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Differential function and regulation of orphan nuclear receptor TR3 isoforms in endothelial cells

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Abstract

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Compliance with ethical standards

Conflicts of interest

None

Informed consent

Consent was obtained from all individual participants included in the study.

TR3 has been reported to be an excellent target for angiogenesis therapies. We reported three TR3 transcript variant messenger RNAs (mRNAs) are expressed in human umbilical vein endothelial cell (HUVEC) and are differentially regulated by vascular endothelial growth factor (VEGF). TR3 transcript variant 1 (TR3-TV1) and variant 2 (TR3-TV2) encoding the same TR3 isoform 1 protein (TR3-iso1) that was named TR3 has been extensively studied. However, the function of TR3 isoform 2 protein (TR3-iso2) encoded by TR3 transcript variant 3 (TR3-TV3) is still not known. Here, we clone and express the novel TR3-iso2 protein and find that expression of TR3-iso2, in contrast to TR3-iso1, inhibits en-dothelial cell proliferation induced by VEGF-A, histamine, and phorbol-12-myristate-13-acetate (PMA). The differential function of TR3-iso2 correlates with the down-regulation of cyclin D1. However, TR3-iso2 plays similar roles in endothelial cell migration and monolayer permeability as TR3-iso1. We further demonstrate that several intracellular signaling pathways are involved in histamine-induced TR3 transcript variants, including histamine receptor H1-mediated phospholipase C (PLC)/calcium/calcineurin/protein kinase C (PKC)/ protein kinase D (PKD) pathway and ERK pathway, as well as histamine receptor H3-mediated PKC-ERK pathway. Further, expressions of TR3-TV1, TR3-TV2, and TR3-TV3 by VEGF and histamine are regulated by different promoters, but not by their mRNA stability.

Keywords

Angiogenesis; Cell signaling; Cell proliferation; Migration; TR3/Nur77 transcription variants

Introduction

Pathological angiogenesis is a hallmark of many diseases including cancer, inflammation, wound healing, and ischemic heart disease. Anti-vascular endothelial growth factor (VEGF) neutralizing antibodies and VEGF receptor (VEGFR) kinase/ multiple kinase inhibitors have been successfully developed and widely used in the clinic (reviewed in [1]). However, in addition to their toxic side effects [2], VEGF-targeted therapies in cancer face the problems of insufficient efficacy [3–12], resistance, and intrinsic refractoriness [10, 13, 14]. Therefore, it is desirable to identify additional angiogenesis targets. Our recent studies demonstrated that TR3 (human: TR3, mouse: Nur77, rat: NGFI-B) is one such promising target [15–17].

TR3/Nur77 is a member of nuclear receptor IV subfamily of transcription factors, without known physiological ligand [18], although several agonists, cytosporone B and a series of methylene-substituted diindolymethanes, were identified [19, 20]. The nuclear receptor IV subfamily members play redundant roles in TCR-mediated apoptosis [21] and brown fat thermogenesis [22, 23]. However, they play different roles in development (reviewed in [24]). TR3/Nur77 also plays important roles in carcinogenesis, inflammation, metabolism diseases, stress, and addiction (reviewed in [25–28]).

Prior to our recent studies [16], little was known about the role of TR3/Nur77 in angiogenesis. Our studies demonstrated that TR3/Nur77 is a critical mediator of angiogenesis. We found that TR3/Nur77 is highly and transiently up-regulated in cultured endothelial cells (EC) and during angiogenesis in vivo. TR3 is induced by angiogenic factors having microvessel permeable activity, including VEGF, histamine, and serotonin, but not by

angiogenic factors that do not have microvessel permeable activity, including bFGF, PlGF, and PDGF [15–17], and in postnatal angiogenesis, such as tumor angiogenesis and skin wound healing [16, 29]. *In gain of function assays*, overexpression of TR3/Nur77 complementary DNA (cDNA) is sufficient to induce endothelial cell proliferation, migration, and tube formation in vitro. Angiogenesis, microvessel permeability, and normal skin wound healing are improved in our transgenic EC-Nur77-S mice, in which the full-length Nur77 cDNA is inducibly and specifically expressed in mouse endothelium [15–17]. The transgenic EC-Nur77-S mice are healthy after Nur77 has been induced for 3 months [29]. *In loss of function assays*, knockdown of TR3 expression by its anti-sense DNA or short hairpin RNA (shRNA) inhibits endothelial cell proliferation, migration, and tube formation induced by VEGF, histamine, and serotonin in vitro. Tumor growth, angiogenesis, and microvessel permeability induced by VEGF, histamine, or serotonin are almost completely inhibited in Nur77 knockout mice [15–17]. Paradoxically, however, Nur77 null mice are viable, fertile, appear to develop a normal adult vasculature, and have no defect in normal skin wound healing [21, 29]. Our studies demonstrated that TR3/Nur77 is an excellent target for pro-angiogenesis and anti-angiogenesis therapies.

In the attempt to study the signaling pathways that regulate the expression of TR3/Nur77 in angiogenesis, we previously reported that there are three TR3 transcript variant messenger RNAs (mRNAs) expressed in endothelial cells [30]. TR3 transcript variant 1 mRNA (TR3-TV1) and variant 2 mRNA (TR3-TV2) encode the same TR3 isoform 1 protein (TR3-iso1) that was named TR3 in previous studies and has been extensively studied. TR3 transcript variant 3 mRNA (TR3-TV3) encodes TR3 isoform 2 protein (TR3-iso2). These three TR3 transcript variant mRNAs are differentially regulated in endothelial cells by VEGF-A through a novel signaling pathway, in which VEGF-A and VEGF-E, but neither VEGF-B, nor PlGF, induce the interaction of VEGFR2/KDR with insulin-like growth factor-1 receptor (IGF-1R), resulting in IGF-1R transactivation [30]. However, the function of TR3-iso2 is not known. In these studies, we clone and express the novel TR3 isoform 2 (TR3-iso2) protein and find that overexpression of TR3-iso2, in contrast to TR3-iso1, inhibits endothelial cell proliferation induced by VEGF-A, histamine, and phorbol-12-myristate-13-acetate (PMA), which correlates very well with the down-regulation of cyclin D1 regulated by TR3-iso2. However, TR3-iso2 plays similar roles in endothelial cell migration and monolayer permeability, as compared to TR3-iso1. We further demonstrate that TR3 transcript variants are regulated by different signaling pathways, and that TR3-TV3 is regulated by a promoter different from that controls TR3-TV1 and TR3-TV2.

Materials and methods

Materials

VEGF was purchased from R&D Systems (Minneapolis, MN). PMA, histamine, and antibody against Flag tag (Cat. No. F-3165) were purchased from Sigma (St. Louis, MO). Antibodies against TR3/Nur77, cyclin D1, VE-cadherin, β-catenin, γ-catenin, p120, and claudin 5 were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Endothelial cell basal medium (EBM) and EGM-MV BulletKit were obtained from Lonza (Allendale, NJ). Vitrogen 100 was purchased from Collagen Biomaterials (Palo Alto, CA,

USA). BAPTA/AM, cyclosporine-A, W-7, KN62, U-73122, GF, CID2011756, PD98059, SB203580, JNK inhibitor II, AG1024, IP3R inhibitor, 2-APB, and rottlerin are the products of Calbiochem (Billerica, MA, USA). Anti-phospho-IGF-1Rβ antibodies were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). Anti-IGF-1Rα antibody was obtained from EMD Millipore (Billerica, MA, USA).

Cell culture

Primary human umbilical vein endothelial cells (HUVECs) purchased from Lonza (Allendale, NJ) were cultured as described previously [16]. Briefly, cells were grown on plates coated with 30 μg/ml vitrogen in endothelial basic medium (EBM) with EGM-MV BulletKit (5 % fetal bovine serum, 12 μg/ml bovine brain extract, 1 μg/ml hydrocortisone, 1 μl/ ml GA-1000, and 10 ng/ml hEGF) that was purchased from Clonetics (Walkersville, MD). HUVECs (passages 5) were used for all experiments.

Clone and expression of TR3 isoform 2

TR3-iso2 cDNA was cloned by reverse transcription PCR (RT-PCR) with RNA isolated from HUVEC with forward prime rTR3-TV3-110F, 5′-CCTCCACCATGGACAGAGGCCAGG-3′, that starts upstream of the translation starting site ATG in the exon 2, and the reverse primer TR3-TV3-785R, 5′-CTGTGGGGGCCCAGAGGCTTT-3′, that locates in the common region of all three TR3 transcript variants. The 650-bp PCR product was used as the template for the 2nd PCR with forward primer TR3-TV3-1F *Xho*I, 5′-AA TTATCTCGAGATGTGGTTGGCCAAGGCCTG-3′, in which an *Xho*I restriction enzyme digestion site (underlined) was added in the 5′-terminal of the TR3-iso2 ATG, and reverse primer TR3-TV1-5′RACE3-718R, 5′-TCAGAGCCACTGGAGGACAG-3′. The 2nd PCR product was digested with restriction enzyme *Xho*I and *Nco*I. The restriction enzyme *Nco*I recognition site is located in the common region of both TR3-iso1 and TR3-iso2. The digested 2nd PCR product was cloned to retrovirus expressing vector pMF by replacing the N-terminal 1–101 bp of TR3-iso1 cDNA in the pMF-TR3 plasmid [16] to generate the pMF-TR3-iso2 that expressing N-terminal Flag-fused TR3-iso2 protein. The plas-mid was confirmed correct by DNA sequencing. Preparation and infection of retrovirus expressing TR3-iso1 and 2 were carried out as described previously [16].

Proliferation assay

HUVECs (2×10^3 cells/well) were seeded in 96-well plates. Twenty-four hours later, cells were transduced with viruses as indicated. Forty-eight hours later, cells were serum starved with 0.1 % FBS in EBM medium for 24 h and then stimulated with or without VEGF, histamine, and PMA as indicated for 30 h; cells were washed with PBS twice and kept in the −80 °C freezer for several hours. A proliferation assay was carried out with CyQuant Cell Proliferation Assay Kit (Invitrogen, California) following the protocol provided by the producer. Data are expressed as the mean±SD of quadruplicate values.

Monolayer migration and monolayer permeability assays

The HUVEC monolayer migration assay and monolayer permeability assay were carried out as described [17]. Confluent HUVECs that were transduced with viruses as indicated on six-well plates were subjected to serum starvation as above. The monolayer was then wounded with a single pass of a 200-μl pipette tip, washed with PBS, and photographed. Cells then were incubated for 16 h, washed with PBS, and photographed with EVOS digital inverted microscope (Thermo Fisher Scientific, Waltham, MA). Data are expressed as the number of cells migrating into the wounded area (20 views from 3 independent experiments).

The HUVEC monolayer permeability assay was carried out as described [17]. HUVECs (3.5×10^5 per 24 wells) were seeded on 0.3-μm pore size polycarbonate membranes in HTS transwell-24 well plates (Corning Incorporated, Corning, NY). Twenty-four hours later, cells were transduced with viruses as indicated. Two days later, culture medium was replaced. Twenty-four hours later, fluoresceinated-dextran, 70 KDa (Molecular Probes, Eugene, Oregon), was added to the transwell at a final concentration of 0.25 mg/ml. FITC-dextran in the bottom wells was read after 1 h with SoftMax Pro program (exCitation 494 nm, emission 518 nm, cutoff 515 nm) in a Spectra Max M5 instrument (Molecular Devices Corporation, Sunnyvale, CA).

Quantitative real-time RT-PCR

RNA was isolated from cells and reverse transcribed. Real-time RT-PCR for TR3 transcription variants were carried as described previously [30].

mRNA stability assay

Serum-starved HUVEC were pretreated with or without a transcription inhibitor, actinomycin D1 (10 μM), for 15 min and then stimulated with VEGF-A (10 ng/ml) or histamine (10 μM) for 0.5, 1, 2, 4, 6, and 8 h. RNA was isolated and subjected to real-time PCR analysis with the primers that are specific to TR3-TV1, TR3-TV2, and TR3-TV3, respectively, as described in previous publication [30].

Construction of TR3 promoter and TR3-TV3 promoter

TR3 promoter that controls the transcription of TR3-TV1 and TV2 were cloned from genomic DNA isolated from HUVEC with forward primer hTR3-luc-F191-1Kpn (−1958) 5′-AAAAAAGGTACCT GGGCGCAGGCAGTGGTCT flanked with Kpn I restriction size (underlined) and reversed primer hTR3-luc-R2249-1Hind 5′-AAAAAAAAGCTTCCGCGTGCGCTCCCGAAGTT, flanked with *Hin*dIII restriction site (underlined). The PCR products were digested with restriction enzymes *Kpn*I and *Hin*dIII and cloned to pGL3-basic luciferase vector (Promega Corporation, Madison, WI) to generate pGL3-TR3p (−1958). The TR3-TV3p promoter, pGL3-TR3-TV3p (−1200), was cloned in a similar way. The PCR product was obtained with forward primer TR3-TV3p (−1200)(*Kpn*I)F 5′-CCAATTGGTACCGCTGCTCCCCTACAGAGTCA flanked with *Kpn*I restriction size (underlined) and reversed primer TR3-TV3p (−22) R, 5′-TGGGAGGCACAGGTCCTTAG, and then digested with *Kpn*I and *Sac*I restriction enzymes. Oligos TR3-TV3p (−1084 *Sac*I1/2F), 5 ′-CCCTCCCACACAGATCCTTGGGACTCTAAATACTGGCCGAACTCGGCCCC-

CTGAGGCTGTGTCTTCTTGGGGAGCTAAGGACCTG-TGCCTCCCACTGGCCCGGAACCACTTTCTGC and TR3-TV3p (−-1084*Sac*I1R), 5′-TCGAGCAGAAAGTGGTTCCGGGCCAGTGGGAGGC-ACAGGTCCTTAGCTCCCAAGAAGACACAGCCTCAGGGGGCCGA-GTTCGGCCAGTATTTATCCCAAGGATCTGTGTGGGA-GGGAGCT, flanked with overhangs of *Sac*I (grayed) and *Xho*I (underlined) restriction enzymes, respectively, were annealed. The PCR product and the annealed oligonucleotide were cloned to pGL3-basic luciferase vector that was digested with *Kpn*I and *Xho*I restriction enzymes. The plasmids were sequenced correct and used for promoter assay.

Promoter assay

HUVEC were transfected with promoter luciferase constructs (four wells per group) as described previously [29]. HUVECs (6×10^4 cells/well) were seeded in a 12-well plate. Twenty-four hours later, cells were washed with minimum essential media (MEM; Life Technologies, Grand Island, NY, USA) three times and transfected with promoter luciferase construct (four wells per group). TR3 and TR3-TV3 promoter construct (990 ng/well), pGL3-TR3p (−1958) and pGL3-TR3-TV3p (−1200), were added to 80 μl MEM, respectively. pRT-SV40 luciferase vector, serving as internal control (10 ng/well), was added to 20 μl MEM, which was added to promoter construct. TransIT2020 (1 μl; Mirus Bio Limited Liability Corporation, Madison, WI, USA) was then added to the DNA mixture. The transfection mixture was incubated at room temperature for 20 to 30 min and added to cells. Four hours later, cells were changed to EBM. Sixteen hours after transfection, cells were stimulated with or without VEGF or histamine. Six hours later after stimulated by VEGF or histamine, cells were lysed and subjected to luciferase analysis with Dual-Luciferase Reporter Assay System (Promega Corporation, Madison, WI, USA), following the instruction provided by the company. The luciferase activity in each well was normalized to the internal luciferase activity.

Statistical analysis

Results are presented as mean±SD. Student's *t* test was employed to determine statistical significance. For signaling pathway studies, one-way ANOVA was used to determine significance. *p* values less than 0.05 were considered to be statistically significant.

Results

Cloning and expression of TR3 isoform 2 protein encoded by TR3-TV3 in HUVEC

TR3 transcript variant 1 (TR3-TV1) consists of exons 3–10, lacking of exons 1 and 2, whereas TR3 transcript variant 2 (TR3-TV2) lacks exons 1, 2, and 4, and is composed of exons 3 and 5–10. TR3 transcript variant 3 (TR3-TV3) contains exons 1, 2, and 5–10, without exons 3 and 4 (Fig. 1a). TR3-TV1 and TR3-TV2 encode the same 59.8-KDa TR3-isoform 1 (TR3-iso1) protein with translation starting site ATG locates in exon 5, whereas TR3-TV3 uses a translation starting site in exon 2, resulting in a 61.2-KDa TR3-isoform 2 (TR3-iso2) protein with 13 amino acids longer than TR3-iso1 protein (Fig. 1a). Except our most recent report [30], all of the studies about TR3 have been obtained with cDNA encoding the TR3-iso1 (TR3 was named in all of the previous publication). Nothing was

known about the function of TR3-iso2. In order to study the function of TR3-iso2, we clone the TR3-iso2 cDNA by RT-PCR with RNA isolated from HUVEC with forward primer that starts upstream of the translation starting site ATG in the exon 2 and the reverse primer TR3-TV3-785R that locates in the common region of all three TR3 transcript variants (Fig. 1a). The 650-bp PCR product was used to clone the open reading frame of TR3-iso2 to retrovirus expressing vector pMF [16] to generate the pMF-TR3-iso2 that expresses N-terminal Flag-fused TR3-iso2 protein as described in detail in “Materials and methods” (Fig. 1b). HUVECs were transduced with or without viruses expressing Lac Z, pMF-TR3-iso2, or pMF-TR3-iso1. Cellular extracts were subjected to immunoblotting with antibodies against the common region of TR3 isoforms and Flag tag. Exogenous Flag-fused TR3-iso2 is detected by antibodies against Flag and TR3 with appearance molecular weight lower than that of TR3-iso1 (Fig. 1c). Our results demonstrate that TR3-iso2 is endogenously expressed in and successfully cloned from HUVEC.

TR3 isoforms differentially regulate HUVEC proliferation, but not migration and monolayer permeability

Previously, we reported that TR3-iso1 regulates the proliferation, migration, and permeability of HUVEC [16, 17]. We would like to test whether TR3-iso2 plays similar roles as TR3-iso1. HUVECs were transduced with viruses expressing Lac Z as control, TR3-iso2, or TR3-iso1. After 2 days, cells were serum starved for proliferation assay. As reported previously, expression of TR3-iso1 significantly increases proliferation as compared to the Lac Z control in HUVEC stimulated with or without VEGF-A (Fig. 2a, 5 vs. 1 and 6 vs. 2, both $*p<0.01$). However, expression of TR3-iso2 has no effect on HUVEC proliferation in the absence of stimulation (Fig. 2a, 3 vs. 1, $*p>0.05$), but inhibits HUVEC proliferation induced by VEGF-A (Fig. 2a, 4 vs. 2, $*p<0.01$). Then, we study whether TR3-iso2 regulates HUVEC migration and permeability. In migration assay, HUVEC were transduced with Lac Z as control, TR3-iso2, or TR3-iso1. Two days after transduction, cells were serum starved for 24 h and wounded with a pipette tip. Twenty-four hours after wounding, cells were fixed and cells migrated to wound area were counted. As shown in Fig. 2, TR3-iso2 induces cell migration, the same as TR3-iso1 (Fig. 2b, 2 and 3 vs. 1, $*p<0.0001$). In monolayer permeability, HUVECs were seeded on 0.3-μm pore size polycarbonate membranes and transduced with Lac Z as control, TR3-iso2, or TR3-iso1 after 24 h. Three days after transduction of viruses, permeability was assayed with fluoresceinated-dextran (70 KDa). The results clearly show that, like TR3-iso1, TR3-iso2 is able to induce cell permeability (Fig. 2c, 2 and 3 vs. 1, $* p<0.001$). These data clearly demonstrate that TR3-iso2 inhibits HUVEC proliferation induced by VEGF-A, but induces cell migration and permeability.

Previously, we reported that TR3-iso1 induces cell proliferation by up-regulation of cyclin D1 [16]. We further study whether TR3-iso2 inhibits the expression of cyclin D1. HUVECs were transduced with Lac Z as control, TR3-iso2, and TR3-iso1. Two days later, cells were serum starved for 24 h and cellular extracts were subjected to immunoblotting with an antibody against cyclin D1. The data show that TR3-iso2 inhibits, while TR3-iso1 increases, the expression of cyclin D1 (Fig. 2d, panel 3). We also reported that TR3-iso1 regulates cell migration and permeability by down-regulation of proteins in VE-cadherin-associated adherences, including VE-cadherin, β-catenin, γ-catenin, p120, and claudin 5 [17].

Immunoblotting analysis with cellular extracts isolated from HUVEC transfected with Lac Z as control, TR3-iso2, and TR3-iso1 shows that expression of VE-cadherin, β-catenin, γ-catenin, p120, and claudin 5 are down-regulated by both TR3-iso1 and TR3-iso2 (Fig. 2d, panels 4–8). The data indicate that TR3-iso2, in contracts to TR3-iso1, decreases the expression of cyclin D1, but down-regulates the expression of junctional proteins, similar to TR3-iso1.

TR3-iso2 in HUVEC stimulated with histamine

Recently, we reported that expression of TR3-iso1 is required for the angiogenesis and microvessel permeability induced by histamine and serotonin [15, 17]. We would like to study whether TR3-iso2 plays a role in histamine-induced angiogenesis. Serum-starved HUVECs were stimulated with or without histamine (10 μM) for 1, 2, and 4 h; RNA was isolated and subjected to real-time PCR analysis with the primers that are specific to TR3-TV1, TR3-TV2, and TR3-TV3, respectively. As shown in Fig. 3, TR3-TV1, TR3-TV2, and TR3-TV3 are induced ~3-, ~120-, and 6-fold, respectively (Fig. 3a, left panel, 5 vs. 6 and 7, $*p<0.05$, right panel, 1 vs. 2 and 3; 5 vs. 6 and 7, all * $p<0.05$). Further, the induction of TR3-TV1 and TR3-TV2 peaks at 1 h after histamine stimulation, but that of TR3-TV3 peaks at 2 h.

We further study whether TR3-iso2 regulates HUVEC proliferation stimulated by histamine. HUVEC were transduced with viruses expressing Lac Z as control, TR3-iso2, or TR3-iso1. After 2 days, cells were serum starved and stimulated with histamine. Similar to its effect on VEGF-A stimulation, expression of TR3-iso2 inhibits HUVEC proliferation induced by histamine (Fig. 3b, 4 vs. 2, $*p<0.01$), while expression of TR3 isoform 1 increases, as reported previously, HUVEC proliferation in the presence and absence of histamine (Fig. 3b, 5 vs. 1 and 6 vs. 2, both * $p<0.001$). Our data showed that TR3-TVs are differentially up-regulated by histamine and that TR3-iso1 and TR3-iso2 play opposite roles in HUVEC proliferation induced by histamine.

Up-regulation of TR3-TV2 and TR3-TV3 by histamine are mediated by various signaling pathways

Most recently, we reported that histamine receptor 1 mediates histamine-stimulated HUVEC proliferation, migration, tube formation in vitro, and angiogenesis in vivo, while histamine receptor 2 mediates proliferation, tube formation, and angiogenesis, but not migration [15]. We test which histamine receptors mediate the expression of TR3-TV2 and TR3-TV3 induced by histamine. Because TR3-TV1 and TV2 encode the same protein and TR3-TV1 expression level is low and is not significantly up-regulated by histamine in HUVEC, we study the signaling pathways by which histamine regulates the expression of TR3-TV2 and TR3-TV3 with real-time PCR. Serum-starved HUVEC were treated with or without histamine receptor antagonists for 10 min and then stimulated with histamine (10 μM) for 1 h. Data in Fig. 4a show that the histamine receptor 1 antagonist almost completely inhibits the expression of TR3-TV2 and TR3-TV3, the histamine receptor 2 antagonist partially inhibits TR3-TV3 expression, but not TR3-TV2 expression, while the histamine receptor 3 antagonist partially inhibits both TR3-TV2 and TR3-TV3 expressions (Fig. 4a, 1 vs. 3; 2 vs. 4, 6, and 8, all * $p<0.05$).

Most recently, we reported that IGF-1R is required for the up-regulation of TR3-TV2 induced by VEGF via its interaction with KDR [30]. We determine whether IGF-1R is involved in the up-regulation of TR3 transcript variants induced by histamine. Two approaches are used, including a pharmacological IGF-1R kinase inhibitor AG1024 and an IGF-1R shRNA (shIGF-1R2) that targets the sequence of 617–635 of IGF-1R mRNA and was reported to knockdown both IGF-1Rα and IGF-1Rβ [30, 31]. Cellular extracts isolated from HUVEC that were transduced with shIGF-1R2 were subjected to immunoblotting with an antibody against IGF-1R. shIGF-1R2 knocks down the expression of IGF-1R (Fig. 4b). HUVEC were treated with or without IGF-1R kinase inhibitor AG1024 or transduced with shIGF-1R2 and then stimulated with histamine. Real-time PCR data show that AG1024 and shIGF-1R2 are unable to inhibit the up-regulation of TR3-TV2 and TR3-TV3 induced by histamine (Fig. 4c, 1 vs. 3 and 5; 2 vs. 4 and 6, all $p>0.05$). In order to confirm that AG1024 and shIGF-1R2 are functional, HUVEC were treated with or without AG1024, or transduced with shIGF-1R2 and then stimulated with VEGF. Real-time PCR data show that AG1024 and shIGF-1R2 significantly inhibit the up-regulation of TR3-TV2, but not TR3-TV3, induced by VEGF-A (Fig. 4c, 7 vs. 9 and 11; 8 vs. 10 and 12, all $*p<0.05$). To further confirm that histamine signaling, in contrast to VEGF signaling [30], does not require IGF-1R, we studied whether histamine cannot induce the phosphorylation of IGF-1R. Serum-starved HUVEC were stimulated with histamine and VEGF-A for 1, 2, and 5 min. Cellular extracts were analyzed by immunoblotting with an antibody against phosphorylated IGF-1Rβ. As shown in Fig. 4, while VEGF-A induces IGF-1R phosphorylation as reported most recently [30], histamine is unable to induce the phosphorylation of IGFR-1R (Fig. 4d).

Because expression of TR3-TV2 and TR3-TV3 by VEGF-A is regulated by different signaling pathways [30], we further study the signaling pathways, by which histamine regulates the expression of TR3-TV2 and TR3-TV3. Serum-starved HUVEC were treated with the inhibitors of several common signaling pathways as indicated and then stimulated with histamine (10 μM). Data in Fig. 4e indicate that BAPTA/AM, an inhibitor that inhibits intracellular Ca^{2+} releasing, and CYA, a calcineurin inhibitor, completely inhibit the expression of TR3-TV2 and TR3-TV3 expression induced by histamine (1 vs. 3 and 5; 2 vs. 4 and 6, all $* p<0.05$). However, calmodulin inhibitors, W-7 and KN62, have no effect on the expression of TR3-TV2 and TR3-TV3 induced by histamine (1 vs. 7 and 9; 2 vs. 8 and 10, all $p>0.05$). Further, phospholipase C (PLC) inhibitor, U-73122, protein kinase C (PKC) inhibitors, GF and PMA, protein kinase D (PKD) inhibitor CID, and MEK inhibitor, PD98059, almost completely inhibit the expression of TR3-TV2 and TR3-TV3 induced by histamine (1 vs. 11, 13, 15, 17, and 19; 2 vs. 12, 14, 16, 18, and 20, all $* p<0.05$). However, IκB shRNA (shIκB) inhibits the expression of TR3-TV2 (1 vs. 21, $* p<0.05$), but inhibitors of JNK and p38 inhibit the expression of TR3-TV3 induced by histamine (2 vs. 24 and 26, $* p<0.05$), respectively. Our data demonstrate that several intracellular signaling pathways are involved in histamine-induced TR3 transcript variants, including H1-mediated PLC/calcium/calcineurin/PKC/PKD pathway and ERK pathway, as well as H3-mediated PKC-ERK pathway.

TR3 transcript variants function in HUVEC stimulated with protein kinase C activator PMA

Since inhibition of PKC blocks histamine-induced TR3 transcript variant expression, we then determine the direct effect of PKC activator PMA on TR3 transcript variants. The data show that PMA (1 mM) induces TR3 transcript variant expression about 79-fold, 443-fold, and 82-fold, respectively, similarly to those induced by histamine and VEGF (Fig. 5a, 1 vs. 3 and 4; 6 vs. 8 and 9; 11 vs. 13 and 14, all * $p<0.05$). Then, we study whether TR3-iso2 plays a role in HUVEC proliferation induced by PMA. HUVEC were transduced with viruses expressing Lac Z as control, TR3-iso2, or TR3-iso1. After 2 days, cells were serum starved and stimulated with PMA for 24 h. Similar to its effect on the stimulation by VEGF and histamine, expression of TR3-iso2 inhibits HUVEC proliferation induced by PMA (Fig. 5b, 4 vs. 2, * $p<0.01$). However, overexpression of TR3 isoform 1 increases the proliferation of HUVEC in the presence or absence of PMA (Fig. 5b, 5 vs. 1; 6 vs. 2, both * $p<0.001$). We further study the signaling pathways that mediate the up-regulation of TR3-TVs induced by PMA. Serum-starved HUVEC were treated with 2-APB (IP3R inhibitor) and rottlerin (PKCdelta inhibitor), and then stimulated with PMA for 1 h. As shown in Fig. 5, 2-APB and rottlerin greatly, inhibit the up-regulation of TR3-TV1, TR3-TV2, and TR3-TV3 induced by PMA (Fig. 5c, 1 vs. 4 and 7; 2 vs. 5 and 8; 3 vs. 6 and 9, all * $p<0.05$). These data further confirm that TR3 transcript variants are regulated by PKC pathway and demonstrate that overexpression of TR3-iso2 inhibits HUVEC proliferation induced by PMA.

VEGF-A and histamine regulate the promoter activities, but not the mRNA stability of TR3 transcription variants

Because TR3-TVs are induced rapidly, we would like to study whether TR3-TVs are regulated by mRNA stability or transcription. Serum-starved HUVEC were pretreated with or without a transcription inhibitor, actinomycin D1, for 15 min and then stimulated with VEGF-A (10 ng/ml) or histamine (10 μM) for 0.5, 1, 2, 4, 6, and 8 h, respectively. RNA was isolated and subjected to real-time PCR analysis with the primers that are specific to TR3-TV1, TR3-TV2, and TR3-TV3, respectively. As shown in Fig. 6, up-regulation of TR3-TV1, TV2, and TV3 are completely inhibited by the treatment of actinomycin D1 (Fig. 6a, * $p<0.05$). We then constructed the luciferase reporters, pGL3-TR3p and pGL3-TR3-TV3p, containing the 1958- and 1200-bp promoter regions upstream of the TR3-TV1 and TR3-TV3 transcription sites, respectively. HUVEC were transfected with plasmids pGL3-TR3p (−1958) and pGL3-TR3-TV3p (−1200), together with internal luciferase control and then stimulated with or without VEGF-A or histamine. The luciferase activities were normalized to internal control. TR3-TV1 and TR3-TV3 promoter activities are up-regulated by VEGF-A or histamine (Fig. 6b, 1 vs. 2 and 3; 4 vs. 5 and 6, all * $p<0.001$). Our data demonstrate that TR3 transcription variants are regulated by different promoters, but not by their mRNA stability.

Discussion

In the present study, we successfully clone TR3 isoform 2 cDNA and express it in HUVEC (Fig. 1). In contrast to TR3-iso1, overexpression of TR3-iso2 inhibits HUVEC proliferation induced by VEGF (Fig. 2a), histamine (Fig. 3b), and PMA (Fig. 5b). Further, expression of TR3-iso2 down-regulates cyclin D1 expression in HUVEC (Fig. 2d). However, TR3-iso2

induces cell migration, permeability, and down-regulation of the proteins in the VE-cadherin-associated adherence, similar to TR3-iso1 (Fig. 2b–d). TR3 transcription variants are differentially regulated by histamine via a signaling pathway of PLC/intracellular Ca^{2+}/calcineurin/PKC/PKD mediated by G protein-coupled histamine receptor H1, and independent of IGF-1R, in HUVEC (Figs. 3a and 4). Moreover, VEGF and histamine up-regulate TR3 transcript variants by their promoter activities, but not by their mRNA stability (Fig. 6).

We find that overexpression of TR3-iso2 plays opposite roles in cell proliferation in endothelial cells, although we are unable to identify, despite extensive effort, the shRNAs that specifically knockdown the expression of each TR3 transcript variants due to their short different regions (data not shown). According to previous publications, TR3/Nur77 exhibits several different functions depending on the cell type and the nature of the stimulus. TR3/Nur77 was initially characterized as a growth factor-inducible gene to regulate fibro-blasts growth induced by mitogenic serum growth factors [32, 33] and pheochromocytoma cell line PC-12 differentiation by nerve growth factor (NGF) and membrane depolarization [34, 35]. Later, it was reported that TR3/Nur77 is required for the growth factor-induced proliferation in lung cancer cells [36]. We were the first to report that TR3/Nur77 is a pro-angiogenic factor to regulate endothelial cell proliferation, migration, and microvessel permeability [16]. Paradoxically, however, Nur77 null mice are viable, fertile, appear to develop a normal adult vasculature, and have no defect in normal skin wound healing [21, 29]. However, Liu et al. and Woronicz et al. first reported a role for TR3/Nur77 in apoptosis; Nur77-expressing transgenic mice show massive thymocyte apoptosis [37, 38]. Further, deoxycholic acid-induced colon carcinogenesis is increased in Nur77−/− mice [39]. It was suggested that these paradoxical effects are due to functional redundancy among the TR3/Nur77 family members or the different subcellular localizations of Nur77 [40]. However, our data that TR3 isoforms play opposite roles in the proliferation of endothelial cells raise another possibility for the TR3/Nur77 paradoxical functions, which leads to a new direction for studying TR3/ Nur77 function in angiogenesis.

Our data that the appearance molecular weight of TR3-iso2 is lower than that of TR3-iso1 may be due to the differential phosphorylation level of these two TR3 isoforms. TR3-iso2 plays an opposite role of TR3-iso1 in proliferation, but a similar role in migration and monolayer permeability, suggesting that differential phosphorylation level of TR3 isoforms is required for HUVEC proliferation, but not migration and monolayer permeability. In the future, we will further study the structural and functional relationship of TR3 isoforms to elucidate the molecular mechanism, by which TR3 isoforms regulate cell proliferation, migration, and monolayer permeability.

Histamine is a biogenic amine with multiple functions in vivo and in cultured cells [41–45]. Many studies have implicated that histamine plays important roles in pathologic angiogenesis. Our previous research demonstrated that histamine is not only a vascular permeable agent, but an angiogenic factor that induces endothelial cell proliferation, migration, tube formation in vitro, and angiogenesis in vivo [15, 17]. Our current research further explores the role of histamine in regulation of TR3-T *Vs.* Similar to VEGF, histamine up-regulates TR3 transcript variants differentially with much higher induction of TR3-TV2

than those of TR3-TV1 and TR3-TV3. The diverse bioactivities of histamine are mediated via various receptors, H1, H2, and H3, that are expressed on a multitude of cell types (reviews in [46, 47]). Histamine H1 receptor and H2 receptor play important roles in angiogenesis [48]. Our previous studies showed that histamine mediates microvascular permeability and angiogenesis through TR3 induction [15, 17]. Here, our data show that up-regulation of TR3-TV2 by histamine is completely or partially inhibited by histamine H1 and H3 receptor antagonists, but not by H2 antagonist. Up-regulation of TR3-TV3 by histamine is inhibited completely by H1 antagonist, but partially by H2 and H3 antagonists, further confirming our results that TR3 isoform 1 and isoform 2 play opposite roles in cell proliferation.

It is well known that histamine triggers microvessel permeability by binding to Gq-couple H1 receptor to activate the downstream pathway of PLC, Ca^{2+} releasing, and protein kinase C [49, 50]. Lahouaria Hadri et al. reported that histamine enhances the promoter activity and expression of SERCA 3 gene via H1 receptor by Ca^{2+}-calcineurin pathway in HUVEC-derived EA.hy926 cells [51]. Further studies suggested that PKD and ERK are downstream targets of PLC-PKC to play a role in histamine signaling pathway because (1) histamine induces tissue factor expression via H1 receptor to activate PKC-PKD phosphorylation in smooth muscle cells [52], (2) histamine induces the expression of early growth response factor 1 (Egr-1) by PKC-ERK, and (3) ERK is essential for histamine-induced cytokine production [53, 54]. Our data indicate that TR3-TV2 and TR3-TV3 are regulated by PLC/Ca^{2+}/calcineurine/PKC/PKD/ERK signaling pathway via G protein-coupled histamine receptor H1. So far, there are a few reports about the histamine H3 receptor in angiogenesis. It was reported that histamine H3 receptor antagonist inhibits the growth of cholangiocarcinoma in vitro and in vivo by increase phosphorylation of PKC and ERK [55]. Our study provides new information about histamine receptor H3 because histamine regulates TR3-TV3 expression via H3 receptor. In our current study, we find that several intracellular signaling pathways are involved in the histamine-induced expression of TR3 transcript variants, including H1-mediated PLC/calcium/calcineurin/PKC/PKD pathway and ERK pathway, and H3-mediated PKC-ERK pathway.

TR3/Nur77 is an orphan nuclear receptor, without physiological ligand, which activities are regulated at transcriptional [24, 56, 57] and post-transcriptional levels [58–60]. In the presence of actinomycin D1, up-regulation of TR3 variants by VEGF and histamine are completely inhibited, indicating that these three TR3 transcript variants are regulated by transcription, which are further confirmed by the promoter luciferase studies. The induction of luciferase activity is much lower than that of mRNA, which may be due to two possibilities: (1) the basal level of luciferase activity is high because transfection of luciferase receptors was carried in the cells without serum starvation, but the mRNA assays were carried out in the cells that were serum starved for 24 h, and (2) there are trans-activating element(s) in the other region(s), such as intron(s), which plays a role in the up-regulation of TR3 transcription variants.

Together, our studies indicate that, similar to VEGF, histamine significantly induces TR3 transcript variants by increasing their transcription. By cloning and expressing TR3-iso2, we find that TR3-iso2 plays an opposite role to TR3-iso1 in regulating HUVEC cell

proliferation. These differential responses are consistent with their effects on cyclin D1 expression. The findings provide new information for therapeutic application of TR3/Nur77 by targeting its isoforms.

Acknowledgments

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Abbreviations

VEGF	Vascular endothelial growth factor
HUVEC	Human umbilical vein endothelial cells
TR3-TV1	TR3 transcript variant 1 mRNA
TR3-TV2	TR3 transcript variant 2 mRNA
TR3-TV3	TR3 transcript variant 3 mRNA
TR3-iso1	TR3 isoform 1 protein
TR3-iso2	TR3 isoform 2 protein
IGF-1R	Insulin-like growth factor-1 receptor
VEGFR	VEGF receptor
shRNA	Short hairpin RNA

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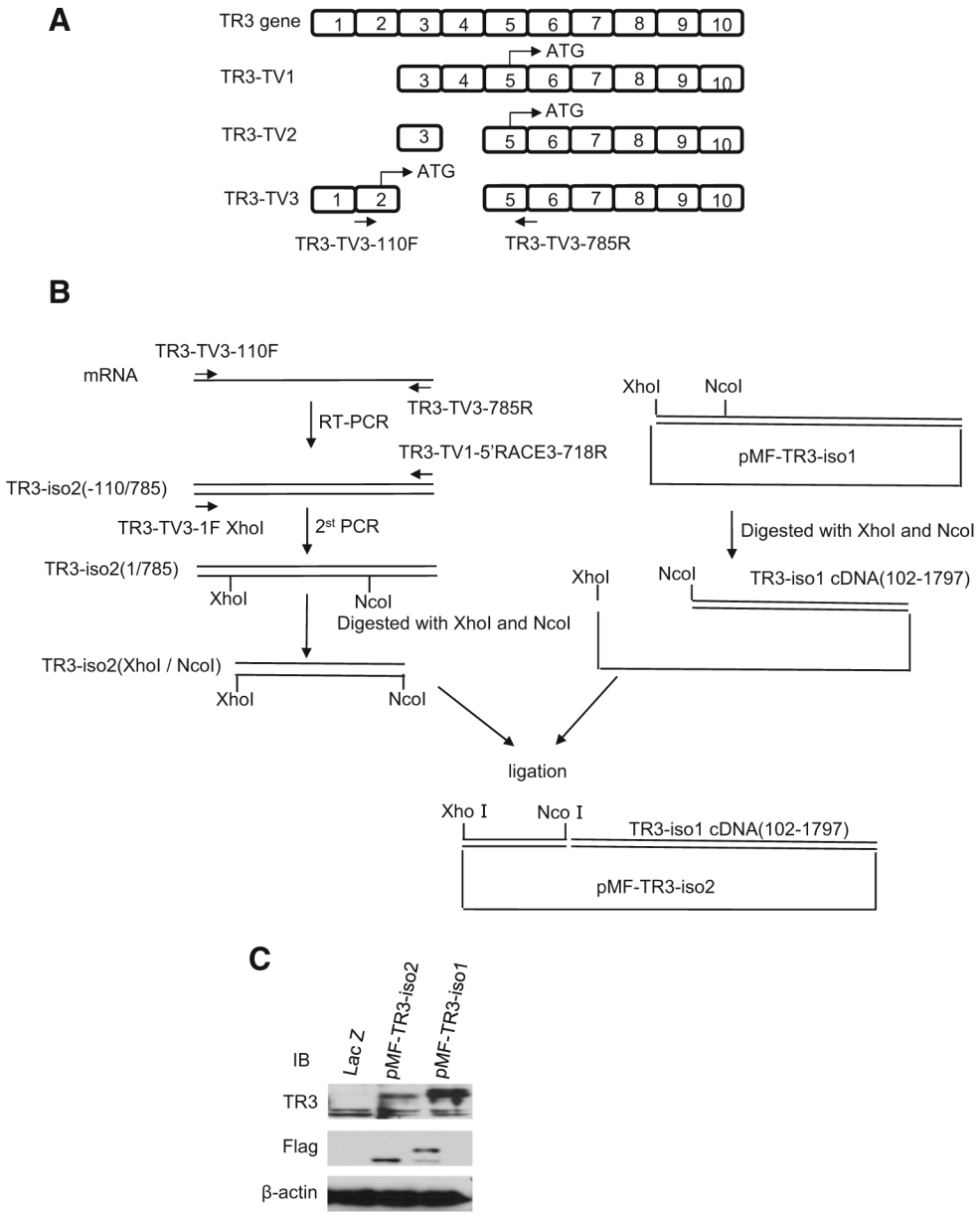

Fig. 1.

Cloning and expression of TR3-iso2 encoded by TR3-TV3 in HUVEC. **a** Schematic representation of TR3-TVs; **b** schematic representation of cloning TR3-iso2 cDNA; **c** cellular extracts isolated from HUVEC transduced with Lac Z, as control, Flag-TR3-iso 2, and Flag-TR3-iso1 were immunoblotted with antibodies against TR3 (*top panel*), Flag (*middle panel*), and β-actin for protein equal loading control (*bottom panel*). Experiments were repeated three times

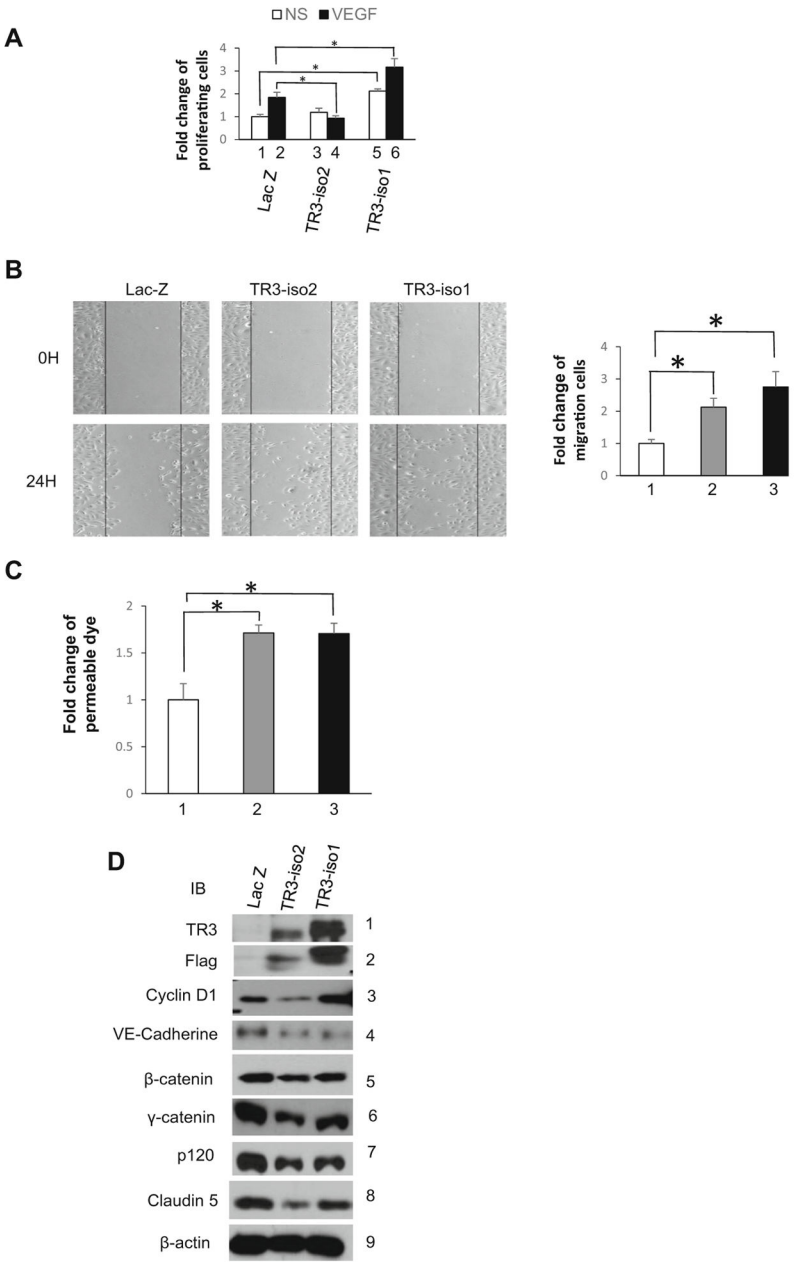

Fig. 2.

TR3 isoforms differentially regulate HUVEC proliferation, but not migration and monolayer permeability. HUVEC were transduced with Lac Z, as control, Flag-TR3-iso2, and Flag-TR3-iso1. After serum starvation, cells were subjected to proliferation assay (n=6) (**a**), migration assay (n=4 views) (**b**), and monolayer permeability assay (n=4) (**c**); **d** cellular extracts isolated from HUVEC transduced with Lac Z, as control, Flag-TR3-iso2, and Flag-TR3-iso1 were immunoblotted with antibodies against TR3 (*panel 1*), Flag (*panel 2*), cell cycle protein, cyclin D1 (*panel 3*), junctional proteins, VE-cadherin, β-catenin, γ-catenin, p120, and claudin 5 (*panels 4–8*), and β-actin for protein equal loading control (*panel 9*). Experiments were repeated three times (*p<0.05)

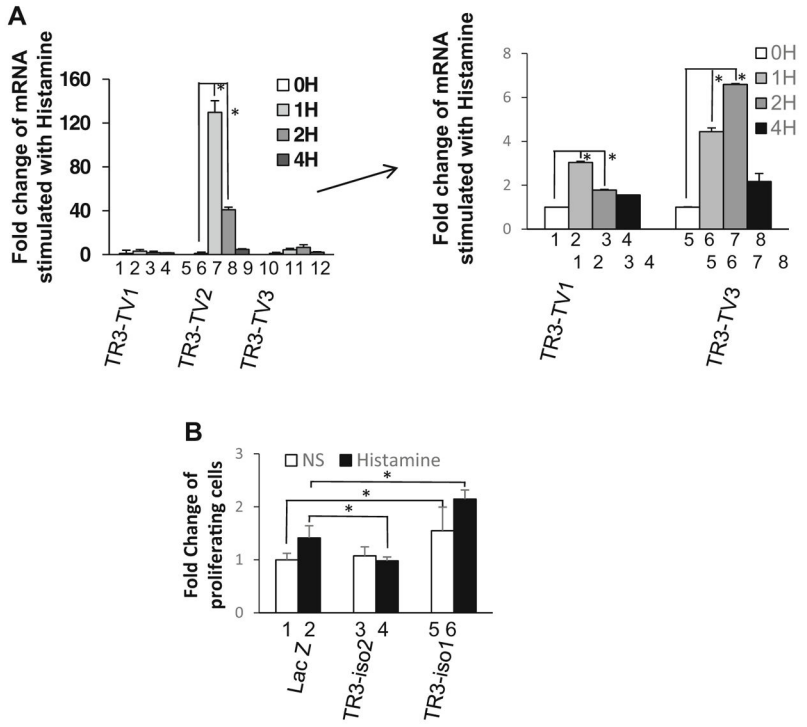

Fig. 3.

TR3 isoform 2 in HUVEC stimulated with histamine. **a** RNA isolated from serum-starved HUVEC that were stimulated with or without histamine for 1, 2, and 4 h were subjected to real-time RT-PCR with specific primers of TR3-TV1, TR3-TV2, and TR3-TV3. *Right panel* is the amplification of boxes in the *left panel* (n=2 for real-time PCR); **b** serum-starved HUVEC that were transduced with Lac Z, as control, Flag-TR3-iso2, and Flag-TR3-iso1 were stimulated with or without histamine for cell proliferation assay (n=6). Experiments were repeated three times (*p<0.05)

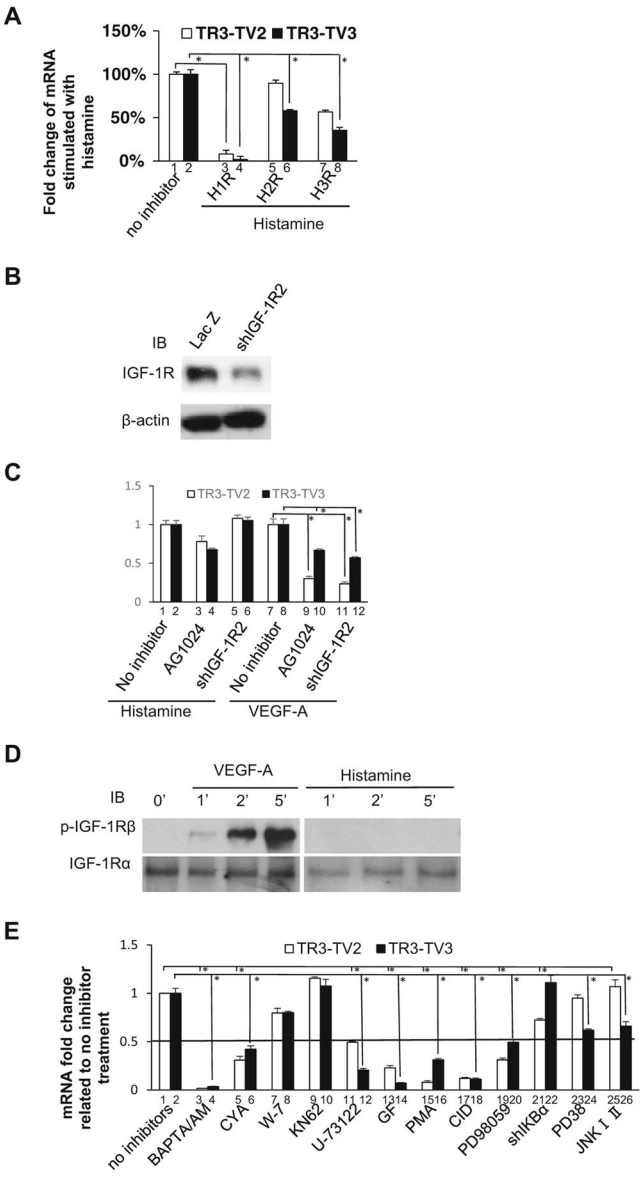

Fig. 4.

TR3-TV2 and TR3-TV3 induced by histamine are mediated by different signaling pathways. **a** Serum-starved HUVEC were pretreated with or without histamine receptor antagonists for 10 min and then stimulated with histamine for 1 h. RNA were isolated and subjected to real-time RT-PCR with specific primers of TR3-TV2 and TR3-TV3 (*n*=2 for real-time PCR); **b** cellular extracts isolated from HUVEC transduced with Lac Z, as control, and shIGF-1R2 were immunoblotted with antibodies against IGF-1R (*top panel*) and β-actin for protein equal loading control (*bottom panel*); **c** serum-starved HUVEC were pretreated with IGF-1R inhibitor, AG1024, or transduced with shIGF-1R2, and then stimulated with histamine or VEGF as indicated. RNA were isolated and subjected to real-time RT-PCR with specific primers of TR3-TV2 and TR3-TV3 (*n*=2 for real-time PCR); **d** cellular extracts isolated from serum-starved HUVEC that were induced with VEGF-A or histamine for 1, 2, and 5

min were immunoblotted with antibodies against phosphorylated IGF-1Rβ (*top panel*) and IGF-1Rα for protein equal loading control (*bottom panel*); **e** serum-starved HUVEC were pretreated with or without inhibitors as indicated for 10 min and then stimulated with histamine for 1 h. RNA were isolated and subjected to real-time RT-PCR with specific primers of TR3-TV2 and TR3-TV3 (n=2 for real-time PCR). Experiments were repeated three times (*p<0.05)

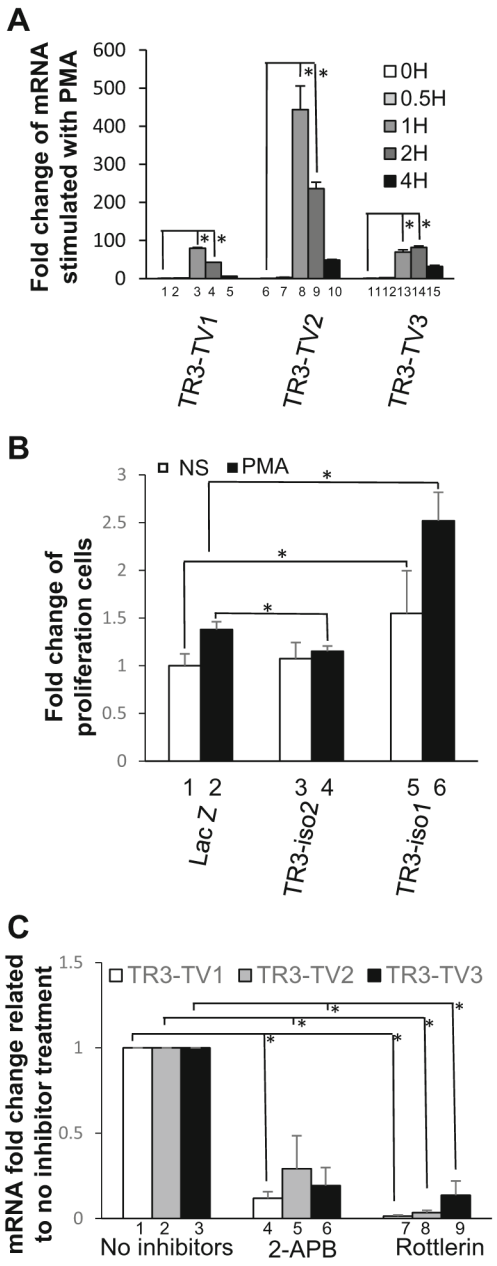

Fig. 5.

TR3 transcript variants function in HUVEC stimulated with protein kinase C activator PMA. **a** RNA isolated from serum-starved HUVEC that were stimulated with or without PMA for 1, 2, and 4 h were subjected to real-time RT-PCR with specific primers of TR3-TV1, TR3-TV2, and TR3-TV3 (n=2 for real-time PCR); **b** serum-starved HUVEC that were transduced with Lac Z, as control, Flag-TR3-iso2, and Flag-TR3-iso1 were stimulated with or without PMA for cell proliferation assay (n=6); **c** serum-starved HUVEC were pretreated with or without inhibitors as indicated for 10 min and then stimulated with PMA for 1 h. RNAwere isolated and subjected to real-time RT-PCR with specific primers of TR3-TV2 and TR3-TV3 (n=2 for real-time PCR); experiments were repeated three times (*p<0.05)

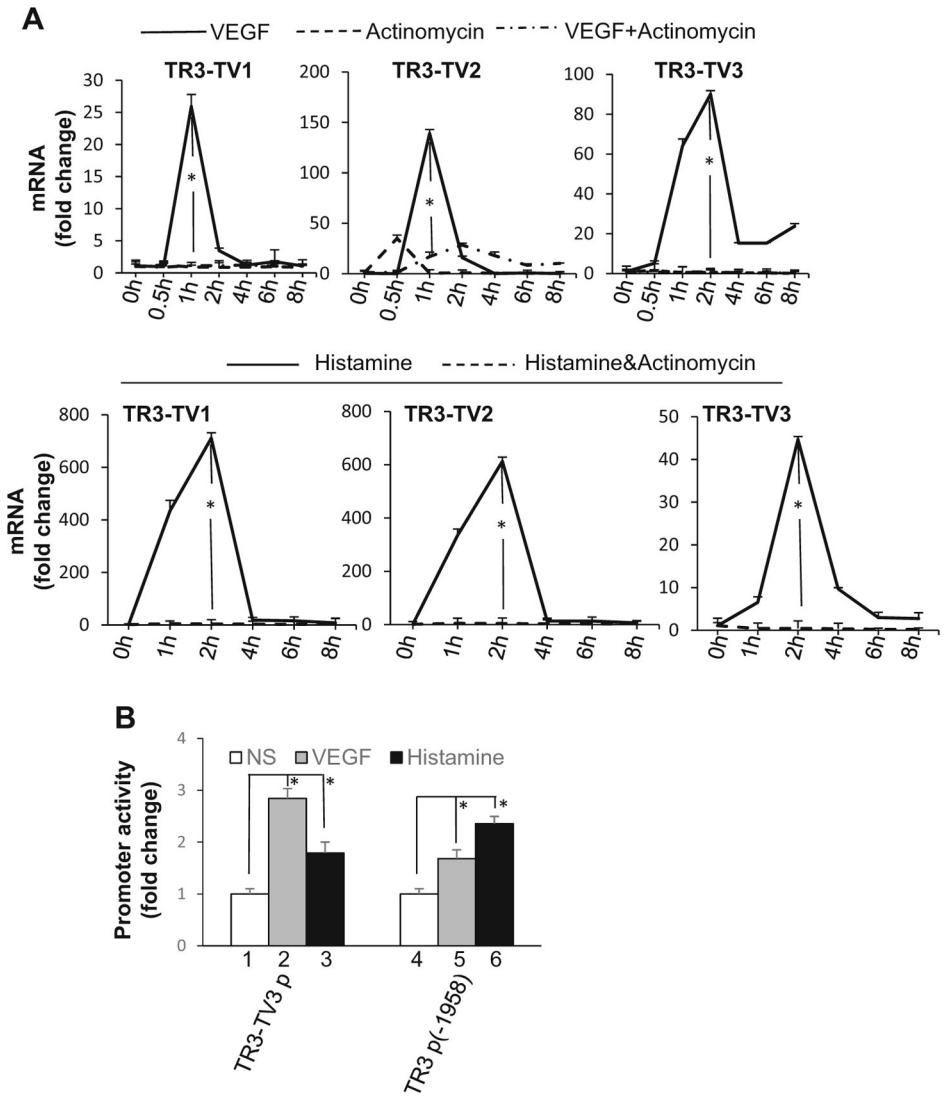

Fig. 6.

VEGF-A and histamine regulate the promoter activities, but not mRNA stability, of TR3 transcription variants. **a** Serum-starved HUVEC were pretreated with or without actinomycin D1 for 15 min and then stimulated with VEGF (*top panel*) or histamine (*bottom panel*) for 0.5, 1, 2, 4, 6, and 8 h. RNA were isolated and subjected to real-time RT-PCR with specific primers of TR3-TV1, TR3-TV2, and TR3-TV3 (n=2 for real-time PCR); **b** HUVECs were transduced with TR3 promoter or TR3-TV3 promoter luciferase constructs and an internal luciferase construct, and then stimulated with or without VEGF-A or histamine for 6 h. Data are presented as fold changes of luciferase activity in cells related to no stimulation control (n=4). Experiments were repeated three times (*p<0.05)