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ORIGINAL PAPER

Transcription of the vascular endothelial growth factor receptor-3 (*VEGFR3*) gene is regulated by the zinc finger proteins Sp1 and Sp3 and is under epigenetic control

Transcription of vascular endothelial growth factor receptor 3

Johannes Hertel · Christoph Hirche · Christoph Wissmann · Matthias P. Ebert · Michael Höcker

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Abstract
Background In the past, the vascular endothelial growth factor receptor-3 (*VEGFR-3*) has been linked to the regulation of lymphangiogenesis and the lymphatic spread of solid malignancies. The molecular mechanisms controlling *VEGFR3* gene expression have, however, remained poorly understood. Here, we aimed at assessing these mechanisms through *VEGFR3* gene promoter analysis and the identification of transcription factors binding to it. In addition, we focussed on epigenetic modifications underlying *VEGFR3* transcription regulation.
Methods 5′ Deletion analyses for the identification of functional promoter elements, electrophoretic mobility shift assays, chromatin immunoprecipitations, methylation-specific PCRs, and Trichostatin A (TSA) and 5-Aza desoxycytidine (5-Aza dC) treatments were performed in this study.
Results Following the isolation of a 2 kb stretch of 5′-flanking DNA of *VEGFR3*, we identified a novel GC-rich element (GRE) spanning −101/−66 sufficient for *VEGFR3* transcription and activated by Sp1 and Sp3, respectively. Histone deacetylase inhibition by TSA led to the accumulation of acetylated histones H3/H4 at the *VEGFR3* gene promoter, up-regulation of its mRNA levels, and transactivation of promoter reporter constructs in endothelial cell lines. Similarly, methylation inhibition by 5-Aza dC triggered up-regulation of *VEGFR3* mRNA levels and increased promoter activity. TSA and 5-Aza-dC did not influence Sp1/Sp3 binding, but increased the transactivating capacity of both transcription factors, suggesting epigenetic modification as an underlying mechanism.
Conclusions Here we describe the identification of regulatory elements controlling human *VEGFR3* gene expression and show that histone acetylation and CpG methylation are important determinants of *VEGFR3* transcription regulation. These findings may facilitate the development of intervention strategies aimed at targeting *VEGFR3*-based tumor lymphangiogenesis and/or lymphatic tumor spread.

Keywords VEGFR-3 · FLT-4 · Sp factors · GC rich element · Epigenetic regulation

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1 Introduction

Angiogenesis, the formation of new blood vessels from endothelial precursor cells, is a prerequisite for the growth and dissemination of solid malignancies [1]. The vascular endothelial growth factor (*VEGF*) superfamily of endothelial growth factors has been identified to critically influence tumor-related angiogenesis [1]. Recently, it became clear that basic mechanisms underlying hemangiogenesis also apply to the lymphatic system, and that *VEGF-C* and its homologue *VEGF-D* are key regulators of lymphangiogenesis and lymphatic tumor spread [2]. The lymphangiogenic effects of *VEGF-C* and *VEGF-D* are mainly mediated by activation of the VEGF receptor-3 (VEGFR3), which is expressed in lymphatic vessels and angiogenic endothelial cells of tumor blood

vessels [2]. The presence of *VEGFR3* in tumor tissues was found to correlate with lymphatic spread, lymphatic tissue invasion, and/or poor prognoses in endometrial [3], lung [4], breast [5], gastric [6] and ovarian cancers [7]. In addition, functional inhibition of *VEGFR3* in a murine gastric cancer model resulted in reduction of tumor size and lymphatic spread, further supporting the involvement of *VEGFR3* in tumor lymphangiogenesis and lymphatic tumor spread [8].

While increasing evidence supports an important role of *VEGFR3* as regulator of tumor lymphangiogenesis and lymphatic dissemination, the current understanding of the molecular determinants and regulatory pathways controlling its expression is very limited. In vitro studies have shown that external growth factors [9], as well as genetic mutations [10], may enhance *VEGFR3* gene expression. In addition, a Notch-dependent pathway of *VEGFR3* promoter regulation has been described [11]. Moreover, a homology region containing multiple conserved binding sites for Sp1-like factors has been identified in the murine *VEGF3* promoter [12]. Despite these molecular insights, however, a detailed analysis of transcription factors and DNA elements regulating the human *VEGFR3* gene has not been performed yet.

The transcription factors Sp1 and Sp3 belong to a superfamily of Sp-like zinc finger proteins [13], which are known to control constitutively expressed 'housekeeping genes' influencing cellular growth and differentiation. More recently, Sp-based transcription was found to be involved in the mechanisms controlling inducible gene expression, including the *VEGF-A* gene family [14–16], and some of our own work has shown that Sp1 and Sp3 may mediate the effects of oxidative stress and *H. pylori* infection on the *VEGF-A* promoter in gastric cancer cells [17, 18].

Regulatory mechanisms comparable to those controlling the lymphangiogenic *VEGF-C/D* genes are expected to play a role in *VEGFR3* transcription regulation, but these have not been reported yet. Therefore, we set out to clone and sequence the human *VEGFR3* gene promoter and, by doing so, identified a minimal regulatory promoter element crucial for *VEGFR3* transcription at −101/−66. This element was found to harbour Sp1 and Sp3 binding sites. In addition, we found that histone de-acetylase inhibition by TSA triggers the accumulation of acetylated histones H3/H4, the up-regulation of mRNA levels, and the transactivation of promoter reporter gene constructs in endothelial cell lines. Similarly, we found that methylation inhibition by 5-Aza dC triggers up-regulation of *VEGFR3* mRNA expression levels and increased promoter activity.

2 Material and methods

2.1 Cell culture and transfection

HMEC-1, MKN-45, MKN-28, Kato-III, Ea.Hy926, HeLa, AGS and SL-2 cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Human Pulmonary Microvascular Endothelial Cell (HPMEC) cell lines were purchased from PromoCell (Heidelberg, Germany). HUVEC cell lines, obtained from in-house preparations, were cultured for no longer than 2 months and regularly replaced by verified frozen stocks. All cells were routinely tested for mycoplasma and found to be free of contamination. HMEC-1 and HPMEC cells were grown in MCDB-131 medium (Invitrogen, Karlsruhe, Germany) supplemented with 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 100 mg/ml human recombinant EGF, 1 mg/ml hydrocortisone and 10 % bovine calf serum in a humidified atmosphere. Ea.Hy926 cells were grown in Dulbecco's modified Eagle medium (Invitrogen, Karlsruhe, Germany) supplemented with 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin and 10 % bovine calf serum in a humidified atmosphere. SL-2 cells were grown as previously described [17]. Transient transfections were carried out using either the Effectene® reagent (Qiagen, Hilden, Germany) according to the manufacturer's instructions, or the calcium phosphate precipitation technique as described before [17]. Luciferase activities were detected using a Dual-Luciferase®-Reporter Assay System as previously described [17]. The incubations were performed in triplicate and the results were normalized for transfection efficiency and calculated as mean +/− standard error of the mean (S.E.M.). The values were expressed as x-fold increases in luciferase activities compared to controls. Statistical significances were calculated using Student's *t*-test (* = $P<0.05$; ** = $P<0.01$; *** = $P<0.001$).

2.2 RNA isolation and quantitative real time PCR

Quantitative RT-PCR analyses were performed as previously described [19]. Briefly, total RNA was isolated from cell lines or tissues using the TRIZOL reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer's guidelines [20]. In addition, RNA was reverse transcribed using oligo-dT primers and SuperScript II polymerase (Invitrogen, Karlsruhe, Germany). cDNAs generated from 50 ng of total RNA were used for quantification using the TaqMan-Universal or SYBRGreen PCR Master Mix (Applied Biosystems, Darmstadt, Germany) in conjunction with an ABI PRISM 7700 Sequence Detection System. Primers and probes were applied as listed in Table 1 for *VEGFR3*, *GAPDH* and *β-actin*.

2.3 DNA constructs and reporter plasmids

Using the Advantage-GC Genomic PCR Kit (Clontech, Heidelberg, Germany) a genomic fragment of 918 base pairs was isolated from the 5′ flanking region of the human *VEGFR3* gene by amplifying human genomic DNA with two gene-specific primers, VEGFR-3 (−859) and VEGFR-3 (+59), located within the first exon of the *VEGFR3* gene

Table 1 Primer and probe sequences for quantitative RT-PCR, immunoprecipitation and CpG methylation analyses

	Forward (5′→3′)	Reverse (5′→3′)	Probe (5′-Fam→3′-Tamra)
RT-PCR			
vegfr-3	*gctgagacccgtggttcct*	*ctatgcctgctctctatctgctcaa*	*acgacctacaaaggctctgtggacaacca*
gapdh	*gaaggtgaaggtcggagtc*	*gaagatggtgatgggatttc*	*caagcttcccgttctcagcc*
β-actin	*tgcattgttacaggaagtccctt*	*gggagaggactgggccat*	*ccatcctaaaagccaccccacttctctcta*
Immunoprecipitation (for amplification)			
vegfr-3	*cgtctgcgttacccgcgt*	*ggctgaaagtgtccgcgc*	
CpG methylation analysis			
vegfr-3			*aaatcccaaacaaaaccacaat*

(NM_002020) (Table 2). The amplified ~0.9 kb DNA fragment was sub-cloned into the promoter-less luciferase reporter gene vector pGL3 (Promega, Mannheim, Germany) yielding the reporter gene construct *VEGFR3*(−859/+59). Using *VEGFR3*(−859/+59) as a template, 5′-deletion constructs were generated using a common 3′ primer and different 5′ primers (Table 2) in PCR reactions as previously described [17]. The *VEGFR3*(−1876/+59) construct was generated by addition of a −1876/−856 fragment to the *VEGFR3*(−859/+59) construct (GenBank accession no. GU733442). The identified *VEGFR3* regulatory elements were studied in the heterologous promoter system vector pT81-Luc as previously described [17], which contains the enhancer-less herpes simplex virus thymidine kinase (TK) promoter. Reporter plasmid Gal4—Luc, in which the reporter gene is driven by a multimer of the Gal4 yeast transcription factor-binding element, as well as the transactivator constructs Gal4—Sp1 and Gal4—Sp3, have been described before [17]. Expression constructs encoding wild type Sp1 or Sp3 (pPac-Sp1 and pPac-Sp3), or their corresponding mutants lacking the transactivation domain (pPac-Sp1-DBD and pPac-Sp3-DBD), have also been described before [17].

2.4 Electrophoretic mobility shift assays

EMSAs were carried out as previously described [17]. In brief, nuclear protein extracts (10 μg) were incubated with [g-^{32}P]ATP-radiolabeled double-stranded oligonucleotides (Table 3), labeled with T4 Polynucleotide Kinase through an Exchange Labeling Reaction as described by the manufacturer's guidelines (Invitrogen, Karlsruhe, Germany). DNA binding reactions were performed in a buffer containing 20 mM HEPES (pH 8.4), 1 μg poly(dI-dC), 15 μg bovine serum albumin, 60 mM KCl, 3 mM dithiothreitol, 1 mM ZnCl2 and 10 % glycerol per gel pocket for 30 min at 30 °C. For competition experiments, nuclear extracts were incubated with 100-fold molar excess of double-stranded, unlabeled competitor oligonucleotides (Table 3). For supershift experiments, nuclear extracts were incubated with antibodies directed against Sp1, Sp3, Sp4, Ap2a, Egr-1 and c-Myc (Santa Cruz Biotechnology, Santa Cruz, CA). DNA-protein complexes were electrophoresed in 5 % non-denaturing polyacrylamide gels containing 0.5 × TBE (50 mM Tris, 50 mM borate, 2 mM EDTA). Finally, gels were dried and exposed to Kodak BioMax MR Films.

2.5 Chromatin immunoprecipitation and quantitative PCR

Chromatin immunoprecipitations were carried out as recommended by the supplier of the anti-acetyl-H3 and anti-acetyl-H4 antibodies (Upstate Biotech). Chromatin was cross-linked by the addition of formaldehyde to a final concentration of 1 % and incubated for 30 min at 37 °C. Cells were washed twice with PBS, counted and 1×10^6 cells were re-suspended in 200 μl of SDS lysis buffer. Recovered cross-linked adducts were sheared by sonication and centrifuged for 10 min at 14.000 g at 4 °C, after which the supernatant was 10-fold diluted in dilution buffer. As input controls, 1 % of each

Table 2 PCR primers for generation of 5′-deletion constructs

	5′ deletion	Sequences (5′-3′)	
vegfr-3 (+59)	agtaccatggagccacagtcgcaggcacag	*vegfr-3* (−198)	agtcagatctgaccctgtggtgcggagcg
vegfr-3 (−1876)	tacgctcgaggctgcggtgagcagatatcatgt	*vegfr-3* (−171)	agtcagatctggagcggcctgaatcc
vegfr-3 (−858)	agtcagatctgtaagaggtgccaagtcagaaag	*vegfr-3* (−113)	agtcagatctccaggccagccggcgc
vegfr-3 (−554)	gtcagatcttctcccgggttcaagcgattc	*vegfr-3* (−94)	cccgcctccggcccccgccccgcccc
vegfr-3 (−490)	agtcagatctcacaaccaagctcggcgtattatt	*vegfr-3* (−42)	agtcagatctgcgcggacactttcagcc
vegfr-3 (−319)	agtcagatctaagagcgcgtgccgcatttt	*vegfr-3* (−5)	agtcagatctgtcggacccacgcgcagc

Table 3 Sequences of probes and competitor oligonucleotides used in EMSA studies and in the heterologous promoter system vector pT81-Luc

Name	Sequences (5′—3′)	^{32}P-probe	Competitor	pT81-Luc
vegfr-3(−101/−32)	tcccgggcccgcctccggcccccgccccgccccgccccgccccaggccagccggcgcccgcgcggacactt	✓	✓	✓
vegfr-3(−101/−66)	tcccgggcccgcctccggcccccgccccgccccgcc	✓	✓	✓
vegfr-3(−65/−32)	ccgccccaggccagccggcgcccgcgcggacact	✓	✓	
vegfr-3(−104/−85)	gaatcccgggcccgcctccg	✓	✓	
vegfr-3(−84/−63)	gcccccgcccccgcccccgcccg	✓	✓	
vegfr-3(−101/−66) mt1	tcccg**tttt**cgc**tat**cggcccccgcccccgcccc gcc	✓	✓	✓
vegfr-3(−101/−66) mt2	tcccgggcccgcctccggc**ttt**cgc**tt**cgc**tt**cgc**t**	✓	✓	✓
vegfr-3(−101/−66) mt3	tcccg**tttt**cgc**tat**cggc**ttt**cgc**tt**cgc**tt**cgc**t**	✓	✓	✓
vegfr-3(−84/−63) mt1	gc**ttt**cgc**tt**cgc**tt**cgc**tt**cg	✓	✓	

Mutations introduced are given in boldface

diluted supernatant was retained at this step. Sheared chromatin was pre-cleared for 1 h at 4 °C with 40 μl salmon sperm DNA/protein A agarose slurry. Acetylated histone H3 (5 μg) and acetylated histone H4 (5 μg) antibodies (Upstate Biotechnologies), rabbit pre-immune sera (60 μg) or no antibody were added to pre-cleared supernatants and left for immunoprecipitation overnight at 4 °C with end-over-end rocking. Immune complexes were incubated for 1 h at 4 °C with 30 μl of salmon sperm DNA/Protein A agarose slurry, then gently centrifuged for 1 min by 2,000 rpm and washed with low salt wash buffer, high salt wash buffer, LiCl wash buffer and TE buffer. DNA-protein complexes were eluted twice in 250 μl fresh 1 % SDS, 0.1 M NaHCO3 and cross-links were reversed at 65 °C for 4 h. After 1 h of proteinase K (10 mg/ml) digestion at 45 °C, the DNA samples were phenol/chloroform extracted before ethanol precipitation. Primers 5′- CGTCTGCGTTACCCGCGT-3′ (forward) and 5′- GGCTGAAAGTGTCCGCGC-3′ (reverse) were used to amplify the *VEGFR3* promoter from immunoprecipitated DNA. Quantitative amplification using a qPCR SYBR Green Core Kit (Eurogentec, Köln, Germany) was performed according to the manufacturer's conditions. The quantity of the PCR products for untreated and TSA treated cells after immunopreciptation with the antibodies or controls were normalized to the input controls.

2.6 Antibody-specific epitope recognition

Anti-Sp1 (PEP2) was raised against amino acids 528–546 mapping within an internal region of Sp1 of rat origin, while anti-Sp3 (D-20) was raised against a peptide mapping at the C-terminus of Sp3 of human origin. Anti-Sp4 (V-20) interacts with a peptide mapping at the C-terminus of Sp4 of human origin and anti-Ap2a (C-18) interacts with the C-terminus of AP-2α of human origin. Anti-Egr-1 (C-19) was applied as a purified rabbit polyclonal antibody raised against a peptide mapping at the C-terminus of Egr-1 of human origin. The anti-c-Myc antibody recognizes an epitope corresponding to amino acids 408–439 within the carboxy terminal domain of human c-Myc. The anti-H3/H4 antibodies were generated against chemically synthesized peptides corresponding to amino acid 219 of *T. thermophila* H4 and amino acid 121 of *T. thermophila* H3, with acetyl groups on lysines 4,7,11 and 9, 14, 15, 29, respectively.

2.7 TSA and 5-Aza dC treatment

Cells were exposed to Trichostatin A (TSA) and/or 5-Aza-2-deoxycytidine (5-Aza dC) (Sigma-Aldrich, Germany) for 24 h and/or 72 h. Agent concentrations differed between experimental settings and are, therefore, mentioned explicitly in the results section. During exposure, medium and 5-Aza dC were replaced every 24 h. Control cultures were incubated with the appropriate solvents for identical time periods. After incubation, cells were washed, harvested in Trizol (Invitrogen, Karlsruhe, Germany) and RNA was prepared for real time RT-PCR and quantification of *VEGFR3* expression as described above. The influence of TSA on the *VEGFR3* promoter was assessed by changing the medium 2 h after transfection, incubation of the cells with 300 ng/ml TSA or vehicle alone for an additional 24 h and, finally, measuring luciferase activity.

2.8 Bisulfide modification of genomic DNA

The CpGenome™ DNA Modification Kit (Chemicon Europe, Hampshire, UK) was used to treat 1 μg genomic DNA extracted from the cell lines HMEC, Ea.Hy926 and AGS according to the manufacturer's recommendations. Modified DNA was re-suspended in 50 μl of 10 mM Tris-buffer and immediately stored at −20 °C for further use.

2.9 CpG methylation analysis of the *VEGF3* promoter

To assess the CpG methylation status of the *VEGFR3* promoter, the sequence from −177 to +72 was amplified by methylation-specific PCR (MSP) in two steps with specific

primers for methylated vs. unmethylated DNA (Table 4). For the first MSP 2 μl bisulfite treated DNA was amplified with 1 unit HotGoldStar DNA-Polymerase (Eurogentec, Seraing, Belgium), 2 mM $MgCl_2$, 10 mM dNTPs and 10 pmol of forward and reverse primers using standard PCR conditions: 10 min at 95 °C, 30 s at 94 °C, 45 s at 60 °C and 60 s at 72 °C (40 cycles) followed by 7 min elongation. Next, 2 μl PCR product from each sample was used for a second PCR reaction to amplify a nested PCR product of 249 bp. For this second MSP reaction we used the forward primer from the first MSP (Meth-forward-vegfr-3 or Unmeth-forward-vegfr-3; Table 4) combined with a new nested (bisulfide conversion-specific) reverse primer (5′AAATCCCAAACAAAACCACAAT 3′) under the same PCR conditions described above. The MSP reaction was always checked by using bisulfite-treated universal methylated DNA (Chemicon Europe, Hampshire, GB) as a positive control. The PCR products were resolved by electrophoresis in 1 % agarose gels containing ethidium bromide. Thereafter, the 249 bp PCR products of the second MSP step were cloned into a pCR®2.1-TOPO® vector using the TOPO TA Cloning Kit (Invitrogen, Karlsruhe, Germany). Plasmid DNA of 4–9 individual clones from each sample were prepared using the QIAprep Spin Miniprep Kit (Qiagen, Hilden, D) and the inserted PCR fragments were sequence verified (JenaGen GmbH, Jena, Germany).

3 Results

3.1 A GC-rich region between −101 and −66 is essential for *VEGF3* promoter activity

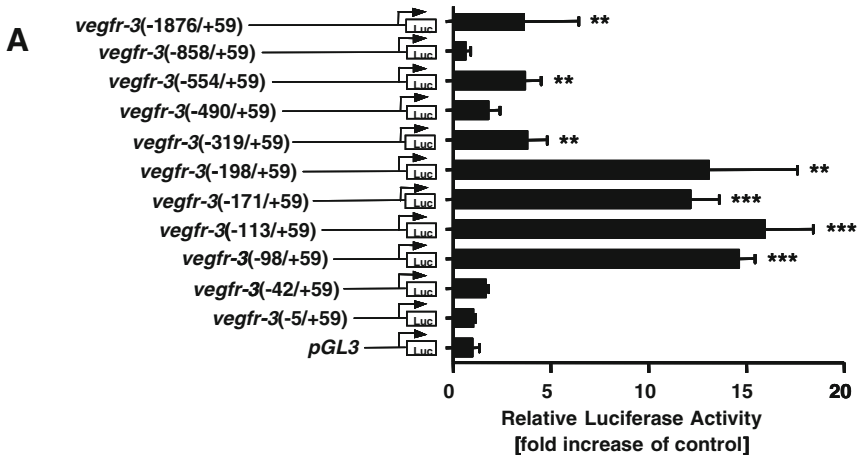
For functional delineation of the proximal promoter region of the human *VEGFR3* gene, luciferase assays in conjunction with 5′ deletion analyses were performed in HMEC-1 cells. By doing so, we found alternating luciferase activity levels corresponding to deletions ranging from −1876 to −319 (Fig. 1a). A further deletion to −198 significantly increased the reporter gene activity (~12-fold), and this activity remained at an elevated level until deletion to −98. Additional loss of 46 bp [*VEGFR3*(−42/+59)] completely abolished *VEGFR3* promoter activity, suggesting the presence of an essential regulatory element controlling its transcription between −98 and −42. Detailed analysis of the sequence from −101 to −32 by a TransFac® database search revealed a GC-rich region with parts of repetitive binding sites for different types of transcription factors (Fig. 1b). Based on this information, we decided to use this DNA fragment as a radiolabeled probe in EMSA assays. Simultaneously, this fragment was sub-cloned into the heterologous, enhancer-less luciferase reporter construct pT81 and functionally analysed through transfection assays in HMEC-1 cells (Fig. 1d). In the EMSA assays, the binding of nuclear proteins led to the formation of two complexes, an upper complex I and a lower complex II (Fig. 1c left, lane 1). For further delineation, the *VEGFR3*(−101/−32) construct was divided into a 5′ (*VEGFR3*(−101/−66)) and a 3′ (*VEGFR3*(−65/−32)) construct and partial sequences (Fig. 1b) that were used as radiolabeled probes in the EMSA assays. Here, only the −101/−32 and −101/−66 stretches formed the two complexes with equal intensities, suggesting equal functional characteristics (Fig. 1c left). Sub-cloning of *VEGFR3*(−101/−66) into the reporter-less empty vector pT81 and subsequent functional analyses through transfection assays resulted in a similar (~25-fold) luciferase activity of *VEGFR3*(−101/−32) compared to the empty pT81 vector, thus underscoring the enhancer characteristics of the element (Fig. 1d). These data strongly suggest the presence of essential enhancer element(s) within the *VEGFR3*(−101/−66) promoter sequence. This observation prompted us to use the latter fragment as a minimal promoter fragment in the following experiments. Site-directed mutagenesis of the 5′ GC-rich region reduced the *VEGFR3* promoter activity by 50 %, whereas disruption of the 3′ GC-rich region led to a reduction to 25 %. Moreover, mutation of both GC boxes completely abolished *VEGFR3* promoter activity (Fig. 1d), thus underscoring the importance of both GC-rich sequences for the transcriptional activity of the *VEGFR3* promoter. These results were confirmed by EMSA competition studies, i.e., *VEGFR3*(−101/−66) mt1 and *VEGFR3*(−101/−66) mt2 both suppressed the formation of complex I and restored complex II, whereas *VEGFR3*(−101/−66) mt3 resulted in the formation of the typical complex pattern (Fig. 1c right, lanes 4–6).

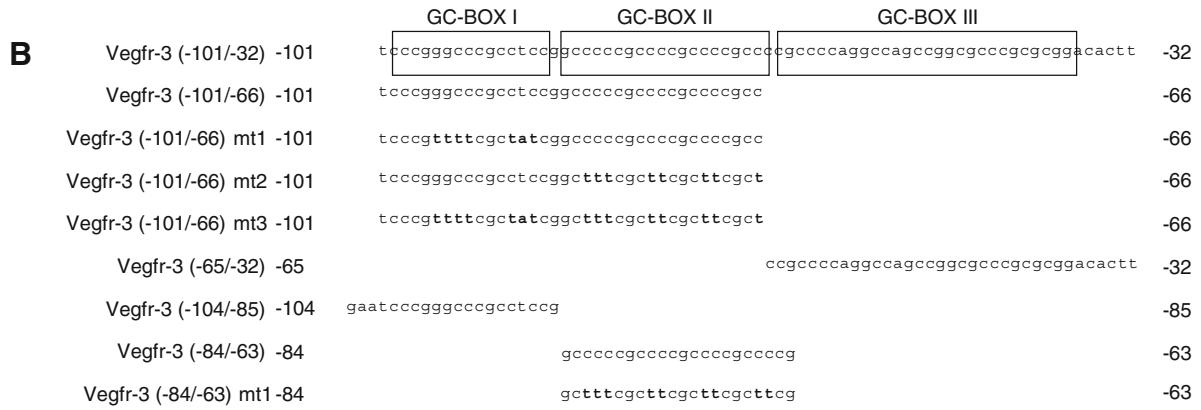
Table 4 Primer sequences used in methylation specific PCR (MSP) for analysis of the CpG methylation status of the *VEGFR3* promoter

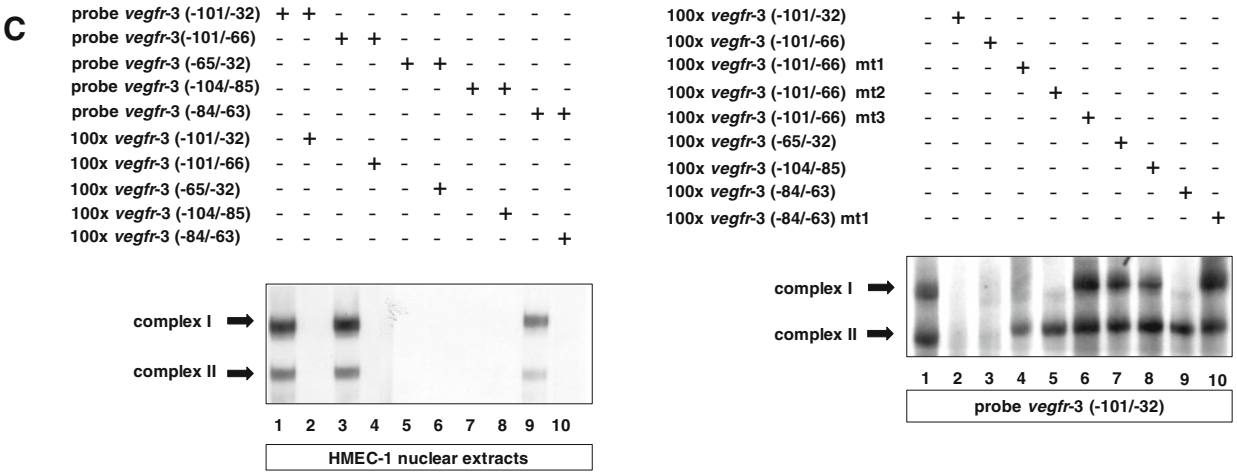
First step MSP-primer (location)	Primer sequence in 5′ → 3′
Meth-forward-vegfr-3	tattgttcgggtgtatcggacgt
Meth-reverse-vegfr-3	ctcaccgtccaaaaatcccaaaca
Unmeth-forward-vegfr-3	tattgtttgggtgtattggatgt
Unmeth-reverse-vegfr-3	ctcaccatccaaaaatcccaaaca

3.2 *VEGFR3* expression and promoter activity in endothelial and epithelial cell lines

In order to identify cell lines suitable for further analysis, *VEGFR3* gene expression levels were determined by quantitative RT-qPCR in the endothelial cell lines HMEC-1, HPMEC, Ea.Hy926 and HUVEC and in the epithelial cell lines AGS, Kato-III, MKN-25 and MKN-48. By doing so, we observed high *VEGFR3* mRNA expression levels in endothelial HMEC-1 cells, moderate expression levels in umbilical HUVECs (~50 % of HMEC-1) and low expression levels in


A
vegfr-3(-1876/+59)
vegfr-3(-858/+59)
vegfr-3(-554/+59)
vegfr-3(-490/+59)
vegfr-3(-319/+59)
vegfr-3(-198/+59)
vegfr-3(-171/+59)
vegfr-3(-113/+59)
vegfr-3(-98/+59)
vegfr-3(-42/+59)
vegfr-3(-5/+59)
pGL3
**
**
**
**
***
***
***
0 5 10 15 20
Relative Luciferase Activity
[fold increase of control]


B
GC-BOX I
GC-BOX II
GC-BOX III
Vegfr-3 (-101/-32) -101 tcccgggcccgcctccggccccgccccgccccgccccgccccaggccagccggcgcccgcgcggacactt -32
Vegfr-3 (-101/-66) -101 tcccgggcccgcctccggccccgccccgccccgcc -66
Vegfr-3 (-101/-66) mt1 -101 tcccgttttcgctatcggccccgccccgccccgcc -66
Vegfr-3 (-101/-66) mt2 -101 tcccgggcccgcctccggctttcgcttcgcttcgct -66
Vegfr-3 (-101/-66) mt3 -101 tcccgttttcgctatcggctttcgcttcgcttcgct -66
Vegfr-3 (-65/-32) -65 ccgccccaggccagccggcgcccgcgcggacactt -32
Vegfr-3 (-104/-85) -104 gaatcccgggcccgcctccg -85
Vegfr-3 (-84/-63) -84 gccccgccccgccccgccccg -63
Vegfr-3 (-84/-63) mt1 -84 gctttcgcttcgcttcgcttcg -63


C
probe vegfr-3 (-101/-32)
probe vegfr-3(-101/-66)
probe vegfr-3 (-65/-32)
probe vegfr-3 (-104/-85)
probe vegfr-3 (-84/-63)
100x vegfr-3 (-101/-32)
100x vegfr-3 (-101/-66)
100x vegfr-3 (-65/-32)
100x vegfr-3 (-104/-85)
100x vegfr-3 (-84/-63)
complex I
complex II
1 2 3 4 5 6 7 8 9 10
HMEC-1 nuclear extracts
100x vegfr-3 (-101/-32)
100x vegfr-3 (-101/-66)
100x vegfr-3 (-101/-66) mt1
100x vegfr-3 (-101/-66) mt2
100x vegfr-3 (-101/-66) mt3
100x vegfr-3 (-65/-32)
100x vegfr-3 (-104/-85)
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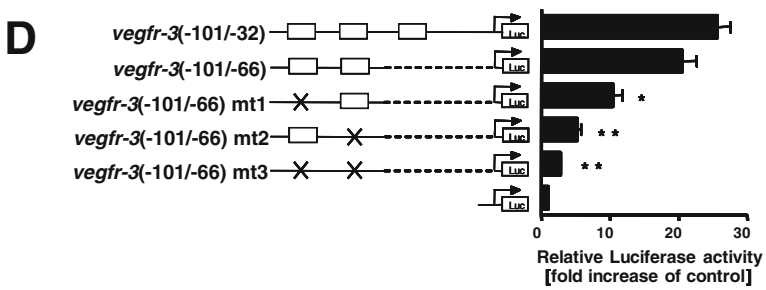
D
vegfr-3(-101/-32)
vegfr-3(-101/-66)
vegfr-3(-101/-66) mt1
vegfr-3(-101/-66) mt2
vegfr-3(-101/-66) mt3
*
**
**
0 10 20 30
Relative Luciferase activity
[fold increase of control]

◀ **Fig. 1** The −101 to −32 sequence is necessary and sufficient for regulating the basal transcriptional activity of the *VEGFR3* gene. **a** HMEC-1 cells were transiently transfected with a pGL3-based series of *VEGFR3* promoter 5′ deletion constructs and luciferase reporter gene activity was determined. Results were normalized for transfection efficiency by co-transfection of equal amounts of a Renilla luciferase reporter gene construct and data are shown as fold increase of activities obtained with the empty vector pGL3. Results are expressed as mean +/− SEM of three separate experiments (*asterisks* indicate statistically significant differences: ** = $P<0.01$; *** = $P<0.001$). **b** Nucleotide sequence of the −101 to −32 *VEGFR3* element and derived wild type and mutant fragments as used in the transcription and EMSA assays. **c** EMSA competition analysis of nuclear proteins binding to the *VEGFR3* sequence. Crude nuclear protein extracts were prepared from HMEC-1 cells and incubated with ^{32}P-labeled double-stranded DNA probes representing the entire −101/−32 *VEGFR3* sequence (*left*), partial wild type or mutated *VEGFR3* sequences (*right*) and indicated competition oligonucleotides, respectively. Complexes were resolved in non-denaturating polyacrylamide gels. **d** The −101/−32 *VEGFR3* sequence confers transcriptional activity to the heterologous promoter system pT81. The entire −101/−32 *VEGFR3* sequence, the minimal promoter element *VEGFR3*(−101/−66) and its mutants were sub-cloned into the heterologous promoter system pT81-Luc and transiently transfected into HMEC-1 cells (*asterisks* indicate statistically significant differences: ** = $P<0.01$). **e** Real-time RT-qPCR quantification of total RNA of human endothelial and epithelial cell lines using *VEGFR3*-specific primers. Expression levels are depicted relative to HMEC-1 cells. **f** Different endothelial and epithelial cell lines were transiently transfected with the *VEGFR3*(−101/−32) promoter construct. Luciferase activity was compared to the empty vector pT81 in each cell line. *Asterisks* indicate statistically significant differences (** = $p<0.01$; *** = $p<0.001$)

pulmonary HPMEC cells (~30 % of HMEC-1). The other cell lines tested, including the endothelial Ea.Hy926 cell line, and

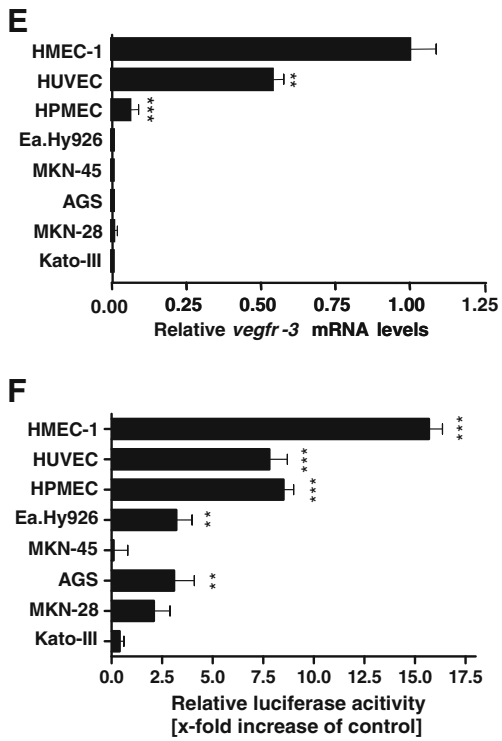

Fig. 1 (continued)

different epithelial tumor-derived cell lines showed no basal expression of *VEGFR3* mRNA (Fig. 1e). Subsequent transfection of the minimal promoter element *VEGFR3*(−101/−32) construct revealed a strong increase of luciferase activity in the highly mRNA expressing HMEC-1 cells (~16-fold). Transfection in HUVECs and HPMEC cells resulted in moderate luciferase activity (~8-fold), whereas transfection in non-expressing cell lines led to low or non-detectable levels of luciferase activity (Fig. 1f).

3.3 Sp1/3 transcription factors bind to the −101/−66 *VEGFR3* promoter sequence

After a TransFac® database search, competition studies of potential transcription factors were carried out using Sp1, Ap2a, Egr-1 and c-Myc consensus binding sequences as unlabeled competition sequences (Table 3). Sp1 (Fig. 2a, lane 3) and Ap2a (Fig. 2a, lane 5) consensus sequences abolished the formation of complex I and II, whereas oligonucleotides with mutated consensus sites restored the formation of complex I and II (Fig 2a, lanes 4 and 6). Oligonucleotides for Egr-1 and c-Myc had no effect on the formation of the respective complexes (Fig. 2a left, lanes 7–10). Application of antibodies directed against Sp1, Sp3, Sp4, Ap2a, Egr-1 and c-Myc in EMSA supershift assays confirmed the obtained results (Fig. 2b). The anti-Sp1 antibody reduced the formation of complex I and produced a supershift, whereas complex II was not affected (Fig. 2b, lane 3). In contrast, addition of the anti-Sp3 antibody abolished the formation of complex II, but had no influence on the shifting of complex I (Fig. 2b, lane 4). The anti-Sp4, anti-Ap2a, anti-Egr-1 and anti-c-Myc antibodies did not influence the complex formation. In order to specify the binding profile of Sp1 and Sp3, radiolabeled *VEGFR3*(−101/−66) mt1 and *VEGFR3*(−101/−66) mt2 were incubated with anti-Sp1 and anti-Sp3 antibodies. The formation of an identical complex pattern suggested the presence of binding motifs for Sp1 and Sp3 in both GC-rich sites of *VEGFR3*(−101/−66). Although the Ap2a consensus sequences resulted in competition of the complexes (Fig. 2a), the anti-Ap2a antibody was not able to supershift or reduce complex formation at probe *VEGFR3*(−101/−66) (Fig. 2b), indicating a cross reactivity of the Ap2a consensus sequence with the Sp1 protein. Therefore, we tested the specificity of the consensus sequences for Sp1 and Ap2a and observed a competition of Sp1 complexes by the Ap2a oligonucleotide and vice versa (Fig. 2d, lanes 3 and 8), using HeLa cells as a reference constitutionally expressing Ap2a (Fig. 2d, lane 10).

In order to investigate the functional impact of the binding of the transcription factors Sp1 and Sp3, transient transfections in *D. melanogaster* SL-2 cells were carried out (Fig. 3). These cells are deficient for several human transcription factors, including Sp1 and Sp3 and, therefore, permit the functional analysis of Sp factors without interfering with

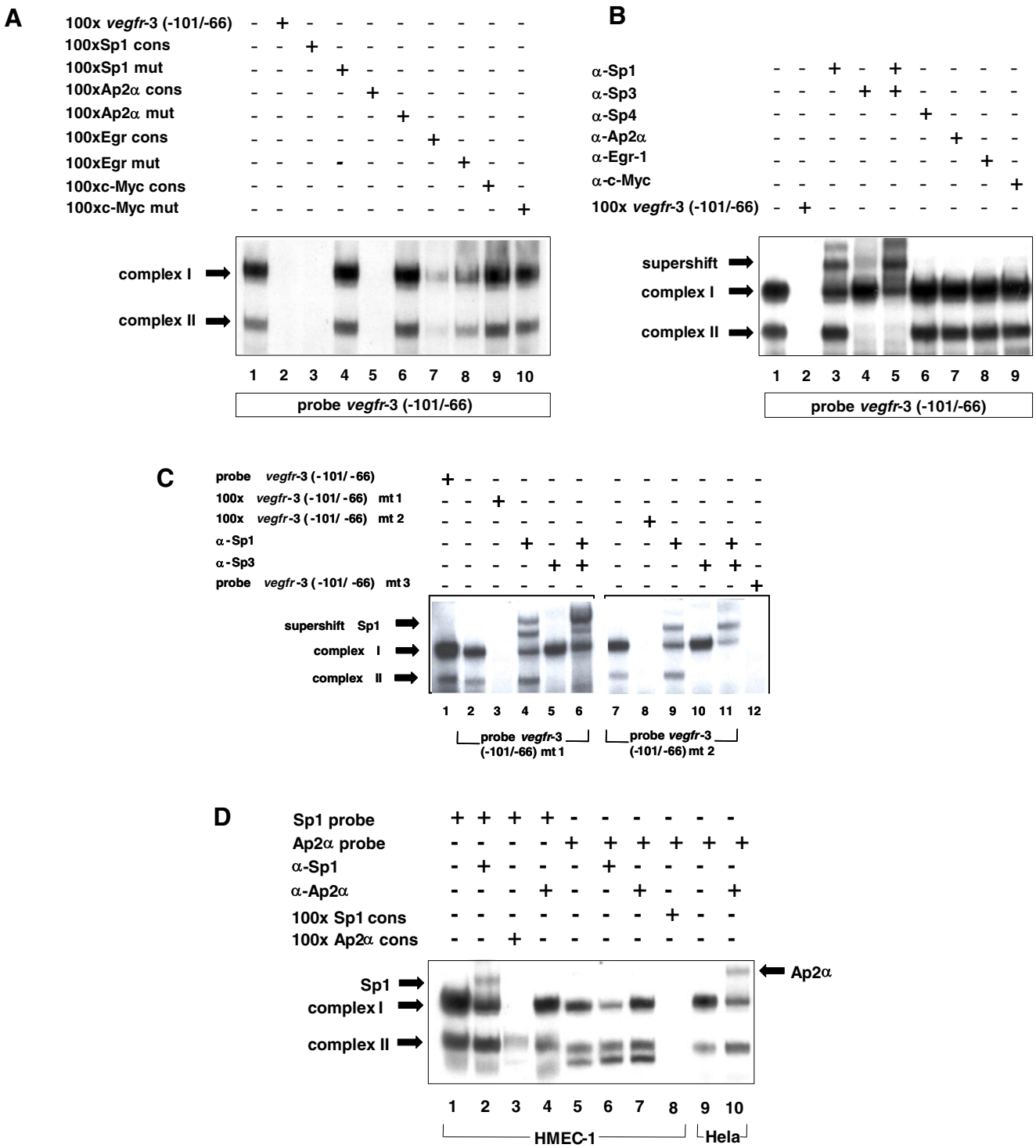

Fig. 2 Transcription factors binding to *VEGFR3*(101/−66). **a** EMSA competition analysis of nuclear proteins binding to the −101/−66 *VEGFR3* sequence. Using ^{32}P-labeled −101/−66 *VEGFR3* nucleotides as a radiolabeled probe, competition studies with consensus binding sites for nuclear hormone receptors were carried out. Unlabeled nucleotides representing consensus and mutant Sp1, AP2α, Egr-1 as well as c-Myc binding motifs were applied in 100-fold molar excess. **b** Supershift analysis of nuclear proteins binding to the −101/−66 *VEGFR3* element. To identify transcription factors binding to the −101/−66 *VEGFR3* sequence, nuclear proteins were prepared from HMEC-1 cells and incubated with ^{32}P-labeled oligonucleotides representing −101/−66 *VEGFR3* as well as specific antibodies as indicated. **c** The mutated *VEGFR3* sequences were used as radiolabeled probes in a competition study with competitor nucleotides as indicated and a supershift analysis with specific antibodies against Sp1 and Sp3. **d** Cross reactivity of Sp1 and Ap2a consensus sequences. Nuclear extracts from HMEC-1 and HeLa cells were probed with radiolabeled Sp1 or Ap2α oligonucleotides and incubated with specific antibodies or competition oliognucleotides for EMSA supershift and competition assays

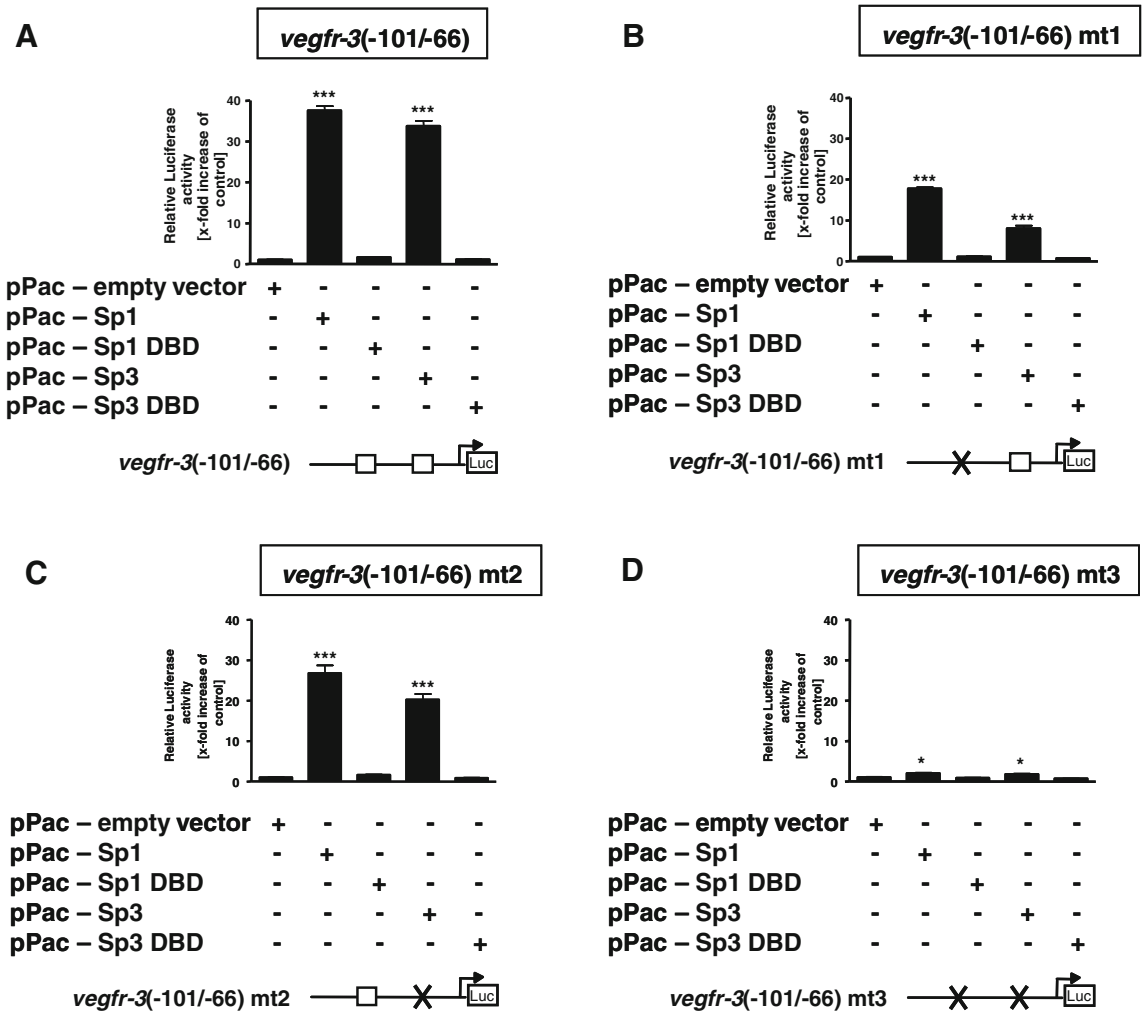

Fig. 3 Sp1 and Sp3 potently activate the *VEGFR3*(−101/−66) element. *D. melanogaster* SL-2 cells were transiently transfected with *VEGFR3*(−101/−66) (**a**), *VEGFR3*(−101/−66) mt1 (**b**), *VEGFR3*(−101/−66) mt2 (**c**) and *VEGFR3*(−101/−66) mt3 (**d**). Additionally, each minimal element was co-transfected with pPac empty vector, pPac–Sp1, pPac–Sp1–DBD, pPac–Sp3, and pPac Sp3–DBD overexpression plasmids. The cells were incubated for 24 h and harvested. The presented data show one typical result of three independent experiments calculated as x-fold increases of presence of pPac empty vector (*asterisks* indicate statistically significant differences: *** = $P<0.001$)

endogenous transcription factors. Co-transfection of Sp1 as well as Sp3 strongly increased the expression of *VEGFR3*(−101/−66) (~35-fold), revealing a potent reactivity of the *VEGFR3* promoter to Sp1 and Sp3 (Fig. 3a). Overexpression plasmid mutants of Sp1 and Sp3 lacking a DNA-binding domain (DBD) were applied as negative controls. These showed no transactivating capacity. The 3′-mutated *VEGFR3*(−101/−66) mt1 revealed significant increases in activity in the presence of both Sp1 (~20-fold) and Sp3 (~10-fold) (Fig. 3b). The 5′-mutated *VEGFR3*(−101/−66) mt2 showed even higher luciferase levels after co-transfection with Sp1 (~30-fold) and Sp3 (~20-fold), respectively (Fig. 3c). Double mutated *VEGFR3*(−101/−66) mt3 revealed slightly positive effects in the presence of Sp1 or Sp3, only reaching luciferase activities comparable to background levels (Fig. 3d).

3.4 The *VEGFR3* gene is regulated epigenetically

In order to assess a putative role of epigenetic modifications on the transcription of the *VEGFR3* gene, the inhibitors Trichostatin A (TSA) and 5-Aza desoxycytidine (5-Aza dC) were applied in stimulation experiments with the endothelial cell lines HMEC-1 and Ea.Hy926, as well as the epithelial cell line AGS. In the endothelial cell line HMEC-1 we found that stimulation with 300 ng/ml TSA for 24 h resulted in a significantly increased expression of *VEGFR3* mRNA compared to the solvent-only treated control (Fig. 4a). Moreover, stimulation of transiently transfected HMEC-1 cells with the *VEGFR3*(−859/+59) reporter construct showed a strong induction by TSA, in contrast to the empty vector control (Fig. 4b), suggesting a direct effect of this HDAC inhibitor on the *VEGFR3* promoter. Furthermore, chromatin

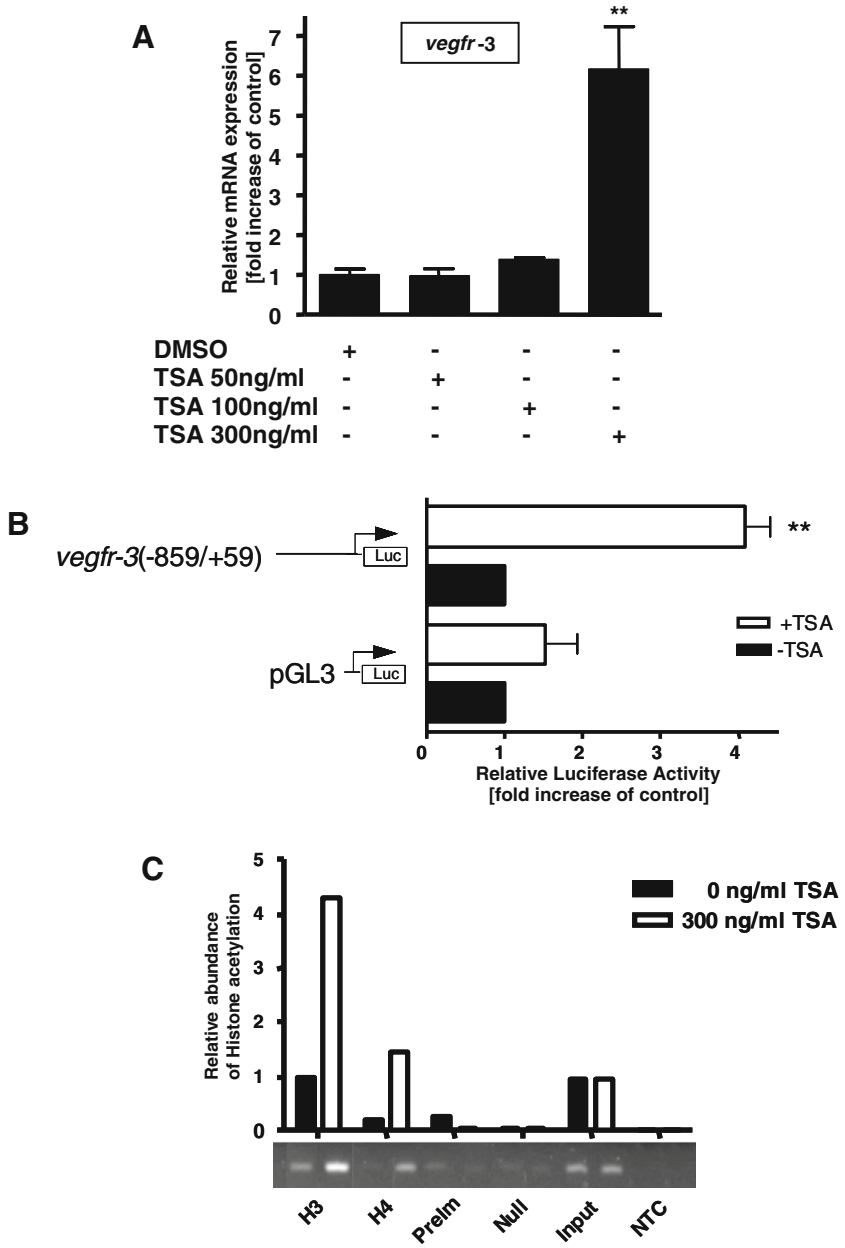

Fig. 4 Influence of promoter acetylation on *VEGFR3* gene expression. HMEC-1 endothelial cells were incubated with different concentrations of TSA for 24 h and *VEGFR3* mRNA levels were determined by RT-qPCR using appropriate primers. *VEGFR3* mRNA abundance is expressed relative to solvent-treated controls and calculated as mean +/− SEM (*asterisks* indicate statistically significant differences: ** = $p<0.01$). Results represent a typical experiment out of three. **b** HMEC-1 cells were transiently transfected with the *VEGFR3*(−859/+59) construct and empty pGL3 vector. After 24 h of TSA treatment cells were harvested and luciferase activity was measured and compared between treated and untreated transfected cells. Results are calculated as mean +/− SEM obtained from three separate experiments (*asterisks* indicate statistically significant differences: ** = $P<0.01$). **c** Quantitative ChIP analysis of Histone H3 and H4 acetylation of the *VEGFR3* promoter. HMEC-1 cells were treated with 300 ng/ml TSA for 24 h or left untreated and DNA-protein complexes were fixed by formaldehyde cross linking, immunoprecipitated with anti-acetylated Histone H3 and H4 antibodies and pre-immune sera (PreIm) or no antibody as controls (Null). Resulting bound DNA was quantitatively amplified by Sybrgreen PCR and normalised to the input control (NTC). Finally, the PCR products were separated in an agarose gel

immunoprecipitation assays (ChIP) revealed a strong increase of the acetylated histones H3 and H4 (~4–6 fold) at the *VEGFR3* promoter (Fig. 4c). To additionally explore a possible transcriptional regulation of the *VEGFR3* gene through promoter methylation, the influence of the demethylating drug 5-Aza dC was tested. In HMEC-1 cells, showing a high basal *VEGFR3* expression level (Fig. 1f), stimulation with 5 μM 5-Aza dC did not result in a significant increase in *VEGFR3*

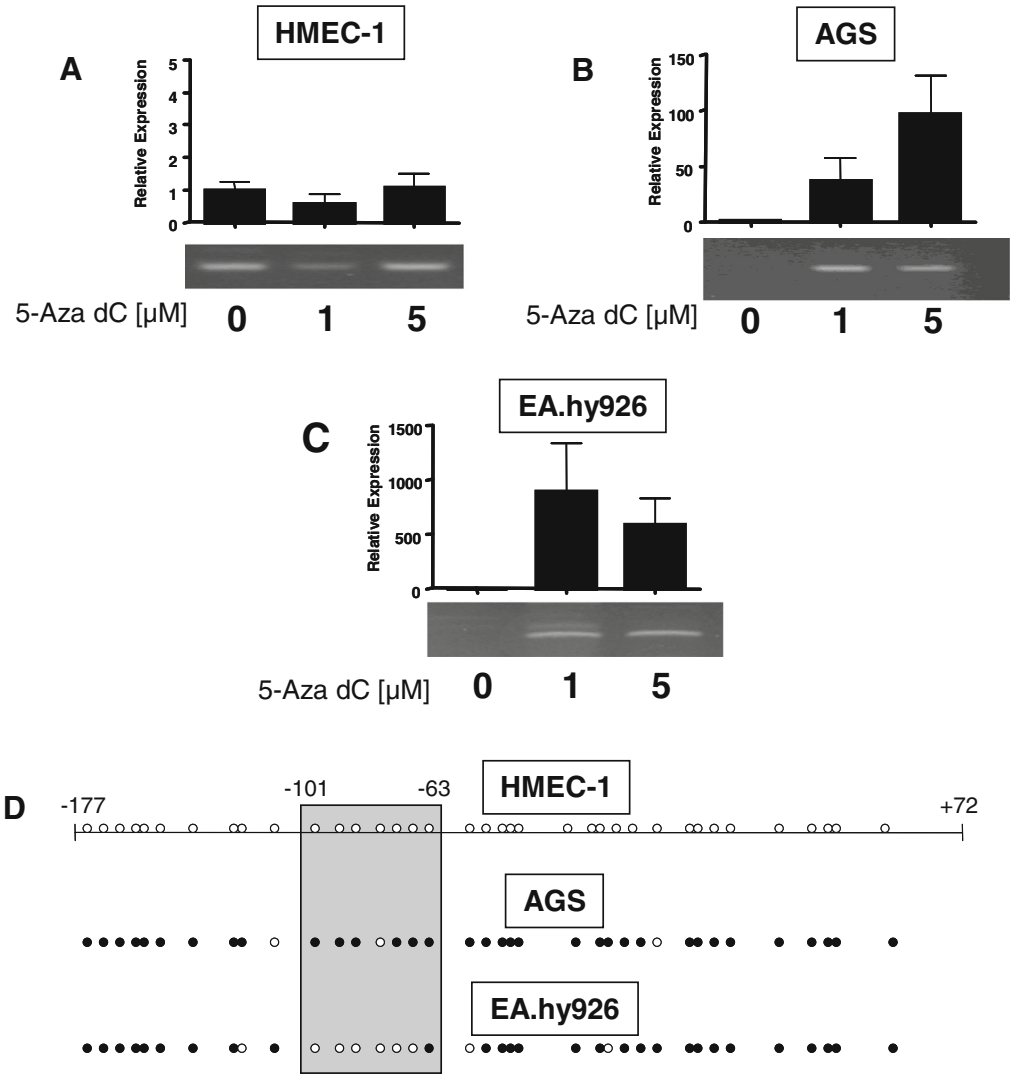

Fig. 5 Influence of promoter methylation on *VEGFR3* gene expression. HMEC-1 (**a**), AGS (**b**) and Ea.Hy926 (**c**) cell lines were incubated 72 h with 5-Aza-dC, after which endogenous *VEGFR3* mRNA levels were determined by quantitative real-time RT-PCR and semi-quantitative RT-PCR, respectively. After normalisation to *ß-actin* as housekeeping gene the expression of *VEGFR3* in each cell line is expressed relative to the expression in solvent treated cells. Results are calculated as mean +/− SEM obtained from three separate experiments. PCR products were separated by agarose gel electrophoresis and presented underneath. **d** MSP Bisulfite sequencing results of genomic DNA from 3 to 8 clones of HMEC-1, AGS and Ea.Hy926 cells. (*black circle*) Methylated and (*white circle*) unmethylated CpG sites are displayed

mRNA level (Fig. 5a). In the epithelial cell line AGS, in which the basal *VEGFR3* level is low, incubation with 1 and 5 μM 5-Aza dC increased the relative expression by a factor 40 and 100, respectively (Fig. 5b). Comparable results were obtained after incubation of the weakly *VEGFR3* expressing endothelial HPMEC cells (data not shown). The endothelial cell line Ea.Hy926, lacking basal *VEGFR3* expression, also showed detectable *VEGFR3* mRNA expression after treatment with 5-Aza dC (Fig. 5c). For verification purposes, we separated the solvent treated and 5-Aza dC stimulated PCR products by agarose gel electrophoresis (Fig. 5a–c). Next, the methylation status of the *VEGFR3* promoter was assessed by methylation-specific PCR (MSP) and bisulfite sequencing of genomic DNA in the HMEC-1, AGS and Ea.Hy926 cell lines (Fig. 5d). While MSP analysis revealed a dense methylation pattern of the *VEGFR3* promoter from −177 to +72 in AGS and Ea.Hy926 cells, in HMEC-1 cells this same promoter sequence showed no methylation. Bisulfite sequencing of 3–8 clones for each cell line confirmed the MSP results. While the AGS cells exhibited a dense methylation pattern in the analysed sequence, HMEC-1 cells did not show any methylated CpGs in the promoter region investigated. In contrast, Ea.Hy926 cells showed a high frequency of methylated CpGs outside the minimal promoter region, while the majority of CpGs inside the minimal promoter sequence was unmethylated.

3.5 The transactivating capacities of Sp1 and Sp3 are affected by epigenetic modifications

By using EMSA binding assays, we compared the formation of complexes using nuclear cell extracts from unstimulated and stimulated HPMEC cells that show a low *VEGFR3* mRNA expression and only a slight formation of EMSA complexes (Fig. 6). The addition of 100 ng/ml TSA to these cells resulted in the formation of typical complexes with a nearly similar pattern intensity (Fig. 6a, lanes 1,2). The addition of specific antibodies directed against Sp1 and Sp3 did not show any significant differences in complex formation (Fig. 6a, lanes 3–6). Simultaneous application of the abovementioned antibodies revealed a slight difference in pattern intensity between unstimulated and stimulated HPMEC cells (Fig. 6a, lanes 7,8). Similar results were obtained by using 5 μM 5-Aza dC as stimulating agent and supershift analyses with the respective antibodies (Fig 6b).

In order to assess the effect of epigenetic modification on the transactivating capacities of Sp1 and Sp3, AGS cells were co-transfected with the 5xGal4—Luc reporter plasmid and Gal4–Sp1 or Gal4–Sp3, respectively (Fig. 6c, d). Whereas individual transfections of 5xGal4—Luc, Gal4—Sp1 or Gal4—Sp3 led to luciferase levels close to the background, co-transfection of the reporter and the transactivating plasmids resulted in readily detectable promoter activity. Stimulation with different concentrations of TSA clearly increased the Sp1 (Fig. 6c) and Sp3 (Fig. 6d) transactivating capacities

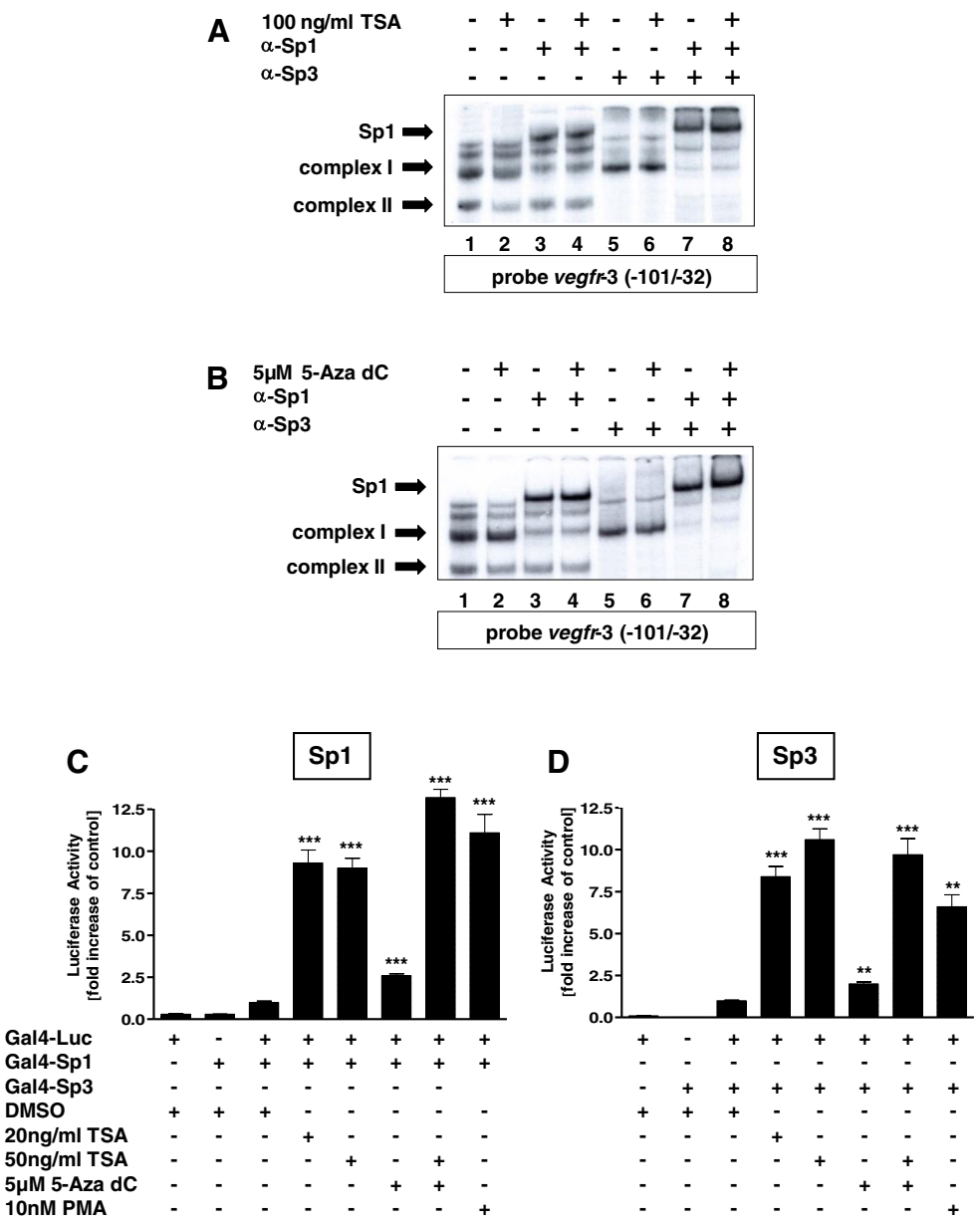

Fig. 6 Influence of epigenetic modifications on binding of and transactivation by Sp1 and Sp3. **a** HPMEC cells were treated with 100 ng/ml TSA for 24 h or left untreated, prepared for nuclear proteins and incubated with radiolabeled *VEGFR3*(−101/−32) probe for EMSA assays. Supershift analysis was carried out using specific antibodies against Sp1 and Sp3 and comparing stimulated and unstimulated nuclear proteins. **b** According to the shift mentioned above HPMEC cells were treated with 5 μM 5-Aza dC for 72 h and nuclear proteins of these cells were used in supershifts comparing stimulated and unstimulated proteins of radiolabeled *VEGFR3*(−101/−32). AGS cells were transiently co-transfected with equal amounts of Gal4–Luc reporter gene and expression plasmids Gal4–Sp1 (**c**) and Gal4–Sp3 (**d**). After transfection for 4 h cells were changed to Ultraculture© media for 20 h and subsequently stimulated with TSA and/or 5-Aza dC for 24 h. As positive control one sample was set to the influence of 10^{-8} M of the PKC activator phorbol 12-myristate 13-acetate (PMA). Values of the stimulated samples are presented as x-fold increases of unstimulated co-transfection of Gal4–Luc and Gal4–Sp1/−Sp3. The data shown represent a typical result out of three experiments

(~10-fold). Slight effects (~2-fold) were noted after stimulation with 5 μM 5-Aza dC, whereas a combination of TSA and 5-Aza dC led to an additional increase of Sp1 (Fig. 6c), but not of Sp3 (Fig. 6d), transactivating capacity.

4 Discussion

In the present study we have uncovered molecular mechanisms underlying transcription regulation of the *VEGFR3* gene, which is intimately implicated in lymphangiogenesis and angiogenesis of normal and malignant tissues. A combination of functional 5′ deletion analyses, systematic core promoter mutagenesis and EMSA studies revealed that two adjacent GC-boxes between −101 and −66 (GC-box I and II) are essential for the regulation of *VEGFR3* promoter activity, and that these two elements are bound and transactivated by the zinc finger transcription factors Sp1 and Sp3. In addition, we found that a third GC-box, located at −65/−32 (GC-box III), is of minor functional importance. GC-rich promoter elements represent typical binding motifs for Sp-like zinc finger proteins, as well as the transcription factors AP2 and Egr-1 (for review see [13]), and have been shown to play essential roles in the regulation of genes controlling cellular growth, proliferation, differentiation and apoptosis [21–24]. In addition to their role as regulators of constitutively expressed 'housekeeping' genes influencing cellular growth and differentiation, Sp1 and Sp3 participate in the regulation of inducible gene expression, and the interaction of Sp1/Sp3 with transcriptional co-factors such as the CREB-binding proteins p300 or CRSP84 may represent an important transcriptional control mechanism [13]. Sp1 can also be regulated through changes in its phosphorylation state [25] and, in addition, different signalling pathways, including RAS-dependent activation of the MEK1/ERK cascade, have been shown to be able to target Sp1 [26–28]. Recent investigations also revealed that the DNA binding activity of Sp1 can be influenced by epigenetic changes brought about through e.g. histone deacetylase (HDAC) inhibition [29].

More recent studies have shown that GC-rich elements are also involved in the regulation of key angiogenesis genes including *VEGF-A*, *VEGFR1* and *VEGFR2* [14–16, 30]. In the *VEGFR2* gene, two GC-rich sites at −60/−37 are critical for the control of its transcriptional activity and, similar to our observations in the *VEGFR3* gene promoter, these proximal GC elements are bound and activated by Sp1 and Sp3 [16], while a third GC-rich motif located at −77/−61 was found to be of minor functional relevance [16]. In contrast to the *VEGFR3* gene, Sp4 was also found to contribute to the regulation of the *VEGFR2* gene. Similar to the findings for *VEGFR2,* several studies including work from our group, showed that for the regulation of the *VEGF-A* gene - which encodes the main ligand for VEGFR2 - two GC-boxes between −88 and −50, which are bound and activated by Sp1 and Sp3, are crucial for regulating its transcriptional activity [17, 18]. In addition to these findings for *VEGF-A* and its receptor *VEGFR2*, a recent study on the *VEGFR1* gene provided evidence that this gene is also regulated through binding of Sp1 and Sp3 to GC-rich elements. A detailed analysis of the regulatory elements involved has, however, not been conducted [15].

Our observations on the *VEGFR3* gene, together with previous findings on the key angiogenesis genes *VEGF-A* and *VEGFR2*, strongly suggest that Sp1/Sp3-dependent activation of proximal GC-elements may represent a core mechanism through which certain genes of the *VEGF* super-family can be controlled. This concept is supported by the finding that in gastric and pancreatic carcinomas the abundance of Sp1 was highly correlated with the expression of the *VEGF-A* gene [31, 32]. Moreover, in both cancer entities a significant correlation between Sp1 abundance and advanced disease stage, lymphatic metastasis and poor survival was found [32, 33], while in gastric adenocarcinomas Sp1 expression was identified as an independent prognostic marker for poor survival [32, 33].

The acetylation/deacetylation of histones, as well as the hypermethylation of gene promoters, plays an important role in the regulation of gene expression [34, 35]. The acetylation status of histones is controlled by the opposing activity of two types of enzymes: histone acetylases and histone de-acetylases [35]. Most of the identified histone acetylases act as transcriptional co-activators, whereas histone de-acetylases function in opposition to histone acetylases by de-acetylating lysine residues on histone tails. Inappropriate recruitment of histone de-acetylases in malignant cells has been found to result in activation of transcriptional programs supporting the onset and progression of cancer. Conversely, histone de-acetylase inhibitors such as TSA have been shown to exhibit potential as anticancer agents [35–37], an effect that could be partially mediated through inhibition of pro-angiogenic factors such as *VEGF-A* [37]. Because previous reports showed that epigenetic modifiers such as TSA can influence the expression of angiogenesis genes including *VEGF-A* [36, 37], we investigated the effect of TSA and 5-Aza dC on the *VEGFR3* gene promoter. Interestingly, we found that the application of TSA stimulated the accumulation of acetylated histones H3 and H4 at the *VEGFR3* promoter and potently induced *VEGFR3* gene transcription, and that 5-Aza dC treatment of cell lines in which *VEGFR3* expression was silenced, resulted in its up-regulation. As TSA and/or 5Aza dC treatment had no influence on the quantitative binding of Sp1 and Sp3 to the *VEGFR3* promoter as shown by detailed EMSA analyses, we employed a Gal4-Sp1/Sp3/Gal4-Luciferase co-transfection system to investigate potential post-translational modifications of both transcription factors in response to epigenetic modification. Here, the luciferase

gene is driven by a multimer of the Gal4 yeast transcription factor binding element, allowing the investigation of direct effects on the transactivation capacities of Sp1 and Sp3 [38]. The transactivation capacities of Sp1 and Sp3 were increased by TSA and to a lesser extent by 5-Aza dC, which strongly suggests that Sp1 and Sp3 are subject to epigenetic modification(s).

Direct effects of epigenetic mechanisms on transcription factors have been investigated before [39], and it has been suggested that TSA can enhance the activity of the *MMP28* gene promoter through direct acetylation of Sp1 and Sp3 [40]. Additionally, the observation that Sp1 associates with HDAC1 at the *NECL1* gene promoter, and that this interaction decreased after TSA treatment, has led to the hypothesis that Sp1, together with its respective binding sites, may represent molecular adapters for HDAC1 [41]. The mechanisms by which DNA methylation can influence Sp-mediated effects are not entirely understood yet, though it is known that methyl groups of CpG sites can directly interfere with binding of Sp1 or Sp3 [42, 43].

In conclusion, we have uncovered molecular mechanisms underlying *VEGFR3* transcription. These mechanisms may play a role in lymphangiogenesis and lymphatic tumor dissemination. We also found that the *VEGFR3* gene is subject to epigenetic control mechanisms mediated by Sp1 and Sp3. Details of these regulatory control mechanisms and the analysis of reversible epigenetic silencing of the *VEGFR3* promoter may contribute to a better understanding of the control over *VEGFR3* expression in the context of vascular differentiation in cancer.

Conflict of interest The authors declare that they have no conflict of interest.

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