DNA methylation regulates expression of VEGF-R2 (KDR) and VEGF-R3 (FLT4)

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Abstract

Background: Vascular Endothelial Growth Factors (VEGFs) and their receptors (VEGF-Rs) are important regulators for angiogenesis and lymphangiogenesis. VEGFs and VEGF-Rs are not only expressed on endothelial cells but also on various subtypes of solid tumors and leukemias contributing to the growth of the malignant cells. This study was performed to examine whether VEGF-R2 (KDR) and VEGF-R3 (FLT4) are regulated by DNA methylation.

Methods: Real-time (RT) PCR analysis was performed to quantify KDR and FLT4 expression in some ninety leukemia/lymphoma cell lines, human umbilical vein endothelial cells (HUVECs) and dermal microvascular endothelial cells (HDMECs). Western blot analyses and flow cytometric analyses confirmed results at the protein level. After bisulfite conversion of DNA we determined the methylation status of KDR and FLT4 by DNA sequencing and by methylation specific PCR (MSP). Western blot analyses were performed to examine the effect of VEGF-C on p42/44 MAPK activation.

Results: Expression of KDR and FLT4 was observed in cell lines from various leukemic entities, but not in lymphoma cell lines: 16% (10/62) of the leukemia cell lines expressed KDR, 42% (27/65) were FLT4 positive. None of thirty cell lines representing six lymphoma subtypes showed more than marginal expression of KDR or FLT4. Western blot analyses confirmed KDR and FLT4 protein expression in HDMECs, HUVECs and in cell lines with high VEGF-R mRNA levels. Mature VEGF-C induced p42/44 MAPK activation in the KDR- /FLT4+ cell line OCI-AML1 verifying the model character of this cell line for VEGF-C signal transduction studies. Bisulfite sequencing and MSP revealed that GpG islands in the promoter regions of KDR and FLT4 were unmethylated in HUVECs, HDMECs and KDR+ and FLT4+ cell lines, whereas methylated cell lines did not express these genes. In hypermethylated cell lines, KDR and FLT4 were re-inducible by treatment with the DNA demethylating agent 5-Aza-2’deoxycytidine, confirming epigenetic regulation of both genes.

Conclusions: Our data show that VEGF-Rs KDR and FLT4 are silenced by DNA methylation. However, if the promoters are unmethylated, other factors (e.g. transactivation factors) determine the extent of KDR and FLT4 expression.

Background

Vascular endothelial growth factors (VEGFs) and their corresponding receptors (VEGF-Rs) are important regulators of angiogenesis and lymphangiogenesis. VEGF-A binds VEGF-R1 (FLT1) and VEGF-R2 (KDR). Both tyrosine kinase receptors are expressed on blood vessel endothelial cells. VEGF-C and VEGF-D bind to VEGF-R3 (FLT4) and the fully processed, mature forms also to KDR. FLT4 is primarily expressed on cells of the lymphatic endothelium [1]. VEGFs and VEGF-Rs are important for vessel formation in healthy individuals, but also for tumor angiogenesis [2]. Moreover, the VEGF-Rs are not only expressed on endothelia, but also on different types of solid tumor cells and on leukemic cells [3-11]. The interaction of receptors with their ligands mediates survival and can lead to proliferation of the malignant cells [2,12].

Even twenty years after their discovery, little is known about the regulation of the three VEGF-Rs. On the transcriptional level, NF-κB and the NF-κB target Proxl have been described as activators of FLT4 in lymphatic endothelial cells [13]. Epigenetic mechanisms contribute
to the regulation of FLT1 and KDR but this is not investigated in great detail [14,15].

We set out to test whether DNA methylation is also responsible for the silencing of FLT4. We determined the methylation status of KDR and FLT4 in human umbilical vein endothelial cells (HUVECs), dermal microvascular endothelial cells (HDMECs) and in a large panel of leukemia and lymphoma cell lines. Confirming that expression of KDR and FLT4 is epigenetically regulated, we observed an inverse correlation between promoter methylation and receptor expression. Furthermore, the demethylating agent 5-Aza-2' deoxycytidine (5-Aza-dC) induced expression of KDR and FLT4 in methylated, but not in unmethylated cell lines.

Methods
Cell lines and primary cell cultures
The cell lines in this study were taken from the stock of the cell bank (DSMZ—German Collection of Microorganisms and Cell Cultures; http://www.dsmz.de). Detailed references and cultivation protocols have been described previously [16]. Primary HDMECs were purchased from Clonetics/Lonza (Verviers, Belgium). Primary HUVECs (pooled) were purchased from PromoCell (Heidelberg, Germany). HDMECs and HUVECs were cultured in endothelial cell growth medium MV (Promo Cell).

CpG island search
CpG island search was done with Methyl Primer Express v1.0 software and EMBOSS CpG plot (http://www.ebi.ac.uk/Tools/emboss/cgplot/index.html). The criteria for an island were: GC content > 50%; CpG observed versus CpG expected ratio > 0.6, length > 100 bp.

Methylation-specific polymerase chain reaction (MSP)
Bisulfite conversion of DNA was performed as described by the supplier (EpiTect Bisulfite Kit, Qiagen, Hilden, Germany). For detecting FLT4 and KDR promoter methylation, we performed nested PCR with first round primers (FLT4 BSP fwd 5'-AAA TAT TTG GGG GAG TTT TAA A-3', FLT4 BSP rev 5'-CCC AAT CTC AAA AAT AAA CAA A-3'; KDR BSP fwd 5'-AAG TGG TTG TTT TGG GAT GTT T-3', KDR BSP rev 5'-AAA TAA TAC AGC GTA CAG-3') amplifying converted DNA independently of the methylation status (bisulfite-specific PCR or BSP; annealing temp.: 54.9°C for FLT4 BSP, 54.7°C for KDR BSP, 35 cycles), and second round primers for M- and U-PCR specifically recognizing the methylated or unmethylated versions of the promoter (FLT4 M fwd 5'-GTC GGT TAT TTC GGG TGT TTC -3', FLT4 M rev 5'-AAT ATC GAC GAA CAA TAT CGA CG-3', FLT4 U fwd 5'-GGG TTG GTT ATT TTG GGT GTT TT-3', FLT4 U rev 5'-ACA CAA TAT CAA CAA ACA ATA TCA ACA-3'; KDR M fwd 5'-CGT TTT CGC GTT TTA GAG TTT C-3', KDR M rev 5'-GCG CAA ATA ATA CCC GAC G-3'; KDR U fwd 5'-TTT TGT TTT TGT GTT TTA GAG TTT T-3', KDR U rev 5'-ACA CAC AAA TAA TAC CCA CAA ACA-3'). PCR products of the initial BSP were diluted 1:100 to 1:4000 for subsequent M- and U-PCR. Annealing temperature was 61.2°C for FLT4 M- and U-PCR, 58°C for KDR M- and U-PCR with 30 cycles each. Epitect PCR Control DNA (Qiagen) was used as control for methylated and unmethylated templates.

Bisulfite sequencing
To confirm the methylation status of the FLT4 and KDR promoters, genomic DNA was bisulfite converted according to the manufacturer’s instructions (Qiagen). Subsequently, amplification of the promoter regions (FLT4: 337 bp; KDR: 612 bp) was performed using BSP primers, specifically binding bisulfite converted DNA (for primer sequence and PCR conditions see MSP section). Resulting PCR products were purified, cloned into the pGEM-TEasy vector (Promega, Madison, WI, USA) and sequenced. Sequences were evaluated using BiQ Analyzer (http://biq-analyzer.bioinf.mpi-sb.mpg.de) and had to conform to at least 90% bisulfite conversion rate. In addition, identical clones were excluded from the analysis.

Gene expression analyses
Quantitative PCR was performed on a 7500 Applied Biosystems (Darmstadt, Germany) real-time PCR system using the manufacturer’s protocol. RNA was prepared using the RNeasy Mini kit (Qiagen). This kit includes a DNase digestion step to avoid false positives resulting from contaminating genomic DNA. For mRNA quantification, reverse transcription was performed using the SuperScript II reverse transcriptase kit (Invitrogen, Karlsruhe, Germany). TaqMan probes (Applied Biosystems) were used to quantify human FLT4 (Hs 01047677 m1) and KDR (Hs 00911700 m1) expression levels with TATA box binding protein (TBP) as endogenous control. For interferon gamma inducible protein 10 (IP-10) and tumor necrosis factor alpha (TNFα), SYTO-82 (Molecular Probes, Leiden, Netherlands) was used as fluorescent dye, Immofix (Biline, Luckenwalde, Germany) as PCR master mix, and ribosomal protein S9 (RPSP9) as endogenous control. The following primers were used: TNFα exon 2 fwd 5'-CCC CAG GGA CCT CTC TCT AA-3', TNFα exon 3 rev 5'-TGG GCT ACA GGC TTG TCA CT-3'; IP-10 exon 1 fwd 5'-GCC ATT CTG ATT TGC TGC CTT A-3', IP-10 exon 2 rev 5'-TGA TGC AGG TAC AGC GTA CAG-3'; RPSP9 exon 2 fwd 5'-GGG TGG TGG TTG GTT ATT TTG GGT GTT TT-3', FLT4 U rev 5'-ACA CAA TAT CAA CAA ACA ATA TCA ACA-3'. Relative
expression levels were calculated using the ΔΔCt-method.

[^3]H]-Thymidine uptake
Assays of [^3]H]-thymidine incorporation were executed as follows: 1.25 × 10^4 cells (in 100 μl) were seeded in triplicate in 96-well flat-bottom microtiter cell culture plates. Inhibitors were added as 2x concentrated solution in a 100 μl volume. For the last 3 h of the incubation period, 1 μCi[^3]H]-thymidine (Hartmann Analytic, Braunschweig, Germany) was added to each well.

Western blot analysis, antibodies, reagents
Samples were prepared as described previously [17]. Anti FLT4, ERK and pERK antibodies were purchased from Santa Cruz (Heidelberg, Germany). Anti IκB, pIκB, KDR, p38 MAPK and pp38 MAPK antisera were obtained from Cell Signalling (New England Biolabs, Frankfurt, Germany). The anti GAPDH monoclonal antibody (mAb) was purchased from Abcam (Cambridge, UK). Specific bands on nitrocellulose membranes were visualized with the biotin/streptavidin-horseradish peroxidase system (Amersham, Freiburg, Germany) in combination with the “Renaissance Western Blot Chemiluminescence Reagent” protocol (Perkin Elmer, Waltham, MA, USA). Synthetic macrophage activating factor of 2 kDa molecular mass (MALP-2) was a gift from P. Mühlradt to H. Weich. The preparation was free of endotoxin.

Analysis of FLT4 and KDR protein expression by flow cytometry
For detection of CD31 (Becton Dickinson Biosciences, Heidelberg, Germany), FLT4 (R&D Systems, Wiesbaden, Germany), KDR (Reliatech, Wolfenbüttel, Germany) and podoplanin (Reliatech) on the cell surface, cells were washed and incubated with the mouse mAb or with the isotope-matched control mouse immunoglobulin (BD Biosciences) for 30 min at 4°C. Subsequently, cells were treated with FITC conjugated anti-mouse secondary Ab (Biozol, Eching, Germany) and propidium iodide. Labeled cells were analyzed on a FACSCalibur (BD Biosciences) using CellQuest Pro software.

Treatment with DNA demethylating agent 5-Aza-2’-deoxycytidine (5-Aza-dC)
5-Aza-dC (Sigma Aldrich, Taukirchen, Germany) dissolved in DMSO was used to verify the effect of methylation on expression of FLT4 and KDR. Cells were seeded at a cell density of 5 × 10^5 cells/ml, 5-Aza-dC was added at a final concentration of 5 μM. Control cells were treated with 0.05% DMSO. After 2 d, half of the medium was replenished with medium with/without 5-Aza-dC (5 μM). After 3 d, cells were harvested to prepare RNA and protein.

Results and Discussion
Expression of KDR and FLT4 in leukemia and lymphoma cell lines
The VEGF-Rs KDR (VEGF-R2) and FLT4 (VEGF-R3) are not only expressed on blood endothelial and lymphendothelial cells, but also on solid tumors and leukemias. Leukemia-derived VEGFs may induce the growth of leu-
kemic cells in an autocrine or paracrine fashion [7,10,18,19]. The promoters of VEGF-Rs and their ligands contain CpG islands, regulatory regions that are typically methylated in epigenetically silenced genes [14]. Recent reports show that expression of FLT1 and KDR are controlled by promoter methylation [14,15]. However, only a limited number of leukemia and lymphoma cell lines have been tested for VEGF-R expression and promoter methylation hitherto.

To find model systems for VEGF-R regulation, we tested some ninety leukemia and lymphoma cell lines for KDR and FLT4 mRNA expression. Both genes were regularly expressed in leukemia but not in lymphoma cell lines: 10/62 (16%) cell lines from various leukemic entities expressed KDR, 27/65 (42%) expressed FLT4 (Table 1). In contrast, 0/30 lymphoma cell lines expressed KDR, and only 1/30 (3%) expressed FLT4 (Table 1). Cell lines with high VEGF-R transcript levels expressed also the corresponding proteins: cell lines CMK, HEL and MEG-01 expressed KDR, whereas cell lines HEL, MHH-CALL2, OCI-AML1 and SUP-B15 were FLT4 positive (Figure 1, Table 2).

OCI-AML1: a model system for VEGF-C induced cell signaling
Cytokine-dependent cell lines have often and successfully been used as model systems for signal transduction studies. In contrast to primary cells, no contaminating cell fraction effects “false” signals in cell lines, and in contrast to cytokine-independently growing cell lines, cytokine starvation silences the relevant enzymes in cytokine-dependent cell lines. We chose cell line OCI-AML1 as this was the only cytokine dependent, FLT4 positive cell line tested (Table 2). The cytokine response profile of this cell line has been published previously [20]. Cell line OCI-AML1 did not show a proliferative response on VEGF-C (data not shown). However, short-term (5 min) stimulation with VEGF-C induced phosphorylation of ERK1/2 (Figure 2). Preincubation with the FLT4 inhibitor MAZ51 inhibited this effect, confirming the specificity of the VEGF-C induced ERK1/2 activation (Figure 2). ERK1/2 phosphorylation was tested because the p42/44 MAPK pathway is a known FLT4 target [21,22]. The results of cell signaling experiments shown in Figure 2 confirm that cell line OCI-AML1 is a model system for FLT4 signaling, especially as KDR, the second receptor for VEGF-C is not expressed in this cell line (Table 2).
To test whether *KDR* is epigenetically regulated, we performed bisulfite sequencing of *KDR* negative and positive cell lines and of primary endothelial cells. The *KDR* negative cell line DOHH-2 had a highly methylated *KDR* promoter, the *KDR* positive cell line HEL was nearly unmethylated (Figure 3). Largely unmethylated were also HDMECs and HUVECs, both expressing *KDR* (Figure 3). To assess the *KDR* methylation status for a larger number of cell lines, we performed methylation-specific PCR (MSP), a technique less costly and laborious than bisulfite sequencing. The majority of *KDR* negative cell lines were methylated, *KDR* positive HUVECs were unmethylated (Figure 4). However, even HDMECs were U-and M-PCR positive although they expressed high *KDR* levels and although only a small minority of clones were methylated according to sequencing analysis (Figures 3 and 4). Apparently, a low proportion of methylated CpGs was sufficient to yield signals in the M-PCR. The same was true for U-PCR: the *KDR* negative cell line DOHH-2 - highly methylated according to the results of bisulfite sequencing - showed signals in M- and in U-PCR (Figure 3, Table 2).

In spite of the high sensitivity – a certain drawback of the PCR-based MSP technique – the accuracy of *KDR* M-PCR was 88% supporting the notion that *KDR* expression is regulated by DNA methylation (Table 2).

**FLT4: promoter methylation and gene expression**

Bisulfite sequencing and BSP analysis were also performed to analyze the methylation status of *FLT4* in cell lines, HUVECs and HDMECs. Results of bisulfite sequencing showed that *FLT4* was largely methylated in the *FLT4* negative cell line EM-2 and unmethylated in the *FLT4* positive cell line SUP-B15 as it was in HUVECs and HDMECs (Figure 5). MSP analysis confirmed that *FLT4* exhibited the inverse correlation between methylation and expression. A high proportion of methylated CpGs was necessary to inhibit *FLT4* expression.

**Table 1 VEGF-R mRNA expression in leukemia and lymphoma cell lines**

|            | KDR | FLT4 |
|------------|-----|------|
|            | +++ | ++   |
| AML        | 0   | 2    |
| pre-B ALL  | 0   | 3    |
| T-ALL      | 0   | 3    |
| NK         | 0   | 0    |
| CML        | 1   | 1    |
| HL         | 0   | 0    |
| ALCCL      | 0   | 2    |
| BL         | 0   | 0    |
| DLBCL      | 0   | 0    |
| FL         | 0   | 0    |
| MCL        | 0   | 0    |
| Σ          | 1   | 2    |

**KDR and FLT4 mRNA expression levels**

|            | mRNA | TBP  |
|------------|------|------|
| AML        | 0    | 6    |
| pre-B ALL  | 0    | 6    |
| T-ALL      | 0    | 6    |
| NK         | 0    | 6    |
| CML        | 0    | 6    |
| HL         | 0    | 6    |
| ALCCL      | 0    | 6    |
| BL         | 0    | 6    |
| DLBCL      | 0    | 6    |
| FL         | 0    | 6    |
| MCL        | 0    | 6    |
| Σ          | 0    | 6    |

*Note: +≥ 5; ++≥ 1; +≥ 0.2; (+) ≥ 0.04; < 0.04.*

KDR and FLT4 mRNA expression levels were determined by quantitative real-time PCR. TBP expression was used as endogenous control and cell lines CMK (KDR) and HEL (FLT4) were used for normalization. Relative quantification: +++ 4; ++ ≥ 1; + ≥ 0.2; (+) ≥ 0.04; < 0.04. AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; NK, natural killer leukemia; CML, chronic myeloid leukemia; HL, Hodgkin lymphoma; ALCCL, anaplastic large cell lymphoma; BL, Burkitt lymphoma; DLBCL, diffuse large B cell lymphoma; FL, follicular lymphoma, MCL, mantle cell lymphoma.

**Figure 1 KDR and FLT4 in HDMECs, HUVECs and leukemia cell lines**

KDR and FLT4 mRNA expression levels were determined by quantitative real-time PCR and indicated underneath the cell line name. TBP expression was used as endogenous control and cell lines CMK (KDR) and HEL (FLT4) were used for normalization. KDR protein expression levels – determined by Western blot analysis – are higher in HDMECs and HUVECs than in positive cell lines (note: 1:10 lysate dilution in primary cells). FLT4 protein expression is higher in HDMECs than in HUVECs (note: 1:10 lysate dilution in HDMECs) and in positive cell lines. Protein expression corresponds to mRNA expression pattern.

**Figure 2**

**Figure 3**

**Figure 4**

**Figure 5**

**KDR: promoter methylation and gene expression**

To test whether *KDR* is epigenetically regulated, we performed bisulfite sequencing of *KDR* negative and positive cell lines and of primary endothelial cells. The *KDR* negative cell line DOHH-2 had a highly methylated *KDR* promoter, the *KDR* positive cell line HEL was nearly unmethylated (Figure 3). Largely unmethylated were also HDMECs and HUVECs, both expressing *KDR* (Figure 3). To assess the *KDR* methylation status for a larger number of cell lines, we performed methylation-specific PCR (MSP), a technique less costly and laborious than bisulfite sequencing. The majority of *KDR* negative cell lines were methylated, *KDR* positive HUVECs were unmethylated (Figure 4). However, even HDMECs were U-and M-PCR positive although they expressed high *KDR* levels and although only a small minority of clones were methylated according to sequencing analysis (Figures 3 and 4). Apparently, a low proportion of methylated CpGs was sufficient to yield signals in the M-PCR. The same was true for U-PCR: the *KDR* negative cell line DOHH-2 - highly methylated according to the results of bisulfite sequencing– showed signals in M- and in U-PCR (Figure 3, Table 2).

In spite of the high sensitivity—a certain drawback of the PCR-based MSP technique—the accuracy of *KDR* M-PCR was 88% supporting the notion that *KDR* expression is regulated by DNA methylation (Table 2).

**FLT4: promoter methylation and gene expression**

Bisulfite sequencing and BSP analysis were also performed to analyze the methylation status of *FLT4* in cell lines, HUVECs and HDMECs. Results of bisulfite sequencing showed that *FLT4* was largely methylated in the *FLT4* negative cell line EM-2 and unmethylated in the *FLT4* positive cell line SUP-B15 as it was in HUVECs and HDMECs (Figure 5). MSP analysis confirmed that *FLT4* exhibited the inverse correlation between methylation and expression. A high proportion of methylated CpGs was necessary to inhibit *FLT4* expression.

**Table 1 VEGF-R mRNA expression in leukemia and lymphoma cell lines**

|            | KDR | FLT4 |
|------------|-----|------|
|            | +++ | ++   |
| AML        | 0   | 2    |
| pre-B ALL  | 0   | 3    |
| T-ALL      | 0   | 3    |
| NK         | 0   | 0    |
| CML        | 1   | 1    |
| HL         | 0   | 0    |
| ALCCL      | 0   | 0    |
| BL         | 0   | 0    |
| DLBCL      | 0   | 0    |
| FL         | 0   | 0    |
| MCL        | 0   | 0    |
| Σ          | 1   | 2    |

**KDR and FLT4 mRNA expression levels**

|            | mRNA | TBP  |
|------------|------|------|
| AML        | 0    | 6    |
| pre-B ALL  | 0    | 6    |
| T-ALL      | 0    | 6    |
| NK         | 0    | 6    |
| CML        | 0    | 6    |
| HL         | 0    | 6    |
| ALCCL      | 0    | 6    |
| BL         | 0    | 6    |
| DLBCL      | 0    | 6    |
| FL         | 0    | 6    |
| MCL        | 0    | 6    |
| Σ          | 0    | 6    |

*Note: +≥ 5; ++≥ 1; +≥ 0.2; (+) ≥ 0.04; < 0.04.* AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; NK, natural killer leukemia; CML, chronic myeloid leukemia; HL, Hodgkin lymphoma; ALCCL, anaplastic large cell lymphoma; BL, Burkitt lymphoma; DLBCL, diffuse large B cell lymphoma; FL, follicular lymphoma, MCL, mantle cell lymphoma.

**Figure 1 KDR and FLT4 in HDMECs, HUVECs and leukemia cell lines**

KDR and FLT4 mRNA expression levels were determined by quantitative real-time PCR and indicated underneath the cell line name. TBP expression was used as endogenous control and cell lines CMK (KDR) and HEL (FLT4) were used for normalization. KDR protein expression levels – determined by Western blot analysis – are higher in HDMECs and HUVECs than in positive cell lines (note: 1:10 lysate dilution in primary cells). FLT4 protein expression is higher in HDMECs than in HUVECs (note: 1:10 lysate dilution in HDMECs) and in positive cell lines. Protein expression corresponds to mRNA expression pattern.
between promoter methylation and gene expression that is indicative for epigenetic regulation (Figure 4, Table 2). However, the accuracy of FLT4 M-PCR (80%) was lower than for KDR M-PCR (88%). Of note was also that TF-1 cells did not express FLT4 although the promoter was unmethylated (Table 2). These data suggested that regulatory mechanisms other than DNA methylation are also important for the regulation of FLT4.

### Effect of DNA demethylating agent 5-Aza-dC on expression of KDR and FLT4

To test whether KDR and FLT4 were silenced by promoter methylation, we treated methylated and unmethylated cell lines with the DNA demethylating agent 5-Aza-dC. In 4/5 KDR-negative cell lines, expression of KDR was induced by DNA demethylation (Table 3). FLT4 expression was upregulated in 4/4 negative cell lines (Table 3). KDR and FLT4 expression in positive cell lines were not affected (Table 3). Although these results confirmed that promoter methylation plays a role for the regulation of these VEGF-Rs, we also noted substantial differences in the levels of 5-Aza-dC-triggered gene induction between different cell lines (Table 3). Furthermore, even in the most sensitive cell lines (HL-60 for KDR induction, EM-2 for FLT4 induction), demethylation did not induce mRNA expression that would translate into protein levels detectable by Western blot analysis (data not shown). These results suggest that other mechanisms than DNA methylation are also involved in the regulation of KDR and FLT4.

Besides DNA methylation, also histone modifications are epigenetic mechanisms that affect the expression of individual genes. Just to mention two examples, acetylated histone H3 (at lysine 9 and 14) is a marker for gene activation [23], tri-methylation of histone H3 lysine 27 stands for gene suppression [24]. Furthermore, epigenetic modifications can influence each other: methylated CpGs in a promoter region can be targeted by proteins that interact with histone deacetylases. The consequence is an inactive chromatin status and transcriptional repression [25,26].

### Table 2 Promoter methylation status and expression levels of KDR and FLT4

|       | KDR MSP mRNA | protein | FLT4 MSP mRNA | protein |
|-------|--------------|---------|---------------|---------|
| 697   | M 0.08 neg   | M/U 0   | neg           | M/U 0   |
| ALL-5L| M 0 neg U    | 0.3 neg | neg           | neg     |
| AP-1060| M/U 0       | n.d. M/U | 0 n.d.       |
| BV-173| M 0 neg U    | 1.4 pos | neg           | neg     |
| CMK   | M/U 1 pos    | M 0.1  | neg           | neg     |
| DOHH-2| M/U 0 neg    | M/U 0  | neg           | neg     |
| EM-2  | M/U 0 neg    | M 0    | neg           | neg     |
| HANK-1| M 0 neg M/U  | 0 neg  | neg           | neg     |
| HEL   | U 2.6 pos    | U 1 pos | pos           | pos     |
| HL-60 | M 0 neg M    | U 0.1  | neg           | neg     |
| JURL-MK1| U 0.15 n.d. | n.d. n.d. | n.d. n.d. |
| L-82  | M 0 n.d. M   | 0 n.d. | neg           | neg     |
| LOUCY | n.d. 0.13 neg | M/U 0 | neg           | neg     |
| M-07e | M/U 0.03 neg | U 0.1  | neg           | neg     |
| MEG-01| U 5.8 pos    | U 0.2  | pos           | pos     |
| MEGAL | M 0 neg M/U  | 0.3 neg | neg           | neg     |
| MHH-CALL2| M/U 0     | neg U   | 1.1 pos       |
| MHH-TALL1| M 0.08 n.d. | n.d. n.d. | n.d. n.d. |
| MOLT-4| M 0 n.d. M   | 0 n.d. | neg           | neg     |
| MUTZ-3| n.d. n.d. U  | 0.6 neg | neg           | neg     |
| MUTZ-8| n.d. n.d. M  | 0.3 neg | neg           | neg     |
| NK-92 | M 0 neg M    | 0 neg  | neg           | neg     |
| OCI-AML1| M/U 0      | neg M   | 1.3 pos       |
| SC-1  | M 0 n.d. M   | 0 n.d. | neg           | neg     |
| SKNO-1| U 0.08 neg   | n.d. n.d. | n.d. n.d. |
| SUP-B15| M 0 neg     | U 1.6  | pos           | pos     |
| TF-1  | U 0.1 pos    | U 0    | neg           | neg     |
| THP-1 | M/U 0 n.d. M/U | 0 n.d. | neg           | neg     |

KDR and FLT4 methylation was determined by MSP. M, positive in M-PCR; U, positive in U-PCR; M/U positive in M- and in U-PCR; n.d., not done. VEGF-R mRNA expression levels were determined by quantitative real-time PCR. Cell lines CMK (KDR) and HEL (FLT4) were used for normalization (set to 1). Protein expression was done by Western blot analysis. The accuracy of KDR M-PCR (mRNA positivity > 0.1) is 88%, the accuracy of FLT4 M-PCR (mRNA positivity > 0.1) is 80%.
However, besides epigenetic mechanisms, also the presence or absence of trans-acting factors may govern the expression of KDR and FLT4. Thus, it has been shown that transcription factor binding sites (Sp1, AP-2 and NF/C20) are essential for the base-line activity of the KDR promoter [27]. Here, we set out to find whether NF/C20 also plays a role for the expression of FLT4.

**Influence of transactivating factors**

During inflammation, new lymphatic vessels are formed. NF-κB is a key mediator of inflammatory processes and has recently been identified as inducer of FLT4 on lymphatic endothelial cells [13]. To test whether NF-κB contributes to FLT4 expression in leukemic cells, we stimulated the FLT4 negative cell line EM-2 and the FLT4 positive cell line OCI-AML1 with synthetic MALP-2. MALP-2 binds to toll-like receptors-2 and -6 [28]. MALP-2 triggers the NF/C20 pathway [29] which leads to the expression of NF/C20 targets like TNFα [30].

Accordingly, MALP-2 (100 ng/ml, 7 min) induced phosphorylation and degradation of the NF-κB inhibitor IκB and stimulated phosphorylation of p38 in cell lines EM-2 and OCI-AML1 (Figure 6). MALP-2 (100 ng/ml, 1 h) triggered expression of the NK-κB targets TNFα (80× in EM-2, 1000× in OCI-AML1) and IP-10 (600× in EM-2, > 1000× in OCI-AML1) in both cell lines. However, the expression of FLT4 was not affected, neither in the FLT4 positive cell line OCI-AML1 nor in the FLT4 negative (methylated) cell line EM-2. MALP-2 did also not increase the FLT4 stimulating effect of 5-Aza-dC on EM-2 cells (data not shown). Thus, our results do not support the view that NF-κB is a transactivator of FLT4.

We observed a 10-fold increase in KDR in cell lines OCI-AML1 and EM-2 (the latter pretreated with 5-Aza-dC). However, as the level reached after stimulation was still extremely low, it appears unlikely that NF-κB is an important regulator for KDR either.
KDR and FLT4 in HDMECs and HUVECs

HDMECs, HUVECs and KDR positive leukemia cell lines exhibited demethylated KDR promoters (Figure 3, Table 2). However, mRNA and protein levels were distinctly higher in the primary cells than in the leukemia cell lines (Figure 1). Results of Western blot analysis were confirmed by flow cytometry (Figure 7). HDMECs and HUVECs expressed the pan-endothelial marker CD31 (Figure 7). HDMECs, primarily consisting of lymphatic endothelial cells, were also positive for the

Table 3 Effect of 5-Aza-dC on expression of KDR and FLT4

|        | KDR induced by Aza | FLT4 induced by Aza |
|--------|--------------------|--------------------|
| CMK    | neg                | -.                 |
| DOHH-2 | neg (+)            | neg ++             |
| EM-2   | neg ++             | pos                |
| HL-60  | neg                | pos +              |
| L-82   | neg                | neg +              |
| SC-1   | neg                | +                  |

Induction of KDR and FLT4 by 5-Aza-dC (5 μM, 3 d) when compared to untreated control cells: - < 2.5-fold; (+) > 2.5-fold; + > 10-fold; ++ > 40-fold; ++ > 160-fold. Expression levels were determined by qRT-PCR. TBP expression was used as endogenous control, DMSO (0.05%) treated cells were used for normalization.

Figure 5 Bisulfite sequencing of the FLT4 promoter. A CpG island is located between -1231 and 769 relative to the ATG codon of FLT4. Part of the promoter region (337 bp, 20 CpG sites) was sequenced from cell lines EM-2 (FLT4 negative) and SUP-B15 (FLT4 positive) as well as from HDMECs and HUVECs. Each line depicts a sequenced clone representing the methylation status of an individual allele. CpGs are represented as open dots (if unmethylated) or filled dots (if methylated). Results of qRT-PCR and methylation specific PCR (MSP) are shown on the left hand side. M: signal in M-PCR; U: signal in U-PCR.

Figure 6 Degradation of IκB and phosphorylation of p38MAPK in MALP-2 responsive cell lines. Cell lines EM-2 (FLT4 negative) and OCI-AML1 (FLT4 positive) were stimulated with MALP-2 (100 ng/ml). The NF-κB inhibitor IκB was phosphorylated (7 min) and degraded (7 min, 21 min) in both cell lines. Likewise, rapid phosphorylation of p38 MAPK was observed in both cell lines by Western blot analysis. GAPDH is shown as loading control.
lymphatic vessel marker podoplanin, HUVECs were podoplanin negative (Figure 7). Both types of primary cells expressed much higher levels of KDR than KDR-positive cell lines (Figure 1). Also FLT4 expression levels varied greatly from one cell type to the other: FLT4 expression of HUVECs was comparable to those of FLT4 positive cell lines, while HDMECs showed much higher FLT4 expression levels (Figures 1 and 7). These results are in line with our data of MSP analyses and 5-Aza-dC experiments suggesting that DNA methylation
is not the only mechanism that controls KDR and FLT4 gene expression.

Conclusions
Our data obtained from primary endothelial cells and from leukemia/lymphoma cell lines show that KDR and FLT4 are epigenetically regulated genes. Both genes can be silenced by methylation. However, if the promoters are unmethylated, other factors are responsible for the extent of KDR and FLT4 expression. Furthermore, we show that the KDR negative/FLT4 positive cell line OCI-AML1 is a model system for FLT4 signal transduction studies.

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Authors’ contributions
HQ designed the study, performed data analyses and wrote the manuscript. SE carried out bisulfite conversion and helped to design primers for M-PCR and U-PCR. JR performed Western blot analyses. HAW supplied antibodies, HDMECs and HUVECs and gave good advice. MZ performed PCR and FACS analyses. HGD provided cell lines and good advice. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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