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C/EBP- β -activated microRNA-223 promotes tumour growth through targeting RASA1 in human colorectal cancer

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Background: Evidences have shown that the RAS signalling pathway plays an important role in colorectal cancer (CRC). Moreover, RAS-GTPase-activating proteins (RASGAPs) as RAS signalling terminators are associated with tumorigenicity and tumour progression. In this study, we used bioinformatics analysis to predict and study important miRNAs that could target RAS p21 GTPase-activating protein 1 (RASA1), an important member of RASGAPs.

Methods: The levels of RASA1 and miR-223 were analysed by real-time PCR, western blotting or *in situ* immunofluorescence analyses. The functional effects of miR-223 and the effects of miR-223-targeted inhibitors were examined *in vivo* using established assays.

Results: Upregulation of miR-223 was detected in CRC tissues ($P < 0.01$) and was involved in downregulation of RASA1 in CRC tissues. Furthermore, the direct inhibition of RASA1 translation by miR-223 and the activation of miR-223 by CCAAT/enhancer binding protein- β (C/EBP- β) were evaluated in CRC cells. An *in vivo* xenograft model of CRC suggested that the upregulation of miR-223 could promote tumour growth and that the inhibition of miR-223 might prevent solid tumour growth.

Conclusions: These results identify that C/EBP- β -activated miR-223 contributes to tumour growth by targeting RASA1 in CRC and miR-223-targeted inhibitors may have clinical promise for CRC treatment via suppression of miR-223.

Colorectal cancer (CRC) is one of the most common malignant tumours worldwide (Jemal *et al*, 2011). Although new and precise molecular mechanisms of CRC are being discovered, it is generally accepted that the development of CRC involves a multistep process involving the accumulation of both genetic and epigenetic changes (Wong *et al*, 2007; Grady and Carethers, 2008). Evidences indicate that the RAS signalling pathway plays an important role in CRC development, progression and resistance to treatment (Haigis *et al*, 2008; Jimeno *et al*, 2009). RAS can serve as a molecular microswitch of the RAS signalling pathway to control cellular functions, whose activity is regulated by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) (Bos *et al*, 2007). Moreover, RAS-GTPase-activating proteins (RASGAPs) as RAS signalling terminators have been reported to

be associated with tumorigenicity and tumour progression (Kofschoten *et al*, 2005; Ohta *et al*, 2009; McLaughlin *et al*, 2013). As an important member of RASGAPs, RAS p21 GTPase-activating protein 1 (RASA1) contains two SH2, SH3, PH and CaLB/C2 domains in N-terminal part. These protein domains allow various functions, such as anti-/pro-apoptosis, proliferation and cell migration, depending on their distinct partners in angiogenesis and cancer (Pamonsinlapatham *et al*, 2009). Lapinski *et al* (2012) revealed that RASA1 could maintain the lymphatic vasculature in a quiescent functional state through its ability to inhibit RAS signal transduction induced by growth factor receptors such as VEGFR-3 in mice. RASA1 gene mutations are associated with capillary malformation–arteriovenous malformations and lymphatic abnormalities in patients (Boon *et al*, 2005;

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Burrows *et al*, 2013). Nevertheless, the role of RASA1 in CRC is rarely reported.

MicroRNAs (miRNAs) have raised considerable attention for their important roles in numerous cellular and developmental processes (Bushati and Cohen, 2007). In particular, miRNAs are key players in human tumours, acting as oncogenes and tumour suppressors (Calin and Croce, 2006). Emerging evidences suggest that a variety of miRNAs are aberrantly expressed in the serum, plasma and tissues of CRC patients, indicating that miRNAs may have vital roles in CRC (Schetter *et al*, 2008; Ng *et al*, 2009; Wang and Gu, 2012). The biological functions of these aberrantly expressed miRNAs involving the RAS signalling pathway in CRC have not been extensively studied (Akao *et al*, 2006; Chen *et al*, 2009; Tsang and Kwok, 2009). Anand *et al* (2010) reported that miR-132 served as an angiogenic microswitch predominantly through targeting RASA1, leading to neovascularisation in an orthotopic xenograft mouse model of human breast carcinoma. Sun *et al* (2013) revealed that miR-31 functioned as an oncogenic miRNA to activate the RAS/MAPK pathway by repressing RASA1 in CRC. However, it is unknown whether other miRNAs could target RASA1 in CRC.

The CCAAT/enhancer binding protein- β (C/EBP- β) is a transcription factor that can either activate or repress transcription (Descombes and Schibler, 1991). Evidences suggest that it plays a critical role in various malignancies, including glioma, pancreatic tumour and skin tumourigenesis (Homma *et al*, 2006; Sterneck *et al*, 2006; Shimizu *et al*, 2007). The C/EBP- β was found to overexpress and induce Cox-2 gene expression in gastric carcinogenesis (Regalo *et al*, 2006). In addition, Fukao *et al* (2007) discovered that there was a C/EBP- β -binding element in pri-miR-223 promoter. Recent studies showed that C/EBP- β could respectively regulate let-7i following microbial infection and miR-145 in cancer cells (O'Hara *et al*, 2010; Sachdeva *et al*, 2012). Although Rask *et al* (2000) reported that C/EBP- β markedly increased in all CRCs compared with normal colon mucosa, it is not clear whether C/EBP- β could regulate miRNAs in CRC.

In this study, we used bioinformatics analysis to predict and find six important miRNAs that could target RASA1 by binding the 3'-UTR of RASA1. The results of *in situ* immunofluorescence analysis and western blotting analysis highlighted that miR-223 and RASA1 demonstrated an inverse correlation in CRC patient tissues. In addition, *in vitro* studies on the direct inhibition of RASA1 by miR-223, the activation mechanism of miR-223 by C/EBP- β in CRC and the potential role of miR-223 to promote colorectal cell proliferation were experimentally investigated. The influence of miR-223 on CRC was further studied in an immunodeficient mouse xenograft tumour model by over- or down-expression of miR-223.

MATERIALS AND METHODS

Clinical samples, cell lines and chemical reagents. Paired CRC and adjacent, nontumour tissue (NAT) samples were obtained from patients who underwent radical resection at Jinling Hospital (Nanjing, Jiangsu, China) from 2011 to 2013. Ethical approval was obtained from the local ethics committee. A total of 24 patients were randomly chosen and numbered for this study. The information of patients is shown in Table 1 and Supplementary Table 1. Caco-2 cells and HT-29 cells were cultured as described previously (Sun *et al*, 2013). All chemical reagents were purchased from Sigma-Aldrich (St Louis, MO, USA).

Assay to detect mutant K-RAS. Paraffin-embedded tumour sections were deparaffinised and air dried, and DNA was isolated using proteinase K and a DNeasy minispin column (Qiagen, Valencia, CA, USA). Then, K-RAS point mutations in codon 12

Table 1. The characteristics of colorectal cancer (CRC) patients and tumours for this study

	Number (n)	Proportion (%)
All cases	24	100
Gender		
Male	14	58.3
Female	10	41.7
Age (years)		
≤60	15	62.5
>60	9	37.5
Primary tumour		
Colon	16	64.0
Rectum	8	36.0
K-RAS status		
Wild type	14	58.3
Mutant	10	41.7
Stage (tumour–node–metastasis (TNM))		
T1	1	4.2
T2	4	16.7
T3	12	50.0
T4	7	29.1
Histology type		
Well differentiated	4	16.7
Moderately differentiated	15	62.5
Poorly differentiated	5	20.8

and codon 13, two hot spots that include >95% of mutations in this gene, were detected as previously reported (Pinto *et al*, 2011).

RNA extraction and quantitative real-time PCR (qRT-PCR) assay. RNA was isolated from tissues and cells and made into complementary DNA using methods previously described (Sun *et al*, 2013). The qRT-PCR for miRNAs and mRNA was performed according to the manufacturer's instructions (Life Technologies, Carlsbad, CA, USA) using the specific primers listed in Supplementary Table 2. The qRT-PCR data for miRNA and mRNA are expressed relative to U6 snRNA or β -actin mRNA, respectively.

Histology and *in situ* immunofluorescence staining assays. The CRC and NAT samples were fixed in 4% (wt/vol) paraformaldehyde. After paraffin embedding, 5- μ m sections were cut and stained with haematoxylin and eosin (H&E; Lerner, New Haven, CT, USA) for histopathological examination. For miRNA *in situ* hybridisation, a 5'-Cy5-conjugated miR-223 probe (Exiqon, Vedbaek, Denmark) was used as previously described (de Planell-Saguer *et al*, 2010)

Protein extraction and analysis. Western blotting was carried out as previously described (Sun *et al*, 2013). Antibodies included: RASA1 (1:200 dilution, Santa Cruz, Santa Cruz, CA, USA), RAS-GTP (1:200 dilution, Thermo Scientific, Hudson, NH, USA), total RAS (1:1000 dilution, BD Biosciences, San Jose, CA, USA), p-MEK1/2, total MEK1/2, p-ERK1/2 or total ERK1/2 (1:1000 dilution, Cell Signaling Technologies, Danvers, MA, USA). We performed the normalisation by blotting the same membranes with an antibody against GAPDH (1:10000 dilution, KangChen Bio-tech, Shanghai, China).

Cell transfection/infection assays. Caco-2 or HT-29 cells were transfected with precursor oligonucleotides (pre-miR-223), anti-sense oligonucleotides (anti-miR-223) (Ambion, Austin, TX, USA) or their corresponding controls (pre-ncRNA or anti-ncRNA) using the Lipofectamine 2000 transfection reagent (Life Technologies) according to the manufacturer's instructions. To obtain HT-29 cells stably expressing miR-223 or ncRNA, HT-29 cells were infected with recombinant lentiviruses respectively carrying

pre-miR-223 precursor (pre-miR-223-LV) or pre-non-coding sequence (pre-NC-LV) (GenePharma, Shanghai, China) in the presence of 8 mg ml⁻¹ polybrene (GenePharma) for 12 h, and the medium was then replaced with fresh medium including 10 µg ml⁻¹ puromycin. The cells stably expressing miR-223 were sorted by a FACS (fluorescent-activated cell sorter, Becton Dickinson, Mountain View, CA, USA) based on the expression of GFP carried by pGLV3 plasmid. The sorted cells were taken as the overexpression model for the following experiments.

Plasmid constructs and Luciferase reporter assay. The RASA1 luciferase reporter plasmid was constructed as previously described (Sun *et al*, 2013). This construct was co-transfected with pre/anti-miR-223 into Caco-2 cells. The binding-site mutant luciferase plasmid (binding site: AACTGAC replaced by TTGACTG) was also transfected as a control.

The full-length human C/EBP-β expression plasmid (pcDNA3.1-C/EBP-β) was obtained from GeneCopeia (Rockville, MD, USA). The C/EBP-β-responsive sequence in the pri-miR-223 promoter (Fukao *et al*, 2007) was cloned into the *MluI/BgII* sites in the pGL3-promoter vector (E1761; Promega, San Luis Obispo, CA, USA) with a luciferase gene, creating pGL3 miR-223⁻¹⁹⁸. The pGL3 miR-223⁻¹⁹⁸ was then co-transfected with pcDNA3.1-C/EBP-β or C/EBP-β siRNA into Caco-2 cells, and the cells were lysed to measure the luciferase activity 24 h later. A plasmid encoding β-gal was co-transfected and used for normalisation.

Cell proliferation assay. The cell proliferation assay was performed as previously described (Sun *et al*, 2013). Briefly, the number of Caco-2 cells with over- or down-expression of miR-223 was determined using WST-8 staining with a Cell Counting Kit-8 (CCK-8, Dojindo, Tokyo, Japan) according to the manufacturer's instructions. Moreover, the growth of Caco-2 cells at 48 h post transfection in the presence or absence of 10 µM U0126 (MEK1/2 inhibitor) was also assessed using the proliferation index KI-67 and PCNA.

The growth of human colorectal tumour in an immunodeficient mouse xenograft model. The 4-week-old, thymic BALB/c male, nude (nu/nu) mice were obtained from Model Animal Research Center of Nanjing University (Nanjing, Jiangsu, China) and maintained under pathogen-limited conditions. All animals received care according to Chinese legal requirements and the animals were equally divided into two groups (seven mice per group). The HT-29 cells that stably overexpressed miR-223 (pre-miR-223-LV) or ncRNA (pre-ncRNA-LV) were subcutaneously injected into their left flanks with the viable cell number of 1 × 10⁷. After subcutaneous (s.c.) implantation of cells, the animals were observed daily for tumour growth as described previously (Sun *et al*, 2013).

Therapeutic effect of a miR-223 inhibitor on an immunodeficient mouse xenograft model. The miR-223 AMOs with full phosphorothioate linkage were designed and synthesised by SBS Genetech Co., Ltd (Shanghai, China), and the most efficient one (5'-GGGGTATTTGACAACTGAC-3') was selected. Polyether-imide (PEI, 25 kDa)/miR-223 AMO complexes were prepared as already reported (Huang *et al*, 2014).

Xenografts were initiated by s.c. injection of 1 × 10⁷ HT-29 cells into the right flanks of nude mice. After 3 weeks, the tumours were aseptically dissected and mechanically minced; and 1 mm × 1 mm diameter tumour pieces were transplanted subcutaneously into the nude mice using a trocar needle. When tumours had grown to a volume of ~100 mm³ on day 10 after tumour transplantation, the mice were randomised into specific treatment group, control treatment group and nontreatment group (7 mice per group). The 10 nmol PEI/miR-223 AMO complexes, PEI/scramble DNA complexes or PEI alone were injected directly into the tumours every 3 days beginning on day 10 after tumour transplantation.

Tumour volumes were monitored every 3 days as indicated in the figures and, upon termination of the experiment, mice were killed and tumours removed. Pieces of each tumour were immediately snap frozen for RNA preparation.

Statistical analysis. All experiments were conducted in triplicate and repeated at least three independent times. Data are expressed as the mean ± s.e.m. The difference among groups was determined using ANOVA analysis, and comparison between two groups was analysed using Student's *t*-test and GraphPad Prism software version 4.0 (GraphPad Software, Inc., San Diego, CA, USA). A value of *P* < 0.05 was considered statistically significant.

RESULTS

Patient characteristics and detection of K-RAS mutations by direct sequencing. Table 1 summarises patient demographic data and CRC characteristics. The cohort in the study consists of 24 CRC samples, including stage T1, T2, T3 and T4 patients. The average patient age is 56.5 years (range, 29–72) and most patients (79.2%) have well or moderately differentiated cancers. Among the 24 patients, 10 patients harbour a somatic K-RAS mutation (41.7%) and of the total K-RAS mutations, 80.0% and 20.0% were observed in codon 12 and codon 13, respectively, that is consistent with the previous report (Samowitz *et al*, 2000) (for patient information, refer to Supplementary Table 1).

MiR-223 is upregulated in human CRC. We performed bioinformatics analysis to search for RASA1-targeted miRNAs. TargetsCan, Miranda, PICTAR5 and PITA were independently used to predict the miRNAs. Combination of these bioinformatics prediction methods revealed that six important miRNAs could target RASA1, including miR-24, miR-132, miR-223, miR-182, miR-31 and miR-335. Therefore, we performed qRT-PCR assay on 24 pairs of CRC tissues and NAT to quantify the expressions of these six miRNAs. As shown in Figure 1A, qRT-PCR analysis revealed that the levels of miR-223 and miR-31 in CRC samples significantly increased (*P* < 0.01); the levels of miR-335 and miR-182 in CRC samples were enhanced (*P* < 0.05); but no significant changes were observed in the levels of miR-24 and miR-132 between CRC and NAT samples (*P* > 0.05). Because miR-31 was found to act as an oncogenic miRNA in CRC in our previous report (Sun *et al*, 2013), we hypothesised that miR-223, 65 times higher than miR-31 in NAT samples as shown in Figure 1B, might play a significant role in CRC. Interestingly, miR-223 was upregulated in 24 CRC samples whereas 10 CRC samples were K-RAS mutated tumours, suggesting that miR-223 could be a broader mechanism for CRC than for K-RAS mutated tumours. The action of miR-223 in CRC was thereby investigated in the following.

Identification of RASA1 as a target of miR-223. The correlation between miR-223 and RASA1 was further examined by using *in situ* immunofluorescence staining, qRT-PCR and western blotting assays in patient samples from sample 1 to sample 12 in Supplementary Table 1. As shown in Figure 2A, following the examination of H&E staining for sample 1, the immunofluorescence staining demonstrated that miR-223 was upregulated whereas the RASA1 protein was downregulated in CRC sample 1 compared with NAT sample 1, suggesting an inverse correlation between miR-223 and the RASA1 in sample 1. The similar results of the immunofluorescence staining were observed in other 11 samples (figures not shown). Consistently, the overall level of miR-223 was increased by 6.85-fold in CRC samples compared with NAT samples in qRT-PCR analysis (Figure 2B), whereas the overall level of RASA1 in CRC samples was 64.9% lower than that in NAT samples (Figure 2C).

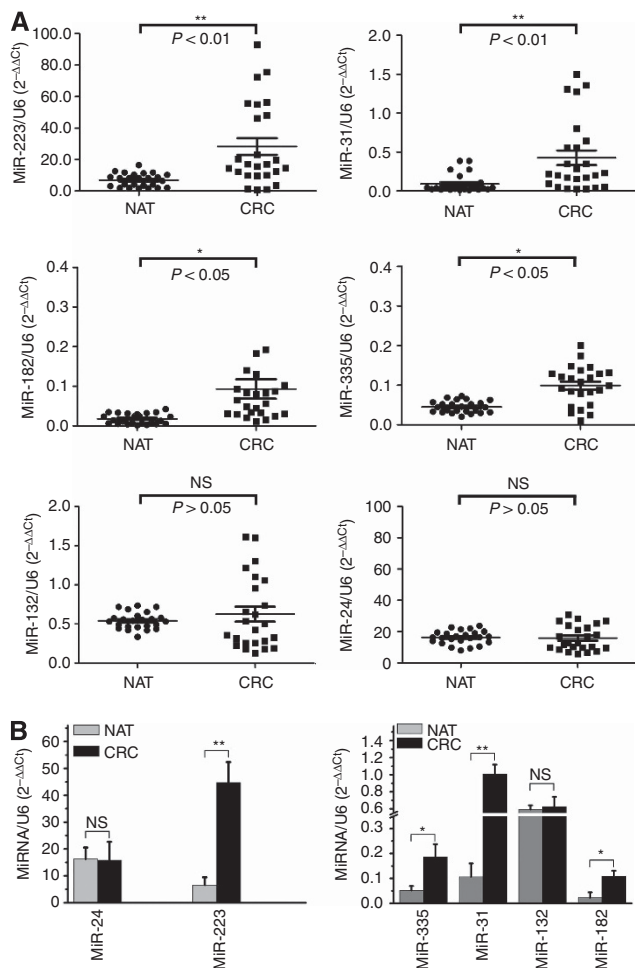


Figure 1. Expression analysis of six RASA1-targeted miRNAs in paired CRC and NAT samples. (A) The levels of six miRNAs tested using qRT-PCR assay and shown as scatter plots. (B) The levels of six miRNAs tested using qRT-PCR assay and shown as histograms. Values are expressed as the means ± s.e. *P < 0.05. **P < 0.01. NS (P > 0.05) = no significant change.

It was not clear whether miR-223 downregulated RASA1 by binding to the 3'-UTR of the RASA1 mRNA; therefore, additional experiments were carried out in CRC cells. As Figure 3A shows, when compared with mock experiments using the luciferase reporter assay, overexpression of miR-223 exerted a repressive effect with ~53.9% reduction in reporter activity. Meanwhile, inhibition of miR-223 resulted in an 18.6% increase in reporter activity compared with the control, and mutation of the miR-223-binding site abolished the above effects. Next, we used western blotting to examine the RASA1 protein level after over- or down-expression of miR-223. As Figure 3B shows, compared with the control, overexpression of miR-223 resulted in a significant decrease in RASA1, whereas downexpression of miR-223 led to an increase in RASA1. However, in contrast with the variation of RASA1 protein, the level of RASA1 mRNA was not repressed by over- or down-expression of miR-223, as shown in Figure 3C. These results demonstrated that miR-223 regulated RASA1 expression only via a translational repression mechanism, rather than by affecting its mRNA stability. Taken together, these results proved that miR-223 might negatively regulate RASA1 in CRC.

C/EBP-β activates miR-223 expression in CRC. In CRC, the expression of miR-223 was upregulated. However, the reason for this is not clear yet. Therefore, we searched for factors that might be responsible for the regulation of miR-223 expression in CRC. As

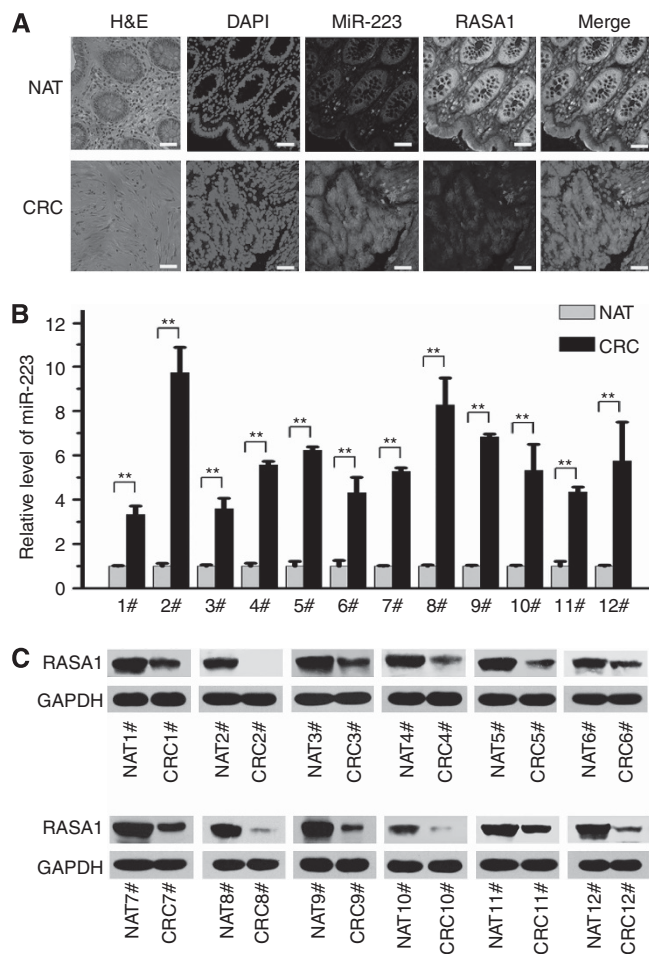


Figure 2. Inverse correlations between RASA1 and miR-223 in paired CRC and NAT samples. (A) Representative photos of three individual experiments of H&E staining and *in situ* immunofluorescence staining for miR-223 and RASA1 for sample 1 (red, miR-223; green, RASA1; blue, DAPI nuclear staining). Pictures were imaged at × 40 magnification on a Nikon confocal microscope. Scale bar, 25 μm. (B) The level of miR-223 in CRC and NAT samples tested using qRT-PCR. The data shown are the means ± s.e. of three individual experiments. (C) The level of RASA1 protein in CRC and NAT samples, as demonstrated using western blotting. The data shown are representative of three individual western blotting analyses. **P < 0.01. A full colour version of this figure is available at the *British Journal of Cancer* journal online.

shown in Figure 3D, we discovered that the C/EBP-β mRNAs were significantly upregulated in 24 CRC samples, indicating a possible positive correlation between miR-223 and C/EBP-β. To further study whether miR-223 could be modulated by C/EBP-β, we generated a miR-223 promoter reporter plasmid carrying luciferase: pGL3 miR-223⁻¹⁹⁸ (Figure 3E). As shown in Figure 3F, the overexpression of C/EBP-β resulted in a 4.8-fold increase in pGL3 miR-223⁻¹⁹⁸ luciferase reporter activity over the control, whereas the inhibition of C/EBP-β with C/EBP-β siRNA resulted in a 62.4% reduction in luciferase activity compared with the control. However, pGL3 basic vector abolished these effects. Consistent with these results, qRT-PCR assay detected an increase of miR-223 level in the cells transfected with pcDNA3.1-C/EBP-β and a significant reduction of miR-223 level in the cells transfected with C/EBP-β siRNA (Figure 3G). In addition, the level of RASA1 protein also obviously changed after over- or down-expression of C/EBP-β (Figure 3H). These results demonstrated that C/EBP-β might be a positive regulator of miR-223, thereby resulting in a significant reduction of RASA1.

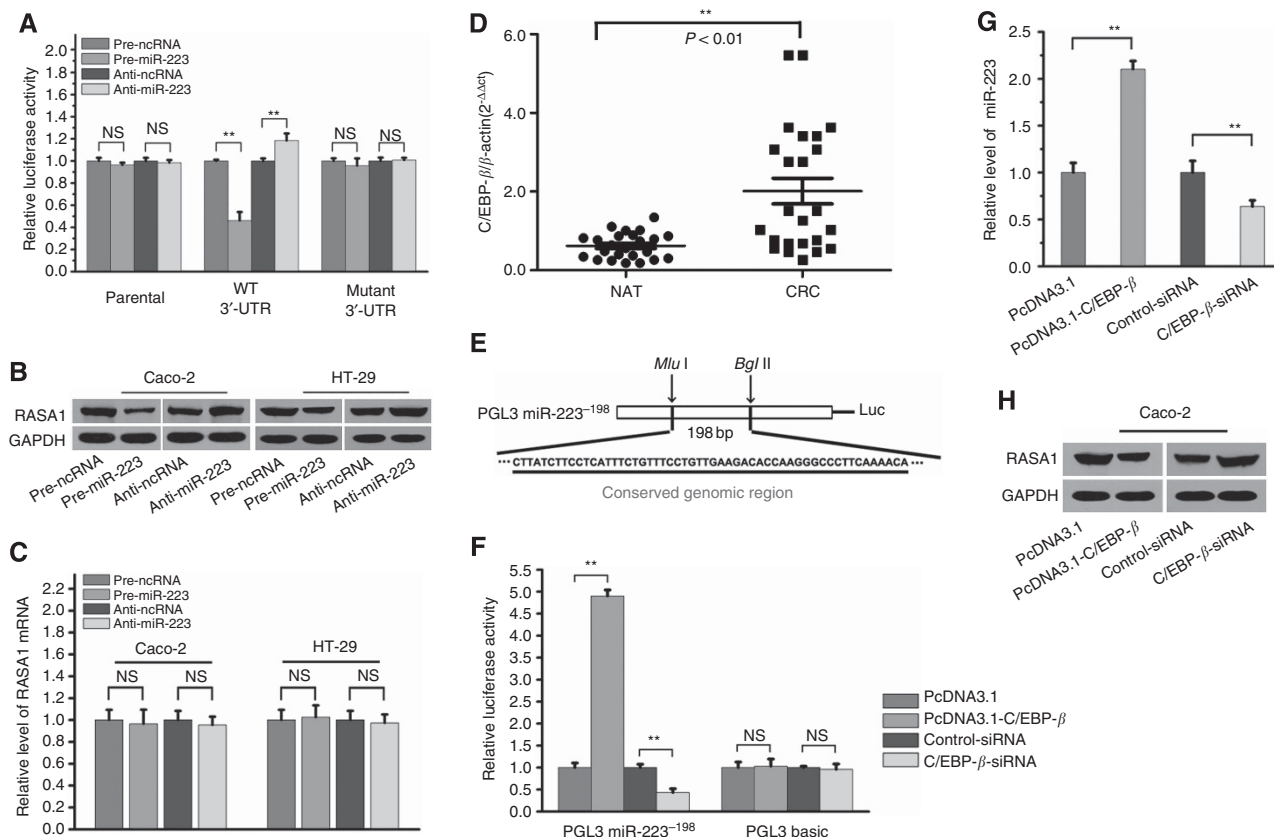


Figure 3. Identification of miR-223-targeting RASA1 and activation mechanism of miR-223 in CRC cells. **(A)** The relative luciferase activity after transfection with pre/anti-miR-223 or the corresponding control in Caco-2 cells. The data shown are the means \pm s.e. of three individual experiments. **(B)** Western blotting analysis of RASA1 in whole-cell lysates from CRC cells transfected with pre/anti-miR-223 or the corresponding control. Left panel: Caco-2 cells; right panel: HT-29 cells. The data shown are representative of three individual western blotting analyses. **(C)** The qRT-PCR analysis of RASA1 mRNA in total RNA from CRC cells transfected with pre/anti-miR-223 or the corresponding control. Left panel: Caco-2 cells; right panel: HT-29 cells. The data shown are the means \pm s.e. of three individual experiments. **(D)** The qRT-PCR analysis of C/EBP- β mRNA in total RNA from CRC and NAT samples. The data shown are the means \pm s.e. of three individual experiments. **(E)** A schematic description of the luciferase reporter construct: pGL3 miR-223⁻¹⁹⁸. The conserved genomic region in pri-miR-223 promoter for C/EBP- β binding is also shown. **(F)** Effect of C/EBP- β on the promoter of miR-223. Caco-2 cells were co-transfected with pGL3 miR-223⁻¹⁹⁸ and pcDNA 3.1-C/EBP- β or C/EBP- β siRNA. After 24 h, the reporter activity was measured using luciferase assay. The data shown are the means \pm s.e. of three individual experiments. **(G)** The qRT-PCR analysis of miR-223 in total RNA from Caco-2 cells transfected with pcDNA 3.1-C/EBP- β or C/EBP- β siRNA at 48 h post transfection. The data shown are the means \pm s.e. of three individual experiments. **(H)** Western blotting analysis of RASA1 in whole-cell lysates from Caco-2 cells transfected with pcDNA 3.1-C/EBP- β or C/EBP- β siRNA at 48 h post transfection. The data shown are representative of three individual western blotting analyses. ** $P < 0.01$. NS = no significant change.

MiR-223 regulates the RAS/MAPK pathway and influences cell proliferation in CRC cells. To study the effect of miR-223 targeting RASA1, we detected the expression of RAS-GTP and total RAS, p-MEK1/2 and total MEK1/2 and p-ERK1/2 and total ERK1/2 using western blotting assay after transfection with pre/anti-miR-223 or the corresponding control. As shown in Figure 4A and B, miR-223 had no obvious effect on the expressions of total RAS, total MEK1/2 or total ERK1/2; however, it strengthened the ability of RAS to bind to GTP and enhanced phosphorylation of the MEK1/2 and ERK1/2 proteins. Overexpression of miR-223 led to a 2.39-fold increase in p-ERK1/2, and downexpression of miR-223 decreased p-ERK1/2 expression by 70.4%. In addition, as shown in Figure 4B, the expression of p-ERK1/2 was not significantly increased or reduced compared with the control after Caco-2 cells were transfected with pre-miR-223 or anti-miR-223 in the presence of U0126, a MEK1/2 inhibitor. In a word, overexpression of miR-223 significantly suppressed RASA1, increased the level of RAS-GTP and activated the MAPK signalling pathway.

Cell proliferation was further monitored and analysed using the CCK-8 kit. Statistical analysis showed that overexpression of miR-223 significantly increased the proliferation of Caco-2 cells,

whereas downexpression of miR-223 obviously inhibited the proliferation of Caco-2 cells (Figure 4C). The qRT-PCR results showed that mRNA levels of KI-67 and PCNA were increased when miR-223 was overexpressed, whereas downexpression of miR-223 reduced mRNA levels of KI-67 and PCNA. However, when Caco-2 cells were treated with U0126 after transfection with pre/anti-miR-223 or the corresponding control, neither overexpression of miR-223 nor downexpression of miR-223 influenced the mRNA levels of KI-67 and PCNA (Figure 4D). Based on the results above, we summarised that overexpression of miR-223 promoted CRC cell proliferation by activating the RAS/MAPK signalling pathway.

The effect of miR-223 on colorectal tumour growth *in vivo*. Taking into account that miR-223 was upregulated in 24 CRC samples and in order to examine the effect of miR-223 on tumour growth *in vivo*, we successfully constructed HT-29 cells stably overexpressing miR-223. The sorted HT-29 cells were observed based on the expression of GFP as shown in Figure 5A, indicating that the cells were successfully infected by pre-miR-223-LV or pre-ncRNA-LV. As shown in Figure 5B, the level of miR-223

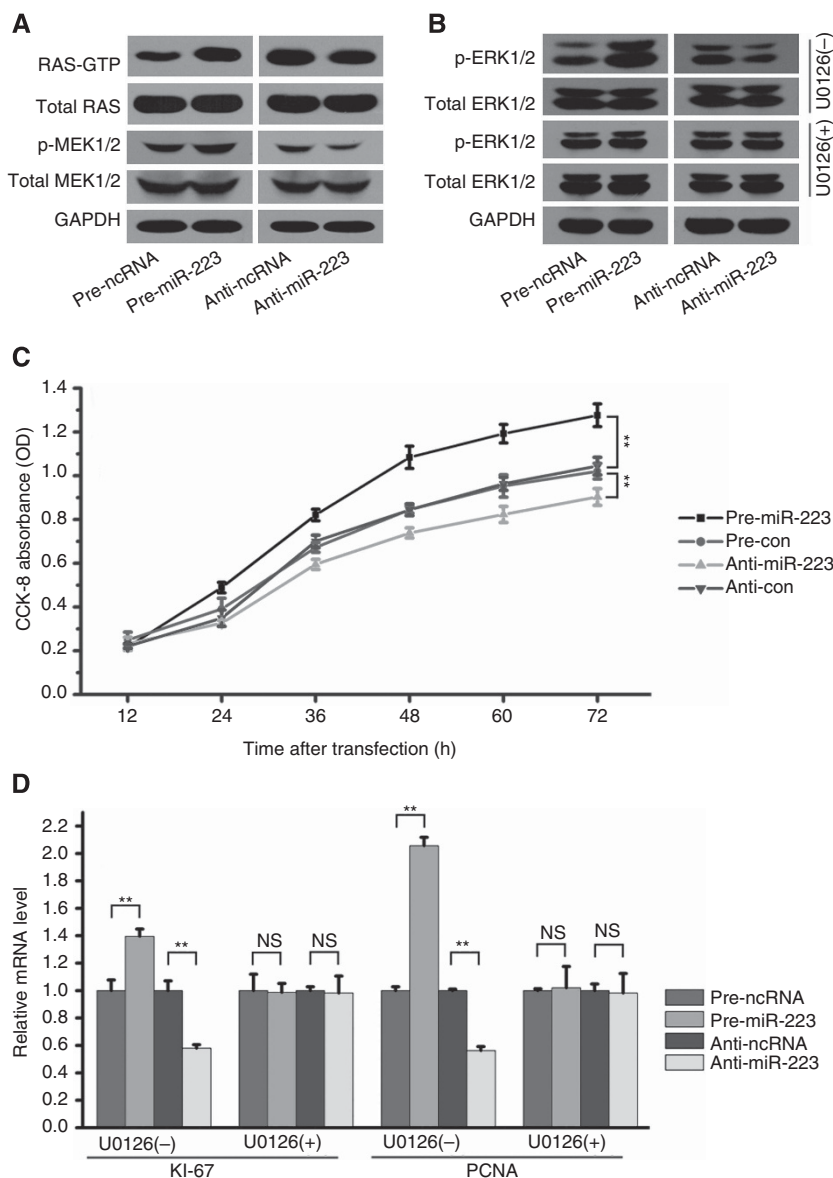


Figure 4. The miR-223 regulates the RAS/MAPK pathway and influences CRC cell proliferation. **(A)** Whole-cell lysates from Caco-2 cells transfected for 48 h with pre/anti-miR-223 or the corresponding control were subjected to western blotting analysis of RAS-GTP, total RAS, p-MEK1/2, total MEK1/2 and GAPDH (as a loading control). The data shown are representative of three individual western blotting analyses. **(B, upper panel)** Whole-cell lysates from Caco-2 cells transfected for 48 h with pre/anti-miR-223 or the corresponding control were subjected to western blotting analysis of p-ERK1/2, total ERK1/2 and GAPDH (as a loading control) without 10 μ M U0126. **(B, lower panel)** Whole-cell lysates from Caco-2 cells transfected for 48 h with pre/anti-miR-223 or the corresponding control were subjected to western blotting analysis of p-ERK1/2, total ERK1/2 and GAPDH (as a loading control) with 10 μ M U0126. The data shown are representative of three individual western blotting analyses. **(C)** Cell proliferation of Caco-2 transfected with pre/anti-miR-223 or the corresponding control was measured using CCK-8. The data shown are the means \pm s.e. of three individual experiments. **(D)** Total RNA from Caco-2 cells transfected for 48 h with pre/anti-miR-223 or the corresponding control were subjected to qRT-PCR analysis of KI-67 and PCNA without or with 10 μ M U0126. The data shown are the means \pm s.e. of three individual experiments. ** $P < 0.01$. NS = no significant change.

in HT-29 cells infected with pre-miR-223-LV was 15.2-fold of the control that resulted in a significant decrease of cellular RASA1 and suggested that the sorted pre-miR-223-LV-infected HT-29 cells could be used as stably overexpressing miR-223 cells in the immunodeficient mouse xenograft tumour model. As shown in Figure 5C, the tumour volumes from pre-miR-223-LV group grew more rapidly than those from pre-ncRNA-LV group, and the excised tumours from pre-miR-223-LV group were 51.2% heavier than those from pre-ncRNA-LV group on day 34 after implantation (Figure 5D), suggesting that overexpression of miR-223 greatly stimulated the growth of HT-29-inducing tumours *in vivo*. Representative photographs of mice bearing the tumours and the

corresponding tumours from each group are shown in Figure 5E and F.

Therapeutic effect of a miR-223 inhibitor *in vivo*. In order to attempt to analyse miR-223 as a therapeutic target in CRC, we designed a miR-223 AMO to inhibit miR-223 expression in the nude mouse xenograft model of human CRC. As shown in Figure 6A and B, when HT-29 cells were transfected with miR-223 AMO, the level of miR-223 declined by 67.3% and the level of RASA1 was enhanced by \sim 41.5% compared with the scramble DNA. To assess the antitumour activity of miR-223 AMO *in vivo*, nude mice with s.c. xenograft tumours of \sim 100 mm³ were treated

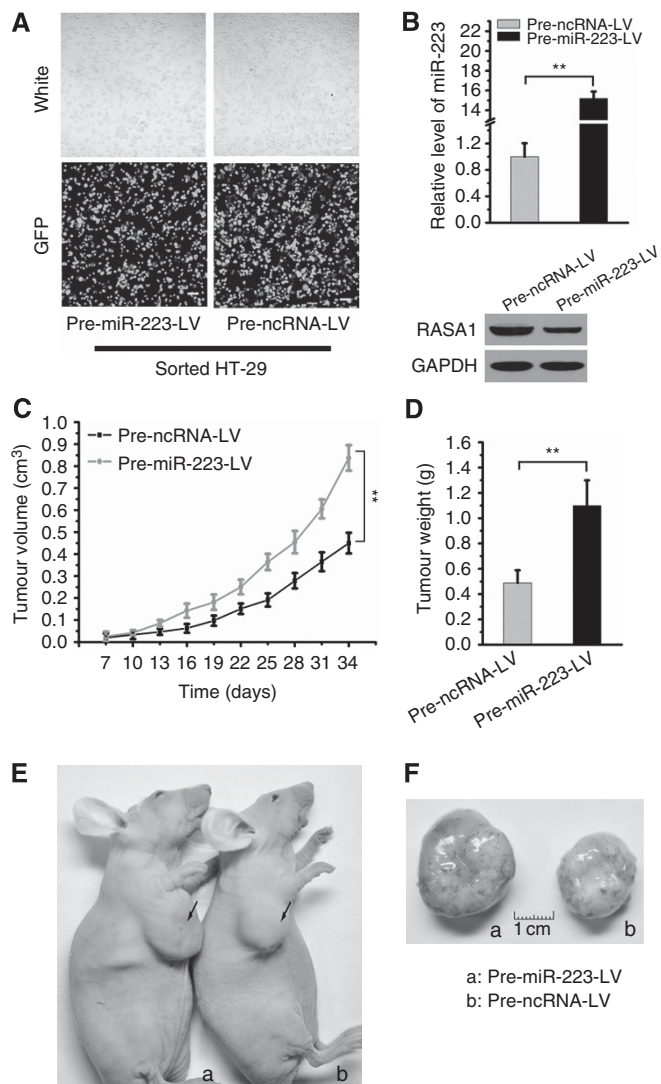


Figure 5. Effect of miR-223 on the growth of colorectal tumour *in vivo*. (A) Representative photos of HT-29 cells infected with pre-miR-223-LV and pre-ncRNA-LV sorted by a FACS based on the GFP expression. Pictures were imaged at $\times 10$ magnification on a Nikon confocal microscope. Scale bar, $100\ \mu\text{m}$. (B, upper panel) The qRT-PCR analysis of miR-223 from HT-29 cells stably expressing miR-223 or ncRNA. The data shown are the means \pm s.e. of three individual experiments. (B, lower panel) Western blotting analysis of RASA1 from HT-29 cells stably expressing miR-223 (pre-miR-223-LV group) or ncRNA (pre-ncRNA-LV group). The data shown are representative of three individual western blotting analyses. (C) The volumes of the xenograft tumours in nude mice derived from s.c. implantation of HT-29 cells stably expressing miR-223 (pre-miR-223-LV group) or ncRNA (pre-ncRNA-LV group). (D) The weights of the xenograft tumours in nude mice 34 days after s.c. implantation of HT-29 cells stably expressing miR-223 (pre-miR-223-LV group) or ncRNA (pre-ncRNA-LV group). (E) A representative photograph of mice bearing tumours 34 days post implantation. (F) A corresponding, representative photograph of excised tumours 34 days post implantation. Values are expressed as the means \pm s.e. $**P < 0.01$.

by intratumoural administration of PEI/miR-223 AMO complexes, PEI/scramble DNA complexes or PEI alone. As shown in Figure 6C, the mice treated with PEI/miR-223 AMO complexes showed the obviously decreased tumour volumes whereas the tumour volumes of mice, among PEI/scramble DNA complexes, PEI alone and control (nontreatment) groups, were not significantly different. Similarly, the tumour weights of PEI/miR-223

AMO complexes-treated mice were 47.2% less than those from other three groups (Figure 6D). Representative photographs of mice bearing the tumours and the corresponding tumours from each group are displayed in Figure 6E and F. In addition, qRT-PCR results showed that the levels of miR-223, KI-67 and PCNA in tumours had a significant decline after the xenografts were treated with PEI/miR-223 AMO complexes, as shown in Figure 6G and H, suggesting miR-223 AMO could be a potentially effective drug against human CRC.

DISCUSSION

Because the tumour suppressor RASA1 and its downstream RAS signalling pathway play crucial roles in controlling cell growth, RASA1-targeted miRNA profiling analyses are very important for a better understanding of the miRNA regulation mechanism through the RAS pathway in CRC. In the present study, we used bioinformatics analyses to find six important miRNAs of miR-24, miR-132, miR-182, miR-335, miR-223 and miR-31 that could target RASA1 depending on the 'seed region' of the RASA1 3'-UTR, but these 'seed regions' for six miRNAs recognition were diverse, suggesting that these miRNAs may play similar or differing roles in CRC. Among the six miRNAs, the significantly upregulated expressions of miR-223 and miR-31 were found in CRC and miR-223 showed very high abundance relative to that of miR-31 in NAT samples. It was also reported that miR-223 was upregulated in larger series of CRC patients than 24 patients (Schetter *et al*, 2008; Zhang *et al*, 2014). Especially, Zhang *et al* (2014) reported that 48 CRC patients with stage T3 or stage T4 presented an even higher expression of miR-223 compared with 42 CRC patients with low-grade stages, indicating that upregulation of miR-223 may play a key role in CRC. The results of *in situ* immunofluorescence analysis and western blotting assay further highlighted the importance of miR-223 in the CRC transformation process. Moreover, emerging evidence shows that miR-223 is a plasma or serum biomarker in gastric cancer and nasopharyngeal carcinoma (Zeng *et al*, 2012; Li *et al*, 2012a), but so far the biological roles of miR-223 in solid tumours are complicated because it may function as an oncogene or as a tumour suppressor, probably associated with the varying pathogenesis of different types of cancers (Wong *et al*, 2008; Li *et al*, 2011a, b, 2012b; Kurashige *et al*, 2012). Therefore, it is very necessary to investigate the role of miR-223 targeting RASA1 in CRC for a clearer view of its dynamic behaviour.

In the present study, we proved that miR-223 might stimulate CRC cell proliferation by targeting RASA1 and, therefore, activating the RAS/MAPK signalling pathway. These results were further supported by the findings that overexpression of miR-223 could promote tumour growth in nude mice. Obviously, miR-223 functions as an oncogenic miRNA by targeting RASA1 in CRC. The other targets of miR-223 have been reported to be FBXW7 in oesophageal squamous cell carcinoma and EPB41L3 in gastric cancer when miR-223 acts as an oncogenic miRNA in solid tumours (Li *et al*, 2011b; Kurashige *et al*, 2012). FBXW7 is another tumour suppressor and thereby negatively regulates oncoproteins, such as cyclin E, c-Myc, c-Jun and Notch in CRC (Akhoondi *et al*, 2007; Iwatsuki *et al*, 2010). FBXW7 was also predicted to affect colorectal cell proliferation probably mainly through the WNT and TGF- β pathways rather than the RAS/MAPK pathway (Cancer Genome Atlas Network, 2012). However, our results demonstrated that the use of a MEK1/2 inhibitor distinctly inhibited miR-223-induced cell proliferation, implying the importance of the RAS/MAPK pathway in CRC. As a possible target of miR-223 in CRC, EPB41L3 expression was not detected in human CRC cells, although EPB41L3 could maintain normal cellular proliferation and adhesion, thereby

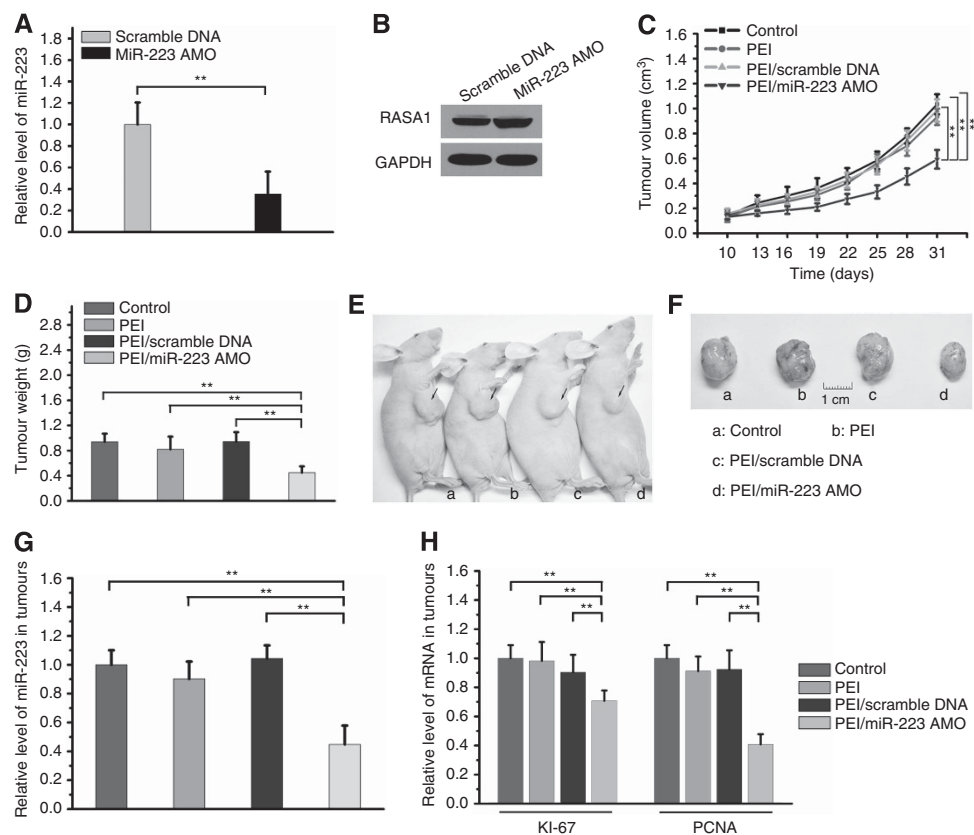


Figure 6. Antitumour activity of miR-223 AMO *in vivo*. **(A)** The qRT-PCR analysis of miR-223 from HT-29 cells transfected with miR-223 AMO or scramble DNA for 48 h. The data shown are the means \pm s.e. of three individual experiments. **(B)** Western blotting analysis of RASA1 from HT-29 cells transfected with miR-223 AMO or scramble DNA for 48 h. The data shown are representative of three individual western blotting analyses. **(C)** The volumes of the xenograft tumours in nude mice from s.c. implantation of HT-29 cells injected with 10 nmol PEI/miR-223 AMO complexes, PEI/scramble DNA complexes, PEI alone or control at indicated time. **(D)** The weights of xenograft tumours in nude mice 31 days after s.c. implantation of HT-29 cells. **(E)** A representative photograph of mice bearing tumours 31 days after s.c. implantation of HT-29 cells. **(F)** Corresponding tumours dissected from mice 31 days after s.c. implantation of HT-29 cells. **(G)** The qRT-PCR analysis of miR-223 from xenograft tumours in nude mice. The data shown are the means \pm s.e. of three individual experiments. **(H)** The qRT-PCR analysis of KI-67 and PCNA from xenograft tumours in nude mice. The data shown are the means \pm s.e. of three individual experiments. ** $P < 0.01$.

preventing malignant transformation of intestinal epithelial cells (Ohno *et al*, 2004). Taken together, upregulation of miR-223 promotes tumour growth in CRC predominantly by targeting RASA1 and activating the RAS/MAPK pathway.

Some transcription factors have been reported to regulate expression of miR-223. For example, C/EBP- α and PU.1 can upregulate the level of miR-223 during granulopoiesis (Fazi *et al*, 2005; Fukao *et al*, 2007). The metastasis-promoting transcription factor Twist can drive miR-223 transcription in gastric cancer (Li *et al*, 2011b). Cell-cycle regulator E2F1 can inhibit miR-223 transcription in acute myeloid leukemia (Pulikkan *et al*, 2010). Among miR-223-regulating transcription factors, C/EBP- β is paid great attention because the increased expression of C/EBP- β is correlated with invasiveness of human CRC (Rask *et al*, 2000). Moreover, the CRC cells display reduced tumorigenic potential when transplanted into the C/EBP- β -deficient animals, suggesting that C/EBP- β is involved in colorectal tumorigenesis (Staiger *et al*, 2009). Our findings in this study further indicate that C/EBP- β serves as a positive regulator for miR-223 expression and has a critical role in RASA1-mediated colorectal tumour growth. Moreover, Xi *et al* (2010) found that C/EBP- β could mediate the activation of miR-31 in normal human respiratory epithelia and lung cancer cells. The miR-31 was demonstrated to directly target RASA1 in CRC in our previous report. Therefore, we speculate that miR-31 may also be regulated by C/EBP- β and play the same role as miR-223 in CRC. Whether miR-223 and miR-31 work

synergistically in CRC and whether the combination with miR-223 and miR-31 inhibitors result in superior reduction of RASA1 would be further studied in the future.

As deregulated cell proliferation is a significant mechanism for neoplastic progression, we speculate that therapies targeting miR-223 might be used to prevent or treat CRC in the future. Especially, miR-223 will implicate as an alternative target for tumour inhibition in CRC patients with a K-RAS mutation because upregulated miR-223 is found in almost all CRC patients with both wild-type K-RAS and K-RAS mutations. Additional findings on miRNA regulation of the gene expression networks of cancers imply the prospect of more therapies. Kota *et al* (2009) found that miR-26a was downregulated in a murine liver cancer model and that treatment with miR-26a overexpressing adenovirus through the tail vein inhibited the growth of hepatocellular carcinoma. In addition, the first cancer-targeted miRNA drug, a liposome-based miR-34 mimic, entered phase I clinical trials in patients with advanced hepatocellular carcinoma (Ling *et al*, 2013). In this study, our results revealed that intratumoural injection of PEI/miR-223 AMO complexes significantly inhibited the growth of colorectal tumours in nude mice because of a significant reduction of miR-223 level, suggesting an important role of miR-223 in CRC and providing a potential therapeutic tool that targets miR-223 for CRC treatment. Therefore, other types of delivery for miR-223 AMO, such as intraperitoneal delivery, can be further investigated for the clinical CRC therapy.

In conclusion, we studied six important miRNAs that could target RASA1 in CRC patients and identified that miR-223 was significantly upregulated and its expression was inversely correlated with RASA1 protein expression. Our findings further revealed that miR-223 activated by C/EBP- β could stimulate colorectal cell proliferation and tumour growth by targeting RASA1 and thereby activating the RAS/MAPK pathway *in vitro* and *in vivo*. The miR-223 AMO significantly inhibited the growth of colorectal tumours in nude mice, providing a promising approach that may be valuable for CRC treatment.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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