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microRNA-200b and microRNA-200c promote colorectal cancer cell proliferation via targeting the reversion-inducing cysteine-rich protein with Kazal motifs

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Abbreviation: 3′-UTR, 3′-untranslated region; CDKN1B, cyclin-dependent kinase inhibitor 1B; CRC, colorectal cancer; EdU, 5-ethynyl-2′-deoxyuridine; EMT, epithelial-mesenchymal transition; miR-200b/c, microRNA-200b and microRNA-200c; miRNA, microRNA; MMP, metalloproteinase; NAT, normal adjacent tissue; ORF, open reading frame; RECK, reversion-inducing cysteiner-ich protein with Kazal motifs; siRNA, small interfering RNA; SKP2, S-phase kinase-associated protein 2; ZEB, zinc finger E-box-binding protein

MicroRNA-200b and microRNA-200c (miR-200b/c) are 2 of the most frequently upregulated oncomiRs in colorectal cancer cells. The role of miR-200b/c during colorectal tumorigenesis, however, remains unclear. In the present study, we report that miR-200b/c can promote colorectal cancer cell proliferation via targeting the reversion-inducing cysteine-rich protein with Kazal motifs (RECK). Firstly, bioinformatics analysis predicted RECK as a conserved target of miR-200b/c. By overexpressing or knocking down miR-200b/c in colorectal cancer cells, we experimentally validated that miR-200b/c are direct regulators of RECK. Secondly, an inverse correlation between the levels of miR-200b/c and RECK protein was found in human colorectal cancer tissues and cell lines. Thirdly, we demonstrated that repression of RECK by miR-200b/c consequently triggered SKP2 (S-phase kinase-associated protein 2) elevation and $p27^{Kip1}$ (also known as cyclin-dependent kinase inhibitor 1B) degradation in colorectal cancer cells, which eventually promotes cancer cell proliferation. Finally, promoting tumor cell growth by miR-200b/c-targeting RECK was also observed in the xenograft mouse model. Taken together, our results demonstrate that miR-200b/c play a critical role in promoting colorectal tumorigenesis through inhibiting RECK expression and subsequently triggering SKP2 elevation and $p27^{Kip1}$ degradation.

Introduction

Recent studies indicate that cancer cells show characteristic microRNA (miRNA) expression profiles. The upregulation of oncogenic miRNAs (targeting tumor-suppressor genes) and the downregulation of tumor-suppressive miRNAs (targeting oncogenes) lead to the dysfunction of cancer cells, including malignant proliferation, invasion and metastasis.[1-3] Among the miRNA correlated with tumorigenesis, miR-200 family is one of the most famous one. The miR-200 family consists of 5 members (miR-200a, miR-200b, miR-200c, miR-141 and miR-429) that are arranged in 2 clusters, with the miR-200b-200a-429 cluster at chromosomal location 1p36 and miR-200c-141 cluster at chromosomal location 12p13 in human. Members from each cluster are largely co-expressed as a result of being processed from the same primary transcript. The miR-200 family can also be divided into 2 presumed functional groups based on their seed sequences, with the seed sequence of miR-200b/200c/429 differing from the seed sequence of miR-200a/141 by only one nucleotide. Although difference in seed sequence is expected to confer largely distinct targeting specificity, it has been shown that these subfamilies have highly overlapping spectrum of targeted genes,[4] suggesting that multiple members of the miR-200 family may target a large common subset of genes to enhance the efficiency of genetic

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regulation. The miR-200 family is well known to play an essential role in tumor suppression by inhibiting epithelial-mesenchymal transition (EMT),[4-6] the initiating step of metastasis. However, conflicting reports regarding the role of miR-200 family in suppressing or promoting tumorigenesis in different cancer types have left unanswered questions. Thus, the underlying molecular mechanisms through which miR-200 family is involved in the development and progression of colorectal cancer remain to be fully elucidated.

The reversion-inducing cysteine-rich protein with Kazal motifs (RECK) is anchored to the cell surface via glycosylphosphatidylinositol and regulate the breakdown of the extracellular matrix by inhibiting several metalloproteinases (MMPs) including MMP-2, MMP-9, MT1-MMP and MMP-7.[7-9] RECK mRNA is easily detectable in normal human organs but is undetectable in a number of cell lines derived from human tumors.[9,10] Downregulation of RECK is found in a wide variety of tumors, and RECK expression levels are predictive in determining the prognosis in a number of common cancers; low levels of RECK are often associated with increased invasiveness and a poor prognosis.[11] Recent research has shown that RECK can suppress cell proliferation and induce cellular senescence when acutely expressed in cancer cells and that at least some of these effects can be attributed to the downregulation of SKP2 (S-phase kinase-associated protein 2) and consequent upregulation of $p27^{Kip1}$ (also known as cyclin-dependent kinase inhibitor 1B, CDKN1B).[12]

In the present study, we predicted that RECK was a target of miR-200b and miR-200c (miR-200b/c). After measuring the expression levels of miR-200b/c and RECK in human colorectal cancer and normal adjacent tissue samples, we detected an inverse correlation between miR-200b/c and RECK protein levels in human colorectal cancer tissues and cell lines. The direct inhibition of RECK translation by miR-200b/c and the potential role of miR-200b/c as a promoter of colorectal tumorigenesis have been experimentally validated.

Materials and Methods

Cells and human tissues

Human colon carcinoma cell lines (Caco-2, HT29 and SW480) were purchased from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). The Caco-2 cells were cultured in DMEM supplemented with 10% fetal bovine serum (GIBCO, CA, USA), and the HT29 and SW480 cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum. All cells were incubated in a 5% CO_2, 37°C, water-saturated atmosphere. The colorectal tumors and paired normal adjacent tissues were derived from patients undergoing a surgical procedure at the First Affiliated Hospital of Nanjing Medical University (Nanjing, China). The epithelium of normal colon was removed from the underlying non-epithelial tissue by scraping and served as control. All of the patients provided written consent, and the Ethics Committee from Nanjing University approved all aspects of this study. Tissue fragments were immediately frozen in liquid nitrogen at the time of surgery and stored at −80°C. The clinical features of the patients are listed in Supplementary **Table 1**.

RNA isolation and quantitative RT-PCR

Total RNA was extracted from the cultured cells and human tissues using TRIzol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Assays to quantify miRNAs were performed using Taqman miRNA probes (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Briefly, 1 μg of total RNA was reverse-transcribed to cDNA using AMV reverse transcriptase (TaKaRa, Dalian, China) and a stem-loop RT primer (Applied Biosystems). The reaction conditions were as follows: 16°C for 30 min, 42°C for 30 min, and 85°C for 5 min. Real-time PCR was performed using a TaqMan PCR kit on an Applied Biosystems 7300 Sequence Detection System (Applied Biosystems). The reactions were incubated in a 96-well optical plate at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. All of the reactions were run in triplicate. After the reaction, the cycle threshold (C_T) data were determined using fixed threshold settings, and the mean C_T was determined from the triplicate PCRs. A comparative C_T method was used to compare each condition to the controls. The relative levels of miRNAs in cells and tissues were normalized to U6. The amount of miRNA relative to the internal control U6 was calculated with the equation $2^{-\triangle\triangle CT}$, in which $\triangle\triangle C_T = (C_{T\ miRNA} - C_{T\ U6})_{target} - (C_{T\ miRNA} - C_{T\ U6})_{control}$. To quantify RECK mRNA, 1 μg of total RNA was reverse-transcribed to cDNA using oligo dT and AMV reverse transcriptase (TaKaRa) in the reaction, which was performed with the following conditions: 42°C for 60 min and 70°C for 10 min. Next, real-time PCR was performed with the RT product, SYBER Green Dye (Invitrogen) and specific primers for RECK and GAPDH. The sequences of the primers were as follows: RECK (sense): 5′-TGGCTGGGTTGGCTT AGG-3′; RECK (antisense): 5′-GCAAACTTTGGAAATAT-CATTCTTTG-3′; GAPDH (sense): 5′-GATATTGTTGC-CATCAATGAC-3′; and GAPDH (antisense): 5′-TTGAT TTTGGAGGGATCTCG-3′. The reactions were incubated at 95°C for 5 min, followed by 40 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 30 s. After the reactions were complete, the C_T values were determined by setting a fixed threshold. The relative amount of RECK mRNA was normalized to GAPDH.

Overexpression of miR-200b/c or knockdown of miR-200b/c

Synthetic pre-miR-200b/c, anti-miR-200b/c and scrambled negative control RNAs (pre-scramble and anti-scramble) were purchased from Ambion (Austin, TX, USA). Cells were seeded in 6-well plates or 60 mm dishes, and were transfected with Lipofectamine 2000 (Invitrogen) the following day when the cells were approximately 70% confluent. In each well, equal amounts of pre-miR-200b/c, anti-miR-200b/c or scrambled negative control RNA were used. The cells were harvested 24 h after transfection for quantitative RT-PCR and Western blotting.

Luciferase reporter assay

To test the direct binding of miR-200b/c to the target gene RECK, a luciferase reporter assay was performed as previously described.[13] The entire 3′-untranslated region (3′-UTR) of human RECK was amplified with PCR using human genomic DNA as a template. The PCR products were inserted into the p-MIR-reporter plasmid (Ambion). The insertion was confirmed as correct by sequencing. To test the binding specificity, the sequences that interact with the miR-200b/c seed sequence were mutated (from CAGUAUU to GUCAUAA), and the mutant RECK 3′-UTR was inserted into an equivalent luciferase reporter. For luciferase reporter assays, cells were cultured in 24-well plates, and each well was transfected with 1 μg of firefly luciferase reporter plasmid, 1 μg of a β-galactosidase (β-gal) expression plasmid (Ambion), and equal amounts (100 pmol) of pre-miR-200b/c, anti-miR-200b/c or the scrambled negative control RNA using Lipofectamine 2000 (Invitrogen). The β-gal plasmid was used as a transfection control. Twenty-four hours post-transfection, the cells were assayed using a luciferase assay kit (Promega, Madison, WI, USA).

Plasmid construction and siRNA interference assay

Three siRNA sequences targeting different sites of human RECK cDNA were synthesized by GenePharma (Shanghai, China). Among the 3 siRNA sequences, one (5′-AAGACC-CAGCCCUUGCCUCAA-3′) was designed according to a previous report.[14] Scrambled siRNAs were included as negative controls. The sequence with the best interfering effect was selected and used in further studies. A mammalian expression plasmid encoding the human RECK open reading frame (pReceiver-M02-RECK) was purchased from GeneCopoeia (Germantown, MD, USA). The mammalian expression plasmids encoding the full length cDNA of human RECK with either wild-type or mutant (binding sites that interact with miR-200b/c were mutated) form of 3′-UTR were purchased from Invitrogen. An empty plasmid served as a negative control. The RECK over-expression vector and the selected RECK siRNA were transfected into Caco-2, HT29, or SW480 cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Total RNA and protein were isolated 24 h post-transfection. The RECK mRNA and protein expression levels were assessed by quantitative RT-PCR and Western blotting.

Construction of miR-200b/c overexpression lentiviral vector

Lentivirus to overexpress miR-200b, miR-200c, or miR-200b plus miR-200c was purchased from Invitrogen. Lentivirus was added to Caco-2 cells at 70% confluence in 6-well plates or 100 mm dishes at an MOI of 10 together with polybrene at a final concentration of 5 μg/mL according to the manufacturer's instructions. Cells were then harvested for quantitative RT-PCR, Western blotting or animal experiments.

Protein extraction and Western blotting

Cells were rinsed with PBS (pH 7.4) and lysed in RIPA Lysis buffer (Beyotime, China) supplemented with a Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific 78440) on ice for 30 min. The tissue samples were frozen solid with liquid nitrogen, ground into a powder and lysed in RIPA Lysis buffer containing the Protease and Phosphatase Inhibitor Cocktail on ice for 30 min. When necessary, sonication was used to facilitate lysis. Cell lysates or tissue homogenates were centrifuged for 10 min (12000 g, 4°C). The supernatant was collected, and the protein concentration was calculated with a Pierce BCA protein assay kit (Thermo Scientific, Rockford, IL, USA). The protein levels of RECK, SKP2, and $p27^{Kip1}$ were analyzed using Western blots with polyclonal anti-human RECK, monoclonal anti-SKP2, and monoclonal anti-$p27^{Kip1}$ antibodies. The protein levels were normalized by probing the same blots with a GAPDH antibody. The antibodies were purchased from the following sources: anti-RECK (Abcam Inc.., Cambridge, MA, USA), anti-SKP2 and anti-$p27^{Kip1}$ (Cell Signaling, Beverley, MA, USA), and anti-GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Images of the Western blot assay were analyzed using Bandscan software (Glyko, Novato, USA).

Cell viability assay

To assess cell viability, Caco-2, HT29 or SW480 cells were seeded in triplicate in 96-well plates at a density of 5×10^3 cells per well in 100 μL of culture medium. The cell proliferation index was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Sigma, USA), which was performed 12, 24, 36, 48, 60 and 72 h after transfection according to the manufacturer's instructions.

EdU proliferation assay

To assess cell proliferation, Caco-2, HT29 or SW480 cells were seeded in 96-well plates. The cells were incubated under standard conditions in complete media. Transfection of the cells was performed the following day as described above. Forty-eight hours after transfection, cell proliferation was detected using the incorporation of 5-ethynyl-2′-deoxyuridine (EdU) with the EdU Cell Proliferation Assay Kit (Ribobio, Guangzhou, China). Briefly, the cells were incubated with 50 μM EdU for 6 h before fixation, permeabilization and EdU staining, which were performed according to the manufacturer's protocol. The cell nuclei were stained with DAPI (Sigma) at a concentration of 1 μg/ml for 20 min. The proportion of the cells incorporated EdU was determined with fluorescence microscopy.

Establishment of tumor xenografts in mice

Four-week-old male C57BL/6J mice were purchased from the Model Animal Research Center of Nanjing University (Nanjing, China) and maintained under specific pathogen-free conditions at Nanjing University. Caco-2 cells were untreated, or infected with the miR-200b/c overexpression lentivirus, or transfected with the RECK overexpression plasmid, or co-transfected with the miR-200b/c overexpression lentivirus plus RECK overexpression plasmid. Cells were injected subcutaneously into C57BL/6J mice (1×10^7 cells per mouse, 5 mice per group). Mice were sacrificed after one month. After the tumors were separated from the animals, the length, width and height of the tumors was measured with digital calipers. The tumor weights were determined

and the ellipsoid volume was calculated using the following formula: Volume = $\pi/6 \times$ (length) $\times$ (width) $\times$ (height). Then, tumor section slides were subjected to immunohistochemical analysis using H&E staining and Ki-67 staining (antibodies-online Inc.., Atlanta, USA) according to the manufacturer's instructions. All animal care and handling procedures were performed in accordance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Review Board of Nanjing University (Nanjing, China).

Statistical analysis

All of the images of Western blotting and the EdU proliferation assay are representative of at least 3 independent experiments. Quantitative RT-PCR, the luciferase reporter assay and the cell viability assay were performed in triplicate, and each experiment was repeated several times. The data shown are the mean $\pm$ SE of at least 3 independent experiments. The differences were considered statistically significant at $P < 0.05$ using Student's t -test.

Results

Upregulation of miR-200b/c levels in human colorectal cancer tissues

We first determined the expression patterns of miR-200b/c in colorectal cancer tissues. As shown in **Fig. 1, A and B**, the expression levels of miR-200b/c were significantly higher in colorectal cancer tissues than in the adjacent normal tissues from the same patients.

Prediction of RECK as a target of miR-200b/c

To explore the potential function of miR-200b/c in colorectal cancer cell biology, we predicted the potential target genes of miR-200b/c using the 3 algorithms TargetScan,[15] PicTar[16] and miRanda[17]. Among the candidate targets of miR-200b/c, RECK, a tumor suppressor gene previously shown to play a critical role in the regulation of cell proliferation, was predicted to be a miR-200b/c target by all 3 of the algorithms and was thus selected for further experimental verification. The predicted interaction between miR-200b/c and the target sites in the RECK 3′-UTR was illustrated in **Figure 1C**. There were 2 hybrids between RECK 3′-UTR and miR-200b/c. The minimum free energy values of these hybrids, −28.1 and −23.9 kcal/mol, were well within the range of genuine miRNA-target pairs. Moreover, there was perfect base-pairing between the seed region (the core sequence that encompasses the first 2–8 bases of the mature miRNA) and the cognate targets. Furthermore, the miR-200b/c binding sequences in the RECK 3′-UTR are highly conserved across species.

An inverse correlation between miR-200b/c and RECK levels in human colorectal cancer tissues and cell lines

Because our quantitative RT-PCR assay identified that miR-200b/c were significantly upregulated during colorectal tumorigenesis, we next investigated whether RECK levels were decreased in colorectal cancer. As shown in **Figure 1D**, RECK protein levels in colorectal cancer tissues were dramatically lower than those in normal adjacent tissues from the same patients. In contrast, no significant change of RECK mRNA levels was observed in colorectal cancer (**Fig. 1E**). The inverse correlation between miR-200b/c and RECK protein levels (**Fig. 1F**) and the disparity between the miR-200b/c and RECK mRNA levels (**Fig. 1G**) were further illustrated using Pearson's correlation scatter plots. In agreement with the notion that animal miRNAs are generally blocking translational processes without affecting transcript levels, the results indicated a miR-200b/c-mediated posttranscriptional regulation mechanism involved in the repression of RECK. Furthermore, we have evaluated the expression levels of miR-200b/c and RECK in 3 colorectal cancer cell lines. As shown in Supplementary **Figure 1, A and B**, the expression levels of miR-200b/c were higher in SW480 cells and lower in Caco-2 cells in comparison with those in HT29 cells. In contrast, the levels of RECK protein were lower in SW480 cells and higher in Caco-2 cells in comparison with those in HT29 cells. In summary, our results revealed an inverse correlation between miR-200b/c and RECK levels in human colorectal cancer tissues and cell lines.

Validation of RECK as a direct target of miR-200b/c

The correlation between miR-200b/c and RECK was further examined by evaluating RECK expression in Caco-2, HT29 and SW480 cells after overexpression or knockdown of miR-200b/c. In these experiments, miR-200b/c overexpression was achieved by transfecting cells with pre-miR-200b/c (synthetic RNA oligonucleotides mimicking miR-200b/c precursors), whereas miR-200b/c knockdown was achieved by transfecting cells with anti-miR-200b/c (chemically modified antisense oligonucleotides designed to specifically target mature miR-200b/c). Efficient overexpression or knockdown of miR-200b/c in Caco-2, HT29 and SW480 cells is shown in Supplementary **Fig.2, A-F**. Clearly, cellular miR-200b/c levels were significantly increased when Caco-2, HT29 and SW480 cells were transfected with pre-miR-200b/c and dropped dramatically when Caco-2, HT29 and SW480 cells were treated with anti-miR-200b/c. The expression of RECK protein was significantly inhibited by the introduction of miR-200b/c in Caco-2 (Fig. 2A), HT29 (Supplementary **Fig.2H**) and SW480 (Supplementary **Fig.2K**) cells, while anti-miR-200b/c significantly increased the RECK protein level in Caco-2 (**Fig. 2B**), HT29 (Supplementary **Fig.2I**) and SW480 (Supplementary **Fig.2L**) cells. To determine at what level miR-200b/c influenced RECK expression, we repeated the above experiments and examined the expression of RECK mRNA after transfection. Although the intracellular level of miR-200b/c was altered significantly after pre-miR-200b/c or anti-miR-200b/c treatment, overexpression or knockdown of miR-200b/c did not affect RECK mRNA level in Caco-2 (**Fig. 2C**), HT29 (Supplementary **Fig.2J**) and SW480 (Supplementary **Fig.2M**) cells. These results demonstrate that miR-200b/c specifically regulate RECK protein expression at the post-transcriptional level, which is a typical animal miRNA-mediated regulation mechanism.

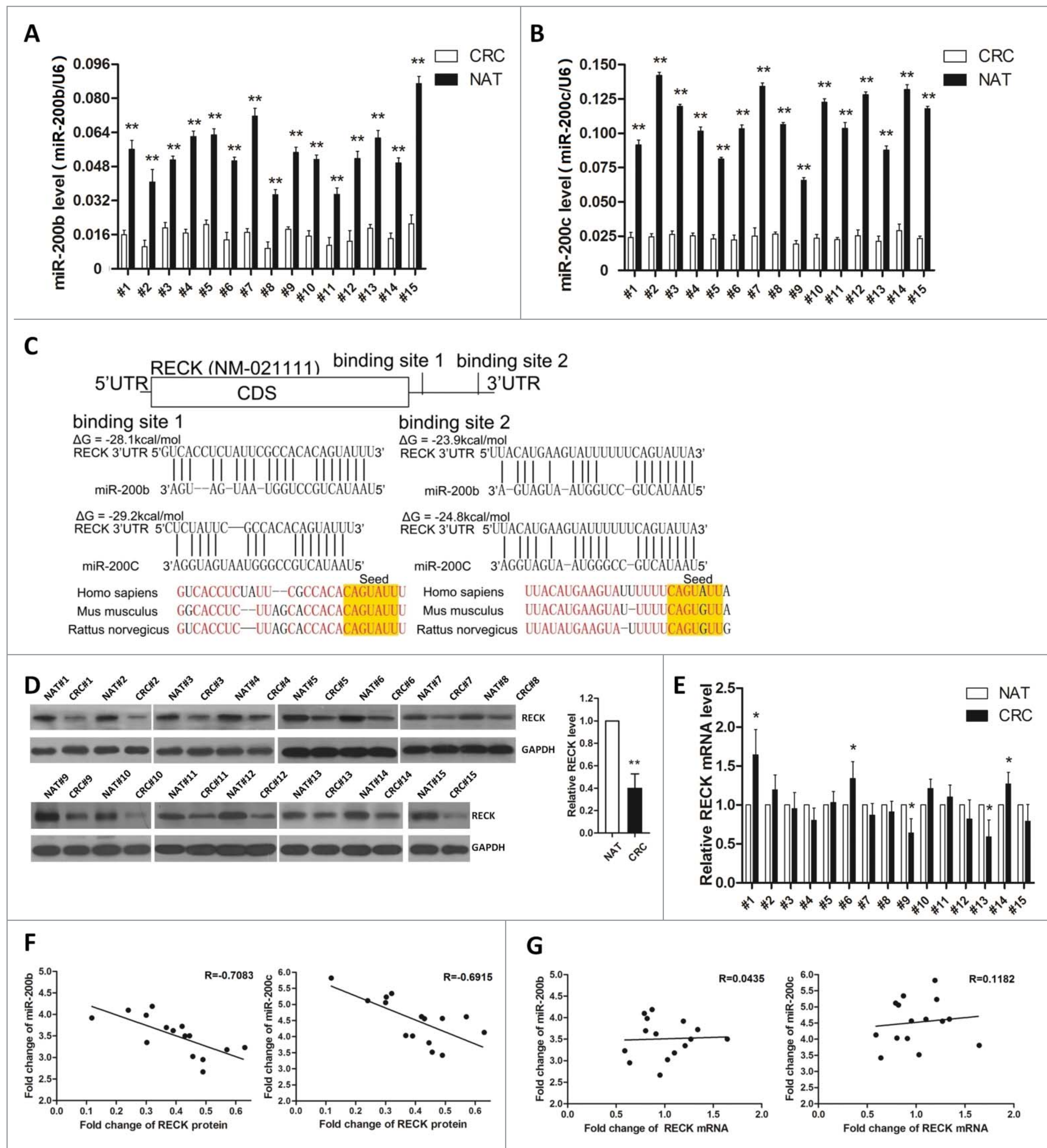

Figure 1. Inverse correlation between miR-200b/c and RECK protein levels in human colorectal cancer tissues. (**A** and **B**) Quantitative RT-PCR analysis of the expression levels of miR-200b/c (in the form of miRNA/U6 ratio) in 15 pairs of colorectal cancer (CRC) and normal adjacent tissue (NAT) samples (**$P < 0.01$). (**C**) Schematic description of the hypothetical duplexes formed by the interactions between the binding sites in the RECK 3′-UTR (top) and miR-200b/c (bottom). The predicted free energy value of each hybrid is indicated. The seed recognition sites are denoted, and all nucleotides in these regions are highly conserved across species, including human, mouse and rat. (**D**) Western blotting analysis of the expression levels of RECK protein in 15 pairs of CRC and NAT samples. (**E**) Quantitative RT-PCR analysis of the relative expression levels of RECK mRNA in 15 pairs of CRC and NAT samples. (**F**) Pearson's correlation scatter plot of the fold change of miR-200b/c and RECK protein in human colorectal cancer tissues. (**G**) Pearson's correlation scatter plot of the fold change of miR-200b/c and RECK mRNA in human colorectal cancer tissues.

To determine whether the negative regulatory effects that miR-200b/c exerted on RECK expression were mediated through the binding of miR-200b/c to the presumed sites in the 3′-UTR of the RECK mRNA, the entire 3′-UTR of RECK containing the 2 presumed miR-200b/c binding sites was fused downstream of the firefly luciferase gene in a reporter plasmid. The resulting plasmid was transfected into Caco-2 cells along with a transfection control plasmid (β-gal) and pre-miR-200b/

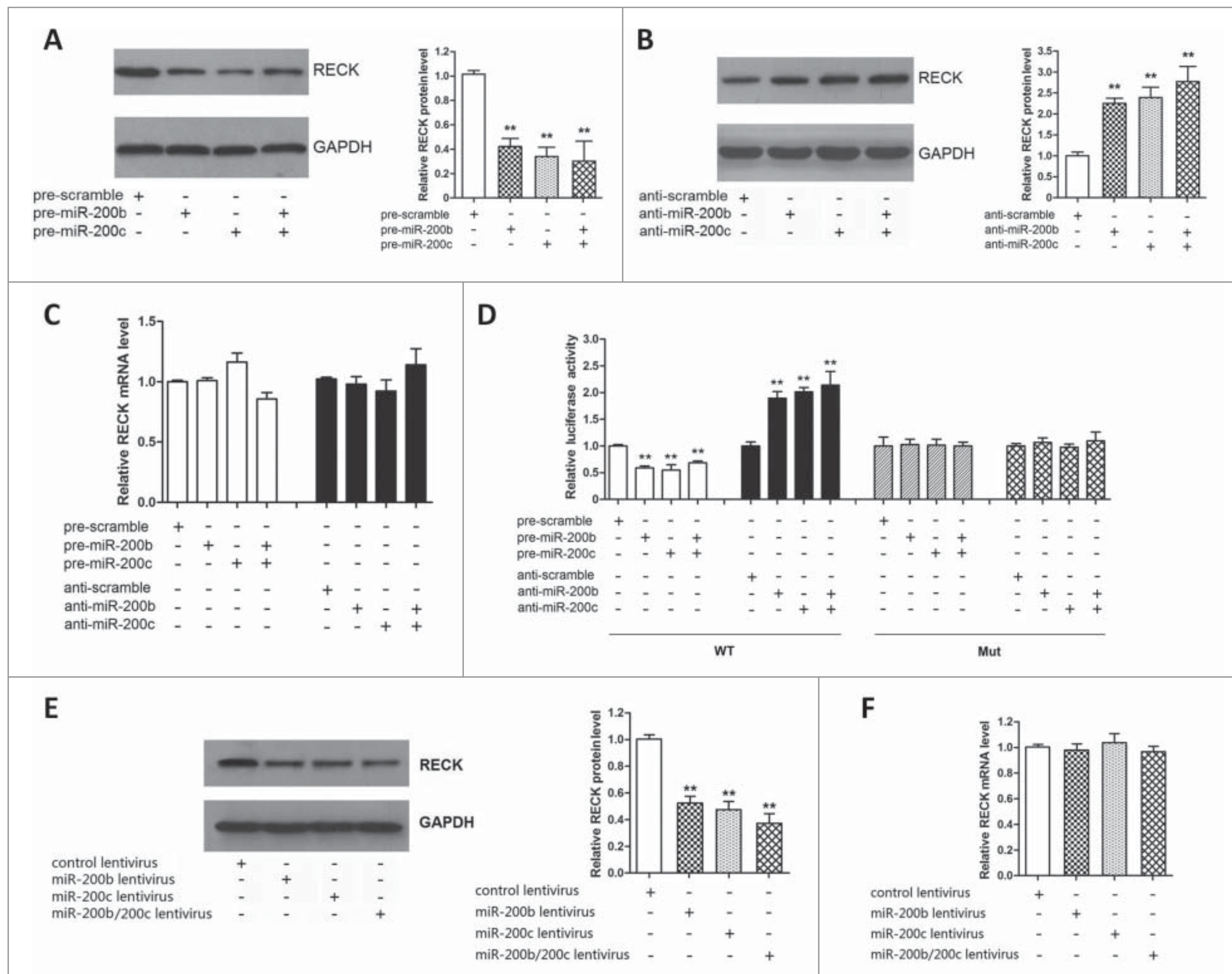

Figure 2. miR-200b/c directly regulate RECK expression at the post-transcriptional level. (**A**and **B**) Western blotting analysis of RECK protein levels in Caco-2 cells treated with pre-scramble, pre-miR-200b, pre-miR-200c, or pre-miR-200b plus pre-miR-200c (in a 0.5:0.5 ratio) and in cells treated with anti-scramble, anti-miR-200b, anti-miR-200c, or anti-miR-200b plus anti-miR-200c (in a 0.5:0.5 ratio). Left panel: representative image; right panel: quantitative analysis (** $P < 0.01$). (**C**) Quantitative RT-PCR analysis of RECK mRNA levels in Caco-2 cells treated with pre-scramble, pre-miR-200b, pre-miR-200c, or pre-miR-200b plus pre-miR-200c (in a 0.5:0.5 ratio) and in cells treated with anti-scramble, anti-miR-200b, anti-miR-200c, or anti-miR-200b plus anti-miR-200c (in a 0.5:0.5 ratio). (**D**) Direct recognition of the RECK 3′-UTR by miR-200b/c. Firefly luciferase reporters containing either wild-type (WT) or mutant (Mut) miR-200b/c binding sites in the RECK 3′-UTR were co-transfected into Caco-2 cells with either the scrambled negative control RNA, pre-miR-200b/c or anti-miR-200b/c. Twenty-four hours post-transfection, the cells were assayed using a luciferase assay kit. The results are calculated as the ratio of firefly luciferase activity in the miR-200b/c-transfected cells normalized to the control cells (** $P < 0.01$). (**E**) Western blotting analysis of RECK protein levels in Caco-2 cells infected with control lentivirus or lentivirus to overexpress miR-200b, miR-200c, or miR-200b plus miR-200c. Left panel: representative image; right panel: quantitative analysis (** $P < 0.01$). (**F**) Quantitative RT-PCR analysis of RECK mRNA levels in Caco-2 cells infected with control lentivirus or lentivirus to overexpress miR-200b, miR-200c, or miR-200b plus miR-200c.

c, anti-miR-200b/c or scrambled negative control RNAs (pre-scramble and anti-scramble). As expected, overexpression of miR-200b/c resulted in a ~40% reduction of luciferase reporter activity compared to cells treated with the pre-scramble control, whereas inhibition of miR-200b/c resulted in a two-fold increase of reporter activity compared to the cells transfected with the anti-scramble control (**Fig. 2D**). Furthermore, we introduced point mutations into the corresponding complementary sites in the RECK 3′-UTR to eliminate the predicted miR-200b/c binding sites. This mutated luciferase reporter was unaffected by both overexpression and knockdown of miR-200b/c (**Fig. 2D**). This finding suggests that the binding sites strongly contribute to the miRNA-mRNA interaction mediating the posttranscriptional repression of RECK expression. In conclusion, our results suggest that miR-200b/c directly recognize and bind to the 3′-UTR of the RECK mRNA transcript and inhibit RECK translation.

To exclude the possibility that the effect of miR-200b/c on RECK repression maybe due to the supraphysiological miRNA level caused by overexpression of miR-200b/c mimic,[18] we constructed lentiviral vector expressing miR-200b/c and infected cell with miR-200b/c-lentivirus to produce miR-200b/c via the endogenous miRNA processing pathway. As shown in Supplementary **Fig.2G**, the expression levels of mature miR-200b and

miR-200c were 4–6-fold higher than the basal levels when Caco-2 cells were infected with miR-200b/c-lentivirus. Infecting Caco-2 cells with miR-200b/c-lentivirus also reduced RECK expression in Caco-2 cells to the same degree as those obtained by using pre-miR-200b/c (**Fig. 2E**). Overexpression of miR-200b/c by lentivirus did not affect RECK mRNA level in Caco-2, too (**Fig. 2F**). The results further verify that miR-200b/c posttranscriptionally regulate RECK expression.

miR-200b/c regulate SKP2 and p27^{Kip1} expression in the RECK signaling pathway

We next analyzed the biological consequences of the decreased RECK expression caused by miR-200b/c in colorectal cancer cells. It was previously reported that acute RECK expression in colon carcinoma cells results in cell cycle arrest accompanied by downregulation of a ubiquitin ligase component, SKP2, and upregulation of its substrate, p27^{Kip1}.[12] p27^{Kip1} regulates the cell cycle and plays an important role in human tumorigenesis. Overexpression of p27^{Kip1} arrests cells in G1, whereas loss of p27^{Kip1} leads to an increase in cell proliferation.[19] p27^{Kip1} is specifically recognized by SKP2, which is a rate-limiting component of the machinery that ubiquitinates and degrades p27^{Kip1}.[20,21] SKP2 upregulation accelerates p27^{Kip1} degradation and leads to cell cycle progression in cancer cells.[22] Here, we investigated whether suppression of RECK expression due to miR-200b/c overexpression affects SKP2 and p27^{Kip1} expression in colorectal cancer. We found that SKP2 protein levels were upregulated and p27^{Kip1} protein levels were downregulated in Caco-2 cells transfected with pre-miR-200b/c (**Fig. 3A**). In contrast, SKP2 protein levels were decreased and p27^{Kip1} protein levels were increased in Caco-2 cells transfected with anti-miR-200b/c (**Fig. 3B**).

Subsequently, we investigated whether forced expression of RECK is sufficient to reverse the inhibitory effects of miR-200b/c on RECK and downstream signaling factors. A plasmid designed to specially express the full-length open reading frame (ORF) of RECK without the miR-200b/c–responsive 3′-UTR was constructed and transfected into Caco-2 cells with either pre-miR-200b/c or the pre-scramble control. Compared to cells transfected with pre-miR-200b/c, the cells transfected with pre-miR-200b/c and the RECK overexpression plasmid exhibited significantly higher levels of RECK (**Fig. 3C**), suggesting that miR-200b/c–resistant RECK rescued the RECK suppression caused by miR-200b/c. Consequently, ectopic expression of RECK also attenuated miR-200b/c–mediated SKP2 upregulation and reversed the miR-200b/c–mediated p27^{Kip1} downregulation in Caco-2 cells (**Fig. 3C**). Taken together, the results suggest that miR-200b/c can affect SKP2 and p27^{Kip1} expression by regulating RECK protein levels.

miR-200b/c promote colorectal cancer cell proliferation via affecting the RECK signaling pathway

We next focused on studying the roles of miR-200b/c in RECK regulation. Because RECK is essential for the regulation of proliferation and cell cycle progression, we evaluated the effects of miR-200b/c on colorectal cancer cell proliferation using the MTT assay. In support of the notion that miR-200b/c function as key oncogenic miRNAs, Caco-2 (**Fig. 4A**), HT29 (Supplementary **Fig.4A**) and SW480 (Supplementary **Fig.4D**) cells transfected with pre-miR-200b/c showed increased proliferation; in contrast, knockdown of miR-200b/c had the opposite effect on cell proliferation in Caco-2 (**Fig. 4B**), HT29 (Supplementary **Fig.4B**) and SW480 (Supplementary **Fig.4E**) cells. Furthermore, we assessed the role of RECK on cell proliferation. To knock down RECK, 3 siRNA sequences targeting different sites of human RECK cDNA were designed (Supplementary **Fig.3A**). The sequence (5′-AAGACCCAGCCCUUGCCUCAA-3′) with the best interfering effect (Supplementary **Fig.3, B and C**) was selected and transfected into Caco-2, HT29 and SW480 cells. To overexpress RECK, a plasmid expressing the RECK ORF was transfected into Caco-2, HT29 and SW480 cells. The efficient overexpression or knockdown of RECK in Caco-2, HT29 and SW480 is shown in Supplementary **Fig.3 D-F**. Consistent with previous studies showing that RECK functions as a proliferation repressor, cells transfected with the RECK overexpression plasmid proliferated at a significantly lower rate, whereas RECK knockdown with the siRNA significantly increased proliferation in Caco-2 (**Fig. 4C**), HT29 (Supplementary **Fig.4C**) and SW480 (Supplementary **Fig.4F**) cells. Thus, the pro-proliferative effect of RECK knockdown was similar to miR-200b/c overexpression. Moreover, compared to cells transfected with pre-miR-200b/c, the cells transfected with pre-miR-200b/c and the RECK overexpression plasmid exhibited significantly lower proliferation rates (**Fig. 4D**), suggesting that miR-200b/c–resistant RECK can attenuate the pro-proliferative effect of miR-200b/c. In addition, Caco-2 cells infected with miR-200b/c overexpression lentivirus showed increased proliferation (**Fig. 4E**). Finally, to avoid the side affect caused by overexpression of RECK with RECK ORF, we constructed a plasmid encoding the full length RECK cDNA with intact 3′-UTR to overexpress RECK. Its mutant form carrying modified miR-200b/c-binding sites served as control. As expected, cells transfected with pre-miR-200b/c proliferated at a higher rate, whereas overexpression of RECK (wild-type or mutant cDNA) significantly decreased cell proliferation (**Fig. 4F**). Compared to cells transfected with pre-miR-200b/c, the cells transfected with pre-miR-200b/c and wild-type or mutant RECK cDNA exhibited lower proliferation rates, and the inhibitory effect was observed to be more significant in cells transfected with mutant RECK cDNA (**Fig. 4F**).

To further test the biological effect of RECK-targeted miR-200b/c on the growth of colorectal cancer cells, the effect of overexpression or knockdown of miR-200b/c and RECK on cell proliferation was examined with the EdU assay, an immunochemical detection of the nucleotide analog incorporated into replicated DNA. Consistent with the results from the MTT assay, the percentage of EdU-positive cells was significantly higher in cells transfected with pre-miR-200b/c and lower in cells transfected with anti-miR-200b/c in Caco-2 (**Fig. 5, A-D**), HT29 (Supplementary **Fig.4, G and H**) and SW480 (Supplementary **Fig.4, J and K**) cells. Similarly, significantly more EdU-positive cells were observed in the RECK siRNA-transfected cells, whereas overexpression of RECK had an opposite effect on cell proliferation in Caco-2 (**Fig. 5, E and F**), HT29 (Supplementary **Fig.4I**)

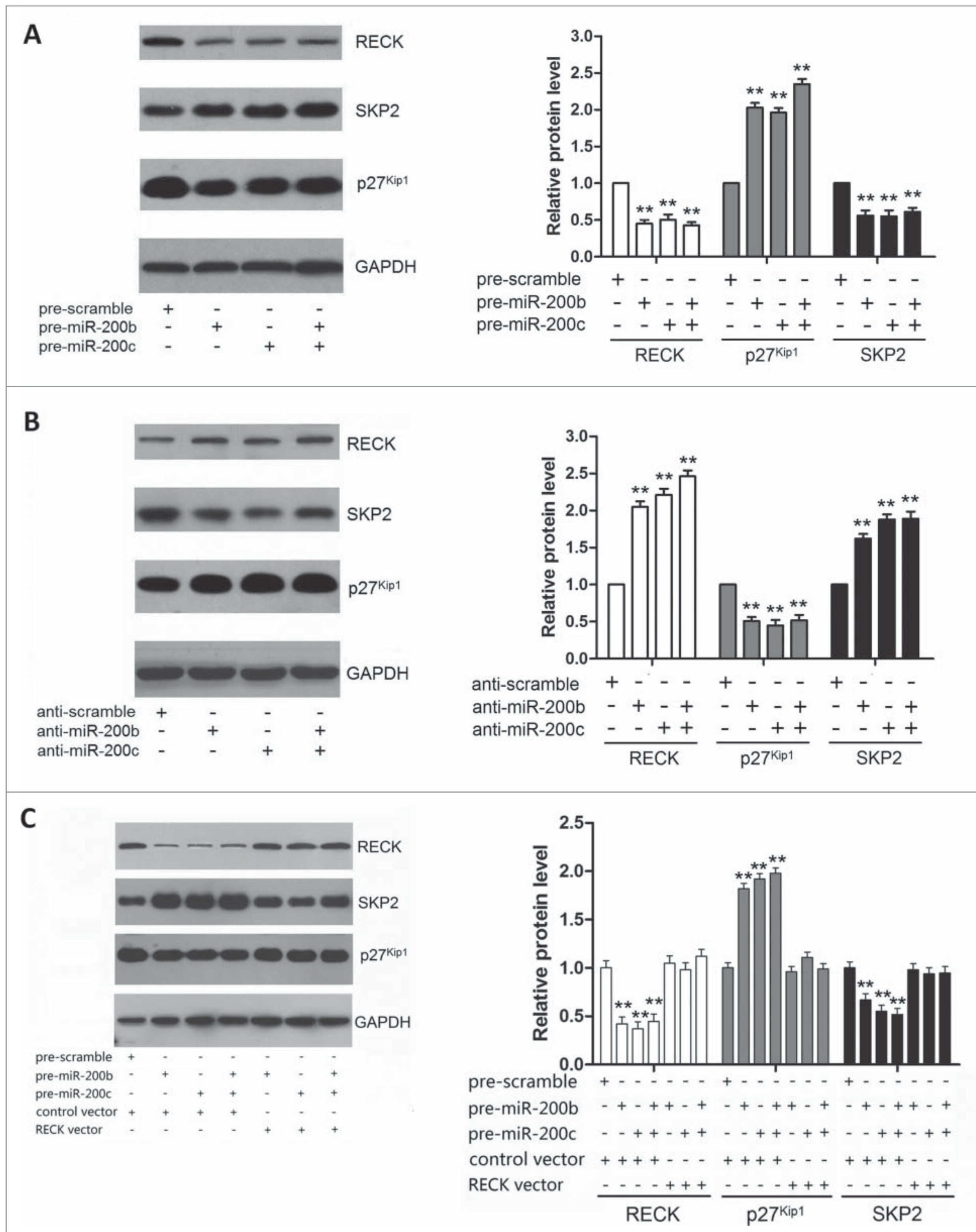

Figure 3. miR-200b/c regulate SKP2 and p27^{Kip1} expression by targeting RECK. (**A** and **B**) Western blotting analysis of the protein levels of RECK, SKP2 and p27^{Kip1} in Caco-2 cells treated with pre-scramble, pre-miR-200b, pre-miR-200c, or pre-miR-200b plus pre-miR-200c (in a 0.5:0.5 ratio) and in cells treated with anti-scramble, anti-miR-200b, anti-miR-200c, or anti-miR-200b plus anti-miR-200c (in a 0.5:0.5 ratio). Left panel: representative image; right panel: quantitative analysis (**$P < 0.01$). (**C**) Western blotting analysis of protein levels of RECK, SKP2 and p27^{Kip1} in Caco-2 cells treated with pre-scramble plus control vector, pre-miR-200b/c plus control vector, or pre-miR-200b/c plus the RECK overexpression vector. Left panel: representative image; right panel: quantitative analysis (**$P < 0.01$).

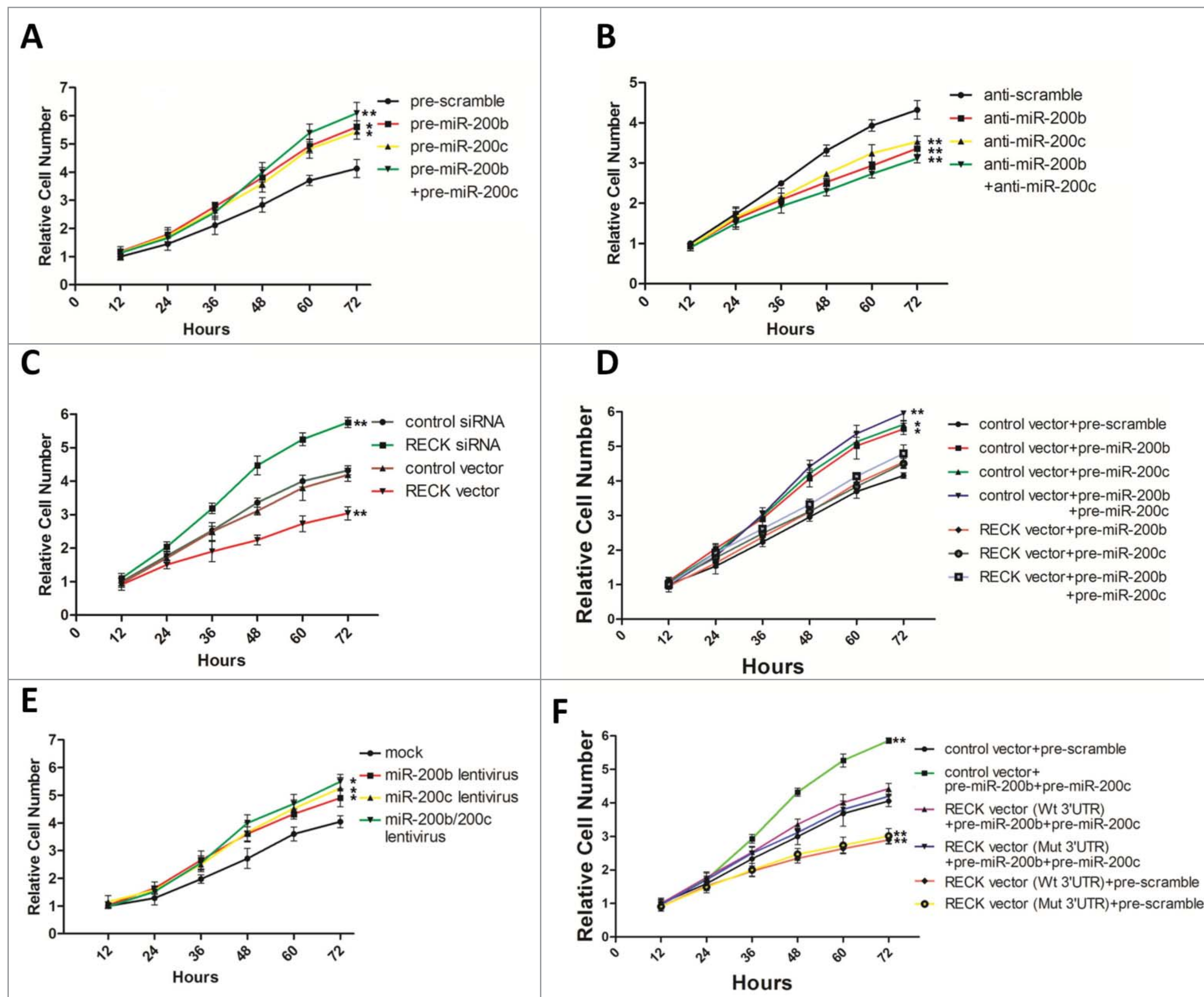

Figure 4. MTT assay analysis of the effect of RECK-targeted miR-200b/c on the growth of colorectal cancer cells. (**A** and **B**) The MTT viability assay was performed 12, 24, 36, 48, 60 and 72 h after the transfection of Caco-2 cells with scrambled negative control RNA, pre-miR-200b/c, or anti-miR-200b/c. (**C**) The MTT viability assay was performed 12, 24, 36, 48, 60 and 72 h after the transfection of Caco-2 cells with scrambled control siRNA, RECK siRNA, control vector or the RECK overexpression vector. (**D**) The MTT viability assay was performed 12, 24, 36, 48, 60 and 72 h after the transfection of Caco-2 cells with pre-scramble plus control vector, pre-miR-200b/c plus control vector, or pre-miR-200b/c plus the RECK overexpression vector. (**E**) The MTT viability assay was performed 12, 24, 36, 48, 60 and 72 h after the infection of Caco-2 cells with control lentivirus or lentivirus to overexpress miR-200b, miR-200c, or miR-200b plus miR-200c. (**F**) The MTT viability assay was performed 12, 24, 36, 48, 60 and 72 h after the transfection of Caco-2 cells with pre-scramble plus control vector, pre-miR-200b/c plus control vector, pre-scramble plus RECK overexpression vector (WT 3′UTR or Mut 3′UTR), or pre-miR-200b/c plus the RECK overexpression vector (WT 3′UTR or Mut 3′UTR).

and SW480 (Supplementary **Fig.4L**) cells. The results again demonstrated that decreased RECK levels yielded the same phenotype generated by miR-200b/c overexpression. To further examine the functional relationship between miR-200b/c and RECK, Caco-2 cells were simultaneously transfected with pre-miR-200b/c and the RECK overexpression plasmid. As expected, overexpression of RECK dramatically attenuated the proliferation induced by miR-200b/c (**Fig. 5, G and H**). Additionally, Caco-2 cells infected with miR-200b/c-lentivirus showed increased cell proliferation (**Fig. 5, I and J**). Finally, when Caco-2 cells were transfected with full length RECK cDNA and its mutant form, it was again observed that RECK overexpression can attenuate the pro-proliferative effect of miR-200b/c, and the inhibitory effect was observed to be more significant in cells transfected with mutant RECK cDNA (**Fig. 5, K and L**). Taken together, the results demonstrate that miR-200b/c promote cell proliferation via silencing RECK.

miR-200b/c promote the growth rate of colorectal cancer cells *in vivo* via regulating RECK

We next evaluated the effects of miR-200b/c or RECK on the growth of colorectal cancer cell xenografts in mice. Caco-2 cells were infected with a lentiviral expression vector to express miR-200b/c, or transfected with a RECK plasmid to overexpress RECK, or co-transfected with the miR-200b/c overexpression lentivirus plus RECK overexpression plasmid. Then cells were implanted subcutaneously into mice, and tumor growth was evaluated on day 28 after cell implantation (**Fig. 6A**). We observed a

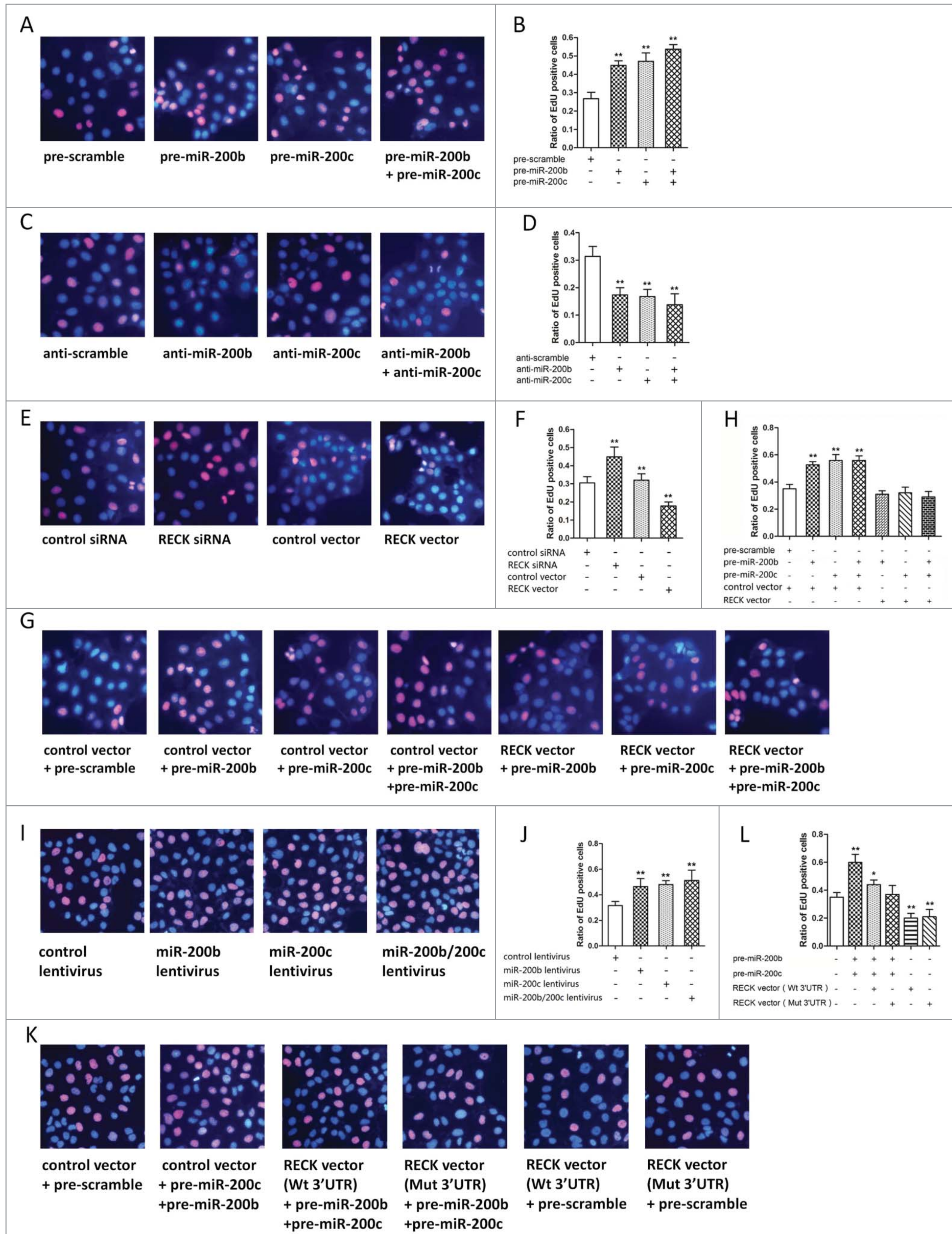

Figure 5. For figure legend, see page 286.

significant elevation in the sizes and weights of the tumors in the miR-200b/c-overexpressing group compared to control group, whereas the size and weight of the tumors in the RECK-overexpressing group was reduced (**Fig. 6, B and C**). In addition, RECK overexpression can attenuate the effect of miR-200b/c on tumor growth (**Fig. 6, B and C**), suggesting that miR-200b/c may promote tumor growth by silencing RECK. Furthermore, H&E staining of xenograft tissues showed more cell mitosis in the miR-200b/c-overexpressing group compared with control group, whereas confluent necrotic areas were observed in xenografts from the RECK-overexpressing group (**Fig. 6D**). Compared to xenografts with miR-200b/c overexpression, xenografts with both miR-200b/c and RECK overexpression exhibited reduced cell mitosis (**Fig. 6D**), suggesting that RECK overexpression can attenuate the pro-proliferative effect of miR-200b/c. In addition, the proliferative activity of tumor cells was assessed by immunocytochemistry with the mouse monoclonal antibody Ki-67, which recognizes a nuclear antigen expressed in all except G0 phase of the cell cycle. The cell proliferation rate, as measured by the percentage of Ki-67-positive tumor cells, was increased in tumors from the miR-200b/c-overexpressing group and decreased in tumors from the RECK-overexpressing group (**Fig. 6E**). Likewise, RECK overexpression can attenuate the pro-proliferative effect caused by miR-200b/c overexpression (**Fig. 6E**). Next, total RNA and protein were extracted from each xenograft and used to evaluate the expression of miR-200b/c and RECK. After 28 d of xenograft growth *in vivo*, tumors from the group with miR-200b/c overexpression showed a significant increase in the expression of miR-200b/c compared to tumors from the control group (**Fig. 6F**). Likewise, RECK mRNA levels were increased in tumors from the RECK-overexpressing group (**Fig. 6G**). Moreover, tumors from the miR-200b/c-overexpressing group displayed reduced RECK protein levels compared to tumors from the control group, whereas the tumors from the RECK-overexpressing group showed elevated RECK protein levels (**Fig. 6H**). Compared to tumors with miR-200b/c overexpression, tumors with both miR-200b/c and RECK overexpression exhibited significantly higher levels of RECK (**Fig. 6H**), suggesting that RECK overexpression can rescue the RECK suppression caused by miR-200b/c. These results, consistent with the findings from the *in vitro* assays, validated the role of miR-200b/c in promoting tumorigenesis through the targeting of RECK.

Discussion

Colorectal cancer is the third most common cancer worldwide, with an estimated one million new cases and half a million deaths each year.[23] It is more common in developed countries.[24] At the molecular level, colorectal cancer arises from a series of genetic and epigenetic alterations that inactivate tumor suppressor genes and activate oncogenes. Currently, the classical tumor suppressors and oncogenes have been expanded to include miRNAs. Studies have demonstrated an extensive alteration of miRNA expression in colorectal cancer, including miR-200 family.[25,26] Consistent with these findings, we found in this study that miR-200b/c were highly expressed in colorectal cancer compared to normal adjacent tissues. Indeed, upregulation of miR-200 family is associated with enhanced tumorigenesis and is significantly correlated with decreased survival in colorectal cancer and some other types of cancer. For example, Kaplan-Meier survival analysis of colorectal cancer patients suggested that high expression of miR-200c was associated with decreased overall survival.[25] Furthermore, it has been shown that miR-200 family is responsible for direct enhancement of distant metastases of breast cancer. Additionally, miR-200 family members are significantly overexpressed in human ovarian cancer.[27] These findings indicate that miR-200 family may be involved in the pathogenesis of human cancer as oncogenes. Conversely, miR-200 family may also act as anti-metastasis genes. We evaluated the effects of miR-200b/c on the migration ability of Caco-2 cells using a standard wound healing assay. The wound healing assay showed that the cell migration was significantly decreased in Caco-2 cells transfected with pre-miR-200b/c and increased in cells transfected with anti-miR-200b/c (Supplementary **Fig.5, A and B**). Additionally, the transfection of RECK siRNA markedly stimulated cell migration, whereas transfection of the RECK-overexpression plasmid significantly reduced the migration ability of Caco-2 cells (Supplementary **Fig.5C**). Thus, both miR-200b/c and RECK showed an inhibitory effect on colorectal cancer cell migration. That is to say, miR-200b/c may inhibit cell migration by silencing genes other than RECK. Actually, miR-200 family has long been known to suppress EMT and metastatic behavior by direct targeting of zing finger E-box-binding protein 1 (ZEB1) and ZEB2, and their downregulation in some tumors promotes invasion and

Figure 5 (See previous page). EdU proliferation assay analysis of the effect of RECK-targeted miR-200b/c on the growth of colorectal cancer cells. The cells with red fluorescence are in the S phase of mitosis, and the cells with blue fluorescence represent all of the cells. (**A**-**D**) The EdU proliferation assay was performed 48 h after the transfection of Caco-2 cells with scrambled negative control RNA, pre-miR-200b/c, or anti-miR-200b/c. A and C: representative image; B and D: ratio of EdU-positive Caco-2 cells (** $P < 0.01$). (**E** and **F**) The EdU proliferation assay was performed 48 h after the transfection of Caco-2 cells with scrambled control siRNA, RECK siRNA, control vector or the RECK overexpression vector. E: representative image; F: ratio of EdU-positive Caco-2 cells (** $P < 0.01$). (**G and H**) The EdU proliferation assay was performed 48 h after the transfection of Caco-2 cells with pre-scramble plus control vector, pre-miR-200b/c plus control vector, or pre-miR-200b/c plus the RECK overexpression vector. G: representative image; H: ratio of EdU-positive Caco-2 cells (** $P < 0.01$). (**I** and **J**) The EdU proliferation assay was performed 48 h after the infection of Caco-2 cells with control lentivirus or lentivirus to overexpress miR-200b, miR-200c, or miR-200b plus miR-200c. I: representative image; J: ratio of EdU-positive Caco-2 cells (** $P < 0.01$). (**K** and **L**) The EdU proliferation assay was performed 48 h after the transfection of Caco-2 cells with pre-scramble plus control vector, pre-miR-200b/c plus control vector, pre-scramble plus RECK overexpression vector (WT 3′UTR or Mut 3′UTR), or pre-miR-200b/c plus the RECK overexpression vector (WT 3′UTR or Mut 3′UTR). K: representative image; L: ratio of EdU-positive Caco-2 cells (** $P < 0.01$).

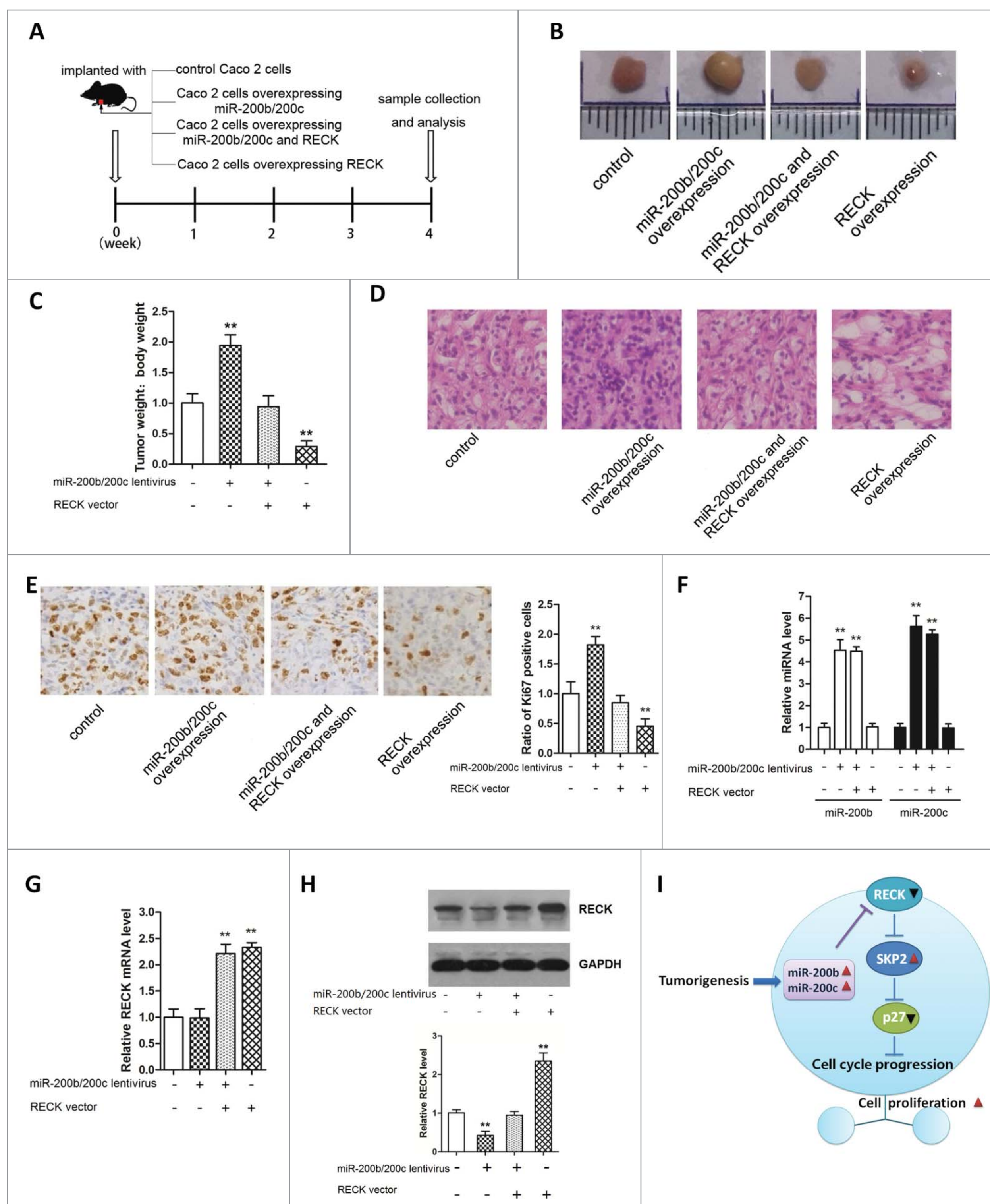

Figure 6. For figure legend, see page 288.

metastasis.[28-30] Thus, whether miR-200 family functions as oncogenes or anti-metastasis genes is dependent on the tumor types and stages. Under different circumstances, miR-200 family may exert different functions. Just like our finding about the role of miR-200b/c in promoting cell proliferation, a previous study has found that, although miR-200c overexpression reduced invasion and migration behaviors in colorectal cancer cell lines, it did result in enhanced cell proliferation.[29] Overall the findings of ours and others highlight distinguishing characteristics of miR-200 family in both

suppressing metastasis and promoting proliferation during colorectal tumorigenesis.

It is well known that a single miRNA can target multiple genes while multiple miRNAs can target a single gene. Thus, miR-200b/c may have multiple different mRNA targets other than RECK, and we cannot rule out the possibility that additional targets are affected by miR-200b/c simultaneously. Therefore, at this stage the most important question is to investigate how critical the new pathway would be in the web of colorectal tumorigenesis. In this study, we found that miR-200b/c overexpression can promote colorectal cancer cell proliferation and that RECK reduction can mimic miR-200b/c induction in promoting colorectal cancer cell proliferation. Interestingly, restoration of RECK expression can reverse miR-200b/c-induced proliferation, suggesting that targeting RECK is one mechanism by which miR-200b/c exert their oncogenic function. Therefore, modulation of RECK by miR-200b/c might explain, at least in part, why the upregulation of miR-200b/c can promote cell growth and colorectal cancer formation. On the other hand, RECK may be regulated by different miRNAs other than miR-200b/c. We have test the effects of miR-21, another miRNA that is known to regulate RECK,[31-34] in combination with miR-200b/c. Although both miR-21 and miR-200b/c can modulate RECK expression, the suppression of RECK by miR-200b/c is obviously more significant than that by miR-21 (Supplementary **Fig.6, A-C**). Moreover, while miR-200b/c can promote the proliferation of colorectal cancer cells, miR-21, by contrast, inhibited cell proliferation (Supplementary **Fig.6D**). Finally, while the expression levels of miR-21 were higher in some colorectal cancer tissues than in the adjacent normal tissues, in others it was lower (Supplementary **Fig.6E**). Taken together, the results suggest that miR-200b/c more efficiently suppress RECK expression in colorectal cancer cells than miR-21, and their effect on cell proliferation is unrelated to miR-21.

It is worth noting that deregulated cell proliferation is a key mechanism for neoplastic progression.[35] The pathways constraining the proliferative response in normal cells are perturbed in most cancers. Previous studies have showed that RECK plays an important role in regulating SKP2 and $p27^{Kip1}$ expression,[12] which is important to control mammalian cell proliferation. Consistent with the negative role of $p27^{Kip1}$ in cell cycle progression, $p27^{Kip1}$ is destabilized in many types of human cancer, and this destabilization correlates with tumor aggressiveness and poor prognosis.[36] Here, we found that miR-200b/c overexpression can increase SKP2 and inhibit $p27^{Kip1}$ expression by targeting RECK. These findings identified a potent link between miR-200b/c and the RECK axis, which may play a vital role in cell proliferation and colorectal tumorigenesis.

On the basis of this study, we propose a consolidated model that illustrates a plausible sequence for the mechanism by which miR-200b/c promote colorectal cancer cell proliferation (**Fig. 6I**). In theory, high levels of miR-200b/c in colorectal cancer cell promoted colorectal tumorigenesis by targeting and downregulating RECK and influencing the downstream RECK pathway, including the upregulation of SKP2 and the consequent downregulation of $p27^{Kip1}$. This study reveals a novel role for miR-200b/c-regulated RECK expression in colorectal tumorigenesis, and provided a potential new target for colorectal therapy in the future.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Figure 6 (See previous page). Effects of overexpression of miR-200b/c or RECK on the growth of colorectal cancer cell xenografts in mice. (**A**) Flow chart of the experimental design. Caco-2 cells were infected with a lentiviral expression vector to simultaneously express miR-200b and miR-200c, or transfected with an RECK plasmid to overexpress RECK, or co-transfected with the miR-200b/c overexpression lentivirus plus RECK overexpression plasmid. Cells (1×10^7 cells per 0.1 mL) were then implanted subcutaneously into 4-week-old C57/BL6 mice (5 mice per group), and tumor growth was evaluated at day 28 after cell implantation. (**B**) Representative image of the tumors from mice implanted with control cells, miR-200b/c-overexpressing cells, RECK-overexpressing cells, or miR-200b/c- and RECK-overexpressing cells. (**C**) Quantitative analysis of the tumor weights in panel B (**$P < 0.01$). (**D**) Representative H&E-stained sections of the tumors from mice implanted with control cells, miR-200b/c-overexpressing cells, RECK-overexpressing cells, or miR-200b/c- and RECK-overexpressing cells. (**E**) Proliferative activity assessed by anti-Ki-67 monoclonal antibody in the tumors from mice implanted with control cells, miR-200b/c-overexpressing cells, RECK-overexpressing cells, or miR-200b/c- and RECK-overexpressing cells. **(F and G)** Quantitative RT-PCR analysis of miR-200b/c (F) and RECK mRNA (G) levels in the tumors from mice implanted with control cells, miR-200b/c-overexpressing cells, RECK-overexpressing cells, or miR-200b/c- and RECK-overexpressing cells. Left panel: representative image; right panel: quantitative analysis (**$P < 0.01$). (**H**) Western blotting analysis of RECK protein levels in the tumors from mice implanted with control cells, miR-200b/c-overexpressing cells, RECK-overexpressing cells, or miR-200b/c- and RECK-overexpressing cells. Upper panel: representative image; lower panel: quantitative analysis (**$P < 0.01$). (**I**) A working model for the role of RECK-targeted miR-200b/c in colorectal cancer. During colorectal tumorigenesis, miR-200b/c activation will directly inhibit RECK expression, consequently triggering SKP2 elevation and $p27^{Kip1}$ degradation, which eventually increase proliferation and result in the malignant transformation of human colorectal cancer.

Authors' Contributions

Study concept and design: Xi Chen. Acquisition of data: Yi Pan, Hongwei Liang, Weixu Chen, Hongjie Zhang. Analysis and interpretation of data: Nan Wang, Feng Wang, Suyang Zhang, Yanqing Liu, Chihao Zhao, and Xin Yan. Drafting of manuscript: Xi Chen. Final approval of manuscript: Junfeng Zhang, Chen-Yu Zhang, Ke Zen, and Xi Chen. Study supervision: Xi Chen.

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