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miR-29b suppresses tumor growth and metastasis in colorectal cancer via downregulating Tiam1 expression and inhibiting epithelial–mesenchymal transition

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Recently, the role of miR-29b in colorectal carcinoma (CRC) development appears to be controversial. Until now, the expression and function of miR-29b in CRC have not been clarified clearly. We showed that decreased expression of miR-29b usually occurred in CRC cell lines and tissue samples. Loss- and gain-of-function assays in vitro revealed suppressive effects of miR-29b on cell proliferation and migration. Endogenous overexpression of miR-29b was sufficient to suppress aggressive behavioral phenotypes in mice. Proteomic analysis showed that miR-29b involved in integrate several key biological processes. In addition, miR-29b mediated the inhibition of epithelial–mesenchymal transition (EMT) and the inactivation of mitogen-activated protein kinase and phosphatidylinositol-4,5-bisphosphate 3-kinase/AKT signal transduction pathway. Further studies found that T lymphoma invasion and metastasis 1 (Tiam1) was identified as a direct target of miR-29b. In contrast to the phenotypes induced by miR-29b restoration, Tiam1-induced cell proliferation and migration partly rescued miR-29b-mediated biological behaviors. Our results illustrated that miR-29b as a suppressor has a critical role in CRC progression, which suggests its potential role in the molecular therapy of patients with advanced CRC.
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Colorectal cancer (CRC) is the third most commonly diagnosed cancer and the third leading cause of cancer death. In China, the incidence of CRC is continually increasing despite advances in treatment and subsequent improvement in prognosis. Metastasis leads to most of the mortalities and has a critical role in the poor prognosis.[1,2] The underlying molecular mechanisms in CRC metastasis are still unclear. Hence, it is urgent to identify important molecules in cancer progression, which may be used to develop new diagnostic strategies and drugs targeting these markers.

MicroRNAs (miRNAs) are a class of diverse, small, noncoding RNAs that are processed from precursors with a characteristic hairpin secondary structure.[3] They usually function as critical gene regulators. In recent years, a large number of studies have confirmed that miRNAs have important roles in tumorigenesis and metastasis by targeting different mRNAs.[4] To date, abnormal expression of several miRNAs, such as miR-21,[5] miR-124,[6] miR-625,[7] miR-339-5p[8] and miR-27b,[9] has been identified in CRC and may contribute to the development and progression of CRC. In our recent study, miR-133a was identified as a tumor-suppressive

miRNA in human CRC that acts by repressing LIM and SH3 protein 1, which has been previously identified as tumor metastasis-associated protein,[10] provides additional evidence of a pivotal role for miRNAs in CRC tumorigenesis and progression.[11]

miR-29b belongs to the miR-29b family that comprises three members: miR-29a, -29b and -29c. Recently, several studies have showed that miR-29b was dysregulated and represses tumor progression in hepatocellular,[12] ovarian,[13] prostate,[14] breast[15] and gastric[16] cancer. In colorectal cancer, increased miR-29b was found in colon cancer cells following exposure to a Hexane extract of American Ginseng (HAG) and suppressed the migration of colon cancer cells.[17] In the other study, increased miR-29b was observed in ulcerative colitis-related CRC compared with ulcerative colitis, suggesting its function as oncogene.[18] Thus, the role of miR-29b in CRC development appears to be controversial. Until now, the expression and function of miR-29b in CRC have not been clarified clearly.

In this study, we detected miR-29b expression in CRC cells and tissue samples. Gain- or loss-of-function assays were

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Abbreviations: Tiam1, T lymphoma invasion and metastasis 1; CRC, colorectal carcinoma; EMT, epithelial–mesenchymal transition; NC, negative control; miRNA, microRNA; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; UTR, untranslated region; MAPK, mitogen-activated protein kinase; ERK, extracellular signal–regulated kinase

used to analyze the effect of miR-29b on cell behaviors. We performed xenograft mice models to investigate its therapeutic role in tumor genesis and metastasis *in vivo*. Finally, we also explored the molecular mechanisms underlying the function of miR-29b and its potential targets.

Results

Decreased expression of miR-29b in CRC tissues and cell lines. We detected miR-29b expression in 24 CRC tissues and matched adjacent normal tissue using real-time PCR. miR-29b was downregulated in 17 of the CRC samples compared with paired adjacent non-cancerous tissues, with up to 12-fold decrease in the CRC tissues (Figure 1a). miR-29b expression levels were significantly lower in CRC tissues than control samples ($P = 0.0228$; Figure 1b). A relatively lower level was found in metastatic CRC compared with non-metastatic CRC ($P = 0.0203$; Figure 1b). In addition, decreased expression of miR-29b was found in all five CRC cell lines compared with the normal human colon epithelial cell line NCM460 or the mean level of the non-cancerous tissue specimens (Figure 1c).

Exogenous miR-29b suppressed CRC cell proliferation and migration *in vitro*. We transfected miR-29b mimic into CRC cell lines SW480 and HCT116 with lower endogenous expression level and evaluated the effects on cellular

behaviors. Real-time PCR was performed to detect the transfection efficiency ($P < 0.05$; Supplementary Figure.1). CCK-8 and soft agar assays showed that miR-29b significantly decreased cell growth and the ability of colony formation in SW480 and HCT116 ($P < 0.05$; Figures 2a and b). Transwell assays revealed a significant reduction in the migration of miR-29b-transfected SW480 and HCT116 cells, respectively ($P < 0.05$; Figure 2c).

On the contrary, anti-miR-29b, as a miRNA inhibitor, was used to investigate the role of miR-29b depletion in CRC cell lines HCT116 and SW620 with relative high endogenous level. Consistent with the above findings, anti-miR enhanced the ability of cell proliferation and colony formation in HCT116 and SW620, respectively ($P < 0.05$; Figures 2a and b). We also found a significant increase in the number of invaded cells after transfection with miR-29b inhibitor ($P < 0.05$; Figure 2c). These findings suggest that miR-29b as a suppressor inhibits aggressive phenotype of CRC cells.

Endogenous overexpression of miR-29b inhibited CRC growth and homing capacity *in vivo*. We stably transfected SW480 cells with pcDNA-GW/EmGFP-miR-29b and then established CRC cell lines with miR-29b overexpression. A qRT-PCR assay showed upregulated 5.8-fold of miR-29b expression in SW480/miR-29b cells compared with SW480/miR-NC cells. Two cell clones were used to perform the following assay (Supplementary Figures 2A).
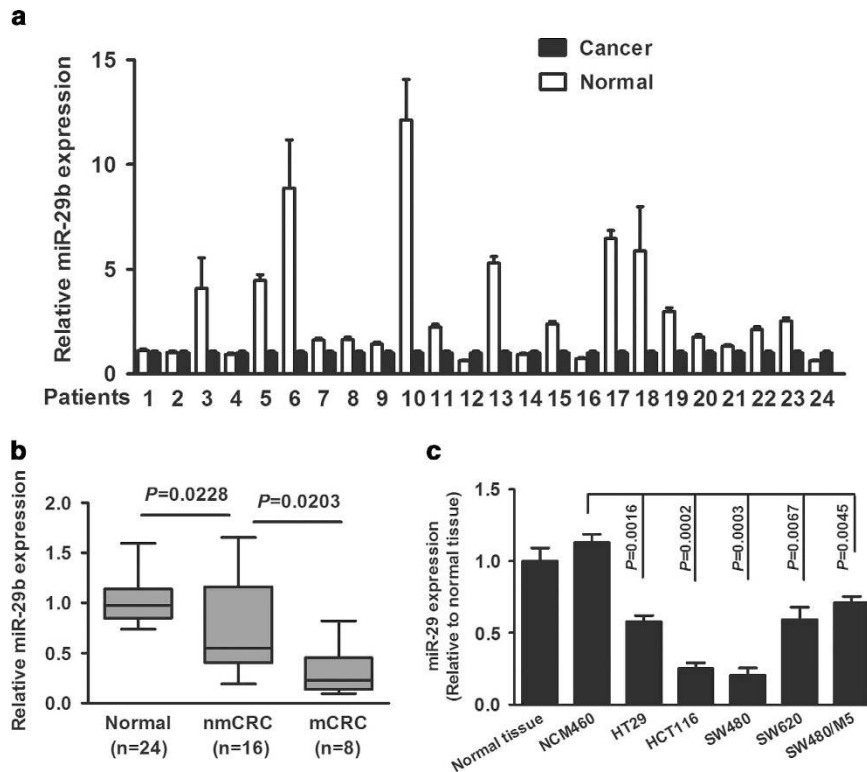
Figure 1 The expression of miR-29b was decreased in CRC tissues and cell lines. (**a**) The histogram indicates the differential expression of miR-29b in cancerous versus non-cancerous tissue using qRT-PCR.(**b**) The miR-29bexpression in CRC tissues with or without metastases relative to match-normal tissues. nmCRC denotes CRC tissues without metastases; mCRC denotes CRC tissues with metastases. (**c**)The relative expression of miR-29b in five CRC cell lines (SW480, SW620, HCT116, SW480/M5 and HT29) was significantly decreased compared with the normal human colon epithelial cell line NCM460 or the mean rate of expression of miR-29b in 24 non-cancerous tissue samples (N-tissue)
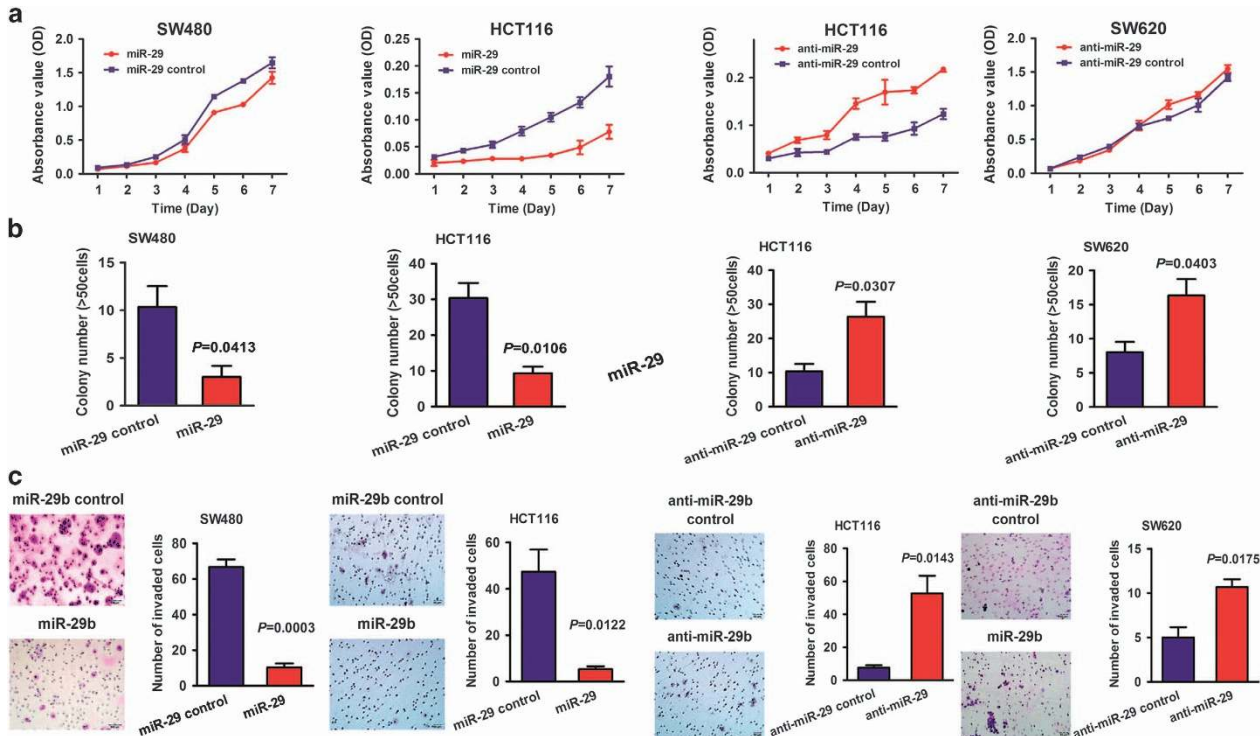
Figure 2 Ectopic expression of miR-29b inhibited aggressive phenotypes in CRC cells. (**a**) The effect of miR-29b on cell proliferation was evaluated by CCK-8 assay after miR-29b or anti-miR-29b transfection of CRC cells. (**b**) Soft agar colony formation assays for CRC cells transfected with miR-29b or anti-miR-29b. The numbers of colonies were scored. Each bar represents the mean of three independent experiments. (**c**) Data of transwell assay for CRC cells transfected with miR-29b or anti-miR-29b. The cells were counted under a microscope in five randomly selected fields. Bars represent the number of cells invaded after transfection with miR-29b

In vitro assay showed that the stable overexpression of miR-29b obviously decreased the potential of cell growth and migration (Supplementary Figures 2B and C). Then, a subcutaneous xenograft model was performed to evaluate the effect of miR-29b overexpression on tumorigenesis of CRC cells. External fluorescent images can provide invaluable real-time data in monitoring tumor formation. As shown in Figure 3a, the tumors in the SW480/miR-29b groups grew more slowly than these in the SW480/miR-NC group, and showed significantly decreased Ki-67 index compared with control (Figure 3c).

To evaluate the effect of miR-29b on lung-homing capacity, we detected lung tumorigenesis after injection through the tail vein. Compared with the control group, we found significantly more and larger tumor nodules in the lung of SW480/miR-29b groups, indicating that miR-29b suppressed the homing ability of CRC cells (Figure 3d).

miR-29b changed the protein expression pattern of CRC cells. To unclose the molecular mechanisms underlying biological functions mediated by miR-29b, we performed two-dimensional differential gel electrophoresis (2D-DIGE)-based proteomics strategy to investigate differential expression proteins after transfection with miR-29b in SW480 and SW620 (Figure 4a). After analysis using DeCyder 5.0 software, 12 and eight differential protein spots were down-regulated in SW480 and SW620 cells transfected with miR-29b, respectively. Among them, total 16 protein spots were successfully identified by matrix-assisted laser

desorption/ionization tandem time-of-flight mass spectrometry (Supplementary Table 1). Interestingly, a candidate protein, identified as T-complex polypeptide 1 subunit beta (CCT2), was simultaneously modulated by miR-29b in SW480 and SW620 cells. The results of western blot analysis demonstrated that the findings of 2D-DIGE-based proteomic analysis are convincing (Figure 4b).

We next investigated the biological processes involved in differential proteins identified using Gene Ontology. All of the proteins regulated by miR-29b were shown to integrate several key biological processes, such as development, response to stimulus, localization and metabolism and so on (Figure 4c). Molecular function annotation revealed that miR-29b may regulate the expression of several important proteins to participate in cell cycle/death (e.g., PSMD11), cell signal transduction (e.g., PDIA3), regulation of gene expression (e.g., ELL, EIF3H) and cytoskeleton reorganization (e.g., KIF5B, KRT1; Figure 4d). This suggests that miR-29b may have an important role in tumor progression through complex mechanisms.

miR-29b mediated the inhibition of epithelial–mesenchymal transition and inactivation of signal transduction pathway. Epithelial–mesenchymal transition (EMT) is an important process during tumor progression by which the epithelial cells acquire mesenchymal properties and show reduced intercellular adhesion and increased motility.[19] Because we have observed miR-29b-mediated reduction of migration ability and gene expression regulation,
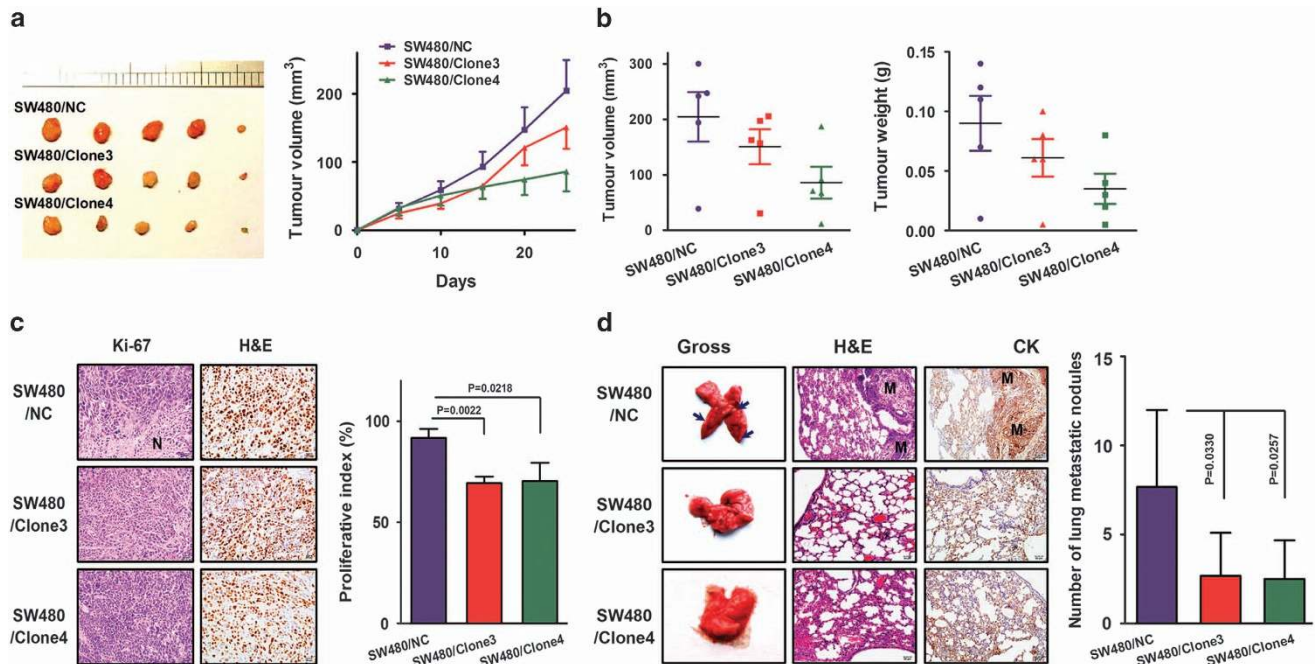
Figure 3 miR-29b inhibited tumor growth and metastasis in nude mice. (**a**) Tumor cells were injected subcutaneously into the back of nude mice to evaluate cancerogenesis. Representative figure of tumors formed. Tumor volume in the back of nude mice injected withSW480/miR-29b and SW480/NC cells was measured. The data of all primary tumors are expressed as mean ± S.D. (**b**) Scatter plots of tumor volume and weight derived from SW480/miR-29b and SW480/NC cells at 30 days after subcutaneous implantation. (**c**) Representative photographs of hematoxylin and eosin (H&E) and immunohistochemical staining of primary cancer tissues were shown (× 400). N, necrotic lesion. Proliferative ability was measured by the Ki-67 proliferative labeling index. (**d**) Tumor cells were injected into nude mice through the tail vein to evaluate the lung metastatic potential of cells. The number of metastatic lung nodules in individual mice was counted under the microscope. Metastatic cancer tissue (H&E staining, × 200).The black arrows indicated metastatic nodes in the liver. M, metastatic lesion

we asked whether miR-29b introduction has an impact on the expression of EMT-associated proteins. Western blot and immunofluorescence assays showed that exogenous miR-29b overexpression resulted in the increase of epithelial markers E-cadherin and β-catenin, and decrease of mesenchymal marker Vimentin (Figures 5a and b). Immuno-histochemistry assays showed that endogenous miR-29b overexpression caused similar changes of EMT markers in vivo (Figure 5c).

Because proteomic studies revealed miR-29b to be involved in signaling transduction pathways, we carried out a western blot analysis of the phosphorylation status of proteins involved in EMT signaling. As shown in Figure 5d, miR-29b significantly suppressed the MAPK pathway through the dephosphorylation of p44/42 MAPK (ERK1/2) and phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/AKT signaling via decreased phosphorylation of AKT at Ser473 and Thr308 in SW480, HCT116 and SW620 cells.

miR-29b directly targets tumor metastasis-related gene Tiam1 in CRC cells. Using a bioinformatic analysis (based on TargetScan Human 6.2, PicTar and miRanda), TIAM1, identified previously as a CRC metastasis-related gene, were predicted as a potential target of miR-29b. Luciferase reporter assay were used to determine whether miR-29b can directly target the 3'UTR region of Tiam1. This involved in cloning the target sequence (wt 3'UTR) or mt sequence (mt 3'UTR) into a luciferase reporter vector, then transfecting 293T cells with the wt or mt 3'UTR vector and miR-29b mimic

or inhibitor (Figure 6a). We observed a significant decrease in the luciferase activity in wt vectors after transfecting with miR-29b mimic. A mutation in the putative binding site in the Tiam1 3'UTR region abrogated this repression, thereby providing additional evidence of direct interaction between miR-29b and Tiam1 (Figure 6a). The mRNA expression by both miR-29b and Tiam1 was analyzed by qRT-PCR in 24 tissue samples obtained from patients with CRC. Tiam1 mRNA levels were higher in CRC tissues than in paired non-cancerous colorectal tissues and had a negative correlation with miR-29b (Figure 6a; $R = - 0.758$, $P = 0.000$). The interaction was confirmed further during our investigation of miR-29b for its ability to repress Tiam1 expression at mRNA and protein level in SW480 and HCT116 cells (Figures 6b and c).

To determine whether Tiam1 rescues the tumor-suppressive effect of miR-29b, we simultaneously co-transfected SW480 and HCT116 cells with miR-29b mimic and pcDNA3-Tiam1 (that contained all but the 3'UTR of Tiam1). We found that the ectopic expression of Tiam1 neutralized the suppression of miR-29b on Tiam1 expression and rescued the miR-29b-mediated inhibition of cell proliferation and migration in SW480 and HCT116 cells (Figure 6d).

Discussion

The expression of miRNAs was deregulated in various kinds of human cancer.[20,21] More and more researches have documented that miRNAs have essential roles in multiple
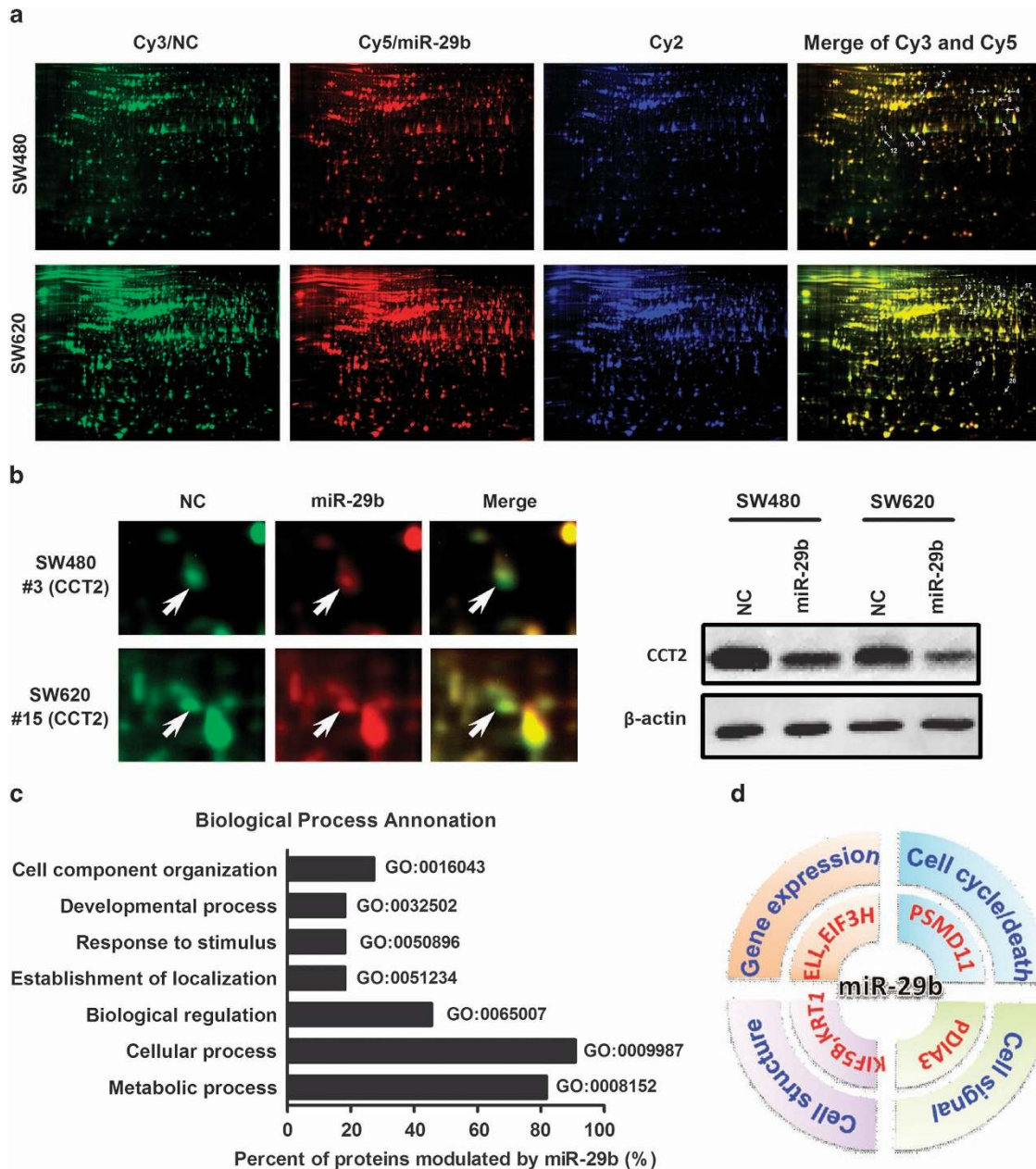
Figure 4 miR-29b altered global protein expression profiles and involved in several key biological processes. (**a**) 2D DIGE images of SW480 (upper) and SW620 (under) cells transfected with miR-29b are shown. Proteins from cells transfected with control were labeled with Cy3. Proteins from cells transfected with miR-29b were labeled with Cy5. Internal standard proteins were labeled with Cy2. Distribution of differentially expressed protein spots in merged images of the Cy-dye labeled images is shown. (**b**) Both enlarged DIGE images (left) and immunoblotting results (right) of acandidate protein, identified as CCT2, are shown. (**c**) The histogram indicated the main biological processes involved in miR-29b using Gene Ontology. (**d**) Pie diagram showed major molecular functions exerted by miR-29b using Gene Ontology

biological processes, including cell differentiation, proliferation, angiogenesis, invasion and migration.[22–25] Recently, several studies have showed the deregulation of miR-29b in many types of tumor, such as gastric,[16] breast,[15] hepatocellular,[12] ovarian[13] and prostate[14] cancer. In colorectal cancer, increased miR-29b was found in colon cancer cells following exposure to a HAG and suppressed the migration of colon cancer cells.[17] In the other study, increased miR-29b was observed in ulcerative colitis-related CRC compared with ulcerative colitis, suggesting its function as an oncogene.[18]

Thus, the role of miR-29b in CRC development appears to be controversial. Our data demonstrated that the downregulation of miR-29b frequently exists in CRC tissue and cell lines, suggesting a tumor suppressive role of miR-29b in CRC development.

Until now, no functional evidence of miR-29b has been documented in CRC. In hepatocellular carcinoma (HCC), miR-29b inhibited the ability of HCC cells to promote capillary tube formation of the endothelial cells and to invade the extracellular matrix gel *in vitro*. Animal models showed that
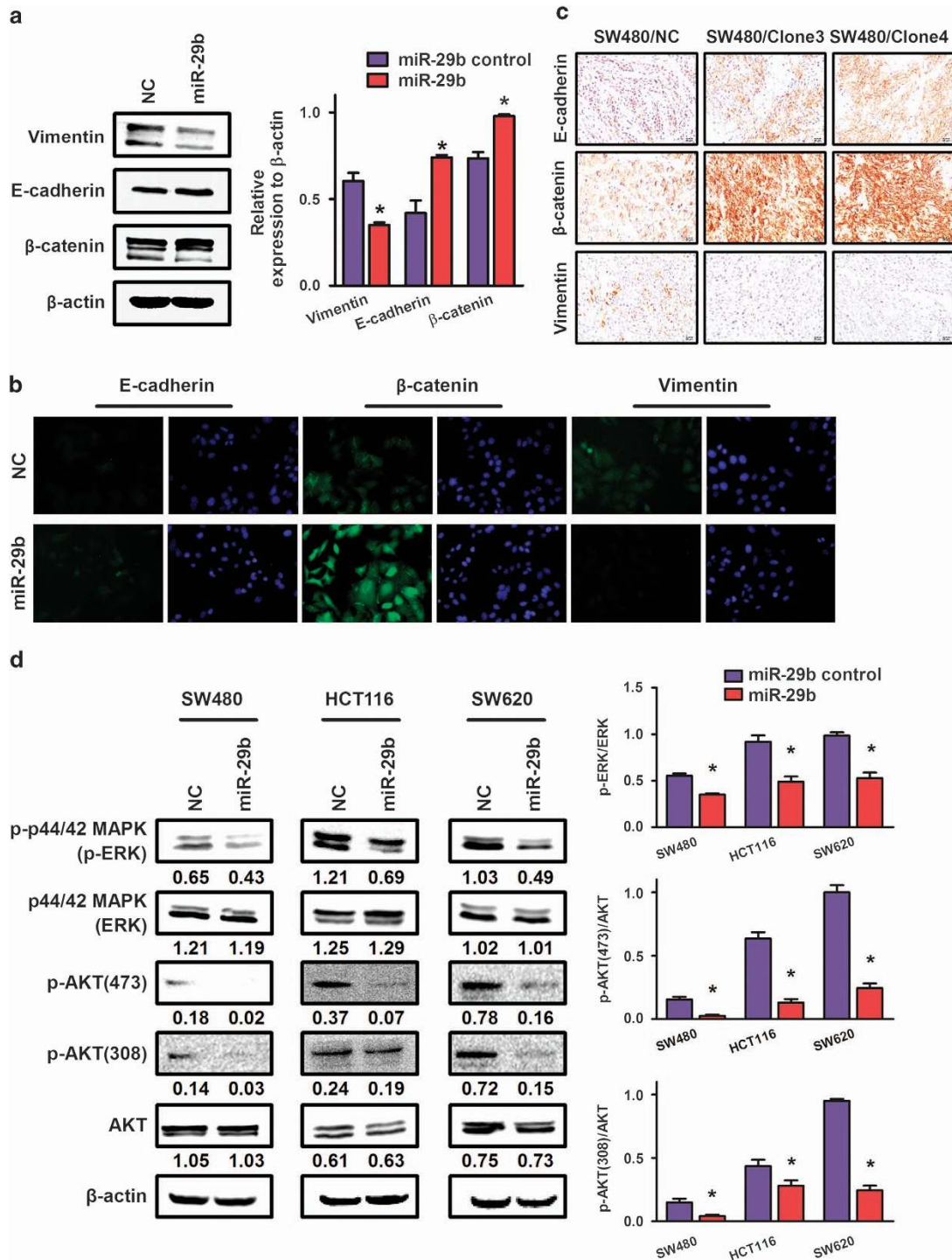
Figure 5 miR-29b inhibited EMT and suppressed phosphorylation of ERK and AKT. (**a**) Western blot analysis was performed to detect the expression of epithelial cell marker E-cadherin, β-catenin and mesenchymal markers Vimentin in SW480 cells transfected with miR-29b. The immunosignal was quantified using densitometric scanning software, and relative protein abundance was determined by normalization with β-actin. (**b**) Immunofluorescence assay was used to show the expression of E-cadherin, β-catenin and Vimentin in SW480 cells transfected with miR-29b. (**c**) miR-29b overexpressing SW480 cells were subcutaneously injected into the mice. The subcutaneous tumors were obtained. Paraffin-embedded tumor sections were stained with anti-vimentin, E-cadherin and β-catenin. Representative figures were shown. (**d**) Phosphorylation levels of ERK and AKT were detected in SW480, HCT116 and SW620 cells transfected with miR-29b (left); the immunosignal was quantified using densitometric scanning software, and the relative protein abundance was determined by normalization with β-actin (right)

miR-29b significantly reduced microvessel density and intrahepatic metastatic capacity.[12] A study on gastric cancer (GC) revealed that the miR-29 family reduced aggressive and progressive phenotypes of GC and inhibitor tumor formation of GC cells in the xenograft mice.[16] Similarly, our gain- and loss-of-function assays showed that miR-29b suppressed cell proliferation and migration *in vitro*, and reduced tumor growth and homing capacity *in vivo*, suggesting its suppressive role in CRC progression.

The molecular mechanisms underlying miR-29b-mediated biological behaviors are still unclear. To comprehensively understand the effect of miR-29b on cancer cells, we performed proteomic analysis to screen the alteration of protein profiling in CRC cells. Using 2-D DIGE, we found a set of proteins that might be directly or indirectly modulated by miR-29b. The candidate proteins have been reported to be involved in tumor development and progression. Bioinformatic analysis revealed that they were involved in several key biological processes, such as development, response to stimulus, localization and metabolism and so on. For example, CCT2, also named as chaperonin-containing T-complex polypeptide 1 subunit beta, was identfied and further confirmed as negatively regulated by miR-29b in SW480 and SW620 cells. Previous studies have showed that CCT2 was overexpressed in CRC and reduced patient survival.[26] Finally, CCT2 was identified as a new modulator of p53-dependent proliferation arrested by RNAi in human cells.[27] These findings are consistent with our proteomic results and a suppressive role of miR-29b in CRC.

EMT has a pivotal role in the initiation of metastasis, a process in which epithelial cells lose adhesion and cytoskeletal components concomitant with a gain of mesenchymal components and the initiation of a migratory phenotype.[28,29] Because the effect of miR-29b on cell migration and gene expression regulation, we detected the change of EMT markers in SW480 cells transfected with miR-29b. The results supported that miR-29b may reverse EMT process to inhibit cell migration. Similarly, miRNA-29b suppresses prostate cancer metastasis by regulating EMT.[14] Recently, the presence of miR-29b was found closely related to DNA methylation by targeting DNA methyltransferase (DNMT), thereby altering the methylation status of tumor-related genes. miR-29b-DNMT signaling is related to the regulation of DNA methylation-related reprogramming events, such as mesenchymal–epithelial transition.[30]

Our proteomic analysis revealed that miR-29b might be associated with the cell signaling pathway. The findings of immunoblot assays demonstrated that miR-29b inactivated MAPK/ERK and PI3K/AKT pathway by dephosphorylation of ERK1/2 and AKT, which is a classical signal transduction pathway and has an essential role in tumor progression. Recent researches revealed that the activation of cap-dependent translation by cooperative ERK and AKT signaling is critical for the promotion of CRC motility and metastasis. The inhibition of either ERK or AKT alone showed limited activity in inhibiting cell migration and invasion, but combined inhibition resulted in a significant impact.[31] To the best of our knowledge, we firstly demonstrated that miR-29b exerted its biological functions by the regulation of MAPK/ERK and

PI3K/AKT pathway, which may also explain the miR-29b-mediated reversion of EMT process.

miRNAs generally exert their biological function by suppressing their specific target genes at a post-transcriptional level. A number of mRNAs were reported as direct targets of miR-29b, such as GATA3,[15] Mcl-1,[32] pre-eclampsia,[33] DNMT3A/DNMT3B,[30] phosphatase and tensin homolog,[34] CCND2[16] and so on. Several studies successively demonstrated that miR-29b suppressed tumor invasion and metastasis by targeting matrix metalloproteinase 2 in hepatocellular,[12] gastric,[16] prostate,[14] and colon cancers.[17] These results suggest that the effects of miR-29b may be tumor specific and highly dependent on its target in each type of cancer. In our research, Tiam1, overexpressed in CRC, was firstly validated as a target of miR-29b by binding directly in the Tiam1 3'UTR. Our previous studies have reported that lentivirus-mediated RNAi resulted in the effective inhibition of *in vitro* cell growth and of the invasive ability of CRC cells. An orthotopic xenograft model confirmed that Tiam1 silence reduces tumor growth and metastais,[35] showing similar biological effects of miR-29b introduction. We also successfully generated Tiam1 transgenic mice, which developed larger and more aggressive neoplasm than wt mice, suggesting its causal role in CRC metastasis.[36] The present study provides novel basis for the abnormal expression of Tiam1 in CRC. We have demonstrated that miR-29b inhibits Tiam1 expression. Tiam1 introduction can rescue miR-29b-mediated biological behaviors. These results suggest that the inhibitory effect of miR-29b is mediated in part through the repression of Tiam1 expression.

Taken together, the identification of miR-29b as a tumor-suppressive miRNA in human CRC that acts by repressing Tiam1 provides additional evidence of a pivotal role for miRNAs in CRC tumorigenesis and progression. Given that miR-29b is downregulated in CRC, the introduction of this mature miRNA into the tumor tissue could serve as a therapeutic strategy by reducing the expression of target genes. miRNA-based therapeutics are still in their infancy; however, our findings are encouraging and suggest that this miRNA could be targeted for the development of a treatment for patients with CRC, especially metastatic CRC, in the future.

Materials and Methods

Cell culture and miRNA transfection. CRC cell lines HT29, HCT116, SW480 and SW620 were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) and maintained as previously described.[10] In addition, a human CRC cell subline with unique liver metastatic potential, designated SW480/M5, was established in our laboratory[37] and used in the analysis. The cells were cultured in RPMI 1640 (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (Gibco-BRL, Invitrogen; Paisley, UK) at a humidity of 5% CO_2 at 37 °C. The normal human colon epithelial cell line NCM460 was provided by Incell Corporation (San Antonio, TX, USA) and cultured in M3 base culture media as previously described.[38]

miRNAs were transfected at a working concentration of 100 nmol/l using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). The miR-29b mimic, a nonspecific miR control, anti-miR-29b, and a nonspecific anti-miR control were all purchased from GenePharma (Shanghai, China). Protein and RNA samples were extracted from subconfluent cells during the exponential phase of growth.

Tumor tissue sample. Fresh primary CRC specimens and paired non-cancerous colorectal tissue were provided by the Tumor Tissue Bank of Nanfang

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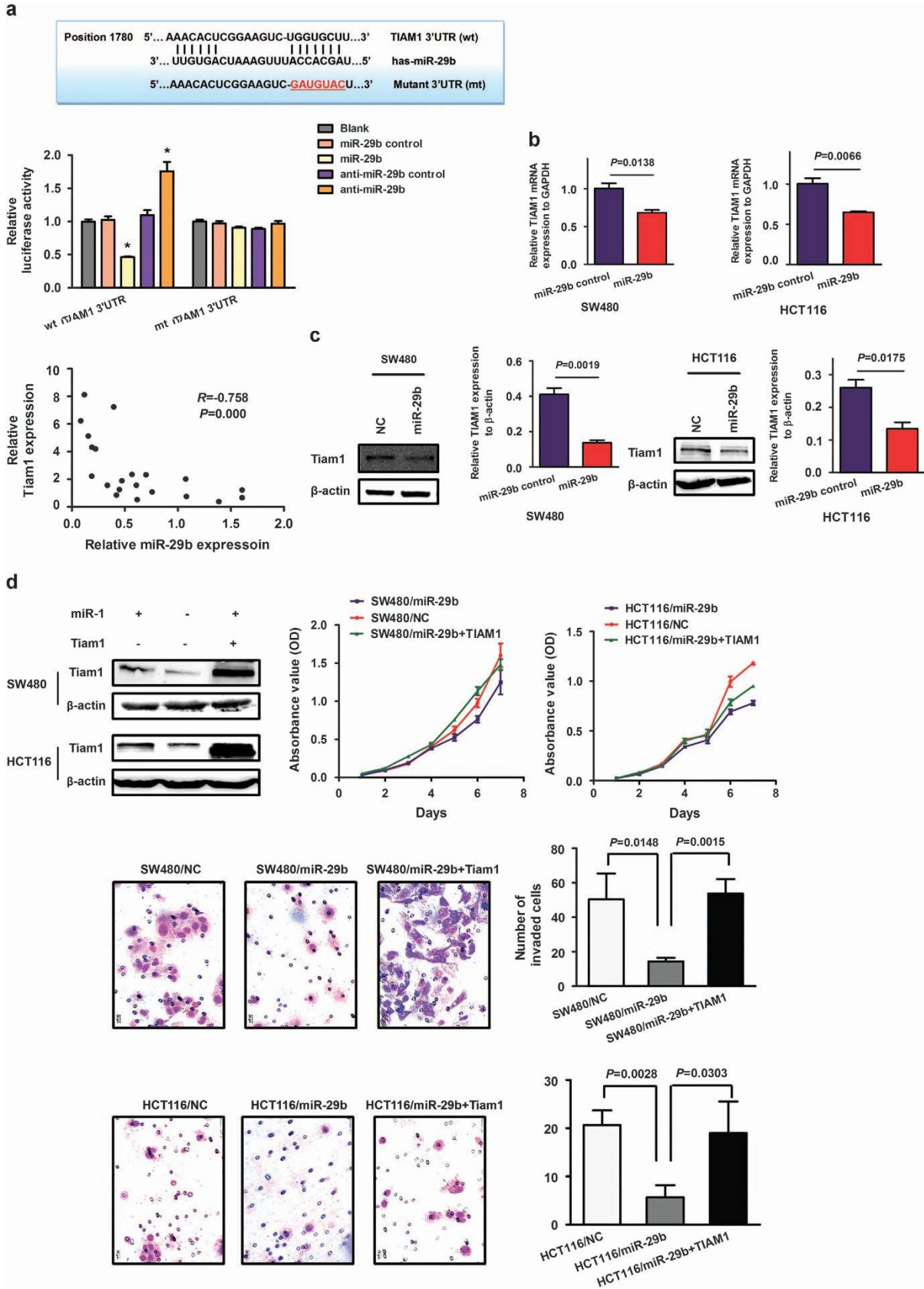
Figure 6 Tiam1 was a direct target of miR-29b in CRC cells. (**a**) Diagram of Tiam1 3'UTR containing putative conserved target site for miR-29b, which were identified using the TargetScan database. Results of luciferase reporter assays in 293T cells, with co-transfection of wt or mt 3'UTR and miR mimic and inhibitor, as indicated (right). The expression of Tiam1 mRNA was compared with GAPDH mRNA in CRC versus non-cancerous tissue by quantitative real-time PCR (qRT-PCR) analysis. A statistically significant inverse correlation between miR-29b and Tiam1 mRNA was observed in CRC specimens. (**b**) Tiam1 mRNA expression in SW480 cells 24 h after transfection with miR-29b was detected by qRT-PCR analysis. (**c**) Tiam1 protein expression in SW480 cells 48 h after transfection with miR-29b was detected by western blot analysis (Left). The immunosignal was quantified using densitometric scanning software, and the relative protein abundance was determined by normalization with β-actin (right). (**d**) SW480 cells transfected with miR-29b mimic and/or Tiam1 cDNA were used to determine the role of Tiam1 in miR-29b-mediated biological behaviors. The expression of Tiam1 protein was detected by western blot analysis (upper left). Cell proliferation was determined by CCK-8 assay (upper right). Results of transwell assay, which was carried out to evaluate the effect of cell migration after transfection are shown (down)

Hospital. In each case, a diagnosis of primary CRC had been made, and the patient had undergone elective surgery for CRC in Nanfang Hospital between 2007 and 2010. The pathological diagnosis was made in the Department of Pathology of Nanfang Hospital of Southern Medical University. The study was approved by the Ethics Committee of Southern Medical University and all aspects of the study comply with the Declaration of Helsinki. Ethics Committee of the Southern Medical University specifically approved that no informed consent was required because data were going to be analyzed anonymously.

Western blot analysis. Protein expression was assessed by immunoblot analysis of cell lysates (20–60 μg) in RIPA buffer in the presence of mouse antibodies to T lymphoma invasion and metastasis 1 (Tiam1), E-cadherin, β-catenin, Vimentin, β-actin (1 : 500; Santa Cruz, CA, USA); and rabbit antibodies to p-Akt (Ser473), p-Akt (Thr308), AKT, p44/42 mitogen-activated protein kinase (MAPK) (extracellular signal–regulated kinase (ERK)1/2), p-p44/42 MAPK (ERK1/2) (1 : 1000; CST, Danvers, MA, USA); and T-complex polypeptide 1 subunit beta (CCT2; 1 : 500; Abcam, Cambridge, UK).

Immunofluorescence. Cells were cultured on cover slips overnight, fixed with 4% paraformaldehyde for 20 min and treated with 0.25% Triton X-100 for 10 min. After blocking in 10% normal blocking serum at room temperature for 10 min, slides were incubated with mouse anti-E-cadherin, anti-β-catenin and anti-Vimentin (1 : 50; Santa Cruz) antibodies at 4 °C overnight followed by washing with PBS three times. Cover slips were then incubated with fluorescein isothiocyanate-conjugated anti-rabbit and Texas Red-conjugated anti-mouse antibodies (1 : 120, Santa Cruz) for 30 min at room temperature, then stained with 6-diamidino-2-phenylindole (Invitrogen).

Bioinformatics. Potential miRNA targets were predicted and analyzed using three publicly available algorithms: PicTar, TargetScan and miRanda.[39] The number of false-positive results was decreased by accepting only putative target genes that were predicted by at least two programs.

miRNA target validation. A 1950-bp fragment of the *Tiam1* 3'untranslated region (3'UTR) was amplified by PCR and cloned downstream of the firefly luciferase gene in the psiCHECK-2 vector (Promega, Madison, WI, USA). This vector was named wild-type (wt) 3'UTR. Site-directed mutagenesis of the miR-29b binding site in the *Tiam1* 3'UTR was carried out using the GeneTailor Site-Directed Mutagenesis System (Invitrogen) and named mutant (mt) 3'UTR. For reporter assays, the wt or mt 3'UTR vector and miR-133a mimic or inhibitor were co-transfected. Luciferase activity was measured 48 h after transfection using the Dual-Luciferase Reporter Assay System (Promega).

Plasmid construction and stable transfection. The precursor sequence of miR-29b was cloned into the pcDNA-GW/EmGFP-miR plasmid expression vector (GenePharma). A construct that included the nonspecific miR-negative control (NC; 99 bp) was used as a NC. The constructed vectors were named pcDNA-GW/EmGFP-miR-29b and pcDNA-GW/EmGFP-miR-NC. pcDNA-GW/EmGFP-miR encodes a red-shifted variant of wt green fluorescent protein (GFP) that has been optimized for brighter fluorescence and greater expression in mammalian cells. SW480 cells were seeded into six-well plates and transfected with the miR-29b–expressing vector or the control vector expressing a nonspecific miR-NC using Lipofectamine 2000 (Invitrogen) then selected using blasticdin (2.5 μg/ml) to generate two stable monoclonal cell lines: an miR-29b stable cell line (SW480/miR-29b) and a control stable cell line (SW480/miR-NC).

Statistical analysis. Data were analyzed using SPSS version 13.0 software (SPSS; Chicago, IL, USA). The Student's *t*-test and the one-way ANOVA test

were carried out for qRT-PCR and CCK-8 analyses and to calculate the tumor growth curve. The correlation between miR-29b and Tiam1 was determined using the Spearman rank correlation test. Statistical significance was established at $P < 0.05$.

Conflict of Interest

The authors declare no conflict of interest.

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