Carcinogenesis vol.35 no.4 pp.886–895, 2014
doi:10.1093/carcin/bgt378
Advance Access publication November 21, 2013

miR-320a suppresses colorectal cancer progression by targeting Rac1

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MicroRNAs (miRNAs) have emerged as critical epigenetic regulators involved in cancer progression. miR-320a has been identified to be a novel tumour suppressive miRNA in colorectal cancer (CRC). However, the detailed molecular mechanisms are not fully understood. Here, we reported that miR-320a inversely associated with CRC aggressiveness in both cell lines and clinical specimens. Functional studies demonstrated that miR-320a significantly decreased the capability of cell migration/invasion and induced G_0/G_1 growth arrest *in vitro* and *in vivo*. Furthermore, *Rac1* was identified as one of the direct downstream targets of miR-320a and miR-320a specifically binds to the conserved 8-mer at position 1140–1147 of *Rac1* 3′-untranslated region to regulate Rac1 protein expression. Over-expression of miR-320a in SW620 cells inhibited Rac1 expression, whereas reduction of miR-320a by anti-miR-320a in SW480 cells enhanced Rac1 expression. Re-expression of Rac1 in the SW620/miR-320a cells restored the cell migration/invasion inhibited by miR-320a, whereas knockdown of Rac1 in the SW480/anti-miR-320a cells repressed these cellular functions elevated by anti-miR-320a. Conclusively, our results demonstrate that miR-320a functions as a tumour-suppressive miRNA through targeting *Rac1* in CRC.

Introduction

Colorectal cancer (CRC) is one of the most prevalent malignancies leading to the cancer-related death in the world (1). The progression of this disease involves multi-step genetic events, among those the tumour cell invasion and metastasis have been acknowledged as the major causes of mortality (2,3). Although many metastasis-related genes have been identified in CRC (4), the epigenetic alterations that promote tumour cell invasion and metastasis are largely unknown. Recent studies have revealed that the non-coding microRNAs (miRNAs) are novel regulators of tumour progression and novel targets for cancer therapy in CRC (5,6).

miRNAs are 22–25 nucleotide short single-stranded non-coding RNAs that directly bind to the 3′-untranslated regions (3′-UTRs) of target messenger RNAs (mRNAs), leading to mRNA degradation or translational suppression (7). miRNAs act as crucial regulators in many physiologic processes, including cellular proliferation, differentiation, apoptosis and metabolism (8–10). In cancer cells, miRNAs play a key role in tumour development by regulating the expression of oncogenes and tumour suppressors or by directly exerting their functions as oncogenes or tumour suppressors (6,11–14). For instance, a group of miRNAs, such as miR-10b and miR-21 are able to initiate invasion and metastasis in breast cancer (15,16), whereas some of other miRNAs including miR-200 family and miR-126 exert their inhibitory effect on invasion and metastasis in breast cancer (17,18). In CRC, the miRNAs, such as miR-200b, miR-106b and miR-21, were found to be highly expressed in aggressive tumours (19,20), whereas the miRNAs, such as miR-320a, miR-218, miR-141 and miR-34a, were down-regulated in this type of tumours (19,21–23). Recently, miRNA expression signatures have been well documented and used as prognostic biomarkers in CRC patients (24–26). Notably, miR-320a was identified as a metastatic suppressor in CRC and high expression of miR-320a in stage II CRC tumours was associated with better disease-free survival in CRC patients (25). Hence, miR-320a is an important suppressive miRNA in CRC development and metastasis.

Mechanistic studies revealed that miR-320a exerts its suppressive function by targeting *neuropilin 1* in CRC cells (19), *aquaporin 1 and 4* in cerebral ischemia (27) and *guanine nucleotide-binding protein G subunit alpha-1* in hepatocellular carcinoma (28). A recent study also showed that miR-320 inhibited Wnt/beta-catenin signaling pathway by targeting the 3′-UTR of *β-catenin* mRNA (29). The characteristic of miR-320a, which regulates the expression of multiple targets, implicates its significance in the regulatory network for disease development. Hence, identification and characterization of its novel downstream targets may provide new insights into understanding the biological and pathological roles of miR-320a in CRC.

In the present study, we demonstrated that miR-320a is significantly down-regulated in CRC progression and the development of metastasis. Ectopic expression of miR-320a in CRC cells suppressed invasion/migration *in vitro* and tumouriogenic capacity *in vivo*. Furthermore, we identified and validated *Rac1* gene as a novel and direct target of miR-320a, as assessed by mutagenesis analysis of 3′-UTR of *Rac1* gene and luciferase activity. Substantial studies indicate that Rac1 plays a critical role in miR-320a-regulated inhibition of cancer cell invasion and metastasis.

Materials and methods

Cell culture

Human colorectal cancer cell lines SW480, SW620, LOVO, SW1116 and HT29 were purchased from American Type Culture Collection. SW480, SW620 and SW1116 were cultured in Leibovitz's L-15 Medium (Corning Cellgro®; Corning, Manassas, VA), LOVO in F-12K Medium (Corning Cellgro®; Corning), and HT29 in McCoy's 5A Medium (Corning Cellgro®; Corning). All culture media were supplemented with 10% foetal bovine serum (Invitrogen, Carlsbad, CA). Cells were maintained at 37°C/5% CO_2 in a humidified incubator.

Clinical specimens

The clinical research protocol was approved by the Ethical Committee of Ruijin Hospital, Shanghai Jiaotong University School of Medicine. Sixty pairs of resected surgical specimens from primary CRC tumour and adjacent non-tumour sites were obtained from Shanghai Minimal Invasive Surgery Center (2011–12) with written consent from all patients. Patients who had received neoadjuvant chemo- and radio-therapy, or had unresectable colorectal cancers, or had tumors of other organs, or were unlikely to be interviewed

Abbreviations: 3′-UTR, 3′-untranslated region; CRC, colorectal cancer; EMT, epithelial–mesenchymal transition; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GTP, guanosine triphosphate; miRNA, microRNA; mRNA, messenger RNA; PBS, phosphate-buffered saline; qRT–PCR, quantitative reverse transcription–polymerase chain reaction; siRNA, small interfering RNA.

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during the follow-up, were excluded. The pathologic classification/staging of tumours was performed in accordance to the Cancer Staging Manual from the International Union Against Cancer (seventh edition, 2009) and the demographic and clinicopathological data are listed in Supplementary Table 1, available at *Carcinogenesis* Online. Primary tumours were classified into two groups: tumours showing metastasis and tumours showing non-metastasis, based on the status of lymph node metastases, which were histologically confirmed and/or distant metastasis diagnosed on ultrasound imaging and computerized tomography scan

RNA and miRNAs extraction and reverse transcription–polymerase chain reaction

Total RNAs from cell lines and tissues were extracted using Trizol reagent (Invitrogen) according to the manufacturer's instruction. Reverse transcription of RNAs was performed using PrimeScript RT Master Mix Perfect Real Time (TaKaRa, Shiga, Japan) as per protocol. The mRNA level of *Rac1* (forward: 5′-CGTGG ATCCG TGTGT GTGAT CAAAG GAC-3′; reverse: 5′-CGGAA TTCAT GGCAG GTGTA AGAGA AAGG-3′) were assessed by semi-quantitative reverse transcription–polymerase chain reaction (semi-qRT-PCR) using *Taq* PCR MasterMix (Tiangen, Beijing, China). Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) (forward: 5′-GGAGC GAGAT CCCTC CAAAAT-3′; reverse: 5′-GGCTG TTGTC ATACT TCTCA TGG-3′) was used as an internal loading control.

Total miRNAs were extracted using mirVana™ miRNA Isolation Kit (Applied Biosystems, Foster City, CA). The expression level of miR-320a was assayed as follows: complementary DNA was reverse transcribed from miRNAs using the specific miRNA primers from the TaqMan MicroRNA Assays (Applied Biosystems) and reagents from the TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems), followed by PCR amplification using the TaqMan MicroRNA Assay together with the TaqMan® Universal PCR Master Mix (Applied Biosystems) in an Applied Biosystems 7500 Fast Real-Time PCR System. The expression level of miR-320a was normalized using U6 small nuclear RNA (RNU6B; Applied Biosystems) by the $2^{-\Delta CT}$ method.

Lentiviral transfection for stable expression clones

Plasmids LV3-pGLV-H1-GFP+Puro with hsa-miR-320a mimics or hsa-miR-320a inhibitor or their respective control oligonucleotides, namely LV-miR-320a and LV-miR-NC (negative control), and LV-anti-miR-320a and LV-anti-miR-NC, were purchased from GenePharma (Shanghai, China). Lentivirus transfection was performed according to the manufacturer's instruction to establish miR-320a-expressing stable clones in SW620 cells (SW620/miR-230a) and anti-miR-320a-expressing stable clones in SW480 cells (SW480/anti-miR-320a). The relative control clones (SW620/miR-NC and SW480/anti-miR-NC) were produced by the similar methods. Expression of miR-320a expression level was examined by qRT–PCR using U6 RNA as an endogenous control.

Protein extraction and immunoblot

Total protein was extracted with radioimmunoprecipitation assay buffer (Solarbio, Beijing, China) containing protease inhibitor cocktail (Roche Applied Science, Basel, Switzerland). Western blot was done by the standard protocol, with primary antibodies against Rac1 (Abcam, Cambridge, UK), vimentin (Abcam), E-cadherin (Cell Signaling Technology, Danvers, MA) or GAPDH (Kangchen, Shanghai, China). Goat anti-mouse or goat anti-rabbit IgG horseradish peroxidase (Upstate Biotechnology, Lake Placid, NY) was used as the secondary antibody. Chemiluminescent signals were visualized using SuperSignal West Pico ChemiluminescentSubstrate (Pierce, Rockford, IL) and the signal intensity was analysed using the Image Lab™ Software Version 4.0.1 (BIO-RAD, Hercules, CA). The experiments were performed in triplicate with GAPDH (Kangchen) as a loading control.

Transient transfection

Small interfering RNA (siRNA) against human *Rac1* and siRNA negative control were supplied from GenePharma. Plasmid pcDNA3-EGFP-Rac1-wt and pcDNA 3-EGFP vector control were donated by Dr Qu Y (Ruijin hospital, Shanghai Jiao Tong University School of Medicine). For transient transfection, cells were seeded in 6-well culture plates and transfection was performed at 60% cell confluence with lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Forty-eight hours after transfection, cells were harvested and the protein levels of the targeted genes were assessed by western blot, with GAPDH as a loading control.

Construction of plasmids and luciferase activity assay

Prediction of miR-320a binding sites was performed using TargetScan software (http://www.targetscan.org). Bioinformatics analysis revealed two putative miR-320a binding sites for miR-320a at 611 and 1140 in *Rac1* 3′-UTR region. Two fragments of mutant *Rac1*-3′-UTR were therefore constructed. The 3′-UTR of *Rac1* containing both miR-320a binding sites was amplified by PCR using its specific forward primer with SpeI restriction site (5′-GG *ACTAGT* CCGAG AAAAT GCCTG CTGTT GT-3′) and reverse primer with HindIII site (5′-TTCTC GCCAG TGAGT TAAGTT GCC *AAGCTT* GG-3′). The PCR product was digested with SpeI/HindIII and inserted at the SpeI and HindIII sites of pMIR-REPORT luciferase vector (Applied Biosystems) to form wild-type construct pMIR/*Rac1*-wt. Mutation of 3′-UTR miR-320a binding sites at 661 (5′-CCAGCUUU-3′ to 5′-CGACCAUA-3′) and 1140 (5′-CAGCUUU-3′ to 5′-GACCAUA-3′) were, respectively, generated to produce mutant constructs, pMIR/*Rac1*-mut611 and pMIR/*Rac1*-mut1140, using a QuickChange Site-Directed Mutagenesis kit (Stratagene, Santa Clara, CA). All constructs were verified by sequencing.

SW620 cells at 60% confluence were transfected with 200 ng luciferase reporter gene construct and 2 ng pRL-TK vector (Promega, Madison, WI) containing Renilla luciferase in combination with miR-320a mimics (GenePharma; final concentration of 100 nM) or miR-NC (100 nM). Transfection was performed with lipofectamine2000 (Invitrogen). Luciferase activity was measured using the Dual Luciferase Reporter Assay (Promega). Firefly luciferase activity was normalized to Renilla luciferase activity for each transfected well. All transfection experiments were conducted in triplicate and repeated three times independently. Data are expressed as the mean ± SD.

Immunofluorescence staining

Immunofluorescence analysis was carried out as described previously (30). In brief, cells growing on coverslips were fixed with 4% paraformaldehyde (Sigma–Aldrich, St Louis, MO), permeabilized for 10 min using 0.1% TritonX-100/phosphate-buffered saline (PBS) and were then blocked with 3% bovine serum albumin in PBS-T (0.2% Tween 20) for 1 h at room temperature. After washing, cells were incubated with primary antibodies against Rac1 overnight in 4°C, followed by incubation with Alex Fluor® 532 goat anti-rabbit IgG (Invitrogen) for 2 h at room temperature. Coverslips were then mounted with antifade reagent with DAPI (Invitrogen) after final washes with PBS. Images were examined and captured using an Olympus Fluoview Confocal Microscope. Rabbit mAb IgG XP isotype (Cell Signaling Technology) was used as negative control.

Migration and invasion assay

Cell invasion and migration activity were assessed using cell culture inserts coated with or without basement membrane matrix (BD Biosciences, Bedford, MA) according to the manufacturer's instruction. Briefly, ~1×10^5 cells in 200 µl non-serum culture medium were placed in triplicate in upper chamber of insert and incubated at 37°C for 48 h in a 5% CO_2 humidified incubator, with medium/10% foetal bovine serum in lower chamber. Cells in upper chamber were then cleared softly with cotton swab and the cells at the bottom of insert were stained in 1% crystal violet for 30 min. Cells were counted under microscope in five random fields and the relative invasion and migration were interpreted as the average number of cells ± standard deviation per field.

Cell proliferation assay

Cell proliferation was examined using water-soluble tetrazolium salt assay using the Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). In short, cells (1.5×10^3/well) were seeded in 96-well culture plates in triplicate and incubated for 4 days at 37°C/5% CO_2 in a humidified incubator. Viable cells were quantified at each 24 h interval by measuring OD_{450} using microplate reader (Epoch; BioTek, Winooski, VT).

Apoptosis and cell cycle analysis

Cells (2×10^6) were fixed with 75% ethanol at 4°C overnight and then washed with cold PBS and treated with RNaseI, followed by staining with propidium iodide for 30 min in dark. Cell cycle analysis was then performed by flow cytometry (FACSCalibur; Becton Dickinson, Sparks, MD). Apoptotic cells were stained using Annexin V-FITC Apoptosis Detection Kit I (BD Biosciences) and analysed by flow cytometry (FACSCalibur) as per manufacturer's instruction.

In vivo tumour growth assay

SW620/miR-320a or SW620/miR-NC cells (5×10^6 in 0.2 ml of sterilized PBS buffer) were implanted subcutaneously into the left flank of 5-week-old male nude mice (8 mice per group). Tumour growth was determined by measuring the tumour volume (mm^3, V = tumour length × tumour width^2/2) every 2–3 days using calipers (30). Mice were euthanized after 8 weeks post-inoculation and the tumours were excised. The animal studies and the experimental protocol were approved by the Institutional Animal Care and Use Committee of the Shanghai Jiao Tong University. All animal experiments were performed according to the guidelines on the care and use of animals for scientific use.

Immunohistochemistry

Tumour tissues were fixed, embedded and stained with haematoxylin and eosin (Sigma–Aldrich). Immunohistochemistry staining was performed as described previously (30) using anti-vimentin (Abcam), anti-E-cadherin (Cell Signaling

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Technology), anti-Ki-67 (Santa Cruz Biotechnology) or anti-Rac1 (Abcam). Negative controls were obtained by excluding the primary antibody. The sections were stained with the 2-Solution DAB Kit (Invitrogen) according to the manufacturer's procedure and were counterstained with Mayer's haematoxylin (Sigma–Aldrich). Staining levels were defined as negative (no or weak staining) and positive (moderate or strong staining) based on the staining intensity in the tumour cells.

Active-Rac1 assay

Cells (SW620/miR-320a, SW620/miR-NC, SW480/anti-miR-320a and SW480/anti-miR-NC) were harvested and lysed with the provided cell lysis buffer in Rac1 Activation Assay Biochem kit (Cytoskeleton, Denver, CO). Active Rac1 [guanosine triphosphate (GTP)-bound Rac1] was enriched and pulled down with PAK-PBD beads as per manufacturer's protocol. Active Rac1 was detected by western blot using the primary antibody against active Rac1 (Abcam). GAPDH was used as a loading control.

Statistical analysis

Student's *t*-test or one-way analysis of variance was used for statistical analysis when appropriate. All statistical analyses were performed using the SPSS 16.0 (SPSS, Chicago, IL). A two-tailed value of $P < 0.05$ was considered statistically significant.

Results

miR-320a inversely associates with aggressiveness and metastasis in CRC

To understand the role of miR-320a in CRC, miR-320a level was examined by qRT–PCR in a series of CRC cell lines and in specimens from a group of CRC patients. As shown in Figure 1A, differential expression of miRNA-320a was observed in a cell line-dependent manner, which show miR-320a was highly expressed in SW480 and HT29 cells, which show lower migratory capacity as indicated in Figure 1B. In contrast, miR-320a showed a relatively lower expression in SW620 and LOVO cells (Figure 1A) with high migratory activity (Figure 1B). Apparently, miR-320a expression showed an inverse relation to the cell migratory ability in these CRC cells ($r = 0.96$, $P < 0.01$). In addition, miR-320a expression also inversely correlated to the cell proliferative ability in these examined CRC cell lines ($r = 0.95$, $P < 0.05$).

To study the association of miR-320a expression with the clinicopathological factors in CRC, we examined the miR-320a expression patterns by qRT–PCR in 60 primary CRC tissues in which 26 tumours have spontaneously developed metastasis (including lymph node and distant metastasis). The miRNA-320a levels in tumours were defined as low or high using the median expression level of miRNA-320a as a cut-off value. As shown in Supplementary Table 2, available at *Carcinogenesis* Online, while the expression of miRNA-320a was not related to age ($P = 0.196$), gender ($P = 0.832$), tumour histology ($P = 0.669$) or tumour site ($P = 0.769$), miR-320a was inversely associated with advanced TNM (Tumour Nodes Metastasis) stage ($P < 0.01$). Further, as shown in Figure 1D, miR-320a expression was significantly reduced in the tumours showing metastasis, compared with those showing non-metastasis ($P < 0.01$). Hence, these results indicate that miR-320a is a suppressive factor in CRC progression.

miR-320a suppresses CRC cell migration/invasion and proliferation in vitro

To further verify the suppressive function of miR-320a in CRC, SW620 cells, which showed a low level of miR-320a (Figure 1A), were transfected with LV-miR-320a mimics to establish SW620/miR-320a stable clones for miR-320a expression. SW620 cells transfected with LV-miR-NC (i.e. SW620/miR-NC cells) were used as control. After confirming the efficacy of transfection by qRT–PCR, the SW620/miR-320a and SW620/miR-NC cells were then applied for invasion and migration assay. As indicated in Figure 2A (left panel), SW620/miR-320a cells showed >2-fold decrease ($P < 0.01$) in both invasive and migratory abilities, compared with the SW620/miR-NC cells.

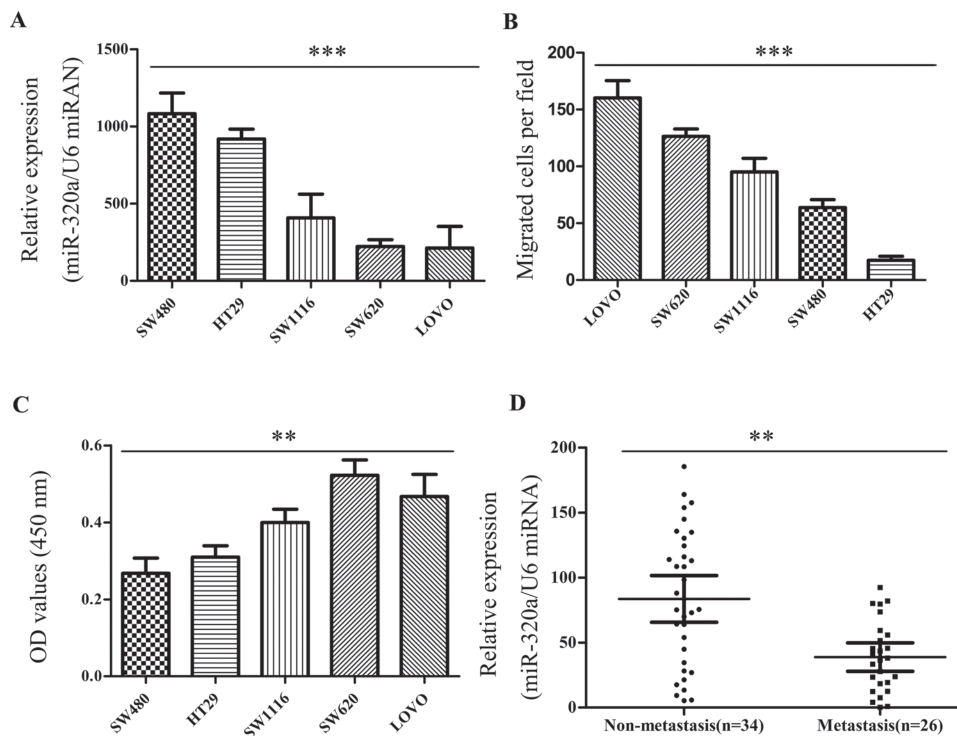

Fig. 1. miR-320a reduction correlates to the increased colorectal cancer aggressiveness. (**A**) Relative expression of miR-320a in five CRC cell lines was examined by qRT–PCR. U6 small nuclear RNA was used as an internal control. (**B**) Migration: migratory abilities of five CRC cell lines were determined by migration assays as described in the Materials and methods. (**C**) Cell proliferation: equal number of cells (1.5×10^3 cells) was seeded in 96-well plate and incubated at 37°C/5% CO_2 in cell culture incubator. Living cells were assayed using water-soluble tetrazolium salt on day 3. (**D**) Levels of miR-320a expression in primary CRCs with or without metastasis. The levels of miR-320a were examined by qRT–PCR using U6 RNA as a control. Data represent the means ± SD from three independent experiments. $^{**}P < 0.01$; $^{***}P < 0.001$.

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On the other hand, SW480 cells that expressed high level of miR-320a were, respectively, transfected with LV-anti-miR-320a and its control LV-anti-miR-NC to produce stable clones, namely SW480/anti-miR-320a and SW480/anti-miR-NC cells. Anti-miR-320a is a complementary fragment to miR-320a and can repress miR-320a's function by direct binding, whereas anti-miR-NC oligonucleotides does not have this function. Therefore, SW480/anti-miR-320a cells should display an elevated invasive and migratory capacity compared with the SW480/anti-miR-NC cells. Indeed, as demonstrated in Figure 2B, SW480/ anti-miR-320a cells showed higher migratory and invasive capacity than the SW480/anti-miR-320a-NC cells ($P < 0.05$). Hence, miR-320a suppresses invasion and migration *in vitro* in CRC cells.

Given the apparent phenomenon that miR-320a level inversely relates to cell proliferative ability in the CRC cell lines examined (Figure 1C), the role of miR-320a in growth repression was assessed. As indicated in Figure 2C (left panel), SW620/miR-320a cells showed a remarkably inhibited growth at day 4 relative to the SW620/miR-NC cells. Conversely, expression of anti-miR-320a in the slow-growing SW480 cells enhanced their growth ability over the SW480/anti-miR-NC cells (Figure 2C, right panel). The effect of miR-320a on cell proliferation was further assessed by cell cycle analysis. As demonstrated in Figure 2D, SW620/miR-320a cells showed an increased cell populations at G_0/G_1 phase and concomitantly a reduction of cell populations at G_2/M phase. On the other hand, the SW480/anti-miR-320a cells displayed a significant reduction of cell populations at G_0/G_1 phase and a relative increase of cell populations at S phase, referring to their respective negative control cells (Figure 2D). These results suggest that miR-320a expression could lead to G_0/G_1 growth arrest. Nevertheless, comparing to their respective controls, expression of miR-320a in SW620 or anti-miR-320a in SW480 cells did not significantly affect cell apoptosis (Supplementary Figure 1, available at *Carcinogenesis* Online). Taken together, miR-320a expression suppresses cell proliferation by affecting cell cycle distribution.

miR-320a inhibits tumour growth and cell invasion in vivo

To further verify the suppressive role of miR-320a in CRC cancer cell aggressiveness, we next performed the animal experiment to evaluate the effect of miR-320a on CRC tumour progression *in vivo*. To this end, the SW620/miR-320a cells and SW620/miR-NC control cells were, respectively, injected into nude mice subcutaneously. As demonstrated in Figure 3, expression of miR-320a in SW620 cells inhibited tumour growth (Figure 3A, left panel) and tumour size (Figure 3A, right panel), compared with the SW620/miR-NC control cells. In addition, haematoxylin and eosin staining of tumour sections showed that the tumours produced from SW620/miR-320a cells were confined within fibrous capsules in tumour specimens (Figure 3B, right panel), indicating a suppression of invasion by miR-320a. In contrast, the tumours formed from the SW620/miR-NC control cells displayed an invasive distribution into the peri-tumour stromal tissues remarkably (Figure 3B, left panel). Therefore, similar to the *in vitro* observation, miR-320a can also inhibit cell invasion *in vivo*.

To understand whether the tumour-suppressive role of miR-320a was caused by affecting cell proliferation as observed in the *in vitro* cell system (Figure 2C), tumour sections were stained with cell proliferation marker Ki-67. The proliferation index was calculated from five randomly selected microscopic fields in each tumour section. As shown in Figure 3C, the percentage of proliferative cells (Ki-67 positive) was dramatically decreased in the tumours produced from SW620/miR-320a cells, relative to the tumours from SW620/miR-NC cells. Hence, a decreased proliferation rate by miR-320a is responsible for tumour suppression *in vivo*.

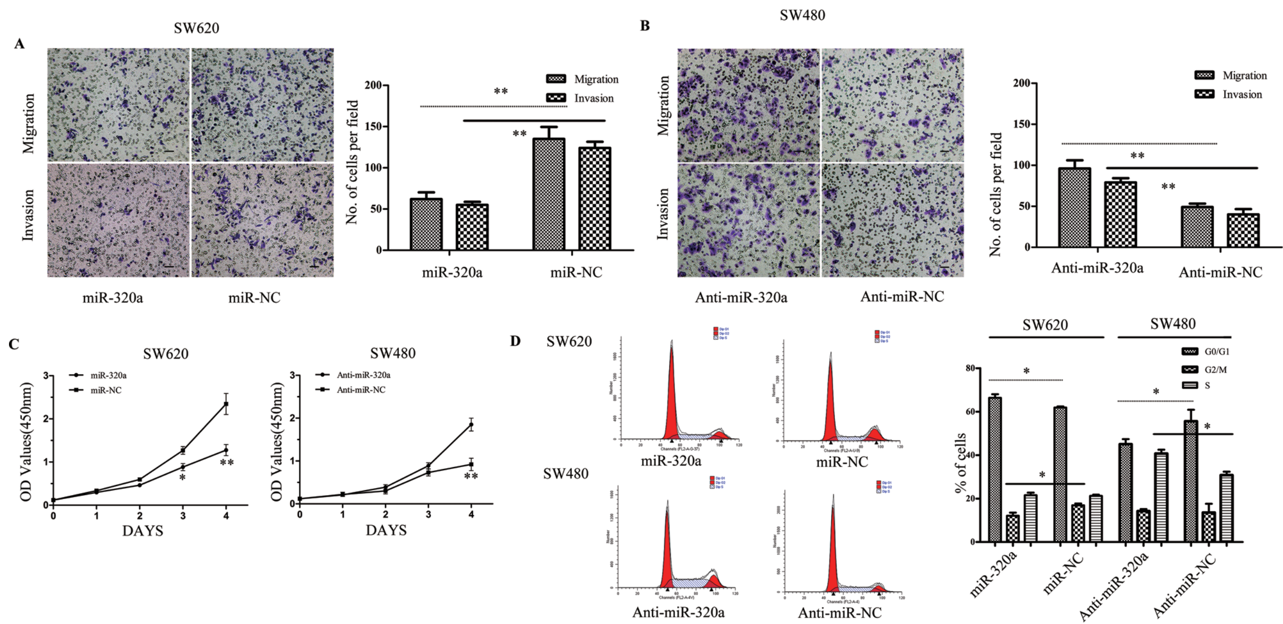

Fig. 2. miR-320a suppresses CRC cell migration/invasion and proliferation *in vitro.* (**A**) miR-320a expression suppresses migration/invasion in SW620 cells. miR-320a-expressing SW620 cells (1×10^5 cells) and control cells were seeded in upper chamber of insert and the migrated/invaded cells were examined after 48 h. (**B**) Anti-miR-320a expression increases cell migration/invasion in SW480 cells. Anti-miR-320a-expressing SW480 cells (1×10^5 cells) and control cells were seeded in upper chamber of insert and the migrated/invaded cells were examined after 48 h post-incubation. Data represent the means ± SD from three independent experiments. Representative photos of stained cells are shown with the original magnification of ×100; scale bars: 50 µm. (**C**) miR-320a inhibits cell proliferation. Cell growth was evaluated by the water-soluble tetrazolium salt assay. miR-320a expression in SW620 cells inhibited cells growth, whereas anti-miR-320a expression in SW480 cells enhanced cell growth, relative to their respective control. Data are expressed with the means ± SD from three independent experiments. (**D**) miR-320a regulates cell cycle. The SW620/miR-320a, SW620/miR-NC, SW480/anti-miR-320a and SW480/anti-miR-NC cells were serum starved for 24 h and used for cell cycle analysis. miR-320a expression in SW620 cells led to relative increase of cells at G_0/G_1 phase and concomitantly a decrease of cells at G_2/M phase. In contrast, anti-miR-320a expression in SW480 cells resulted in a reduction of cells at G_0/G_1 phase and an increase of cells at S phase. Data are interpreted as the means ± SD from three independent experiments. $^*P < 0.05$, $^{**}P < 0.01$.

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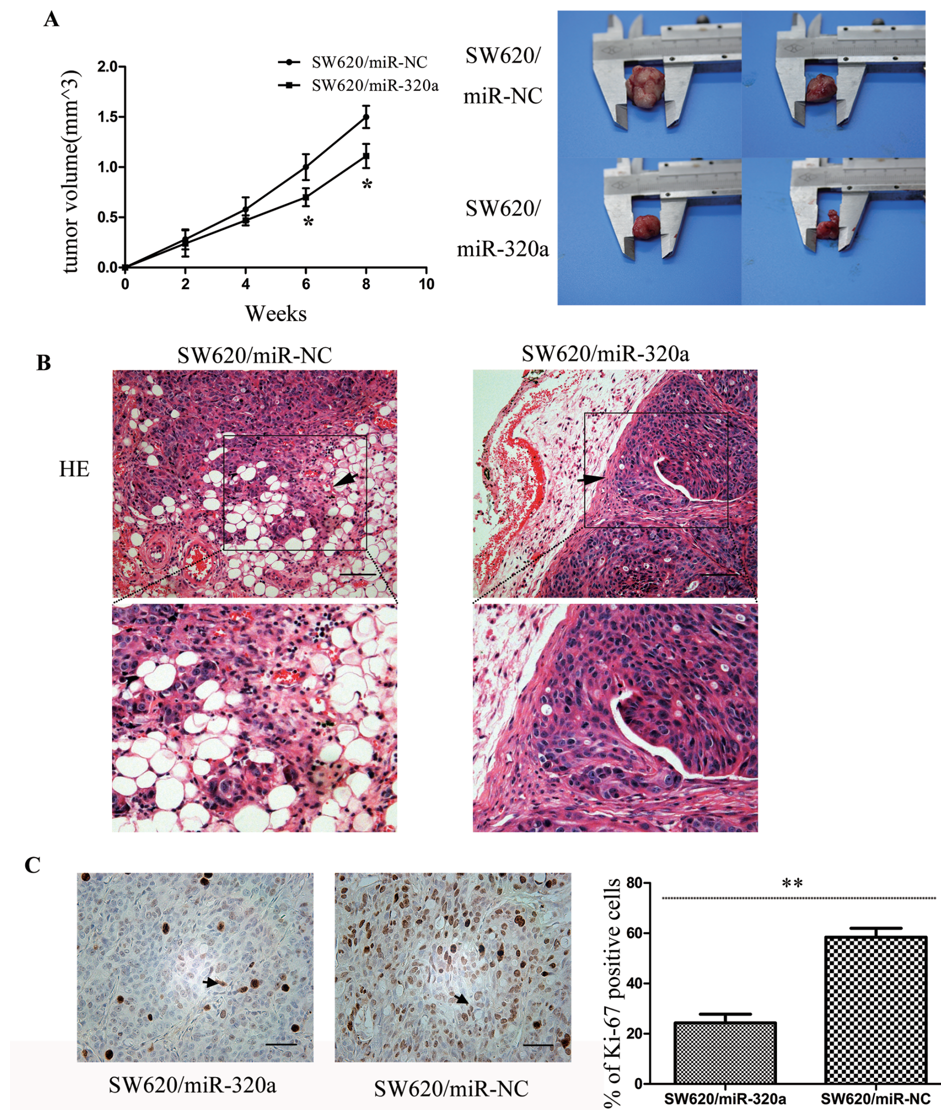

Fig. 3. miR-320a expression inhibits tumour growth, cell invasion and proliferation *in vivo*. (**A**) miR-320a suppresses tumour growth. Tumour volume was examined every 2 days after implantation of SW620/miR-320a and SW620/miR-NC (negative control) cells. (**B**) miR-320a inhibits tumour cell invasion. Tumour sections were stained with haematoxylin and eosin and tumour cell invasion was observed in the tumours produced from the SW620/miR-NC cells (indicated by arrows). An representative haematoxylin and eosin-stained section from each group is shown with an original magnification of ×200; scale bars: 50 µm. (**C**) Proliferation of tumour cells *in vivo*. The effect of miR-320a on cell proliferation in tumours was assessed by examining the proliferative index of Ki-67. Immunohistochemistry staining showed that the Ki-67 staining was significantly decreased in the SW620/miR-320a tumours compared with the SW620/miR-NC tumours. Magnification ×200. Scale bars: 50 µm. *$P < 0.05$, **$P < 0.01$.

miR-320a inhibits the epithelial–mesenchymal transition

Given the fact that the epithelial–mesenchymal transition (EMT) program is the crucial process in the acquisition of malignant traits in cancer progression (31–33), and that miR-320a expression in SW620 cells inhibited the cellular migration and invasion *in vitro* and suppressed tumour formation *in vivo*, it is assumed that miR-320a may involve in EMT program in CRC cells. Indeed, the expression of epithelial cell marker E-cadherin was significantly enhanced ($P < 0.05$) and the expression of mesenchymal cell marker vimentin was decreased ($P < 0.01$) in the SW620/miR-320a cells, compared with the SW620/miR-NC control cells (Figure 4A). On the other hand, SW480/anti-miR-320a cells expressed a remarkably reduced level of E-cadherin ($P < 0.01$) and an elevated level of vimentin ($P < 0.05$), relative to the SW480/anti-miR-NC cells (Figure 4A). Hence, the *in vitro* study indicates a repressive function of miR-320a in EMT in CRC cells. Using the mice tumour specimens, we also observed that the tumours produced from SW620/miR-320a cells showed relative high staining intensity of E-cadherin and weak staining intensity of vimentin, relative to the tumours formed from the SW620/miR-NC (Figure 4B). Hence, both the *in vitro* and *in vivo* studies indicate a significant repression of miR-320a in EMT in CRC cells.

miR-320a directly targets Rac1 3′-UTR to reduce Rac1 expression

To further identify new targets of miR-320a and understand the mechanisms underlying its tumour suppressive function in CRC, the bioinformatics tools, TargetScan (http://www.targetscan.org), was used for target search. We identified that the genes including *Rac1*, *MTDH*, *TRIM2*, *SNX4* and *CYTIP* are the potential downstream targets of miRNA-320a. Of these genes, the gene *Rac1* (gene ID: 5879) showed the highest score and is a focus of this study. Importantly, Rac1 plays a critical role in regulating cell migration and invasion (34–36).

In 3′-UTR of *Rac1* mRNA, two binding sites for miR-320a were identified: a conserved 8-mer at position 1140–1147 of 3′-UTR and a poorly conserved 7-mer-m8 at position 611–617 of 3′-UTR (Figure 5A). To understand whether miR-320a directly binds 3′-UTR of *Rac1*, we constructed luciferase reporter system with the full-length

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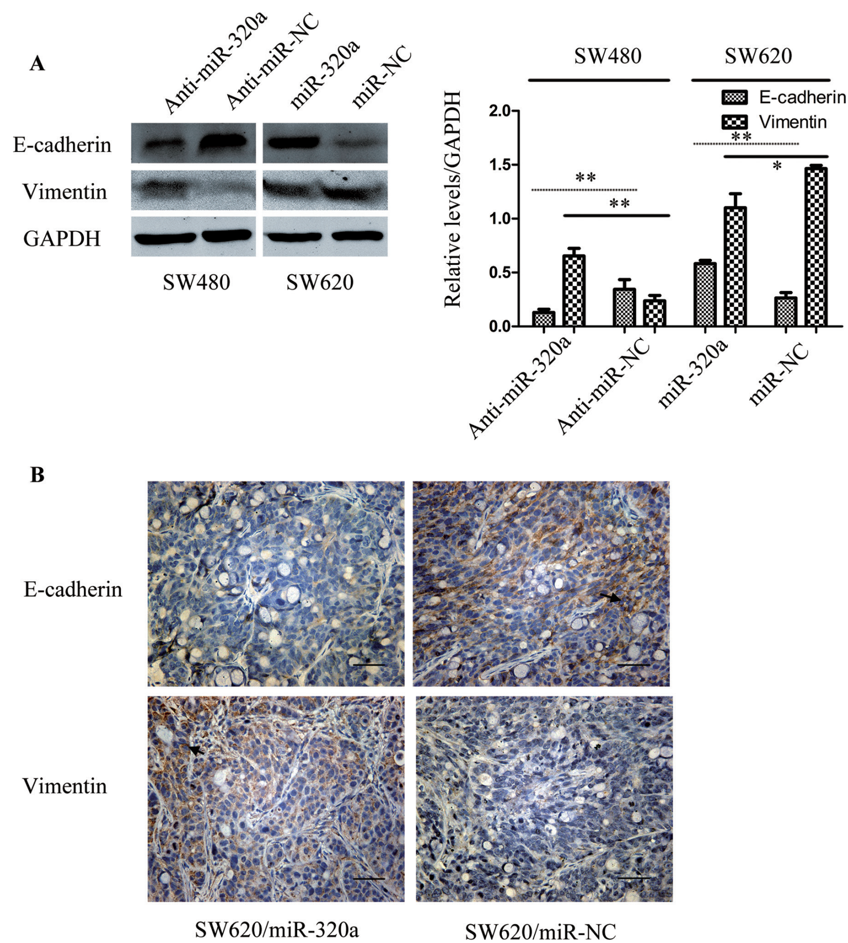

Fig. 4. miR-320a expression inhibits EMT in CRC cells *in vitro* and *in vivo*. (**A**) Effect of miR-320a on the expression of EMT markers. miR-320a expression enhanced the expression of E-cadherin and attenuated the expression of vimentin in SW620 cells. Anti-miR-320a expression decreased the level of E-cadherin and elevated the level of vimentin in SW480 cells. (**B**) EMT marker expression in tumours. Immunohistochemistry analysis showed that E-cadherin (indicated by arrows) was highly expressed, whereas vimentin (indicated by arrow) was reduced in the SW620/miR-320a tumours, compared with the SW620/miR-NC tumours. Data are expressed as the means ± SD from three separate experiments. Magnification ×200, Scale bars: 50 µm. $^{*}P < 0.05$, $^{**}P < 0.01$.

wild-type or two mutant fragments of 3′-UTR of *Rac1* mRNA, namely pMIR/*Rac1*-wt, pMIR/*Rac1*-mut611 and pMIR/*Rac1*-mut1140 (Figure 5A). These vectors were co-transfected with pRL-TK vector into SW620 cells, in combination with miR-320a or miR-NC, to examine the luciferase activity. As demonstrated in Figure 5B, compared with the miR-NC, miR-320a treatment significantly reduced the relative luciferase activity of the wild-type 3′-UTR (pMIR/*Rac1*-wt) and mutant 3′-UTR (pMIR/mut611) of *Rac1* ($P < 0.05$). However, miR-320a and miR-NC induced a relative similar luciferase activity in the cells transfected with pMIR/Rac-mut1140, suggesting that the conserved 8-mer at position 1140–1147 of 3′-UTR is essential for specific binding of miR-320a in *Rac1* mRNA. These data indicate that miR-320a can specifically bind to the 3′-UTR of *Rac1* mRNA.

To examine the regulatory effect of miR-320a in Rac1 expression, both the mRNA and protein levels were analysed in these established cell models. As shown in Figure 5C (left panel), expression of miR-320a in SW620 cells or anti-miR-320a in SW480 cells did not significantly affect *Rac1* transcription, relative to their respective controls. Instead, compared with SW620/miR-NC control, the SW620/miR-320a cells showed a significant reduction of both total Rac1 and active Rac1 (GTP-bound Rac1) protein levels (Figure 5C, right panel). In contrast, SW480/anti-mR-320a cells displayed an enhanced active Rac1 expression over the SW480/anti-miR-NC cells. These results suggest that miR-320a translationally regulates Rac1. The inhibitory effect of miR-320a on Rac1 was further verified by immunofluorescence analysis examined with Rac1 antibody (Supplementary Figure 2, available at *Carcinogenesis* Online). Furthermore, in the xenograft tumours, immunohistochemistry analysis revealed that Rac1 expression was significantly suppressed in the SW620/miR-320a tumours compared with the staining intensity of Rac1 in the SW620/miR-NC tumours (Figure 5D). Taken together, miR-320a expression attenuates Rac1 protein expression *in vitro* and *in vivo*.

Overexpression of Rac1 restores the miR-320a-induced inhibition of cell migration/invasion and proliferation

We showed that miR-320a stable expression in SW620 cells remarkably suppressed cell migration and invasion (Figure 2A) and decreased the expression of total and active Rac1 (Figure 5C, right panel). Given that Rac1 is a direct downstream target of miR-320a, we then investigated whether the decreased Rac1 is responsible for the miR-320a-induced inhibition of cell aggressiveness. To this end, Rac1 was transiently overexpressed in SW620/miR-320a cells by introducing Rac1-expressing vector pcDNA3-EGFP-Rac1-wt. Vector pcDNA3-EGFP-Rac1-wt encodes the entire coding region of *Rac1* without 3′-UTR element, yielding an mRNA that is resistant to miR-320a-mediated inhibition of translation of EGFP-Rac1 mRNA. Rac1 was fused with EGFP and expressed as a fusing protein as verified by western blot in Figure 6A. Functional studies revealed that, compared with the SW620/miR-320a cells transfected with pcDNA3-EGFP vector control, ectopic expression of Rac1 in SW620/miR-320a cells significantly enhanced the capabilities of cell migration and invasion to the similar levels as observed in SW620/miR-NC cells (Figure 6B, left panel). In addition, because the SW620/miR-320a cells showed a growth arrest at G_0/G_1 phase

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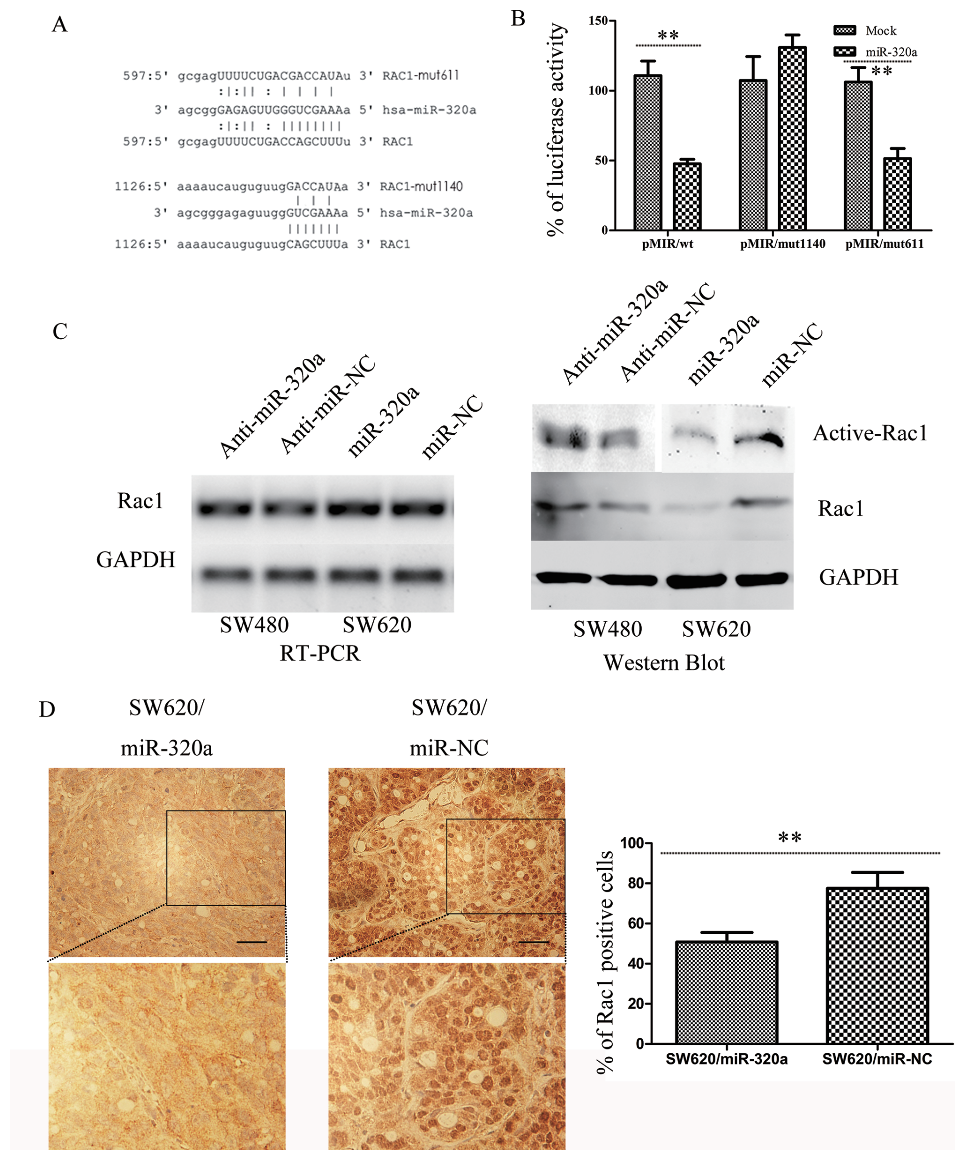

Fig. 5. *Rac1* is a direct target gene of miR-320a. (**A**) miR-320a binding sites and mutation of 3′-UTR of *Rac1* mRNA. (**B**) Functional identification of miR-320a binding sites in 3′-UTR of *Rac1* mRNA. Luciferase activity was used as a reporter for the determination of miR-320a binding site in 3′-UTR. Luciferase-expressing vectors (i.e. pMIR/wt, pMIR/mut1140 and pMIR/mut611) were transfected into SW620 cells in combination with miR-320a or miR-NC and the luciferase activity was assessed. Mutation of 3′-UTR at the miR-320a binding site (1140–1147) is unable to decrease the luciferase activity by miR-320a, compared with the cells transfected with pMIR/wt or pMIR/mut611. (**C**) miR-320a regulates Rac1 protein expression. Semi-RT–PCR analysis showed that miR-320a or anti-miR-320a did not affect the mRNA levels of *Rac1*. Instead, miR-320a expression in SW620 cells suppressed, whereas anti-miR-320a expression in SW480 cells increased, the protein expression of total and active Rac1, relative their respective controls. (**D**) Immunohistochemical staining of Rac1 in tumour sections. The level of Rac1 expression was evaluated based on the staining intensity. Data represent the means ± SD from at least three separate experiments. Magnification ×200, Scale bars: 50 μm. $^{**}P < 0.01$.

compared with SW620/miR-NC cells (Figure 2D), expression of Rac1 in SW620/miR-320a cells led to a reduction of cell populations at G_0/G_1 phase with a proportional increase of cells at G_2/M phase to a similar level as observed in SW620/miR-NC cells (Figure 6B, right panel). Taken together, these results suggest that Rac1 expression could restore the suppressed migration/invasion and cell growth induced by miR-320a in SW620 cells.

Knockdown of Rac1 antagonizes the anti-miR-320a-induced cell migration/invasion and proliferation

We also demonstrated that when the SW480 cells were transfected with anti-miR-320a, both the migration and invasion of SW480 cells were significantly increased (Figure 2B) and the level of active Rac1 was highly elevated (Figure 5C, right panel), relative to the SW480/anti-miR-NC cells. Similarly, to examine the role of the elevated Rac1 in the SW480/anti-miR-320a cells, Rac1 expression was efficiently repressed by Rac1 siRNA and the efficient suppression of Rac1, relative to the SW480/anti-miR-320a cells treated with control siRNA, was confirmed by western blot (Figure 6A). As shown in Figure 6C (left panel), reduction of Rac1 in these cells suppressed the capability of cell migration and invasion, compared with the SW480/anti-miR-320a cells treated with control siRNA, leading to a reduction of migration/invasion to a similar level as observed in SW480/anti-miR-NC cells (Figure 6C, left panel).

Because SW480/anti-miR-320a cells showed a decreased population of cells at G_0/G_1 phase and an increased cell population at S phase over the SW480/anti-miR-NC cells (Figure 2D), the enhanced Rac1 level was depleted by Rac1 siRNA in SW480/anti-miR-320a cells and the cell cycle was analysed. As demonstrated in Figure 6C (right panel), knockdown of Rac1 siRNA resulted in cell cycle shift

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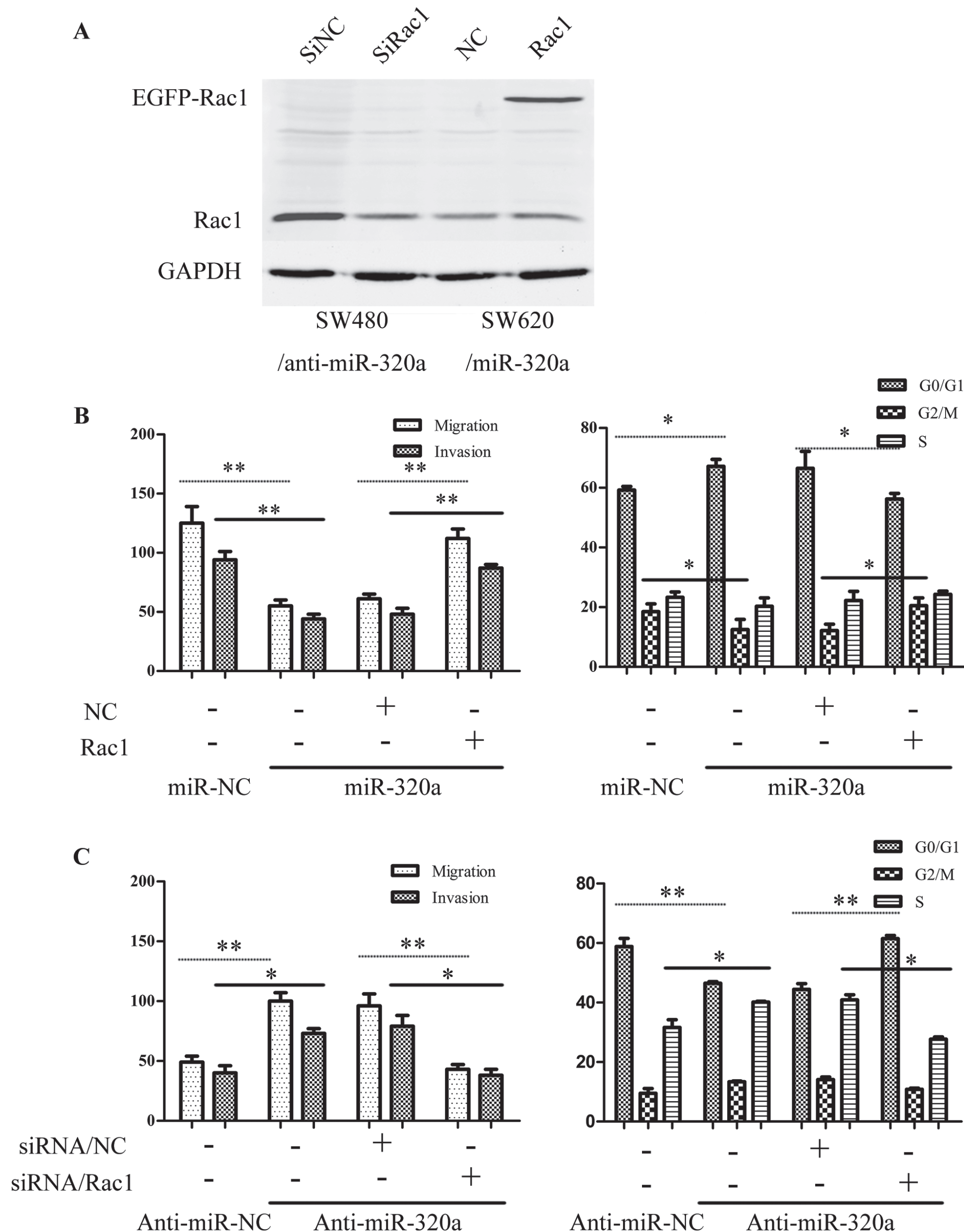

Fig. 6. Rac1 mediates miR-320a's function in CRC cells. (**A**) Exogenous expression of Rac1 was performed by transfecting the pcDNA3-EGFP-Rac1 vector into the SW620/miR-320a cells. Knockdown of Rac1 was carried out by transfecting siRNA/Rac1 into the SW480/anti-miR-320a cells. The expression level of Rac1 was examined by western blot. (**B**) Rac1 expression in SW620/miR-320a cells restored cell migration/invasion inhibited by miR-320a to a similar level as observed in SW620/miR-NC cells. In addition, Rac1 expression reduced the cell population at G_0/G_1 phase and concomitantly increased the population at G_2/M phase. (**C**) Rac1 knockdown by siRNA in SW480/anti-miR-320a cells suppressed cell migration/invasion elevated by anti-miR-320a to a similar level as observed in SW480/anti-miR-NC cells. Reduction of Rac1 increased the cell population at G_0/G_1 phase and reduced the population at S phase. Independent triplicate experiments were performed and data are interpreted as the means ± SD. $*P < 0.05$, $**P < 0.01$.

in which the cells at G_0/G_1 phase was increased, whereas the cells at S phase was concomitantly reduced, compared with the SW480/anti-miR-320a cells treated with control siRNA. This data suggest that the enhanced Rac1 by anti-miR-320a in SW480 cells plays a critical role in the regulation of cell cycle.

Discussion

Colorectal carcinogenesis is a multi-step process mediated by complex cascades of molecular events governing genomic stability and cell proliferation (37). Recently, miRNAs in CRC progression and metastasis have been well studied and demonstrated an oncogenic or suppressive function (6,12,38,39). In particular, miR-320a is a novel tumour and metastasis suppressor by directly targeting mRNAs of *neuropilin 1*, *guanine nucleotide-binding protein G subunit alpha-1* and *β-catenin* (19,28,29). Here, we identified Rac1 as a new direct target of miR-320a and miR-320a exerts its tumour-suppressive function *via* down-regulating *Rac1* oncogene.

miR-320a has emerged as a regulator of glycolysis and was dysregulated in myasthenia gravis, cerebral ischemia and cancers (25,27,40,41). Human miR-320a gene is localized at chromosome 8p21.3, which is frequently lost due to deletion of chromosome 8p during the CRC progression (42). This region of chromosome 8p21-22 is also identified as a liver metastatic susceptibility locus (43). Hence, miR-320a is likely to be a factor defining tumour progression and metastasis. Indeed, miR-320a was reported to be down-regulated in the metastatic liver tissues compared with the primary CRC

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tumours in CRC patients (19) and is a potential prognostic miRNA in CRC (25). In supporting of this, we also found that miR-320a expression was significantly decreased in the metastatic CRC tissues, indicating a negative relation to the CRC progression. Functional studies demonstrated that overexpression of miR-320a in more aggressive SW620 cells led to a significant inhibitory effect on the cell migration/invasion and proliferation, whereas expression of anti-miR-320a in less aggressive SW480 cells resulted in a more aggressive phenotype. These results, together with other studies, indicate miR-320a is a tumour-suppressive miRNA in CRC.

We showed that miR-320a could induce G_0/G_1 arrest in SW620 cells. This was further supported by the fact that the expression of anti-miR-320a in slow-growing SW480 cells reduced the cell population at G_0/G_1 phase and thereof increased cell growth. Apparently, the miR-320a-induced G_0/G_1 arrest prevented tumour growth *in vivo* as evidenced by assessing the proliferative index Ki-67 in tumours. This may provide, at least in part, insights into the tumour-suppressive mechanism of miR-320a in CRC. Meanwhile, we also found that miR-320a has a potential regulatory role in EMT. Our studies showed that miR-320a expression in SW620 cells induced the expression of epithelial marker E-cadherin and suppressed the expression of mesenchymal marker vimentin, and *vice versa* in anti-miR-320a-expressing SW480 cells. The EMT program plays an important role in tumour progression and metastasis (31,44). The miR-320a-inhibited EMT could substantiate its tumour-suppressive function in cancer. Recently, the involvement of miRNAs in controlling EMT has been emphasized (45,46). To date, the miR-200 family has been shown to be major regulators of EMT through silencing the EMT transcriptional inducers, ZEB1 and ZEB2, which in turn repress miR-200f in a double-negative feedback loop (47,48). The recent finding indicated that miR-320a could directly bind to the 3′-UTR of *β-catenin* mRNA to regulate Wnt/β-catenin signaling, leading to inhibition of prostate tumour-initiating cells (29). However, the detailed molecular regulation of miR-320a in EMT remains elusive and is of interest to investigate.

To understand the functional mechanism of miR-320a as a tumour-suppressive miRNA, we performed bioinformatics analysis to identify the downstream genes of miR-320a. We demonstrated *Rac1* as one of the newly identified downstream targets of miR-320a and Rac1 expression was regulated by miR-320a *via* direct binding to 3′-UTR of *Rac1* mRNA. This was evidenced by the fact that miR-320a expression repressed the luciferase activity and Rac1 protein expression, whereas anti-miR-320a expression elevated the Rac1 protein expression. Rac1 is a GTPase that belongs to the RAS superfamily of small GTP-binding proteins and regulates multiple cancer-associated cellular phenotypes including cell proliferation, motility and other cellular responses such as secretory processes, phagocytosis of apoptotic cells, epithelial cell polarization and growth factor-induced formation of membrane ruffles (for review, see ref. 49). The role of Rac1 in cancer progression has been well documented, with an emphasis on its role in the acquisition of migratory, invasive and metastatic phenotypes (50–53). In the present study, miR-320a suppressed, whereas anti-miR-320a enhanced, the expression of total and active Rac1. This finding indicates a significant role of Rac1 in the miR-320a-regulated inhibition of cell migration/invasion. Indeed, re-expression of Rac1 in the SW620/miR-320a cells restored the capability of cell migration/invasion suppressed by miR-320a. In contrast, knockdown of Rac1 by siRNA in the SW480/anti-miR-320a cells repressed the capacity of cell migration/invasion elevated by anti-miR-320a. Hence, Rac1 plays a critical role in mediating miR-320a's function as a tumour-suppressive miRNA in CRC.

In summary, we present evidence that miR-320a plays a tumour-suppressive role in CRC cells. The suppressive effects of miR-320a were exerted by down-regulating the protein expression of its direct target Rac1. Although miR-320a's function in cancer has not been fully understood, our findings provide a new molecular target and mechanism of action for miR-320a as a novel suppressive miRNA. Therefore, miR-320a could be regarded as a new target for CRC prevention and therapy.

Supplementary material

Supplementary Tables 1 and 2 and Figures 1 and 2 can be found at http://carcin.oxfordjournals.org/

Funding

National Natural Science Foundation of China (30873000).

Conflict of Interest Statement: None declared.

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Received July, 6, 2013; revised November 10, 2013; accepted November 15, 2013

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