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ORIGINAL ARTICLE

Adenomatous polyposis coli (APC) regulates miR17-92 cluster through β-catenin pathway in colorectal cancer

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Adenomatous polyposis coli (APC) mutation is the most common genetic change in sporadic colorectal cancer (CRC). Although deregulations of miRNAs have been frequently reported in this malignancy, APC-regulated miRNAs have not been extensively documented. Here, by using an APC-inducible cell line and array analysis, we identified a total of 26 deregulated miRNAs. Among them, members of miR-17-92 cluster were dramatically inhibited by APC and induced by enforced expression of β-catenin. Furthermore, we demonstrate that activated β-catenin resulted from APC loss binds to and activates the miR-17-92 promoter. Notably, enforced expression of miR-19a overrides APC tumor suppressor activity, and knockdown of miR-19a in cancer cells with compromised APC function reduced their aggressive features *in vitro*. Finally, we observed that expression of miR-19a significantly correlates with β-catenin levels in colorectal cancer specimens, and it is associated to the aggressive stage of tumor progression. Thus, our study reveals that miR-17-92 cluster is directly regulated by APC/β-catenin pathway and could be a potential therapeutic target in colon cancers with aberrant APC/β-catenin signaling.

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INTRODUCTION

Colorectal carcinoma (CRC) is one of the leading causes of cancer death in the developed world, with a disease-specific mortality rate estimated around 33%.[1] Over 85% of sporadic CRCs have loss-of-function mutations of the adenomatous polyposis coli (*APC*) gene occurring at an early stage of cell transformation.[2] *APC* is classified as tumor suppressor gene and has a critical role in several cellular processes including cell division, adhesion and migration.[3–5] At biochemical level, *APC* has been shown to integrate to canonical Wnt pathway whose stimulation triggers the translocation of the oncoprotein β-catenin from cell membrane to the cytoplasmic and nuclear compartments. Nuclear β-catenin acts as co-activator of T-cell and lymphoid enhancer (TCF/LEF) factors in the transcriptional activation of target genes,[6] although a role of accumulated cytoplasmic β-catenin as stabilization factor of mRNA molecules has been recently suggested.[7] In epithelial cells, β-catenin associates at the cellular membrane with the adhesion molecule E-cadherin. Free cytoplasmic β-catenin is phosphorylated and targeted for ubiquitination-dependent degradation by a protein complex formed by APC, GSK-3, CKIα and Axin.[8,9] Mutations of the *APC* gene in colorectal carcinomas results in unrestrained β-catenin signaling and contributes to a proinvasive gene expression profile along with cellular transformation.[10,11] It is worth noticing that nearly half of the colorectal tumors with intact *APC* genes were found to contain activating mutations of β-catenin.[6,12] Thus, mutation of APC or β-catenin represents the most common genetic change (>90%) in CRC, pointing to a driver activity of APC/β-catenin signaling in colon cancer development.

MiRNAs are short noncoding RNA molecules implicated in several cellular processes such as development, differentiation, proliferation, cell cycle progression, apoptosis, inflammation and stress responses.[13,14] MiRNAs mechanism of action relies on inhibition of translation or induction of degradation of target mRNAs through direct binding to their 3′-untranslated region.[15] Given their propensity to regulate numerous processes by targeting mRNAs, it is not surprising that aberrant expression of miRNAs has been linked to numerous pathological conditions.[16–19] Several studies observed a frequent upregulation of miR-17-92 cluster, miR-31, miR-21 and miR-200 cluster in colorectal carcinoma, suggesting an oncogenic role of these miRNAs in this malignancy.[19,20] Although a recent study reported miRNA profile in tumor from APC(Min/+) mouse,[21] APC-regulated miRNAs remain largely uncharacterized in human CRC.

In this study, we profiled miRNA changes upon induction of APC expression in colorectal cancer cells. We found that mir-17-92 cluster, frequently upregulated in CRC, is significantly repressed by APC through induction of β-catenin degradation. We further unveiled that knockdown of miR-19a reduces aggressive features (cell growth, migration and invasion) in cancer cells with compromised APC function. Our study demonstrates that the reduction in miR-19a expression levels is a major mechanism by which APC exerts its tumor suppressor activity and suggests that miR-19a could be a potential therapeutic target in colon cancers with aberrant APC/β-catenin signaling.

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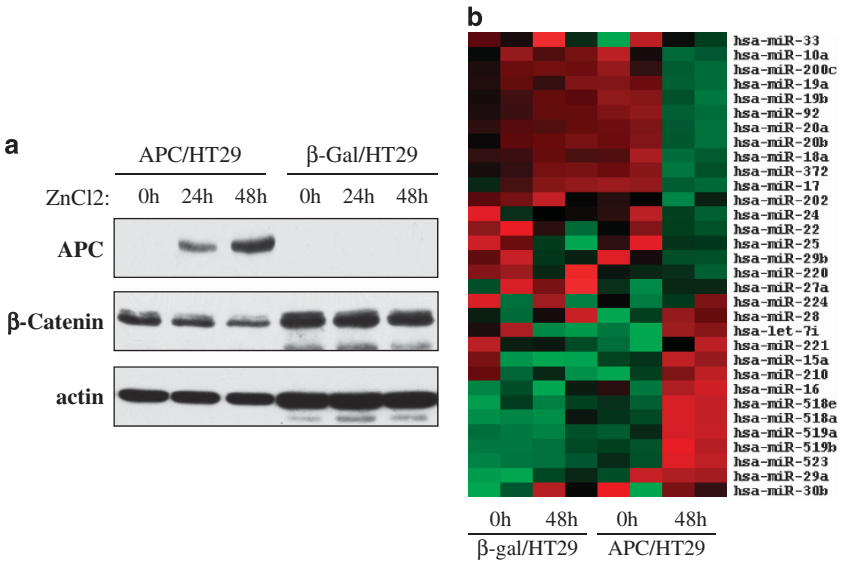
RESULTS

MiRNA expression profile in APC-inducible expression colorectal cancer cells

In HT29 cells, the endogenous *APC* gene is mutated and not functional. In an attempt to identify the miRNAs regulated by APC in CRC, we analyzed miRNA profile in HT29 cells with $ZnCl_2$-inducible APC expression (APC/HT29 cells). β-Gal/HT29 cells were used as controls. APC induction following $ZnCl_2$ treatment for 0, 24 and 48 h was validated by increased levels of full-length APC protein as well as by decreased β-catenin protein levels (Figure 1a). Following these conditions, RNA was isolated and hybridized to a custom miRNA array platform[22–24] that covers up to 650 miRNAs. More than a dozen miRNAs were shown to be significantly differentially expressed between APC-induced and ctrl cells (Figure 1b). Of these deregulated miRNAs (⩾1.5 fold), 16 were reduced and 10 were induced by APC (Figure 1c). Interestingly, APC repressed the expression levels of several members of miR-17-92 (for example, miR-17, miR-18, miR-219, miR-20 and miR-92) and miR-200 (for example, miR200a, miR200b and miR200c) families and increased the expression levels of miR-518 cluster (for example, miR-518a/e, miR-519a/b, miR-523 and miR-526) and miR-15/16. These findings suggest that each cluster shares the same promoter, under the control of APC pathway. Previous studies have shown frequent upregulation of miR-17-92 family in human colorectal carcinoma.[25] The ability of APC to repress the expression of members of miR-17-92 cluster was confirmed by northern blot and/or qRT–PCR (quantitative reverse transcriptase PCR) analyses (Figures 2a and b). In addition, we



c **List of APC regulated miRNAs**

miRNA	Fold	P value	Chr. location
Down regulated miRNA			
hsa-miR-10a	1.53	0.019	17q21.32
hsa-miR-17-5p	1.85	0.012	13q31.3
hsa-miR-18a	1.75	0.011	13q31.3
hsa-miR-19a	1.80	0.014	13q31.3
hsa-miR-19b	1.82	0.012	13q31.3
hsa-miR-20a	1.75	0.03	13q31.3
hsa-miR-20b	1.75	0.006	Xq26.2
hsa-miR-92	1.61	0.026	13q31.3
hsa-miR-181a	1.71	0.021	1q31.3
hsa-miR-196a	1.62	0.017	17q21.32
hsa-miR-200a	1.67	0.013	1p36.33
hsa-miR-200b	1.7	0.025	1p36.33
hsa-miR-200c	1.73	0.01	12p13.31
hsa-miR-206	1.8	0.001	6p12.2
hsa-miR-335	1.49	0.025	7q32.3
hsa-miR-380-5p	1.46	0.007	14q32.31
Up regulated miRNA			
hsa-miR-518a	0.66	0.017	19q13.42
hsa-miR-518e	0.66	0.027	19q13.42
hsa-miR-523	0.52	0.014	19q13.42
hsa-miR-526a	0.46	0.001	19q13.42
hsa-miR-210	0.66	0.028	11p15.5
hsa-miR-519a	0.87	0.017	19q13.42
hsa-miR-519b	0.71	0.029	19q13.42
hsa-miR-15a	0.62	0.021	13q14.2
hsa-miR-16	0.65	0.022	13q14.2
hsa-let-7i	0.61	0.037	12q14.1

Figure 1. Profile of APC-regulated miRNAs. (**a**) Western blot. APC/HT29 and β-Gal/HT29 cells were treated with $ZnCl_2$ for indicated times and then subjected to immunoblot analysis with indicated antibodies (note: expression of APC leads to decrease in β-catenin level). (**b** and **c**) Heatmap (**b**) and table (**c**) show the miRNAs significantly regulated by expression of APC.

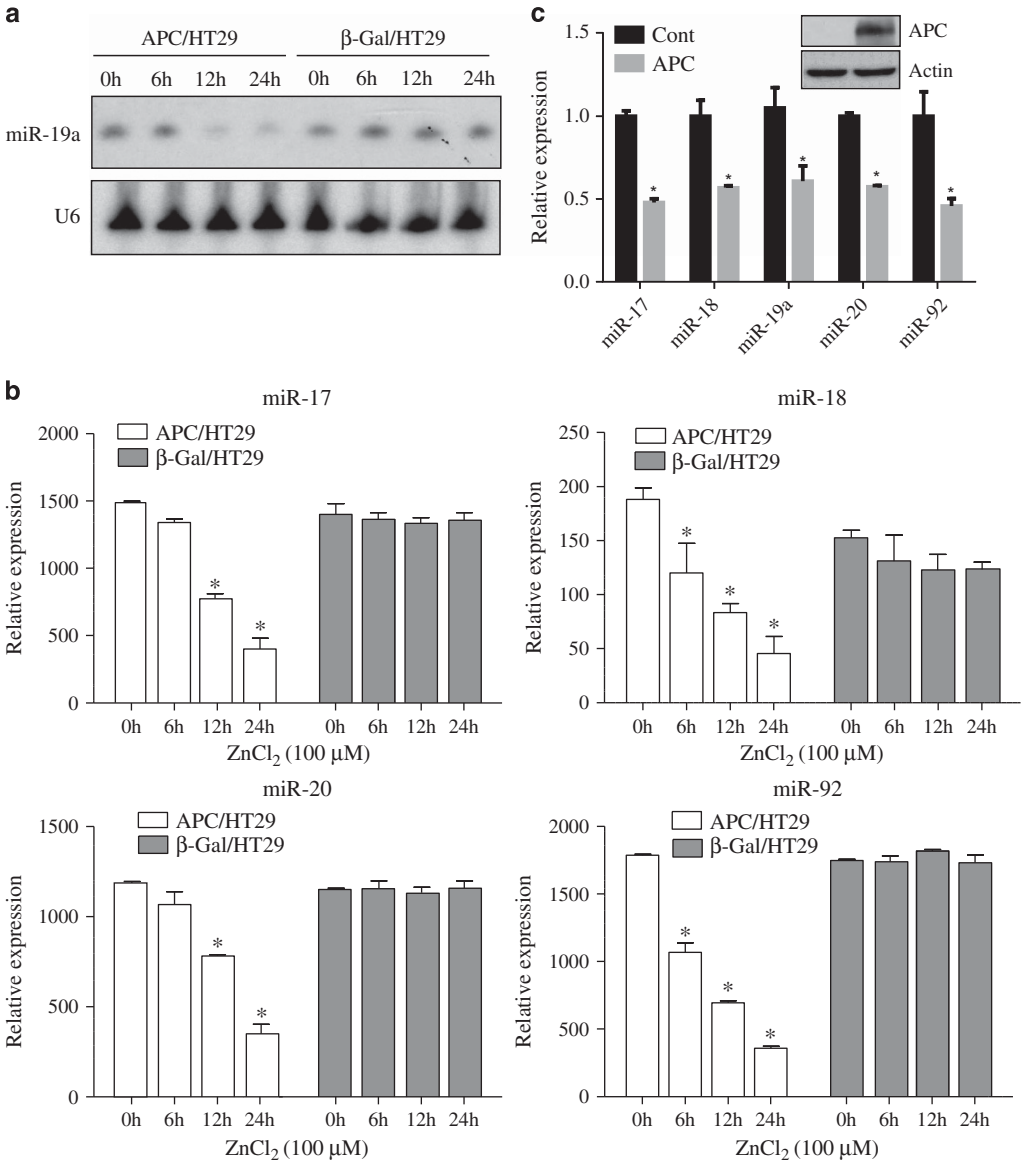


Figure 2. MiR-17-92 cluster is repressed by APC. Northern bot (**a**) and real-time PCR (**b**) analysis revealed that expression of members of miR-17-92 cluster was decreased in APC/HT29 but not β-Gal/HT29 cells upon induction of APC expression. (**c**) HCT15 cells were transfected with APC and vector control. After incubation for 48 h, the cells were subjected to immunoblot (insert panel) and real-time PCR analysis of levels of miR-17-92 cluster. $^*P < 0.05$.

expressed APC in another APC-mutant cell line HCT15 and found that miR-17-92 levels were significantly reduced in APC-transfected cells when compared with vector-treated cells (Figure 2c).

APC repressed miR-17-92 expression through induction of β-catenin degradation

APC exerts tumor suppressor function by targeting oncoprotein β-catenin to degradation. This process requires β-catenin forming a destruction complex with APC, Axin and GSK-3β as well as phosphorylation by GSK-3β and CK1 at four serine/threonine sites within N-terminal armadillo domain of β-catenin.[26] Mutation of these serine/threonine residues occurs in CRC and leads to stabilization and cytoplasmic-nuclear translocation of β-catenin with subsequent activation of TCF/LEF transcription machinery. Therefore, we further investigated the involvement of β-catenin in APC-induced repression of miR-17-92. To this end, wild-type and mutant β-catenin were introduced into $ZnCl_2$-inducible APC/HT29 cells. Following APC induction, mutant β-catenin levels were unaffected, in contrast to wild-type β-catenin levels that were reduced (Figure 3a, left panel). Consequently, APC failed to repress miR-19a expression in cells transfected with mutant β-catenin (Figure 3a, right panel). These data suggest that miR-17-92 cluster downregulation is the consequence of APC-induced β-catenin degradation. We further investigated whether ectopic expression of β-catenin induces miR-17-92 production in wild-type APC cells. As most CRC cell lines have either APC or β-catenin mutation, we initially transfected HEK293 cells, in which neither APC nor β-catenin is mutated, with different amounts of wild-type or constitutively active mutant β-catenin (Figure 3b). Real-time PCR analysis revealed that the members of miR-17-92 family were induced by enforced expression of wild-type and mutant β-catenin (Figure 3c). To confirm these results in colon cells, we repeated the experiments in NCI-H508 cells, in which both APC and β-catenin are wild type, and found that the levels of miR-17-92 family members were significantly elevated upon expression of wild-type or constitutively active mutant β-catenin

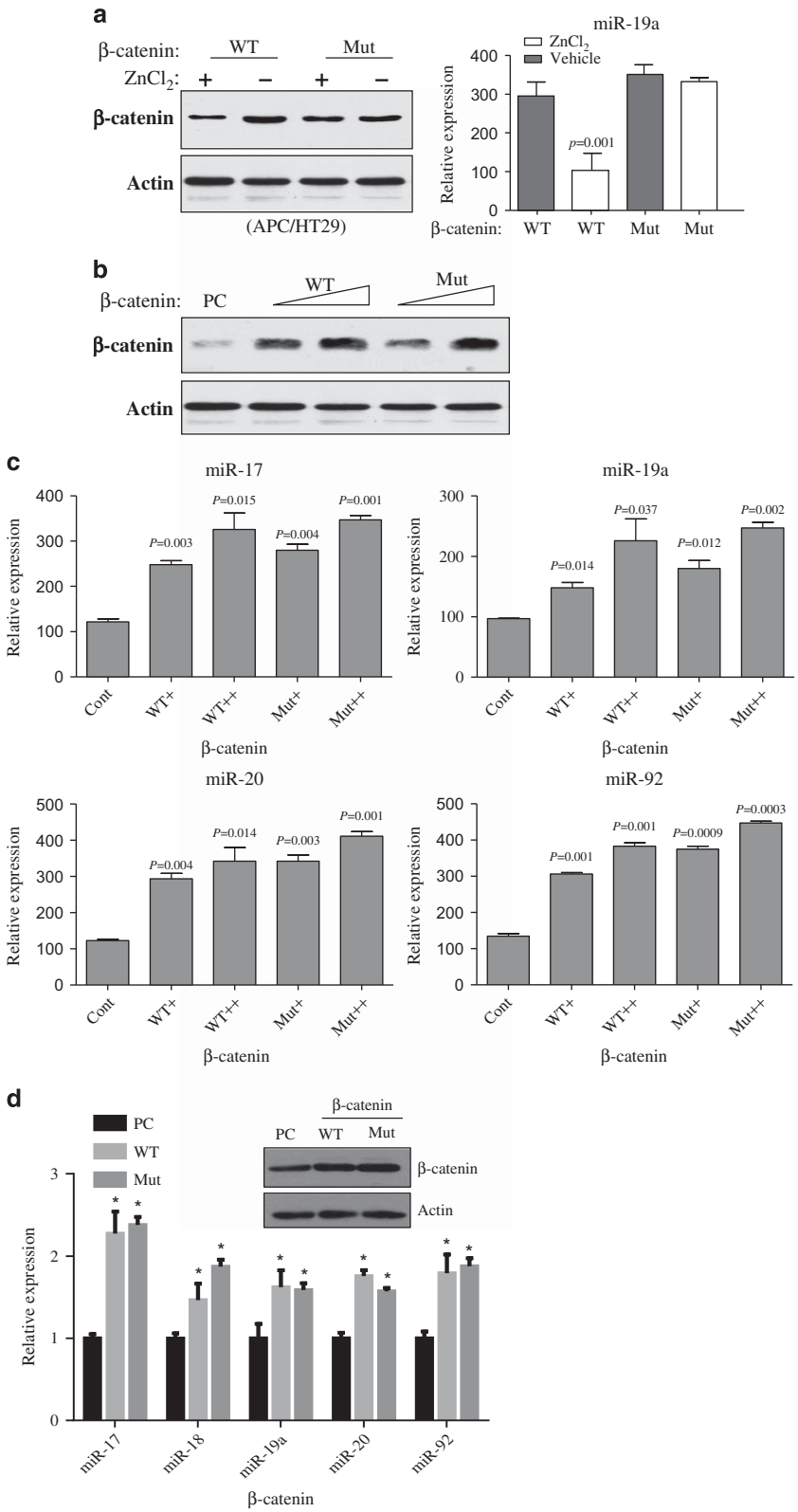


Figure 3. β-catenin induces miR-17-92 cluster expression and mediates the APC action. (**a**) APC/HT29 and β-Gal/HT29 cells were transfected with wild-type (WT) and constitutively active mutant (Mut) β-catenin and then treated with or without $ZnCl_2$ to induce APC for 48 h. β-catenin protein levels (left panel) and mirR-19a (right panel) were analyzed by western blot (left) and qRT–PCR (right) analyses. (**b**) Western blot analysis of HEK293 cells that were transfected with increasing amounts of wild-type and constitutively active mutant β-catenin. (**c** and **d**) Expression of miR-17-92 members in β-catenin-transfected HEK293 (**c**) and NCI-H508 (**d**) cells was analyzed by qRT–PCR. $^*P < 0.05$.

(Figure 3d). These data suggest that β-catenin induces miR-17-92 expression and that APC inhibition of miR-17-92 is mediated by β-catenin degradation.

APC inhibits β-catenin binding to and transcription of miR-17-92 promoter

β-catenin is a co-activator of TCF/LEF transcription factors. In that regard, by bioinformatics analysis, we identified two putative TCF/LEF-binding consensus motifs within the human miR-17-92 promoter (Figure 4a). We next examined whether β-catenin activates miR-17-92 promoter activity. For this purpose, we transfected HEK293 cells with miR-17-92 promoter luciferase reporter construct along with different amounts of wild-type or constitutively active β-catenin. Luciferase activity was assessed after 48 h of incubation. Our data show that both wild-type and constitutively active β-catenin induce miR-17-92 promoter activity in a dose-dependent manner (Figure 4b). Consistent with these data, APC induction reduced miR-17-92 promoter activity (Figure 4c). Moreover, APC expression was unable to inhibit miR-17-92 promoter activity induced by mutant β-catenin (Figure 4d). We next investigated the effect of APC on the affinity of β-catenin/TCF binding to miR-17-92 promoter. As two β-catenin/TCF/LEF binding sites (−2079/−2072 and −2005/−1998) are close to each other (Figure 4a), we performed chromatin immunoprecipitation (ChIP) with a set of primers flanking both binding motifs. HEK293 cells were transfected with β-catenin or myc-TCF4 together with and without APC induction. ChIP assay was carried out with β-catenin and TCF4 antibody; IgG was used as control. Our data show that both β-catenin and TCF4 bind to miR-17-92 promoter and that enforced expression of APC significantly inhibits this binding (Figure 4e). As β-catenin is a co-activator of TCF/LEF transcriptional factors, we further examined the requirement of TCF/LEF binding

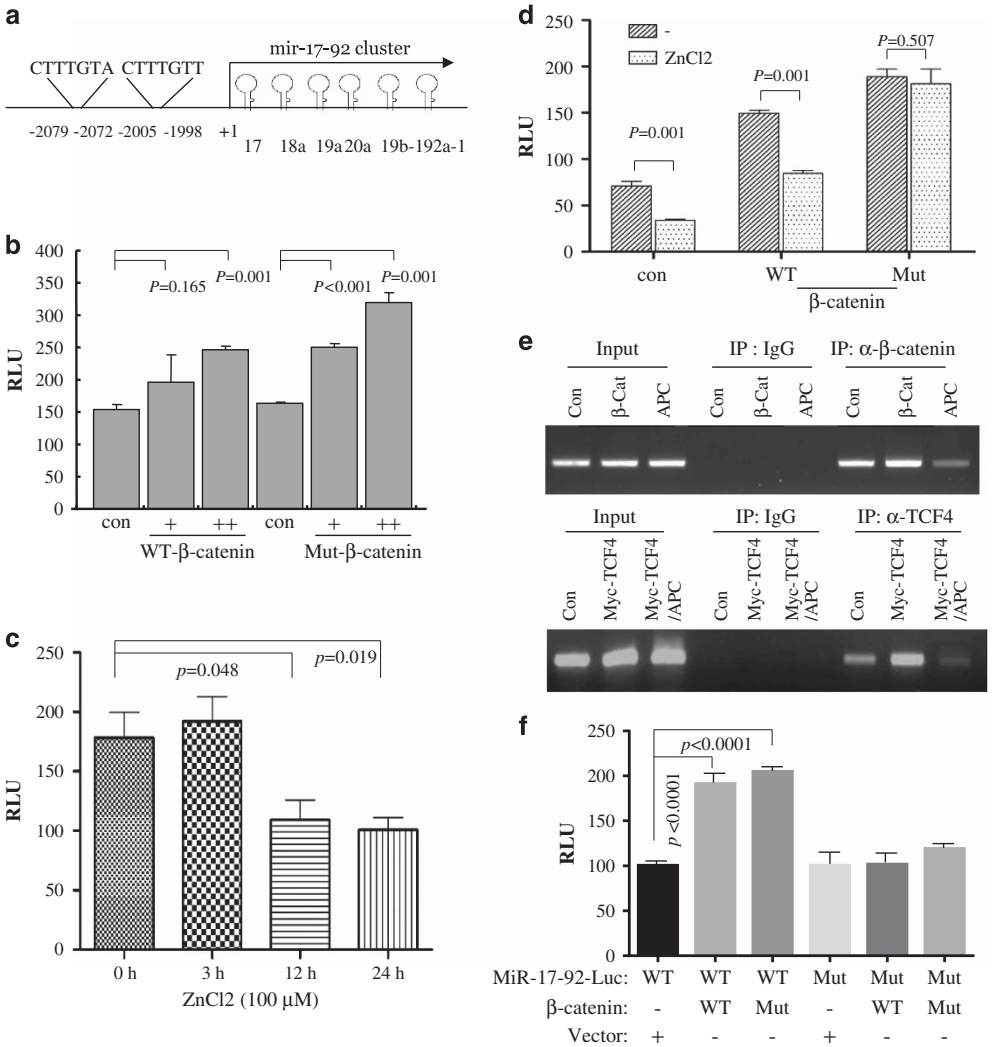


Figure 4. MiR-17-92 promoter is induced by β-catenin which is inhibited by APC. (**a**) Diagram represents miR-17-92 promoter with the two TCF/LEF binding sites indicated. (**b**) β-catenin induces miR-17-92 luciferase promoter activity. HEK293 cells were transfected with pGL3-miR-17-92-Luc together with or without WT and Mut β-catenin. Luciferase assay was performed after 48 h incubation. (**c**) APC inhibits miR-17-92 promoter activity. pGL3-miR-17-92-Luc was introduced into APC/HT29 cells. Following the induction of APC expression by $ZnCl_2$, luciferase activity was measured. (**d**) APC represses wild-type but not constitutively active mutant β-catenin induced miR-17-92 promoter activity. Luciferase assay was carried out in APC/HT29 cells that were transfected with indicated plasmids and treated with/without $ZnCl_2$. (**e**) β-catenin/TCF/LEF directly binds to miR-17-92 promoter, which is inhibited by APC. HEK293 cells were transfected with vector control, β-catenin or Myc-TCF4 together with and without APC, and then subjected to ChIP assay with β-catenin and TCF4 antibodies. IgG was used as control. (**f**) Mutation of TCF/LEF binding sites of the miR-17-92 promoter largely abrogates the miR-17-92 promoter activity induced by β-catenin. Following transfection of wild-type and mutant miR-17-92-Luc together with and without WT and constitutively active Mut β-catenin for 48 h, luciferase assay was performed.

sites to activate miR-17-92 promoter by β-catenin. We mutated the TCF/LEF binding sites within the miR-17-92 promoter and showed that both wild-type and constructively active β-catenin failed to induce the mutant miR-17-92 promoter activity (Figure 4f).

Enforced expression of miR-19a overrides APC tumor suppressor activity and knockdown of miR-19a in cancer cells with compromised APC function reduces aggressive features *in vitro*

MiR-19 has been shown to be a major oncogenic member of miR-17-92 cluster.[27] Furthermore, a recent study showed that expression of miR-19 in the context of the miR-17 ~ 92 cluster at medium levels promoted colon tumor progression.[28] By examination of a panel of colon cancer cell lines, we observed increase of miR-19a expression in either APC mutation or β-catenin mutation lines with variable levels when compared with wild-type APC/β-catenin NCI-H508 cell line (Figure 5a). As APC represses miR-17-92 at transcription level, we next asked whether ectopic expression of miR-19a family member(s) is able to override APC tumor suppressor activity. Thus, we transfected APC/HT29 cells with pre-miR-19a. In agreement with previous reports,[29] induction of APC expression with $ZnCl_2$ in APC/HT29 cells inhibited cell proliferation, cell migration and invasion. However, in pre-miR-19a-treated cells, these APC-induced effects were strongly diminished (Figures 5b and d). As controls, we expressed miR-19a in β-Gal/HT29 cells and found no effect on colony growth and cell migration following treatment with $ZnCl_2$ (Supplementary Figure 1). To further confirm these results, we introduced APC together with and without pre-miR-19a into HCT15 cells and observed that expression of APC alone repressed colony growth, cell migration and invasion. These phenotypes were largely overridden by co-expression of miR-19a (Supplementary Figure 2). On the other side, knockdown of miR-19a in parental HT29 cells reduced cell growth, migration and invasion (Figures 5c and e). We further verified these findings by knockdown of miR-19a in APC-mutant HCT15 cells (Supplementary Figure 3) and ectopic expression of miR-19a in wild-type APC/β-catenin NCI-H508 cells (Supplementary Figure 4). Collectively, these findings suggest that reduction in miR-19a expression levels is a major mechanism by which APC exerts its tumor suppressor activity.

Co-expression of miR-19a and β-catenin in CRC

Mutations of APC/β-catenin pathway have been observed in more than 90% of sporadic CRCs. These mutations lead to stabilization and accumulation of β-catenin.[30] Having demonstrated that miR-17-92 is induced by APC mutations through β-catenin transcriptional activity, we further examined whether this regulation occurs *in vivo*. We examined 117 colorectal tumors and 42 normal colon tissues for expression of miR-19a and β-catenin (Figures 6a and c). Among the 117 colorectal tumors, 85 had overexpression of miR-19a and 92 had overexpression of β-catenin. Among the 92 tumors with elevated β-catenin, 79 (86%) also had elevated miR-19a levels ($P < 0.0001$). These data suggest that there is a significant relationship of co-expression of β-catenin and miR-19a, which further support the findings of biochemical and functional links between APC/β-catenin and miR-17-92 cluster (Figure 7).

DISCUSSION

Mutation of APC and elevated levels of miR-17-92 cluster have been frequently detected in CRC.[25,31,32] In this report, we demonstrated a direct link between APC and miR-17-92. APC represses miR-17-92 through inhibition of β-catenin. Mutation of APC leads to stabilization of β-catenin, which in turn binds to and activates miR-17-92 promoter. Enforced expression of miR-19a largely overrides APC-inhibited cell growth, migration and invasion and knockdown of miR-19a in cancer cells with compromised APC function reduces aggressive features *in vitro*. Moreover, elevated level of β-catenin is significantly correlated with miR-19a overexpression in human colorectal carcinoma. These findings suggest that miR-17-92 cluster is one of key effects of APC/β-catenin/TCF4/LEF pathway (Figure 7).

Accumulating studies have shown that miR-17-92 cluster is one of the most elevated miRNA families in this malignancy.[33,34] Furthermore, this cluster has been suggested not only to be involved in progression of colorectal adenoma to adenocarcinoma but also to have diagnostic value in CRC.[35] For instance, analysis of the exfoliated colonocytes isolated from feces of CRC patients and healthy volunteers showed that the expression of the miR-17-92 cluster was significantly higher in CRC patients than in healthy volunteers.[36] In addition, upregulation of miR-92 in serum was demonstrated to be a predictive value for CRC with a sensitivity of 89% and a specificity of 70%.[37] Increased miR17-92 expression in CRC has also been shown to be associated to DNA copy-number gain of miR-17-92 locus on 13q31 and c-MYC expression.[25] Although MYC induces miR-17-92 promoter activity and expression,[38,39] the mechanism of elevated miR-17-92 in CRC remains elusive. We showed in this study that APC mutation induced miR-17-92 expression. Mechanistically, APC mutation leads to stabilization and accumulation of β-catenin, which in turn binds to miR-17-92 promoter and promotes its expression. Indeed, we also document significant co-overexpression of miR-17-92 and β-catenin in CRC. Thus, we provided the evidence of miR-17-92 as a direct target of APC/β-catenin pathway in CRC.

It has been well documented that the genes regulated by β-catenin/TCF4/LEF such as CCND1, c-MYC, ID2 and MMP7, mediate APC cellular function.[40] Depletion of CCND1 or c-MYC significantly reduces phenotypes resulted from mutation of APC.[41] Previous studies identified miR-19 as the most important miRNA of the miR-17-92 cluster in c-*Myc*-induced lymphomagenesis.[27,42] A recent report showed that quantitatively controlling the expression of miR-17 ~ 92 determines colon tumor progression. Expression of miR-19 in the context of the miR-17 ~ 92 cluster at medium levels promoted tumor metastasis through induction of Wnt/β-catenin-mediated epithelial–mesenchymal transition by targeting to PTEN, whereas higher levels of miR-18a in the context of the miR-17 ~ 92 cluster inhibited tumor growth and metastasis by directly targeting β-catenin.[28] These findings not only further highlight the importance of miR-19 in promoting colon cancer progression but also suggest a fine-tone regulation between miR-17-92 and β-catenin to maintain certain levels of miR-19 and β-catenin in colon carcinogenesis. We demonstrated in this report that elevated levels of miR-19a were correlated with APC mutation and β-catenin expression in colon cancer cell lines and primary tumors, respectively (Figures 5a and 6). Enforced expression of miR-19a largely overrides the cellular phenotypes induced by wild-type APC. Further, we provide evidences that knockdown of miR-19a in APC-mutant cancer cells reduces cell growth, migration and invasion (Figure 5). Collectively, these data suggest that miR-19 is functionally important in APC pathway.

Although alteration of APC is a very common event in CRC, no effective targeted therapy has been developed for APC mutation patients. Considerable efforts have been made to identify small molecule compounds that antagonize Wnt/β-catenin signaling.[43,44] Previous studies reported that xanthothricin inhibits β-catenin/TCF4 transcriptional activity by disrupting β-catenin/TCF4 binding.[45] Furthermore, a series of acyl hydrazones destabilize β-catenin acting downstream of the β-catenin destruction complex.[46] However, these compounds have not clinically been tested in patients with APC mutation. Recent studies have shown that function of miRNAs can be efficiently and specifically inhibited by chemically modified anti-miR oligonucleotides, supporting their potential as targets for the development of novel therapies. In fact, antisense of miR-122, a key miRNA-promoting HCV replication, is currently in phase II clinical trials for patients with chronic HCV infection.[47] Our data show that miR-17-92 is a downstream target

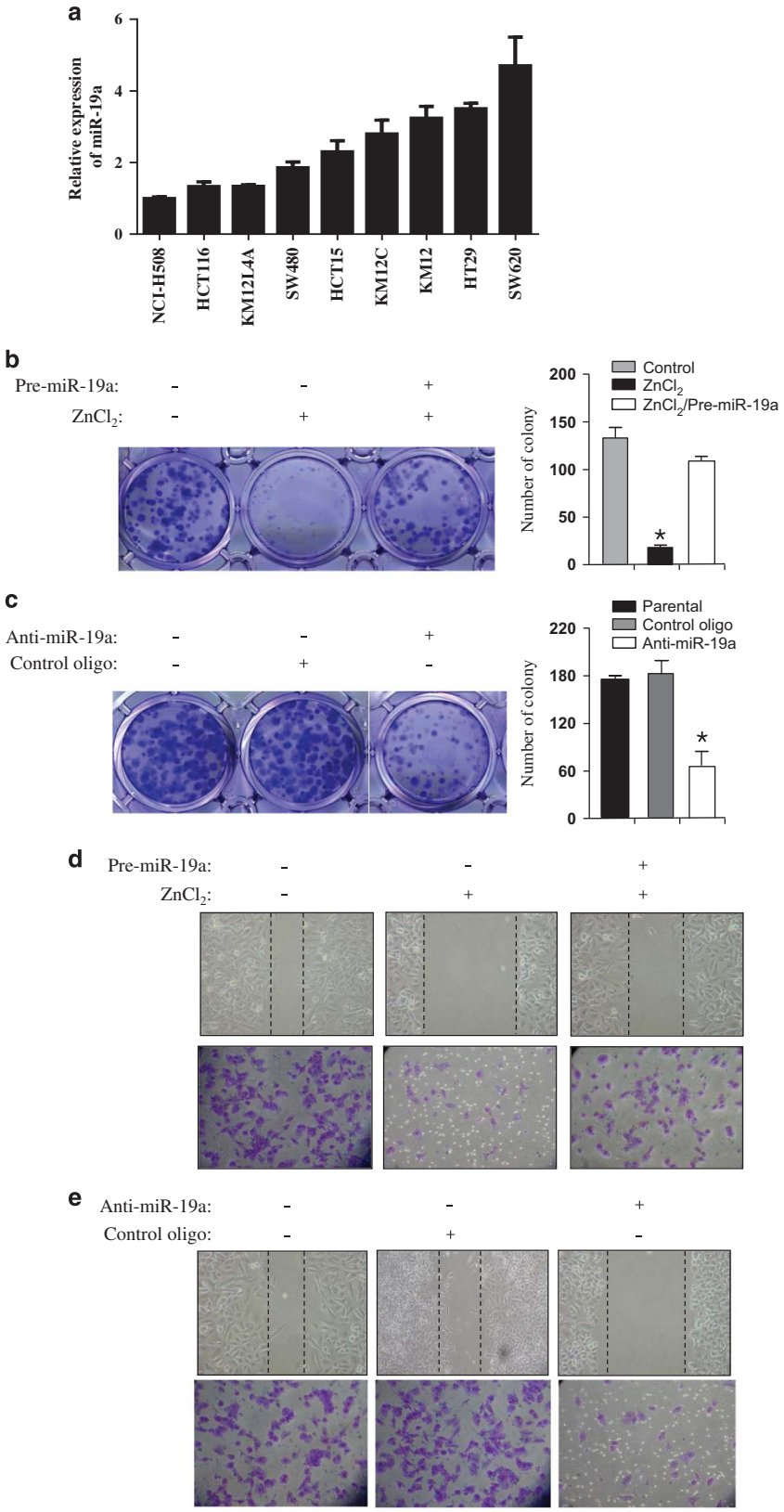
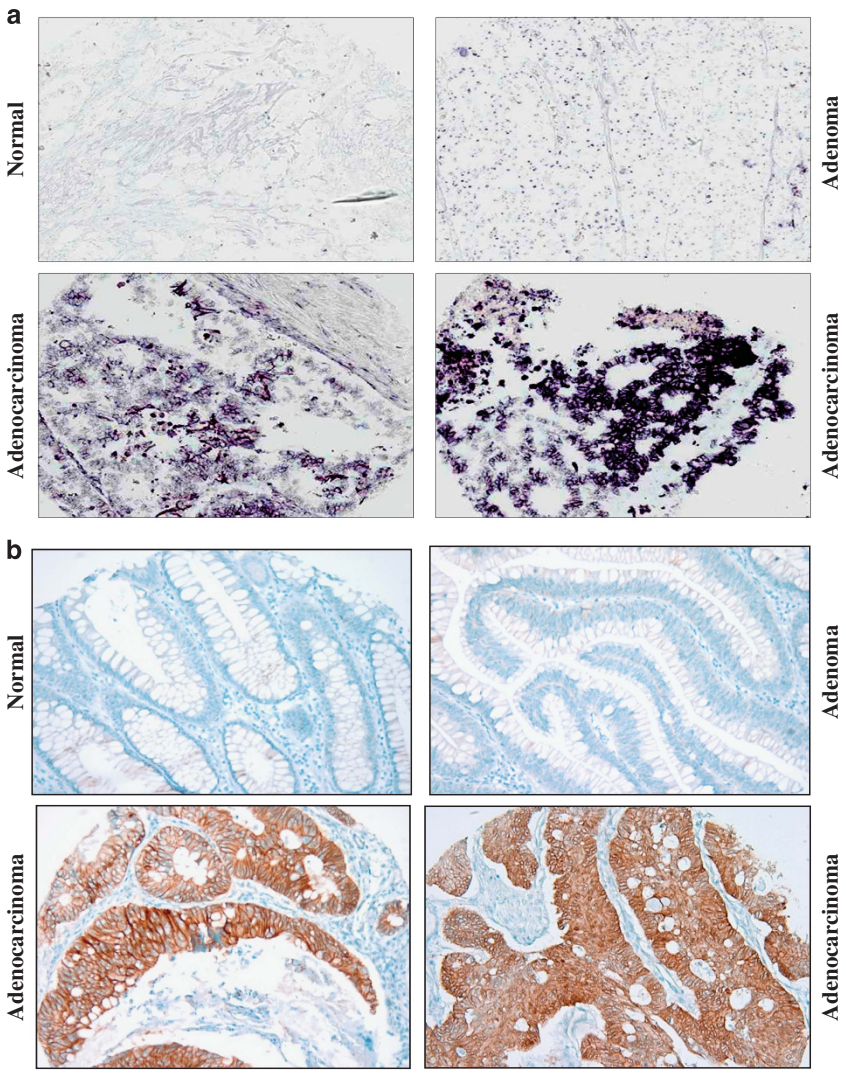


Figure 5. MiR-19a mediates APC cellular function. (**a**) Real-time qPCR analysis of miR-19a expression in a panel of colon cancer cell lines. (**b** and **c**) Colony formation was performed in APC/HT29 cells that were transfected with pre-miR-19a, anti-miR-19a or control oligo and then treated with (**b**) or without (**c**) $ZnCl_2$. The number of colonies was quantified (right). (**d** and **e**) Cell migration and invasion assay. APC/HT29 cells were transfected and treated as above and subsequently subjected to cells migration (wound healing) and invasion assays. $^*P < 0.05$.



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Expression of miR-19a and β-catenin in colorectal cancer

		miR-19a		*p*-value
		Low	High	
β-catenin	Low	19	6	<0.0001
	High	13	79	

Figure 6. Co-expression of miR-19a and β-catenin in human colorectal cancer. (**a**) LNA-miR-19a *in situ* hybridization analysis of colorectal cancer TMA. (**b**) Immunohistochemical staining of same colorectal cancer TMA with β-catenin antibody. Note: elevated β-catenin locates either cytoplasm or nucleus in tumors. (**c**) Overexpression of miR-19a is significantly correlated to elevated levels of β-catenin, $P < 0.0001$. TMA, tumor tissue microarray.

of APC and mediates APC cellular function. Thus, inhibition of miR-17-92, especially miR-19, using anti-miRs could be a potential therapeutic strategy for CRC patients with APC mutation.

MATERIALS AND METHODS

Cell lines, culture, plasmid, transfection and antibodies

All the colon cancer cell lines were obtained from ATCC (Manassas, VA, USA). $ZnCl_2$ inducible and β-Gal/HT29 cell lines, kindly provided by Bert Vogelstein,[29] were maintained in McCoy's SA media (GIBCO/BRL) supplemented with 10% fetal bovine serum. NCI-H508 cell line was a gift from Dr Thomas Ried (National Cancer Institute, USA). The rest of the colorectal cancer cell lines and HEK293 cells were obtained from the American Type Culture Collection and cultivated following the manufacturer's instruction. The firefly luciferase reporter vectors pGL3 basic, pGL3 promoter and *Renilla* luciferase vectors pRL-SV40 are from Promega (Madison, WI, USA). The pGL3-miR-17-92 reporter plasmid was obtained from Dr Tomas Stopka. Mutation of TCF/LEF binding sites of the miR-17-92 promoter was generated using QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA). Wild-type and constitutively active mutant β-catenin and Myc-TCF4 are from Addgene. Transfection was performed using Lipofectamine reagents (Life Technologies, Carlsbad, CA, USA). APC antibody was from Santa Cruz (C-20; Catalog #sc-896,

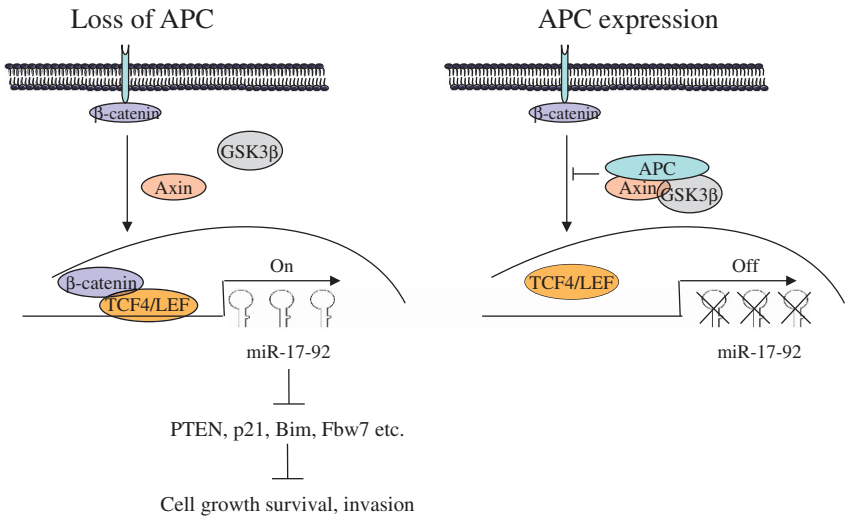

Figure 7. Diagram represents the proposed model of APC regulation of miR-17-92 cluster.

Santa Cruz, CA, USA). Antibodies against β-catenin (Catalog #9562S) and TCF4 (Catalog #2565S) were purchased from Cell Signaling (Danvers, MA, USA).

MicroRNA microarray analysis

Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA). MiRNA profiling was performed as previously described.[48] Briefly, miRNA array was hybridized with [γ-^{32}P]ATP–labeled small RNA probes. To ensure the accuracy of the hybridizations, each labeled RNA sample was hybridized with three separate arrays and quantified. In addition, eight oligonucleotides with any nonmatching known miRNA were used as hybridization controls. Hybridization signals for each spot of the array and background values at 15 empty spots were measured. The signals that fail to exceed the average background value by more than 3 s.d. were excluded. All statistical analyses on microarray data were performed using R software v2.5.0 http://www.r-project.org/ and the Bioconductor software package http://www.bioconductor.org/. The microarray data were initially background-corrected using a normal plus exponential convolution model, normalized (a) within arrays using a method that normalizes the M-values for each single microarray using robustly fitted regression splines for each print-tip group and an empirical Bayesian approach to shrink the individual print-tip curves towards a common value, and subsequently (b) between arrays using a method which ensures that the A-values (average intensities) have the same empirical distribution across arrays, leaving the M-values (log-ratios) unchanged.[49] After the normalization step, the probes were pre-filtered on the basis of empty spots and negative control intensity distribution over all the arrays.

MiRNA qRT–PCR detection and quantification

Expression of miR-17-92 family members and U6 was detected using TaqMan microRNA Reverse Transcription kit (Applied Biosystem, Foster City, CA, USA). Briefly, 200 ng of total RNA from each cell line and tumor RNA were used for primer-specific reverse transcriptase (RT) in both miR-17-92 and U6, and then 2 μl of the RT product was used for subsequent qPCR. The qPCR was performed on ABI HT9600 (ABI, Foster City, CA, USA) and data were collected and analyzed using ABI SDS version 2.3. To calculate relative concentration, miR-17-92 and U6 C_T values for all the samples were obtained. A normalized expression for each sample was obtained by subtracting C_T of has-miR by the same sample's U6 CT and designated as ΔC_T. This value is then transformed by performing $2^{-(\Delta CT)}$.

Cell proliferation, migration and invasion assay

Cell growth was determined using colony formation assay. Cell migration was evaluated by wound-healing assay. Cell invasion assay was performed as we previously described.[14] Briefly, β-Gal/HT29 cells were transfected with anti-miR-19a, whereas APC/HT29 cells were treated with pre-miR-19a. As controls, β-Gal/HT29 and APC/HT29 cells were transfected with control oligonucleotides against GFP. Following culture with and without additional $ZnCl_2$ for 24 h, cells were seeded in the upper chamber of Boyden Chambers coated with Matrigel. All the chambers contained normal culture medium containing $ZnCl_2$. After incubation for 48 h, invasion and migration were examined under a Nikon inverted light microscope (Tokyo, Japan).

Luciferase and ChIP assay

Luciferase assay was performed using the Luciferase Assay System (Promega), and activities were normalized to β-galactosidase activity. ChIP assay was performed essentially as previously described.[14] Solubilized chromatin was prepared from a total of 2×10^7 asynchronously growing cells. The chromatin solution was diluted 10-fold with ChIP dilution buffer, and precleared with protein-A beads blocked with 2 μg of sheared salmon sperm DNA and preimmune serum. The precleared chromatin solution was divided and utilized in immunoprecipitation assays with anti-β-catenin, -TCF4 antibodies or IgG. Following wash, the antibody-protein–DNA complex was eluted from the beads by resuspending the pellets in 1% SDS, 0.1 M $NaHCO_3$ at room temperature for 20 min. After cross-linking, protein and RNA were removed by digestion. Purified DNA was subjected to PCR with primers specific for two putative TCF/LEF binding sites within the human miR-17-92 promoter region (forward miR-17-92 chip-F 5′-AGCG CCTCCAGAACAAAGCGGC-3′, miR-17-92 chip-R 5′-TCCCGCGCCACACTCCCA GCAA-3′). Amplified PCR products (343 bp) were resolved by 1.5% of agarose gel electrophoresis and visualized by ethidium bromide staining.

Colorectal tumor tissue microarray, miRNA-locked nucleic acid *in situ* hybridization and immunohistochemical staining

Primary colon cancer, adenoma and normal tissue specimens were obtained from patients who underwent surgery at H. Lee Moffitt Cancer Center and approved by the Institutional Review Board and in accord with an assurance filed with and approved by the US Department of Health and Human Services. Informed consent was obtained from all the subjects. MiRNA *in situ* hybridization was performed in colorectal cancer tumor tissue microarrays, which contains 117 colon cancers and 20 adenomas, and analyzed as described previously.[23,50] The probe sequences used were as follows: LNA-miR-19a; 5′-digoxigenin-tCAgTTtTGcatAGatttgcaca-3′. Immunohistochemistry analysis and immunofluorescence staining were performed following our routine procedures.[22] Immunohistochemistry analysis of colorectal cancer tumor tissue microarrays with β-catenin antibody was carried out as previously described.[14]

Statistical analysis

Statistical comparisons were based upon unpaired Student's *t*-test. Correlation of expression of miR-19a and β-catenin was analyzed with Chi-square test. $P\leqslant0.05$ was considered to be statistically significant.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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