The microRNA-30 family targets DLL4 to modulate endothelial cell behavior during angiogenesis

Gemma Bridge,1 Rui Monteiro,2 Stephen Henderson,1 Victoria Emuss,1 Dimitris Lagos,3 Dimitra Georgopoulou,1 Roger Patient,2 and Chris Boshoff3

1Cancer Research UK Viral Oncology Group, UCL Cancer Institute, University College London, London, United Kingdom; 2MRC Molecular Haematology Unit, The Weatherall Institute of Molecular Medicine, University of Oxford, Oxford, United Kingdom; and 3Centre for Immunology and Infection, Department of Biology and Hull York Medical School, University of York, York, United Kingdom

Introduction

DLL4 plays a fundamental role in vascular development and angiogenesis.1,2 DLL4 haploinsufficiency results in extensive arterial defects and embryonic lethality,3 indicating that the developing vasculature is sensitive to minor alterations in DLL4 dosage. DLL4 expression is mainly restricted to the endothelium of nascent vessels, particularly the tip cells, where it maintains stalk cell identity in neighboring cells, thereby regulating vessel sprouting and branching in response to angiogenic stimuli.4 The importance of optimal DLL4 expression in physiologic angiogenesis is illustrated through its regulation of intersegmental vessel (ISV) development in zebrafish. Morpholino (MO) knockdown of dll4 in zebrafish results in an increased number of endothelial cells within the ISVs and ectopic ISV branching from the dorsal aorta (DA) because of overactivation of Vegfa signaling.5,6

DLL4 is relevant in pathologic angiogenesis and is overexpressed in human tumors, often in association with markers of inflammation, hypoxia and angiogenesis.7,8 Inhibition of DLL4 suppresses experimental tumor growth by inducing nonproductive, deregulated angiogenesis.9,10 We and others have shown that DLL4 expression is up-regulated in lymphatic endothelial cells (LECs) after infection by Kaposi sarcoma herpesvirus (KSHV).12,13 An oncogenic γ-herpesvirus that is the etiologic agent of Kaposi sarcoma (KS), KS is an angioproliferative neoplasm composed of cells of endothelial origin.14 Although accurate regulation of DLL4 levels is a hallmark of angiogenesis, the mechanisms that finely regulate DLL4 expression are not completely defined. Therefore we hypothesized that, in addition to well-known transcriptional mechanisms that affect DLL4 expression, DLL4 is regulated at the posttranscriptional level.

Microinjection of miR-30 mimics into zebrafish embryos resulted in suppression of dll4 and subsequent excessive sprouting of intersegmental vessels and reduction in dorsal aorta diameter. Use of a target protector against the miR-30 site within the dll4 3′UTR up-regulated dll4 and synergized with Vegfa signaling knockdown to inhibit angiogenesis. Furthermore, restoration of miR-30b or miR-30c expression during Kaposi sarcoma herpesvirus (KSHV) infection attenuated viral induction of DLL4. Together these results demonstrate that the highly conserved molecular targeting of DLL4 by the miR-30 family regulates angiogenesis. (Blood. 2012;120(25):5063-5072)

Methods

Cell culture

LECs were purchased from Promocell and grown in endothelial growth medium MV (Promocell) supplemented with 10 ng/mL VEGF-C (R&D Systems). HUVECs were purchased from Promocell and grown in endothelial growth medium MV2 (Promocell). For both LECs and HUVECs, experiments were performed before passage 8. BCBL-1 cells, latently infected with recombinant GFP-KSHV,22 were cultured as previously described.23 293T and immortalized human fibroblast cells were grown in DMEM (Invitrogen), supplemented with 10% FBS.

The online version of this article contains a data supplement.
KSHV production and infection of LECs

KSHV was produced and used to infect LECs as previously described.23 This procedure reproducibly resulted in 30% to 50% of LECs expressing GFP 3 days after infection.

MicroRNA mimics and inhibitors

LEC and HUVECs were seeded in 6-well plates at 5 × 10^5 cells per well and 293T were seeded in 12-well plates at 2.5 × 10^5 cells per well, 16 hours before transfection. miRDIAN miRNA mimics and inhibitors for hsa-miR-30b, hsa-miR-30c, and the negative control No. 1 (nontargeting control; Thermo Scientific) were transfected at 100 nM, unless otherwise stated. Cells were harvested for RNA or protein, used for the hanging drop assay, or transfected with luciferase reporter plasmids 48 hours after transfection.

Western blotting

LEC or HUVECs were lysed in Pierce M-PER buffer (ThermoScientific). Equal amounts of protein were resolved on a 10% polyacrylamide gel. Antibodies against DLL4 (Cell Signaling Technology), GAPDH (Monoclonal 6C5, Advanced Immunochemical) and α-tubulin (Monoclonal B-5-1-2, Sigma-Aldrich) were detected with HRP-conjugated secondary antibodies and were quantified using ECL or ECL Plus (GE Healthcare).

Lentivirus production and infection of LECs and HUVECs

Genomic fragments containing pre-miR-30b and pre-miR-30c-1 were cloned into LECs and were expressed using a modified pSIN-MCS lentiviral vector as described,23 subsequently referred to as pSIN_30b and pSIN_30c, respectively. The number of lentiviral copies per cell was determined by qPCR and miRNA expression was confirmed by qRT-PCR. Cells were infected in suspension. Experiments were performed 2 to 3 days after infection.

Quantitative PCR and quantitative RT-PCR

Genomic DNA for qPCR was extracted using the QIAamp DNA mini-kit (QIAGEN). The number of lentiviral copies per cell (c/c) was determined as previously described.23 Total RNA was extracted using the mirNeasy mini-kit (QIAGEN) and subjected to DNase I treatment (QIAGEN). For cDNA synthesis using the SuperScript II reverse transcriptase (Invitrogen). The number of lentiviral copies per cell was determined by qPCR and miRNA expression was confirmed by qRT-PCR.

Luciferase reporter assay

The reporter plasmids (50 ng), either empty vector (pEZK-MT01) or the DLA 3'UTR containing plasmid (pEZK-DLL4), were transfected into 293T cells, 16 hours after transfection with miRNA mimic. Cells were harvested 24 hours after transfection according to the Dual-Luciferase Reporter assay system (Promega). Luciferase activity was measured using a Fluoroskan Ascent FL luminometer (ThermoScientific). Firefly activity was normalized to internal renilla luciferase levels.

3-D spheroid in vitro angiogenesis assay

HUVECs were transfected with miRNA mimic (ThermoScientific) and after 24 hours spheroids were generated as previously described.24 One hundred spheroids were generated per condition, collected after 24 hours and embedded in matrigel basement membrane matrix (BD Bioscience). Spheroids were monitored for 120 hours and photographs were taken on an Axiovert 100 microscope (Zeiss) using an AxioCam (Zeiss) and acquired using AxioVision software (Zeiss). To analyze average sprouts per spheroid, sprouts were counted using Adobe Photoshop CS2 (n = 60). Average sprout length was measured using the segmented lines tool in ImageJ (National Institutes of Health). Five sprouts were measured per spheroid (n = 20).

Embryo manipulation and in situ hybridization procedures

Zebrafish embryos were obtained by natural spawning of adult zebrafish. Embryos were raised and maintained at 28.5°C in system water and staged as described.23 Tg(kdrl:EGFP)26 and Tg(fli1a:EGFP)27 lines were used to monitor blood vessel development. Antisense MOs (GeneTools) and miRNA mimics (Thermo Fischer Scientific) were injected into 1 to 4-cell-stage embryos. The MOs used in this work were MO1-dll4 (5 ng),6 dll4-TPmiR-30 5'-TGTAACAAATCAGAAAATGATT-3' (10 ng unless otherwise stated), dll4-TPcontrol 5'-ATAGCCTCTTATATTACTCCTTTAATGTT-3' (10 ng unless otherwise stated), kdrl MO38 (4.5 ng), and kdrl MG38 (4.5 ng). dll4-TPmut-30 was designed so that the 3' end binds to the miR-30 target site within the dll4 3' UTR, whereas the 5' region binds to the downstream flanking sequence, as per Choi et al.29 dll4-TPmut-30 was designed so that it binds to another unrelated region of the dll4 3' UTR (596-620 nt), which is not predicted to contain any other miRNA binding sites, as per Choi et al.29 miRNA mimics were injected in the quantities stated. In situ hybridization was performed as described.30 RNA probes were labeled with digoxigenin (Roche) and detected using BM Purple (Roche). Images of in situ hybridizations were taken using a Nikon 1200F camera on a Nikon E1500 dissecting scope and acquired using ACT-1 software (Nikon). Fluorescent images of the Tg(kdrl:EGFP) and Tg(fli1a:EGFP) embryos were taken using an AxioCam (Zeiss) on an Axiovision Lumar V2 dissecting scope (Zeiss) and acquired using AxioVision Release 4.8.0 software. Figures were generated with Adobe Photoshop CS4.

Statistical and bioinformatics analysis

All experiments were performed in independent replicates and error bars correspond to SEM unless otherwise stated. Statistical significance (P value) was calculated with a 2-sided unpaired student t test unless otherwise stated. Processing and statistical analysis of the KLEC gene expression microarray (GEM) data (GSE17016) was performed using Bioconductor packages (affy, limma) for the R programming language. GEM and RNAseq data for analysis of the correlation between DLL4 and miR-30 in tumors compared with normal tissue were obtained from The Cancer Genome Atlas (www.cancergenome.nih.gov) data portal (https://tcga-data.nci.nih.gov/tcga). As data for each sample were individually processed rather than normalized as a batch and because data from different platforms and data types were analyzed, both the miRNA and gene expression data were scaled (ie, between 0 and 1) and centered (ie, approximately 0) before merging. For a full list of the sample numbers, data types, and platforms, please see supplemental Table 1 (available on the Blood Web site; see the Supplemental Materials link at the top of the online article).

Results

KSHV regulates expression of a miRNA family predicted to target DLL4

KSHV infection of endothelial cells, including LECs, is a tractable model to study aspects of endothelial cell biology.31,32 DLL4 is one of the most significantly up-regulated genes in KLECs.12 We therefore examined the most down-regulated cellular miRNAs in KLECs to determine whether we could identify miRNAs that regulate DLL4.26 We reanalyzed our microarray data with respect to down-regulated miRNAs and found that the miR-30 family is
down-regulated in KLECs 72 hours PI (Figure 1A). The most significantly suppressed probes corresponded to miR-30b and miR-30c and the microarray data were validated by qRT-PCR (Figure 1B). The mature miR-30c miRNA detected by the microarray can be produced from 2 distinct precursor hairpins (pre–miR-30c-1 and pre–miR-30c-2) and we confirmed down-regulation of both sources in KLECs (supplemental Figure 1A).

We used the TargetScan prediction algorithm to identify miR-30 targets and ranked them according to total context score.33 We analyzed this list with respect to genes significantly altered in KLECs and discovered that the 3’UTR of DLL4 scored favorably (total context score 0.39). TargetScan analysis of the DLL4 3’UTR sequence indicated that the miR-30 family is the best scoring miRNA for this 3’UTR (Figure 1C).

miR-30 CONTROLS ANGIOGENESIS BY REGULATING DLL4

To validate our target predictions for miR-30b and 30c, we measured DLL4 expression after transfecting synthetic miR-30b and miR-30c mimics into LECs. DLL4 mRNA and protein levels

Figure 1. KSHV regulates expression of the miR-30 family, which is predicted to target DLL4. (A) Heatmap representing relative changes in expression of hsa-miR-30 family members in LECs after KSHV infection. Red and yellow denote low and high expression, respectively. Four replicates of LECs and KLECs are shown. Two to 3 probes are shown for each member of the miR-30 family. Probes for hsa-miR-30b and hsa-miR-30c showed significant changes in expression (**Q < .01; ***Q < .001). Original GEM data from Lagos et al.20 (B) Down-regulation of miR-30b and miR-30c in KLECs, confirmed by qRT-PCR (means SEM, n = 3). Expression is relative to LECs. Differences between LECs and KLECs were significant (**P < .01). (C) Complementarity between miR-30 family members and the DLL4 3’UTR. Black lines indicate canonical Watson and Crick base-pairing, gray lines indicate G:U wobbles. The predicted target site within the DLL4 3’UTR, positions 59 to 66, is shown in red; miR-30 seed region is shown in green. (D) Heatmap table displaying the correlation coefficient R between expression of DLL4 and each member of the miR-30 family in the indicated tumor types. GEM and RNAseq data were obtained from The Cancer Genome Atlas (www.cancergenome.nih.gov) data portal (www.tcga-data.nci.nih.gov), as described in “Methods.” Because of the large difference in the number of replicates for each tumor type, the significance of R was calculated for each miR-30 versus DLL4 combination. The values and fill-in color indicate the degree of negative correlation. Nonsignificant correlations are grayed out; significant correlations are marked with an asterisk (*P < .05; **P < .01). Tumor types included are breast invasive carcinoma (BRCA), colon adenocarcinoma (COAD), glioblastoma multiforme (GBM), renal clear cell carcinoma (RCC), lung adenocarcinoma (LUAD), rectum adenocarcinoma (READ), and ovarian serous cystadenocarcinoma (OV).
were significantly reduced in LECs expressing either mimic (Figure 2A-B) and this effect was dose-dependent (supplemental Figure 2A). Coexpression of miR-30b and miR-30c, at an equivalent total concentration, did not increase DLL4 repression, suggesting that there are no additive or synergistic effects between these miR-30 family members (supplemental Figure 2A). These findings correspond with our target prediction studies, which indicate only miR-30 family members (supplemental Figure 2A). These findings indicating that there are no additive or synergistic effects between these mimics on global mRNA levels.37

As the highly stable mimics led to a supraphysiologic up-regulation of miR-30 of more than 1000-fold (data not shown), we also used lentiviruses to overexpress miR-30 in its pre-miRNA form. Infection of LECs with lentiviruses expressing miR-30b or miR-30c (pSiN_30b or pSiN_30c) suppressed DLL4 (Figure 2C). This was confirmed in another type of endothelial cell, HUVECs (Figure 2D). We confirmed expression of the mature miRNAs in LECs and HUVECs transduced with these viruses (supplemental Figure 2B-C) and DLL4 suppression increased with increasing viral copies per cell (supplemental Figure 2B). Conversely, transfection of hairpin inhibitors against miR-30b and miR-30c into LECs led to an increase in DLL4 protein levels (Figure 2E). Taken together, these data indicate that the miR-30 family regulates endogenous DLL4.

To confirm that these miRNAs act through the DLL4 3'UTR we used a vector with the luciferase coding sequence up-stream of the DLL4 3'UTR (DLL4_wt). We expressed this construct in the presence of miR-30b or miR-30c mimics (Figure 2F) and observed a 50% reduction in luciferase activity. The control vector maintained luciferase activity in the presence of exogenous miR-30b and miR-30c (means ± SEM, n = 3). Firefly expression was normalized to renilla expression to give the relative light units (RLU), which are shown relative to NTC mimic. MT01 is a control reporter, lacking a 3'UTR sequence but containing the firefly and renilla luciferase genes. Related statistically significant values are indicated by horizontal bars. In all panels, statistical significance denoted by *P < .05; **P < .01; ***P < .001.

miR-30 targeting of DLL4 influences endothelial cell behavior in vitro

DLL4 expression influences angiogenic pathways in endothelial cells and contributes to the angiogenic signature of KSHV-infected endothelial cells.12,38 We therefore used KSHV-infection of LECs
to investigate the effect of miR-30 on DLL4 levels. (Figure 3A-B). DLL4 mRNA expression was increased 3-fold in KLECs compared with LECs and this induction was attenuated to 2-fold in KLECs expressing exogenous miR-30, suggesting that miR-30 can suppress DLL4 levels in a dynamic system where it is normally up-regulated (Figure 3A). This was also reflected at the protein level where KSHV infection induced a 5-fold increase in DLL4 that was reduced to 4-fold in pSIN_30c transduced cells (Figure 3B). These findings reveal an additional miRNA-controlled layer of DLL4 regulation in human endothelial cells and are consistent with our previous work showing that activation of the extracellular-signal regulated kinase (ERK) pathway also drives DLL4 induction in KSHV-infected endothelial cells.12

During pathologic and physiologic angiogenesis, endothelial cells expressing DLL4 stimulate Notch signaling in adjacent cells and are specified as “tip” cells. Tip cells localize to the apex of the developing sprout, excluding the signal-receiving “stalk” cells, which contribute to the body of the developing vessel. Suppression of Notch signaling leads to excessive sprouting and multiple vessel branches because the tip cell phenotype is not restricted.2 We used an in vitro model of sprouting angiogenesis to investigate whether DLL4 targeting by miR-30b affected normal tip-cell behavior.24

HUVECs expressing either miR-30b mimics or nontargeting control (NTC) were induced to form spheroids which were then embedded in matrigel. Those spheroids composed of miR-30b–overexpressing HUVECs displayed an increased propensity to form sprouts of greater length (Figure 3C) indicating that miR-30 overexpression promotes angiogenic sprouting. miR-30 mimics down-regulated DLL4 mRNA in this cell type (supplemental Figure 3A-C).

**Exogenous expression of miR-30 induces hyperbranching of intersegmental vessels in zebrafish**

The developing zebrafish vasculature is an established model of angiogenic processes. Zebrafish DLL4 regulates angiogenic sprouting of ISVs and is detectable from 8 hours postfertilization (hpf) by RT-PCR5; using qRT-PCR, we observed dll4 induction between 6 hpf and 12 hpf in zebrafish embryos (supplemental Figure 4A). Expression of miR-30b and miR-30c homologs is found during development and in the adult fish.39 We observed a correlation between expression of miR-30 and dll4 from 18 hpf to 30 hpf (supplemental Figure 4A) corresponding with the temporal window during which angiogenic sprouting occurs.20
These temporally coincident changes in dll4 and miR-30 suggest a functional interaction that may contribute to tight control of DLL4 expression during vascular development. We investigated this relationship by increasing miR-30 expression through microinjection of miR-30 mimics. To reduce nonspecific effects of exogenous miR-30, microinjected mimic was titrated to levels where overall embryo morphology was normal and miR-30 expression was detectable at a physiologically relevant level (Figure 4A). We considered an up-regulation of 20- to 25-fold to be within physiologic levels, as changes of this magnitude are seen for individual miRNAs during zebrafish development.41 We confirmed the down-regulation of dll4 expression in the vasculature by in situ hybridization (Figure 4B). dll4 expression titrated with levels of miR-30 and was reduced by 20% to 30% compared with control embryos at the lowest mimic concentrations (Figure 4C). These data indicate that dll4 mRNA levels can be disrupted in vivo by exogenous expression of miR-30.

It was previously shown that dll4 silencing using MOs induces excessive ISV branching.5,6 We investigated the effect of reduced dll4 expression by miR-30 on endothelial cell behavior in Tg(kdrl:EGFP) zebrafish, using dll4 MO-injected embryos as a positive control (Figure 4E, supplemental Figure 4B). Compared with uninjected control embryos, we found that embryos expressing miR-30b or miR-30c mimics showed vessel-free, hyper-migratory endothelial cells, ISVs at a more advanced stage of sprouting and premature dorsal longitudinal anastomotic vessel (DLAV) formation at 25 hpf (Figure 4E, supplemental Figure 4B left panels yellow arrowheads). With increasing amounts of miR-30 mimic, a higher percentage of embryos displayed advanced sprouting and a greater proportion of these exhibited premature DLA V formation and hyper-migratory behavior (supplemental Figure 5A). This phenotype was also present in dll4 MO-injected embryos (supplemental Figure 4B), suggesting that miR-30 overexpression phenocopies the dll4 morphant phenotype. At 25 hpf, the diameter of the DA was significantly reduced in dll4 morphants and in miR-30–overexpressing embryos compared with WT embryos (Figure 4D). This reduction in vessel diameter concurs with previous work showing that DLL4 up-regulation in tumors correlates with vessel...
Injected with embryos lacking 1 ISV but with a minimum of 6 ISVs. Severe phenotype: embryos with 5 or less ISVs. To determine statistical significance, each observation of no phenotype, of the MOs and/or TPs indicated. Columns are the average of 2 independent experiments with 15 to 30 embryos counted per sample per experiment. Moderate phenotype:

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To confirm that the miR-30 family targeted dll4 in vivo, embryos were coinjected with a target protector MO (TP) designed to bind to a region within the dll4 3’UTR containing the miR-30 target site (dll4-TpR-30). Coinjection of dll4-TpR-30 with miR-30b or miR-30c mimics led to a partial rescue of the miR-30–induced ISV hyperbranching (Figure 4E), with the advanced sprouting phenotype being reduced by approximately one-half in coinjected embryos (supplemental Figure 4B). These findings indicate that dll4 down-regulation is a significant contributing factor to the advanced sprouting and excessive branching phenotypes observed. These data suggest that miR-30 can regulate endothelial cell behavior in vivo and that the targeting of dll4 by miR-30 is functionally relevant during vascular development.

Increased dll4 expression synergizes with partial VegFA signaling to inhibit angiogenesis

To investigate the endogenous role of miR-30 in the regulation of dll4, we injected dll4-TpR-30 or dll4-TpR control alone and examined dll4 expression at 28 hpf. Consistent up-regulation of dll4 in dll4-TpR-30 injected embryos was observed by in situ hybridization (Figure 5A) and qRT-PCR (Figure 5B). When Tg(fli1a:EGFP) zebrafish were injected with dll4-TpR-30 alone, we observed an increase in the percentage of embryos with shorter or missing ISVs at 28 hpf (Figure 5C, vii, D), suggesting that elevating dll4 levels partially blocked ISV angiogenesis.

Notch signaling via DLL4 negatively regulates expression of VEGFR2, the receptor through which VEGFA signals. Microarray and qRT-PCR analyzes of HUVECs transduced with empty vector and DLL4-encoding retroviruses have previously shown that VEGFR2 is down-regulated in DLL4-expressing HUVECs, whereas HUVECs cultured on recombinant DLL4-coated plates display reduced VEGFR2 protein levels. This down-regulation is known to be caused by DLL4 signaling through NOTCH as HUVECs cultured on recombinant DLL4-coated plates in the presence of the γ-secretase inhibitor DAPT did not show VEGFR2
Kdr, MO, and Figure 5D. We propose that this is caused by impairment in Vegfa. Knockdown of which ensues prevents normal intersegmental vessel sprouting and the DLAV does knockdown, the restriction of tip cell specification and inhibition of Vegfa signaling occurs. (C) When miR-30 regulation of increased loss of ISV sprouting compared with the levels of Vegfa signaling by negatively regulating 5E. This suggested that the miR-30 family is indirectly regulating dll4 expression (Figure 6C). If kdr is also suppressed, the subsequent lack of Vegfa signaling results in impaired ISV sprouting (Figure 6C).

Discussion

The 5 members of the miR-30 family all share the same seed sequence and are encoded by 6 genes located on human chromosomes 1, 6, and 8. This level of redundancy would suggest a critical functional role for this miRNA family. Members of the miR-30 family have been implicated in osteoblast differentiation, adipo genesis, epithelial-to-mesenchymal transition (EMT), Xenopus proephros development, cellular senescence, myocardial matrix remodeling, and cancer. Genes that have been identified as targets of 1 or more miR-30 family members include snail homolog 1 (drosophila; SNA1)46; LIM homeobox 1 (lhx1)47; B-cell lymphoma 6 (BCL6)48; v-myb myeloblastosis viral oncogene homolog (avian)–like 2 (MYBL2)49; and runt-related transcription factor 2 (RUNX2).50 Our study is the first to identify and validate DLL4 as a target of miR-30 and to demonstrate a key role for miR-30 in angiogenesis.

For sprouting angiogenesis to occur successfully a balance must be maintained between the number of tip cells, which lead the nascent sprouts, and stalk cells which will make up the endothelium of the new vessel. DLL4 plays a crucial role in regulating the ratio of tip cells to stalk cells. Specifically, expression of DLL4 by the tip cells stimulates Notch signaling in the adjacent cells, thereby maintaining stalk cell identity and restricting tip-cell specification. When DLL4 signaling or expression is inhibited, tip-cell specification is not controlled, leading to excessive sprouting from existing vessels. This can be seen during zebrafish ISV development, when knockdown of dll4 causes the formation of an aberrant network of interconnected branches. It is also relevant to the control of tumor vasculature as the use of blocking antibodies against DLL4 has been shown to promote angiogenesis, but the increased tumor vascularity is leaky and hence nonproductive, leading to the inhibition of tumor growth. We hypothesized that DLL4 is not only regulated at the transcriptional level but that its expression is subject to fine-tuning by posttranscriptional mechanisms, such as miRNAs, to maintain optimal levels for successful angiogenesis.

We used KSHV infection of LECs as a model with which to study DLL4 regulation, as DLL4 is one of the most significantly up-regulated genes in KLECs. We observed significant down-regulation of miR-30b and miR-30c in KLECs, which was of interest given that the miR-30 family was predicted to target DLL4 through a specific site within the 3'UTR. The simultaneous up-regulation of DLL4 and suppression of miR-30 in KLECs suggested a possible regulatory relationship between DLL4 and miR-30 in endothelial cells. This was further supported by our observation that DLL4 and miR-30 are negatively correlated in several tumor types that are moderately or highly angiogenic.

We confirmed that DLL4 is a target of miR-30 and that miR-30 actively regulates endogenous DLL4 in human vascular and lymphatic endothelial cells in vitro and during zebrafish vascular development in vivo. We have shown, both in vitro and in vivo, that miR-30 controls DLL4 expression through a specific site within the DLL4 3'UTR. This regulation is also relevant to oncogenic virus infection, as the up-regulation of DLL4, which is normally seen in KLECs, was attenuated by the application of exogenous miR-30.

Our study has revealed a functional role for miR-30 in the regulation of angiogenesis via DLL4 targeting. Overexpression of
miR-30 in endothelial cells promotes angiogenic sprouting in vitro. The introduction of exogenous miR-30 into zebrafish embryos led to aberrant migration of endothelial cells and longer sprouts at 25 hpf which developed into excessive ISV branching at 72 hpf, phenotypes also observed after dll4 knockdown. We show that the excessive vascular sprouting caused by miR-30 overexpression was by way of dll4 targeting through the use of a TP, specific to the miR-30 target site within the dll4 3‘UTR. Recently it has been shown that knockdown of miR-27b causes defective vascular sprouting in developing zebrafish embryos. Rescue studies using knockdown of dll4 and spry2 implicated these 2 targets as the causative agents of this phenotype; however, TP studies were not used to fully delineate the respective roles of dll4 and spry2. The angiogenesis inhibitor SEMA6A has also been identified as a target of miR-27b, and could account for the phenotype seen on miR-27b inhibition in zebrafish embryos.

The TP also provided us with a useful tool with which to explore the endogenous role of miR-30 during zebrafish vascular development. We used it to specifically block the interaction of miR-30 with dll4 during development, while still allowing miR-30 to interact with its other targets. We have shown that inhibiting the normal control of dll4 by miR-30 elevates dll4 levels, which partially blocks ISV angiogenesis. This would suggest that during normal ISV sprouting miR-30 is actively suppressing dll4, helping to maintain tight control of dll4 expression and hence angiogenesis. However, further investigations revealed a more intricate regulatory pathway. VEGFR2 expression has been shown to be inhibited by Notch signaling via DLL4. When dll4 levels were increased using the TP, we observed down-regulation of kdr, a functional ortholog of VEGFR2. Vegfa signaling in zebrafish is mediated through the synergistic action of Kdrl and another Vegfa receptor, Kdr. When injection of dll4-TPmimic was combined with knockdown of kdr, ISV sprouting was moderately to severely inhibited in the majority of embryos, as seen when the expression of both kdr and kdr is blocked using MOs. This work has therefore revealed that the miR-30 family indirectly regulates Vegfa signaling by controlling dll4 expression and that this regulatory axis confers robustness to Vegfa-mediated angiogenesis.

We have identified and validated DLL4 as a novel target of the miR-30 family and demonstrated the important role of these miRNAs during sprouting angiogenesis. We show that miR-30 regulates vascular development in vivo and provides robustness to Vegfa signaling by indirectly influencing kdr expression. Our findings indicate that the manipulation of miR-30 in the setting of pathologic vascularization could represent a new therapeutic approach.

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The authors thank Prof A. Harris and Dr E. Bridges for advising on the 3D spheroid in vitro angiogenesis assay. This work was funded by a United Kingdom MRC PhD Studentship (G.B.), an MRC Program Grant (C.B. and D.L.), a BHF project grant (R.M. and R.P.), an MRC Unit Grant (R.M. and R.P.), and by Cancer Research UK (S.H., V.E., and C.B.).

Authorship

Contribution: G.B. designed and performed the experiments, analyzed the data, and wrote the paper; R.M. designed and performed the zebrafish experiments and assisted in preparation of the paper; S.H. provided the bioinformatics analysis and statistical assistance; V.E. designed experiments and assisted with paper preparation; D.L. performed the miRNA microarray and assisted with paper preparation; D.G. performed some of the KSHV experiments; R.P. assisted with paper preparation; and C.B. designed the experiments, supervised the project, and wrote the paper.

Conflict-of-interest disclosure: G.B., V.E., and C.B., are joint inventors on a patent application for DLL4 targeting by the miR-30 family, which was filed at the UKPTO on 09.02.11, application No. 1102283.7. The remaining authors declare no competing financial interests.

Correspondence: Chris Boshoff, Cancer Research UK Viral Oncology Group, UCL Cancer Institute, University College London, London, WC1E 6BT, United Kingdom; e-mail: c.boshoff@ucl.ac.uk.

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Correspondence: Chris Boshoff, Cancer Research UK Viral Oncology Group, UCL Cancer Institute, University College London, London, WC1E 6BT, United Kingdom; e-mail: c.boshoff@ucl.ac.uk.


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The microRNA-30 family targets DLL4 to modulate endothelial cell behavior during angiogenesis

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