstream roles in regulating angiogenesis. We performed DNA microarrays on cultured human umbilical vein endothelial cells (HUVECs) stimulated with VEGF-A^{165} and found that several genes were up-regulated. Among the most highly induced was TR3 (mouse homologue Nur77), an immediate-early response

gene and member of the class IV subfamily of the orphan nuclear receptor superfamily of transcription factors (6). TR3/Nur77 was known as an important regulator of cell growth and apoptosis in tumor cells, lymphocytes, and neurons (7, 8), but a role in angiogenesis had not been anticipated. We here report that TR3/Nur77 is both necessary and sufficient for VEGF-A–induced proliferation and survival of cultured endothelial cells and for angiogenesis in vivo.

RESULTS

Expression and function of TR3 in vitro

Up-regulation of TR3 in HUVECs. Serum-starved HUVECs were stimulated with 10 ng/ml VEGF-A165 for 1 h, and RNA was isolated and its expression assessed on Affymetrix DNA microarray chips. In agreement with a recent report (9), VEGF-A165 highly up-regulated TR3 and we confirmed this finding by quantitative real-time RT-PCR and immunoblotting (Fig. 1, A and B). TR3 was also induced, although to a slightly lesser extent, by another VEGF-A isoform, VEGF-A120, but not by several other angiogenic growth factors including bFGF, platelet-derived growth factor, and placenta growth factor, another member of the VPF/VEGF family (Fig. 1 C).

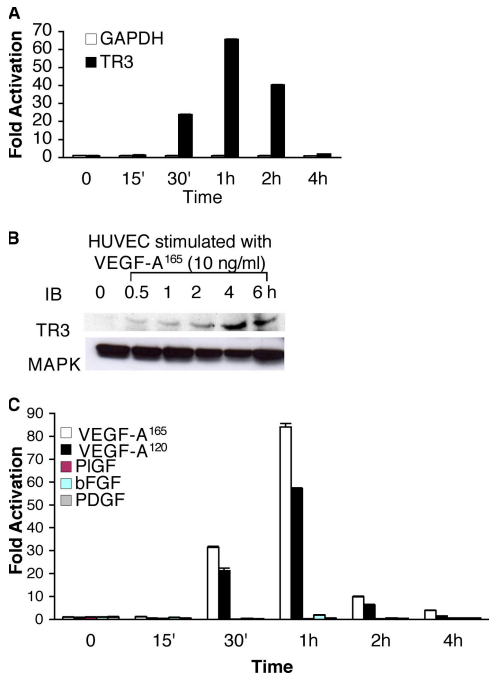

Figure 1. VEGF-A induces TR3 expression in cultured HUVECs. (A) Quantitative real-time RT-PCR to demonstrate TR3 and control GAPDH mRNA expression after stimulation with 10 ng/ml VEGF-A^{165} at times indicated. (B) Immunoblot of TR3 protein expression in VEGF-A^{165}–stimulated HUVECs (top). Bottom shows MAPK protein loading control. (C) TR3 mRNA expression in HUVECs stimulated with 10 ng/ml VEGF-A^{165}, VEGF-A^{120}, placenta growth factor, platelet-derived growth factor (PDGF), or with 25 ng/ml bFGF over time as determined by quantitative real-time RT-PCR ($n = 2$).

Cloning and expression of TR3 sense and antisense cDNAs and mutants. To elucidate TR3/Nur77's role in angiogenesis-related events, we constructed TR3-sense (TR3-S) and TR3-antisense (TR3-AS) DNAs. In addition, TR3/Nur77, like other members of the orphan nuclear receptor superfamily, has three functionally distinct domains: transactivation, DNA binding, and ligand binding (10, 11). Therefore, we engineered mutant forms of TR3/Nur77 that lacked each of these domains, as previously described (Fig. 2 A; reference 11). Each of these constructs was fused with the Flag tag and then transduced into HUVECs using an efficient retroviral system that yielded almost 100% infection (12). HUVECs retrovirally transfected with TR3-S DNA exhibited a three- to fourfold increase in TR3 protein expression as compared with untransfected cells or HUVECs transduced

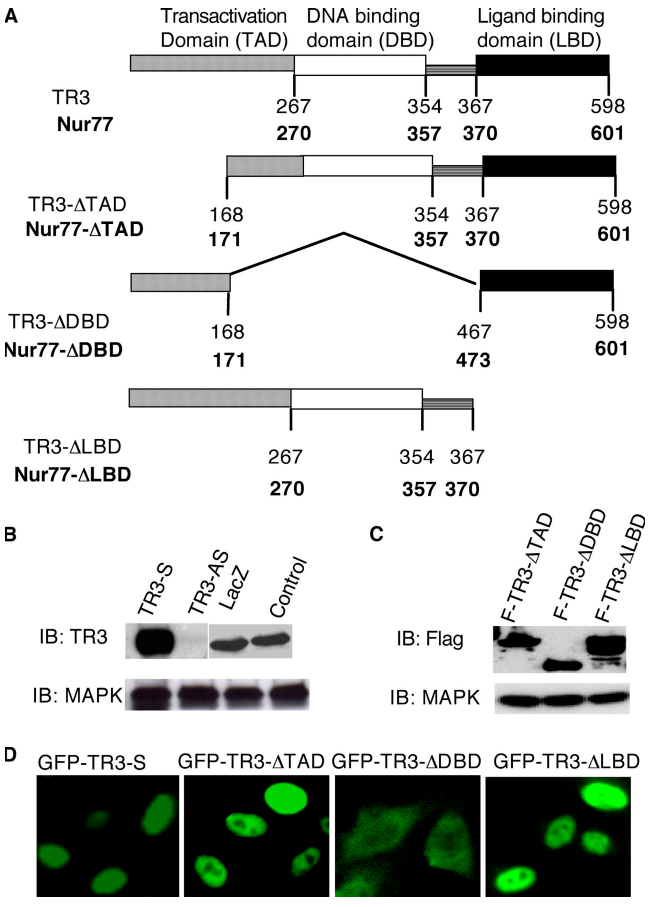

Figure 2. TR3 and Nur77 mutants. Expression of TR3/Nur77 sense, antisense, and mutant proteins in transfected HUVECs. (A) Schematic structure of TR3 and Nur77 genes and mutants constructed to lack TAD, DBD, or LBD domains. (B) Expression of TR3 protein in control HUVECs and in HUVECs transfected with TR3-S, TR3-AS, or LacZ cDNAs. TR3-S–transfected HUVECs expressed three- to fourfold more TR3 protein than untransfected or LacZ-transfected cells. Endogenous TR3 protein expression was strongly inhibited in cells transfected with TR3-AS. Bottom shows MAPK protein loading control. (C) Expression of Flag-TR3-ΔTAD, Flag-TR3-ΔDBD, and Flag-TR3-ΔLBD in HUVECs. (D) Subcellular localization of GFP-fused TR3 mutants in HUVECs.

with LacZ. On the other hand, HUVECs transfected with TR3-AS expressed greatly diminished amounts of TR3 protein (Fig. 2 B). All three mutant forms of TR3 were expressed and to similar extents (Fig. 2 C). GFP fusion proteins were prepared with each mutant and their subcellular distribution followed after introduction into HUVECs. GFP-TR3-ΔDBD protein was localized to the HUVEC cytosol, whereas GFP-TR3-ΔTAD and GFP-TR3-ΔLBD, like GFP-TR3-S, were confined to the nucleus (Fig. 2 D). We then tested each of these transfected cells using in vitro angiogenesis assays that measured cell proliferation, survival, and tube formation.

Endothelial cell proliferation assay. Serum-starved HUVECs transduced with LacZ (control), TR3-S, or TR3-AS DNAs were cultured for 24 h with or without VEGF-A^{165}, and cell proliferation was assessed by 4-h [^{3}H]thymidine incorporation (12). As shown in Fig. 3 A, HUVECs transduced with TR3-S strongly incorporated [^{3}H]thymidine (lane 3 vs. lane 1; $P < 0.001$) in the absence of added VEGF-A^{165} and in amounts equivalent to those induced by VEGF-A^{165} in LacZ-transduced cells (lane 3 vs. lane 2; $P > 0.5$). Incorporation was not enhanced further when VEGF-A^{165} was added (Fig. 3 A, lane 4 vs. lane 3; $P>0.5$). HUVECs transduced with TR3-AS incorporated [^{3}H]thymidine at baseline levels (Fig. 3 A, lane 5 vs. lane 1), but incorporation was not increased by the addition of VEGF-A^{165} (lane 6 vs. lane 2; $P < 0.001$).

We next investigated [^{3}H]thymidine incorporation in HUVECs transduced with mutant TR3s that lacked each of its three domains. As shown in Fig. 3 A, HUVECs transfected with TR3 mutants lacking the transactivation (ΔTAD) or DNA binding (ΔDBD) domains incorporated [^{3}H]thymidine at baseline levels but were not stimulated to increased incorporation by VEGF-A^{165}. These data suggest that TR3 transcriptional activity is required for VEGF-A^{165}–induced HUVEC proliferation. However, HUVECs transfected with the TR3 mutant that lacked the ligand binding domain (ΔLBD) behaved like HUVECs transfected with full-length TR3-S, exhibiting greatly increased [^{3}H]thymidine incorporation in the absence of added VEGF-A^{165}. In addition, incorporation was not further enhanced by the addition of VEGF-A^{165}. Thus, the LBD is apparently not required for the enhanced proliferative response observed after TR3 transfection.

Endothelial cell survival assay. HUVECs undergo apoptosis when cultured in the absence of serum and VEGF-A^{165} is known to protect endothelial cells from apoptosis (13). After 3 d of culture in the absence of serum and VEGF-A^{165}, >70% of LacZ-transfected HUVECs underwent apoptosis (Fig. 3 B, lane 1). As expected, the addition of VEGF-A^{165} protected such HUVECs, reducing apoptosis by approximately one half (Fig. 3 B, lane 2 vs. lane 1; $P < 0.001$). HUVECs transfected with TR3-S DNA, without added VEGF-A^{165}, also showed reduced apoptosis, similar to that of LacZ-transfected HUVECs treated with VEGF-A^{165} (Fig. 3 B, lane 3 vs. lane 2; $P > 0.05$); the addition of VEGF-A^{165} reduced apoptosis slightly further (lane 4). However, in HUVECs transduced

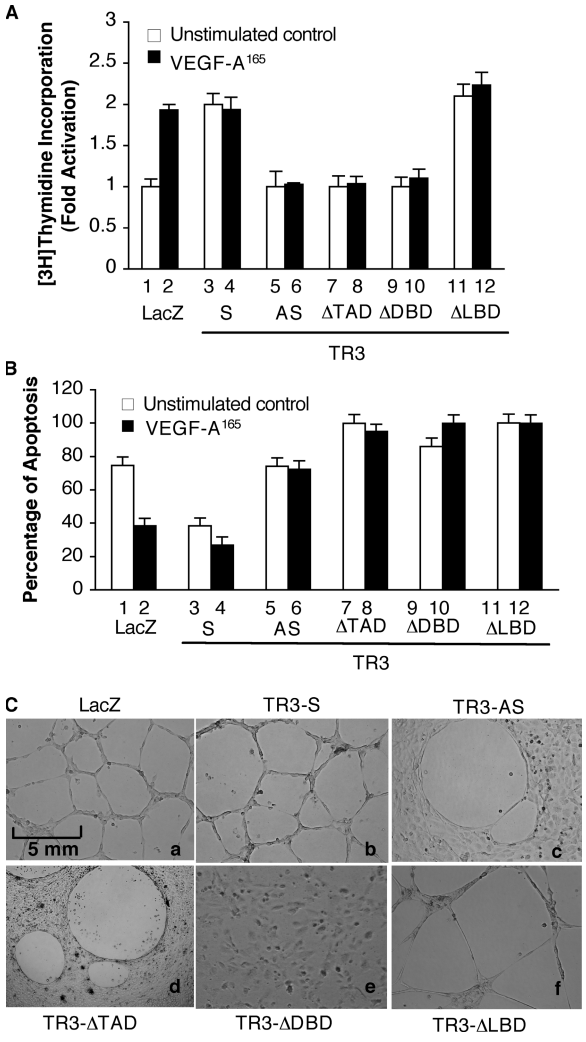

Figure 3. Assays measuring functional capabilities of cultured HUVECs transfected with TR3-S, TR3-AS, and TR3 mutant cDNAs, with or without VEGF-A^{165} stimulation. (A) [^{3}H]thymidine incorporation (reference 12; $n = 4$). (B) Cell survival assay ($n = 4$). (C) Tube formation on Matrigel.

with TR3-AS DNA, nearly 80% of cells were apoptotic and the addition of VEGF-A^{165} did not significantly improve cell survival (Fig. 3 B, lanes 5 and 6; $P > 0.05$). HUVECs transduced with any of the mutant TR3 DNAs underwent nearly 100% apoptosis and could not be rescued by the addition of VEGF-A^{165} (Fig. 3 B, lanes 7–12; $P > 0.05$). Thus, overexpression of full-length TR3, but not mutants lacking any of its domains, protects HUVECs from apoptosis in the absence of added VEGF-A^{165}. In addition, VEGF-A^{165} was not able to protect from apoptosis HUVECs that had been transfected with any of the domain-lacking mutants.

Tube formation assay. HUVECs or HUVECs transfected with LacZ form characteristic tubal networks when cultured on Matrigel in growth medium for 18 h (Fig. 3 C, a). TR3-S–transfected HUVECs formed similar networks (Fig. 3 C, b).

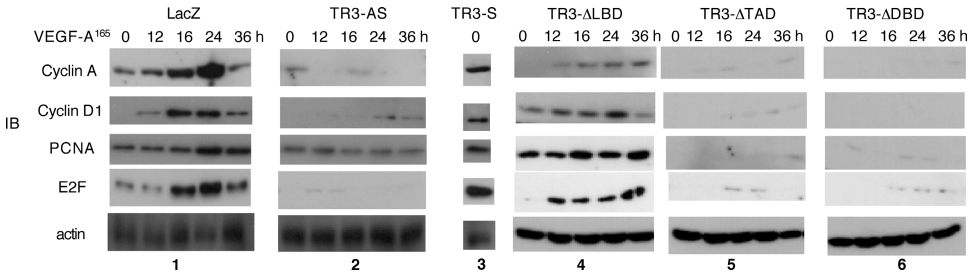

Figure 4. TR3 regulation of cell cycle gene expression in HUVECs. Immunoblots of cell extracts from HUVECs transfected with LacZ, TR3-S, TR3-AS, and TR3 mutant DNAs, with or without VEGF-A^{165} stimulation for indicated times. Actin expression serves as a protein loading control.

However, HUVECs transfected with TR3-AS or with TR3-ΔTAD formed greatly reduced numbers of tubes (Fig. 3 C, c and d). TR3-ΔLBD–transfected HUVECs approximated the normal network pattern but formed a somewhat looser meshwork (Fig. 3 C, f). Finally, cells transfected with TR3-ΔDBD remained as a confluent monolayer and exhibited no evidence of network formation (Fig. 3 C, e). These data indicate that TR3 has an important role in HUVEC tube formation and that the ΔTAD and especially the ΔDBD domain are particularly important in this process.

TR3 regulates expression of cell cycle–related genes. VEGF-A^{165} is known to induce the expression of several cell cycle–related genes in HUVECs (14). Therefore, we investigated whether TR3 had a similar effect. We found that VEGF-A^{165} induced the expression of cyclins A and D1, PCNA, and E2F in HUVECs transduced with LacZ (Fig. 4, panel 1) and also in untransfected HUVECs (not depicted). In HUVECs transduced with TR3-AS, baseline expression of these genes was reduced or similar to that of LacZ-transduced cells, and expression was either not increased or increased to a lesser extent after the addition of VEGF-A^{165} (Fig. 4, panel 2). In addition, expression of all four of these genes was increased in TR3-S–transduced cells in the absence of VEGF-A^{165} (Fig. 4, panel 3). In TR3-ΔLBD–transfected cells, expression of these genes was roughly similar to that of LacZ-transduced cells and was comparably stimulated upon exposure to VEGF-A^{165} (Fig. 4, panel 4). However, HUVECs transduced with TR3-ΔTAD or TR3-ΔDBD did not express detectable amounts of any of these genes, with or without the addition of VEGF-A^{165} (Fig. 4, panels 5 and 6). Consistent with these data, HUVECs transfected with TR3-AS, TR3-ΔTAD, or TR3-ΔDBD grew much more slowly in culture than control or Lac-Z–transfected cells.

Expression and function of Nur77 (the mouse TR3 homologue) in vivo

Expression of Nur77 in angiogenesis assays in vivo. Our experiments with cultured endothelium suggested that TR3 could have an important role in regulating angiogenesis. To test this possibility, we investigated whether Nur77, the mouse homologue of TR3, was induced in the course of VEGF-A–mediated angiogenesis in vivo. We first injected an adenoviral vector expressing VEGF-A164 (the murine equivalent of human VEGF-A165) into nude mouse ear skin to generate a strong local angiogenic response (Fig. 5 A) (15). We found that Nur77 mRNA and protein expression were strongly up-regulated in a time-dependent fashion (Fig. 5, B and C) that correlated temporally with the early angiogenic response, which is characterized by the formation of enlarged, pericyte-poor "mother" vessels (15). Healing skin wounds and TA3/St and mouse ovarian tumor (MOT) ascites tumors induced a similar, VEGF-A–driven angiogenic response (16, 17), and we found that Nur77 expression was up-regulated in all three of these examples (Fig. 5 D). However, Nur77 was not detected in immunoblots of TA3/St or MOT tumor cell extracts (not depicted). Collectively, these data demonstrate that Nur77 is strongly up-regulated in several different examples of VEGF-A–induced angiogenesis.

Nur77 regulates angiogenesis in Matrigel assays in vivo. To elucidate Nur77's mechanism of action, we made use of a recent modification of the Matrigel assay to introduce genes of interest into vascular endothelium in vivo (18). SK-MEL-2 melanoma cells that had been transfected to overexpress VEGF-A^{165} (SK-MEL/VEGF cells) (18), and PT67 cells packaging retroviruses that expressed LacZ, Nur77-sense (S), or Nur77-antisense (AS), were incorporated into Matrigel plugs that were injected into the s.c. space of nude mice. The VEGF-A^{165} secreted by SK-MEL/VEGF cells induces nearby vascular endothelial cells to divide and therefore to become susceptible to infection with retroviruses secreted by PT67 packaging cells (18).

Initial experiments confirmed that Nur77-S and Nur77-AS RNAs were expressed by cells in Matrigels (Fig. 6 A, panels A–D). We then evaluated the angiogenic response that developed after implantation of Matrigel plugs containing various cell mixtures on days 1 (not depicted) and 3 (Fig. 7 A). Angiogenesis was assessed by macroscopy (Fig. 7 A, top panels) and by histology and immunohistochemistry for the endothelial cell marker CD31 (bottom panels). Plugs containing only PT67 cells that packaged LacZ-expressing retroviruses (PT67/LacZ cells) did not induce considerable angiogenesis. However, strong angiogenesis was induced in plugs containing SK-MEL/VEGF cells, whether alone or combined with PT67/LacZ cells. The angiogenic response was characterized

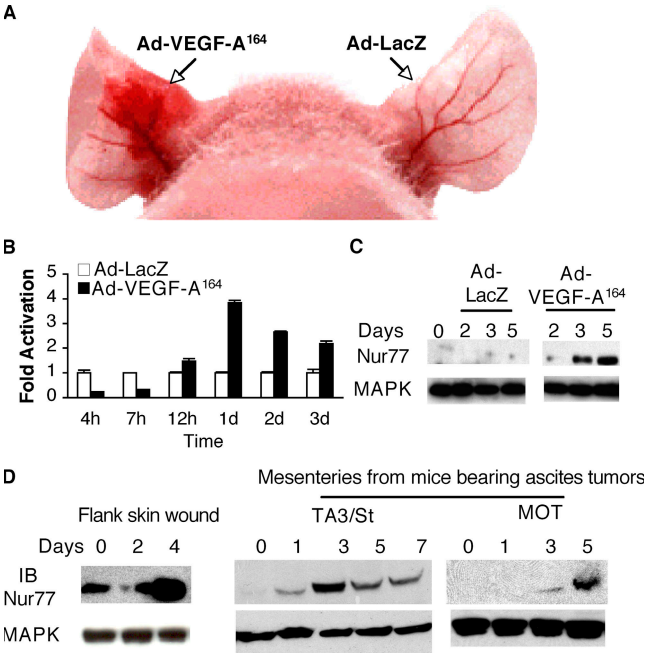

Figure 5. Increased expression of Nur77 in VEGF-A–induced angiogenesis. (A) Angiogenic response in nude mouse ears 5 d after intradermal injection of Ad-VEGF-A^{164} (left ear). Ad-LacZ was injected in right ear as a negative control (reference 15). (B) Fold activation of Nur77 mRNA over time in mouse ears after intradermal injection of Ad-VEGF-A^{164} or Ad-LacZ as determined by quantitative real-time RT-PCR. (C) Immunoblots of Nur77 protein expression in uninjected mouse ears or in ears injected with Ad-LacZ or Ad-VEGF-A^{164} (top). Bottom shows MAPK protein loading control. (D) Immunoblots of Nur77 protein expression (top) in healing skin punch biopsy wounds and in mesenteries of nude mice bearing TA3/St and MOT ascites tumors. Bottom shows MAPK protein loading controls.

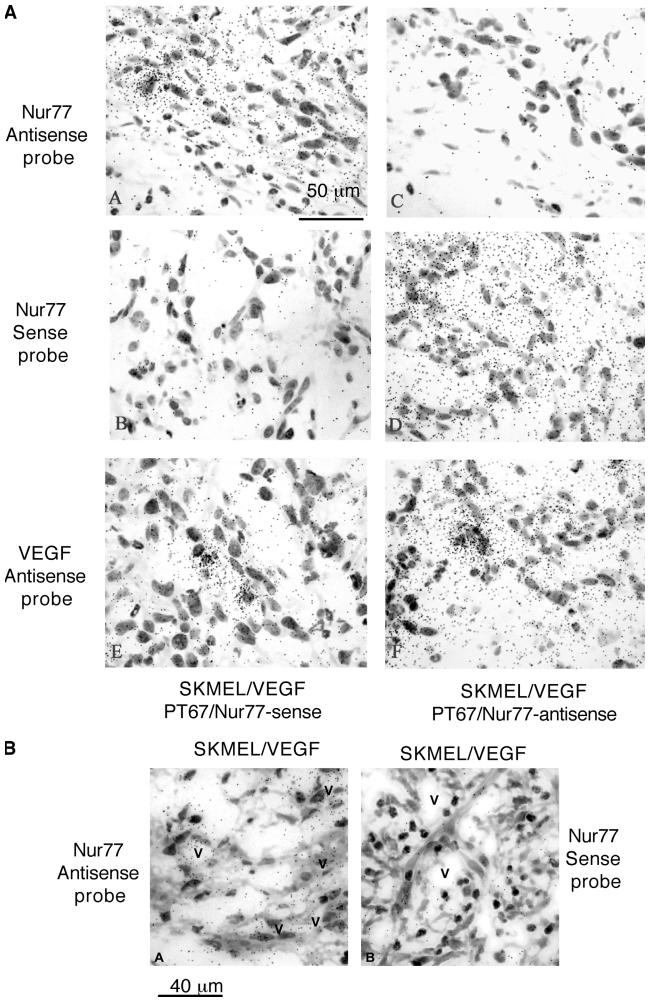

Figure 6. Nur77 and VEGF-A expression in Matrigel assays. (A) In situ hybridization performed on Matrigels containing SK-MEL/VEGF cells and PT67 cells packaging either Nur77-S (left) or Nur77-AS–expressing retrovirus (right) cDNAs. Probes were Nur77-AS (A and C), Nur77-S (B and D), and VEGF-A^{165}-AS (E and F). (B) In situ hybridization demonstrating Nur77 expression in newly formed blood vessels in Matrigels containing SK-MEL/VEGF cells hybridized with Nur77-AS (A) or Nur77-S (B) probes. v, vessel.

by the formation of mother vessels, enlarged, thin-walled, pericyte-poor vessels typical of early VEGF-A–induced angiogenesis (15). In situ hybridization confirmed that Nur77 was induced in gels containing SK-MEL/VEGF (Fig. 6 B). When PT67/Nur77-AS cells were included in the Matrigel, the angiogenic response induced by SKMEL/VEGF cells was strikingly inhibited (Fig. 7 A). The effect of Nur77-AS DNA was not attributable to inhibition of VEGF-A^{165} expression by tumor cells (Fig. 6 A, panels E and F). Furthermore, overexpression of TR3/Nur77-AS DNA in either SKMEL/VEGF or PT67 cells did not affect proliferation as measured by [^{3}H]thymidine incorporation (not depicted).

To further characterize the role of Nur77 in angiogenesis, we incorporated PT67/Nur77-S packaging cells into the Matrigel plugs, alone or along with SK-MEL/VEGF cells. Strong angiogenesis with mother vessel formation developed when PT67/Nur77-S cells were included along with SK-MEL/VEGF cells (Fig. 7 A). Of particular interest, PT67/Nur77-S cells induced significant angiogenesis with the formation of mother vessels even in the absence of SK-MEL/VEGF cells (Fig. 7 A), likely because Matrigel implants, as a foreign matrix, induced sufficient endothelial cell proliferation to allow some retroviral uptake. Collectively, these data indicate that Nur77 is able to induce typical angiogenesis in vivo in the absence of exogenous VEGF-A^{165}.

Quantitation of angiogenesis induced by Nur77. To quantitate the angiogenic response, we measured the intravascular volume of plasma contained within Matrigel plug-associated blood vessels. Evan's blue dye was injected i.v. into mice 1 and 3 d after implanting Matrigel plugs containing various cell mixtures. This dye binds to plasma proteins and therefore the amount of plasma within the Matrigel-associated vasculature can be calculated from simultaneous measurements of dye concentration in peripheral blood plasma. Matrigel plugs were harvested 5 min after i.v. dye injection, when blood vessels were filled with dye–plasma protein complexes but before there was time for significant extravasation.

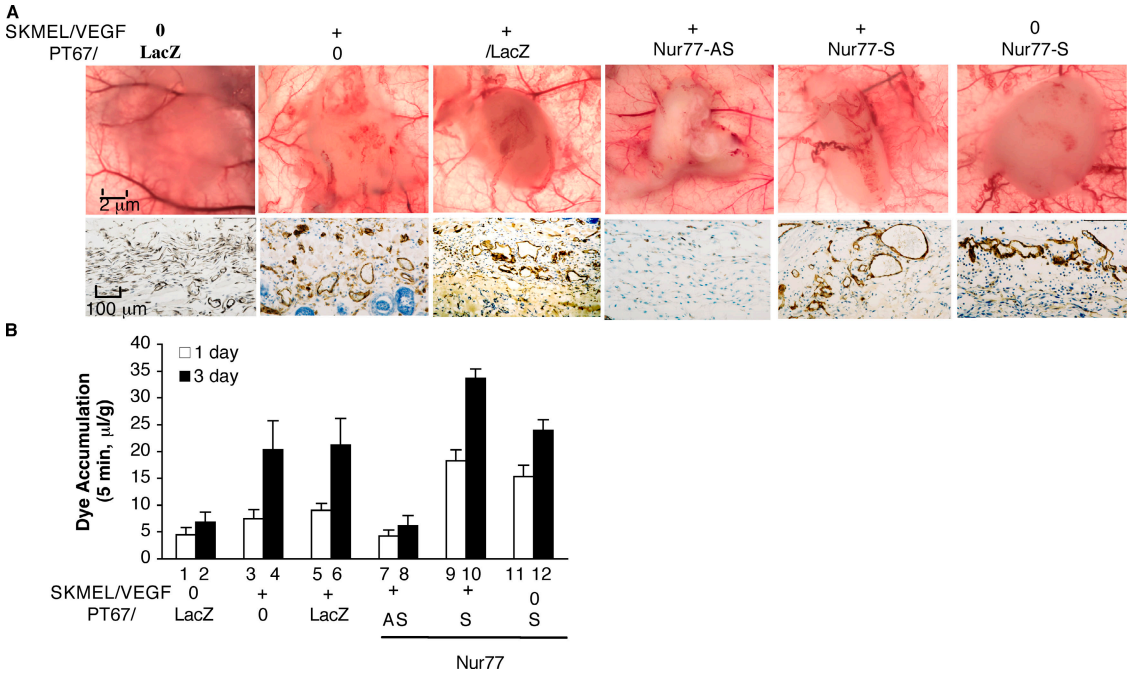

Figure 7. Angiogenic response induced by Nur77-S or Nur77-AS in Matrigel assays in vivo. (A) Macroscopic (top) and CD-31–stained microscopic (bottom) images of the angiogenic response induced 3 d after implantation of Matrigels with indicated contents of VEGF-A^{165}–secreting SKMEL/VEGF cells and PT67 cells packaging LacZ, Nur77-S, or Nur77-AS. (B) Quantitative measurement of intravascular plasma volumes (μl/g) in Matrigels containing indicated cell mixtures at 1 and 3 d after implantation as determined by accumulation of Evan's blue dye administered i.v. 5 min before killing.

Intravascular dye accumulation in Matrigel plugs containing VEGF-A^{165}–expressing SKMEL/VEGF cells (alone or with PT67/LacZ cells) increased more than twofold above baseline levels at 3 d in Matrigels lacking SKMEL/VEGF cells (Fig. 7 B, lanes 4 and 6 vs. lane 2; $P < 0.001$); the increase at 1 d was smaller and not statistically significant (lanes 3 and 5 vs. lane 1; $P > 0.05$). Inclusion of PT67 cells packaging Nur77-AS DNA completely blocked the response of SKMEL/VEGF cells on day 3 (Fig. 7 B, lane 8 vs. lanes 4 and 6; $P < 0.001$). Dye accumulation in Matrigel plugs increased still further to more than threefold on day 3 when SKMEL/VEGF cells were included along with PT67/Nur77-S cells (Fig. 7 B, lane 10 vs. lanes 4 and 6; $P < 0.01$). The increment in dye accumulation also achieved statistical significance on 1 d (Fig. 7 B, lane 9 vs. lanes 1, 3, and 5; $P < 0.001$). However, when PT67/Nur77-S cells were incorporated in Matrigel plugs in the absence of SKMEL/VEGF cells, dye accumulation increased significantly by approximately twofold on day 1 (Fig. 7 B, lane 11 vs. lanes 1, 3, and 5; $P < 0.001$). Dye accumulation in these plugs increased further at 3 d, similar to that of 3 d plugs containing SKMEL/VEGF cells, alone or with PT67/LacZ cells (Fig. 7 B, lane 12 vs. lanes 4 and 6; $P > 0.05$). These data indicate that inclusion of cells packaging Nur77-S not only induces angiogenesis but does so more rapidly than that induced by SKMEL/VEGF cells, consistent with the activity of Nur77 being downstream that of VEGF-A. The quantitative measurements of vascular plasma volumes presented in Fig. 7 B therefore confirm the qualitative measures of angiogenesis presented in Fig. 7 A.

Inhibition of tumor growth in Nur77-null mice. To determine whether TR3/Nur77 is required for tumor-induced angiogenesis, we studied the growth of B16F1 melanoma in $Nur77^{-/-}$ mice. 5×10^5 tumor cells were injected s.c. in wild-type and $Nur77^{-/-}$ mice, and tumor growth was monitored daily. As shown in Fig. 8, A and B, a, tumors grew to large size in wild-type mice but growth in null mice was greatly inhibited ($P < 0.001$). Inhibition of tumor growth in $Nur77^{-/-}$ mice was associated with reduced angiogenesis and particularly with a reduction in the generation of large mother vessels (Fig. 8 B, b–e). B16F1 melanoma cells are syngeneic in C57Bl6 mice and therefore do not generate a cellular immune response. Nonetheless, because TR3/Nur77 has been implicated in apoptosis of T lymphocytes (8), we made a careful search for inflammatory cells. Small numbers of macrophages were found infiltrating tumors in both wild-type and $Nur77^{-/-}$ mice, but lymphocytes were not found. Therefore, it is unlikely that the inhibition of tumor growth in $Nur77^{-/-}$ mice is attributable to a T lymphocyte defect.

Mechanisms by which Nur77 induces angiogenesis in vivo

Nur77 acts independently of VEGFR-2/KDR. The data presented above provided evidence that the angiogenic

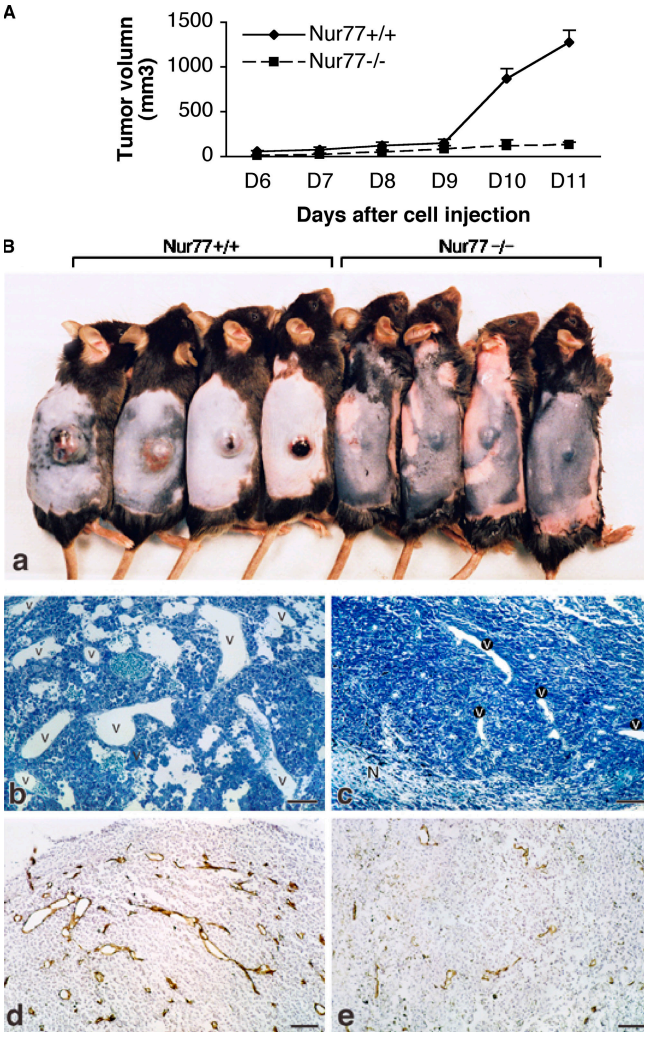

Figure 8. Growth of B16F1 melanoma is inhibited in Nur77$^{-/-}$ mice. (A) Growth of B16F1 melanomas in wild-type and Nur77$^{-/-}$ mice (mean + SD, $n = 4$). (B, a) Gross appearance of tumors at 11 d. Histology (b and c) and CD31 immunohistochemistry (d and e) of B16F1 melanomas growing in wild-type (b and d) and Nur77$^{-/-}$ (c and e) mice at day 11. Tumors in wild-type mice have numerous, large mother vessels (v), whereas vessels in Nur77$^{-/-}$ mice are much less numerous and considerably smaller in size. N, necrosis; bar, 100 μm.

response induced by Nur77 developed earlier than that induced by VEGF-A165, suggesting that Nur77 functioned downstream of VEGF-A165. Nonetheless, it was possible that Nur77 acted in part by inducing VEGF-A expression. To test that possibility, we made use of SU1498, a VEGFR-2/KDR kinase inhibitor that is known to inhibit VEGF-A165–induced angiogenesis (19). In Matrigel assays, SU1498 strongly inhibited the angiogenic response induced by SKMEL/VEGF cells (i.e., by VEGF-A165) but had no inhibitory effect on the angiogenesis induced by PT67/Nur77 cells (Fig. 9, A and B). Thus, the angiogenesis induced by Nur77 was not mediated through VEGFR-2/KDR and therefore not through VEGF-A.

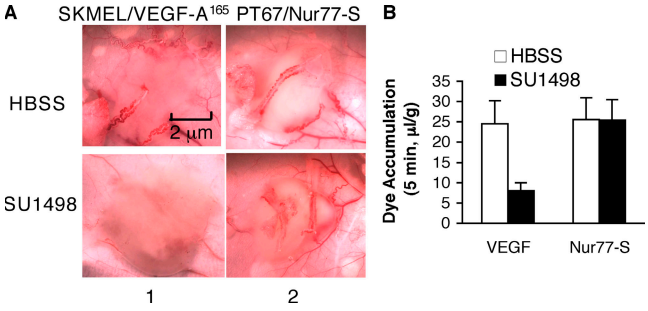

Figure 9. Nur77 functions downstream of VEGFR-2/KDR in Matrigel assays in vivo. (A) SU1498, a VEGFR-2/KDR kinase inhibitor, inhibited angiogenesis induced by VEGF-A^{165}–expressing SKMEL/VEGF-A cells (lane 1) but not that induced by Nur77 (lane 2). (B) Quantitative measurement of intravascular plasma volumes (μl/g) in Matrigels containing indicated cell mixtures ± SU1498 as determined by accumulation of Evan's blue dye administered i.v. 5 min before killing.

Importance of Nur77's transcriptional activity for inducing angiogenesis. TR3/Nur77 is known to be a transcription factor, but at least some of its activities, such as those involving neuron differentiation and T cell apoptosis, occur independently of its transcriptional activity (11). To elucidate the mechanisms by which Nur77 mediates angiogenesis, we tested our Nur77 deletion mutants (Fig. 2 A) in Matrigel assays in vivo. Angiogenesis did not develop in Matrigels that included only PT67 cells packaging Nur77-ΔTAD or Nur77-ΔDBD (Fig. 10). However, PT67 cells packaging Nur77-ΔLBD induced angiogenesis similar to that induced by SKMEL/VEGF cells or by full-length Nur77. Furthermore, inclusion of PT67 cells packaging Nur77-ΔTAD or Nur77-ΔDBD PT67 markedly inhibited the angiogenic response induced by SKMEL/VEGF cells, whereas Nur77-ΔLBD PT67 packaging cells had no inhibitory effect. These data indicate that both the transactivation and DNA binding domains are required for Nur77-mediated angiogenesis but that the LBD is not necessary.

DISCUSSION

TR3 and its mouse (Nur77) and rat (NGFI-B) homologues play important roles in tumor, lymphocyte, and neural

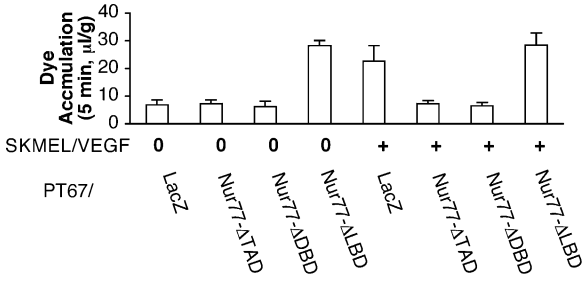

Figure 10. The transactivation and DNA binding domains of Nur77 are required to induce angiogenesis in Matrigel assays in vivo. Quantitative measurement of intravascular plasma volumes (μl/g) in 3 d Matrigels containing indicated cell mixtures as determined by accumulation of Evan's blue dye administered i.v. 5 min before killing.

growth and survival (8, 20–22). TR3/Nur77/NGFI-B is differentially expressed in brain and in many other tissues during development and is constitutively expressed at low levels in several adult tissues (23–27). It is highly induced as an immediate-early response gene in the adult nervous system by growth factors, membrane depolarization, seizures, and by a variety of drugs and other stimuli (27–33). TR3 and/or its mouse or rat homologues are also induced in antigen-induced glomerulonephritis, in the regenerating liver, and by diverse stimuli in a variety of cultured cells (34, 35). Recently it has been implicated in the pathogenesis of hepatocellular carcinoma induced by hepatitis B virus X protein (36) and was found to be one of nine genes whose down-regulation in solid tumors correlated with reduced metastasis (37). In addition to regulating cell growth and differentiation, TR3/Nur77/NGFI-B has also been implicated in apoptosis in several types of cells, including T lymphocytes, the adult retina, and vascular smooth muscle cells, and several carcinoma cell lines (8, 20–22). TR3 has also been described as a death receptor in Alzeimer's disease where it is expressed at high levels in neurons undergoing degeneration (38–41). Despite these important functions, Nur77 null mice lack a developmental phenotype, perhaps reflecting compensation by closely related family members such as NOR1 and NOT (10).

The data presented here extend the role of TR3-Nur77 to a new field, that of VEGF-A–induced pathological angiogenesis. Two isoforms of VEGF-A, but not several other growth factors, induced TR3 expression in cultured vascular endothelial cells (Fig. 1). HUVECs in which TR3 was overexpressed incorporated increased amounts of [^{3}H]thymidine, were protected from apoptosis, and induced several cell cycle genes, all responses that are induced by VEGF-A in control HUVECs (Figs. 3 and 4). On the other hand, overexpression of antisense TR3 prevented HUVECs from responding to VEGF-A in these same assays and greatly diminished HUVEC tube formation on Matrigel. These in vitro findings were extended in vivo to demonstrate that Nur77 was up-regulated in several examples of VEGF-A–mediated angiogenesis, including those induced by Ad-VEGF-A^{164}, wound healing, and the growth of two VEGF-A–expressing tumors, MOT and TA3/St (Fig. 5). In addition, making use of a recently modified Matrigel assay for expressing genes in endothelial cells in vivo, we demonstrated that introduction of Nur77-S DNA induced a typical angiogenic response that was qualitatively and quantitatively equivalent to that induced by VEGF-A–expressing cells (Fig. 7). On the other hand, overexpression of Nur77-AS abrogated the angiogenic response expected from VEGF-A in this assay. The possibility that Nur77 was acting through VEGF-A was excluded by experiments demonstrating that a VEGF-A receptor inhibitor, SU1498, blocked the angiogenic response induced by VEGF-A but not that induced by Nur77 (Fig. 9). These findings, along with the finding that the angiogenic response induced by Nur77 developed earlier than that induced by VEGF-A, indicate that Nur77 acts independently and downstream of VEGF-A. Finally, tumor growth and associated angiogenesis and mother vessel formation were significantly reduced in Nur77 null mice (Fig. 8). Collectively, these data indicate that TR3-Nur77 is necessary and sufficient for VEGF-A–induced angiogenesis, both in vitro and in vivo.

TR3/Nur77/NGFI-B is an orphan member of the steroid/thyroid/retinoid superfamily, whose members act mainly as transcription factors that induce or repress gene expression (10). However, several groups (11, 42, 43) have recently shown that TR3's ability to induce apoptosis in tumor cells is independent of transcription and occurs when TR3 transmigrates from the nucleus to the cytoplasm. Like other members of its superfamily, TR3/Nur77 has three major domains that regulate different functions (Fig. 2). These include NH_2-terminal transactivation and DNA binding domains that are essential for regulating gene transcription, as well as a COOH-terminal LBD. Following the lead of Lin et al. (11), we prepared mutant forms of TR3/Nur77 that deleted each of these domains and transfected each of them into HUVECs. The different mutant forms exerted different effects on HUVECs in different in vitro assays (Figs. 3 and 4). HUVECs transduced to express Nur77 mutants lacking the TAD or DBD domains incorporated [^{3}H]thymidine at normal baseline levels but could not be stimulated to increased incorporation by VEGF-A^{165}. However, TR3-ΔLBD mutants incorporated [^{3}H]thymidine in similar amounts as full-length TR3, both without and with added VEGF-A^{165}.

Overexpression of TR3-S was able to rescue HUVECs from apoptosis as effectively as VEGF-A^{165}. However, TR3 mutants lacking any of the three domains were not able to do so (Fig. 3 B). These data therefore differ from those of Lin et al. (11) who found that the TAD and DBD domains were not required for inducing apoptosis in tumor cells. TR3-ΔLBD HUVECs developed fairly good tube formation on Matrigel, whereas TR3-ΔTAD–transfected HUVECs showed reduced tube formation and TR3-ΔDBD–transfected HUVECs were unable to form tubes; i.e., exhibited a stronger inhibitory response than that of HUVECs transfected with Nur77-AS (Fig. 3 C). Finally, mutant HUVECs lacking the TAD or DBD domains expressed undetectable amounts of cell cycle genes under baseline conditions, and these genes were only minimally induced by the addition of VEGF-A^{165} (Fig. 4). However, ΔLBD mutants behaved much like LacZ transfectants, expressing low levels of cell cycle genes under baseline conditions and greatly increased amounts upon stimulation with VEGF-A^{165}. These data indicate that the transactivation and DNA binding domains of TR3 are required for TR3-mediated HUVEC proliferation, expression of cell cycle genes, and tube formation, functions that are therefore likely attributable to TR3's transcriptional activity. This finding is not unexpected in the case of the ΔDBD mutant that was localized to HUVEC cytoplasm and therefore not in position to perform transcriptional activity (Fig. 2 D). In agreement with these in vitro data, the introduction of PT67 cells packaging Nur77 mutants lacking the

TAD or DBD domains was unable to induce an angiogenic response in the Matrigel assay, either alone or together with SKMEL/VEGF cells. However, PTK67 cells packaging Nur77-ΔLBD, like those packaging Nur77-S, did induce angiogenesis in this assay, even in the absence of SKMEL/VEGF cells. These data indicate that the transactivation domain and the DNA binding domain, but not the LBD, are needed for angiogenesis in vivo and are in agreement with a recent report indicating that the DNA binding and transactivation domains of TR3/Nur77 are required for induction of cell proliferation in lung cancer cells (42).

Collectively, we have shown that TR3/Nur77 has an essential downstream role in VEGF-A signaling in endothelial cells and have begun to dissect the roles of its several domains in regulating different endothelial cell functions. As far as we know, TR3/Nur77 is the first transcription factor found to regulate VEGF-A–mediated angiogenesis. As such, it could provide a useful target for positive and/or negative regulation of pathological angiogenesis.

MATERIALS AND METHODS

Cell culture. HUVEC culture and proliferation assays were performed as described previously (12). In brief, after 24 h of serum starvation, culture was continued with or without the addition of 10 ng/ml VEGF-A^{165} for an additional 20 h, followed by a final 4 h of culture with the addition of [^{3}H]thymidine.

Construction of TR3/Nur77 mutants. TR3 and Nur77 cDNAs were obtained by RT-PCR with RNA isolated from HUVEC and mouse mesentery RNA, respectively. Mutants were generated by PCR-based mutagenesis. After sequence confirmation, DNAs were fused with Flag tag or GFP to create Flag or GFP fusion proteins, respectively.

Endothelial cell tube formation assay. 10^5 HUVECs transduced with LacZ, TR3-S, TR3-AS, TR3-ΔTAD, TR3-ΔDBD, and TR3-ΔLBD cDNAs were seeded on Matrigel with endothelial cell growth medium (Clonetics Co.) and photographed after 16 h. Experiments were repeated three times.

Cell survival assay. HUVECs transduced with LacZ, TR3-S, TR3-AS, TR3-ΔTAD, TR3-ΔDBD, or TR3-ΔLBD were seeded on 100-mm tissue culture dishes. After 2 d, cells were changed to 0.1% BSA in EBM medium, with or without 50 ng/ml VEGF-A^{165}, for 3 d. Cells were trypsinized, washed with PBS, and stained with propidium iodide solution (50 μg/ml propidium iodide in 0.1% sodium citrate–0.1% Triton X-100) for 1 h. Apoptotic cells were quantified by flow cytometry. Experiments were repeated four times.

Quantitative real-time RT-PCR. Applied Biosystems software was used to design optimal primer pairs for real-time RT-PCR and data calculation. The forward and reverse primers for TR3 were 5′-AGCATTATGGTGTCCGCACAT-3′ and 5′-CTTGGCGTTTTTCTGCACTGT-3′, respectively. 5′-/5TET/TGAGGGCTGCAAGGGCTTCTTCAA/36-TAMNph/-3′ served as an internal probe for TR3. The primers for Nur77 were 5′-ATGCCTCCCCTACCAATCTTC-3′ and 5′-CAGTGCTAGGCC CGGAGTC-3′, respectively. The Nur77 internal probe was 5′/5TET/CACTTCCCTCATCCGGGCACA CTT/36-TAMNph/-3′. GAPDH served as an internal control. Experiments were repeated three times, in duplicate.

In vivo angiogenesis models. 4–5-wk-old female Nu/Nu mice (National Institutes of Health [NIH]) were injected in ear skin with 10^8 PFU of nonreplicating adenoviral vectors expressing mouse VEGF-A^{164} (Ad-VEGF-A^{164}) or LacZ (Ad-LacZ; reference 15). Other nude mice received 4-mm punch biopsies on their flanks or were injected i.p. with 10^6 MOT or TA3/St mammary tumor cells (17, 44, 45). Ears and mesenteries were collected at indicated times, and RNA and protein were isolated with the QIAGEN kit or the T-PER tissue protein extraction reagent (Pierce Biotechnology, Inc.), respectively. Experiments were repeated in triplicate.

In situ hybridization and immunohistochemistry. Tissues were harvested, photographed, and fixed in 4% paraformaldehyde. In situ hybridization was performed as described previously (15) using Nur77-S and Nur77-AS probes representing the NH_2-terminal 344 nucleotides. Immunohistochemistry was performed on frozen sections as described previously using rat anti-CD31 antibody (15).

Matrigel angiogenesis assays (18). 10^7 SK-MEL/VEGF cells, alone or mixed with 10^7 PT67 packaging cells infected with retroviruses expressing LacZ, Nur77-S or Nur77-AS cDNAs or Nur77 mutants, were suspended in 0.5 ml of growth factor–reduced Matrigel (BD Biosciences) and injected s.c. into Nu/Nu mice. In some experiments, the KDR inhibitor SU1498 (Calbiochem) was incorporated into Matrigel plugs (40 μg/ml Matrigel) and also injected i.p. daily (1 mg/kg) after Matrigel implantation. Each experiment was replicated on eight mice.

Quantitative analysis of plasma volumes in Matrigel assays. Mice (four per group) implanted with various cell combinations in Matrigel were anesthetized with Avertin (200 mg/kg tribromoethanol) and injected i.v. via the tail vein with 0.2 ml of Evan's blue dye (5 mg/ml in saline). After 5 min, blood was collected in heparin by cardiac puncture and centrifuged at 14,000 rpm for 10 min to obtain platelet-poor plasma, which was diluted in formamide for measurement of Evan's blue dye concentration. Animals were killed by CO_2 narcosis and Matrigel plugs were dissected free by cautery to prevent blood loss, weighed, and extracted with 2 ml formamide at room temperature for 3 d. Dye in plasma or extracted from Matrigels was measured at 620 nM in a Thermo Max microplate reader (Molecular Devices) using Softmax 881 software. Standard curves were generated by measurement of serial dilutions of Evan's blue dye in formamide (μg/ml). Intravascular plasma volumes (μl per gram Matrigel) were calculated on the basis of Evan's blue dye concentrations in blood plasma to provide an absolute measure of the volume of plasma in the vascular bed.

Tumor growth in wild-type and Nur77$^{-/-}$ mice. Nur77$^{-/-}$ mice were provided by J. Milbrandt (Washington University School of Medicine, St. Louis, MO). 5×10^5 B16F1 melanoma cells (American Type Culture Collection) were injected into the flank skin of wild-type (C57BL/6) and Nur77$^{-/-}$ mice. Tumor size was measured daily and animals were killed on day 11 when tumors in control animals had reached a size of nearly 1,500 mm^3. Tumor size was calculated as the product of $\pi/6$ times the measures of the tumor's length, width, and height. Tissues were prepared for 1 μm Giemsa-stained Epon sections and for immunohistochemistry as described previously (15).

Statistics. Analysis of variance and the Tukey-Kramer multiple comparisons test were used to determine statistical significance.

Animal welfare. All animal experiments were performed in compliance with the Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committee.

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