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A Novel Intracellular Isoform of VEGFR-1 Activates Src and Promotes Cell Invasion in MDA-MB-231 Breast Cancer Cells

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ABSTRACT

Two types of VEGFR-1 receptors have been characterized: a full-length transmembrane receptor and a truncated extracellular soluble isoform (sVEGFR-1). We report here the characterization, in normal and cancer cells, of a new family of intracellular isoforms of VEGFR-1 resulting from alternative initiation of transcription in intronic sequences of the gene. While the classical isoforms of VEGFR-1 were barely detectable in MDA-MB-231 breast cancer cells, one of the intracellular isoforms transcribed from intron 21 (i_{21}VEGFR-1) was the main isoform expressed in these cells. The new transcript encodes for a protein that contains only the phosphotransferase domain and the carboxyterminal tail of VEGFR-1. Treatment of MDA-MB-231 cells with siRNA specific for the tyrosine domain of VEGFR-1 suppressed the expression of i_{21}VEGFR-1, downregulated phosphorylation of Src at tyrosine 418, and reduced markedly the invasion capacity of these cells in vitro. Accordingly, overexpression of transfected i_{21}VEGFR-1 in MDA-MB-231 cells upregulated the active form of Src and increased invasiveness of MDA-MB-231 cells. The expression of i_{21}VEGFR-1 in MDA-MB-231 cells was inhibited by retinoic acid. Both, activation of Src and downregulation by retinoic acid, have been reported in other intracellular members of the Fms/Kit/PDGFR family of tyrosine kinases, particularly in the intracellular isoform of c-kit, analogous structurally to i_{21}VEGFR-1 and frequently expressed in cancer cells. J. Cell. Biochem. 110: 732–742, 2010.

KEY WORDS: VEGFR-1; Flt-1; MDA-MB-231 CELLS; Src; INVASION

The proteins of the VEGF family bind and activate three tyrosine kinase receptors: VEGFR-1 (Flt-1), VEGFR-2 (KDR/Flk-1), and VEGFR-3 (Flt-4). These proteins are essential regulators of angiogenesis, vasculogenesis, and lymphangiogenesis [Ferrara, 2002; Lohela et al., 2009] and, in addition, promote invasion of tumor cells through autocrine and paracrine mechanisms [Bachelder et al., 2002]. Both VEGFR-1 and VEGFR-2 have seven Ig-like extracellular domains, a single-transmembrane region, and several intracellular domains including a consensus tyrosine kinase sequence, interrupted by a kinase-insert domain [Shibuya et al., 1990; Terman et al., 1991]. A truncated extracellular isoform of VEGFR-1, named soluble VEGFR-1, consists essentially of six of the seven extracellular domains of the molecule [Kendall and Thomas, 1993].

The biological significance of VEGFR-1 and VEGFR-2 receptors has been demonstrated by gene-targeting studies. VEGFR-1 and VEGFR-2 knockout mice die in utero by embryonic day 8.5–9.5. VEGFR-2-defective mice die in the absence of endothelial and hematopoietic cells [Shalaby et al., 1995]. Mice defective in VEGFR-1 die with an excess of endothelial cells that cannot form functional vessels [Fong et al., 1995]. While mice defective in the whole length VEGFR-1 receptor are not viable, animals defective just in the tyrosine kinase domains of the receptor are able to develop normal vessels and survive [Hiratsuka et al., 1998]. However, the monocytes–macrophages of these animals do not migrate toward VEGF [Barleon et al., 1996]. Another interesting observation is the attenuation of pathological angiogenesis in these animals and the decrease of lung-specific metastasis [Hiratsuka et al., 2002; Dawson et al., 2009a,b]. It has been demonstrated that bone-marrow-derived hematopoietic progenitor cells that express VEGFR-1 home to tumor-specific premetastatic sites and form cellular clusters before the arrival of tumor cells. Preventing VEGFR-1 function using antibodies or by the removal of VEGFR-1^+ cells from the bone marrow of wild-type mice abrogates the formation of these premetastatic clusters and prevents tumor metastasis [Kaplan et al., 2005]. Furthermore, VEGFR-1 promotes migration and invasion of colorectal and pancreatic cancer cells [Fan et al., 2005; Wey et al., 2005] and mediates the epithelial–

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mesenchymal transition in these cells [Bates and Mercurio, 2005; Yang et al., 2006].

In endothelial cells, the Src family kinases, Src and Yes are required for VEGF-induced vascular permeability and survival [Eliceiri et al., 1999]. Likewise, VEGF-induced Src activation has been reported in Kaposi's sarcoma cells [Munshi et al., 2000]. Activation of Src has been implicated in progression and metastasis of a variety of solid tumors [Summy and Gallick, 2003]. VEGF stimulation of colorectal cancer cells resulted in VEGF-enhanced VEGFR-1/Src complex formation, Src phosphorylation at tyrosine 418, and cellular migration [Lesslie et al., 2006]. Recently, it has been reported that breast cancer cells that lodge in the bone marrow succumb in this environment when deprived of Src activity [Zhang et al., 2009].

Here we report the characterization of a new family of intracellular isoforms of VEGFR-1, lacking the extracellular domains of the molecule. These isoforms are expressed in MDA-MB-231 mammary carcinoma cells and also in normal endothelial cells, macrophages, and fibroblasts. After silencing the isoforms of VEGFR-1 expressed in MDA-MB-231 cells, the invasion capacity of these cells in vitro decreases and phosphorylation of Src at tyrosine 418 also decreases. Accordingly, transfection of the most abundant isoform expressed in MDA-MB-231 cells increases invasiveness and phosphorylation of Src at tyrosine 418. These results indicate that the intracellular VEGFR-1 isoform expressed in MDA-MB-231 breast cancer cells promotes invasion through an Src-dependent pathway.

MATERIALS AND METHODS

CELL CULTURE

MDA-MB-231 cells, obtained from the American Type Culture Collection (ATTC) (Manassas, VA) were maintained in Dulbecco's modified Eagle medium/Ham's F-12 (1:1) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 50 U/ml penicillin G, and 50 μg/ml streptomycin sulfate. MCF7 cells were obtained from ATTC and maintained in Dulbecco's modified Eagle medium supplemented with 10% FCS, 50 U/ml penicillin G, and 50 μg/ml streptomycin sulfate. Human microvascular dermal neonatal endothelial cells (HMVEC-d-Neo) and human umbilical vein endothelial cells (HUVEC) were obtained from Clonetics (Lonza, Walkersville, MD) (cc-2516 and cc-2517) and cultured following the provider's recommendations and media. Human U-937 macrophages were obtained from ATCC and maintained in RPMI 1640 with 2 mM L-glutamine, supplemented with 10% FBS, 50 U/ml penicillin G, and 50 μg/ml streptomycin sulfate. U-937 macrophages were incubated 72 h with phorbol 12-myristate 13-acetate (Sigma, St. Louis, MO) 10 ng/ml and stimulated with lipopolysaccharide L-2887 (Sigma) 500 ng/ml for 12 h, in order to express VEGFR-1. Human nasal mucosa fibroblasts were kindly provided by Dr. C. Picado (IDIBAPS, Barcelona, Spain) and maintained in DMEM supplemented with 10% FBS, 50 U/ml penicillin G, 50 μg/ml streptomycin sulfate, and 0.002 mg/ml amphotericin. Cells were maintained in a 5% CO_2 atmosphere at 37°C. All cell lines were used at low passage (<20) and regularly tested against mycoplasma.

SRC INHIBITOR

The selective Src family-tyrosine kinase inhibitor, 4-amino-5- (4-chlorophenyl)-7-(*t*-butyl) pyrazolo (3,4-d) pyrimidine (PP2) (Calbiochem, San Diego, CA) [Hanke et al., 1996] suspended in 1% dimethyl sulfoxide (DMSO) was added to the culture medium at a concentration of 10 μM.

CELL PROLIFERATION AND CELL INVASION ASSAYS

For proliferation assays, MDA-MB-231 cells were seeded at a density of 5×10^4 cells/ml in triplicate 35-mm plates. At various times, cells were collected and trypan blue was added. Live cells were counted using a hemocytometer. Cell invasion assays were done using BD BioCoat Matrigel Invasion Chamber (BD Biosciences) (polycarbonate filter of 8 μm pore size). Forty-eight hours after transfection, MDA-MB-231 cells were trypsinized and a suspension of 7×10^5 cells in serum-free medium was layered in the upper compartment. The lower chamber contained culture medium with 5% FBS as a chemoattractant. After 4 or 24 h, the cells on the upper surface of the membrane were removed with a cotton swab and the invading cells on the underside of the membrane were fixed, stained, and counted in seven random fields at 40× or 10× magnification. The total number of cells recovered from the lower compartment was counted on a hemocytometer as an additional control.

PREPARATION OF RNA, ELECTROPHORESIS, NORTHERN HYBRIDIZATION, AND PROBES

Cellular RNA was obtained using the RNeasy (total RNA) (Qiagen, Hilden, Germany) and Oligotex (poly-A mRNA) (Qiagen) following the manufacturer's instructions. The procedures used for electrophoresis, Northern blot analysis, and preparation of DNA probes have been previously described [Mezquita et al., 1999, 2000]. Human VEGFR-1 kinase domain primers were used to obtain a probe that hybridized with the full-length VEGFR-1 mRNA [Mezquita et al., 2003]. Human VEGFR-1 N-terminal primers were used to obtain a probe that hybridized to both full-length and soluble VEGFR-1 forms [Mezquita et al., 2003]. Total RNA from term human placenta was prepared with the TriPure Isolation Reagent from Roche (Mannheim, Germany), according to the specifications of the manufacturer.

RACE-5′

The BD-Smart Race protocol from BD Biosciences Clontech (Palo Alto, CA) was followed. The method uses PowerScript Reverse Transcriptase, a variant of MMLV reverse transcriptase, with terminal transferase activity. This activity adds several C's at the end of the first-strand product, allowing annealing of a poly-G-tailed oligonucleotide that is copied by the reverse transcriptase, creating an extended sequence that is used for amplification. First-strand synthesis was done with total RNA (1 μg), the BD Smart IIA oligonucleotide, and the 5′ coding sequence (CDS) BD primer as recommended by the company. PCR amplification was done with specific primers (HR5 and nested HR5N or HF521 and nested HF522; Table I), in conjunction with provided universal primers. PCR conditions were as follows: 3 min at 94°C; 28–35 cycles of 30 s at 94°C, 30 s at 65°C, and 3 min at 68°C; final extension, 3 min at 68°C.

TABLE I. Primers Used for RT-PCR, RACE5′, DNA Cloning and Sequencing

Name	Sequence	Sequence position	Direction
H16U	ATGCACCTGTGTGGCTGCGA	2289–2308[a]	Forward
H28U	CAAGTTCATGAGCCTGGAAAGAAT	3651–3674[a]	Forward
HAF	CCTCGCCTTTGCCGATCC	27–44[b]	Forward
HAR	GGATCTTCATGAGGTAGTCAGTC	632–652[b]	Reverse
HCID	CAGCTGGAATGGGAGAAACTGG	3869–3890[a]	Reverse
H16-1	GGTACCATGAAAAGGTCTTCTTCTGA	nt 17–26, 2347–2366[a]	Forward
H16-2	CCGAGACTTGGTACCATGAAAAGGTCT	nt 16–27, 2347–2358[a]	Forward
HF18E-AT	ACAGAATTCGATGACTGAGCTAAAAATC	nt 1028, 2625–2643[a]	Forward
HF18U	GTTAGTGAAGCAACCGGCTGAG	Intron 18, 9883977–9883956[c]	Forward
HF21D	CCTCCAGCCCACTTTATCCAAGC	Intron 21, 9876663–9876685[c]	Reverse
HF21D2	CTGTTTGTAATGGCTCTTGTTATCC	Intron 21, 9876519–9876543[c]	Reverse
HF21E-AT	ATACAGAATTCTATGGAAGATCTGATTTC	nt 11–29, 2984–3002[a]	Forward
HF21U	TGACAGGGAACTGTACCGTTTTCTC	Intron 21, 9876754–9876730[c]	Forward
HF521	TCCGTAAGACCACACGTCGCTCTTGG	3233–3258[a]	Reverse
HF522	CACAAATCTTCACCACGTTGTTCTCAG	3092–3118[a]	Reverse
HFFA	CAAGAGGGCCCTCACTTATCGTCGTCATCCTTGTA	Flag sequence	Reverse
HFFp	GTCGTCATCCTTGTAATCGATGGGTGGGGTGGAG	nt 19–34, 3999–4014[a]	Reverse
HFFw	GATGTTGAGGAAGAGGAGGATT	2935–2956[a]	Forward
HFI15U	TTGGTCTTGGAGGAACAGTGCTCAG	Intron 15, 9900408–9900384[c]	Forward
HFIB	ATACAGGATCCGATGGGTGGGGTGGAG	nt 12–27, 3999–4014[a]	Reverse
HFrev	AAGCTAGTTTCCTGGGGGTATA	4059–4080[a]	Reverse
HR5	ACCATCAGAGGCCCTCCTTGCTTGG	2696–2720[a]	Reverse
HR5N	GCTCAGTCATCAGAGCTTTGTACTCGCTG	2607–2635[a]	Reverse

[a]Nucleotide position taking the A in the ATG codon of full-length VEGFR-1 as number 1.
[b]Nucleotide position in β-actin sequence (accession number BC 016045).
[c]Nucleotide position in VEGFR-1 genomic contig (accession number NT_024524).

RT–PCR

Enhanced Avian Reverse Transcriptase and Accu Taq LA Polymerase mix, with enzyme proofreading activity (Sigma), was used, as well as OneStep RT-PCR (Qiagen). Total RNA (0.3–1 μg) was reverse transcribed with either: random nanomers, anchored oligo$(dT)_{23}$ (Sigma) or the antisense-specific primer, and amplified by PCR for 33 cycles (VEGFR-1) or 28 cycles (β-actin). Annealing temperature and extension times were done, respectively, according to the T_m of the primers and length of expected PCR products. PCR conditions were as follows: 45 s at 94°C; 28–33 cycles of 45 s at 94°C, 45 s at 55–62°C, and 1–2 min at 72°C; final extension, 10 min at 72°C. From a total volume of 50 μl, 10 μl (VEGFR-1) or 5 μl (β-actin) was loaded on agarose gels stained with ethidium bromide. DNA markers used were EcoLadder (Bioline, London, UK) or BioRad (Hercules, CA). The specific primers used for intracellular VEGFR-1 (iVEGFR-1) were HF15U, HF18U, HF21U, HF522, HFFw, HFrev, H16U, H28U (Table I) and for β-actin, HAF and HAR (Table I).

DNA SEQUENCING

PCR products were completely washed of dNTPs and primers, and concentrated, with Microcon centrifugal filter devices (Millipore, Bedford, MA). Thirty to sixty nanograms of PCR product or 400–600 ng of cloned DNA was used. Sequencing was conducted with the BigDye Terminator v3.1 (Applied Biosystems, Foster City, CA) and sequences were analyzed in an Applied Biosciences Sequencer.

CLONING, ELECTROTRANSFORMATION, AND DNA PREPARATION

The whole coding region of the i_{21}VEGFR-1, followed at the C-terminal end by the tag-Flag sequence, was cloned in the pcDNA3.1(+) vector (Invitrogen, San Diego, CA). The hybrid sequence was obtained by two consecutive PCR amplification steps to incorporate the Flag sequence and unique restriction sites (*Kpn*1 and *Apa*1, respectively) at each end for directional cloning. The primers pairs HF16-1/HFFp and HF16-2/HFFA (Table I) were used. i_{18}VEGFR-1 and i_{21}VEGFR-1 were cloned in the pEGFP-C1 vector (Clontech) containing the green fluorescence protein and the neomycin resistant gene. The primers pairs H18E-AT/HFIB and HF21E-AT/HFIB (Table I), containing restriction sites (*Eco*R1 and *Bam*H1) at the ends for directional cloning, were used to amplify, by RT-PCR, the whole coding region of each of the isoforms i_{18}VEGFR-1 and i_{21}VEGFR-1. Primers were removed from DNA using microcon columns (Millipore), and the DNA was digested with the appropriate restriction enzymes and ligated to the corresponding vector. Electrocompetent bacterial cells were electroporated for colony screening of positive clones. The recombinants, having the expected insert size, were confirmed by sequencing, and expanded for DNA preparation (Qiagen) to obtain high-purity DNA for eukaryotic transfection.

WESTERN BLOTTING

Cells were lysed on ice in NP40 lysis buffer: 150 mM NaCl, 20 mM HEPES (pH 7.5), 0.5% NP-40, a cocktail of protease inhibitors (Complete, Roche) and phosphatase inhibitors (Calbiochem). Cell extracts were separated on 10% SDS–PAGE, transferred to PVDF membranes, and probed with antibodies. Antibodies for VEGFR-1 proteins (C-17) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and from Cell Signaling Technology (Beverly, MA). Antibodies for Src and phospho-Src (PY 418) were from Invitrogen.

TRANSFECTION OF siRNA AND PLASMID DNA

Stealth RNAi (Invitrogen) and Dharmacon ON-TARGET plus SMART pool plus siRNAs (Thermo Scientific, Lafayette, CO) were used for the interference of iVEGFR-1. The siRNA sequences (sense strand) targeting iVEGFR-1 were: Stealth RNAi (Invitrogen) A

(GGUUCAAGCAUCAGCAUUUGGCAUU), B (GGCCUGGAACAAGGCAAGAAACCAA), C (GGAAAGUAUUUCAGCUCCGAAGUUU), D (AGGAGUAGAGUACUCAGGAGCUCUC), and ON-TARGET plus SMART pool plus siRNAs (Dharmacon) 1 (GGAAATAGTGGGTTTACAT), 2 (CGTGTGGTCTTACGGAGTA), 3 (TTGAAGAACTTTTACCGAA), and 4 (GAGAAAAGGAGATACTCGA). Scrambled stealth RNAi and ON TARGET plus non-targeting pool were used as negative controls. Transfections were carried using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. The transfection efficiency, between 80% and 90% was assessed using the BLOCK-iT Fluorescent Oligo (Invitrogen). Transfection of iVEGFR-1 plasmid was carried using Lipofectamine (Invitrogen). For stable transfection, cells were selected with geneticin (Invitrogen)-containing medium and subcloned by limiting dilution.

STATISTICAL ANALYSES

Values were expressed as means ± standard error. Comparisons were made using the two-tailed Student's *t*-test.

RESULTS

CHARACTERIZATION OF NOVEL INTRACELLULAR ISOFORMS OF VEGFR-1

VEGFR-1 in humans consists of 30 exons spanning more that 193 kb [Kondo et al., 1998]. A truncated soluble form was found to be derived from the first 13 exons and an additional sequence derived from intron 13 [Kendall and Thomas, 1993]. Here we report the nucleotide sequence of five novel intracellular isoforms of VEGFR-1 that initiate transcription within intronic sequences. These isoforms have lost the extracellular domains and possess either all the intracellular domains or just an incomplete kinase domain and the C-terminal sequence (Fig. 1). The new intracellular isoforms were named iVEGFR-1. As iVEGFR-1 started transcription within intron sequences, they were given the name of the intron number from where they started transcription. Isoforms i_{15}VEGFR-1 and i_{15as}VEGFR-1 started in intron 15; isoform i_{18}VEGFR-1 in intron 18; and isoforms i_{21}VEGFR-1 and i_{21as}VEGFR-1 in intron 21. The isoforms i_{15as}VEGFR-1 and i_{21as}VEGFR-1 resulted from alternative splicing (as) of i_{15}VEGFR-1 and i_{21}VEGFR-1, respectively. The five isoforms incorporated additional 5′ leader sequences from the corresponding 5′ intron.

In a previous article [Mezquita et al., 2003] we observed, in Northern blot analysis of VEGFR-1 expression in endothelial cells, the presence of a band of higher mobility than the corresponding to the full-length human VEGFR-1 transcript. We report here that this band, which hybridizes to probes from the kinase domain, but does not hybridize to probes from the N-terminal part of the VEGFR-1 transcript, is present in endothelial cells, macrophages, fibroblasts, breast cancer MDA-MB-231 cells, and also in tissue samples such as human placenta, confirming the expression of the isoform in vivo (Fig. 2A). The expression of i_{21}VEGFR-1 in human placenta also has been determined by RT-PCR using primers that span from intron 21 (Fig. 2C). An additional evidence of the expression in vivo of i_{21}VEGFR-1 has been obtained in mature mouse testis, where i_{21}VEGFR-1 is the main VEGFR-1-expressed isoform (unpublished results).

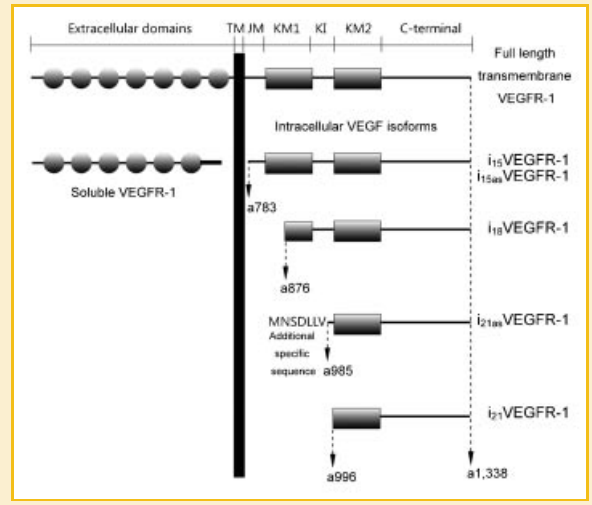


Fig. 1. Schematic representation of full-length VEGFR-1, soluble VEGFR-1, and intracellular isoforms of VEGFR-1. The intracellular isoforms lack the extracellular and the transmembrane domains. Isoform i_{15}VEGFR-1 possesses all the intracellular domains. Isoform i_{18}VEGFR-1 consists of part of the ATP-binding domain (without the ATP-binding sequences), the kinase insert, the phosphotransferase domain, and the C-terminal tail. Isoform i_{21}VEGFR-1 possesses the phosphotransferase domain and the C-terminal tail. Isoform i_{21as}VEGFR-1 possesses the phosphotransferase domain, plus 11 amino acids of the kinase insert, the additional specific sequence MNSDLLV and the C-terminal tail. TM, transmembrane domain; JM, juxtamembrane domain; KM1, ATP-binding domain; KI, kinase insert; KM2, phosphotransferase domain; a, amino acid.

To characterize the new intracellular isoforms expressed in MDA-MB-231 breast cancer cells we performed RACE experiments using primers within the tyrosine kinase domains (HFR5, HFR5N, HFR521, and HFR522) and analyzed the 5′-UTR and the coding region by sequencing. These experiments revealed the expression of at least five intracellular isoforms of VEGFR-1.

ISOFORM i_{15}VEGFR-1

Isoforms i_{15}VEGFR-1 and i_{15as}VEGFR-1 started in nucleotide 11265 of intron 15. They differed by an alternative splicing of intron 15. Both isoforms used the same 3′ acceptor site of exon 16 of the complete VEGFR-1 but different 5′ donor sites. One of the cDNAs, after creating a new exon 1 (N1) of 73 nucleotides, jumped to exon 16 and eliminated an intron of 665 nucleotides. The other cDNA shared the same exon N1 of 73 nucleotides, but then jumped twice, first to a new acceptor site in intron 15, creating a second exon (N2) of 62 nucleotides, and then to the established acceptor site of exon 16. Isoform i_{15as}VEGFR-1 eliminated two introns of 375 and 228 nucleotides. The new donor and acceptor sites in i_{15}VEGFR-1 isoforms were either canonical sites or similar to other splice sites of the VEGFR-1 gene. Exon N1 donor site had the canonical AGgt site; exon N1 acceptor sites were either agGA (exon 19) or agCT. Exon N2 donor site was ATgt. Identical sites to the new donor (ATgt) and acceptor (agCT) sites were found in exons 23, 25 and 28 (ATgt) and in exons 26 and 30 (agCT) of the full-length DNA.

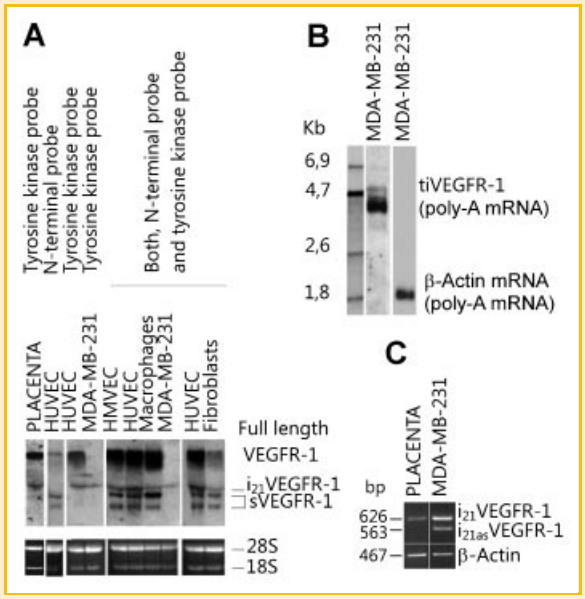


Fig. 2. Expression of VEGFR-1 isoforms in human MDA-MB-231 breast cancer cells, human placenta, endothelial cells, macrophages, and fibroblasts. A: Northern blot analysis of total RNA (20–40 μg) from term human placenta, HUVEC, MDA-MB-231 cells, HMVEC, U-937 macrophages, and fibroblasts. Specific probes for detecting N-terminal sequences (full-length VEGFR-1 and sVEGFR-1) and tyrosine kinase sequences (full-length VEGFR-1 and iVEGFR-1) were used as described in the Materials and Methods Section. Panels with ribosomal RNAs show the equalization of RNA samples used for analysis. B: Northern blot analysis was also performed from poly-A-mRNA (3–5 μg). The blot was reprobed with β-actin to ensure RNA integrity. C: RT-PCR analysis of i_{21}VEGFR-1 expression in human placenta and MDA-MB-231 cells. β-Actin amplification has been used as a control. Results are representative of three independent experiments.

The two i_{15}VEGFR-1 isoforms had in common exon N1 and exons 16–30 of the complete VEGFR-1. Exon 16 in the membrane-spanning receptor coded for the transmembrane domain, but in the new variants, the first ATG translation initiation site of the open-reading frame (ORF) was located close to the end of exon 16. The resulting CDS would be then identical in the two i_{15}VEGFR-1 isoforms, and when translated, the protein product would include the juxtamembrane domain, the two kinase subdomains (the ATP-binding domain and the phosphotransferase domain) as well as the kinase insert of VEGFR-1. The putative translation product would be a protein, missing all the extracellular domains and retaining the intracellular domains that confer the protein tyrosine kinase activity. Putative protein i_{15}VEGFR-1 had 556 amino acids and the sequence was identical to the amino acids 783–1338 (AF063657) of the full-length VEGFR-1, except for a Phe that substituted a Leu due to a C/T change in nucleotide 739 of the CDS (nucleotide 3085 of the full-length CDS). The 5′-UTR of the two i_{15}VEGFR-1 isoforms started with a polypyrimidine tract, and a putative IRES was present in exon 1 (Inserm Ires Databank). The presence of the polypyrimidine tracts, as well as the putative IRES in exon 1, could allow these two variants to be translated under stress or regulatory conditions, when other cap-dependent translation mRNAs are not translated [Avni et al., 1994; Stein et al., 1998]. In addition, the 5′-UTR sequences of both i_{15}VEGFR-1 isoforms contain two and three upstream open-reading frames (uORFs). The presence of uORFs in the 5′-UTR of mRNAs is known to regulate translation in eukaryotes; this is a common feature of RNAs coding for proto-oncogenes and transcription factors [Morris and Geballe, 2000].

ISOFORM i_{18}VEGFR-1

The i_{18}VEGFR-1 isoform started in nucleotide 4170 of intron 18. Its 5′-UTR, of 159 nucleotides, was composed of the last 127 nucleotides of exon 18, plus the first 32 nucleotides of exon 19 of the full-length VEGFR-1 and had two uORFs. The first ATG translation initiation site in frame with VEGFR-1 was at position 33 of exon 19, and the resulting CDS would translate a protein product without the extracellular, transmembrane, and juxtamembrane domains, conserving 283 of the total 332 amino acids of the kinase domain. The putative translation product in i_{18}VEGFR-1 would be a protein that, although retaining most of the protein kinase domain, would have lost the ATP-binding sequences of the kinase domain. This protein would have 463 amino acids and would be identical to amino acids 876–1338 (AF063657) of the full-length VEGFR-1.

ISOFORM i_{21}VEGFR-1

The two i_{21}VEGFR-1 isoforms started at nucleotide 157 of intron 21. These two isoforms differed by introducing or not a splicing at the end of intron 21. Isoform i_{21}VEGFR-1 had no splicing, and its 5′-UTR, of 306 nucleotides, comprised the last 274 nucleotides of intron 21 plus the first 32 nucleotides of exon 22 of the full-length VEGFR-1. i_{21as}VEGFR-1, instead, eliminated, by splicing, the last 96 nucleotides of intron 21, creating a new consensus 5′ splice site (TGgt), and using the same 3′ acceptor site of exon 21 of the complete VEGFR-1. The 5′-UTR in i_{21as}VEGFR-1 would have 156 nucleotides. i_{21}VEGFR-1 had six uORFs, while i_{21as}VEGFR-1 conserves only the first three uORFs. In isoform i_{21}VEGFR-1, the first ATG translation initiation site in frame with VEGFR-1 was located at nucleotide 33 of exon 22. Isoform i_{21as}VEGFR-1 incorporates 54 new coding nucleotides (18 amino acids) due to the presence of an ATG site in frame from intron 21. Thus, i_{21as}VEGFR-1 putative coding region would start with the specific MNSDLLV sequence, followed by the whole CDS of exon 22. The protein i_{21}VEGFR-1 would have 343 amino acids and the sequence would be identical to the amino acids 996–1338 (AF063657) of the full-length VEGFR-1. Putative protein i_{21as}VEGFR-1 would have 360 amino acids and the sequence would be identical to the amino acids 986–1338 (AF063657) of the full-length VEGFR-1. These isoforms conserved 163 (i_{21}VEGFR-1) and 174 (i_{21as}VEGFR-1) of the 332 amino acids of the kinase domain, including none (i_{21}VEGFR-1) or 11 amino acids (i_{21as}VEGFR-1) of the kinase insert. Both i_{21}VEGFR-1 isoforms had lost, like i_{18}VEGFR-1, the ATP-binding domain (Fig. 1). A nucleotide change C/T at positions 960 in i_{21}VEGFR-1, 864 in i_{21as}VEGFR-1, and 1173 in i_{18}VEGFR-1 does not introduce any change in translation (nucleotide 3739 of the full-length CDS). After the differential processing of introns 15, 18, or 21, the new cDNAs processed all the other exons as does full-length VEGFR-1 mRNA.

The nucleotide sequences of VEGFR-1 isoforms have been deposited in the GenBank database under accession numbers DQ836394 (i_{15}VEGFR-1), DQ836395 (i_{15as}VEGFR-1), EF491868 (i_{18}VEGFR-1), EF491869 (i_{21}VEGFR-1), and EF491870 (i_{21as}VEGFR-1).

EXPRESSION OF VEGFR-1 ISOFORMS IN BREAST CANCER CELLS

Breast cancer tumors expressing estrogen receptors (ER^+), such as ER^+ MCF-7, are histologically more differentiated and have a lower metastatic potential than ER^- tumors, such as ER^- MDA-MB-231 [Fisher et al., 1981]. The full-length VEGFR-1 receptor and the soluble form (sVEGFR-1) were not detected in MDA-MB-231 by Northern blot analysis of total RNA (Fig. 3A), in accordance with a previous report [Soker et al., 1998]. However, both forms were barely detectable by RT-PCR (Fig. 3B). Western blot analysis showed high expression of the full-length VEGFR-1 receptor in endothelial cells but not in MDA-MB-231 cells (Fig. 3B). Only one band of higher mobility than the corresponding to the full-length VEGFR-1 transcript was detected by Northern blot in MDA-MB-231 cells but not in MCF-7 cells (Fig. 3A). To identify the major band detected by Northern blot in MDA-MB-231 cells, we performed RNA interference experiments using oligonucleotides located in exons 18(A), 21(B), 25(D), and 27(C). When assessed by RT-PCR, oligonucleotide A interfered isoform i_{15}VEGFR-1, and oligonucleotide B interfered isoforms i_{15}VEGFR-1 and i_{18}VEGFR-1, while oligonucleotides C and D were the only ones able to interfere all the isoforms (Fig. 4A). The Northern band characteristic of MDA-MB-231 cells and a 39 kDa protein detected by Western blot disappeared when interference was carried out by oligonucleotides C and D or alternatively with Dharmacon oligonucleotides (Fig. 4B). As oligonucleotides C and D, but no oligonucleotide A or B, interfered the band detected in Northern analysis, this band should correspond to i_{21}VEGFR-1.

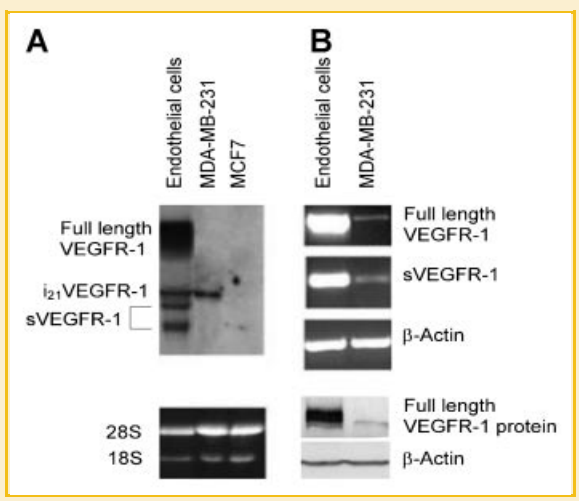


Fig. 3. Expression of VEGFR-1 in endothelial cells and breast cancer cells. A: Northern blot analysis of total RNA (20–40 μg) from endothelial cells, MDA-MB-231 cells, and MCF-7 cells. Total RNA was obtained as described under the Materials and Methods Section. Ribosomal RNAs (bottom) were used as a loading control and control of integrity. B: Top: RT-PCR analysis of full-length VEGFR-1 and sVEGFR-1 expression in endothelial cells and MDA-MB-231 cells. β-Actin amplification has been used for equalization of RNA samples. Bottom: Western blot analysis of full-length VEGFR-1 protein. β-Actin has been used for equalization of protein samples. For Western blot analyses, 100 μg of protein was loaded into the gels. Results are representative of three independent experiments.

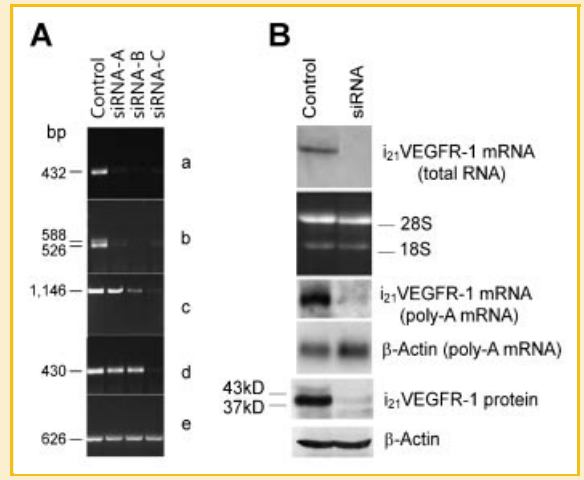


Fig. 4. Silencing of iVEGFR-1 in MDA-MB-231 cells. A: Agarose gel electrophoresis of RT-PCR from total RNA of MDA-MB-231 cells treated with siRNA. From left to right: RT-PCR from RNA of control scrambled RNA-treated cells, RT-PCR from RNA of siRNA-A-treated cells, RT-PCR from RNA of siRNA-B-treated cells, and RT-PCR from RNA of siRNA-C-treated cells. From top to bottom: (a) primers HF16U and HFR5, expanding exons 16–19 of the full-length VEGFR-1, amplify isoform i_{15}VEGFR-1, but not isoforms i_{18}VEGFR-1 or i_{21}VEGFR-1. The amplified isoforms are targets of siRNA-A, -B, and -C. b: Primers HF15U and HFR5 selectively amplify isoform i_{15}VEGFR-1, a target of siRNA-A, -B, and -C. c: Primers HFFw and HFrev, expanding exons 21–30, amplify isoforms i_{15}VEGFR-1 and i_{18}VEGFR-1 but not i_{21}VEGFR-1. Isoform i_{18}VEGFR-1 is not targeted by siRNA-A. d: Primers HF28U and HFrev, expanding exons 28–30 (430 bp), amplify all the isoforms, and only siRNA-C has a major interfering effect, indicating that i_{21}VEGFR-1 is the main isoform expressed. e: β-Actin amplification used for equalization of RNA samples. RT-PCR has been carried out for 33 cycles (VEGFR-1) or 28 cycles (β-Actin), and 10 μl (VEGFR-1) or 5 μl (β-Actin) aliquots has been loaded on the gel. B: Top and middle panels: Northern blot analysis of VEGFR-1 mRNA from MDA-MB-231 total RNA (40 μg) and poly-A-mRNA (5 μg). From left to right: Dharmacon ON TARGET plus non-targeting pool used as the negative control and Dharmacon ON-TARGET plus SMART pool plus siRNAs, which targets VEGFR-1. Below each panel ribosomal RNAs and β-Actin used as loading and integrity controls. Bottom panel: A protein of 39 kDa, the putative translation product of i_{21}VEGFR-1, was recognized by an antibody that detects the C-terminus of human VEGFR-1 and is downregulated by silencing of i_{21}VEGFR-1. β-Actin has been used for equalization of protein samples. For Western blot analyses, 100 μg of protein was loaded into the gels. Results are representative of three independent experiments.

The amount of i_{21}VEGFR-1 expressed in MDA-MB-231 cells varies with cell culture conditions. When MDA-MB-231 culture medium was changed every day, the amount of i_{21}VEGFR-1 was barely detectable. However, the amount increased markedly when cells were maintained for 5–6 days without any change of the culture medium (Figs. 5 and 6).

EFFECT OF RETINOIC ACID ON THE EXPRESSION OF i_{21}VEGFR-1

Previous studies have shown that other intracellular isoforms of the same family of type III tyrosine kinases, such as c-kit and platelet-derived growth factor (PDGF)-α-receptor are expressed while cells

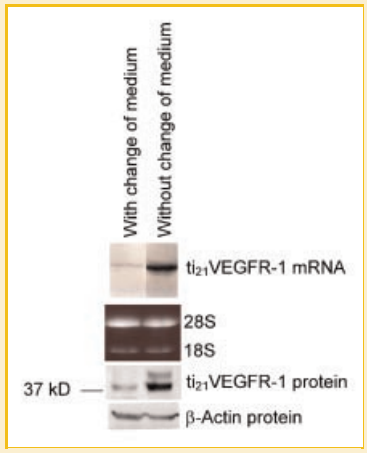


Fig. 5. Expression of i_{21}VEGFR-1 in MDA-MB-231 cells in different cell culture conditions. Northern blot analysis of total RNA (40 μg) from MDA-MB-231 cells cultured for 5 days with daily changes of culture medium (1) or without changes in culture medium (2). Ribosomal RNAs show the equalization of RNA samples used for analysis. Bottom panel: Western blot analysis of cell lysates (100 μg of protein) obtained from MDA-MB-231 cells cultured for 5 days with daily changes of culture medium (1) or without changes in culture medium (2). β-Actin has been used for equalization of protein samples. For Western blot analyses, 100 μg of protein was loaded into the gels. Results are representative of three independent experiments.

are in an undifferentiated state and are downregulated after retinoic acid treatment [Mosselman et al., 1994; Zayas et al., 2008]. When MDA-MB-231 cells were cultured for 2, 4, and 6 days, without adding retinoic acid to the culture medium, the expression of i_{21}VEGFR-1, assayed by RT-PCR, increased with time markedly (Fig. 6). No increase of expression was observed in the presence of 5 μM retinoic acid (Fig. 6).

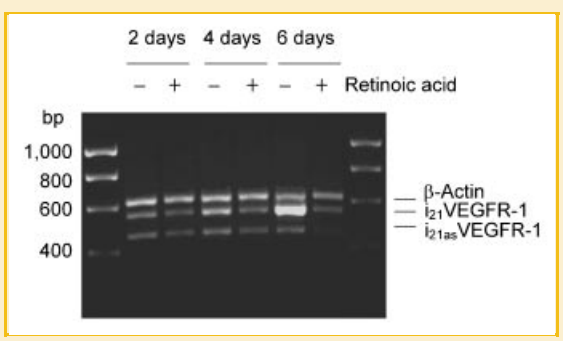


Fig. 6. Effect of retinoic acid on i_{21}VEGFR-1 expression in MDA-MB-231 cells. Cells were cultured for 2, 4, and 6 days in the absence or the presence of 5 μM retinoic acid. The expression of i_{21}VEGFR-1, assayed by RT-PCR, increases markedly in the absence of retinoic acid. In the presence of retinoic acid no upregulation of i_{21}VEGFR-1 was observed. Left and right panels: DNA markers: β-Actin (626 bp), i_{21}VEGFR-1 (563 bp), i_{21as}VEGFR-1 (467 bp). Results are representative of three independent experiments.

SILENCING i_{21}VEGFR-1 EXPRESSION DECREASES CELL INVASIVENESS, WHILE TRANSFECTION OF i_{21}VEGFR-1 INCREASES THE CAPACITY OF MDA-MB-231 CELLS TO PASS THROUGH A MATRIGEL BARRIER

Since i_{21}VEGFR-1 is the main VEGFR-1 isoform expressed in MDA-MB-231 breast cancer cells, our initial approach to study the function of this isoform consisted in inhibiting its expression by RNA interference or overexpressing the intracellular isoform by transfection of i_{21}VEGFR-1. Silencing or overexpressing i_{21}VEGFR-1 did not change the proliferation rate of MDA-MB-231 cells significantly (Fig. 7). However, the ability of MDA-MB-231 siRNA-transfected cells to invade through Matrigel was substantially decreased as compared to control cells and markedly increased after overexpression of i_{21}VEGFR-1 (Fig. 7).

SILENCING i_{21}VEGFR-1 EXPRESSION DECREASES SRC PHOSPHORYLATION AT TYROSINE 418, WHILE TRANSFECTION OF i_{21}VEGFR-1 INCREASES THE ACTIVE FORM OF SRC

It has been reported that the full-length VEGFR-1 receptor associates with Src and induces Src phosphorylation at tyrosine 418 [Lesslie et al., 2006]. To determine if Src is activated in a similar way by the intracellular isoform i_{21}VEGFR-1 we performed experiments of interference or overexpression of i_{21}VEGFR-1 in MDA-MB-231 cells. Silencing i_{21}VEGFR-1 by RNA interference decreases Src phosphorylation at tyrosine 418 as demonstrated by Western blot analysis with an specific antibody against Y418-Src peptide (Fig. 8B). To test further the effect of i_{21}VEGFR-1 on Src phosphorylation at tyrosine 418 we transfected MDA-MB-231 cells with i_{21}VEGFR-1. Cells stably or transiently transfected with i_{21}VEGFR-1 upregulate the active form of Src (Fig. 8C,D). Src activation has been implicated in cell invasion and could be a potential mechanism to explain the increase of cell invasiveness produced by i_{21}VEGFR-1. As demonstrated in Figure 7A (bottom panel) Src kinase inhibition by PP2 produces a similar effect to silencing i_{21}VEGFR-1, decreasing the capacity of MDA-MB-231 cells to pass through a Matrigel barrier.

DISCUSSION

In this article we characterize a new family of five intracellular isoforms of VEGFR-1 expressed in normal and cancer cells. The most abundant form in MDA-MB-231 cells, i_{21}VEGFR-1, has lost all the extracellular domains and contains only the phosphotransferase domain and the carboxyterminal tail of VEGFR-1. To our knowledge, the expression of alternative mRNA isoforms of VEGFR-1, which initiate within intronic sequences of the gene, has not been previously reported. Alternative isoforms of other members of the family of type III tyrosine kinase receptors also initiate in intronic sequences. A form of c-kit (tr-kit) that has lost all the extracellular domains and contains only the phosphotransferase domain and the carboxyterminal tail of c-kit [Rossi et al., 1992] is expressed in ≈30% of gastrointestinal and hematopoietic tumor cell lines examined [Takaoka et al., 1997] and it is present in 66% of advanced primary prostate tumors [Paronetto et al., 2004]. Interestingly, tr-kit is also expressed in normal cells at postmeiotic stages of mouse

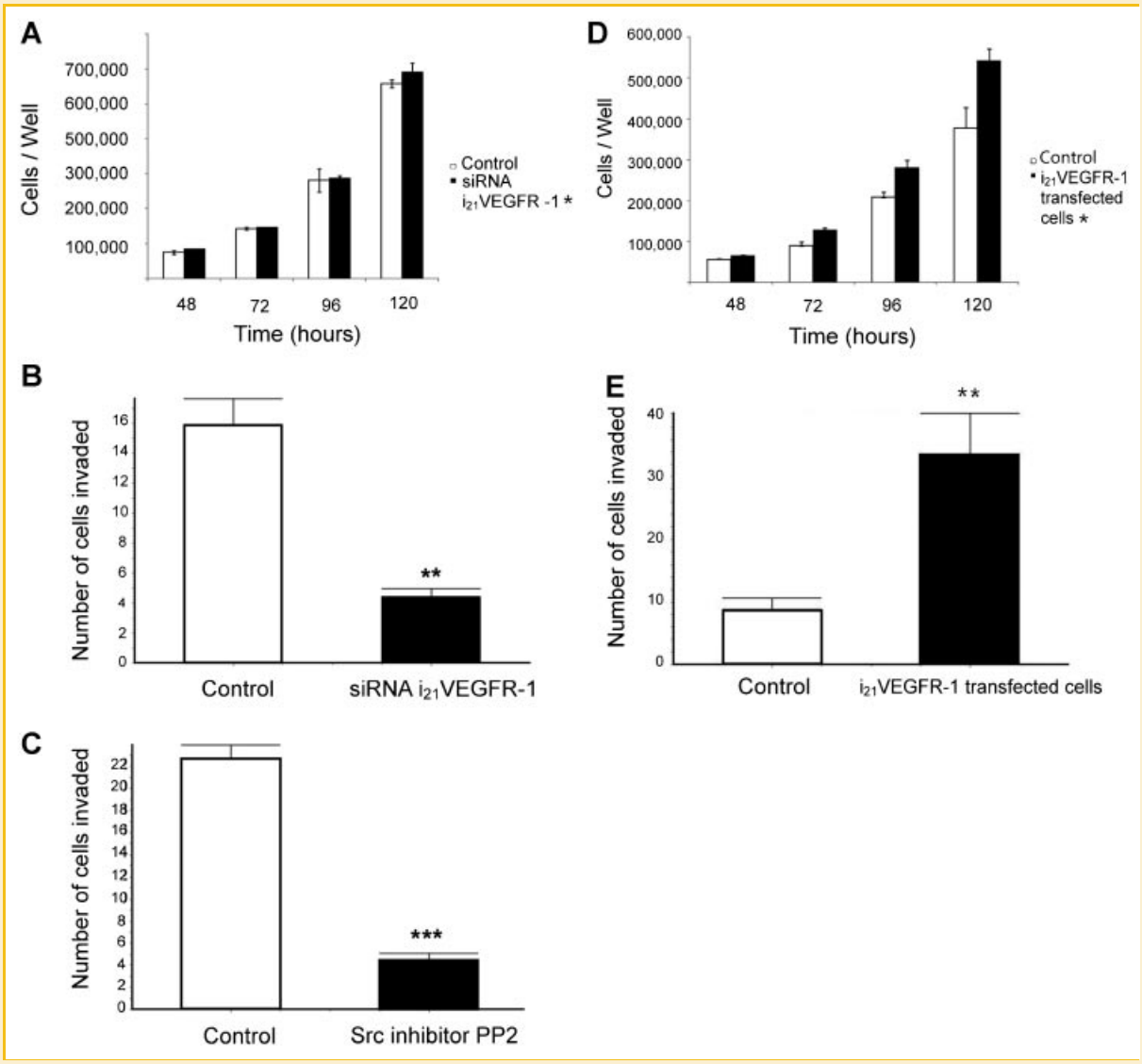
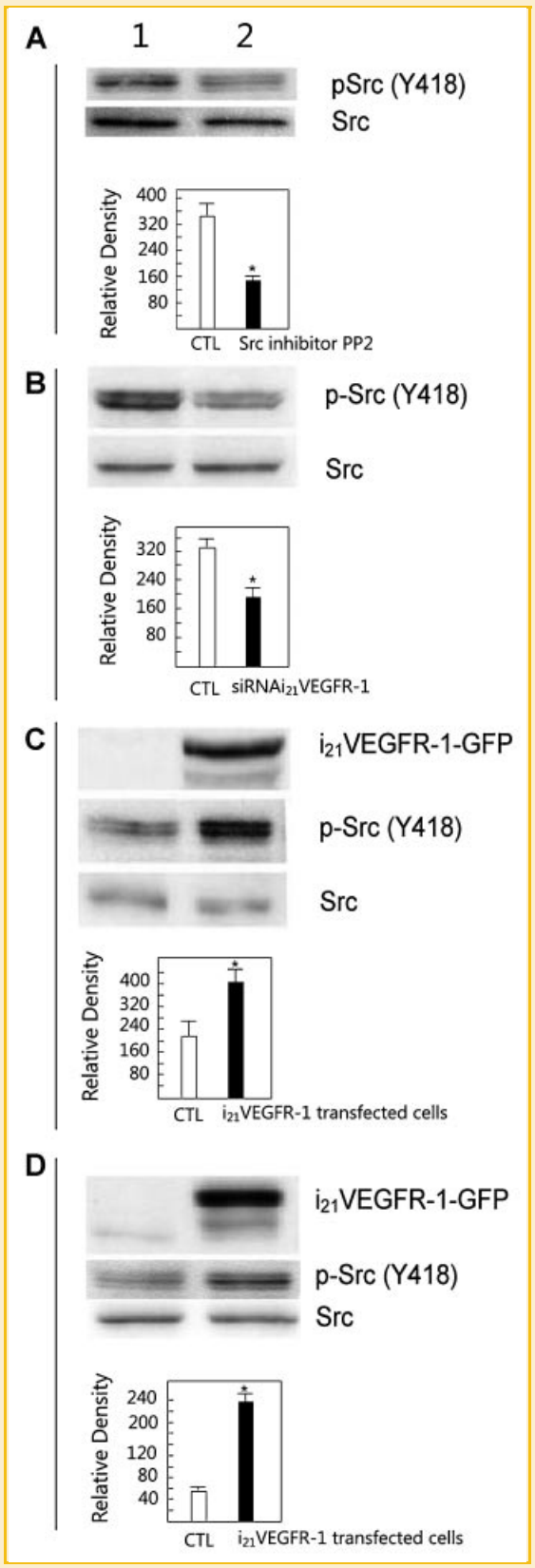


Fig. 7. Effect of silencing or overexpression of i_{21}VEGFR-1 on in vitro proliferation and invasiveness of MDA-MB-231 breast cancer cells. A: Cells were transfected with control or i_{21}VEGFR-1 siRNAs, harvested at different times, and counted as indicated in the Materials and Methods Section. Treatment with siRNA i_{21}VEGFR-1 did not change significantly proliferation ($^{*}P > 0.235$). B: For in vitro invasion assays, MDA-MB-231 cells were seeded on polycarbonate filters coated with Matrigel, as described in the Materials and Methods Section, and incubated for 24 h. Quantification of invaded cells represents the mean number of cells per field counting seven random fields at 40× magnification. Treatment with siRNA i_{21}VEGFR-1 significantly decreased cell invasion ($^{**}P < 0.0001$ vs. control cells). C: Treatment with the Src inhibitor PP2 (10 μM; $^{***}P < 0.0001$ vs. control cells). D: Cells stably transfected with i_{21}VEGFR-1 were harvested at different times and counted as indicated in the Materials and Methods Section. Overexpression of i_{21}VEGFR-1 did not change quite significantly proliferation ($^{*}P > 0.08$). E: For in vitro invasion assays, MDA-MB-231 cells were seeded on polycarbonate filters coated with Matrigel, as described in the Materials and Methods Section, and incubated for 4 h. Quantification of invaded cells represents the mean number of cells per field counting seven random fields at 10× magnification. Overexpression of i_{21}VEGFR-1 increased significantly cell invasion ($^{**}P < 0.0008$). Bars indicate standard error. Results are representative of three independent experiments.

spermatogenesis [Rossi et al., 1992]. Other members of type III tyrosine kinase receptors, such as PDGFR-α and -β, also possess intracellular isoforms that initiate transcription within intronic sequences and are expressed in human germ cell tumors [Vu et al., 1989; Mosselman et al., 1994; Mosselman et al., 1996; Wang and Culty, 2007]. Our observation that retinoic acid downregulates i_{21}VEGFR-1 expression has also been reported for the intracellular isoforms of PDGFR-α and c-kit, which are also downregulated by retinoic acid [Vu et al., 1989; Zayas et al., 2008].

In the present study we show that the full-length and the soluble isoforms of VEGFR-1 are barely detectable in MDA-MD-231 cells, whereas the new intracellular isoform i_{21}VEGFR-1 is preferentially expressed in these cells. While our observations indicate that endothelial cells, fibroblasts, and macrophages express all the isoforms of VEGFR-1, the preferential expression of i_{21}VEGFR-1 in MDA-MB-231 makes these cells particularly interesting to study the function of this intracellular isoform. Consistent with our findings, lack of VEGFR-1 expression in MDA-MB-231 cells has been



previously reported at the mRNA level [Soker et al., 1998; Fitzpatrick et al., 2003; Liang and Hyder, 2005] and at the protein level [Barr et al., 2005]. However, the expression of the transmembrane VEGFR-1 in MDA-MB-231 cells [Wu et al., 2006] and of a full-length iVEGFR-1 [Lee et al., 2007] also has been reported. Some of the primers used to assay the expression of VEGFR-1 by RT-PCR [Yang et al., 2006] cannot discriminate between the full-length VEGFR-1 and the intracellular isoform we report here. Using a specific primer, which cannot detect the intracellular isoform i_{21}VEGFR-1, the expression of the full-length VEGFR-1 was not detected in MDA-MB-231 cells [Liang and Hyder, 2005].

Our observation that the main isoform of VEGFR-1 expressed in MDA-MB-231 breast cancer cells increases invasiveness of MDA-MB-231 cells without changing cell proliferation is in accordance with the effect of activation of the full receptor VEGFR-1 in colorectal carcinoma cells [Lesslie et al., 2006]. In these cells VEGF stimulation enhanced VEGFR-1/Src complex formation, Src phosphorylation at tyrosine 418, and cellular migration, which was effectively blocked by pharmacologic inhibition of VEGFR-1 or Src kinase [Lesslie et al., 2006]. We do not know the mechanism involved in Src activation by i_{21}VEGFR-1. One possibility is direct interaction of i_{21}VEGFR-1 with the SH2 domain of Src kinases, as has been postulated for tr-kit [Sette et al., 2002; Paronetto et al., 2004]. The direct interaction of tr-kit with the SH2 domain of Src displaces the autoinhibitory constraint caused by binding of this domain to the C-terminal tail of Src [Sette et al., 2002]. Another interesting possibility could be the involvement of the phosphatase SHP-2, a known activator of Src kinases [Zhang et al., 2004]. Yeast two-hybrid analysis has shown that the phosphatase SHP2 binds to tyrosine 1213 at the C-terminal end of VEGFR-1 [Igarashi et al., 1998; Ito et al., 1998].

Activation of Src by the intracellular isoform i_{21}VEGFR-1 may contribute to the malignant phenotype of MDA-MB-231 breast

Fig. 8. Effect of i_{21}VEGFR-1 on Src activity in MDA-MB-231 cells. A: MDA-MB-231 were incubated in the absence (1) or in the presence (2) of Src inhibitor PP2 (10 μM). Cell lysates were subjected to Western blotting with the phospho-specific anti-Src Y418 antibody. Western blotting with anti-Src antibodies demonstrates equivalent total Src expression. Phosphorylation was quantified by densitometric scanning. The double bands of p-Src(Y418) most likely represent the heterogeneity of the Src family kinases. Due to the high sequence homology of the members of this family the p-Src(Y418) antibody reacts with Src and with Src-related kinases such as Fyn or Yes. B: MDA-MB-231 cells were treated with ON TARGET plus non-targeting pool as a negative control (1) or Dharmacon ON-TARGET plus SMART pool plus siRNAs (2). Cell lysates were subjected to Western blotting with the phospho-specific anti-Src Y418 antibody. Western blotting with anti-Src antibodies demonstrates equivalent total Src expression. Phosphorylation was quantified by densitometric scanning. C: Lysates of cells stably transfected with the empty vector (1) or with i_{21}VEGFR-1-GFP (2) were subjected to Western blotting with the anti-VEGFR-1 antibody (top panel), and with the phosphotyrosine specific anti-Src Y418 antibody (middle panel). Western blotting with anti-Src antibodies (bottom panel) demonstrates equivalent total Src expression. Phosphorylation was quantified by densitometric scanning. D: Same as panel C using transient transfection with i_{21}VEGFR-1-GFP. Columns indicate mean of three experiments. Bars indicate standard error. $^{*}P < 0.008$ significant change in phosphorylated Src was determined by Student's *t*-test.

cancer cells activating invasion. Further studies will show if endogenous activation of Src kinase by i_{21}VEGFR-1 could make cancer cells independent of the corresponding extracellular ligands and resistant to anti-ligand and anti-receptor therapies.

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