Latency-Associated Nuclear Antigen of Kaposi Sarcoma–Associated Herpesvirus Promotes Angiogenesis through Targeting Notch Signaling Effector Hey1

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Abstract
Notch signaling has been implicated in the pathogenesis of Kaposi sarcoma. Kaposi sarcoma is an angioproliferative neoplasm that originates from Kaposi sarcoma–associated herpesvirus (KSHV) infection. Previously, we showed that the KSHV LANA protein can stabilize intracellular Notch in KSHV-infected tumor cells and promote cell proliferation. However, whether Notch signaling functions in pathologic angiogenesis of Kaposi sarcoma remains largely unknown. Hey1, an essential downstream effector of the Notch signaling pathway, has been demonstrated to play a fundamental role in vascular development. In the present study, we performed whole transcriptome, paired-end sequencing on three patient-matched clinical Kaposi sarcoma specimens and their corresponding adjacent stroma samples, with an average depth of 42 million reads per sample. Dll4, Hey1, and HeyL displayed significant upregulation in Kaposi sarcoma. Further verification based on immunohistochemistry analysis demonstrated that Hey1 was indeed highly expressed in Kaposi sarcoma lesions. Using the Matrigel plug assay, we showed that downregulation of Hey1 and γ-secretase inhibitor treatment caused dramatic reduction in the formation of new blood vessels in mice. Interestingly, LANA was responsible for the elevated level of Hey1 through inhibition of its degradation. Importantly, Hey1 stabilized by LANA promoted the neoplastic vasculature. Taken together, our data suggest that hijacking of the proangiogenic property of Hey1 by LANA is an important strategy utilized by KSHV to achieve pathologic angiogenesis and that Hey1 is a potential therapeutic target in Kaposi sarcoma. Cancer Res; 74(7); 2026–37. ©2014 AACR.

Introduction
Angiogenesis performs a fundamental role in the development of cancer. Both the growth and the metastasis of cancer need blood vessels to provide nutrients and oxygen, and to remove metabolic wastes (1). Kaposi sarcoma is a vascular neoplasm of viral origin (2, 3). Given the highly vascularized nature of Kaposi sarcoma tumors even at early stages, it has been proposed that Kaposi sarcoma–associated herpesvirus (KSHV) may induce angiogenesis directly (3, 4). This indicates that Kaposi sarcoma may provide an ideal model for the study of angiogenesis mediated by tumor viruses.

The mechanisms by which KSHV induces vascular remodeling have been identified to some extent: a large number of angiogenic factors such as hypoxia-inducible factor-1α and VEGF-A can be unregulated during infection with KSHV (5, 6).

Meanwhile, KSHV also promotes angiogenesis through repression of angiogenic inhibitors such as thrombin (7). Moreover, the KSHV genome itself encodes viral cytokine- and chemokine-like factors that can activate endothelial cells and stimulate angiogenesis (4, 8–12). However, although it is a well-defined angiogenesis-associated pathway, whether the Notch signaling pathway can be manipulated by KSHV in Kaposi sarcoma angiogenesis, is largely unknown (13, 14).

The role of the Notch signaling pathway in Kaposi sarcoma tumorigenesis has been confirmed over recent decades (15). Importantly, inhibition of Notch signaling has been shown to inhibit tumor growth (16, 17). Notch signaling is targeted by KSHV through vFLIP (latent) and vGPCR (lytic), thus contributing to cell proliferation (8, 17, 18). Cheng and colleagues found that numerous downstream targets of the Notch signaling pathway, such as Hey and Hes, were upregulated in three-dimensional cultures of KSHV-infected cells (18). However, whether Notch signaling also functions in the angiogenic process of Kaposi sarcoma remains further investigation (4, 8, 9).

It should be noted that, besides the previously reported function of the Notch ligands DLL4 and Jagged1 and its receptor Notch4 in cancer angiogenesis (16, 19–23), there are no data about other members, particularly Hey1. In fact the role of Hey1 in embryonic cardiovascular development has been well depicted (24–26). Recently, Hey1 has been identified as a potential focal point, integrating bone morphogenetic proteins and the Notch signaling pathway, which both function
in angiogenesis (27, 28). Furthermore, more than 500 target genes can be regulated by Hey1 and Hey2, as demonstrated by microarray analysis; thus, the biologic function of Hey1 was once underestimated (29).

As a key component of the Notch signaling pathway, the possibility that Hey1 is manipulated by LANA cannot be ruled out because it is widely accepted that Hey1 plays a critical role in the development of embryonic vasculature. We hypothesize that KSHV may manipulate Hey1 through LANA to promote angiogenesis in Kaposi sarcoma. In this study, we show that the Notch signaling pathway is highly activated in Kaposi sarcoma lesions and its downstream effector Hey1 is specifically expressed in Kaposi sarcoma and promotes the formation of new blood vessels. Importantly, LANA hijacks the proangiogenic capability of Hey1 to promote angiogenesis through enhancement of its stabilization. Our current study provides a better understanding of Kaposi sarcoma angiogenesis, with potential implications for new Kaposi sarcoma therapy.

Materials and Methods

Ethics statement

The animal experiments in this study were performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Shanghai Government and national law. Mice were handled according to all applicable provincial and national animal care guidelines, under animal care protocols approved by the Institutional Animal Care and Use Committee of the Institut Pasteur of Shanghai, Chinese Academy of Sciences (Shanghai, China; Animal protocol # A2012007). The clinical section of the research was reviewed and ethically approved by the Institutional Ethics Committee of the First Teaching Hospital of Xinjiang Medical University (Urumqi, Xinjiang, China; Study protocol # 20082012). Written informed consent was obtained from all participants, and all samples were anonymized.

Cell lines, antibodies, and reagents

Primary human umbilical vein endothelial cells (EGM HUVECs, CC-2517) were purchased from Lonza company and were cultured in EGM2MV medium (Lonza). Doxycycline-inducible iSLK cells were provided by Dr. Jae Jung at the University of Southern California (Los Angeles, CA). The HEK 293T and 293:219 (a 293 cell line that harbors the KSHV genome) cells were a gift from Gamem D (Microbiology and Medicine University of California, San Francisco, CA). The KSHV-positive (KSHV-BJAB) and -negative (BJAB) cell lines were generously provided by Erle S. Robertson (University of Pennsylvania, Philadelphia, PA). All cells were cultured at 37°C in the presence of 5% CO2.

A mouse monoclonal antibody (mAb) against LANA and polyclonal antibody (pAb) against Hey1 were prepared by immunizing mice with recombinant LANA or Hey1 expressed in Escherichia coli, respectively. Anti-HA, Anti-His, and anti-Flag (H9658, F3165) mAbs and rabbit anti-tubulin (A2066) pAb, γ-secretase inhibitor (GSI), proteasome inhibitor MG132 and cycloheximide (CHX), were all purchased from Sigma. Recombinant basic fibroblast growth factor (bFGF) was purchased from R&D Systems. Growth factor-reduced Matrigel was purchased from BD Biosciences.

Plasmids, shRNA, and transfection

The pA3F-LANA, which encodes the full length of LANA-1, has been described previously (30). Human Hey1 was amplified from a 293T cell cDNA library and cloned into pCDNA3.1-HA (Invitrogen), pCDNA3.1-Myc-His, and lentivector pCDH-CMV-IREs-Blast plasmid, respectively. The short hairpin RNAs (shRNA) complementary to LANA (5'-TGCCCCACAGTGGTCA-CATCCGGGC-3') or Hey1 (5'-CGCATCTCAAAACTAGCTT-3') were cloned into the pLKO.1 vector, respectively. A pLKO.1 vector with scrambled sequence against LANA was used as control (5'-TGGCTTCTGATGATACCAAC-3'). Mouse shRNAs were purchased from GenePharma, including shDLL4 (5'-GCA-ACTGTCTTATGCGCTTGG), shNotch1 (5'-GCTTCTTCTTAC-TGGCAATGT), and shHey1 (5'-GCTTCTATCTCCAGATCC-ATG'). They were cloned into the pGPU6/GFP/Neo vector. The negative control was a mus-scrambled sequence, namely 5'-GTTCTCCAGACGTTCAGC'.

The 293T cells were transiently transfected using the polyethylenimine method as described previously, with minor modifications (31). LANA, Hey1, and shHey1 were packaged into lentivirus following the manufacturer’s protocol. To transduce primary human umbilical vein endothelial cell (HUVEC) and iSLK:219 cells, the lentiviruses were ultracentrifuged collected. The results shown represent experiments performed in triplicate.

RNA isolation, library preparation, sequencing, and analysis

Total RNA was isolated from three fresh-frozen Kaposi sarcoma lesions and the corresponding samples of normal tissue, with the use of an RNeasy Fibrous Tissue Mini Kit (Qiagen). Isolation of the mRNA was achieved by Oligo(dT) magnetic bead selection three times to ensure pure poly(A)+ populations. The samples were subjected to second-strand cDNA synthesis. After end repair, adenylation of 3' ends, adapter ligation, isolation of 300 bp cDNA fragments, and subsequent PCR amplification, 100 bp paired-end sequencing reads were obtained with the Illumina HiSeq platform. RNA-Seq reads were aligned to the human reference sequence National Center for Biotechnology Information (NCBI) hg19 with TopHat2. The RefSeq and Genocode databases were used as reference annotations to calculate the values of fragments per kilobase of transcript per million mapped reads for known transcripts (FPKM). Differential expression was determined using a series of two-class SAM analyses. A two-class comparison of the pool of the Kaposi sarcoma lesions with their corresponding normal tissues was performed to determine differentially expressed genes; the P value of fold-change for significance was set at <0.05 (Wald score >1.96 or <−1.96) in Wald test analysis.

Histopathology and immunohistochemical analysis

The clinical tissue specimens from 28 patients with Kaposi sarcoma were collected from Xinjiang, northwestern China.
One pair-matched Kaposi sarcoma case with its adjacent stroma serving as negative control was analyzed by both hematoxylin and eosin staining (H&E) and immunohistochemistry (IHC). The expression of LANA and Hey1 in other specimens was analyzed by IHC. The slides were counterstained with hematoxylin. Matrigel plugs from the mice were removed and fixed, paraffin embedded, and stained with H&E (32, 33).

**In vivo Matrigel plug angiogenesis assay**

Female C57Bl/6 mice, 6 to 8 weeks of age, were purchased from Shanghai SLAC Laboratory Animal Co. Ltd. and maintained at the Institut Pasteur of Shanghai, Chinese Academy of Sciences. There were four mice in each group. Growth factor-reduced Matrigel (500 μL) alone or mixed with an increasing dosage of bFGF (R&D Systems) at a concentration of 100 and 300 ng/mL was injected subcutaneously into the flanks of the mice. In the GSI treatment group, Matrigel was premixed with 300 ng/mL bFGF and different dosages of GS. Similarly, 100 or 300 μg shDIH, shNotch1, or shHey1 plasmid and the scrambled control were mixed with bFGF (300 ng/mL) in advance. The mice were injected with individual mixtures as described above. On day 14, the mice were killed and the Matrigel plugs were removed, as previously reported.

To study angiogenesis regulation by LANA targeting of Hey1, the Matrigel was premixed with plasmids diluted by Dulbecco’s Phosphate Buffered Saline (DPBS), with four groups: empty plasmid, pA3F-LANA, pA3F-LANA plus shRNA control vector, and pA3F-LANA plus shHey1 plasmid. These were hydrodynamically injected into mice. On postinjection days 3, 7, and 10, the mice were repeatedly hydrodynamically injected with plasmids in each group as above. The amount of LANA and its control empty vector used was 50 μg each time; for shHey1 and scrambled shRNA, 100 μg was used. On day 14, the Matrigel plugs were removed and photographed. After the removal of debris by centrifugation, the hemoglobin concentration was measured using Drabkin’s reagent (Sigma-Aldrich) in each group. Alternatively, they were fixed in 4% paraformaldehyde at 4°C overnight, and subsequently embedded and dissected for H&E analysis (32–34).

**Capillary tube formation assay**

Overexpression of Hey1 in HUVEC cells was induced by transduction with the corresponding lentivirus for 48 hours. The cells were seeded in Matrigel-coated plates at a density of \(2 \times 10^5\) cells in 100 μL EGM medium. The assembled assays were allowed to incubate to time points of 0, 15, 30, 60, 120, and 240 minutes. Photographs were obtained and the total RNA of the cells was collected using TRIzol. The mean loop area, indicating the capability of HUVEC cells to induce capillary tube formation, was calculated by ibidi Company (Germany). Independent experiments were performed in triplicate.

**Inhibitor treatment and Western blot analysis**

GSI at 0, 20, 40 and 0, 10, 40, μmol/L was used to treat the B and 293T cells for 60 or 72 hours. CHX (100 μg/mL) was used to inhibit further protein synthesis and the cells were harvested at the indicated time points. The proteasome inhibitor MG132 at 0, 1, 5, and 10 μg/mL was used on the 293T cells in the presence or absence of ectopic LANA. The final concentration of MG132 at 10 μg/mL was utilized for 3 hours before harvesting the cells for the ubiquitination assay. Following incubation, the cells were harvested and lysed, and the cell lysates were collected. The protein was then heated in SDS–mercaptoethanol lysis buffer and analyzed by SDS-PAGE. Antibodies against tubulin were involved as a loading control.

**Transwell invasion assay and cell counting**

Transwell invasion assays were performed using Transwell chambers (8 mm polycarbonate membrane, Corning). The iSLK.219 cells were treated with shLANA and control vector for 48 hours. After this, Hey1 lentivirus was transduced into the corresponding cells for another 24 hours. The shHey1 transduction was performed on HUVEC cells for 48 hours. Typically, the cells were seeded in the upper chamber with basic medium at 1 × 10^6 cells/well. Migration and invasion were allowed to proceed for 24 hours at 37°C, and the cells were fixed with 70% methanol and stained with 0.5% crystal violet solution. The number of migrated cells was determined by counting stained cells from multiple randomly selected microscopic visual fields using ImageJ software. Photographs were obtained and independent experiments were performed in triplicate.

**Statistical analysis**

SPSS 11.5 (SPSS Inc.) was used for all statistical analyses. Data are shown as mean values with SEM. The level of significance was set at \(P < 0.05\), as determined by Student t tests with GraphPad Prism 5 software (\(n = 4\) for each group).

**Results**

LANA and Hey1 were highly expressed in Kaposi sarcoma pathologic vasculature

To characterize the Notch signaling pathway–associated RNA expression in Kaposi sarcoma, we performed paired-end, high-throughput sequencing with the Illumina HiSeq platform on malignant Kaposi sarcoma as well as patient-matched normal tissues (Fig. 1A). We obtained an average of 42 million reads for each sample, 95% of which were aligned with the human genome (35). To assess protein-coding gene expression in Kaposi sarcoma, we assigned sequencing reads to coding transcripts using RefSeq annotations. Genes (\(n = 665\)) were differentially expressed in all patients; of these, 645 were upregulated and 20 were downregulated. This shared signature of differentially regulated genes included several well-established angiogenesis-associated molecules, as shown in Fig. 1A. Of these, components of the Notch signaling pathway, including DI4, Hey1, and HeyL, were significantly upregulated in Kaposi sarcoma lesions, implying their involvement in Kaposi sarcoma pathogenesis. Furthermore, both LANA and Hey1 were specifically distributed in Kaposi sarcoma tumor tissue, in contrast with normal tissues (Fig. 1B). To ensure that this finding was common in Kaposi sarcoma lesions, rather than a specific case, more paraffin-embedded Kaposi sarcoma tissues were used in this study. Of those, four slides were randomly selected for presentation in Fig. 1C. Interestingly, expression of LANA and Hey1 was observed in vascular channels that were centered on collagen bundles and spindle cells that formed...
short fascicles (Fig. 1C). Enlarged images of the Hey1 staining indicated its expression in inner raised and inflated endothelial cells lining the irregularly shaped vessels. These data suggested the correlation of LANA and Hey1 in the Kaposi sarcoma clinical samples and the possible clinical involvement of these two molecules in Kaposi sarcoma angiogenesis.

**GSI treatment and repression of the Notch pathway impaired new blood vessel formation**

The above results suggested a possible role of Hey1 in tumor angiogenesis. To confirm this, we next explored the Hey1-induced phenotype using the *in vivo* Matrigel plug assay. Being a strong growth factor, bFGF induces angiogenesis in a dose-dependent manner, as expected (Fig. 2A). Surprisingly, GSI treatment significantly reduced the number of neoplastic blood vessels in Matrigel plugs stimulated by bFGF when compared with control treatment, which can nonselectively block the Notch signaling pathway activation. (Fig. 2A). This indicated the critical proangiogenic feature of the Notch signaling pathway *in vivo*. Next, knockdown of the ligand Dll4, receptor Notch1, and effector Hey1 led to an identical phenotype with GSI treatment (Fig. 2B). These findings were confirmed by further histologic analysis of the number and size of blood vessels in each group (Fig. 2B). To illustrate that the granular features formed in Matrigels were indeed neoplastic vessels, the hemoglobin content was measured. The measurements totally coincided with the results from H&E analysis, suggesting that the vasculature was functional (Fig. 2C and D). Taken together, these results suggest that Notch signaling is a key pathway promoting the angiogenesis mediated by Hey1.

**Hey1 exhibited proangiogenic properties, validated by capillary tube formation assay**

To delineate the pattern of Notch pathway players during vessel development, a capillary tube formation assay was

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Figure 1. LANA and Hey1 are highly expressed in pathologic vascular endothelial cells in Kaposi sarcoma (KS) lesions. A, heatmap representation of differentially regulated genes performed on RNA extracted from three Kaposi sarcoma lesions and their patient-matched normal tissues. The genes were ranked according to absolute values of \( \log_2 \) relative change. B, LANA and Hey1 were highly specifically distributed in Kaposi sarcoma tumor rather than its adjacent stromal tissue on immunohistochemical analysis. Original magnification, \( \times 5 \) in H&E and \( \times 40 \) in IHC. C, representative immunohistochemical analysis of LANA and Hey1 colocalization in pathologic vessels in Kaposi sarcoma lesions. Original magnification, \( \times 40 \) in all examinations.
performed using HUVEC cells; the cells were photographed and collected at various time points. Ectopic expression of Hey1 resulted in greatly increased amounts of tubes in comparison with the control cells at 120 and 240 minutes, respectively, after induction (Fig. 3A). In addition, quantitative analysis of mean loop area, indicating angiogenic capability, was carried out between groups (Fig. 3B). When the transcriptional expression level was measured using quantitative PCR, it was clearly shown that Dll4 and Hey1 play critical roles in blood vessel formation, in contrast with the almost stable status of Jagged1, Hes1, and VEGFR2. The expression of Dll4 and Hey1 was dynamically changed during different stages of endothelial cell initiated vessel formation (Fig. 3C). Moreover, the ability of Hey1 to repress transcription during the expression of VEGFR2 in angiogenesis coincided with previous observations (36, 37), indicating that the function of Hey1 oscillates between endothelial cell migration/proliferation and tube alignment/forma-

tion (Fig. 3C).

LANA was the major component that upregulated Hey1 expression during KSHV infection

Multiple KSHV-encoded viral proteins have been demonstrated to activate the Notch pathway (3, 6, 12, 16–18, 38–40). We reported previously that KSHV LANA stabilizes ICN and contributes to cell proliferation (30). Herein, we chose to identify the major component of KSHV that manipulates Hey1, and to elucidate the mechanism further. The Notch pathway was activated in B cells and the level of expression of intracellular Notch (ICN) and Hey1 were both increased by KSHV infection (Fig. 4A). Moreover, LANA repression in KSHV-positive 293T cells greatly reduced the endogenous expression of Hey1, suggesting that it takes major responsibility for Hey1 during KSHV infection (Fig. 4B). In a further attempt to determine whether the modulation of Hey1 by LANA necessarily depends on Notch signaling pathway activation, the cells were treated with GSI inhibitor for 3 days, followed by LANA repression for another 48 hours. Subsequently, the level of expression of ICN and Hey1 was measured and compared between the GSI treated and untreated groups when both of them were LANA repressed. It was shown that the decreased level of Hey1 after GSI treatment was considerably higher than that of ICN when LANA was repressed in both groups (Fig. 4B). This indicated that LANA may upregulate Hey1, but that this does not necessarily depend on activation of the Notch signaling pathway. In addition, the expression of Hey1, whether endogenous or ectopic, was positively correlated with the LANA dosage in 293T cells (Fig. 4C and D). These data
In this system, hexahistidine (His6)-tagged Hey1 was utilized in its degradation, the ubiquitination assay was carried out. (Fig. 6A). To confirm that LANA promotes Hey1 degradation, the ten-dosed transduced HUVEC cells formed more tubes than the control cells, especially during the phases of tube alignment and maturation. Original magnification, ×40. B, the index of mean loop area, indicating the angiogenic capability of HUVEC cells, was measured and compared between the two groups. C, Hey1 and Dll4 were significantly activated during vessel development, in contrast with the almost invariant behavior of Jagged1 and Hes1. The mRNA level was detected by quantitative real-time PCR. Data, mean ± SEM, n = 4.

Figure 3. Dll4 and Hey1 take major responsibility for vessel formation in the capillary tube formation assay. A, Hey1 has a proangiogenic effect in vitro. Hey1-transduced HUVEC cells formed more tubes than the control cells, especially during the phases of tube alignment and maturation. Original magnification, ×40. B, the index of mean loop area, indicating the angiogenic capability of HUVEC cells, was measured and compared between the two groups. C, Hey1 and Dll4 were significantly activated during vessel development, in contrast with the almost invariant behavior of Jagged1 and Hes1. The mRNA level was detected by quantitative real-time PCR. Data, mean ± SEM, n = 4.

LANA increased Hey1 expression by prolonging its half-life

The above experiments showed that the upregulation of Hey1 during KSHV infection contributed to LANA; we subsequently explored the mechanism. The protein synthesis inhibitor CHX was used to determine the half-life of endogenous Hey1 in the presence or absence of LANA. Interestingly, KSHV infection greatly extended the half-life of endogenous Hey1 in 293T cells from 3 hours to more than 12 hours (Fig. 5A). Moreover, when endogenous LANA was repressed and this was followed with CHX treatment, a significant reduction in Hey1 expression was observed at the very beginning of the treatment. In addition, the half-life of Hey1 was decreased from 12 hours to less than 6 hours (Fig. 5B). We suggested that LANA increased Hey1 expression by prolonging its half-life, thus initiating a hypothesis that LANA may stabilize Hey1 by inhibiting its degradation.

LANA stabilized Hey1 by preventing its degradation

Having demonstrated the ability of LANA to increase Hey1 expression, we further examined the possibility that LANA may inhibit the degradation of Hey1 thus contributing to its accumulation. In the present study, endogenous Hey1 was increased by using the proteasome inhibitor MG132 in a dose-dependent manner, indicating that Ub modification takes major responsibility for Hey1 degradation. The tendency was enhanced further by cotransfection with LANA (Fig. 6A). To confirm that LANA stabilized Hey1 by preventing its degradation, the ubiquitination assay was carried out. In this system, hexahistidine (His6)-tagged Hey1 was utilized as a purification reagent for Ub–protein conjugates. Cell lysates were made under denaturing conditions (6 mol/L guanidine HCl). Hey1 was degraded increasingly with respect to its ectopic expression level. The degradation was inhibited dramatically by cotransfection with LANA, in a dose-dependent manner (Fig. 6B). In detailed analysis, the lysine mutations at K27R and K63R of Ub displayed total dysfunction for Hey1 degradation (Fig. 6C). In further attempt to identify whether the two sites of Ub were functional of LANA to stabilize Hey1, wild-type Ub, K27A, or K63A was transiently transfected into 293T cells at the presence of LANA or not. The expression of endogenous Hey1 was partially rescued when K27A was cotransfected with LANA plasmid, indicating LANA inhibiting Hey1 degradation partially through this site (Fig. 6D). In summary, we demonstrated that LANA stabilizes Hey1 by preventing its degradation. On the basis of the above results, the hypothesis is theoretically reasonable that LANA increases Hey1 expression during KSHV infection, which provides Hey1 with an opportunity to function in angiogenesis, and the effect ultimately benefits the pathogenesis of Kaposi sarcoma.

LANA promoted the angiogenesis mediated by Hey1

To illustrate the possibility that the angiogenic capability of Hey1 can be hijacked by LANA, we performed hydrodynamic injection of mice with either a control vector or a LANA plasmid in PBS. Surprisingly, the LANA plasmid stimulated more new vessel formation in subcutaneous explanted Matrigel in mice than vehicle vector treatment (Fig. 7A). This result was validated by the formation of more and larger erythrocyte infilling in blood vessels, observed on histologic analysis (Fig. 7A). However, the increased vessel formation resulting from LANA injection was impaired by subsequent injection of the

[Figure 3, 4, 5, 6, 7: Visual representations of the data and analysis presented in the text.]
shHey1 plasmid into mice (Fig. 7A). A similar phenotype was observed in the in vitro tube formation assay (Fig. 7B). Next, we studied the ability of LANA to promote cell migration and invasion, which are also required by tumor cells during angiogenesis. The number of Transwelled iSLK.219 cells was significantly decreased when LANA was repressed, compared with control shRNA treatment (Fig. 7C). With further transduction of Hey1 lentivirus, the number of cells increased tremendously, when compared with the control group (Fig. 7C). The absolute area of Transwelled cells was calculated using ImageJ software, and the P values related to LANA repression/shRNA control and LANA repression rescued by Hey1/vector control were 0.0006 and 0.0019, respectively (Fig. 7D). Furthermore, Hey1 exhibited the ability to promote cell invasion in primary HUVEC cells, which indicates that this is more than a cell-type–specific effect (Fig. 7E). In summary, we conclude that LANA promotes the angiogenesis and cell migration mediated by Hey1. The angiogenic property of Hey1 hijacked by LANA is an essential strategy of KSHV to establish pathologic vasculature in Kaposi sarcoma.

Discussion

Blood vessel formation supports tissue growth and organ function in development, physiology, and disease. An insufficient supply of nutrients and oxygen prompts the formation of new vessels from the walls of existing vessels, in a process termed angiogenic sprouting (41). Kaposi sarcoma is a typical angioproliferative neoplasm characterized by large amounts of neoangiogenesis throughout its pathologic process (3, 4). It is commonly agreed that Notch signaling plays critical roles in Kaposi sarcoma tumorigenesis (8, 15–18, 30). However, whether it is involved in Kaposi sarcoma angiogenesis is far from fully understood (9, 17). Hey1, a downstream effector of the Notch pathway, which plays a critical role in embryonic vascular development, was a candidate for elucidating the role of the Notch signaling pathway in Kaposi sarcoma angiogenesis (25, 26). Moreover, the mediation of Hey1 by multiple KSHV components indicates its significance to clinical treatment of Kaposi sarcoma (8, 12, 15, 18, 42). However, these studies have generally delineated the involvement of Notch signaling,
including Hey1, during KSHV infection, but have omitted functional study of Hey1 in Kaposi sarcoma.

Using high-throughput RNA-Seq and histologic examination, we showed that Hey1 is closely associated with Kaposi sarcoma angiogenesis, as demonstrated by its significant upregulation in Kaposi sarcoma lesions compared with normal tissues and further verification of its specific expression at the sites of pathologic vessels in Kaposi sarcoma specimens. In addition, Hey1 exhibited a remarkable ability to promote angiogenesis, validated by the Matrigel plug assay in mice and the capillary tube formation assay. Interestingly, GSI treatment and knockdown of Dll4 and Notch1 dramatically decreased the number of newly formed vessels. More importantly, the mechanism by which LANA stabilizes Hey1 is by prevention of its degradation. Finally, the repression of Hey1 leads to a great reduction in induction of new vessel formation by LANA, which indicates that LANA may hijack the angiogenic property of Hey1 to perform its own role. Our novel finding that KSHV LANA manipulates the Notch effector Hey1 to promote Kaposi sarcoma angiogenesis indicates that Hey1 is a promising therapeutic target in clinical treatment of Kaposi sarcoma. To our knowledge, this is the first report to indicate the angiogenic phenotype of the Notch signaling pathway and its downstream effector Hey1 in Kaposi sarcoma on the basis of in vivo assay.

Although the Notch pathway seems to be proangiogenic in Kaposi sarcoma, as indicated by our research and others, its precise function in tumor angiogenesis is difficult to pinpoint. In situations in which Notch signaling is absent or reduced, for example in Dll4 heterozygous animals, no functional vessel patterning and perfusion can occur and hypoxia/ischemia persists (43). The first phase I clinical research that utilized a GSI inhibitor to treat patients with incurable tumors presented excellent medical effect (44). On the other hand, several reports have indicated that Notch signaling pathway functions to inhibit angiogenesis (20, 45, 46). The concordant proangiogenic role of the pathway in Kaposi sarcoma counters the consensus that the Notch signaling pathway functions in both pro- and antiangiogenic ways. We have proposed here that VEGFR3 may account for this. The molecule was significantly upregulated in Kaposi sarcoma, as determined by RNA-Seq, in the present study. Benedito and colleagues previously reported that Notch-dependent VEGFR3 upregulation prompts angiogenesis without the requirement for VEGF–VEGFR2 signaling, suggesting that more studies on VEGFR3 in Kaposi sarcoma should be performed in the future (21).

In the present study, great efforts were made to define accurately the pattern of Notch signaling in vessel development by using the capillary tube formation assay. Interestingly, Dll4 and Hey1 were absent from migrating and proliferating cultures of endothelial cells but were rapidly induced during capillary-like network formation. However, other Notch signaling pathway components, including Jagged1 and Hes1, remained almost quiescent throughout the time course of the experiment. Most surprisingly, the pattern of VEGFR2 remains

Figure 5. LANA increases Hey1 expression by prolonging its half-life. A, KSHV infection was crucial for endogenous Hey1 stabilization by prolonging its half-life. The half-life of Hey1 was expanded from 3 hours to more than 12 hours under KSHV presence. B, LANA is the key component responsible for Hey1 stabilization. The half-life of Hey1 was shortened when LANA was repressed. Protein levels were determined by Western blotting and densitometry. α-Tubulin and GAPDH were used as a loading control.
constant irrespective of the presence or absence of ectopic Hey1. However, Hey1 seems to act as a transcriptional repressor to VEGFR2 dynamically, which is in line with the results reported by Henderson and colleagues (36, 37). The mechanism needs further investigation. Taken together, these results have demonstrated that Hey1 and other Notch signaling pathway components such as Dll4 are angiogenic molecules capable of inducing the formation of new blood vessels.

The mechanism by which LANA regulates Hey1 was elucidated in our study. We demonstrated that KSHV infection unregulated the level of expression of endogenous Hey1 in B and 293T cells and LANA takes major responsibility for it. Our results showed that Hey1 was degraded by the ubiquitin-proteasome system, as indicated by other research (47, 48). Ectopic expression of LANA significantly inhibited the degradation of Hey1, in a dose-dependent manner. Thus, we validated the fact that LANA upregulates Hey1 through prevention of its degradation. In our previous work, we demonstrated that LANA stabilized Notch ICD, facilitating cell survival (30). Here, we have demonstrated that LANA hijacks the Notch signaling pathway, leading to pathologic angiogenesis in Kaposi sarcoma, by targeting Hey1.

In the study described herein, LANA itself promoted vascular formation to some extent in murine models, and subsequent injection of shHey1 impaired it, thereby indicating that LANA promotes angiogenesis mediated by Hey1. Accordingly, KSHV LANA targets Hey1 to promote pathologic angiogenesis.
Figure 7. LANA promotes the angiogenesis mediated by Hey1. A, LANA promotes angiogenesis mediated by Hey1 in vivo. Histologic images are shown below. Original magnification, ×40. B, LANA promotes angiogenesis mediated by Hey1 in vitro. Original magnification, ×40. C, LANA promotes cell migration and invasion through targeting Hey1. D, ImageJ was used to calculate the absolute area of Transwell cells. Significance levels for the indicated comparisons: **, P = 0.0019; ***, P = 0.0006. E, Hey1 facilitates cell invasion in primary HUVEC cells in a noncell-type-specific way. Mean and SDs from three independent transfections are shown.

Angiogenesis becomes possible. Although there is limited evidence validating Hey1 as an oncogene (27, 49, 50), the molecule exhibits outstanding angiogenic capacity and tumorigenicity, as featured in our research. It should be noted that, although visible new blood vessels were formed as a result of hydrodynamic injection of LANA, the amount of vessels did not reach that stimulated by bFGF. A nude mouse model instead of C57BL/6 mice should be used to study LANA-induced angiogenesis in future. Our research has extended the knowledge of the Notch pathway in Kaposi sarcoma to a more functional and mechanical level. A deeper understanding of the fundamental principles of KSHV and the Notch pathway in the host will undoubtedly aid in the development of new avenues for the treatment of blood vessel-related pathologies.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: X. Wang, H. Wen, K. Lan

Development of methodology: X. Li, K. Lan

Acquisition of data (provided animals, collected and managed patients, provided facilities, etc.): X. Wang, K. Lan

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): X. Wang, Z. He, X. Lin, K. Lan

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Xing Wang, Zhiheng He, Tian Xia, et al.


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