Leukemia (2011) 25, 1882–1890

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ORIGINAL ARTICLE

Hypoxia-microRNA-16 downregulation induces VEGF expression in anaplastic lymphoma kinase (ALK)-positive anaplastic large-cell lymphomas

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The anaplastic lymphoma kinase (ALK), tyrosine kinase oncogene is implicated in a wide variety of cancers. In this study we used conditional onco-ALK (NPM-ALK and TPM3-ALK) mouse MEF cell lines (ALK+ fibroblasts) and transgenic models (ALK+ B-lymphoma) to investigate the involvement and regulation of angiogenesis in ALK tumor development. First, we observed that ALK expression leads to downregulation of miR-16 and increased Vascular Endothelial Growth Factor (VEGF) levels. Second, we found that modification of miR-16 levels in TPM3-ALK MEF cells greatly affected VEGF levels. Third, we demonstrated that miR-16 directly interacts with VEGF mRNA at the 3′-untranslated region and that the regulation of VEGF by miR-16 occurs at the translational level. Fourth, we showed that expression of both the ALK oncogene and hypoxia-induced factor 1α (HIF1α) is a prerequisite for miR-16 downregulation. Fifth, *in vivo*, miR-16 gain resulted in reduced angiogenesis and tumor growth. Finally, we highlighted an inverse correlation between the levels of miR-16 and VEGF in human NPM-ALK+ Anaplastic Large Cell Lymphomas (ALCL). Altogether, our results demonstrate, for the first time, the involvement of angiogenesis in ALK+ ALCL and strongly suggest an important role for hypoxia-miR-16 in regulating VEGF translation.
Leukemia (2011) **25,** 1882–1890; doi:10.1038/leu.2011.168; published online 22 July 2011
Keywords: onco-ALK; lymphomas; microRNA; VEGF

Introduction

The role of Vascular Endothelial Growth Factor (VEGF) in solid tumor pathologies has been extensively investigated and evidence has recently emerged that it may also have a role in hematological malignancies.[1,2] VEGF expression by neoplastic cells has been demonstrated in non-Hodgkin lymphomas and has been correlated with tumor progression and poor survival rates.[3] VEGF promotes the formation of new blood vessels by stimulating endothelial cell division and migration; this neo-vascular network allows tumor growth and metastasis.[4] Regulation of VEGF occurs at multiple levels, including transcription, mRNA stabilization and splicing, and translation.[5,6]

MicroRNAs (miRNAs) are 20–25-nucleotide-long non-coding RNAs that act as negative regulators of gene expression. They bind to a specific sequence of mRNA, usually located in the 3′-UTR (untranslated region). This interaction can lead either to targeted mRNA cleavage or to repression of mRNA translation, in both cases resulting in reduced levels of the encoded protein.[7] miRNAs are important in the regulation of cellular differentiation, proliferation and apoptosis.[8] Some miRNAs are considered to be oncogenes or tumor suppressors and are aberrantly expressed in tumors including hematological malignancies.[9,10] Aberrant expression of miRNAs can arise via a number of different mechanisms, such as genomic abnormalities or epigenetic modifications.[9–11] Recently, several miRNAs have been found to regulate angiogenic processes.[12] Notably, miR-15a and miR-16-1 have an important role via regulation of VEGF expression.[13] miR-16, is downregulated by hypoxia,[14] supporting the notion that a hypoxia-induced reduction of miR-16 levels contributes to an increase in VEGF expression.

Anaplastic lymphoma kinase (ALK) fusion proteins (onco-ALK) have been detected in several human malignancies, including Anaplastic Large Cell Lymphomas (ALCL),[15] diffuse large B-cell lymphomas[16,17] and a wide variety of solid cancers, such as inflammatory myofibroblastic tumors[18] and non-small cell lung cancers.[19] The mechanisms of malignant cell transformation mediated by oncogenic ALK tyrosine kinase expression are not clearly understood. Recently, Marzec *et al*. reported that ALK-positive (ALK+) ALCL strongly express hypoxia-induced factor 1α (HIF1α) mRNA, even under normoxic conditions. They found that inhibition of HIF1α expression markedly suppressed cell growth and proliferation and decreased VEGF synthesis in ALCL cell line.[20] As lymphoma tissues are predominantly under hypoxic conditions, it is possible that HIF1α has an important role in the pathogenesis of ALK+ ALCL.

Compared with ALK-negative (ALK−) ALCL, ALK+ ALCL have a better prognosis when undergoing CHOP-based chemotherapy,[21,22] however, ALK+ ALCL relapses after chemotherapy are very invasive and have a worse prognosis.[23,24] We recently developed conditional mouse models for NPM- and TPM3-ALK-induced lymphomagenesis using the tetracycline system.[25] In these models, ALK oncogenes induce B lymphoma formation associated with a robust angiogenesis. Although these models do not truly reproduce the human disease, they represent a powerful

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Received 1 February 2011; revised 18 April 2011; accepted 30 May 2011; published online 22 July 2011

tool to investigate the molecular mechanisms involved in ALK-associated disorders.

Until now, the role of VEGF in ALCL development and post-treatment relapse has not been studied. Here, we used onco-ALK (NPM-ALK and TPM3-ALK) mouse conditional MEF cell line (ALK+ fibroblasts),[25] as well as transgenic models (ALK+ B-lymphoma),[26] to both determine whether ALK oncogenes regulate VEGF levels and tumor angiogenesis and investigate the mechanisms involved.

Materials and methods

Conditional murine models

Generation of conditional transgenic mice and MEF Tet-OFF cells (Clontech, Saint Quentin Yvelines, France) for expression of NPM-ALK or TPM3-ALK oncogenes was described by Giuriato *et al*.[25,26] The tetracycline regulatory system was used to control the transgene transcription. The addition of doxycycline (an analog of tetracycline) allowed silencing of onco-ALK expression (OFF condition, ALK–), whereas doxycycline removal permitted onco-ALK expression (ON condition, ALK+). Lymph nodes from ALK+, ALK− and healthy mice, that is, normal littermate transgenic mice were used for RNA and DNA extractions.

Tumor and normal samples

The study was carried out in accordance with the institutional review board-approved protocols and the procedures followed were in accordance with the Helsinki Declaration of 1975, as revised in 2000. The diagnosis of ALCL was based on morphologic and immunophenotypic criteria as described in the last WHO classification.[27,28] The percentage of malignant cells was assessed by ALK1 or CD30 staining and was greater than 80% for all selected cases. Frozen tumor samples from 20 ALK+ ALCL, 6 ALK− ALCL, and 5 reactive lymph nodes were retrieved from the tumor bank from Toulouse CHU and used for RNA and DNA extractions. A total of 100cases with available paraffin blocks, of which 86 cases were positive for the ALK protein, were used to prepare tissue microarrays. Sera from ALCL patients (ALK+: $n=17$ and ALK−: $n=16$) and five healthy donors were used to perform VEGF ELISA.

RNA preparation

Total RNA was prepared using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The concentration of RNA was quantified using a NanoDrop Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). RNA integrity was evaluated using an Agilent 2100 BioAnalyzer (Agilent Technologies, Palo Alto, CA, USA). RNAs with an RNA integrity number >7.5 were used for miRNA and mRNA quantifications.

Real-time RT-PCR assays

All qPCR were performed using an ABI 7300 Real-Time instrument (Applied Biosystems, Foster, CA, USA). TaqMan miRNA assays (Applied Biosystems, CA, USA) were used to measure miR-15a and miR-16 expression following manufacturer's instructions. RNU24 for human and SnoRNA202 for mice served as internal controls.

Expression of *S14*, *HIF1α* and *VEGF* genes were estimated by qPCR using a primer pair designed as follows: for human or mouse VEGF, the forward primer was 5′-CGAGATAGAGTA CATCTTCAAGC-3′ and the reverse primer was 5′-TTGATCCG CATGATCTGCATGG-3′. For human HIF1α, the forward primer was 5′-CATAAAGTCTGCAACATGGAAGGT-3′ and the reverse primer was 5′-ATTTGATGGGTGAGGAATGGGTT-3′. Human or mouse S14 was used as reference or housekeeping gene; the forward primer 5′-ATCAAACTCCGGGCCACAGGA-3′ and the reverse primer was 5′-CTGCTGTCAGAGGGGATGGGG-3′ for human and the forward primer was 5′-GGTGGCTGAGG GAGAGAATG-3′ and the reverse primer was 5′-CTCGGCA GATGGGTTTCCTTG-3′ for mouse. cDNA was synthesized from total RNA using M-MLV reverse transcriptase (Promega, Madison, WI, USA). qPCR was performed using SYBR green real-time PCR master mix (Eurogentec, Angers, France). The PCR mixture (25 μl) consisted of 12.5 μl master mixture, 0.75 μl for each PCR primer at 10 μM, 5 μl diluted cDNA and 6 μl DNase and RNase free water. qPCR was done using 40 cycles of 15 s at 90 °C and 1 min at 60 °C.

Relative fold changes of miRNA and target transcript expression were calculated by the ΔΔCT method and the values were expressed as $2(-\Delta\Delta CT)$. Detected target transcripts were normalized to the endogenous housekeeping gene *S14*.

miRNA northern blotting

Northern blots were performed using 10 μg of total RNA on denaturing PAGE. After blotting on Amersham Hybond-N+, miR oligonucleotides complementary to miR-15a and miR-16 5′-end-labeled using T4 polynucleotide kinase and γ-^{32}P ATP, were used as a probe.

miRNA transient transfections and target validations

MEF-TPM3-ALK cells[25] were transfected with 50 nM of pre-miR-15a (miR-15a) *or* pre-miR-16 (miR-16) (Ambion, Applied Biosystems, Austin, TX, USA), negative control miRNA (pre-miR control no. 1 or scramble; Ambion, Applied Biosystems), locked nucleic acid (LNA) (5′-CGCCAATATTTACGTGCTGCTA-3′) (LNA-16) (Sigma-Aldrich Chimie, Saint-Quentin Fallavier, France) or anti-miR-16 (Ambion, Applied Biosystems), using MEF transfection reagent (Altogen, Las Vegas, NV, USA) following the manufacturer's protocol. The expression of miR-16 was detected by RT-qPCR 48 h after transfection.

MEF-TPM3-ALK cells were transfected with 20 ng of pRLCMV-VEGF 3′-UTR wild-type (pRL-VEGF 3′-UTR)[29] and 250 ng of pCMV-β-Gal control vector serving as transfection control in a 12-well plate. For the co-transfection of miR-16, LNA-16, anti-miR-16 or scrambled miRNA was added with the reporter vectors, 50 nM of each miRNA or 50 nM of each inhibitor or control was transfected using Lipofectamine 2000 (Invitrogen, Cergy Pontoise, France). Cell lysate was collected and assayed 48 h after transfection. *Renilla* luciferase (LucR) activities were measured with a luminometer (Centro LB960, Berthold, Berthold Technologies, Thoiry, France) using the Luciferase Reporter Assay (Promega) and galactosidase activity was measured using the β-galactosidase enzyme assay system (Promega) according to the manufacturer's instructions. The results are expressed as relative luciferase activity/galactosidase activity.

All transfections and measurements were performed in triplicate and repeated at least three times.

VEGF ELISA

In all 96-well plates coated with anti-mouse or -human VEGF monoclonal antibody (Calbiochem, Merck KGaA, Darmstadt, Germany) were used to measure VEGF secretion following the manufacturer's guidelines. Absorbance was measured at 450 nm

with a wavelength correction at 570 nm. Each experiment was performed in triplicate and repeated three times.

VEGF and HIF1α immunohistochemistry

Sections (5 μM) were cut from each tissue microarray, deparaffinized, subjected to heat antigen retrieval and stained with rabbit polyclonal anti-VEGF antibody (A20, 1/40 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-HIF-1α antibody (clone H1α67, 1/100, Novus Biologicals, Cambridge, UK). *Antibody binding was detected with Dako REAL Detection System* (Code K5001, Dako France, Trappes, France). The percentage of positive cells was evaluated using a Leica DMR microscope (Leica Geosystems France, Le Pecq, France) equiped with a DFC300FX camera and a ×200/0.85 NA objective lens. Cases were considered positive when more than 10% of neoplastic cells were stained. Image processing was performed using the IM50 software from Leica Geosystems France.

DNA methylation analysis

We searched the human and mouse genome database (http://genome.ucsc.edu/) (University of California Santa Cruz, Genome Bioinformatics) for the existence of miRNAs embedded in a CpG island. Genomic DNA samples were modified by sodium bisulfite using the CpGenome DNA modification kit (Chemicon, Temecula, CA, USA) following the manufacturer's instructions. The DNA methylation status was analyzed by methylation-specific PCR after sodium bisulfite modification of DNA. Human male and mouse genomic DNA universally methylated for all genes (Intergen Company, Purchase, NY, USA) was used as a positive control for methylated alleles. Water blanks were included with each assay. The *deleted in leukemia* and *structural maintenance of chromosomes-4* genes are the host genes for miR-16-1 and miR-16-2, respectively. Primer sequences of methylation-specific PCR of miR-16 are described in Supplementary Table S2. Following amplification, PCR products were subjected to gel electrophoresis through a 2.5% agarose gel and were visualized by ethidium bromide staining and UV transillumination.

Tumorigenicity assays

MEF-TPM3-ALK cells[25] were transfected, as described above, with pre-miR-16 or scrambled control miRNA (pre-miR control no. 1), resuspended in PBS (2×10^6 cells/site) and then injected subcutaneouslyinto female Balb/c nu/nu mice, 4–6 weeks old (Iffa Credo, L'Arbresle, France). Five mice were used for each tested condition. Tumor volume was calculated as $v =$ length (mm) × width (mm) × thickness (mm) × 0.52 and measured every 2 days.

Statistical analysis

Values were expressed as mean ± s.d. Non-parametrical Mann–Whitney test and/or unpaired *t*-test or two-way ANOVA were applied to analyze the differences between groups. Analyses were performed using GraphPad Prism version 4.00 for Windows (San Diego, CA, USA). $P<0.05$ was considered to be significant.

Results

VEGF overexpression and miR-16 downregulation in murine onco-ALK models

We have previously developed conditional transgenic mice that express onco-ALK (NPM-ALK and TPM3-ALK) and exhibit robust angiogenesis (Supplementary Figure S1a). These mice were used to investigate whether ALK oncogene expression induces an increase in VEGF mRNA levels using RT-qPCR. We observed that the level of VEGF mRNA was significantly upregulated in lymph nodes isolated from mice with ALK+ tumors (Supplementary Figure S1b) when compared with lymph nodes isolated from normal littermate transgenic mice (normalized to 1) and from onco-ALK OFF healthy mice (Supplementary Figure S1b). We next checked whether the ALK oncogene-mediated increase in VEGF mRNA was followed by an increase in VEGF protein levels. The amount of secreted VEGF was measured by performing ELISA assays on mice sera. We observed that VEGF secretion was significantly upregulated in sera prepared from mice with ALK+ tumors in comparison with sera prepared from onco-ALK OFF healthy mice (*P*: 0.0173 for NPM-ALK; *P*: 0.0303 for TPM-ALK) and from control littermate animals (Supplementary Figure S1c).

Since miR-15 and miR-16 contribute to an increase in VEGF,[14] we used northern-blotting to compare miR-15a and miR-16 expression levels in tumor lymph nodes isolated from onco-ALK transgenic mice (ALK+) with lymph nodes isolated from doxycycline-treated animals (ALK−) (Supplementary Data). *To test for significant differences in expression of miR-15 and miR-16, two statistical tests* (the two-sample *t*-test and the Mann–Whitney test) *were performed* (Supplementary Table S1). In onco-ALK mice (ALK+), we found that miR-15a and miR-16 were significantly downregulated in lymphoma cells when compared with lymph node cells isolated from onco-ALK OFF healthy mice (ALK−) (0.471 ± 0.086 vs 0.761 ± 0.058 mean expression for miR-15a; 0.493 ± 0.025 vs 0.863 ± 0.044 mean expression for miR-16) (Supplementary Table S1).

To further confirm the upregulation of VEGF levels and the downregulation of miR-15a and miR-16 in onco-ALK-expressing cells *in vitro*, we used another murine onco-ALK model: MEF cells conditionally expressing the TPM3-ALK oncogene (MEF-TPM3-ALK cells).[25] Using ELISA assays, we observed a substantial increase in secreted VEGF (mean: 617 ± 126.33 pg/ml; p: 0.0318) in the supernatants of MEF TPM3-ALK-positive cells (ALK+) when compared with MEF TPM3-ALK-negative cells (ALK−) (Figure 1a). These data are consistent with the results obtained in transgenic mice harboring onco-ALK lymphomas (Supplementary Figures S1b and c and Supplementary Table S1). No significant variation in VEGF mRNA levels was observed between ALK+ and ALK− conditions (Figure 1b). We therefore used RT-qPCR, to analyse the expression levels of miRNA and observed that ALK+ cells express reduced levels of miR-15a and miR-16 when compared with ALK− cells (Figure 1c), however only miR-16 downregulation was significant ($P<0.05$) (Figure 1c). Altogether, these results suggest that miR-16 downregulation contributes to a decrease in VEGF levels in ALK+ cells.

VEGF mRNA is an miR-16 target in MEF TPM3-ALK cells

To understand the role of miR-16 in controlling VEGF levels, we looked at the consequences of either over-expressing or silencing miR-16 in ALK+ cells. The amount of miR-16 in transfected ALK+ cells was more than 700 fold higher than in control cells (Supplementary Figure S2). Transfection with miR-16, but not with the negative controls, resulted in a significant decrease in secreted VEGF protein levels 48 h after transfection (Figure 2a). Conversely, when endogeneous miR-16 was downregulated using an antisense locked nucleic acid to miR-16 (LNA-16), we observed an increase in VEGF secretion

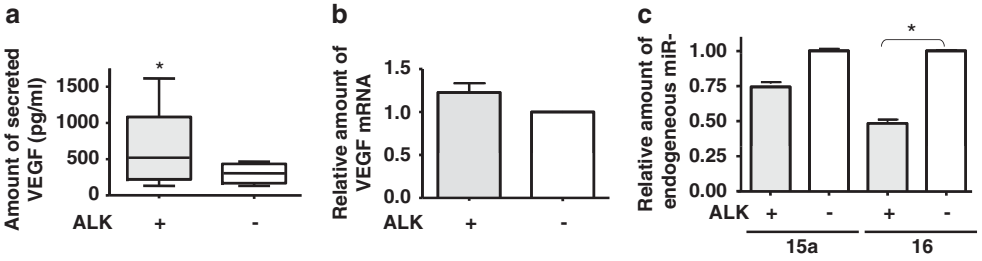

Figure 1 miR-16 down- and VEGF upregulation in conditional TPM3-ALK MEF cells. (**a**) ELISA and (**b**) RT-qPCR assays were performed to determine VEGF expression at the protein and mRNA level. (**c**) Endogeneous miR-15a and miR-16 levels were evaluated by RT-qPCR. miR-16 and miR-15a were normalized to Sno202 and VEGF mRNA to S14 rRNA. Bars represent standard deviation and asterisks depict statistically significant differences (*$P<0.05$; Student's *t*-test). Doxycycline was added or not to repress (ALK−) or induce (ALK+) TPM3-ALK expression. Each experiment was performed in triplicate and repeated three times.

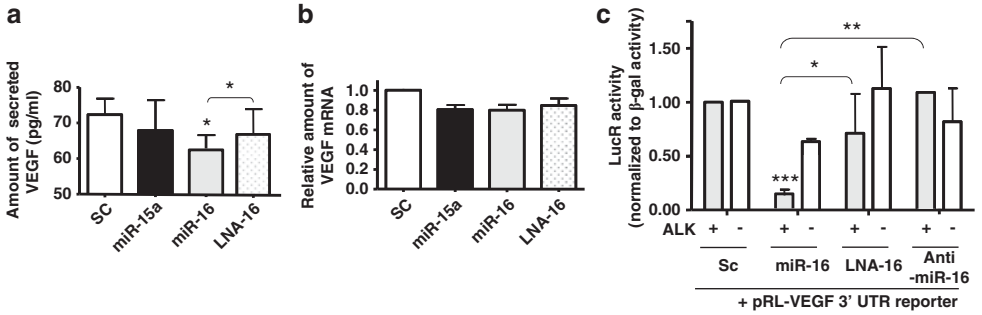

Figure 2 VEGF mRNA is an miR-16 target in TPM3-ALK MEF cells. (**a**, **b**) The effect of miR-16 overexpression on VEGF protein and mRNA levels was analysed in MEF TPM3-ALK cells transfected with pre-miR-16 (miR-16). The following negative controls were used: pre-*miR-15a* (miR-15a), a scrambled miRNA control (Sc) and an antisense LNA (LNA-16). (**c**) pRL-VEGF 3′-UTR construct was co-transfected with miR-16, antisense LNA-16 or miR-16 inhibitor (anti-miR-16) in TPM3-ALK MEF cells. Doxycycline was added or not to repress (ALK−) or induce (ALK+) TPM3-ALK expression. LucR activities were normalized to the cotransfected β-galactosidase activities. ELISA (**a**) and RT-qPCR (**b**, **c**) were performed to detect changes in VEGF expression levels 48 h after transfection. First, miR-16 was normalized to SnoRNA202 and VEGF mRNA to S14 rRNA then both were normalized to Sc experiments (**b**, **c**). Bars represent SD and asterisks depict statistically significant differences compared with scramble (*$P<0.05$, **$P<0.01$, ***$P<0.001$; Student's *t*-test). Each experiment was performed in triplicate and repeated three times.

(Figure 2a). Importantly, RT-qPCR did not show any differences in VEGF mRNA levels under all transfection conditions (Figure 2b). Thus, miR-16 levels inversely correlate with VEGF protein levels with mRNA levels unaffected. We therefore postulated that, in ALK+ cells, VEGF is controlled by miR-16 at a translational level.

To investigate further, we used a LucR reporter construct to examine whether miR-16 regulated VEGF translation through binding to its target site in the VEGF mRNA 3′-UTR. LucR activity was 6.5-fold lower in ALK+ cells transfected with miR-16 when compared with a scrambled miRNA control normalized to 1 ($P<0.001$, two-way ANOVA) (Figure 2c). Transfection with LNA-16 ($P<0.05$, two-way ANOVA) or a chemically modified inhibitor against miR-16 (anti-miR-16) ($P<0.01$, two-way ANOVA) led to a significant increase in the normalized LucR activity in ALK+ cells when compared with miR-16 transfection (Figure 2c). In ALK-inactivated cells (ALK−), reporter activity was not significantly changed under all conditions (Figure 2c). We conclude that, in ALK+ cells, VEGF mRNA is a target for miR-16 binding and that VEGF expression is controlled, at least in part, by the amount of miR-16.

miR-16 downregulation is not dependent on DNA loss and epigenetic modifications in murine TPM3-ALK models

In an attempt to identify the mechanisms involved in miR-16 downregulation in murine onco-ALK models, we first searched for DNA loss by high-resolution array-based comparative genomic hybridization array analysis (see Supplementary Data), on tumoral lymph nodes from TPM3-ALK transgenic mice. DNA karyotype changes did not seem to be involved in miR-16 downregulation (data not shown).

Mus musculus miR-16 is expressed from two loci, *deleted in leukemia 2* located on chromosome 3 and *structural maintenance of chromosomes-4* located on chromosome 14; these code for miR-16-1 and miR-16-2, respectively. We sought to determine whether aberrant methylation of the putative promoter regions of *deleted in leukemia 2* and *structural maintenance of chromosomes-4* was responsible for miR-16 downregulation through analysis of their methylation profile by methylation-specific PCR on DNA isolated from MEF TPM3-ALK-positive cells before (ALK+) and after (ALK−) ALK inactivation by doxycycline. As shown in Figure 3a, the miR-16-1 putative promoter was found non-methylated in both ALK+ and ALK− cells. Our data show that miR-16 downregulation is not mediated by an epigenetic mechanism.

ALK oncogene tyrosine kinase activity and hypoxia marker HIF1α may be involved in miR-16 downregulation in MEF TPM3-ALK cells

We next analysed whether regulation of miR-16 and VEGF was dependent upon functional ALK activity. First, we confirmed that TPM3-ALK kinase activity was inhibited following treatment with the ALK tyrosine kinase inhibitor, crizotinib,[30] by using

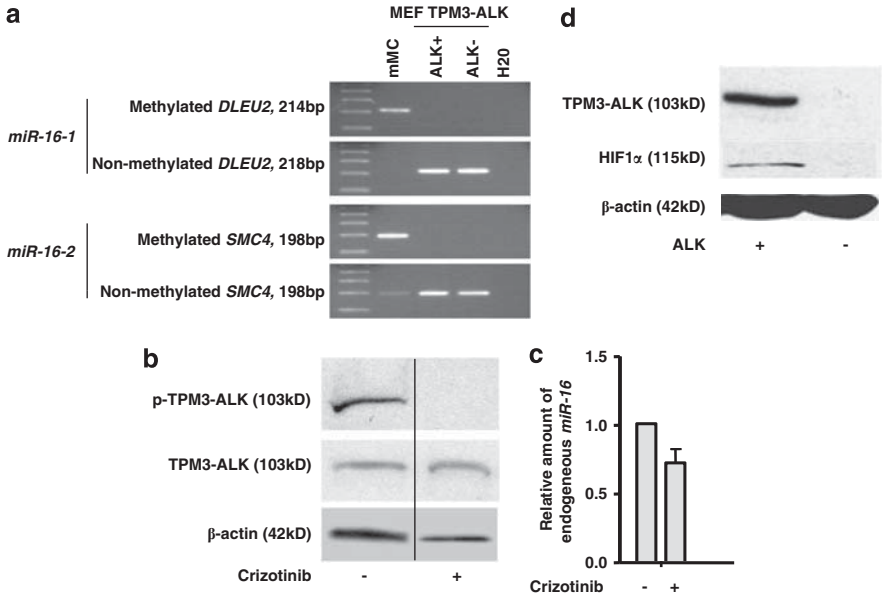

Figure 3 Mechanisms involved in miR-16 downregulation in TPM3-ALK-positive MEF cells. (**a**) Methylation-specific PCR (MSP) analysis of the mouse miR-16-1 (mmu-miR-16-1/*deleted in leukemia 2 (DLEU2) host gene*) and miR-16-2 (mmu-miR-16-2/*structural maintenance of chromosomes-4* (*SMC4*) *host gene*) CpG island regions were performed on DNA prepared from TPM3-ALK MEF cells, treated or not with doxycycline to repress (ALK−) or induce (ALK+) TPM3-ALK expression, and from mouse positive methylated control (mMC). H_20 indicates water blanks. (**b**, **c**) TPM3-ALK MEF cells were treated (+) or not (−) with 1 μM of Crizotinib. (**b**) Protein lysates (60 μg) were subjected to western-blotting analysis using anti-ALKc, anti-tyrosine and anti-β-actin antibodies. (**c**) RT-qPCR assays were performed to evaluate endogeneous miR-16 expression levels. MiR-16 was normalized to SnoRNA202. Bars represent SD. Each experiment was performed in triplicate and repeated three times. (**d**) Protein lysates (60 μg) isolated from TPM3-ALK MEF cells treated (ALK−) or not (ALK+) with doxycycline, were subjected to western-blotting analysis using anti-ALKc, anti-HIF1α and anti-β-actin antibodies.

western-blotting to confirm loss of TPM3-ALK autophosphorylation (Figure 3b and Supplementary Data). Using RT-qPCR we also observed that, in cells treated with crizotinib, miR-16 levels were decreased, although not significantly, following ALK tyrosine kinase inhibition (Figure 3c). This result suggests that ALK tyrosine kinase activity may partially contribute to under-expression of miR-16.

As hypoxia and HIF1α are known regulators of miR-16 expression, we analysed the expression profile of HIF1α in ALK+ and ALK− MEF cells. ALK inactivation via crizotinib resulted in significantly lower HIF1α expression (Figure 3d) when compared with ALK-expressing cells (ALK+). Together, these results suggest that the expression of ALK and HIF1α is a prerequisite for miR-16 downregulation in ALK-positive cells.

In vivo *repression of onco-ALK tumor growth and angiogenesis by miR-16 over-expression*

To provide *in vivo* evidence for a negative impact of miR-16 on tumor growth and angiogenesis, we subcutaneously transplanted MEF TPM3-ALK cells transfected with either miR-16 or a scrambled miRNA control into athymic nude mice. In miR-16-transfected cells tumor growth was reduced, as assessed by both tumor volume (Figures 4a and b) (P at 26 days: <0.001, two-way ANOVA test) and weight measurements (Figure 4c) (P at 26 days: 0.0255) VEGF serum levels were also reduced in tumors from ALK+ MEF cells transfected with miR-16 when compared with tumors from ALK+ MEF cells transfected with a scrambled miRNA control (Figure 4d). We performed CD34 immunostaining to examine vascular morphology in both cases. In control tumors, a high density of CD34 immunostaining and collapsed vessels were apparent (Figure 4e, upper panel, arrow). In contrast, decreased microvessel density and open lumen vessels (Figure 4e, lower panel, asterisk), were observed in subcutaneous tumors induced by ALK+ MEF cells transfected with miR-16. Altogether, these results demonstrate that miR-16 over-expression is able to reduce tumor growth and angiogenesis. Thus, our results strongly suggest that miR-16 down-regulation is a key event in ALK tumoral angiogenesis.

Association between miR-16 expression and VEGF levels in human ALK-positive lymphomas

To investigate a possible association between miR-16 expression and VEGF levels in human ALK+ cells, we looked at whether miR-16 was downregulated in ALCL tumor samples using RT-qPCR. We observed miR-16 levels to be significantly lower in ALK-positive ALCL lymph node biopsies ($n=20$) when compared with ALK-negative ALCL biopsies ($n=6$) (P: 0.0068) (Figure 5a). We then sought to identify the mechanisms involved in the reduction of miR-16 mRNA in ALK-expressing cells by searching for DNA structural or epigenetic modifications. As observed in the mouse models, the miR-16 downregulation in ALCL patients was not related to DNA promoter epigenetic methylation (Supplementary Figure S3) or DNA deletion; in the 32 ALK+ ALCL cases tested, comparative genomic hybridization array analysis (Supplementary Data) shows and only one case with a deletion of the 13q14.2 region coding for miR-16-1 and no deletion of the miR-16-2 region coding, 3q25.33 (data not shown).

Next, we wanted to investigate VEGF mRNA and protein expression in ALK+ human cells. VEGF mRNA amounts were evaluated by RT-qPCR on lymph node biopsies from ALCL patients. Our results show that levels of VEGF mRNA were increased in ALK+ ALCL lymph node biopsies ($n=20$), when compared with ALK− ALCL biopsies ($n=6$) or reactive lymph

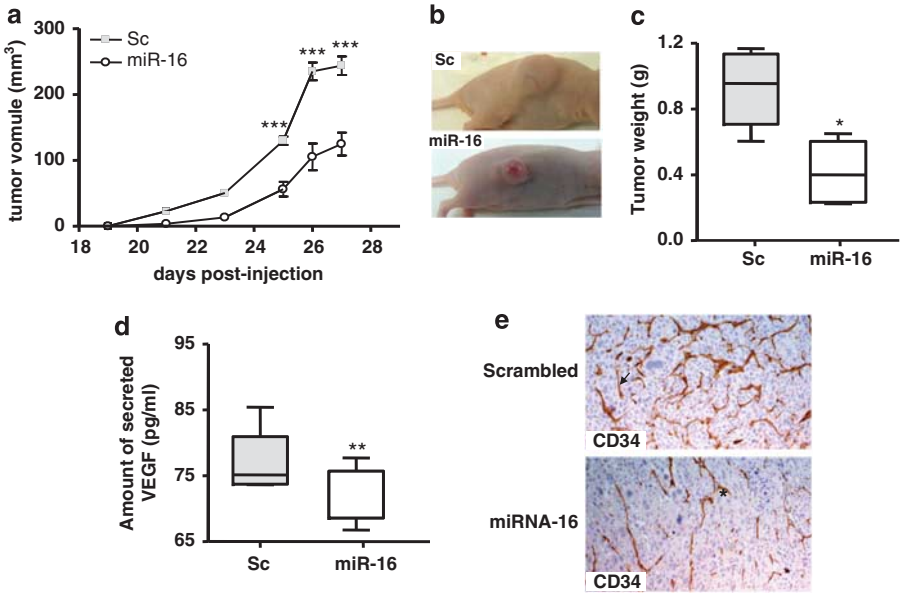

Figure 4 *In vivo* repression of tumor growth and angiogenesis by miR-16 gain of function. TPM3-ALK MEF cells, transfected with pre-miR-16 (miR-16) or scrambled pre-miRNA control (Sc), were injected subcutaneously into five female Balb/c nude mice for each tested condition. (**a**) Tumor volume was measured every 2 days using callipers. (**b**) Macroscopic observation of tumor-bearing mice 27 days post subcutaneous injection. (**c**) Tumor weight at necropsy. (**d**) ELISA assays were performed on mice sera to measure VEGF levels. (**c, d**) Bars represent SD and asterisks depict statistically significant differences compared with scrambled control (*$P<0.05$, **$P<0.01$ and ***$P<0.001$; Student's *t*-test). (**e**) Microvessel density was evaluated by CD34 immunoperoxidase staining 27 days post subcutaneous injection. (→) collapsed vessel, (*) open lumen vessel. Original magnification 400 × 0.85.

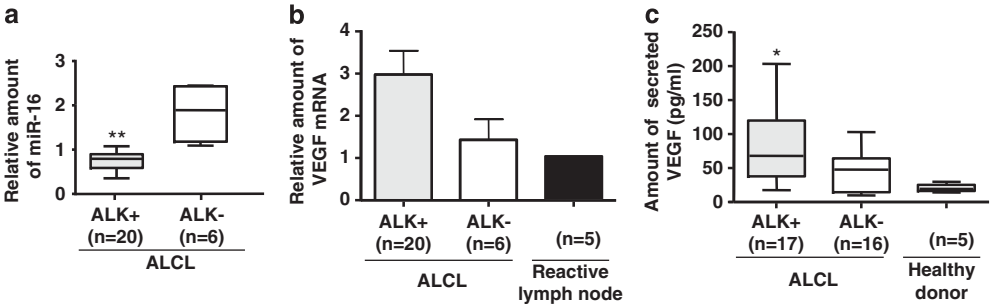

Figure 5 Endogeneous miR-16 down- and VEGF upregulation in human ALK-positive ALCL. (**a**, **b**) Endogeneous miR-16 mRNA was evaluated by RT-qPCR on biopsies. (**c**) VEGF expression was measured using an ELISA assay on sera (**c**) from ALK+ and ALK− ALCL. VEGF mRNA amounts were normalized to reactive lymph nodes. Bars represent SD and asterisks depict statistically significant differences (*$P<0.05$ and **$P<0.01$; Student's *t*-test). Each experiment was performed in triplicate. The number of biopsies in each group is denoted by the *n* value.

nodes ($n=5$) (Figure 5b). VEGF protein expression was tested using ELISA assays on ALCL sera and immunohistochemistry staining on tissue microarrays from lymph node biopsies of ALCL patients. We observed that VEGF protein expression was significantly enhanced in sera from ALK+ ALCL patients ($n=17$, p: 0.0242) when compared with sera from healthy donors ($n=5$) (Figure 5c). Immunohistochemistry staining was performed on 86 ALK+ ALCL samples and 14 ALK− ALCL samples. Endothelial cells, myofibroblasts and rare reactive lymphocytes admixed with lymphoma cells were used as an internal positive control and, as expected, were positive for VEGF (data not shown). In the tumor cells, we found no significant variation in VEGF expression between ALK+ and ALK-samples. Indeed, VEGF staining was detected in 68% (59 of 86) of ALK+ patients and in 57% of ALK− patients (8 of 14). However, a low VEGF staining intensity was predominantly seen in ALK− ALCL, while variations in the intensity of the staining was observed in ALK+ ALCL samples; some ALK+ ALCL showed strong VEGF staining, whereas other showed a moderate to weak staining, as illustrated for 8 cases in Figure 6a. Importantly, we also observed HIF1α protein expression in ALK+ ALCL lymph nodes biopsies (Supplementary Figure S4). Together, these results suggest that the ALK oncogene is able to induce VEGF expression, probably through both transcriptional and translational regulation, and involving both transcription factor HIF1-α and miR-16.

Finally, we used RT-qPCR to search for a correlation between VEGF protein levels, as assessed by immunohistochemistry, and the amount of endogeneous miR-16 in ALK+ ALCL cells. The percentage of tumoral cells expressing VEGF was graded as follows: 0: negative (0%); 1: <50%; 2: 50–75% and 3: >75%. We noted a strong inverse correlation between the amount of miR-16 and the VEGF grade of ALK+ tumoral cells (Pearson's correlation test, $R^2=0.9768$, Figure 6b). In the majority of the cases, low and intermediate miR-16 expression (relative amount <2) was associated with more than 50% of tumoral cells

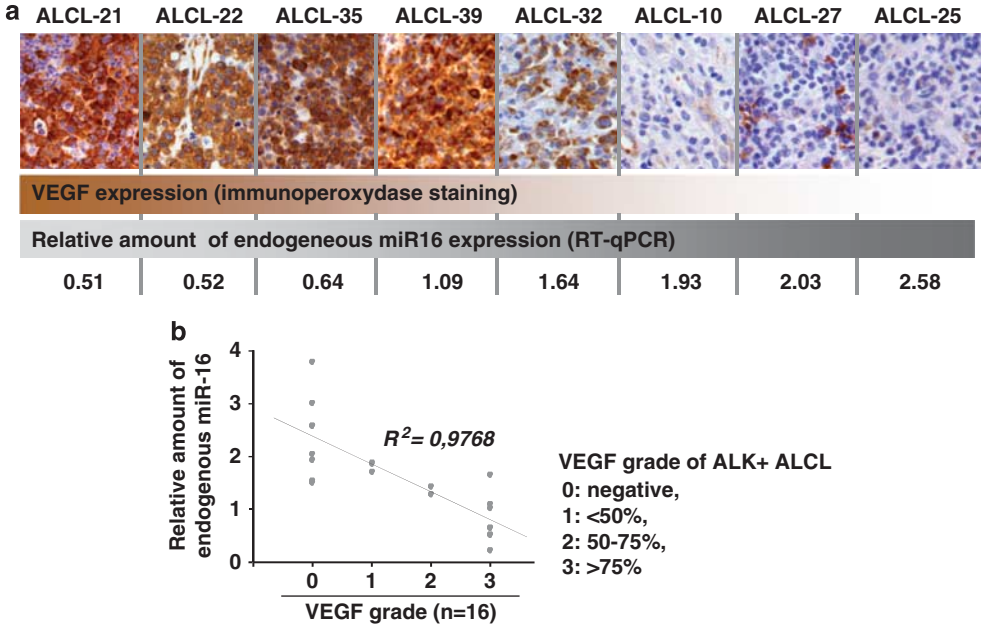

Figure 6 Inverse correlation between VEGF expression and relative amount of endogenous miR-16 in ALK-positive ALCL. (**a**) Immunohistochemistry (immunoperoxidase staining, original magnification, 400 × 0.85) and RT-qPCR were performed to determine VEGF and miR-16 expression in 8 human ALK+ lymphomas (ALCL-10, -21, -22, -25, -27, -32, -35 and -39). (**b**) Pearson's correlation test in 16 ALK+ ALCL. The percentage of tumoral cells expressing VEGF was graded as negative (0); 1: <50%, 2: 50–75% and 3: >75%.

expressing VEGF (Figure 6b). In contrast, enhanced miR-16 expression was associated with a low percentage or no ALK+ tumoral cells expressing VEGF (Figure 6b). Collectively, these results suggest that a decrease in miR-16 may be associated with an increase in VEGF protein levels in a substantial proportion of human ALK+ ALCL.

Discussion

Various hematological disorders and epithelial malignancies such as non-small cell lung cancer are associated with translocation of the *ALK* gene, located on chromosome 2p23. Around 17 chimera proteins have been characterized so far.[15,16,18,19,31] The majority of ALK translocations lead to diffuse large B-cell lymphomas and ALCL, a peripheral T-cell-derived malignancy. NPM-ALK and TPM3-ALK are the main translocations recorded in ALCL.[32]

A direct role for NPM-ALK in cellular transformation has been shown both *in vitro and in vivo*, and such studies have shed light on the mechanisms of malignant transformation by this oncoprotein.[33,34] These mechanisms include activation of several downstream signal-transduction pathways, which regulate cell survival, proliferation, migration and, more recently, hypoxia.[20,24] Although hypoxia has been reported to upregulate HIF-1α and VEGF in ALK-positive (ALK+) ALCL,[20] so far no studies have implicated it in angiogenesis in ALK tumor development.

In this study, we have used onco-ALK (NPM-ALK and TPM3-ALK) cellular and mouse models conditional for ALK expression[25,26] to report for the first time, an increase in VEGF secretion both *in vitro* and *in vivo*. The increase in VEGF secretion in these models was not seen in normal cells, that is, following doxycycline-induced onco-ALK inactivation. Importantly, high VEGF serum levels were also detected in ALK+ ALCL patients when compared with healthy donors. These results led us to search for the underlying mechanism for this VEGF upregulation following ALK expression.

Extensive literature has shown that VEGF levels are tightly regulated at transcriptional, post-transcriptional and translational levels.[5,6] Recently, miR-16 was shown to regulate VEGF mRNA stability and protein expression levels.[14,29] In this study we have demonstrated that miR-16 is downregulated upon ALK expression both *in vitro* and *in vivo* using our onco-ALK conditional cellular and mouse models for ALK tumorigenesis.[25,26] Importantly, a weaker expression of miR-16 was also observed in ALK+ ALCL patients when compared with ALK− ALCL patients.

The mechanisms for regulation of miRNA levels are not yet understood, despite the wealth of publications about the biological effects of miRNAs. A few reports have shown that downregulation of some miRNAs in haematopoietic cancer occurs through epigenetic factors or by genomic abnormalities. This has been observed for the miR-16 sequence at chromosomal locus 13q14, which is deleted in more than 50% of chronic lymphocytic leukemia or multiple myeloma patients.[9,10] ALK+ ALCL harbor genomic alterations including 13q losses but the locus 13q14 is rarely lost.[35,36] In accordance with these data, we did not observe any abnormalities on chromosome 13 in ALK lymphomas from onco-ALK conditional transgenic mouse models and in only one of the human ALK+ ALCL samples. We also found that miR-16 downregulation was not related to DNA promoter epigenetic methylation changes in both the onco-ALK mouse models and in the human ALK+ ALCL samples. As we found an over-expression of HIF1α in ALK+ tumoral cells, we cannot rule out a possible hypoxia-mediated mechanism for miR-16 downregulation, as previously described by Hua *et al.*[14] However, a mechanism for this hypoxia-miR-16 regulation is not yet known.

To confirm the close relationship between miR-16 and VEGF levels in ALK tumors, we performed miR-16 gain expression in TPM3-ALK-positive MEF cells and studied the subsequent levels of VEGF secreted *in vitro* as well as the cells' ability to induce subcutaneous tumor growth in nude mice. Our results indicate that VEGF mRNA is a target of miR-16 and that forced

expression of miR-16 leads to a reduction in tumor growth, microvessel density and serum VEGF levels in MEF TPM3-ALK cells engrafted into nude mice. In human ALK+ tumors, we were able to demonstrate for the first time a strong inverse correlation between miR-16 and VEGF expression levels. Nevertheless, as the increase in VEGF mRNA was detected in ALK+ lymphoma cells, that is, in conditional transgenic models as well as ALCL patients, we suggest that VEGF is regulated at both transcriptional, as reported by Marzec *et al.*,[20] and post-transcriptional levels in ALK+ ALCL.

Several trials have been opened for ALK+ tumors (http://www.ClinicalTrials.gov). Only one agent, PF-02341066/crizotinib, an inhibitor of the tyrosine kinase activity of both ALK and the MET oncogene,[30] has recently reached the clinical arena in the treatment of ALK+ non-small cell lung cancer.[37] Relapsed advanced ALK+ ALCL are also sensitive to ALK inhibition by crizotinib.[38] However, recently two secondary mutations were reported following ALK inhibitor treatment.[39] They were within the kinase domain of the EML4-ALK oncogene involved in non-small cell lung cancer, and conferred marked resistance to crizotinib.[39]

As ALK signaling activates multiple downstream pathways, it is reasonable to speculate that small molecules, targeting key effectors within these pathways, will represent valuable targets to kill ALK+ cancer cells. Therefore, the development of combined therapies should help prevent the occurrence of resistance.[40] To this end, we recently demonstrated the efficiency of an antibody against CD160 in TPM3-ALK-induced fibroblast tumors in athymic nude mice.[41] This unusual anti-angiogenic therapeutic is a glycophosphatidylinositol-anchored protein which is expressed by growing but not quiescent endothelial cells.[42] We found that the antibody not only inhibited tumor vascular density but also slowed down the growth of ALK tumors.[41]

Anti-angiogenic strategies have become an important therapy for solid tumors[43] as well as hemato-lymphoid malignancies.[44] Importantly, an improvement in response, progression-free survival and/or overall survival has been demonstrated when conventional therapy was supplemented by VEGF inhibitors. As miRNAs can act both as oncogenes and tumor-suppressor genes, a very appealing therapeutic option is the combination of miRNAs with chemotherapy.[45] Indeed, several studies *in vitro* and *in vivo* using anti-miRNA molecules (antagomirs), LNA-anti-miRNA oligonucleotides or anti-miRNA oligonucleotides, have validated the efficiency of such agents in both solid tumors[46] and haematopoietic malignancies.[47] Importantly, the first clinical trial applying anti-miRNA agents as drugs has already been launched.[48,49]

In conclusion, our results report a new fundamental process for ALK-mediated tumorigenesis involving angiogenesis and VEGF upregulation through, at least in part, miR-16 down-regulation. These new data should encourage further work to optimize multi-target therapies and increase the chances of eradicating ALK+ tumors.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

The authors thank, Dr L Vandel for providing the pCMV-β-Gal vector and, for their excellent technical assistance: F Capilla and D Lestrade (Plateau technique d'histopathologie expérimentale, CPTP-UMR1043, Toulouse); M March (Laboratoire d'Anatomie et Cytologie pathologiques, CHU Purpan, Toulouse), C Lopez (CRCT-UMR1037, Toulouse) and S Quentin (Plateforme Génomique IUH, Hôpital Saint-Louis, Paris). We thank Dr N Jabrane-Ferrat for critical reading. English proofreading was performed by Scientific Scripts (http://scientificscripts.com). This work was supported by grants from the INSERM, 'Association pour la Recherche sur le Cancer', no. A09/1/5073 (FM), Cancéropôle Grand-Sud-Ouest (FM), CITTIL (Coopération Transpyrénéenne de recherche de traitements innovants contre la leucémie) and INCa (projet PAIR Lymphomes). ED was supported by a doctoral fellowship from the 'Association pour la Recherche sur le Cancer' and MF was under a contract supported initially by the 'La Fondation de France' then 'La Fondation pour la Recherche Médicale'. This work is dedicated to my 'Dou'.

References

1 Moehler TM, Neben K, Ho AD, Goldschmidt H. Angiogenesis in hematologic malignancies. *Ann Hematol* 2001; **80**: 695–705.
2 Salven P. Angiogenesis in lymphoproliferative disorders. *Acta Haematol* 2001; **106**: 184–189.
3 Ruan J, Hajjar K, Rafii S, Leonard JP. Angiogenesis and antiangiogenic therapy in non-Hodgkin's lymphoma. *Ann Oncol* 2009; **20**: 413–424.
4 Roskoski Jr R. Vascular endothelial growth factor (VEGF) signaling in tumor progression. *Crit Rev Oncol Hematol* 2007; **62**: 179–213.
5 Bastide A, Karaa Z, Bornes S, Hieblot C, Lacazette E, Prats H *et al.* An upstream open reading frame within an IRES controls expression of a specific VEGF-A isoform. *Nucleic Acids Res* 2008; **36**: 2434–2445.
6 Touriol C, Bornes S, Bonnal S, Audigier S, Prats H, Prats AC *et al.* Generation of protein isoform diversity by alternative initiation of translation at non-AUG codons. *Biol Cell* 2003; **95**: 169–178.
7 Krol J, Loedige I, Filipowicz W. The widespread regulation of microRNA biogenesis, function and decay. *Nat Rev Genet* 2010; **11**: 597–610.
8 Croce CM, Calin GA. miRNAs, cancer, and stem cell division. *Cell* 2005; **122**: 6–7.
9 Garzon R, Calin GA, Croce CM. MicroRNAs in cancer. *Annu Rev Med* 2009; **60**: 167–179.
10 Lee YS, Dutta A. MicroRNAs in cancer. *Annu Rev Pathol* 2009; **4**: 199–227.
11 Saito Y, Jones PA. Epigenetic activation of tumor suppressor microRNAs in human cancer cells. *Cell Cycle* 2006; **5**: 2220–2222.
12 Wang S, Aurora AB, Johnson BA, Qi X, McAnally J, Hill JA *et al.* The endothelial-specific microRNA miR-126 governs vascular integrity and angiogenesis. *Dev Cell* 2008; **15**: 261–271.
13 Roccaro AM, Sacco A, Thompson B, Leleu X, Azab AK, Azab F *et al.* MicroRNAs 15a and 16 regulate tumor proliferation in multiple myeloma. *Blood* 2009; **113**: 6669–6680.
14 Hua Z, Lv Q, Ye W, Wong CK, Cai G, Gu D *et al.* MiRNA-directed regulation of VEGF and other angiogenic factors under hypoxia. *PLoS One* 2006; **1**: e116.
15 Morris SW, Kirstein MN, Valentine MB, Dittmer KG, Shapiro DN, Saltman DL *et al.* Fusion of a kinase gene, ALK, to a nucleolar protein gene, NPM, in non- Hodgkin's lymphoma. *Science* 1994; **263**: 1281–1284.
16 Arber DA, Sun LH, Weiss LM. Detection of the t(2;5)(p23;q35) chromosomal translocation in large B-cell lymphomas other than anaplastic large cell lymphoma. *Hum Pathol* 1996; **27**: 590–594.
17 Delsol G, Lamant L, Mariame B, Pulford K, Dastugue N, Brousset P *et al.* A new subtype of large B-cell lymphoma expressing the ALK kinase and lacking the 2; 5 translocation. *Blood* 1997; **89**: 1483–1490.
18 Griffin CA, Hawkins AL, Dvorak C, Henkle C, Ellingham T, Perlman EJ. Recurrent involvement of 2p23 in inflammatory myofibroblastic tumors. *Cancer Res* 1999; **59**: 2776–2780.

19 Rikova K, Guo A, Zeng Q, Possemato A, Yu J, Haack H *et al.* Global survey of phosphotyrosine signaling identifies oncogenic kinases in lung cancer. *Cell* 2007; **131**: 1190–1203.
20 Marzec M, Liu X, Wong W, Yang Y, Pasha T, Kantekure K *et al.* Oncogenic kinase NPM/ALK induces expression of HIF1alpha mRNA. *Oncogene* 2010; **30**: 1372–1378.
21 Brugieres L, Quartier P, Le Deley MC, Pacquement H, Perel Y, Bergeron C *et al.* Relapses of childhood anaplastic large-cell lymphoma: treatment results in a series of 41 children–a report from the French Society of Pediatric Oncology. *Ann Oncol* 2000; **11**: 53–58.
22 Williams DM, Hobson R, Imeson J, Gerrard M, McCarthy K, Pinkerton CR. Anaplastic large cell lymphoma in childhood: analysis of 72 patients treated on The United Kingdom Children's Cancer Study Group chemotherapy regimens. *Br J Haematol* 2002; **117**: 812–820.
23 Armstrong F, Lamant L, Hieblot C, Delsol G, Touriol C. TPM3-ALK expression induces changes in cytoskeleton organisation and confers higher metastatic capacities than other ALK fusion proteins. *Eur J Cancer* 2007; **43**: 640–646.
24 Palmer RH, Vernersson E, Grabbe C, Hallberg B. Anaplastic lymphoma kinase: signalling in development and disease. *Biochem J* 2009; **420**: 345–361.
25 Giuriato S, Faumont N, Bousquet E, Foisseau M, Bibonne A, Moreau M *et al.* Development of a conditional bioluminescent transplant model for TPM3-ALK-induced tumorigenesis as a tool to validate ALK-dependent cancer targeted therapy. *Cancer Biol Ther* 2007; **6**: 1318–1323.
26 Giuriato S, Foisseau M, Dejean E, Felsher DW, Al Saati T, Demur C *et al.* Conditional TPM3-ALK and NPM-ALK transgenic mice develop reversible ALK-positive early B-cell lymphoma/leukemia. *Blood* 2010; **115**: 4061–4070.
27 Delsol GFB, Mûller-hermelink HK, Campo E, Jaffe ES, Gascoyne RD, Stein H *et al. Anaplastic Large Cell Lymphoma, ALK-Positive*. IARC Press: Lyon, 2008, pp 312–316.
28 Mason DYHN, Delsol G, Stein H, Campo E, Kinney MC, Jaffe ES *et al. Anaplastic Large Cell Lymphoma, ALK-Negative*. IARC Press: Lyon, 2008, pp 317–319.
29 Karaa ZS, Iacovoni JS, Bastide A, Lacazette E, Touriol C, Prats H. The VEGF IRESes are differentially susceptible to translation inhibition by miR-16. *Rna* 2009; **15**: 249–254.
30 Christensen JG, Zou HY, Arango ME, Li Q, Lee JH, McDonnell SR *et al.* Cytoreductive antitumor activity of PF-2341066, a novel inhibitor of anaplastic lymphoma kinase and c-Met, in experimental models of anaplastic large-cell lymphoma. *Mol Cancer Ther* 2007; **6** (12 Pt 1): 3314–3322.
31 Perez-Pinera P, Chang Y, Astudillo A, Mortimer J, Deuel TF. Anaplastic lymphoma kinase is expressed in different subtypes of human breast cancer. *Biochem Biophys Res Commun* 2007; **358**: 399–403.
32 Piccaluga PP, Gazzola A, Mannu C, Agostinelli C, Bacci F, Sabattini E *et al.* Pathobiology of anaplastic large cell lymphoma. *Adv Hematol* 2010; **2010**: 345053.
33 Chiarle R, Voena C, Ambrogio C, Piva R, Inghirami G. The anaplastic lymphoma kinase in the pathogenesis of cancer. *Nat Rev Cancer* 2008; **8**: 11–23.
34 Turner SD, Alexander DR. What have we learnt from mouse models of NPM-ALK-induced lymphomagenesis? *Leukemia* 2005; **19**: 1128–1134.
35 Salaverria I, Bea S, Lopez-Guillermo A, Lespinet V, Pinyol M, Burkhardt B *et al.* Genomic profiling reveals different genetic aberrations in systemic ALK-positive and ALK-negative anaplastic large cell lymphomas. *Br J Haematol* 2008; **140**: 516–526.
36 Youssif C, Goldenbogen J, Hamoudi R, Carreras J, Viskaduraki M, Cui YX *et al.* Genomic profiling of pediatric ALK-positive anaplastic large cell lymphoma: a Children's Cancer and Leukaemia Group Study. *Genes Chromosomes Cancer* 2009; **48**: 1018–1026.
37 Kwak EL, Bang YJ, Camidge DR, Shaw AT, Solomon B, Maki RG *et al.* Anaplastic lymphoma kinase inhibition in non-small-cell lung cancer. *N Engl J Med* 2010; **363**: 1693–1703.
38 Gambacorti-Passerini C, Messa C, Pogliani EM. Crizotinib in anaplastic large-cell lymphoma. *N Engl J Med* 2010; **364**: 775–776.
39 Choi YL, Soda M, Yamashita Y, Ueno T, Takashima J, Nakajima T *et al.* EML4-ALK mutations in lung cancer that confer resistance to ALK inhibitors. *N Engl J Med* 2010; **363**: 1734–1739.
40 Wodarz D, Komarova NL. Emergence and prevention of resistance against small molecule inhibitors. *Semin Cancer Biol* 2005; **15**: 506–514.
41 Chabot S, Jabrane-Ferra N, Bigot K, Provost A, Golzio M, Tabiasco J *et al.* A novel antiangiogenic and vascular normalization therapy targeted against human CD160 receptor. *J Exp Med* 2011; **208**: 973–986.
42 Fons P, Chabot S, Cartwright JE, Lenfant F, L'Faqihi F, Giustiniani J *et al.* Soluble HLA-G1 inhibits angiogenesis through an apoptotic pathway and by direct binding to CD160 receptor expressed by endothelial cells. *Blood* 2006; **108**: 2608–2615.
43 Palazzo A, Iacovelli R, Cortesi E. Past, present and future of targeted therapy in solid tumors. *Curr Cancer Drug Targets* 2010; **10**: 433–461.
44 Medinger M, Mross K. Clinical trials with anti-angiogenic agents in hematological malignancies. *J Angiogenes Res* 2010; **2**: 10.
45 Iliopoulos D, Lindahl-Allen M, Polytarchou C, Hirsch HA, Tsichlis PN, Struhl K. Loss of miR-200 inhibition of Suz12 leads to polycomb-mediated repression required for the formation and maintenance of cancer stem cells. *Mol Cell* 2010; **39**: 761–772.
46 Hebert C, Norris K, Scheper MA, Nikitakis N, Sauk JJ. High mobility group A2 is a target for miRNA-98 in head and neck squamous cell carcinoma. *Mol Cancer* 2007; **6**: 5.
47 Agirre X, Vilas-Zornoza A, Jimenez-Velasco A, Martin-Subero JI, Cordeu L, Garate L *et al.* Epigenetic silencing of the tumor suppressor microRNA Hsa-miR-124a regulates CDK6 expression and confers a poor prognosis in acute lymphoblastic leukemia. *Cancer Res* 2009; **69**: 4443–4453.
48 Henke JI, Goergen D, Zheng J, Song Y, Schuttler CG, Fehr C *et al.* microRNA-122 stimulates translation of hepatitis C virus RNA. *Embo J* 2008; **27**: 3300–3310.
49 Jopling CL. Regulation of hepatitis C virus by microRNA-122. *Biochem Soc Trans* 2008; **36** (Pt 6): 1220–1223.

Supplementary Information accompanies the paper on the Leukemia website (http://www.nature.com/leu)