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miR-320a/SP1 negative reciprocal interaction contributes to cell growth and invasion in colorectal cancer

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Abstract

Background: It has demonstrated that transcription factors (TFs) could be engaged reciprocal regulative circuits with some miRNAs to maintain the cellular homeostasis. Disequilibrium of the reciprocities by certain tumor-related stimuli, may give rise to deregulation of downstream cellular signaling pathways, thus promoting malignant phenotypes in tumor.

Methods: We performed bioinformatics analysis, quantitative polymerase chain reaction (qRT-PCR), immunoblotting, dual-luciferase reporter assay, and a series of functional assays in vitro and in vivo, to describe a novel SP1/miR-320a reciprocal interaction in colorectal cancer (CRC).

Results: Firstly, we found that miR-320a was statistically downregulated in CRC tissues and cell lines. Consistent with findings in other cancers, miR-320a exhibited inhibitory effects on cell growth and invasion of CRC in vitro and in vivo. Moreover, we identified Specificity Protein 1 (SP1), a well-known transcription factor, as a target gene of miR-320a, and ectopic SP1 expression partly abolished miR-320a-induced inhibitory effects. Reversely, we confirmed SP1 to interact with miR-320a promoter thus leading to depression of miR-320a. It hence illustrated a double-negative feedback loop engaging miR-320a with SP1. Additionally, on the basis that SP1 promoted MACC1 transcription, we by immunoblotting assay dissect that, the oncogenic signaling MACC1/MET was inactivated in the context of miR-320a-induced SP1 downregulation.

Conclusion: Taken together, our study newly describes the miR-320a/SP1 negative reciprocal interaction contributes to cell growth and invasion in CRC, through modulating MACC1/MET signaling pathway.

Background

Colorectal cancer (CRC) represents the third most common cancer and leads to the fourth most common cancer-related mortality worldwide. It is estimated that global CRC burden would increase by 60% to more than 2.2 million new cases and 1.1 million deaths by 2030 [1]. In the past few years, growing advances in our understanding of molecular signaling pathways have provided novel targets or thoughts for anti-CRC therapy. However, from the perspective of required effectiveness, it is still necessary to extend research on molecular regulatory mechanism underlying tumorigenesis and metastasis, in order to excavate more promising therapeutic targets and efficient approaches.

MiRNAs represent a cluster of small non-coding RNAs acting canonically as negative regulator of gene expression via inducing mRNA degradation or inhibiting mRNA translation [2]. Emerging studies have indicated that miRNAs exert promotive or suppressive influence on cancerous physiological processes, depending on the targeting genes and downstream signaling pathways [3]. miR-320a, previously named miR-320, is first identified to be correlated with the probability of recurrence-free survival of CRC in 2008 [4]. Since then, miR-320a is often reported to be downregulated and associated with patient survival, tumor stage, in an extended spectrum of cancer types, including breast, liver, prostate, lung cancer and

glioma [5–9]. Mechanistically, miR-320a could inhibit FOXM1 expression thus suppressing FOXM1-induced epithelial-mesenchymal transition and metastasis [10, 11]. In another way by blocking HMGB1 or STAT signaling, miR-320a negatively regulates invasion of tumor cells [6, 12]. Besides, silence of miR-320a is found to associate with Imatinib-resistant in gastrointestinal stroma [13], or exacerbate chemo-resistance of breast cancer to Tamoxifen [14]. Considering the versatility of miR-320a as a cancer-related miRNA, it is significative to explore the potential way of miR-320a being regulated and identify novel miR-320a-targeting genes.

Recent studies have increasingly demonstrated the role of miR-320a in CRC progression. Similar to other mentioned tumors, miR-320a is downregulated in CRC tissues compared with normal colonic epithelia [15, 16], and is associated with tumor stage and lymphatic metastasis [17, 18]. Moreover, miR-320a is correlated with sensitivity to preoperative chemoradiotherapy, while restoration of miR-320a in CRC cells could induce a shift of sensitivity [19]. In mechanism, absence of miR-320a in CRC is responsible for enhanced cell growth, invasion, chemo-resistance, by unfreezing activity of a series of tumor-promotive genes such as RAC1, SOX4, FOXM1, FOXQ1, and Wnt/β-catenin signaling [15, 16, 20]. Notably, a genome-wide miRNA expression profiling-based analysis propose increased expression pattern of miR-320a in colorectal adenoma with higher histologic grade [21], which indicates its regulatory effects may differentiate in precancerous stage of CRC.

Dysregulation of transcription factors (TFs) in tumor texture, induce altered gene expression and downstream signaling transduction, thus representing rational targets for anti-cancer therapy. SP1 is the most representative member of tumor-related transcription factors, which is usually over-expressed and negatively associated with poor prognosis in varies of tumors [22]. Studies have disclosed that SP1 could transactivate oncogenic genes including FAS, EGFR, VEGF, MET, etc., consequently leading to enhanced activity of signaling pathways in charge of cell proliferation, apoptosis, angiogenesis and metastasis [23–26]. On the other hand, SP1 interacts with genes with regulatory activity, such as SMAD3, XIAP, etc., to form transcription complex and mediate downstream target gene expression [26, 27]. Remarkedly, recent reports indicate non-coding RNA comprised of miRNA and lncRNA, are also transcription targets of SP1 as well as encoding genes [28–30], suggesting a more complex SP1-centered tumor molecular regulation network than the way it seemed before.

Referring to the regulatory relationship between TFs and miRNAs, especially a recent disclosed SP1/miR-22 feedback loop existing in CRC that facilitating tumor progression by suppressing downstream PTEN/AKT signaling [31], we by bioinformatic analysis predict several SP1-binding sites within miR-320a promoter, and in turn, SP1 is a predicted target gene of miR-320a. We hence hypothesize a reciprocal regulatory link between SP1 and miR-320a that participates in CRC development.

Methods

Bioinformatic analyses

The expression pattern of miR-320a in colon and rectal cancer tissues was analyzed following instruction of an online software OncomiR (http://www.oncomir.org/) [32], whose data resource is obtained from The Cancer Genome Atlas (TCGA). The putative binding activity of miR-320a to 3'UTR of SP1 mRNA was predicted by using online tools TargetScan (http://www.targetscan.org/vert_72/) and Diana microT-CDS (http://diana.imis.athena-innovation.gr/DianaTools/index.php?=microT_

CDS/index). The analyses of GEP datasets were performed using bioinformatic tool R2 (http://r2.amc.nl/).

Tissue samples and cell lines

A cohort of 12 pairs of primary CRC tissues and their matched adjacent colonic mucosa were obtained from patients who underwent surgical resections at the First People's Hospital of Yunnan Province, and were snap-frozen in liquid nitrogen, followed by storing at -80˚C for further use. All patients whose tissue samples were collected for the study signed the informed consent. This project was approved by the Ethics Committee of the First People's Hospital of Yunnan Province.

7 Human CRC cell lines, including HT29, SW480, SW620, LoVo, DLD-1, SW1116, HCT116, were purchased from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China. The human normal colonic epithelial cell line FHC, was kindly donated by Dr. Liang Peng from Guangzhou Medical University (Guangzhou, China). CRC cells were cultured routinely in RPMI-1640 medium (Invitrogen, Waltham, US) supplemented with 10% FBS (Invitrogen, Waltham, US) and 1% penicillin-streptomycin solution. FHC cells were maintained in Dulbecco's modified Eagle's medium (DMEM): F12 medium (Invitrogen, Waltham, US) with supplements following ATCC protocol. 293T cells were maintained in DMEM medium containing high glucose supplemented with 10% FBS. All the cells mentioned were cultured in a 37˚C humidified atmosphere containing 5% CO_2.

Oligonucleotide, plasmids and cell transfection

Hsa-miR-320a mimics and its negative control oligonucleotide were synthesized by Shanghai Genepharma Co., Ltd. (Shanghai, China). For transfection, SW480 or SW620 cells were seeded into 6-well clusters at a density of 3×10^6 cells per well then transfected using Lipofectamine® 2000 (Invitrogen, Waltham, US) according to the manufacturer's protocol, with 30 nM miR320a mimics or scramble controls for 48 h at 37˚C before further experimentation in assays or RNA/protein extraction.

RNA extraction and qPCR analysis

Total RNA was isolated from CRC tissues and cells using the TRIzol® reagent (Invitrogen, Waltham, US). Mature miR320a expression in cells was determined using a Hairpin-it™ miRNAs qPCR kit (Genepharma, Shanghai, China). RNU6B was used as an endogenous control. SP1 mRNA expression was determined by using SYBR green qPCR assay (Takara, Dalian, China). The thermocycling conditions were as follows: Denaturation at 95˚C for 3 min, followed by 40 cycles of amplification at 95˚C for 12 sec and extension at 62˚C for 40 sec. GAPDH was used as the endogenous control. Data was analyzed using the $2^{\Delta\Delta Ct}$ method.

Cell function assays

Cell growth level was determined by using Cell Counting Kit-8 method (MCE, Monmouth Junction, US) according to the manufacturer's protocol. For colony formation assay, cells were trypsinized and seeded on 6-well plates at a density of 3 × 102 cells per well and cultured at 37˚C for 10 days. The colonies were stained with 0.1% crystal violet solution containing 80% methanol for 5 min at room temperature. The number of colonies defined as > 50 cells/colony were counted at × 40 magnification by using a light microscope. For cell invasion ability, it was measured using Transwell inserts with 8 μm pores (Corning, US). Cell density was adjusted to 10^6/ml in serum-free RPMI-1640 medium. 200μL of cell suspension was added into each upper insert pre-coated with Matrigel matrix (BD, US), while 500 μL RPMI-1640 medium containing 10% FBS was added into a matched lower chamber. After a 48-h incubation, the non-invaded cells were removed from the upper surface of the transwell membrane with a cotton swab, while the invaded cells on the lower membrane surface were fixed in methanol, stained with 0.1% crystal violet, photographed and counted. Six random fields at × 100 magnification for each insert were observed.

Immunoblotting analysis

Cultured cells were lysed in RIPA buffer with 1% phenylmethylsulphonyl fluoride (PMSF). Protein was loaded onto an SDS-PAGE Minigel and transferred onto a PVDF membrane. After being probed with primary antibody at 4˚C overnight, the blots were subsequently incubated with HRP-conjugated anti-IgG (Cell Signaling, Danvers, US). Signals were visualized using ECL substrates (Invitrogen, Waltham, US). GAPDH was used as an endogenous protein for normalization.

Rabbit antibodies against SP1 (#9389S), MACC1 (#86290S), c-MET (#8198S), p-AKT (#4060S), p-ERK1/2 (#4370S), ERK1/2 (#4095S), GAPDH (#2118S) were purchased from Cell Signaling. Mouse antibody against AKT (#60203-2-Ig) was purchased from Proteintech, US. All the primary antibodies were 1:1000 diluted for incubation.

Lentivirus packaging and stable cell line establishment

miR-320a-expressing lentivirus particles were packaged by co-transfecting the miR-320a-expressing clone with lentiviral packaging plasmids into 293T cells, using Lenti-Pac™ HIV Expression Packaging Systems (GeneCopoeia, Rockville, US) according to the manufacturer's instructions. For the establishment of stable miR-320a-expressing cells, SW620 cells were incubated with viral supernatant in the presence of 8 μg/ml Polybrene for 24 h, followed by puromycin (Invitrogen, Waltham, US) selection until drug-resistant colonies became visible.

Luciferase reporter assays

To identify the complementary binding of miR-320a to 3’UTR of SP1 mRNA, fragments of 3’UTR of SP1 containing the putative miR-320a binding site or mutant counterpart were amplified by PCR and subcloned into a pMIR-REPORT vector immediately downstream of the luciferase gene sequence. 293T cells were plated in 24-well clusters at a density of 1 × 10^5 cells/well, then co-transfected with pMIR-REPORT constructs and miR-320a expressing vector or control vector. Cells were harvested after 48 h

incubation at 37°C for further detecting luciferase activity using a dualluciferase reporter assay system (Promega, Madison, US) and normalized by Renilla activity.

To detect binding activity of SP1 to promoter region of miR-320a or MACC1, we amplified the fragments in 2000 bp length upstream TSS of miR-320a and 1000 bp for MACC1, the respectively subcloned them into pGL4.10 vector (Promega, Madison, US) downstream of the luciferase gene sequence. 293T cells were plated in 96-well clusters at a density of 5×10^3 cells/well, then co-transfected with pGL4.10 constructs and SP1 expression vector or control vector. Cells were harvested after 48 h incubation at 37°C for further detecting luciferase activity using a dualluciferase reporter assay system (Promega, Madison, US) and normalized by Renilla activity.

Mouse xenograft model

Female BALB/C nude mice (4–6 weeks old) were purchased from Hunan SJA Laboratory Animal Co., Ltd (Changsha, China). The animal experiment was conducted according to protocols approved by the Committee on the Ethics of Experimental Animal of the First People's Hospital of Yunnan Province. A total of 3×10^6 cells was injected subcutaneously into the flank of mice to generate xenograft model (n = 4 per group). Measurement of tumor size started at 10 days after cell injection when the xenografts became measurable. Tumor volume was measured every 5 days and calculated as (length [mm] × width^2 [mm^2])/2. The mice were sacrificed and tumors were collected at day 30.

Statistical analysis

Statistical analyses were performed using the SPSS software, version 15.0 (SPSS, Inc., Chicago, US) and GraphPad Prism software version 6.01 (Graphpad Software, Inc., La Jolla, US). Comparisons of miR-320a expression between CRC tissues and paired adjacent colonic tissues were performed using a Wilcoxon's paired test. All data are presented as the mean ± standard deviation. Comparisons among multiple groups were performed using one-way analysis of variance followed by Tukey's post hoc test. $P < 0.05$ was considered to indicate a statistically significance.

Results

miR-320a is aberrantly downregulated in CRC

miR-320a has been demonstrated to be decreased in multiple cancer types. We thus first analyzed miR-320a expression data in colorectal cancer from TCGA by using OncomiR bioinformatic tool. As shown in Fig. 1A, miR-320a was commonly upregulated in both colon cancer (COAD) (P = 6.67e-04) and rectal cancer (READ) (P = 9.57e-03) cohorts compared with matched normal adjacent epithelial. We further conducted qPCR to determine miR-320a expression in a panel of 12 pairs of clinical CRC tumor and adjacent normal epithelial specimens, and it showed that miR-320a expressed in significantly high level in CRC tissues compared to normal epithelial (P = 0.005, Fig. 1B), which is in accordance with that observed in TCGA data. We also examine miR-320a expression in CRC cell lines by qPCR. The expression pattern of miR-320a across the 7 tested CRC cell lines, was commonly lower than that in normal colonic

epithelial cell line FHC (Fig. 1C). Out of those low-miR-320a expressing CRC cell lines, SW620 and SW480 were selected for further experiments.

miR-320a inhibits CRC cell growth and invasion in vitro and tumor growth in vivo

To dissect the functional relevance of miR-320a expression in modulating CRC cell malignant phenotypes, we performed in vitro gain-of-function assays by upregulating miR-320a via introducing mimics into SW480 and SW620 cells. Upon restoration of miR-320a, the growth rate was significantly inhibited in both SW480 and SW620 cells compared with control counterparts (Fig. 2A). Likely, restoration of miR320a induced a significant decrease of colony formation ability in SW480 ($P = 0.006$) and SW620 cells ($P = 0.0302$) (Fig. 2B). In addition, we observed less invasive SW480 cells ($P = 0.0145$) and SW620 cells ($P = 0.0018$) adhering on lower membrane of transwell inserts, in the context of miR-320a restoration (Fig. 2C). In a xenograft mouse model ($n = 4$ per group), compared with negative control group, the group of mice bearing stable miR-320a-expressing SW620 cells, exhibited significant reduction of tumor volume *ex vivo* and tumor growth trend *in vivo* (Fig. 2D, E). These above results indicate that miR-320a may exert suppressive effects on CRC progression.

SP1 is a direct target gene of miR-320a in CRC

It is well known that miRNAs modulate cellular processes in tumor through negatively regulate target genes. We sought to identify the downstream target genes practically regulated by miR-320a. By utilizing online algorithms (TargetScan/Diana microT-CDS), we performed bioinformatic prediction for potential target gene of miR-320a, and it was demonstrated that there was a predicted binding site for miR-320a within 3'UTR of SP1 mRNA (Fig. 3A). We thus speculated miR-320a may negatively regulate SP1 expression in CRC. To identify it further, miR-320a mimics was introduced into SW480 and SW620 cells. As predicted, SP1 was demonstrated to be decreased significantly in both mRNA (Fig. 3A, B) and protein level (Fig. 3C) upon upregulation of miR-320a. To further investigate if the predicted binding site of miR-320a within 3'UTR of SP1 mRNA is responsible for the decreased SP1 expression, we constructed a luciferase plasmid containing wild type 3'UTR region of SP1 (SP1-3'UTR) and a counterpart containing mutant binding site for miR-320a (SP1-3'UTR-mut). SP1-3'UTR was co-transfected with miR-320a mimic or negative control oligonucleotide (NC) respectively into 293T cells in a luciferase reporter assay. It showed that miR-320a induced decreased luciferase activity compared to the negative control oligonucleotide ($P = 0.000$, Fig. 3D), while the decreased luciferase activity mediated by miR-320a was abolished upon introducing the mutant binding site ($P = 0.000$, Fig. 3D), thus indicating that miR-320a could directly target SP1 by binding to 3'UTR of SP1 mRNA.

miR-320a inhibits CRC cell growth, colony formation and invasion ability through directly targeting SP1

Considering that SP1 usually transactivates oncogenic gene and signaling pathways, we anticipated that miR-320a-induced SP1 downregulation was responsible for its suppressive effects on CRC cells. We performed a function rescue experiment by introducing SP1-expressing vector into SW480 and SW620 cells to restore SP1 expression in the presence of ectopic miR-320a expression (Fig. 4A). Expectedly, during 96 h observation, we found that restoration of SP1 partly relieved miR-320a-induced cell growth inhibition in SW480 and SW620 cells by a CCK-8 assay (Fig. 4B). Similarly, it was also observed that SP1 reversed miR-320a-induced inhibition of colony formation and invasion ability of CRC cells in a certain

extent (Fig. 4C, D). These data hence illuminate that miR-320a could inhibit cell growth, colony formation and invasion ability of CRC cells, at least in part, through directly targeting SP1.

SP1 suppress miR-320a expression transcriptionally to promote CRC cell growth, colony formation and invasion ability

Previous studies have reported that SP1 as a TF, could regulate multiple tumor-related miRNAs expression in tumorigenesis, which represents a crucial aspect of SP1-dominated tumor modulatory mechanism. By qPCR assay we observed downregulation of miR-320a upon with SP1 upregulation in SW480 and SW620 cells induced by SP1-expressing vector transfection (Fig. 5A), implying potential transcriptional effects of SP1 on miR-320a. Since SP1 protein usually recognizes and binds to a consensus GC box 5'-(G/T) GGGCGG (G/A) (G/A) (C/T)-3' which termed as SP1-binding site within promoter region of target genes, we by using JASPAR online tool found 4 putative SP1 binding sites within 1000 bp of promoter region of miR-320a upstream the TSS. To confirm if the binding activity of SP1 to miR-320a promoter is practical, we generated a luciferase reporter containing the 4 putative binding sites which co-transfected together with SP1-expressing vector in 293T cells. It was determined that SP1 significantly impeded luciferase activity when it recognized the promoter of miR-320a ($P < 0.0001$, Fig. 5B). These findings suggest that SP1 protein could directly interact with the promoter and depress transcription of miR-320a.

To further investigate if SP1 modulates cellular process of CRC via regulating miR-320a expression, we performed another rescue assay by introducing miR-320a into SW480 and SW620 cells in the context of SP1 upregulation. By immunoblotting, the upregulation of SP1 in both of the CRC cells, was observed to be depressed again with miR-320a introduction (Fig. 5C). Consequently, in function assays, upregulation of SP1 promoted cell growth, colony formation and invasion ability significantly in the CRC cells, which was reversed partly by miR-320a restoration (Fig. 5D, E, F).

Our results collectively describe a reciprocal loop formed by miR-320a and SP1 that plays a regulatory role in CRC.

miR-320a depresses oncogenic MACC1/MET signaling pathway through SP1 in CRC

We sought to disclose the regulatory mechanism underlying miR-320a/SP1 feedback loop by exploring its downstream effectors or signaling. Metastasis Associated in Colon Cancer-1 (MACC1) has been elucidated to keep tumor cell growth, activate invasion and metastasis, mainly depending on its promotive modulating HGF/MET oncogenic pathway. Moreover, the MACC1 promoter contains binding elements of SP1, which contributes to the expression of MACC1[33]. Initially, we cloned luciferase reporters containing a fragment of wild type MACC1 promoter up to 1000 bp from TSS and a mutant counterpart, and introduced respectively them together with SP1-expressing vector in 293T cells. In the detection, SP1 was evidently observed to motivate the luciferase activity of wild type promoter but not for the mutant one, thus confirming the transactivation of SP1 to MACC1 ($P < 0.001$, Fig. 6A). We next examined a series of crucial MACC1 downstream effectors activity in the context of miR-320a-induded SP1 inhibition by immunoblotting. As presented in Fig. 6B, upon with miR-320a restoration, suppression of SP1 led to depression of MACC1, c-MET expression subsequently, and eventually impaired

phosphorylated activity of ERK1/2 and AKT (Fig. 6B), which suggested miR-320a depresses MACC1/MET signaling pathway by directly negatively targeting SP1.

Discussion

Here in our study, we find miR-320a is commonly downregulated in CRC tissues compared with normal colonic epithelial. Restoration of miR-320a suppresses cell growth, clone formation ability, invasiveness of CRC cells *in vitro*, and tumor growth in xenografted mice model. miR-320a is predicted *in silico* and confirmed by luciferase reporter assay and western blotting, to target SP1 by binding to its mRNA 3'UTR. Rescue of SP1 expression in CRC cells could partly abrogate miR-320a-induced inhibition of cell behaviors, indicating that miR-320a exert tumor-suppressive role through at least partly, inhibiting SP1 activity. On the other hand, we show that SP1 induces downregulation of miR-320a by functioning as a transcription repressor binding to miR-320a promoter. Targeting SP1 using shRNA leads to restoration of miR-320a and concomitantly repression of cell growth, clone formation ability, invasiveness of CRC cells, which is partly antagonized upon miR-320a inhibitor treatment. Moreover, in mechanism analysis, we show that MACC1, previously described as a master regulator of oncogenic HGF/MET signaling [34], is a transcription target of SP1. By western blotting, we observe MACC1 expression is expectedly repressed along with SP1 inhibition induced by miR-320a. As downstream effectors of MACC1, MET expression and ERK1/2, AKT phosphor-activity are concomitantly observed being attenuated. Our data hence illustrate a double-negative feedback loop between SP1 and miR-320a in CRC cells, which retains miR-320a expressing at low level but elevating level of SP1, thus leading to malignant cell phenotypes through inducing enforced oncogenic MACC1/MET signaling.

Since most of coding mRNAs harbor different complementary seed sequences for miRNAs recognition, a cluster of mRNAs may be regulated by a single miRNA, and *vice versa*. Moreover, different miRNAs could co-target cooperatively a cluster of protein coding mRNAs with relevant function or belonging to a cellular pathway [35]. On the other hand, from the perspective of gene expression regulated by TFs, miRNA as a sort of non-coding genes, is presumably transcriptionally regulated by TFs. Therefore, a substantial number of regulatory interactions between TFs and miRNAs are presumably conserved across cancer entities. Recent studies have experimentally disclosed a number of TF-miRNA reciprocities involved in tumorigenesis of multiple cancers. For example, miR-23 ~ 24 ~ 27 cluster, upregulated as an oncogene in breast cancer, can target directly HIC, while HIC can in turn repress miR-23 ~ 24 ~ 27 cluster transcription, thus indicating a double negative feedback loop that promote tumor growth [36]. In liver cancer, EZH2 is engaged a reciprocal negative circuit with miR-101-1, thus attenuating tumor-suppressive role by inducing downregulation of miR-101-1 [37]. In addition, interplay that implicates more than a single TF or miRNA, is often described in diverse cancerous processes, especially in EMT-regulating mechanism. It has been reported that EMT-inducing TFs such as ZEB1/2, Snail, Slug, etc., often form reciprocal regulatory network with some certain miRNAs consist of miR-34, miR-200 and miR-17-92 cluster [38]. Take miR-200 family for example: p53-inducible miR-200 family is the first identified EMT-inhibiting miRNAs, mainly through targeting EMT-inducing TFs ZEB1/2 by posttranscriptional modification, whereas ZEB1/2 can bind to

promoter of miR-200 family thus directly repressing expression of them, which means frequent loss of p53 and/or miR-200 family in tumor shift the equilibrium of the reciprocal regulation among p53, miR-200 and ZEB1/2, towards mesenchymal state thereby confer tumor cell with disseminated feature [39].

It is well characterized that SP1 and its modification (phosphorylation, acetylation, glycosylation) are crucial for maintaining cell homeostasis and early development of embryo by retaining basal transcriptional machinery [40]. We therefore speculate SP1 and miR-320a reciprocity represent a manner of sustaining conventional adjustive activity of SP1 and/or miR-320a in normal cellular processes. However, in tumor context, the equilibrium may be shifted by certain tumor-related stimuli, thus giving rise to elevating level and activity of SP1, and miR-320a expressing in low level, eventually promoting malignant feature of tumor cells. This role change in question implies SP1 a non-oncogene addiction (NOA) gene [40], and underscores one of causes for miR-320a downregulation in CRC.

MACC1 has been established as a key player and biomarker in tumor progression first in CRC [34], then across multiple tumor entities [41]. Recent mechanism studies have unveiled possible upstream transcriptional regulation of MACC1 by β-catenin, YB1 [42, 43], while TWIST1/VEGFA, Nanog/Oct4 axis, and SPON2 [44–46] as its downstream effectors, hence illustrating a MACC1-centered regulatory network in tumor.

In our study, we newly describe a regulatory approach upstream MACC1. It is illustrated that, deregulation of SP1 in CRC due to disequilibrium of SP1/miR-320a negative reciprocity, promotes MACC1 transcription, thus leading to activation of MACC1-mediated c-MET and downstream signaling pathway, eventually influencing malignant features of CRC (Fig. 7). Targeting either SP1 or miR-320a may presumably represent potential strategy to prevent MACC1-driven tumor progression.

Conclusion

miR-320a forms a negative reciprocal interaction with SP1, thus potentiating cell growth and invasion of CRC, through modulating MACC1/MET signaling pathway.

Declarations

Ethics approval and consent to participate

Specimen collection and animal experiments were approved by the Ethics Committee of the First People's Hospital of Yunnan Province.

Consent for publication

Not applicable

Availability of data and materials

All data generated or analysed during this study are included in this published article.

Competing interests

The authors declare no competing interests.

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Authors' contributions

WZ and QG designed the study. WZ, HY and YW performed most of the assays in the research. ZW performed the bioinformatic analysis. JW and GD collected and treated CRC tissues in prior to qPCR assay. YZ analyzed the data statistically and wrote the manuscript.

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Figures

A

miRNA	Cancer	T-test *P*-value	T-test FDR	Upregulated in	Tumor log2 mean expression	Normal log2 mean expression
miR-320a	COAD	6.67e-04	2.53e-03	Normal	7.56	10.39
miR-320a	READ	9.57e-03	4.41e-02	Normal	6.59	9.88

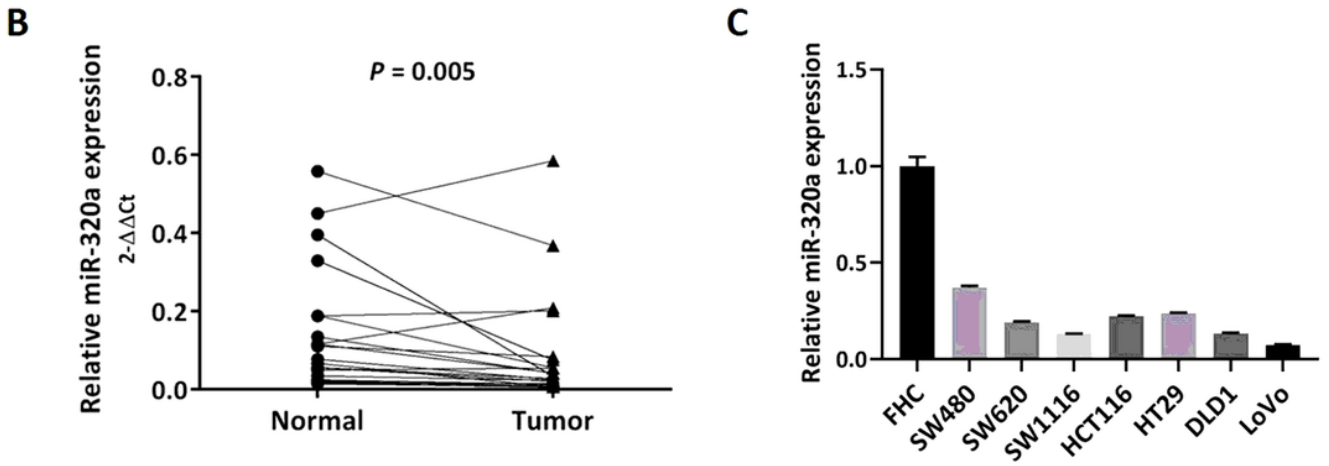

Figure 1

miR-320a is aberrantly downregulated in CRC tissues and cell lines. (A) Both in colon cancer (COAD) and rectal cancer (READ), the expression of miR-320a is analyzed to be significantly decreased in CRC tissues. The expression data of miR-320a in CRC is extracted from The Cancer Genome Atlas (TCGA), and calculated by OncomiR online software. (B) downregulation of miR-320a is observed in CRC tissues compared to their matched normal epithelial in a cohort of 12 primary CRCs (P = 0.005). (C) Compared with normal human colonic epithelial cell FHC, it is commonly exhibited a pattern of decreased miR-320a expression in 7 enrolled CRC cell lines. Of those, SW480 and SW620 cells are chosen for further assays.

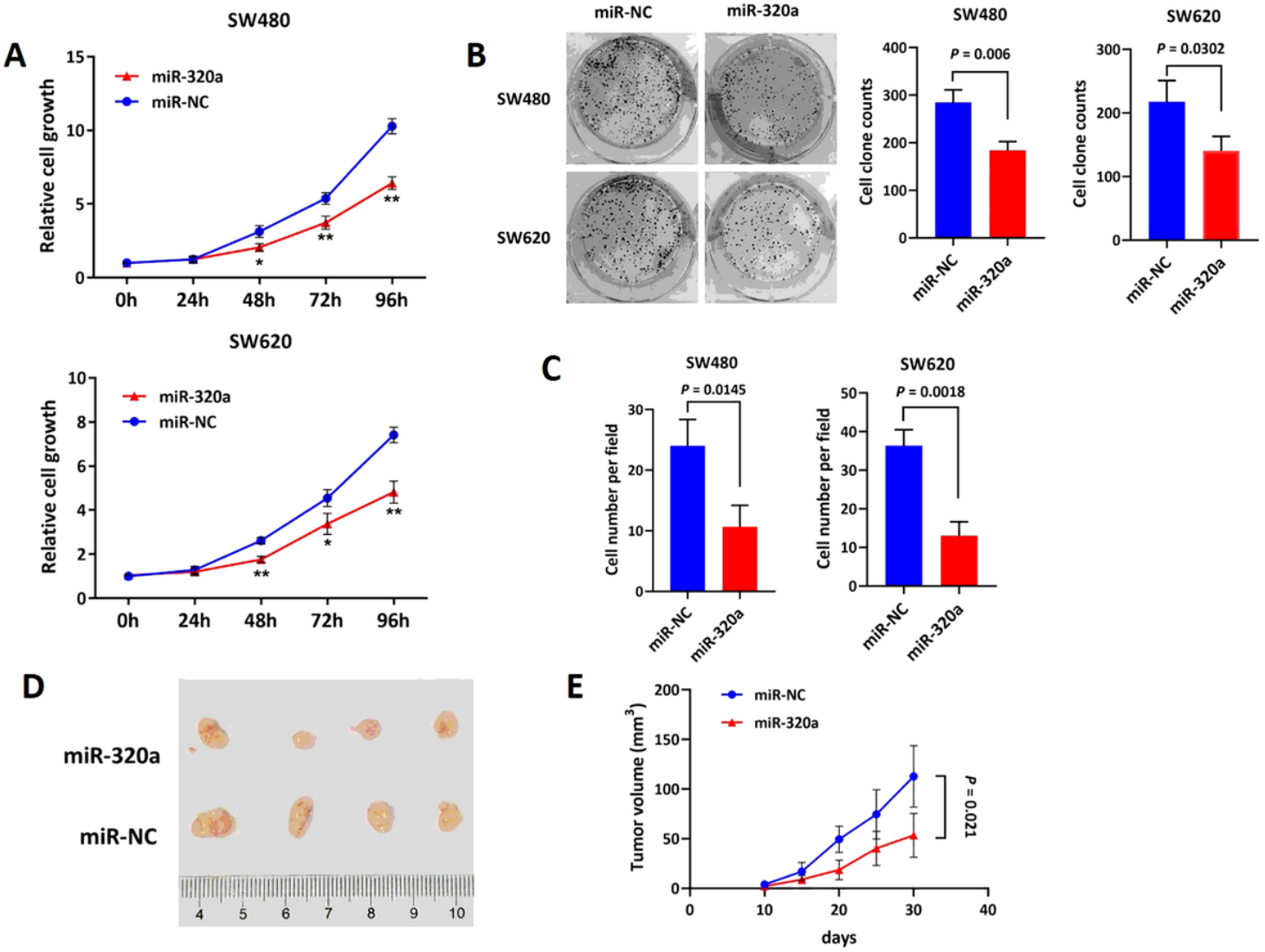

Figure 2

miR-320a inhibits CRC cell growth and invasion in vitro and tumor growth in vivo. (A) Restoration of miR-320a by introducing mimics significantly inhibits cell growth both in SW480 and SW620 cells during a 96h observation. * P < 0.05 vs miR-NC; ** P < 0.01 vs miR-NC. (B) In colony formation assay, restoration of miR-320a exhibits inhibitory effects on cell colony formation ability both in SW480 (P = 0.006) and SW620 (P = 0.0302) cells compared with negative control. (C) Restoration of miR-320a significantly inhibits cell invasion ability in SW480 (P = 0.0145) and SW620 (P = 0.0018) cells. In xenograft mouse model (n = 4 per group), compared with negative control group, the group of mice bearing stable miR-320a-expressing SW620 cells, exhibited significant reduction of tumor volume ex vivo (D) and tumor growth trend in vivo (P = 0.021) (E).

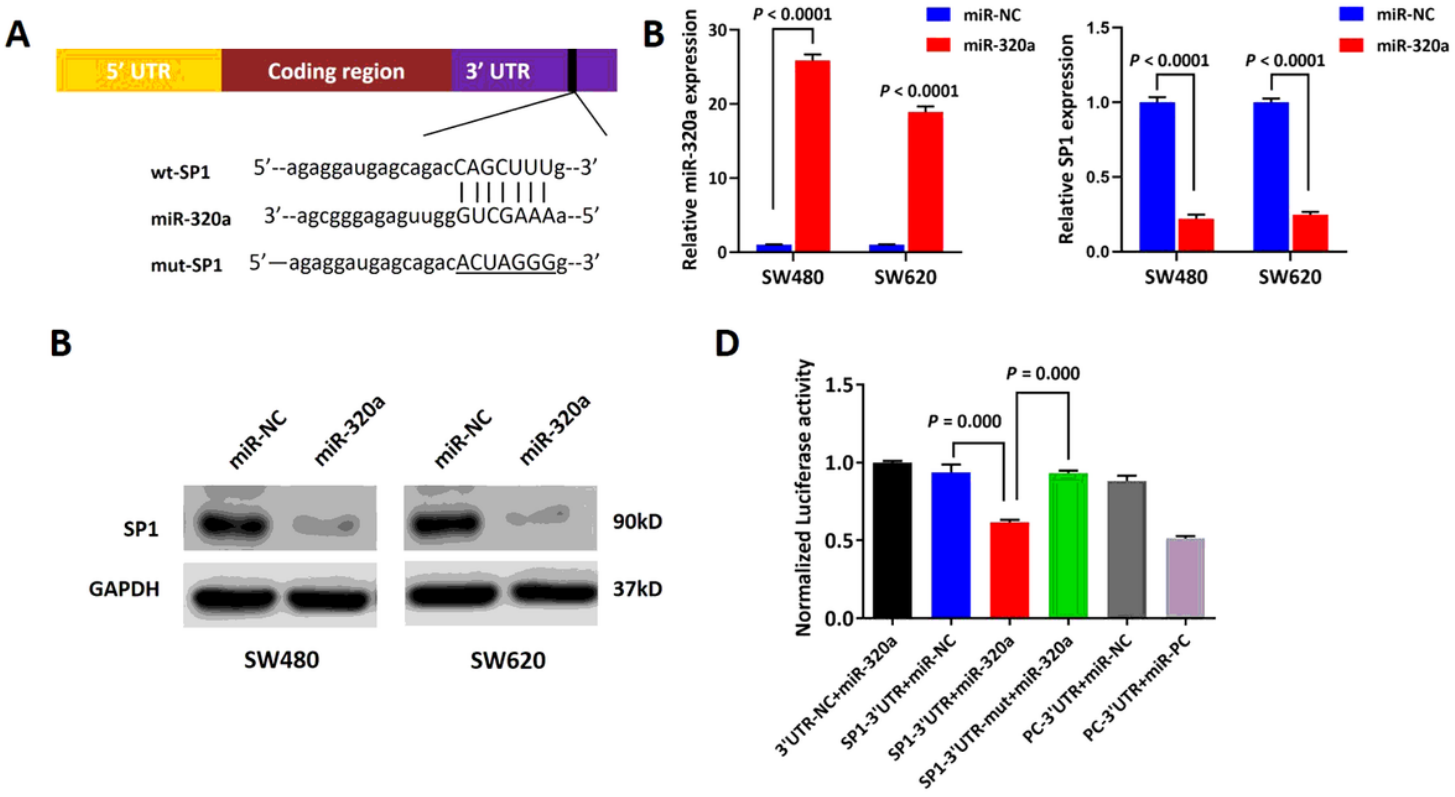

Figure 3

SP1 represents a direct target gene of miR-320a. The predicted miR-320a binding site within SP1 3′UTR and its mutated version are as presented. (B, C) Restoration of miR-320a in SW480 and SW620 cells induces decreased expression of SP1 mRNA by qPCR assay. (D) By immunoblotting assay, restoration of miR-320a in SW480 and SW620 cells suppresses SP1 expression at protein level. (E) The repression of luciferase activity by SP1-3′UTR is dependent on miR-320a (P = 0.000), while 3′UTR-mut of SP1 abrogates miR-320a-mediated repression of luciferase activity (P = 0.000) in 293T cells. In this luciferase reporter assay, miR-146b and TRAF6-3′UTR were used respectively as a positive miRNA and a positive target gene 3′UTR. PC, positive control; UTR, untranslated region; Mut, mutant.

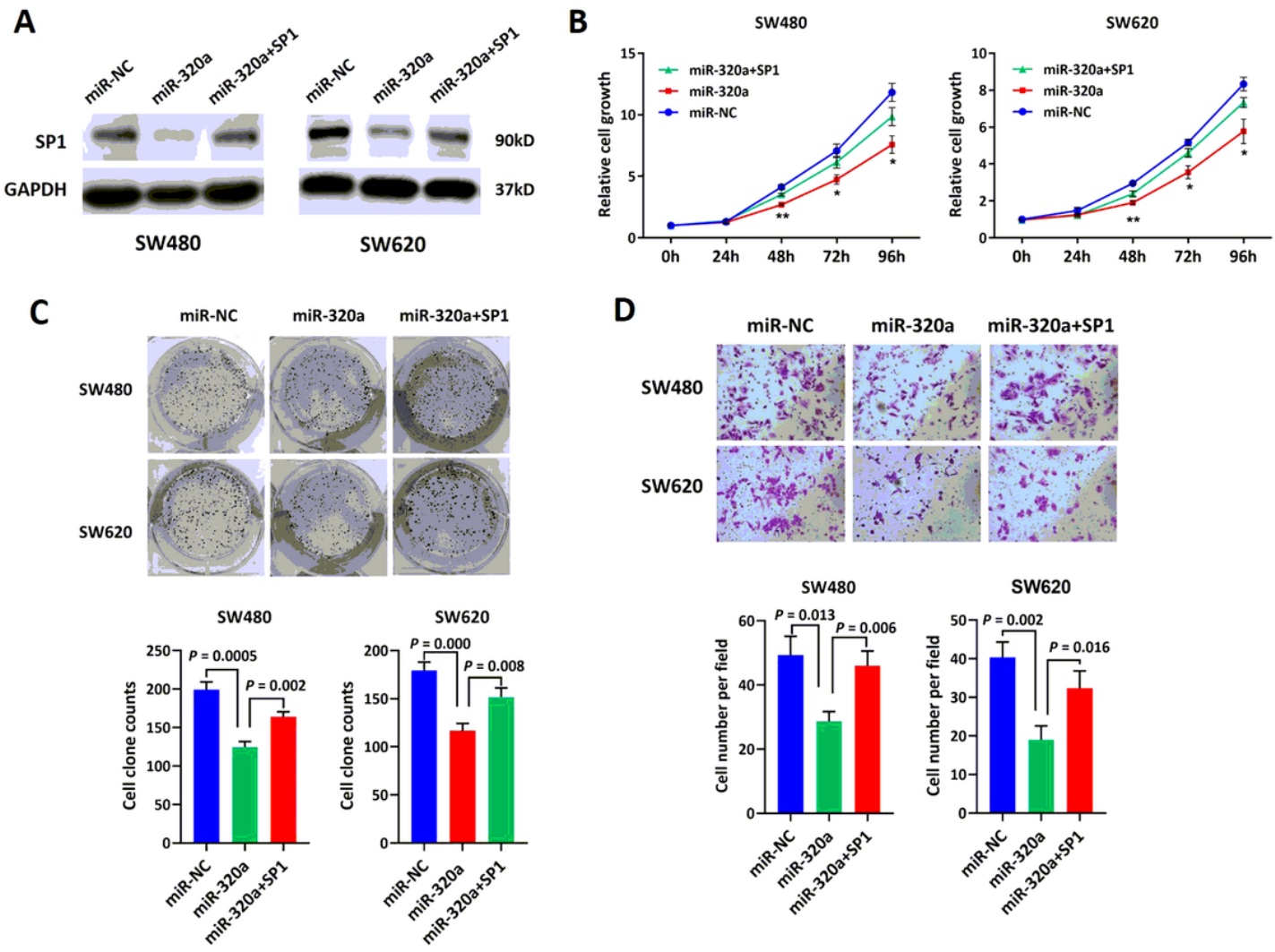

Figure 4

miR-320a inhibits CRC cell growth, colony formation and invasion ability through directly targeting SP1. A rescue experiment is performed to determine if miR-320a exhibits tumor suppressive features through targeting SP1. (A) In immunoblotting assay, downregulation of SP1 expression induced by ectopic miR-320a, is abrogated by transfecting SP1-expressing vector into both SW480 and SW620 cells. Consequently, restoration of SP1 partly reverses that inhibitory influences of miR-320a on cell growth (B) (* $P < 0.05$; ** $P < 0.01$), colony formation (C) and invasion ability (D).

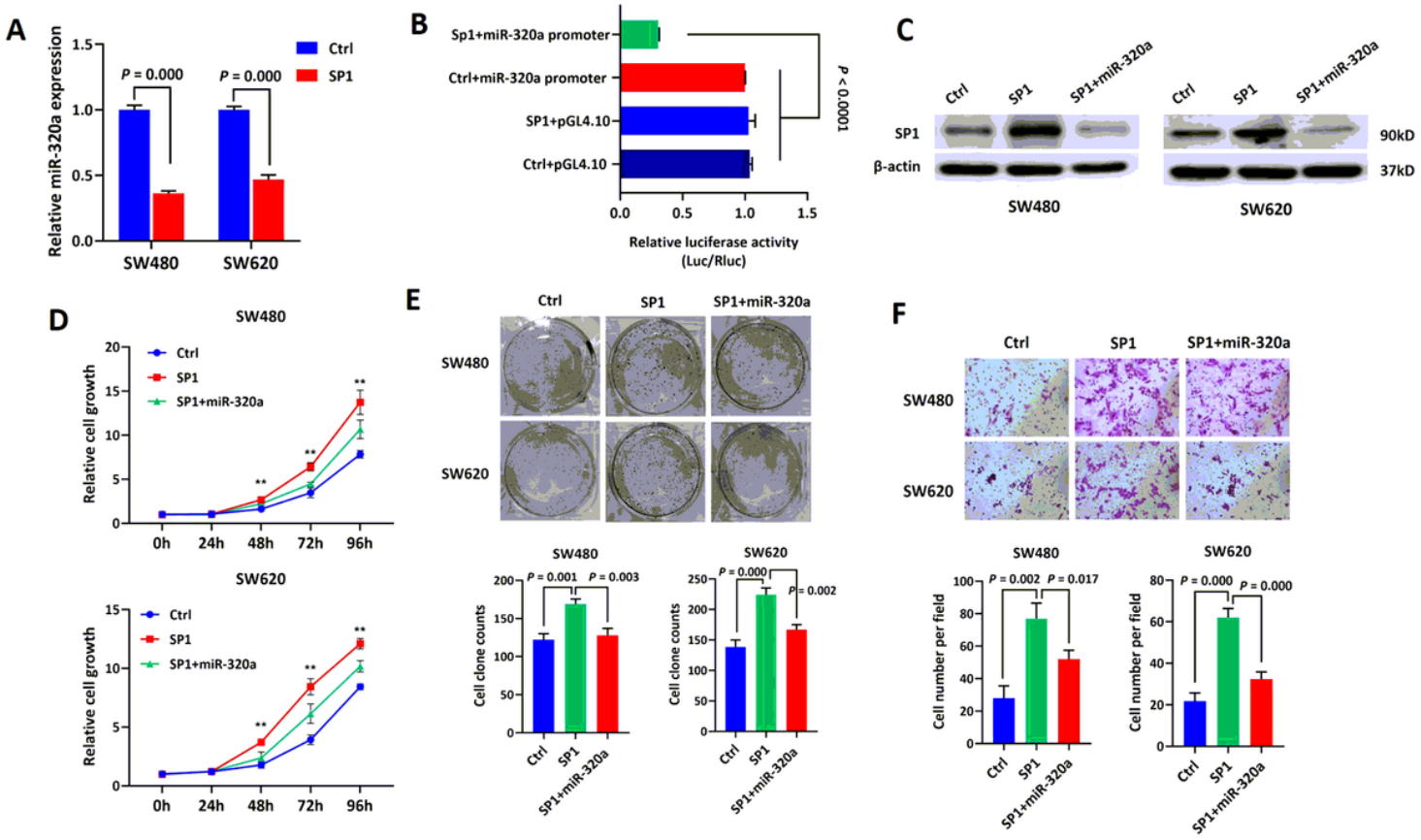

Figure 5

SP1 transcriptionally suppresses miR-320a expression to promote CRC progression in vitro. (A) It is observed in qPCR experiment that miR-320a is downregulated upon with SP1 upregulation induced by SP1-expressing vector. (B) By luciferase reporter assay, SP1 specifically represses luciferase activity of miR-320a promoter compared with other control groups (P < 0.0001), indicating SP1 could inhibit miR-320a transcription. Another rescue experiment is conducted by introducing miR-320a mimics into SP1-expressing SW480 and SW620 cells. (B) By immunoblotting assays, ectopic miR-320a expression successfully suppresses SP1 expression in the presence of SP1-expressing vector. Consequently, SP1-promotes cell growth (C) (* P < 0.05; ** P < 0.01), colony formation (D) and invasion ability (E) in SW480 and SW620 cells, is in turn partly abrogated by ectopic miR-320a expression.

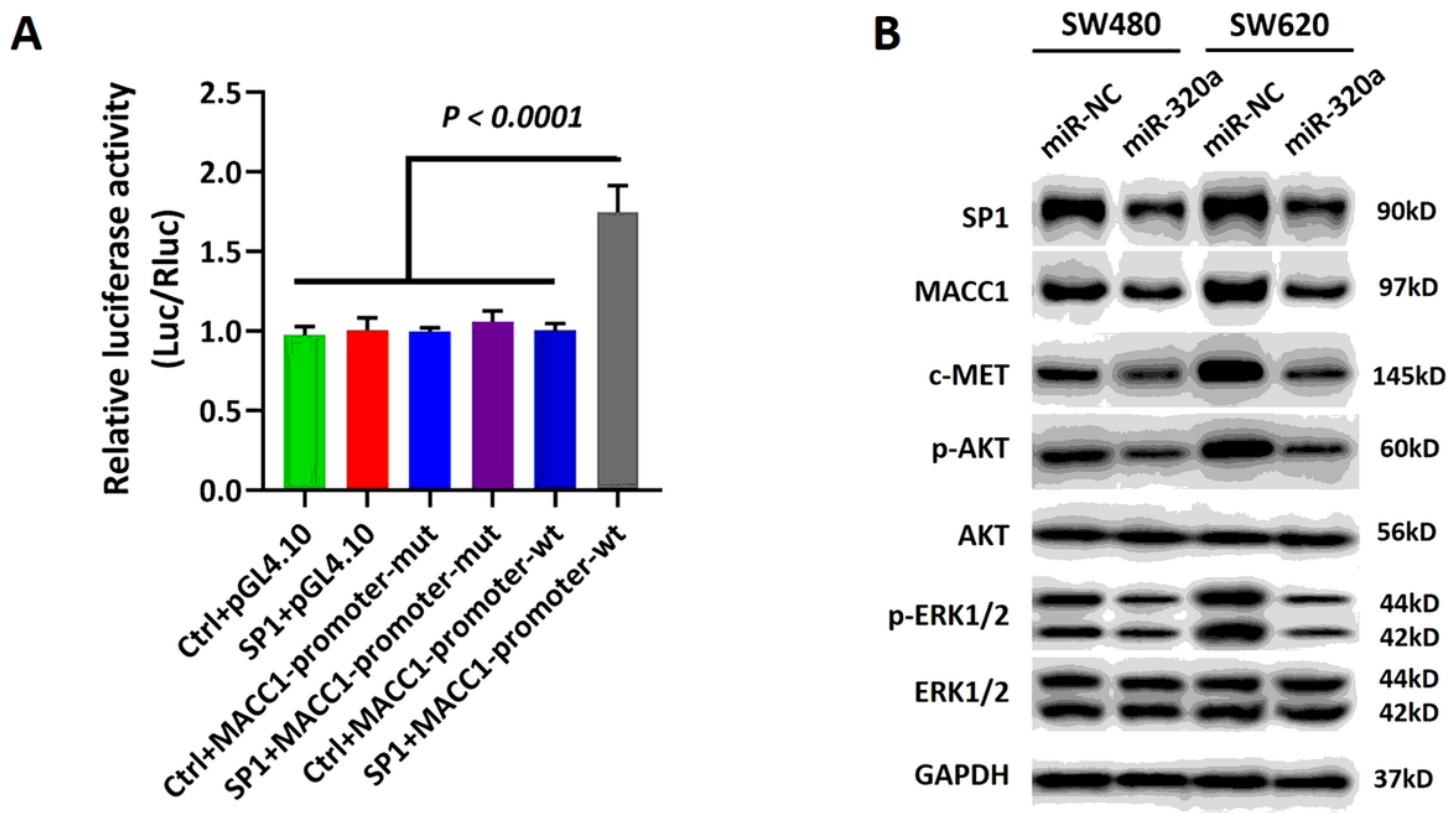

Figure 6

miR-320a modulates oncogenic MACC1/MET signaling pathway through SP1. Previous study identifies SP1-binding elements within MACC1 promoter region. We thus perform a luciferase reporter assay to confirm if SP1 modulates MACC1 transcriptionally. (A) The increased luciferase activity of MACC1-promoter-wt is dependent on SP1 ($P < 0.0001$, vs control groups), indicating SP1 transactivates MACC1 expression. (B) In immunoblotting assay, ectopic miR-320a expression apparently inhibits SP1 expression followed by MACC1 inhibition. Besides, as downstream effectors of MACC1, c-MET, p-ERK1/2, p-AKT are all shown to be repressed along with ectopic miR-320a expression.

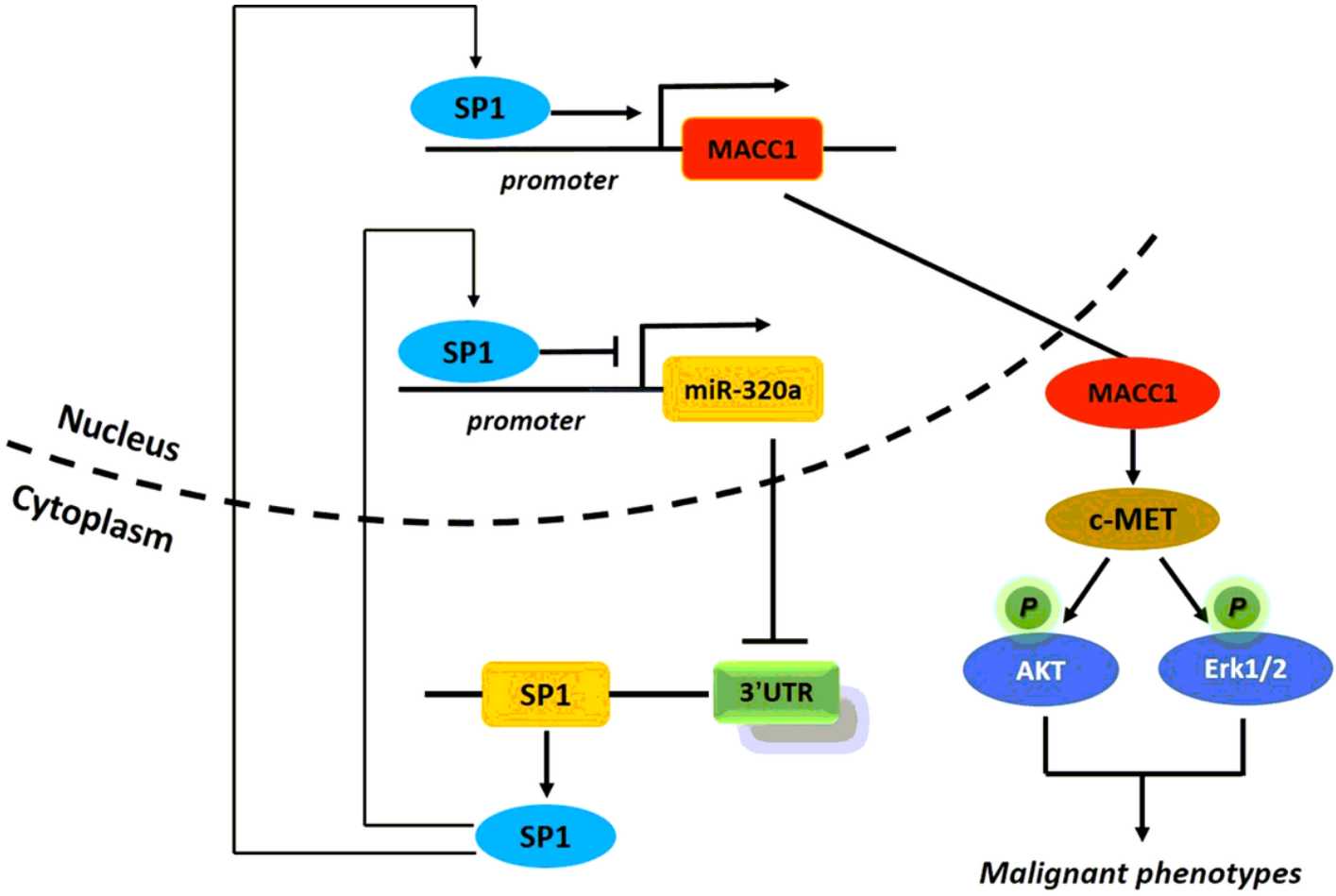

Figure 7

A schematic of miR-320a/SP1 reciprocity engaged in modulating MACC1/MET signaling in CRC.