RESEARCH ARTICLE

MicroRNA-197 induces epithelial-mesenchymal transition and invasion through the down-regulation of HIPK2 in lung adenocarcinoma

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ABSTRACT

Background: The major cause of cancer-related deaths in patients with lung adenocarcinoma (LAD) is due to distant metastasis. Many reports have indicated that miRNA plays a key role in tumor metastasis. The expression of miR-197 is correlated with LAD progression, however, it is still unknown the mechanism of miR-197 in the processing of LAD. **Methods:** A Boyden chamber migration/invasion assay for the metastatic function study in vitro. Real-time PCR and Western blot assays to analyze the EMT hallmark changes in both the mRNA and protein levels. 3'-UTR reporter luciferase assay to show HIPK2 is a direct target of miR-197. **Results:** miR-197 enhances LAD cell migration and invasion miR-197. The down-regulation of miR-197 suppresses the EMT and migration ability. HIPK2 is a direct functional target of miR-197 in LAD metastasis. **Conclusions:** In summary, miR-197 controls EMT and metastasis by directly silencing HIPK2. The findings suggest that interfering with the miR-197-dependent regulation of HIPK2 could be a useful approach for the treatment of patients with late stage metastatic LAD.

BACKGROUND

Lung cancer is the leading cause of cancer-related deaths in men worldwide, accounting for 1.8 million deaths annually. The most

common type, lung adenocarcinoma (LAD), constitutes almost 40 percent of lung cancers. Approximately 90% of patients with LAD develop distant metastasis at the advanced stage (1-3). Therefore, to design effective therapeutic strategies for patients with LAD, it is very important to understand the molecular mechanisms underlying distant metastasis. EMT (epithelial-mesenchymal transition) is an evolutionarily conserved developmental process during which epithelial cells lose polarity and then acquire a mesenchymal phenotype, and this transition has been involved in the initiation of metastasis (4,5).

MicroRNAs (miRNAs) are small non-coding RNAs that up-regulate or down-regulate gene expression post-transcriptionally. It is well-known that miRNAs play a central role in tumour metastasis. For example, the miR-200 family members attenuate the EMT through targeting the repressors of E-cadherin that induce epithelial differentiation, or by targeting the EMT activators known as ZEB1/2 genes (6-8). Previously, miR-197 has reportedly been aberrantly expressed in different types of tumors. It plays a dual role by regulating tumor growth according to the tumor types, as oncogenes or tumor suppressors. On the one hand, miR-197 induces apoptosis, suppresses tumorigenicity, and inhibits cell proliferation and migration (9-13). On the other hand, miR-197 can suppress the expression of the tumour suppressor gene FUS1, and its expression is correlated with poor clinical outcomes in patients with LAD (14, 15). In these patients, miR-197 expression was significantly higher in tumours than in normal tissue and was associated with advanced tumour stage, poor overall survival and higher recurrence rates (14, 15), which suggest that miR-197 is important for LAD development. However, no studies have systematically determined the role of miR-197 in the development of metastatic disease in LAD.

In this study, we demonstrated the function of miR-197 in LAD and found that miR-197 strongly activates EMT and ultimately promotes LAD metastasis by targeting HIPK2. These observations suggest that miR-197 could be a therapeutic target for preventing LAD metastasis.

MATERIALS AND METHODS

Cell culture

The human LAD cell lines A549 and PC9 were obtained from the American Type Culture Collection (ATCC). The cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% foetal bovine serum (FBS), and they were incubated at 5% CO_2 at 37°C.

Real-time PCR

Total RNA was extracted from the LAD cells by using TRIzol (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. Quantification was determined by using NanoDrop 1000 spectrophotometer (Thermo Scientific, Pittsburgh, PA, USA). Reverse transcription of RNA was performed using the ImProm-II reverse transcription system (Promega, Madison, WI, USA) following the manufacturer's instructions. Real-time PCR was performed in an ABI7500 Prism Sequence Detection System (Applied Biosystems, Foster City, CA) by using a SYBR Green kit (TaKaRa, Tokyo, Japan), and the relative changes were quantified. The $2^{-\Delta\Delta CT}$ method was used to measure gene expression. Each experiment was repeated at least three times.

RNA interference

LAD cells were stably infected with the pre-microRNA expression construct known as the lenti-miR expression plasmid, which contained the full-length miR-197 in the H1-MCS-CMV-EGFP vector (GeneChem, Shanghai, China). The sh-miR-197 was cloned into the H1-MCS-CMV-EGFP vector (GeneChem, Shanghai) to generate H1-MCS-CMV-EGFP-sh-miR-197 (GeneChem, Shanghai, China). A non-targeting sequence was used as a lentivirus negative control and was purchased from GeneChem (GeneChem, Shanghai, China).

Migration and invasion assays

A migration assay was performed using 24-well culture inserts with porous polycarbonate membranes (8.0 μm, Millipore, Billerica, MA). For the Matrigel invasion assay, the filters were pre-coated with 30 μl of Matrigel (BD Biosciences, USA) for 4 hours. In brief, 5×10^4 cells in 200 μl of serum-free medium were added to the upper chamber, and 800 μl of medium with 10% serum was placed in the lower chamber. The plates were incubated for 24 hours at 37°C in 5% CO_2. Cells that did not migrate or invade through the pores were removed with a cotton swab. Cells on the lower surface of the membrane were examined and counted under a microscope. Each experiment was repeated at least three times.

Western blot assay

LAD cells were washed with PBS, collected, and lysed with RIPA lysis buffer (Cell Signaling Technology). Protein concentrations were measured with Qubit Protein Assay Kit and Qubit 2.0 Fluorometer (Invitrogen). Approximately 50μg of total protein was loaded on a 10% or 12% SDS-PAGE gel, and transferred to polyvinylidene difluoride membranes for immunoblotting. Membranes were blocked in 5% milk in TBST and incubated with primary antibody at 4°C overnight, followed with horseradish peroxidase (HRP)-linked secondary antibody, and then detected with Pierce ECL western blotting substrate (Thermo Scientific). The following antibodies were used: E-cadherin (1:1000, Abcam, Cambridge), snail (1:1000, Abcam, Cambridge), vimentin (1:1000, Abcam, Cambridge), HIPK2 (1:1000, Cell Signaling Technology, USA), and β-actin (1:5000, Boster, China).

3’-UTR luciferase reporter assay

Lipofectamine-2000 (Invitrogen) was used to transfect A549 cells with luciferase vectors (an empty luciferase vector, a luciferase vector containing the 3’-UTR of the wild-type target gene, and a luciferase vector containing the 3’-UTR of the mutant-type target gene) for HIPK2 together with miR-197 or the negative control. After 48 hours, transfected A549 cells in each well of 24-well plates

were harvested with 100 μL of lysis buffer. 10 μL of the cell extract was used to measure luciferase activity by using the Dual-Luciferase Reporter Assay System (Promega, Madison, USA). All assays were performed in triplicate and the data were presented as the ratios between firefly and *Renilla* fluorescence activities.

Statistical analyses

All data were presented as the means ± SD. The statistical analyses were performed using Student's t-test. A difference was considered statistically significant when $p < 0.05$. All of the statistical analyses were performed with SPSS 13.0 software.

RESULTS

miR-197 enhances LAD cell migration and invasion

First, we analysed the role of miR-197 in metastasis by migration and invasion studies. We generated LAD cell lines with stable miR-197 overexpression in A549 and PC9 cells using lentivirus transfection. Next, we performed a Boyden chamber migration/invasion assay to study metastatic function in vitro. The ectopic expression of miR-197 significantly increased the migratory and invasive abilities of both miR-197-over-expressing A549 and PC9 cells (Figures 1A-1B). Collectively, our data suggested that miR-197 overexpression significantly increased the migratory and invasive abilities of LAD cells in vitro.

miR-197 promotes LAD metastasis through EMT

EMT plays an critical role in the metastasis process, which provides the motility, invasion and migration properties of cancer cells [4, 5]. Therefore, we further explored whether miR-197-promoted LAD metastasis is mediated through the EMT. To confirm this hypothesis, we first used miR-197-overexpressing A549 and PC9 cells to analyse the hallmark EMT changes at both the mRNA and

protein levels. As shown in Figures 2A-2B, the epithelial marker E-cadherin was dramatically decreased after miR-197 overexpression. However, the mesenchymal markers vimentin and snail were increased in miR-197-overexpressing cells compared to the vector cells. Taken together, these results demonstrate that miR-197 enhances the EMT process in LAD cells.

The down-regulation of miR-197 suppresses the EMT and migration ability

Having shown that miR-197 overexpression could enhance the EMT process in LAD cells, we next employed a loss-of-function approach by using shRNA to investigate the role of miR-197 in the EMT process. As anticipated, the migratory and invasive capabilities of both A549 and PC9 cells were significantly reduced by miR-197 inhibition (Figures 3A–3B). In addition, as shown in Figures 3C–3D, the epithelial marker E-cadherin was increased, and the mesenchymal marker vimentin was decreased in sh-miR-197-transfected A549 and PC9 cells compared with the vector groups. Collectively, our findings suggest that miR-197-promoted LAD metastasis is mediated by EMT.

HIPK2 is a direct target of miR-197 in LAD cells

To investigate the roles for the target gene of miR-197 in the EMT process and metastasis in LAD, we first performed a miRNA target gene prediction with Targetscan database. We found that HIPK2 exhibited miR-197-binding sequences in its 3'-UTR regions (Figure 4A). Next, the luciferase activity was decreased by miR-197 over-expression when the Luc-HIPK2-wt was present, compared with the luciferase activity in the Luc-HIPK2-mu, suggesting that miR-197 reduced the luciferase activity of Luc-HIPK2-wt but had no effect on Luc-HIPK2-mu (Figure 4B). Taken together, our results suggest that miR-197 promotes LAD metastasis by directly targeting HIPK2.

HIPK2 is a direct functional target of miR-197 in LAD metastasis

We further tested whether HIPK2 is functionally regulated by miR-197 in LAD metastasis. Interestingly, the miR-197 over-expression-associated migration and invasion changes could be partially rescued by HIPK2 overexpression (Figures 5A–5B). Collectively, these results demonstrated that miR-197 promotes LAD metastasis by targeting HIPK2.

DISCUSSION

Many reports have demonstrated that the dysregulation of miRNAs contributes to cancer metastasis (16-18). However, it is not clear whether miRNAs play a key role in the regulation of LAD metastasis. The present study identified that miR-197 is involved in the promotion of LAD metastasis. For the first time, we found that miR-197 promoted the migratory and invasive abilities of LAD cells with two different cell lines and with overexpression and knockdown experiments. In this study, we provide strong evidence of the role of miR-197 through experiments performed in two different LAD cell lines. Moreover, we demonstrated that miR-197 enhanced EMT of LAD cells, an important process for metastasis, supporting the role of miR-197 as a factor that promotes LAD metastasis. This finding supports a previous report showing that miR-197 induces epithelial-mesenchymal transition by targeting p120 catenin in pancreatic cancer cells(19).

The EMT process is a crucial step in initiating the metastatic spread of many tumour cells into distal organs in a variety of cancers (20, 21). Importantly, miRNAs have been shown to regulate EMT (22-24). Here, we found that miR-197 expression in involved in inducing the EMT process, as demonstrated by the gain of mesenchymal markers and loss of epithelial markers. Moreover, overexpression of miR-197 greatly promoted EMT in LAD cells, while knock down of miR-197 by shRNA inhibited EMT. Importantly, a high level of miR-197 expression was significantly associated with low E-cadherin expression and high vimentin and snail expression, which supports the loss- and gain-of-function studies we performed in LAD tumour cell lines. To our knowledge, this

study is the first to demonstrate that miR-197 enhances the EMT process in LAD.

It is well established that miRNA regulates a target gene expression to perform its function (25, 26). Therefore, we investigated, in this study, the functional target gene for miR-197 that was relative to LAD metastasis regulation. HIPK2 was predicted to be a functional target gene of miR-197 by a TargetScan database analysis. Moreover, we found that miR-197 directly bound to the 3'-UTR region of HIPK2 and suppressed HIPK2 expression. Previously, there was no direct evidence of a relationship between miR-197 and HIPK2 in cancer cells. This study provides the first evidence demonstrating that miR-197 directly regulates HIPK2 in LAD cells.

CONCLUSIONS

Collectively, our study has significant implications for understanding the underlying mechanisms of how miR-197 contributes to tumor progress in LAD. miR-197 controls EMT and metastasis by directly silencing HIPK2. The findings from this study suggest that interfering with the miR-197-dependent regulation of HIPK2 could be a useful approach for the treatment of patients with late stage metastatic LAD.

LIST OF ABBREVIATION

LAD, lung adenocarcinoma

EMT, epithelial-mesenchymal transition

MiRNAs, MicroRNAs

CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

CONTRIBUTIONS

NZ carried out most of experiments and participated in drafted the manuscript. LT carried out migration and invasion assays. ZM participated in the design of the study and performed the statistical analysis. NG conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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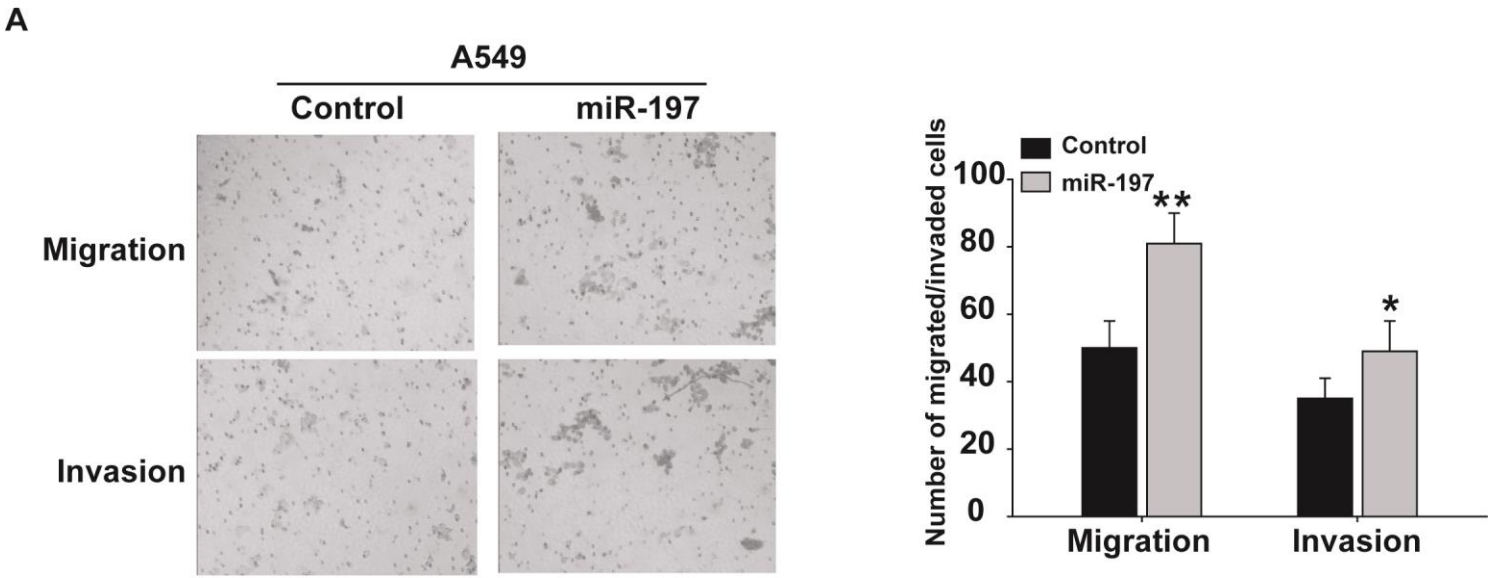
Figure legends

Figure 1: miR-197 enhances LAD cell migration and invasion in vitro. A and B. The migration and invasion abilities of A549 (A) and PC9 (B) cells when transfected with the miR-197 over-expression vector or an empty vector as assessed by Transwell migration and Matrigel invasion assay for 24 hours. All experiments were performed at least three times, and the data are expressed as means ± SD. The statistical significance of differences was measured by unpaired student's t-test. *P < 0.05, **P < 0.01.

Figure 1



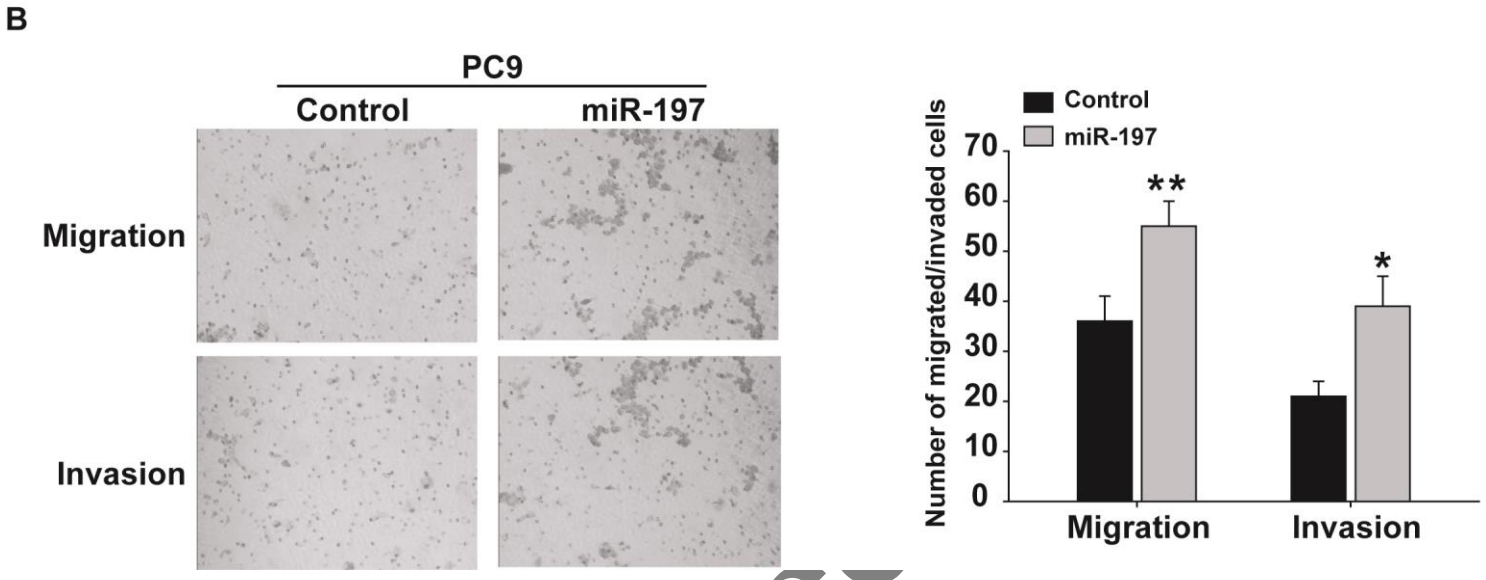
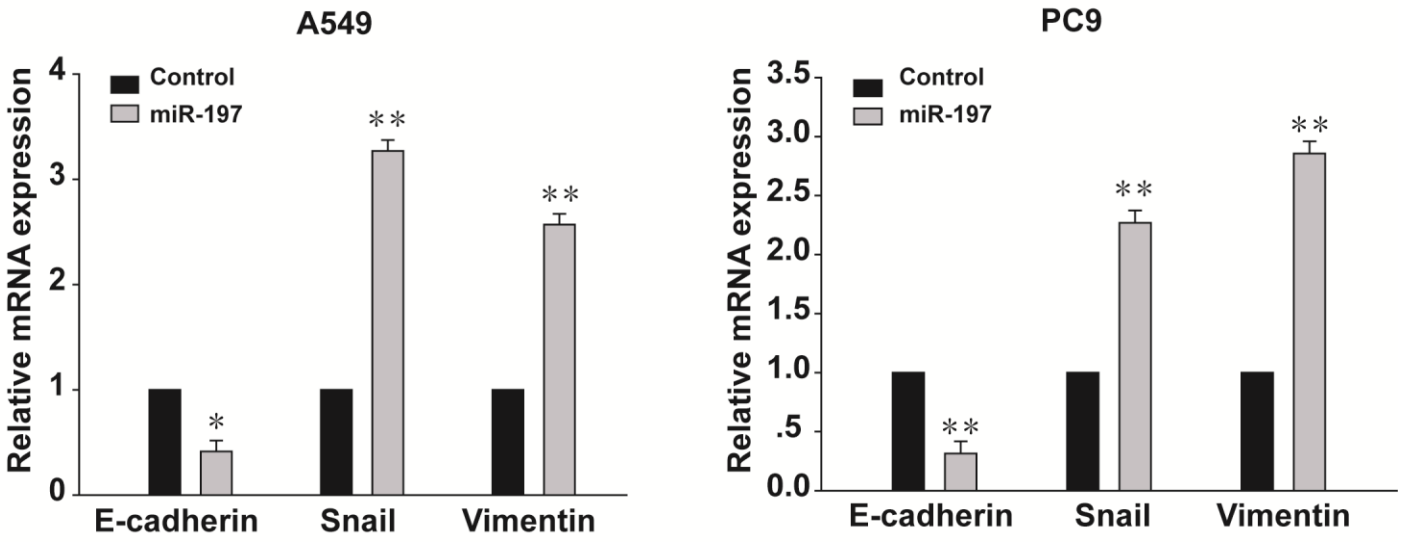


Figure 2: miR-197-promoted LAD metastasis is mediated by the EMT. A. The expression of EMT markers as analyzed in miR-197-over-expressed LAD cell lines by real-time PCR. B. EMT markers in two miR-197 over-expressed LAD cell lines as analyzed by western blots. All experiments were performed at least three times and the data are expressed as means ± SD. The statistical significance of differences was measured by unpaired student's t-test. *P < 0.05, **P < 0.01.

Figure 2

A



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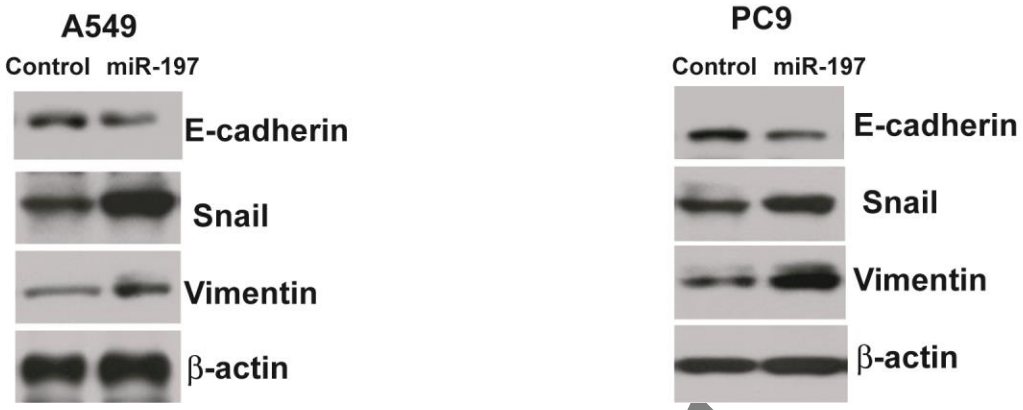


Figure 3: The down-regulation of miR-197 suppresses the EMT and migration ability in LAD cells. A. and B. The migration and invasion abilities of A549 (A) and PC9 cells (B) as transfected with the sh-miR-197 vector or an empty vector and assessed by Transwell migration and Matrigel invasion assay for 24 hours. C–D. The expression of EMT markers analyzed in sh-miR-197-transfected A549 cells (C) and PC9 cells (D) by real-time PCR. All experiments were performed at least three times and the data are expressed as the means ± SD. The statistical significance of differences was measured by unpaired student's t-test. *P < 0.05, **P < 0.01.

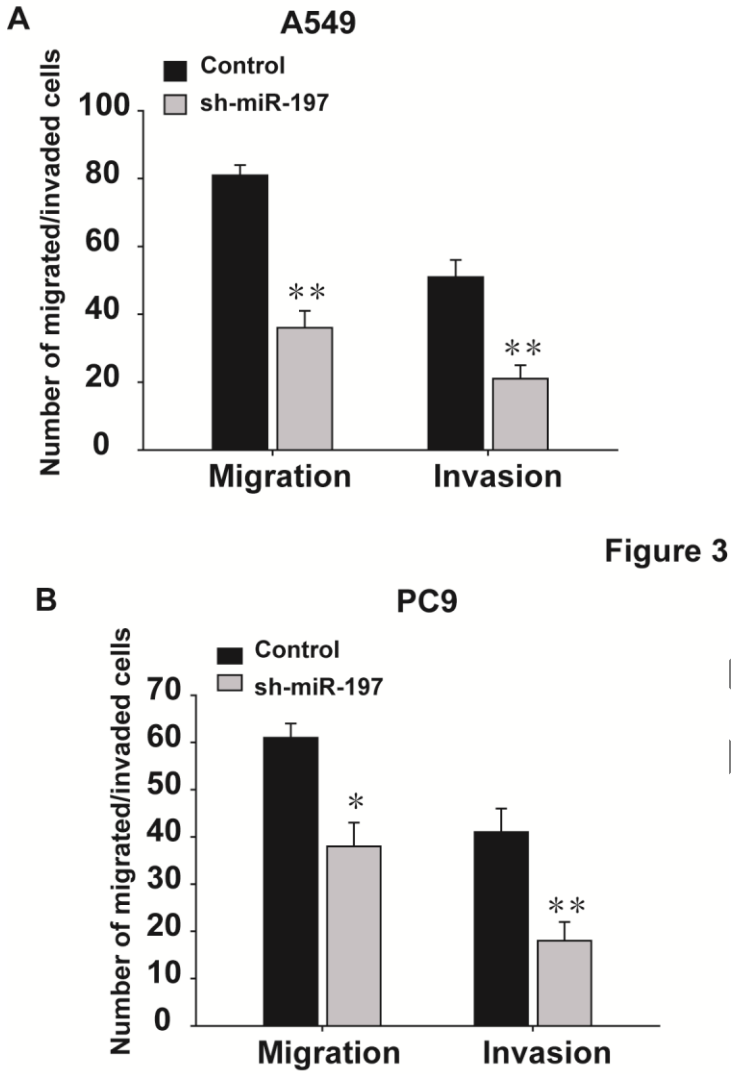
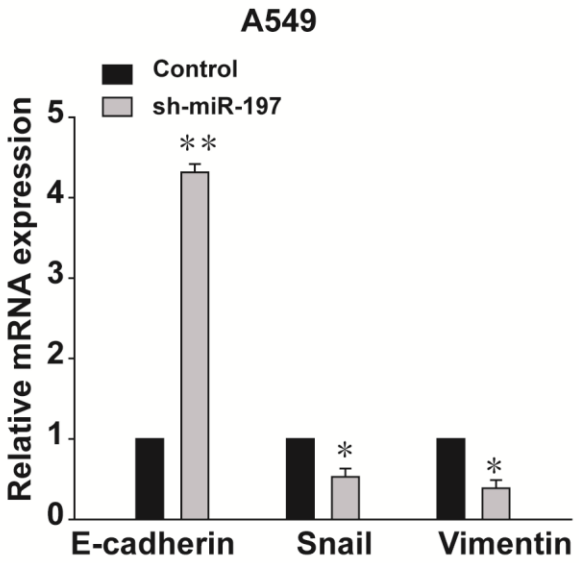


Figure 3

C



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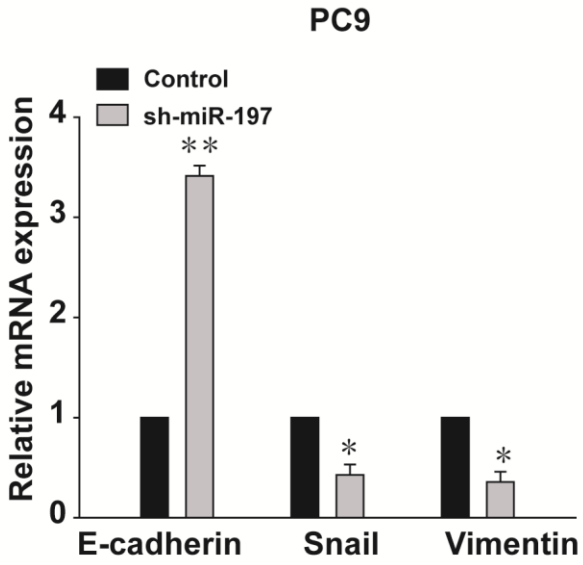

Figure 4: HIPK2 is a direct target of miR-197 in LAD cells. A. A schematic representation of putative miR-197 binding in the 3' UTR of HIPK2. B. Dual-luciferase assays showing the repression of wild-type UTR (HIPK2-3'UTR) or mutant UTR (HIPK2-3' UTR mut) following the transfection of the vector or miR-197 over-expression vector. All experiments were performed at least three times and the data are expressed as the means ± SD. The statistical significance of differences was measured by unpaired student's t-test. **P < 0.01.

A

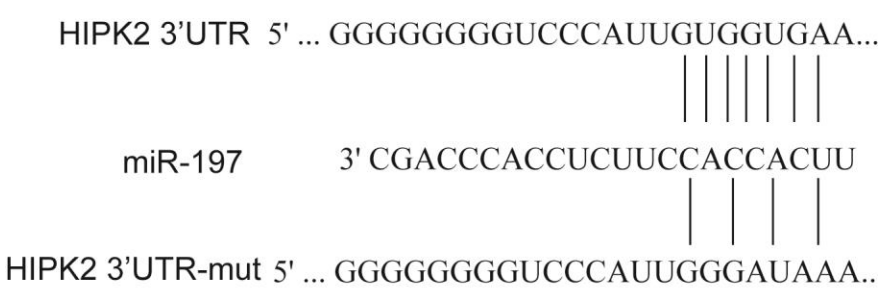

Figure 4

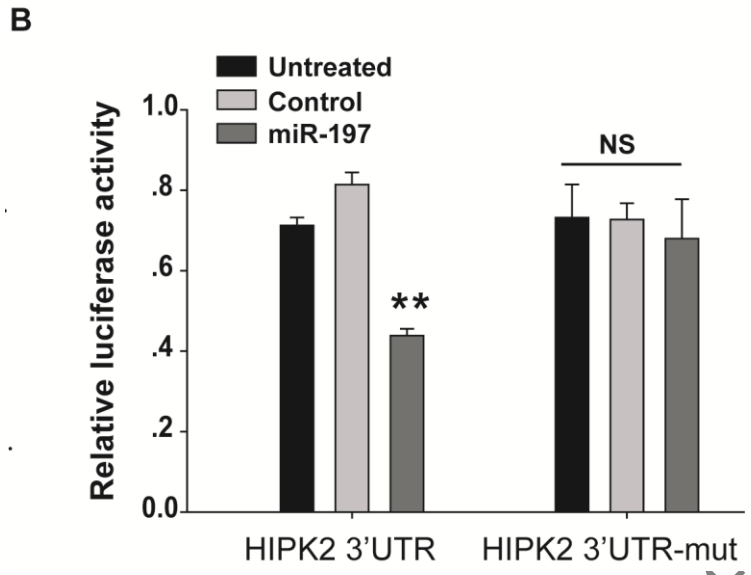

Figure 5: HIPK2 was a direct functional target of miR-197 in LAD metastasis. A. The migration capacities of A549 cells after the indicated treatment as assessed by Transwell migration assay. *P < 0.05, **P < 0.01. B. The invasive capacities of A549 cells after the indicated treatment as assessed by Matrigel invasion assay. All experiments were performed at least three times and the data are expressed as the means ± SD. The statistical significance of differences was measured by unpaired student's t-test. *P < 0.05, **P < 0.01.

A

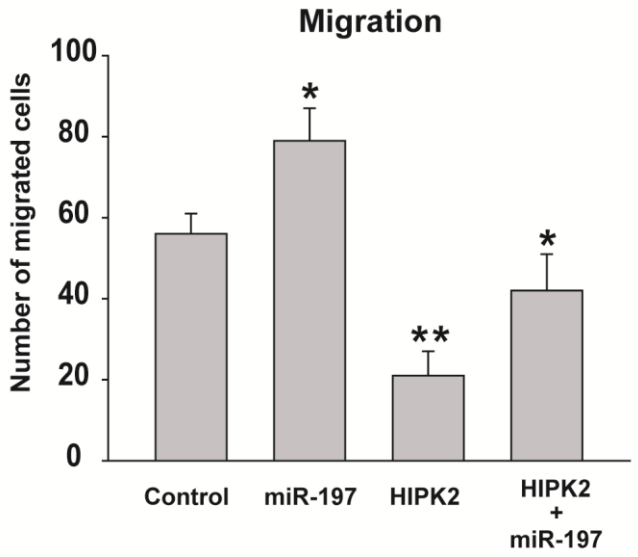

Figure 5