Aberrant ceRNA-mediated regulation of KNG1 contributes to glioblastoma-induced angiogenesis

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ABSTRACT

Glioblastoma is a highly vascularized brain tumor that causes high mortality. Kinogen-1 (KNG1) has demonstrated both tumor suppressor and antiangiogenesis properties in glioblastoma cells. We analyzed the microarray and proteomic profiles of tumor tissues from glioblastoma patients (N = 180), and identified potential RNA regulators of the KNG1. Validation experiments in U87 glioblastoma cells showed that the regulation of KNG1 by CTU1, KIAA1274, and RAX was mediated by miR-138. The siRNA-mediated knockdown of CTU1, KIAA1274, or RAX in U87 cells and immortalized human endothelial cells (iHECs) significantly reduced KNG1 expression (P < 0.05 for all), which resulted in the upregulation of oncogenic EGFR signaling in both cell lines, and stimulated angiogenic processes in cultured iHECs and zebrafish and mouse xenograft models of glioblastoma-induced angiogenesis. Angiogenic transduction of iHECs occurred via the uptake of U87-derived exosomes enriched in miR-138, with the siRNA-mediated knockdown of KNG1, CTU1, KIAA1274, or RAX increasing the level of miR-138 enrichment to varying extents and enhancing the angiogenic effects of the U87-derived exosomes on iHECs. The competing endogenous RNA network of KNG1 represents potential targets for the development of novel therapeutic strategies for glioblastoma.

INTRODUCTION

Primary brain tumors, known as gliomas, are some of the most highly vascularized tumors that occur in humans [1], exhibiting sustained neovascularization [2, 3] that often consists of initial vascular co-option and subsequent angiogenesis [4]. Glioblastoma (GBM), also known as glioblastoma multiforme, is the most common type of glioma. The prognosis for GBM is most often poor. Rapid tumor progression and resistance to chemotherapy and radiotherapy are common in GBM [4], resulting in a 1-year survival rate of approximately 23% [5, 6].

Our preliminary data showed that the tissue and serum levels of kinogen-1 (KNG1), are often elevated in GBM patients. The full length kinogen-1 polypeptide, known as high molecular weight kinogen (HK), undergoes proteolytic cleavage to release the nonapeptide, bradykinin, from domain six of HK. Bradykinin promotes the invasion of gliomas [7] by stimulating the B2 receptor [8, 9] and epidermal growth factor receptor (EGFR) signaling pathways [10, 11], which promote angiogenesis through the upregulation of VEGF expression. Bradykinin antagonists suppress the growth of glioma tumor cells [12–14], and peptide analogs of domain 5 of KNG1 inhibit angiogenesis [15] and metastasis [16].

Sequence homology within microRNA (miRNA) response elements (MREs) in different mRNAs result in competition between different microRNAs (miRNAs) and...
competing endogenous RNAs (ceRNAs) [17, 18]. This functional overlap forms the combinatorial foundation of ceRNA crosstalk, and results in a multitude of coregulatory interactions that influence gene expression at the post-transcriptional level [18]. The dysregulation of ceRNA-miRNA networks has been linked to a variety of diseases, including various types of cancer [19–21].

Based on the hypothesis that perturbations in the ceRNA-miRNA network regulating KNG1 influence angiogenesis in GBM, we examined the RNA and proteomic profiles of tumor tissues from GBM patients, and identified species relevant to the post-transcriptional regulation of KNG1 based on a computational analysis of our findings. Predictions of the roles of these species in the dysregulation of KNG1 in GBM-induced angiogenesis were validated in the U87 GBM cells and xenograft models of GBM-induced angiogenesis in zebrafish and mice. Our findings suggest that the ceRNA-mediated downregulation of KNG1 expression contributes to angiogenesis in GBM.

RESULTS

The ceRNA network of KNG1 regulates angiogenesis in GBM via EGFR signaling

The GBM samples (N = 180) subjected to proteomic profile analysis were selected based on mean vascular density (MVD), which was assessed based on the simultaneous immunofluorescence detection of CD31 and CD34 (Figure 1A). The proteomic profiles of the 20 GBM samples (Figure 1B) that exhibited the highest and lowest fluorescence intensity (MVD high and MVD low groups, respectively; n = 10 in each group) were analyzed using mass spectrometry, and 37 differentially expressed proteins were identified based on a Benjamini-Hochberg P-value < 0.05 and fold change (FC) > 3 or < -3. Among these, null expression of the KNG1 protein was identified in the high MVD group and the serum samples of the corresponding patients (Supplementary Figures S1A–S1C, Supplementary Table S1). In a previous study, the proteolytically cleaved fifth domain of KNG1 inhibited the migration and invasion of prostate tumors via EGFR signaling [22]. We found that the production of EGFR protein inversely correlated (Benjamini-Hochberg P < 0.05 and FC > 3 high/low MVD) with that of KNG1 in GBM tissues, and that reduced KNG1 expression resulted in the upregulation of EGFR signaling and angiogenesis (Figure 1C). These results suggested that the regulation of KNG1 influences angiogenesis in GBM.

Using the RNA22 program and a cutoff of 2 or more shared MREs between KNG1 and its putative ceRNAs, we analyzed a dataset of 17 genes and 23 transcripts (Table 1), which were derived from our mRNA microarray data and the results of the quantitative RT-PCR (qRT-PCR) analysis to predict ceRNAs that target miR-532, miR-138, and miR-454-3p and act as transregulators of KNG1. We used the RNA22 program because previous studies using RNA22 have reported low false prediction rates [23]. We generated MRE enrichment scores (Table 1), as described previously [24], and validated the 16 putative KNG1 ceRNAs with the highest MRE enrichment scores and their shared miRNA targets in the GBM MVD high and low groups (Tables 2, 3). The integrated analysis indicated that miR-140, miR-138, and miR-454-3p and the following predicted ceRNAs were components of the crosstalk network regulating KNG1 in both the KNG1 low and high groups and the MVD low and high groups: CTU1, DUSP9, KIAA1274, TMEM105, RAX, NCCR1P1, NET1, KRTAP10-7, ENPP7, KRTAP10-1, and CPNE2 (Table 4).

The 3'-UTR of murine KNG1 was predicted to contain MREs for 3 different miRNAs (Table 1). In our initial experimental validation, we examined whether the KNG1 3'-UTR physically associated with endogenous miRNAs in cultured cells because these interactions served as key selection criteria in our computational analyses. We used RNA immunoprecipitation (RIP) to pull down endogenous miRNAs associated with the KNG1 3'-UTR, and demonstrated that immunoprecipitates containing the KNG1 3'-UTR from U87 and immortalized microvascular endothelial-like cells (iHECs) were significantly enriched for miR-532, miR-138, and miR-454-3p (Supplementary Figure S2D), compared to the empty MS2 vector and miR-240 (non-KNG1-targeting miRNA control). These results showed that miR-532, miR-138, and miR-454-3p are KNG1-targeting miRNAs, and supported the findings of the overexpression and knockdown experiments. Therefore, the roles of miR-532, miR-138, and miR-454-3p in KNG1 regulation were analyzed further.

Transcripts within ceRNA networks are coregulated. Therefore, we investigated whether coexpression patterns existed between KNG1 and the putative ceRNAs in GBM tissue samples and the TCGA GBM data (Supplementary Table S2). A stratified analysis based on KNG1 levels revealed significant correlations between the differential expression of CTU1, DUSP9, KIAA1274, TMEM105, RAX, NCCR1P1, NET1, ENPP7, KRTAP10-1, and CPNE2 and the level of KNG1 expression in the tumor tissues from GBM patients and the TCGA GBM data (Table 4; Supplementary Figure S3; Supplementary Table S3). We investigated whether the levels of miRNA expression correlated with those of KNG1 and its putative ceRNAs. We stratified our analysis of the expression levels of the 10 validated KNG1-targeting ceRNAs, with one group associated with miRNAs expressed at a lower level in GBM tissues, compared to the controls, and the other group associated with miRNAs expressed at relatively higher levels. We observed an increase in the Pearson correlation coefficients between the expression levels of CTU1, KIAA1274, RAX, NET1, and TMEM105 and the level of KNG1. The levels of the NCCR1P1 and DUSP9 candidate ceRNAs
Figure 1: Proteomics Profile of Antiangiogenesis genes in glioblastoma. A. Twenty GBM tissue samples were selected for proteomic profile analysis based on MVD, which was assessed by simultaneous immunofluorescence detection of CD31 (red) and CD34 (green). Fluorescence intensity was recorded at λ = 571 nm, which greatly reduced the intensity of GFP emission in the overlapping fluorescence emission spectra. B. Of the 180 GBM samples examined, those with the highest and lowest MVD values were assigned to the MVD high and low groups (n = 10 each group), respectively. C. Western blotting (left) was performed to measure the expression levels of KNG1, pEGFR, and EGFR protein in GBM samples in the MVD low group (lanes 4–8) and MVD high group (lanes 9–13) in which KNG1 was differentially expressed (MVD of GBM samples increasing from left to right). The blots shown are representative of at least 4 independent experiments. Densitometry measurements of western blots were used to plot the expression level of KNG1 and the p-EGFR/total EGFR ratio (EGFRr) to quantify differential expression relative to the mean level of expression in the control tissue samples (right; *P < 0.05; **P < 0.01).
were consistent with the level of miRNA expression in the array results (Supplementary Table S4), but were not significantly different between the GBM and control tissues (Table 4; Supplementary Figures S3C, S3D).

Table 1: Analysis of miR-532, miR-138, and miR-454-3p MREs in ceRNA candidates

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<th>Rank</th>
<th>Gene</th>
<th>Transcript</th>
<th>MutamMe Score</th>
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<th>miR-140-5p</th>
<th>miR-138-5p</th>
<th>No. miRNAs</th>
<th>No. MREs</th>
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<td>2</td>
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</table>

Note: Validated KNG1-targeting miRNAs were used to predict putative KNG1 ceRNAs. Candidates sharing at least 2 miRNAs were considered putative KNG1 ceRNAs.

Table 2: Validation of shared miRNAs from microarray results based on qRT-PCR analysis of GBM MVD high/low groups

<table>
<thead>
<tr>
<th>miRNA</th>
<th>TCGA (KNG1 High/Low)</th>
<th>Study Cohort (MVD High/Low)</th>
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<tr>
<td></td>
<td>FC (-log2)</td>
<td>Normalized</td>
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<td>hsa-miR-138</td>
<td>2.26</td>
<td>5.10/3.93</td>
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<td>hsa-miR-140</td>
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<td>7.95/7.57</td>
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<td>hsa-miR-454-3p</td>
<td>1.83</td>
<td>5.20/4.32</td>
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*By logist test, *By t-test

We also identified KNG1-coexpressed mRNAs that are targeted by KNG1-targeting miRNAs, and confirmed that the KNG1-targeting miRNAs and their putative ceRNAs were expressed in U87 cells, iHECs,
Table 3: Validation of candidate ceRNAs from microarray results base on qRT-PCR analysis of GBM MVD high/low groups

<table>
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<tr>
<th>Gene</th>
<th>FC (log_2)</th>
<th>Mean ± SD</th>
<th>Score</th>
<th>P-value*</th>
<th>P-value#</th>
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<tr>
<td>CTU1</td>
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<tr>
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<td>-0.16</td>
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<tr>
<td>DUSP9</td>
<td>-1.29</td>
<td>6.7 ± 2.83 / 7.46 ± 0.96</td>
<td>46.71</td>
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<td>0.08</td>
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<td>KIAA1274</td>
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<td>5.17 ± 2.01 / 4.9 ± 3</td>
<td>50.14</td>
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<td>0.00</td>
</tr>
<tr>
<td>TMEM105</td>
<td>-2.46</td>
<td>3.67 ± 2.01 / 8.1 ± 0.42</td>
<td>30.36</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>MARCH6</td>
<td>-0.05</td>
<td>6.04 ± 2.63 / 9.51 ± 2</td>
<td>1.63</td>
<td>0.92</td>
<td>0.36</td>
</tr>
<tr>
<td>SERF2</td>
<td>-3.60</td>
<td>10.07 ± 4.83 / 10.91 ± 1.7</td>
<td>56.79</td>
<td>0.00</td>
<td>0.00</td>
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<tr>
<td>KIAA1274</td>
<td>-3.69</td>
<td>5.17 ± 2.01 / 4.9 ± 3</td>
<td>50.14</td>
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<td>SERF2</td>
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*By logist test, #By t-test

Table 4: Coexpression analysis of KNG1 and candidate ceRNAs in the GBM MVD high/low groups

<table>
<thead>
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<th>Gene</th>
<th>GBM TCGA</th>
<th>GBM Study Cohort</th>
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<td>All samples</td>
<td>miRNA down</td>
</tr>
<tr>
<td></td>
<td>FC</td>
<td>t-test</td>
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<tr>
<td>CTU1</td>
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<td>DUSP9</td>
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<tr>
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<tr>
<td>TMEM105</td>
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<td>0.00</td>
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<tr>
<td>RAX</td>
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<td>0.00</td>
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<td>NCCRP1</td>
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<td>NET1</td>
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GBM-associated vascular endothelial cells (VECs), and DICER iHEC and U87 cell lines expressing siRNA complementary to the N-terminal coding exon of the human DICER-1 gene [25] (Supplementary Figure S2A). The expression of miR-138 appeared to be cell-type specific, as the level of miR-138 was significantly higher in U87 cells (Supplementary Figure S2A). The qRT-PCR analysis showed that expression of each candidate ceRNA was significantly reduced by its respective siRNA in both the wild-type and DICER iHEC and U87 cell lines (Supplementary Figure S2B). In RIP experiments using the 3'-UTRs of CTU1, KIAA1274, RAX, or NET1 as baits (Supplementary Figure S2D), miR-138 was enriched >2-fold in RAX precipitates. The siRNA mediated knockdown of CTU1, KIAA1274, or RAX increased miRNA availability, as evidenced by the increased levels of miR-532, miR-138, and miR-454-3p associated with the MS2-KNG1 3'-UTR bait mRNA (Supplementary Figures S2C, S2D). These results indicated that CTU1, KIAA1274, and RAX sequester miR-532, miR-138, and miR-454-3p to regulate KNG1 levels in U87 cells, and implied that their downregulation increased the availability of miR-140 and miR-138 in iHECs.

Effects of ceRNA-mediated KNG1 regulation in GBM-associated vascular endothelial cells

We investigated the ability of the putative KNG1 ceRNAs to modulate KNG1 by knocking down the expression the ceRNAs in U87 cells and iHECs. These experiments used siRNA pools (4 different siRNAs each), and included BMP4 as a control because it was not predicted to be a ceRNA target (Figure 2). Western blotting showed that depletion of the CTU1, KIAA1274, or RAX transcripts significantly reduced KNG1 protein levels in U87 cells, whereas only the depletion of KIAA1274 reduced KNG1 protein expression in iHECs (Figures 2D, 2H).

To determine whether the effects of the siRNA-mediated knockdown of the ceRNA required the KNG1 3'-UTR, we constructed a chimeric luciferase reporter construct that included the KNG1 3'-UTR (Luc-KNG1 3'-UTR) downstream of the luciferase coding region. The siRNA-mediated depletion of CTU1, KIAA1274, and RAX in separate experiments significantly reduced luciferase activity in the U87 cells expressing the Luc-KNG1 3'-UTR (Figure 2A). The knockdown of CTU1, KIAA1274, and RAX transcript levels was confirmed in U87 cells transfected with siCTU1, siKIAA1274, and siRAX, respectively (Supplementary Figure S2B). Only the depletion of KIAA1274 reduced luciferase activity in iHECs, which was consistent with the western blotting results, whereas knockdown of the other candidate ceRNAs had no significant effect in iHECs (Figure 2E). The ectopic overexpression of ceRNA 3'-UTRs, which were constructed based on the location of their predicted MREs, increased the expression of both the Luc-KNG1 3'-UTR reporter and the endogenous KNG1 protein in U87 cells, with a similar effect on the level of KNG1 in U87 cells overexpressing the KNG1 3'-UTR. Only the depletion of KIAA1274 significantly reduced KNG1 expression in iHECs (Figures 2F, 2G). The correlation analysis showed that the reduced miRNA dependency of ceRNA-KNG1 regulation likely contributed to the abrogation of endogenous miR-138 in iHECs, whereas endogenous miR-140 and miR-454-3p were not significantly dependent on ceRNA-mediated KNG1 regulation (Figure 2, Supplementary Figures S2C, S2D).

We also investigated the miRNA dependency of ceRNA-mediated KNG1 regulation in DICER iHEC and U87 cell lines. Because not all miRNAs are DICER dependent, we also examined whether the maturation of the miRNAs was inhibited in DICER iHEC and U87 cell lines. The qRT-PCR analysis showed that significantly lower levels of the miRNAs were present in the DICER cell lines (Supplementary Figure S2A), indicating that siRNA-mediated gene silencing occurred independent of DICER in the DICER iHEC and U87 cell lines (Supplementary Figures S2B, S2C). The siRNA-mediated knockdown of CTU1, KIAA1274, or RAX significantly reduced the level of KNG1 protein in wild-type U87 cells, and the downregulation of KNG1 expression caused by the depletion of the ceRNAs was significantly attenuated in the si-RNA DICER U87 cells (Figure 2D), which suggested that mature miRNAs are required for ceRNA mediated regulation of KNG1. Only the knockdown of KIAA1274 reduced the KNG1 protein level in DICER iHECs, compared to that in wild-type iHECs (Figure 2H).

To further assess the miRNA dependency of ceRNA-mediated KNG1 regulation, we examined whether the expression profiles of miR-532, miR-138, and miR-454-3p were influenced by that of DICER. Samples were categorized as low or high, based on DICER expression. Supplementary Figure S3C and S3D show that an increase in DICER expression was accompanied by increases in miRNA expression and decreases in ceRNA expression. The negative correlation between miRNA and ceRNA expression was, however, attenuated in samples with relatively low DICER expression. These results are consistent with those of the aforementioned validation experiments in which the ceRNAs were overexpressed or knocked down, justifying their inclusion in our analysis.

Having shown that the CTU1, KIAA1274, and RAX ceRNAs regulate KNG1 expression in a miRNA-dependent manner, we investigated possible associations between miR-138, miR-140, and miR-454-3p. The CTU1, KIAA1274, and RAX 3'-UTRs were each cloned based on the location of their predicted MREs. The ectopic expression of miR-138, miR-140, or miR-454-3p significantly reduced the luciferase activity of Luc-CTU1-3'-UTR, Luc-KIAA1274-3'-UTR, and Luc-RAX-3'-UTR in U87 cells (Supplementary Figures S4A–S4C).
Figure 2: Putative ceRNAs Modulate KNG1 Expression. A. Luciferase activity in U87 cells cotransfected with luc-KNG1 3'-UTR reporter construct and siKNG1, siCTU1, siKIAA1274 (siKIA), siRAX, or siBMP4 (siNC). B. Luciferase activity of U87 cells cotransfected with a luciferase-KNG1 3'-UTR reporter construct and plasmids overexpressing the 3'-UTR of the ceRNAs. C. Western blot showing KNG1 expression in U87 cells overexpressing the 3'-UTR of the ceRNAs. D. Western blot for KNG1 levels in wild type and DICER⁻ U87 cells transfected with siKNG1, siCTU1, siKIAA1274, siRAX, or siNC. E. Luciferase activity in iHEC cells cotransfected with ceRNA specific siRNA and a luciferase-KNG1 3'-UTR reporter construct. F. Luciferase activity in iHECs cotransfected with plasmids expressing the 3'-UTRs of ceRNAs and a luciferase-KNG1 3'-UTR reporter construct. G. Western blot showing KNG1 expression in iHECs overexpressing the 3'-UTRs of ceRNAs. H. Western blot for KNG1 expression in wild type and DICER⁻ iHECs transfected with ceRNA specific siRNA or siBMP4 (siNC). (A–H) Mean ± SD; n ≥ 4; *P < 0.05; **P < 0.01.
However, in iHECs, the effect of miR-138 was attenuated, compared to that of miR-140 and miR-454-3p in iHECs. Consistent with these results, the overexpression of miR-138, miR-140, or miR-454-3p significantly reduced the ectopic expression of CTU1, KIAA1274, and RAX in iHECs and U87 cells (Supplementary Figures S4D–S4I). These results collectively confirm that miR-138, miR-140, and miR-454-3p regulate CTU1, KIAA1274, and RAX levels via their 3’-UTRs in both U87 cells and iHECs, and indicate that miR-138 plays a more prominent role in ceRNA-mediated regulation of KNG1, as evidenced by the effects of miR-140 and miR-454-3p in iHECs being consistently less profound than that in U87 cells. These results also suggest that the cell-specificity of ceRNA-mediated KNG1 regulation is due, at least in part, to the abrogation of endogenous miR-138 and the weaker influences of miR-140 and miR-454-3p on the expression of KNG1 and its ceRNAs in iHECs, compared with that in U87 cells.

Aberrant activation of EGFR signaling might account, at least in part, for the tumorigenic and angiogenic effects of the loss of KNG1 expression. The proteomic analysis of GBM tissues (low/high MVD) identified an association between ceRNA-mediated KNG1 regulation and EGFR signaling. Therefore, we examined how ceRNA-mediated regulation of KNG1 influences EGFR signaling by knocking down KIAA1274 expression in serum-starved wild-type and DICER− iHEC and U87 cell lines before treatment with EGF (Figures 3A–3F). The siRNA-mediated depletion of KIAA1274 significantly elevated the level of phosphorylated EGFR (p-EGFR) levels in the wild-type U87 cells, with 1.8- and 2.7-fold increases at 5 and 15 min, respectively, relative to the negative control (Figure 3C). Similar effects were observed in wild-type iHECs, with 2.6- and 3.6-fold increases at 5 and 15 min, respectively (Figure 3F). The effect of KIAA1274 depletion on p-EGFR was, however, abrogated in the DICER− iHEC and U87 cell lines, relative to the negative control (Figures 3C, 3F). These results suggested that KIAA1274 knockdown activated the EGFR pathway in a miRNA-dependent manner, which is consistent with the role of KIAA1274 in maintaining KNG1 expression in U87 cells and iHECs.

Based on our analysis of the ceRNAs and EGFR signaling, we investigated the effect of knocking down KNG1 and its ceRNAs on angiogenesis. The individual depletion of KNG1, CTU1, KIAA1274, and RAX significantly increased the proliferation of wild-type U87 cells and iHECs (Figures 3C, 3F). The effect of the siRNA-mediated knockdown of CTU1, KIAA1274, and RAX was significantly attenuated in both of the DICER− cell lines, whereas the knockdown of KNG1 significantly reduced proliferation in both DICER− cell lines (Figures 3C, 3F). The proliferation of wild-type U87 cells was significantly reduced by individual overexpression of the 3’-UTRs of KNG1, CTU1, KIAA1274, and RAX, whereas only the overexpression of KNG1 and KIAA1274 reduced the proliferation of wild-type iHECs (Figures 3A, 3D). The effects of KNG1-3’-UTR, CTU1-3’-UTR, KIAA1274-3’-UTR, and RAX-3’-UTR overexpression were attenuated in both DICER− cell lines (Figures 3B, 3E). These data indicated that the observed phenotype was at least partially independent of the coding sequences of KNG1 and KIAA1274, and were consistent with the EGFR signaling data, suggesting that the KNG1-ceRNA network suppressed GBM-induced angiogenesis.

**GBM-derived exosomes restores ceRNA regulation of KNG1 in VECs**

Having characterized the angiogenic effects of KNG1 and its ceRNA in U87 cells and iHECs, we examined the properties of each using an *in vitro* endothelial tubule formation assay [26]. After the iHECs were cultured without (control) or with U87 cells to induce angiogenesis, miRNA expression was examined, as described previously [27]. Consistent with the effect of KIAA1274 on KNG1 protein levels and the proliferation of iHECs, the suppressive effects of siCTU1, CTU1-3’-UTR, siRAX, and RAX-3’-UTR expression on the proliferation and angiogenesis of iHECs were significantly lower than that of siKIAA1274 or KIAA1274-3’-UTR expression (Figure 3G).

We investigated whether exosomes secreted by wild-type U87 cells (exo-U87s) influenced the miRNA dependency of ceRNA-mediated KNG1 regulation in iHECs using tubule formation assays. The dynamic light scattering analysis showed that the physical properties of the purified exo-U87s (mean diameter ± SEM = 70.29 ± 16.95 nm) were consistent with those of exosomes [28], and incubation in medium containing exo-U87s significantly increased tubule formation in iHECs cultured with U87 cells in Matrigel (data not shown). The Spearman correlation coefficient between KNG1 expression in iHECs and that of the ceRNAs significantly increased with increasing exo-U87 titer (Figure 4B). We examined the contents of the exo-U87s using qRT-PCR and western blotting. The level of miR-138 in exo-U87s was 2.9 fold higher than that in the U87 cells (Figure 4C). In U87 cells in which miR-138 was ectopically overexpressed, the level of miR-138 in exo-U87s increased to a proportionately greater extent than that observed in the U87 cells (Figure 4D). The siRNA-mediated knockdown of DICER in the U87 cells, reduced the level of miR-138 in their exo-U87s to a greater extent than that observed in the U87 cells (Figure 4C). These results suggested that exo-U87-induced angiogenesis in iHECs was mediated by miR-138 that was previously transcribed in the parent U87 cell.

The overexpression of the 3’-UTR of KNG1, CTU1, KIAA1274, or RAX in U87 cells reduced the amount of miR-138 in the exo-U87s to a level less than
Figure 3: Depletion of KNG1-targeting ceRNAs activates EGFR Pathway and stimulates proliferation in cultured cells. A, B. Proliferation curve of wild type and DICER⁻ U87 cells transfected with plasmids overexpressing the 3’-UTRs of KNG1 or the ceRNAs ($P < 0.01$ for all vs. pcDNA control). (C, left) Western blot showing pEGFR and total EGFR expression in wild type and DICER⁻ U87 cells following serum starvation and transfection with siKNG1, siKIAA1274, or siBMP4 (siNC), with densitometric quantification of p-EGFR, relative to p-EGFR at 0 min for siNC, shown below the blot and (C, right) proliferation curves of wild type and DICER⁻ U87 cells in response to siRNA transfection. D, E. Proliferation curves of wild type and DICER⁻ iHEC in response to transfection with plasmids overexpressing the 3’-UTRs of KNG1 or the ceRNAs ($P < 0.01$ for both vs. pcDNA control). (F, left) Western blots showing pEGFR and total EGFR expression in wild type and DICER⁻ iHECs following serum starvation and transfection with siKNG1, siCTU1, siKIAA1274, siRAX, or siBMP4 (siNC), with densitometric quantification of p-EGFR, relative to p-EGFR at 0 min for siNC, shown below the blot. (F, right) Proliferation curves of wild type and DICER⁻ iHECs in response to siRNA transfection. (Continued)
that in the parent U87 cells (Figure 4C), which inhibited the proliferation of iHECs cultured with them, compared with that of iHECs cultured in the presence of control U87 cells (Figure 4H). The siRNA-mediated knockdown of the KNG1, CTU1, KIAA1274, or RAX mRNAs in the U87 cells increased the amount of miR-138 in the exo-U87s to a level higher than that in the parent U87 cells (Figure 4D), and stimulated the proliferation of iHECs cultured with them, compared with that of iHECs cultured in the presence of control U87 cells (Figure 4G). Quantification of miR-138, either normalized to Let-7a-5p or based on absolute copy-number, showed that the level of miR-138 in exo-U87s was inversely proportional to the intracellular levels of the 3'-UTRs of KNG1, CTU1, KIAA1274, and RAX (Figures 4C, 4D). No significant difference in proliferation was observed between iHEC lines incubated in culture medium from which exo-U87s had been removed following transfection of the U87 cells with the ceRNA specific siRNAs or plasmids expressing the 3'-UTR of KNG1 or the ceRNAs with visualization based on pEGFR expression (green fluorescence) in iHEC and (G, right) relative quantification based on average cell length (mean ± SD; n ≥ 5; *P < 0.05, **P < 0.01 compared with pcDNA or siNC).

We investigated whether the level of miR-138 in multivesicular bodies (MVBs) was influenced by the levels of miR-138 and ceRNAs. Nine distinct subcellular fractions from U87 cells were analyzed by western blotting. We found that the MVBs in fraction 3 and, to a lesser extent, fractions 2 and 4, were enriched with the tetraspanin, CD81. Fractions 4 to 9 were enriched in endoplasmic reticulum components, as indicated by the presence of calnexin, whereas fractions 1 and 2 were enriched in the P bodies markers, AGO2, GW182, and DCP1A (Figure 5A). In U87 cells in which an miR-138 mutant (miR-138-mut) was overexpressed using an lentiviral vector (LV), endogenous miR-138 was detectable at varying levels in each fraction (Figure 5B). Overexpressing wild type miR-138 increased its levels in all subcellular fractions (data not shown), with the level of miR-138 in each fraction increasing with increased MVB enrichment. By contrast, overexpressing CTU1-3'-UTR, KIAA1274-3'-UTR, or RAX-3'-UTR significantly increased miR-138 levels in fraction 1 of the U87 cells, compared to cells overexpressing a scrambled RNA control (Figure 5B). These data suggest that increasing the expression of miR-138 or the ceRNAs increased miR-138 enrichment in MVBs (miRNA secretory pathway) and P bodies (miRNA activity pathway), respectively, and that the subcellular localization of miRNAs can be altered by changes in the levels of its target ceRNAs, shifting the balance between intracellular miRNA activity and the secretion of miRNA via exosomes.

We compared the endogenous ceRNA of GBM tumor samples (n = 357) with paired control samples (n = 357) from TCGA. The copy number of KIAA1274 in GBM samples was significantly lower than that in the controls (P = 0.015; FC: 1.39 vs. 2.01; Supplementary Figure S5). We also compared the ceRNA profiles of GBM samples with low (n = 217) and (n = 140) high KNG1
Figure 4: Changes in ceRNA levels regulate miR-138 enrichment of exo-U87s and the proliferation of recipient iHECs in cell culture. A. Size distribution of U87-derived exosomes determined using dynamic light scattering. B. Plot of the Spearman R² of correlation between expression of KNG1 and ceRNAs vs. exo-U87 titer. C, D. Assessment of miR-138 expression (mean ± SEM, n = 5) relative to that of Let-7a-5p in exo-U87s and wild type U87 cells transfected with plasmids overexpressing the 3’-UTR of KNG1 or the ceRNAs or (D) wild type U87 cells transfected with plasmids overexpressing the 3’-UTR of KNG1 or the ceRNAs or (D) wild type U87 cells transfected with plasmids overexpressing the 3’-UTR of KNG1 or the ceRNAs or empty vector (pcDNA) or (F) wild type U87 cells transfected with siKNG1, siCTU1, siKIAA1274, siRAX, siBMP4 (siNC), or miR-138. E, F. Proliferation curves of iHECs incubated in exo-U87 depleted culture medium collected from (E) DICER− U87 cells or wild type U87 cells transfected with plasmids overexpressing the 3’-UTR of KNG1 or the ceRNAs or empty vector (pcDNA) or (F) wild type U87 cells transfected with siKNG1, siCTU1, siKIAA1274, siRAX, siBMP4 (siNC), or siNC. G, H. Proliferation curves of iHECs cultured in medium collected from (G) DICER− U87 cells or wild type U87 cells transfected with plasmids overexpressing the 3’-UTR of KNG1 or the ceRNAs or pcDNA or (H) wild type U87 cells transfected with siKNG1, siCTU1, siKIAA1274, siRAX, or siNC.
Figure 5: Subcellular localization of KNG1-ceRNAs. A. Western blotting analysis of P body, MVB, and ER markers in subcellular fractions obtained from U87 cells. (B, top to bottom) Level of miR-138 in subcellular fractions obtained from U87 cells transduced with LVs overexpressing miR-138 or miR-138-mut (top) or transfected with plasmids overexpressing the 3'-UTR of KNG1, CTU1, KIAA1274, or RAX (bottom) and a 3'-UTR control, with levels shown as ΔCt values (Ct of the fraction minus mean Ct of all fractions).
levels. The copy numbers of RAX and CTU1 in the KNG1 low subgroup were lower than those in the KNG1 high subgroup (Supplementary Figure S5). These results suggest that reduced levels of KNG1-regulating ceRNAs are associated with tumor angiogenesis.

**KNG1-ceRNA levels in U87 cells regulate angiogenesis in vivo**

We established a xenograft tumor model in transgenic zebrafish (Danio rerio) to investigate the effects of perturbations of the KNG1-ceRNA network. Matrigel plugs containing red fluorescent protein (RFP)-expressing iHECs cells were inoculated with U87 cells transfected with one of the following: (A) a plasmid overexpressing KNG1-3'-UTR, CTU1-3'-UTR, KIAA1274-3'-UTR, RAX-3'-UTR, or miR-138; (B) empty vector (control); or (C) siKNG1, siCTU1, siKIAA1274, siRAX, or an siRNA negative control (siNC) complementary to BMP4 (Figure 6). Zebrafish embryos were injected with equivalent volumes of Matrigel plugs, and the number of iHECs was measured at 6 days postinjection using a digital three-dimensional reconstruction. The overexpression of siCTU1, siRAX, or siKIAA1274 in the U87 cells upregulated the proliferation of iHECs in zebrafish somite, compared with the siNC. The expression of miR-138 or siKNG1 U87 in the U87 cells increased the proliferation of iHECs to a greater extent than that of siCTU1, siRAX, or siKIAA1274 (Figure 6A), whereas the expression of CTU1-3'-UTR, RAX3'-UTR or KIAA1274 3'-UTR in the U87 cells reduced the proliferation of iHECs, relative to the control (Figure 6B). The expression of the KNG1-3'-UTR in the U87 cells resulted in no detectable RFP in iHECs, indicating that miR-138 was transferred from U87 cells to iHECs in vivo.

We also established a mouse xenograft model of GBM-induced angiogenesis. Matrigel plugs containing luciferase-expressing iHECs were inoculated with U87 cells overexpressing the 3'-UTR of KNG1. Nonobese diabetic, severe combined immunodeficiency (NOD-SCID) mice were injected subcutaneously with equivalent volumes of the Matrigel plugs. The NOD-SCID strain lacks T, B, and natural killer lymphocytes, which prevented an effective immune response to the human exo-U87s. The transplant NOD-SCID mice were also injected via the tail vein every 2 days with exo-U87s from U87 cells expressing siKNG1, siCTU1, siRAX, siKIAA1274, or siNC. The proliferation of xenografted iHECs was assessed based on luciferase expression. On day 9, the xenografted iHECs exhibited significantly enhanced proliferation in mice that received intravenous injections of exo-U87s from U87 cells expressing the siCTU1, siRAX, siKIAA1274, or siKNG1, compared to that in mice that received wild type exo-U87s (Figures 6C, 6D). These data suggest that the proangiogenic activity of miR-138 was transferred from U87 cells to iHECs in exo-U87s in vivo.

**DISCUSSION**

We characterized the regulation of KNG1 expression in GBM cells. Our microarray analysis of GBM tumor tissues identified putative ceRNAs that were relevant to KNG1 expression. Cell culture experiments showed that the regulation of KNG1 by the CTU1, KIAA1274, and RAX ceRNAs was mediated by miR-138. The ceRNA-mediated downregulation of KNG1 resulted in the upregulation of proangiogenic EGFR signaling in iHECs and U87 cells, and stimulated angiogenic processes in iHECs and xenografted zebrafish and mice. The siRNA-mediated knockdown of the KNG1 mRNA and the ceRNAs in U87 GBM cells significantly increased miR-138 loading into exosomes, and the miR-138-enriched, GBM-derived exosomes induced the proliferation of VECs and angiogenesis in the xenograft models.

Our results suggest that RAX functions in the KNG1 ceRNA network as an miRNA sponge, as the downregulation of RAX increased the availability of miR-140 and miR-138 in iHECs, which is consistent with previous reports of RAX gene hypermethylation and the loss of RAX expression in human astrocytomas [29]. Our results also indicate that high-level expression of miR-454-3p might represent a novel diagnostic biomarker for GBM. The other KNG1-regulating ceRNA identified, KIAA1274 (PALD1), is expressed primarily in VECs and vascular smooth muscle cells [30]. Although the expression of KIAA1274 transcripts in the U87 cells used in our experiments was relatively low, a previous study showed that the expression of the PALD1 protein, a putative phosphatase, is restricted to VECs and GBM tumor cells in humans [30]. The direct relationship between the expression levels of KNG1 and KIAA1274 in U87 cells demonstrate its importance in regulating miR-138 in VEC-transducing exosomes. This is the first report of KNG1-KIAA1274 regulation of miR-138 exosome enrichment in GBM-induced angiogenesis.

The upregulation of EGFR signaling in U87 cells and iHECs in response to KNG1 knockdown (Figures 3C, 3F) is consistent with the findings of previous studies of the binding of HK and cleaved HK (HKa) to endothelial cells. Both HK and HKa bind to a protein complex consisting of globular C1q receptor, cytokeratin 1, and urokinase plasminogen activator receptor (uPAR) on the surface of human endothelial cells, with HK and HKa demonstrating specific binding to each [31, 32]. Liu et al. showed that HKa blocked the colocalization of uPAR and EGFR and subsequent EGFR phosphorylation in prostate cancer cells, which also develop into highly vascularized tumors, but HK was not tested [22]. Therefore, it is possible that the siRNA-mediated knockdown of KNG1 in U87 cells and iHECs...
Figure 6: Altering KNG1-ceRNA levels in U87 cells induces angiogenesis in zebrafish and mouse xenograft models of GBM. (A, B; left panels) Zebrafish embryos following injection with Matrigel plug containing RFP-expressing iHECs cocultured with (A) U87 cells transduced using an LV expressing miR138; U87 cells transfectected using siKNG1, siCTU1, siKIAA1274, siRAX, or siBMP4 (negative control); (B) U87 cells transfected with plasmids expressing the 3'-UTR of KNG1 or the ceRNAs or pcDNA (EV); or DICER U87 cells. Relative quantification of RFP mean fluorescence intensity (MFI) is shown in the chart in the right panels (P < 0.01 for all compared with siNC or EV).

(C) NOD-SCID mice following a single subcutaneous injection of Matrigel plug containing luciferase-expressing iHECs cocultured with U87 cells expressing the 3'-UTR of KNG1 and intravenous injections of exo-U87s from wild type U87 cells or exosomes from U87 cells transfected with siKNG1, siCTU1, siRAX, or siKIAA1274 via the tail vein every 2 days (P < 0.01 for all compared with wild type exo-U87s).

(D) Time course of iHEC luciferase intensity quantification for the mouse xenografts.
activated EGFR signaling via the colocalization of uPAR and EGFR. Our findings warrant further study of the effects of ceRNA-regulated KNG1 expression on EGFR signaling in GBM and endothelial cells.

Vascular co-option occurs early in GBM progression, and is followed in later stages by angiogenesis [4, 33, 34]. Bradykinin, which is proteolytically cleaved from HK, has been shown to enhance the B2 receptor-mediated migration of glioma cells toward blood vessels in vitro [7]. The need to suppress angiogenesis early in tumor development is consistent with baseline KNG1 expression inhibiting the proliferation of iHECs, which is demonstrated by the increase in proliferation of iHECs in which the KNG1 mRNA and its ceRNAs were depleted (Figure 3F). As the GBM cells proliferate, hypoxic conditions develop in daughter cells that are pushed further away from the blood vessel, requiring the induction of angiogenesis to maintain tumor growth. This scenario is consistent with the effects of siRNA-mediated knockdown of KNG1 expression in U87 cells, which increased the miR-138 enrichment of proangiogenic exosomes (Figure 4D). Our findings warrant future investigations of the effects of hypoxia on KNG1 expression in GBM cells.

Although vascular endothelial growth factor (VEGF) pathways have been shown to contribute to angiogenesis independent of hypoxia, the importance of vascular co-option in early GBM tumor development might explain the suboptimal effectiveness of VEGF antagonists and other antiangiogenesis therapies for the treatment of GBM [35, 36]. Therefore, our findings have important clinical implications for the development of interventions targeting HK stimulation of B2-mediated vascular co-option to be used in combination with antiangiogenesis interventions in an effort to inhibit both forms of neovascularization in GBM patients.

MATERIALS AND METHODS

Compliance with ethical standards

All procedures performed involving human participants were performed in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Our study protocols were approved by the institutional review boards of the participating hospitals, and written informed consent was obtained from all of the patients prior to their participation. All procedures involving animals were performed in accordance with the ethical standards of the participating institution and the Guidelines for the Humane Treatment of Laboratory Animals (Ministry of Science and Technology of the People’s Republic of China, Policy No. 2006 398), and were approved by the Institutional Animal Care and Use Committee of each participating institution.

Patients and tissue samples

GBM tumor tissue samples were surgically resected from 180 patients who had received a histologically confirmed diagnosis of recurrent GBM by a neuropathologist at Beijing Tiantan Hospital or Peking Union Medical College Hospital. The patients had previously undergone identical regimens of radiation therapy, alkylating agent-based chemotherapy using temozolomide, and VEGF-based antiangiogenesis therapy. For each patient, 10 control tissue samples were resected from unaffected areas adjacent to the tumor, and blood samples were collected by venipuncture. All of the tissue samples were collected and quality assessed according to the standards of TCGA database (Supplementary Table S2). Only tissue samples that were > 95% tumor tissue were included in our analysis. The MVD was quantified based on CD31 and CD34 as described previously [37]. The clinical and pathological characteristics of the patients from whom samples were obtained are presented in Table 1.

Simultaneous isolation of mRNA, miRNA, and protein

Proteins and RNAs were simultaneously isolated from frozen tissues, cultured cells, and exosomes using the AllPrep DNA/RNA/Protein Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer’s instructions. Enriched miRNAs were extracted and purified using the Ambion mirVana miRNA Isolation Kit (Life Technologies, Carlsbad, CA, USA), and the integrity and concentration of the eluates were assessed using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA).

Selection of GBM tissue samples for proteomic profiling

The GBM tissue samples subjected to proteomic profile analysis were selected based on the extent of angiogenesis present, which was assessed by calculating MVD as described previously [37]. To determine MVD, the GBM tissues were incubated in a solution containing a 1:100 dilution of anti-CD31 rat monoclonal antibody (BD Biosciences, San Jose, CA, USA) and a 1:200 dilution of anti-CD34 rabbit monoclonal antibody (Abcam, Cambridge, MA, USA). Primary antibody reactivity was detected using a DyLight 549-labelled anti-rat Ig secondary antibody and a GFP-labeled anti-rabbit secondary antibody. Only vessels in which the expression of both markers was detected were used to determine MVD. Three-dimensional reconstructions were analyzed using the ZEN LE fluorescence microscope and digital imaging software (Zeiss), and MVD was calculated based on the maximum fluorescence intensity of the sample field (IF max) and the mean fluorescence intensity of the corresponding 10 control tissue samples (IF control).
Tissue proteomic profiling

The MVD high and MVD low GBM tissue samples were subjected to two-dimensional gel electrophoresis (2DE). Protein pellets from the AllPrep extractions were solubilized in 0.1 mL of 7M urea, 2M thiourea, 4% CHAPS, 20 mM DTT, and 2% ammonium (pH 3–10). The first dimension separation used the ISODALT system (Life Technologies) with ampholines (pH 3–10). The second dimension separation was performed using sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) with an 11%–19% linear acrylamide gradient. Protein spots were visualized by silver staining, and the wet silver-stained gels were scanned using a Pharmacia Image Scanner at 300 dpi and 16-bit. Gel reproducibility was evaluated based on triplicate 2DE samples. The Progenesis SameSpot, version 4.0, software (Nonlinear Dynamics, Newcastle upon Tyne, UK) was used for gel alignment, spot detection, spot quantification, total spot volume normalization, and statistical analysis. Alignment was performed after manually assigning 20 landmark vectors.

Protein bands were manually excised from the SDS-PAGE gels. The cysteine residues were reduced with dithiothreitol, and alkylated using iodoacetamide. The gel slices were treated with sequencing-grade trypsin (Promega, Madison, WI, USA) in 40 mM ammonium bicarbonate at 37°C overnight with gentle shaking. The reaction was stopped using 0.1% tetrafluoroacetic acid at 30°C for 15 min. The peptides were extracted and desalted using ZipTip C18 columns (EMD Millipore, Billerica, MA, USA). Following elution, the peptides were crystallized in 50% (v/v) acetonitrile in a saturated aqueous solution of alfa-cyano-4-hydroxycinnamic acid. The mass spectra for the peptides were obtained using matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS) in a Reflex IV TOF mass spectrometer (Bruker, Ettlingen, Germany) equipped with a nitrogen laser set at an emission wavelength of 337 nm. The instrument was set in positive ion reflectron mode with delayed extraction and an accelerating voltage of 20 kV.

An average mass accuracy > 20 ppm was maintained by using a mixture of 7 peptide standards for an external calibration for each measurement, and all of the spectra were recorded using a minimum of 250 laser shots. The peak m/z values and intensities were recorded over a mass range of 0.800 to 20.0 kDa. The ClinProTools, version 2.0, software (Bruker) was used for baseline subtraction, spectra normalization based on total ion current, and the determination of peak m/z values and intensities over a mass range of 0.800 to 10.0 kDa. The signal-to-noise ratio was > 5, and a mass shift ≤ 0.1% was used for spectral alignment. The peak area was used for the quantitative standardization. The ClinProTools genetic algorithm was used to establish the best pattern for distinguishing between the GBM and control tissues. A 20% leave-out cross-validation was performed for each individual profile, and the relative peak intensities were compared. Trypsin autolysis products were used for internal calibration.

Peptide matching and protein searches were performed based on comparison to the NCBI nr and/or SWISS PROT databases using the MASCOT and ProFound search engines. The main search parameters were as follows: no restriction on molecular weight (MW) or isoelectric point, taxonomy, mouse, one missed cleavage, cysteine carboxymethylation, methionine oxidation, and peptide mass tolerance of 50 to 100 ppm. A threshold score of \( P < 0.05 \) in MASCOT was considered to indicate a significant protein match.

Serum proteomic profiling

All the serum samples were fractionated using the ClinProt MB-WCX Profiling Kit, according to the manufacturer’s standard protocol (Bruker). The eluted sample was diluted 1:10 in freshly prepared matrix solution, which contained 0.3 g/L \( \alpha \)-cyano-4-hydroxycinnamic acid in 2:1 ethanol and acetone. Approximately 1 \( \mu \)L of the eluate was spotted onto an AnchorChip MALDI-TOF-MS target (Bruker), and the target was dried at room temperature before analysis. The MALDI-TOF-MS measurements were performed using an Autoflex TOF mass spectrometer (Bruker) to determine the serum protein profiles. For quality control, 11 peptide standards and 13 reference sera were used as external standards for baseline calibration and recalibration after every eight samples to maintain an average deviation in MW ≤ 100 \( \mu \)g/g and a coefficient of variability < 30%. Profile spectra were acquired from an average of 400 laser shots per sample. The remaining parameters were identical to those used for tissue profiling.

Serum biomarker identification

Proteins identified as being up/down-regulated by serum protein profiling were subjected to liquid chromatography and tandem MS for confirmation of protein identification using a LTQ Orbitrap XL mass spectrometer (Thermo Scientific) with the following parameters: nano ion source, spray voltage 2.0 kV, scanning time 60 minutes, data dependent and dynamic exclusion MS mode, mass range of 0.300-2.00 kDa, Orbitrap for MS1 at a resolution of 1 × 10^6, and LTQ for collision induced dissociation and
MS2. The six most intense ions were selected as the parent ions. Chromatograms were analyzed using the BioworksBrowser, version 3.3.1, software (SP1), and database searches were performed using Sequest (IPI Human Database, build 3.45) based on the mass lists produced. The parent ion and fragment mass relative accuracies were set at 50 µg/g and 1 D, respectively, for generating peak lists.

Western blotting

Total protein concentrations were determined spectrophotometrically using a Bradford assay dye (Bio-Rad Laboratories, Hercules, CA, USA). Samples containing 10 µg of total protein were subjected to SDS-PAGE using a 4% to 12% acrylamide gradient in NuPAGE MOPS SDS running buffer (Life Technologies), and the fractionated protein bands were electrophoretically transferred to Whatman nitrocellulose membranes (GE Healthcare Life Sciences) in NuPage transfer buffer (Life Technologies) with 10% methanol. The membranes were probed with the primary and secondary antibodies. The anti-KNG1 antibody (66123-1-lg, Proteintech Group, Chicago IL, USA); anti-CTU1 antibody (OAAAB00356, Aviva Systems Biology); anti-KIAA1274 antibody (ABIN447393, Antibodies-online.com, Aachen, Germany); anti-RAX antibody (H00030062-M02, Abnova, Taipei, Taiwan); anti-TMEM105 antibody (sc-248856, Santa Cruz Biotechnology, Dallas, TX, USA) were used as primary antibodies. Primary antibody reactivity was visualized using an enhanced chemiluminescence detection system (GE Healthcare Life Sciences). The protein bands were quantified by densitometry and normalized to a β-Actin standard using the Quantity One software (Bio-Rad).

Double-immunofluorescence assay

The double immunofluorescence analysis of iHECs and U87 cells was performed using a modified MVD assay, as described previously [39]. Cells were treated with a solution of 1:100 anti-CD31 rat monoclonal antibody and 1:200 anti-CD34 rabbit monoclonal antibody, followed by treatment with GFP-labeled anti-rabbit and DyLight 549-labelled anti-rat Ig secondary antibodies. Three-dimensional reconstructions were analyzed using a ZEN LE fluorescence microscope.

Quantitative reverse transcription and polymerase chain reaction

For the qRT-PCR analysis, total RNA was isolated using the Ambion mirVana RNA Isolation Kit, and purified using the RNaseasy Mini Kit (Qiagen). First-strand cDNA synthesis was performed using the Applied Biosystems High Capacity cDNA Archive Kit (Life Technologies), according to the manufacturer’s instructions. The mRNA reverse transcription was performed using the Applied Biosystems MicroRNA Reverse Transcription Kit (Life Technologies), according to manufacturer’s instructions. Real-time PCR was performed using the LightCycler 480 System (Roche Molecular Systems, Pleasanton, CA, USA) with the following primers: KNG1-Forward, CTTTGGAAATGTGTACCCG; KNG1-Reverse, GCATACTCGTGTAGTTG; CTU1-Forward, GCGTGACTTGTAGGACACAC; CTU1-Reverse, GTGGGTGGACACAGAAATAGG; KIAA1274-Forward, GTTGAGCTTGCTGACCTCCT; KIAA1274-Reverse, CGA GCCTAAACAAAAAGCAGG; RAX-Forward, ATATTGGA GTACGGGAGGAG; RAX-Reverse, GCAGCGAAGTGT TCCCTAGAA; TMEM105-Forward, GTCCCCATCAGC TCAAAGAA; and TMEM105-Reverse, ACACCGCACA CTGAAAAACCC. GAPDH-Forward 5’-CGGAGTCAACG GATTGGTCTGTAT-3’; GAPDH-Reverse 5’-AGCTCCTCTC CATGGTGGGAGAC-3’; EGFR/EGFRvIII-Forward 5’- AAACGCTAAAGGCAAATACAGG-3’; EGFR/EGFRvIII Reverse 5’-TCCAAGGGACAGGAATATGG-3’; and previously described Gluc primers [40]. Thermal cycling was performed using an initial denaturation at 94°C for 30 s, followed by 40 cycles of 94°C for 3 s, 60°C for 30 s, and a melt curve stage of 94°C for 15 s, 60°C for 1 min, 95°C for 15 s and 60°C for 15 s.

Computational analyses

To better understand the biological functions and/or diseases that were most relevant to the data sets and facilitate our understanding of functional links to GBM-induced angiogenesis, an IPA was used to compare the different types of cellular interactions involved, including gene-gene interactions and gene-miRNA interactions. The IPA evaluates the biological significance of a given canonical pathway or biological function as the probability that the pathway or function is associated with the dataset by random chance. The mRNA gene identifiers and the corresponding fold changes were imported into the Pathway Studio. Each gene identifier was mapped to the corresponding gene object. The TCGA dataset also was used as the training set for ceRNA signature. The P-value was calculated using the right-tailed Benjamini-Hochberg multiple testing correction, with P < 0.05 indicating a statistically significant result. Interconnections between the identified proteins, mRNA, and miRNAs associated with GBM-induced angiogenesis were evaluated using the Core, Metabolomics Comparison Analysis, and Path eExplorer option of IPA. Gene symbols and gene ontology terms were assigned using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) web-based computational tool (http://david.abcc.ncifcrf.gov/), and the Rna22 program was used to predict miRNA targets (https://cm.jefferson.edu/rna22v2.0-homo_sapiens/).

Coexpression of KNG1 and ceRNA was evaluated using the publicly available TCGA and GEO databases. From these datasets, whole-transcript and miRNA expression data for 323 GBM tissue samples and 10 TCGA controls
were analyzed. The GeneSpring GX software (Agilent Technologies) was used to process and normalize the level 1 TCGA expression data, and entrez gene ID annotations were made using the Platform Design utility of TCGA (https://tcga-data.nci.nih.gov/tcga/tcgaPlatformDesign.jsp). The expression data of candidate mRNAs and miRNAs from GBM patients were used as the test group. The dataset included mRNA and miRNA from 40 GBM patients and 10 controls that had been validated by qRT-PCR.

Group classification for differential expression analysis was performed as described by Tay et al 2011 [24]. Samples were subdivided into two subsets according to KNG1 expression. The KNG1-low subset exhibited a level of KNG1 expression below that of the controls, and the KNG1-high subset exhibited a higher level of KNG1 expression than the controls (Benjamini-Hochberg \( P < 0.05, FC < \pm 2 \)). We focused our analysis on miRNAs that potentially downregulate KNG1, and used the Rna22 miRNA target prediction algorithm to predict the miRNA-Target (KNG1 or ceRNA) interactions. After the miRNA interactions were experimentally validated, samples with validated lower or higher levels of miRNA expression were selected to analyze the coexpressed KNG1 ceRNAs further, with at least 1 out of 3 KNG1-targeting miRNAs demonstrating a level of expression one standard deviation lower or higher than that in the control. Samples inconsistent or variable levels of expression among the KNG1-targeting miRNAs were discarded.

Coexpression between KNG1 and the ceRNAs was analyzed in multiple tissues, and a specific annotated database (http://www.cbu.mbcunito.it/ts-coexp) was used to generate normal tissue-specific human-mouse conserved and human-specific coexpression networks. The level of coexpression was considered significant if it ranked in the top 1% of genes coexpressed with KNG1. Genomic status was assessed using the Affymetrix Genomewide SNP_6.0 raw data from the TCGA database, and the Genotyping Console Software (Affymetrix, Santa Clara, CA, USA) was used for the genotyping analysis and visualization.

Cell culture and transduction

The U87 glioma cell line was maintained in Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies) supplemented with 10% FBS and 1% sodium pyruvate. The iHECs (ACBRI-376, Cell Systems, Kirkland, WA, USA), which were derived from human primary brain VECs, and the U87 cells were used to examine exosome transfer. The iHECs were transduced with 107 TU/mL SFFV:Tag LV, and purified using flow cytometry based on RFP fluorescence. Doses of SFFV:Tag LV-RFP for transducing other cells ranged from 103 to 107 TU/mL, depending on the cell type. In dose-response experiments, U87 cells were transduced with LV doses ranging from 106 to 107. The iHECs were transduced with LV doses ranging from 105 to 106 TU/mL.

Transduced cells were cultured on Matrigel-coated 24-well plates in basal medium (Lonza Biologies, Portsmouth, NH, USA) supplemented with hydrocortisone, EGF, FGF, VEGF, insulin-like growth factor (IGF), ascorbic acid, and FBS (Lonza) only or with exo-U87s (7 μg/well), followed by incubation at 37°C in a humidified atmosphere of 1% \( O_2 \), 94% \( N_2 \), and 5% \( CO_2 \). The culture medium was replenished every 3 days. At 16 h post-treatment, tubule formation was assessed by light microscopy, and images were recorded using a ZEN LE microscope and digital imaging software. Culture media were replaced every 3 days. In some experiments, sequential transduction (super transduction) was performed, in which cells were transduced with LV; washed and replated, and transduced with LV again 5 to 7 days after the first transduction.

The U87 cells and iHECs were transduced with the LVs at 3 to 4 wk before lysis. Approximately 1 × 10⁶ U87 cells and iHECs were fractionated by iodixanol gradient ultracentrifugation as described previously [41, 42]. For RNA extraction and miRNA expression analysis, RNA was extracted from 200 μL of each fraction using 700 μL of Qiazol. For protein extractions, 1 mL of each fraction was concentrated using 30 μL the StrataClean Resin (Agilent Technologies, Santa Clara, CA, USA), according to the manufacturer’s instructions, and the concentrated samples were subjected to SDS-PAGE for western blotting as described previously [42].

For siRNA transfections, the nontargeting siRNA control (siNC); the siRNA for KNG1 (siKNG1), CTU1 (siCTU1), KIAA1274 (siKIAA1274), RAX (siRAX), and TMEM105 (siTMEM105); the siGENOME siRNA reagents, and DharmaFect 1 reagent were purchased from GE Healthcare Life Sciences (Pittsburgh, PA, USA). The U87 and iHECs cells were transfected with 100 nM siRNA in 12-well culture dishes at a density of 1 × 10⁶ cells per well. To generate the DICER-iHEC and U87 cell lines, the U87 and iHECs were seeded in 6-well culture dishes at a density of 3 × 10⁵ cells per well before transduction using an LV expressing RNA complementary to exon 1 of the human DICER gene. For LV transduction, the U87 and iHECs were seeded in 12-well culture dishes at a density of 1.3 × 10⁶ cells per well. Cells were trypsinized and seeded for the various assays no sooner than 16 h post-transduction.

Plasmids

The 3'-UTRs of KNG1, CTU1, KIAA1274, RAX and TMEM105 were amplified by PCR from the genomic DNA of U87-2M1 cells and were cloned into pcDNA3.1+ according to standard protocols. The KNG1 3'-UTR was then subcloned into psiCHECK-2 (Promega) using XhoI and NotI restriction sites. KNG1 3'-UTR was also subcloned into the pm52 vector for RIP analysis. Primer sequences are as follows: KNG1 3'-UTR-F, TTTAAGTGGCTATGGGTA; KNG1 3'-UTR-R, TTTTCCGTACATCGTGGTGTTTAT; CTU1 3'-UTR1-F, GGTCAACACGCGACGACA; CTU1
3’UTR1-F, AATGGGACATGAAAGGGCTAA; KIAA1274 3’UTR2-F, GAGACTGAGCCGGAGTTGG; KIAA1274 3’UTR2-R, CTGTGAGTGGTAGGCAGCAAG; RAX 3’UTR-F, CGCCGTTGGGCCCCGCTGCA ACC; RAX 3’UTR-R, ACAGGCGGTCTCCATCTTC; TEMEM105 3’UTR-F, CTATACACGAGGTATGGAACTG; and TEMEM105 3’UTR-R, TTGGATGAGG GCAGGGGAG. For U87 and iHECs cell transduction, an RFP reporter gene was cloned into a previously described LV expressing the SV40 large T antigen under the transcriptional control of the SFFV promoter [43] using the BamHI and SalI restriction enzyme sites to produce the SFFV.Tag LV plasmid.

Exosome purification

Exosomes were purified from U87 cells and iHECs using the ExoQuick TC Kit (System Biosciences, Mountain View, CA, USA) or by ultracentrifugation as described previously [42]. Exosomes from the serum of healthy controls and GBM patients were diluted in 13 mL of PBS, and filtered sterilized sterile before the final ultracentrifugation. To degrade any extra-exosomal RNA, RNase A (Thermo Scientific) was added to the purified exosomes to a final concentration of 100 μg/mL, and the suspensions were incubated for 15 min at 37°C immediately use or storage at –80°C. Total RNA was purified from the exosomes using the Ambion MirVana RNA Isolation Kit, according to the manufacturer’s protocol. The concentration and quality of RNA were finally determined using a NanoDrop ND-1000 spectrophotometer. An 40-μL aliquot of purified exosomes diluted in PBS to a final volume of 100 μL, and exosome size was determined by dynamic light scattering using 40 μL cuvettes and a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK), according to the manufacturer’s instructions. Three sets of 80 sequential acquisitions were performed for each exosome preparation.

Matrigel assay and in vivo miRNA activity analysis

The DICER-U87 cells and DICER-iHECs were seeded in 24-well plates, and transduced with the miR-138 reporter LV. At on week post-transduction, 2 × 10⁶ DICER-iHECs were embedded in Matrigel with or without 1 × 10⁶ RFP-U87. Matrigel plugs were prepared, and implanted subcutaneously into 8 week-old female Swiss nu/nu mice as described previously [42]. The Matrigel implants were harvested for flow cytometric analysis on day 8 post-injection.

Flow cytometry

Flow cytometry was performed using a FACS Aria II flow cytometer (BD Biosciences) based on GFP and RFP fluorescence, as described previously [42]. Fluorochrome-conjugated antibodies were added the cell suspensions, and suspensions were incubated on ice. The cells were washed, and suspended in PBS with 2% FBS and 7-amino-actinomycin D (7-AAD) to stain nonviable cells. Matrigel implants were excised and made into single-cell suspensions by collagenase IV (0.2 mg/mL, Worthington), dispase (2 mg/mL, Life Technologies) and DNaseI (0.1 mg/ml, New England BioLabs) treatment in IMDM medium. After 30 min at 37°C in a shaking thermoblock, the cell suspensions were filtered and washed in PBS containing 2 mM EDTA and 2% FBS. Matrigel-cell suspensions were incubated with rat anti-human CD31 and CD34 blocking primary antibodies (4 μg/mL each), and stained with 7-amino-actinomycin D (7-AAD) to label nonviable cells.

Luciferase reporter assay

A previously described method was used for the miRNA target validation [24]. The iHECs and U87 cells were cotransfected with 100 ng of empty psiCHECK-2 vector or psiCHECK-2+KNG1 3’-UTR and 100 nM siRNA or 1 mg of the vector constructs using Lipofectamine 2000 (Life Technologies), according to manufacturer’s instructions. Firefly and Renilla luciferase activities were measured using the dual-luciferase reporter system and a luminometer (both Promega) at 72 h post-transfection as described previously [24].

Cell proliferation assay

The proliferation of U87 cells and iHECs cells was evaluated as described previously [24]. At 8 h post-transfection, the U87 cells and iHECs were trypsinized. The suspended were seeded into 4 separate 12-well plates at a final density of 2 × 10⁴ cells/well. Each day, one plate was selected for analysis. The cells were washed with PBS, and fixed in 10% formalin at room temperature for 10 min. Following formalin fixing, the cells were stained with crystal violet, and lysed with 10% acetic acid before measuring optical density at 595 nm.

Improved endothelial tubule formation assay

The improved endothelial tubule formation assay was performed as described previously [26]. Transgenic U87 cells (1 × 10⁴) were cultured in confocal dishes. The U87 cells were overlaid with a 3-mm layer of Matrigel containing 3 × 10⁴ iHECs in 100 μL of conditioned medium. A cocktail of the following angiogenic factors was added: hydrocortisone, EGF, FGF, VEGF, IGF, ascorbic acid, FBS, and heparin (in singlequots from Lonza). The cells were grown under hypoxic conditions (94% N₂, 5% CO₂, and 1% O₂) for 72 h at 37°C before imaging.

RIP assay

Following transfection with MS2 expression plasmids, the cells were lysed, and RIP was performed as
profiling data were considered statistically significant when categorical serum profiling data. Intergroup differences in the distribution. A chi-squared test was used to evaluate the test was used for the continuous data without a normal distribution. A Student’s t-tests, with the level of statistical significance set at $P < 0.05$, and the results are presented as the mean ± standard deviation (SD). The association between gene expression and cell proliferation was assessed using a univariable Cox regression analysis and a permutation test performed using the Biometric Research Branch-Array Tools as described previously [46]. Unless otherwise stated, all experiments were performed at least three times.

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CONFLICTS OF INTEREST

The authors have declared that no conflict of interest exists.

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