ZNF148 modulates TOP2A expression and cell proliferation via ceRNA regulatory mechanism in colorectal cancer

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Abstract

Background: Competing endogenous RNA (ceRNA) regulation is a novel hypothesized mechanism that states RNA molecules share common target microRNAs (miRNAs) and may competitively combine into the same miRNA pool.

Methods: Zinc finger protein 148 (ZNF148) and TOP2A expression were analyzed in 742 colorectal cancer (CRC) tissues using immunohistochemistry (IHC). ZNF148 mRNA, TOP2A mRNA, miR101, miR144, miR335, and miR365 expression were estimated in 53 fresh frozen CRC tissues by reverse transcription polymerase chain reaction. Mechanisms underpinning ceRNA were examined using bioinformatics, correlation analysis, RNA interference, gene over-expression, and luciferase assays.

Results: Protein levels of ZNF148 and TOP2A detected by IHC positively correlated (Spearman correlation coefficient $[r_s] = 0.431$, $P < 0.001$); mRNA levels of ZNF148 and TOP2A also positively correlated ($r = 0.591$, $P < 0.001$). Bioinformatics analysis demonstrated that ZNF148 and TOP2A mRNA had 13 common target miRNAs, including miR101, miR144, miR335, and miR365. Correlation analysis demonstrated that levels of ZNF148 mRNA were negatively associated with levels of miR144, miR335, and miR365. Knockdown and overexpression tests showed that ZNF148 mRNA and TOP2A mRNA regulated each other in HCT116 cells, respectively, but not in Dicer-deficient HCT116 cells. Luciferase assays demonstrated that ZNF148 and TOP2A regulated each other through 3′UTR. Overexpression of ZNF148 mRNA and TOP2A mRNA caused significant downregulation of miR101, miR144, miR335, and miR365 in the HCT116 cells. We also found that knockdown of ZNF148 and TOP2A significantly promoted cell growth, and overexpression of ZNF148 and TOP2A inhibited cell proliferation, which was abrogated in Dicer-deficient HCT116 cells.

Conclusion: ZNF148 and TOP2A regulate each other through ceRNA regulatory mechanism in CRC, which has biological effects on cell proliferation.

Abbreviations: ceRNA = competing endogenous RNA, CRC = colorectal cancer, IHC = immunohistochemistry, miRNAs = microRNAs, RT-PCR = reverse transcription polymerase chain reaction, ZNF148 = zinc finger protein 148; TOP2A = topoisomerase II alpha.

Keywords: colorectal neoplasms, competing endogenous RNA, microRNA, TOP2A, ZNF148

1. Introduction

Colorectal cancer (CRC) is a multistep disease that develops from the accumulation of genetic and epigenetic aberrations,[1,2] resulting in a network of complex interactions. The understanding of these networks and mechanisms may contribute to the optimization of diagnostic and prognostic parameters, as well as the development of novel targeted therapies.[1]

Recently, a novel regulatory mechanism, known as competing endogenous RNA (ceRNA), was proposed by Pandolfi in 2011.[3] This hypothesis suggests that microRNAs (miRNAs), approximately 22 nucleotides in length, bind to miRNA response elements (MREs) in target mRNA molecules, resulting in decreased stability of target mRNAs and repressed expression of target mRNAs.[3] Many studies have shown that in addition to conventional miRNA to mRNA regulation, a reverse mRNA to miRNA mechanism also exists.[4] On the basis of these phenomena, the concept of a ceRNA regulation mechanism emerged, which hypothesizes that RNAs that share the same MREs may regulate each other by competitively combining into the same miRNA pool.[3,5] The ceRNA regulation mechanism contributes to a large-scale regulatory network across transcriptomes, greatly expanding the functional genetic information of the human genome and may play an important role in various pathological conditions, such as cancer.[3] A large number of studies have reported that varying types of RNA (including pseudo-mRNA,[6] long noncoding RNA,[7,8] and mRNA)[9] function as ceRNAs and play an important role in differentiation,[8] tumorigenesis,[7] tumor development,[9] and metastasis.[10] On the basis of the ceRNA regulation mechanism theory, if 2 mRNAs regulate each other, then their expression levels would positively correlate.

Zinc finger protein 148 (ZNF148), also known as ZBP89, ZBP-89, or ZFP148, is a multifunctional zinc-finger transcription factor associated with signaling pathways upstream of cellular proliferation, embryogenesis, differentiation, growth arrest, and apoptosis.[11] It plays a significant role in the regulation of cell growth, apoptosis, and carcinogenesis in many types of cancer.[12] But its role and mechanism in CRC is still unclear.[13] Topoisomerases are nuclear enzymes, which regulate DNA topology by action on the temporary cleavage and ligation cycle of DNA.[14] Among all forms of topoisomerases, topoisomerase II alpha (TOP2A) is extensively associated with cell proliferation and therefore is an important therapeutic target in diseases that involved cellular proliferation such as cancers.[15] TOP2A is over-expressed in various carcinomas, and it helps a lot in diagnosing cancer and observing disease progression and prognosis prediction.[14,16,17]

In our previous study, we detected DNA TOP2A expression in 490 CRC tissues, and found that overexpression of TOP2A was related with a lower T stage, a lower N stage, and a lower recurrence rate. Furthermore, we also found that the expression level of TOP2A was an independent prognostic indicator for CRC.[2] In another previously published study, we found that lower expression levels of ZNF148 were a predictive factor of poor overall survival and disease-free survival.[11] However, the mechanism regulating ZNF148 and TOP2A expression is poorly understood. We, therefore, sought to examine whether the expression of these 2 tumor suppressor genes are regulated by the ceRNA mechanism.

2. Methods

This study was approved by the Committee on Ethics of Biomedical Research, Changhai Hospital, Second Military Medical University (Shanghai, China). Written informed consent was obtained from all patients included in this study. The criteria of patient selection and clinicopathological variables of the included 742 CRC patients were the same as our previously published study.[11]

2.1. Immunohistochemistory

The procedure and protocol of immunohistochemistry (IHC) was described previously.[11] Paraffin-embedded, formalin-fixed CRC tissues were immunostained for ZNF148 and TOP2A proteins. The signal was amplified with horseradish peroxidase-conjugated secondary antibody, followed by counterstaining with hematoxylin. Anti-ZNF148 monoclonal antibody (1:100) was purchased from Abcam (Cambridge, MA, USA), (ab69933) and anti-TOP2A monoclonal antibody (1:40) was purchased from DAKO (Copenhagen, Denmark), Denmark, respectively. Expression levels of ZNF148 and TOP2A were estimated using the criterion given on the ATLAS website,[18] based on the percentage and intensity of stained tumor cells.

2.2. miRNA target prediction

The miRanda software, which was downloaded from the website "http://www.miRNA.org/miRNA/getDownloads.do," was used to predict target miRNA with the default parameters.

2.3. RNA extraction and RT-PCR

Total RNA and miRNA were extracted from CRC tissue using Trizol reagent (Invitrogen, Los Angeles, CA, USA) and reverse transcribed using M-MLV-RTase (Promega, Madison, WV, USA), according to the manufacturers' instructions. The resulting complementary DNA was used for real-time polymerase chain reaction (PCR) using the SYBR-Green Master PCR Mix (Applied Biosystems, Foster, CA, USA) in triplicate. PCR and data collection were performed on the TP800 qPCR System (Takara, Tokyo, Japan). All data were normalized to actin expression as an endogenous control for mRNA or U6 for miRNA. The relative expression level of the target gene compared to the control was expressed as $2^{-(Ct-Cc)}$ (Ct and Cc were the mean threshold cycle differences after normalizing to actin or U6). Primers used in real-time PCR were shown in Table 1.

Table 1

Primers used in real-time reverse transcription polymerase chain reaction.

Gene	Forward (5′–3′)	Reverse (3′–5′)
ZNF148	CGGTATCTTCAAGCAGCAAGTAAC	GTGCCTGTGACTCTAATATGGATG
TOP2A	CCTTTGCCAATGCTTCCAAGTTAC	GTGTCTTCTCGGTGCCATTCAAC
Actin	GTGGACATCCGCAAAGAC	AAAGGGTGTAACGCAACTA
miR-101	ACACTCCAGCTGGGTACAGTACTGTGATA	TGGTGTCGTGGAGTCG
miR-144	ACACTCCAGCTGGGTACAGTATAGATGA	TGGTGTCGTGGAGTCG
miR-335	ACACTCCAGCTGGGTCAAGAGCAATAACGAA	TGGTGTCGTGGAGTCG
miR-365	ACACTCCAGCTGGGTAATGCCCCTAAAAAT	TGGTGTCGTGGAGTCG
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT

2.4. Cell culture

HCT116, Dicer-deficit HCT116, HT-29, and HEK293T cell lines were purchased from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Science (Shanghai, China) within 1 month of starting in vitro study. These cell lines were maintained in Dulbecco Modified Eagle Medium supplemented with 10% fetal bovine serum at 37°C in a humidified incubator with a constant air flow of 5% CO_2 and 95% O_2.

2.5. Lentivirus packaging

The ZNF148 siRNA lentivirus (shZNF14) was constructed according to the previous protocol,[19] the target sequence for human ZNF148 (NM_021964.2) was 5′-CTAGCCGGGACATT-GATCAGGTTGATAATCTCGGATTATCAACCTGATCAATG-TCTTTTTTAAT-3′, and the negative control sequence was 5′-AAAAAA CCTGTGCATAGTAGTACTAATCTCGAGATTAG-TACTACTATGCACAGGCC GG-3′. Oligonucleotides were cloned into the vector pFH-L (Shanghai Preii, Shanghai, China).

To construct the ZNF148 overexpression lentivirus (oeZNF14), the coding sequence of ZNF148 was amplified from genomic DNA and cloned into the slow virus vector plasmid pCDH using *Eco*RI and *Xba*I sites. The primer sequences were ZNF148-F, 5′-TCAGG-GATCCATGAACATTGACGACAACTGG-3′ and ZNF148-R, 5′-TTTTCCCGGGGCCAAAAG TCTGGCCAGTTG-3′. Similarly, shTOP2A and oeTOP2A were constructed with the same methods.

2.6. Transfection

Lentivirus packaging and infection were performed according to the manufacturer's instructions. In brief, 48 h after 293T cells were co-transfected with shZNF148 or oeZNF148 expressing plasmids and 2 helper plasmids, pCMVΔR8.92 and pVSVG-I (Shanghai Preii) using Lipofectamin2000 (Invitrogen). Culture media containing packaged lentivirus was harvested and concentrated.

For cell infection, a concentration of 5×10^4/well HT-29 cells was incubated with shZNF148, oeZNF148, shTOP2A, or oeTOP2A with a multiplicity of infection of 50 for 24 h. After 24 h, the culture medium was replaced. Knockdown efficiency was validated by real-time PCR and Western blot analysis at day 5 post-transduction. After confirming knockdown efficiency, cells were seeded into the 96-well plates for methylthiazolete-trazolium (MTT) cell proliferation assay.

2.7. Luciferase assays

Luciferase assays were used to validate whether TOP2A was regulated by ZNF148. HCT116 cells were seeded 24 h before transfection at a density of 5×10^4 cells per well in 24-well plates. The 3′UTRs of TOP2A and ZNF148 were obtained by reverse transcription PCR (RT-PCR) amplification. psiCHECK-2 and TOP2A 3′UTR were co-transfected with ZNF148 shRNA with Lipofectamine 2000 according to the manufacturer's instructions. In all experiments, firefly luciferase gene in psiCHECK-2 was used as a normalization control for transfection efficiency. At 48 to 72 h after transfection, firefly and Renilla luciferase activities were measured consecutively with the Dual-Luciferase reporter assay system using a luminometer (Promega).

2.8. MTT cell proliferation assay

Cells infected with shZNF148, oeZNF148, shTOP2A, oeTOP2A, or nonsilencing siRNA lentivirus (Con) were seeded in 96-well plates at a density of 2×10^3 cells per well. At indicated time points, 20 μL MTT solution (5 mg/mL) was added into each well. After 4 h of incubation at 37°C, 150 μL dimethyl sulfoxide was added to dissolve crystals. After 10 min at room temperature, absorbance was recorded at 490 nm.

2.9. Statistical analysis

Student *t* test, 1-way analysis of variance, and nonparametric analysis were used to detect the difference between the different groups. Association of 2 variables was analyzed by Pearson correlation coefficient (r) or Spearman correlation coefficient (r_s). P value < 0.05 (2-sided) was considered to be statistically significant. All statistical analyses were conducted using Statistical Package for Social Sciences version 17.0 statistical software.

3. Results

3.1. Correlation between ZNF148 and TOP2A protein expression

Expression levels of ZNF148 and TOP2A were detected by IHC in 742 CRC tissue samples. Representative microphotographs of positive staining amounts of ZNF148 and TOP2A in CRC tissues are shown in Fig. 1. The relationship between ZNF148 staining and TOP2A staining is summarized in Table 2. ZNF148 and TOP2A expression levels positively correlated ($r_s = 0.431$, $P < 0.001$).

3.2. Bioinformatics analysis

MiRanda software was used to predict target miRNA of mRNA. Bioinformatics analysis showed that TOP2A mRNA had 26 possible target miRNAs, ZNF148 mRNA had 70 possible target miRNAs, and TOP2A mRNA and ZNF148 mRNA had 13 common target miRNAs (Fig. 2A). The 13 common miRNAs included miR26a, 26b, 101, 144, 203, 335, 365, 377, 410, 411, 495, 543, and 1297. Of the 13 common target miRNAs, expression of the first 7 miRNAs (miR26a, 26b, 101, 144, 203, 335, 365) had been reported in CRC tissues or cell lines in the literature.[20–27] Our RT-PCR experiments showed the presence of miR101, miR144, miR335, and miR365 in human HCT116 colon cells, and hence these 4 miRNAs were selected for analysis in this study.

3.3. Relationship between expression levels of ZNF148 mRNA, TOP2A mRNA, miR101, miR144, miR335, and miR365 in 53 CRC tissues

Expression levels of ZNF148 mRNA, TOP2A mRNA, miR101, miR144, miR335, and miR365 were assessed by RT-PCR in 53 fresh frozen CRC tissues. The relationship between 2 RNAs is listed in Table 3. No significant correlation was revealed between TNM stage and expression level of any identified RNA. Pearson correlation analysis indicated that expression levels of ZNF148 mRNA and TOP2A mRNA positively correlated ($r = 0.591$, $P < 0.001$, Fig. 2B). Expression level of ZNF148 mRNA was negatively correlated with miR144 ($r = -0.326$, $P = 0.017$), miR335 ($r = -0.406$, $P = 0.003$), and miR365 ($r = -0.350$, $P = 0.010$). Expression levels of TOP2A mRNA were negatively correlated with miR335 ($r = -0.343$, $P = 0.012$) and miR365 ($r = -0.338$, $P = 0.013$).
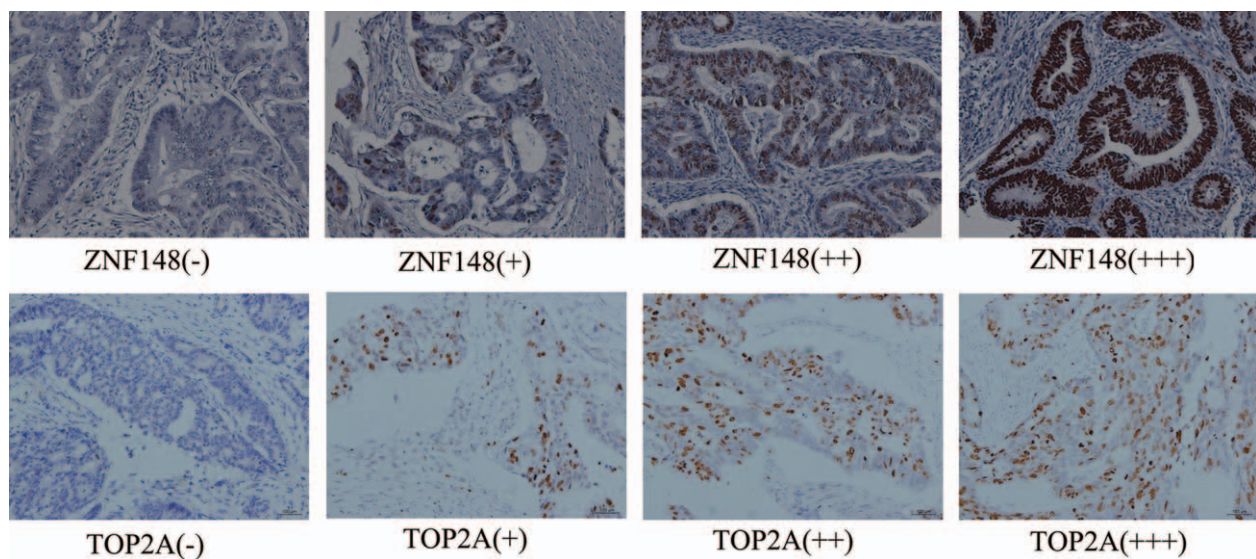
Figure 1. Representative immunohistochemistry of ZNF148 and TOP2A expression in colorectal cancer tissue (×200).

3.4. ZNF148 and TOP2A regulate each other via ceRNA mechanism

To confirm our hypothesis, we utilized anti-ZNF148 siRNA knockdown of ZNF148 or overexpression of ZNF148. ZNF148 expression was down regulated by lentivirus-mediated shRNA. At the highest infection efficiency, Green Fluorescent Protein was identified in more than 90% of HT-29 cells (Fig. 3A). After infection for 5 days, mRNA levels of ZNF148 in HT-29 cells infected with ZNF148 lentiviral shRNA decreased significantly (Fig. 3B). Western blot analysis also revealed ZNF148 protein levels decreased significantly (Fig. 3C). In HCT116 cells, down-regulation of ZNF148 mRNA (Fig. 3D) led to downregulation of TOP2A mRNA (Fig. 3E); however, in Dicer-deficit HCT116 cells (HCT116−/− cells do not express the majority of mature miRNAs), levels of TOP2A mRNA were not affected (Fig. 3F), indicating that mature miRNAs are essential for the regulation between ZNF148 and TOP2A. In HCT116 cells, overexpression of ZNF148 mRNA (Fig. 3G) led to upregulation of TOP2A mRNA (Fig. 3H); but in Dicer-deficit HCT116 cells, levels of TOP2A mRNA were not affected (Fig. 3I). To ascertain whether this observed effect is dependent upon TOP2A 3′UTR, we constructed a chimeric luciferase plasmid tagged with the TOP2A 3′UTR.

In the contrary, we also found that, in HCT116 cells, knockdown of TOP2A mRNA (Fig. 4A) led to downregulation of ZNF148 mRNA (Fig. 4B); but not in Dicer-deficient HCT116

cells (Fig. 4C). And overexpression of TOP2A mRNA (Fig. 4D) led to upregulation of ZNF148 mRNA (Fig. 4E), but not in Dicer-deficient HCT116 cells (Fig. 4F).

Luciferase assays showed that luciferase activity decreased significantly in the "psiCHECK-2 + TOP2A 3′UTR + ZNF148 shRNA" group, indicating that ZNF148 regulates TOP2A expression through TOP2A 3′UTR (Fig. 5A). In the contrary, we also found that luciferase activity decreased significantly in the "psiCHECK-2 + ZNF148 3′UTR + TOP2A shRNA" group, indicating that TOP2A regulates ZNF148 expression through ZNF148 3′UTR (Fig. 5B).

3.5. ZNF148 and TOP2A mRNA regulate the levels of miR101, miR144, miR335, and miR365

To verify the miRNAs that mediate ZNF148/TOP2A ceRNA modulation, we used the miRanda algorithm to predict common targeting miRNAs. Predicted miRNAs (miR101, miR144, miR335, and miR365) were expressed in HCT116 cells. Overexpression of ZNF148 mRNA (Fig. 5C) and TOP2A mRNA (Fig. 5D) caused a significant downregulation of miR101, miR144, miR335, and miR365 in HCT116 cells.

3.6. Regulation of cell proliferation by ZNF148 and TOP2A

To evaluate the effects of anti-ZNF148 siRNA or overexpression of ZNF148 on proliferation, a cell proliferation assay was

Table 2

Relationship between ZNF148 staining and TOP2A staining detected by immunohistochemistry in 742 colorectal cancer tissues ($r_s = 0.431$, $P < 0.001$).

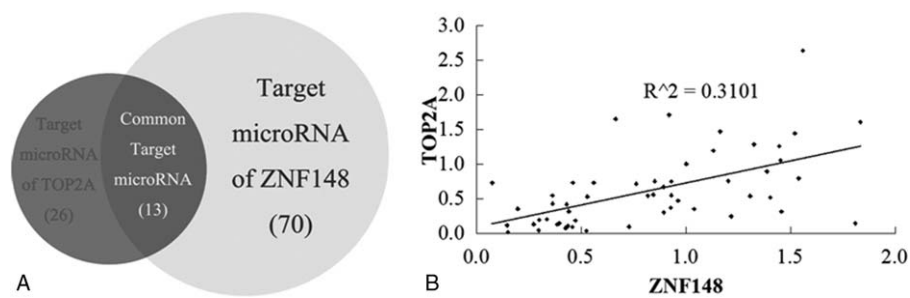|	TOP2A				
ZNF148	(−)	(+)	(++)	(+++)	Total
(−)	155	177	62	29	423
(+)	12	49	24	4	89
(++)	13	29	83	11	136
(+++)	7	22	27	38	94
Total	187	277	196	82	742

Figure 2. Relationship between ZNF148 and TOP2A. (A) ZNF148 mRNA and TOP2A mRNA have 13 common target miRNAs predicted by miRanda software. (B) The relationship between ZNF148 and TOP2A mRNA levels in 53 colorectal cancer tissues ($r = 0.591$, $P < 0.001$).

performed. The results revealed that knockdown of ZNF148 in HCT116 cells significantly promoted cell growth (Fig. 6A). In contrast, overexpression of ZNF148 and ZNF148 3′UTR in HCT116 cells inhibited proliferation (Fig. 6A). However, overexpression of ZNF148 3′UTR in Dicer-deficit HCT116 cells did not inhibit cell proliferation (Fig. 6B). And similarly, we also found that the alteration of TOP2A and TOP2A 3′UTR affected the proliferation in HCT116 cells (Fig. 6C and D).

4. Discussion

ZNF148 is a zinc-finger transcription factor that is universally expressed at low levels in nearly all mammalian cells,[28] including colorectal mucosa.[29] It has been demonstrated that ZNF148 is involved in the regulation of cell growth and cell death. Indeed, ZNF148 is usually over-expressed in tumor cell lines and human tumor tissues, including CRC,[30] gastric cancer, and hepatocellular carcinoma.[31] In our previous study,[11] we demonstrated that ZNF148 is over-expressed in CRC tissue, compared to normal colorectal mucosa and colorectal adenomas. Further analyses showed that ZNF148 expression levels decreased consistently from stage I to stage IV CRC.[11] Reduced expression of ZNF148 in tumors was found to be significantly associated with lymph node metastases, advanced TNM disease stage, poor differentiation, higher rates of disease recurrence, shorter overall survival rates, and shorter disease-free survival.[11]

Our results were consistent with the findings of Bandres[32] and Law.[33] However, the exact mechanism of action of ZNF148 remains elusive.[30] Our previous studies involving TOP2A exhibited tumor suppressor properties similar to ZNF148.[2] This led us to hypothesize that TOP2A and ZNF148 may influence each other through a ceRNA regulation mechanism. Furthermore, ceRNA regulation mechanism has also been reported to play an important role in cell proliferation of esophageal cancer and CRC.[34,35] ZNF148 is over-expressed in CRC, and it inhibits cell proliferation in CRC cells. The underlying mechanism is still unknown. The ceRNA regulation mechanism provides us a new way to clarify the mechanism of ZNF148 regulating cell proliferation.

According to the ceRNA hypothesis,[3] if 2 genes regulate each other by through a ceRNA mediated mechanism, the mRNA levels of these 2 genes would be negatively associated with the levels of target miRNAs, and the mRNA levels of these 2 genes would be positively correlated with each other. For example, if gene A and gene B regulate each other through ceRNA, and their common target miRNAs were miRNA1 and miRNA2; when the expression of gene A is increased, miRNA1 and miRNA2 would decrease, and the expression of gene B would increase. Therefore, gene A is negatively correlated with miRNA1, gene A is positively correlated with gene B, and miRNA1 is positively correlated with miRNA2.

In this study, ZNF148 positively correlated with both TOP2A protein levels ($r_s = 0.431$, $P < 0.001$) and mRNA levels ($r = 0.591$,

Table 3

Relationship of expression level between ZNF148 mRNA, TOP2A mRNA, and miR101, 144, 335, and 365 in 53 colorectal cancer tissues.

	N	r	P
mir101*mir144	43	0.582	**0.000**
mir101*mir335	43	0.683	**0.000**
mir101*mir365	43	0.569	**0.000**
mir101*TOP2A	43	−0.119	0.448
mir101*ZNF148	43	−0.237	0.126
mir144*mir335	53	0.812	**0.000**
mir144*mir365	53	0.869	**0.000**
mir144*TOP2A	53	−0.202	0.147
mir144*ZNF148	53	−0.326	**0.017**
mir335*mir365	53	0.882	**0.000**
mir335*TOP2A	53	−0.343	**0.012**
mir335*ZNF148	53	−0.406	**0.003**
mir365*TOP2A	53	−0.338	**0.013**
mir365*ZNF148	53	−0.350	**0.010**
TOP2A*ZNF148	53	0.591	**0.000**

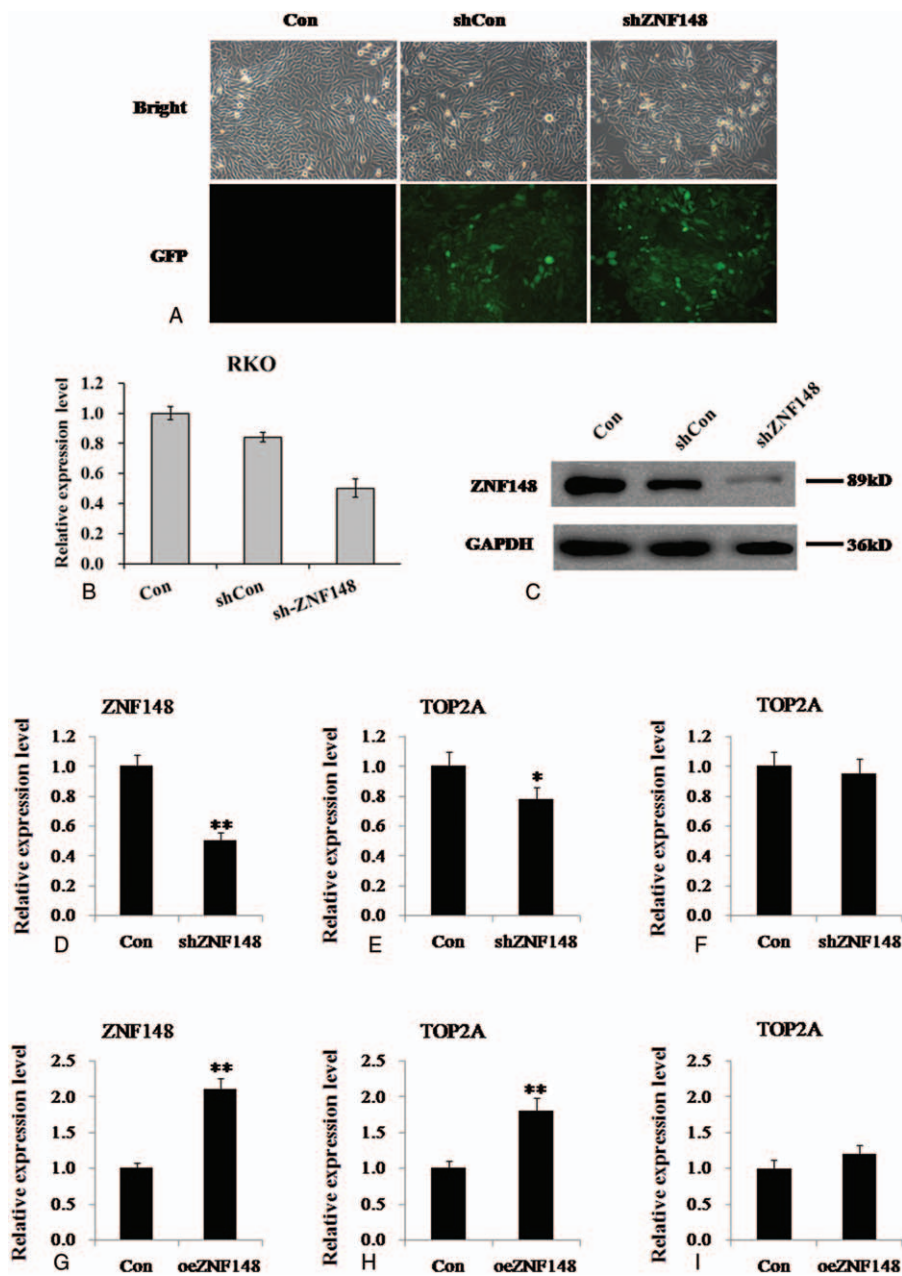Bold values indicate statistical significance of $P < 0.05$.

Figure 3. ZNF148 modulates TOP2A expression as ceRNA. Green Fluorescent Protein (GFP) was identified in more than 90% of HT-29 cells (A). ZNF148 mRNA in HT-29 cells infected with ZNF148 lentiviral shRNA decreased significantly (B). Western blot revealed ZNF148 protein level decreased significantly (C). In HCT116 cells, downregulation of ZNF148 mRNA (D) led to downregulation of TOP2A mRNA (E); however, in Dicer-deficit HCT116 cells, levels of TOP2A mRNA were not affected (F). Overexpression of ZNF148 mRNA (G) led to upregulation of TOP2A mRNA (H); however, in Dicer-deficit HCT116 cells, levels of TOP2A mRNA were not affected (I). $^*P < 0.05$; $^{**}P < 0.01$.

$P < 0.001$). Bioinformatics analysis identified 13 common miRNA targets for ZNF148 and TOP2A. RT-PCR experiments showed the presence of miR101, miR144, miR335, and miR365 in human HCT116 colon cells, and hence these 4 miRNAs were selected for analysis in this study. Furthermore, our Pearson correlation analysis indicated that the expression level of ZNF148 mRNA was negatively associated with expression levels of miR144, miR335, and miR365, and the expression level of TOP2A mRNA was negatively correlated with miR335 and miR365.

The roles of miR101, 144, 335, and 365 in CRC are still poorly understood.[20–27] Our study showed that there were no significant correlations between UICC TNM stage and expres-

sion levels of miR101, 144, 335, and 365, consistent with results in the literature.[20–27] Our study also indicated that the expression levels of miR101, 144, 335, and 365 were positively correlated, with all correlation coefficients (r) higher than 0.4. Therefore, this suggests that these 4 miRNAs play synergistic roles in the ceRNA-mediated regulation between ZNF148 and TOP2A.

On downregulation of ZNF148 mRNA by lentivirus mediated shRNA, TOP2A mRNA expression reduced in HCT116 cells, but was not reduced in Dicer-deficit HCT116 cells, indicating that mature miRNAs are essential for the regulation between ZNF148 and TOP2A. Similarly, overexpression of ZNF148
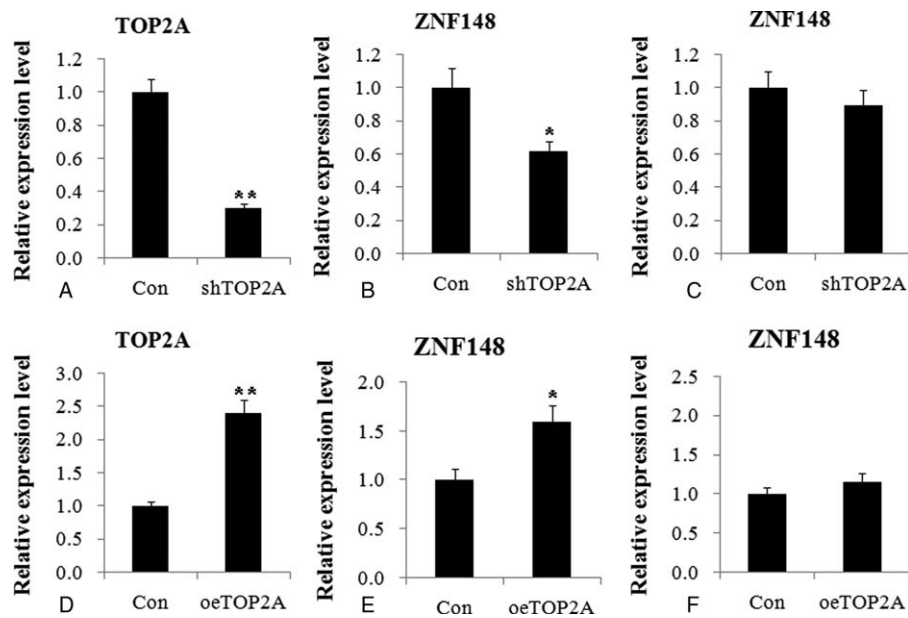
Figure 4. TOP2A modulates ZNF148 expression as ceRNA. In HCT116 cells, downregulation of TOP2A mRNA (A) led to downregulation of ZNF148 mRNA (B); however, in Dicer-deficit HCT116 cells, levels of ZNF148 mRNA were not affected (C). Overexpression of TOP2A mRNA (D) led to upregulation of ZNF148 mRNA (E); however, in Dicer-deficit HCT116 cells, levels of ZNF148 mRNA were not affected (F). $^{*}P < 0.05$; $^{**}P < 0.01$.

mRNA led to upregulation of TOP2A mRNA in HCT116 cells; however, levels remained unchanged in Dicer-deficit HCT116 cells, which further suggested that ZNF148 regulates TOP2A in the mediation of miRNAs. Luciferase assays demonstrated that ZNF148 regulated TOP2A expression through TOP2A 3′UTR. Conversely, TOP2A mRNA could regulate ZNF148 mRNA expression via ZNF148 3′UTR in HCT116 cells, but not in Dicer-deficient HCT116 cells. Moreover, we found that overexpression of ZNF148 mRNA and TOP2A mRNA caused significant downregulation of miR101, miR144, miR335, and miR365 in HCT116 cells, which indicates that these 4 miRNAs mediate ZNF148/TOP2A ceRNA modulation.

In HCT116 cells, knockdown of ZNF148 significantly promoted cell growth and overexpression of ZNF148 or
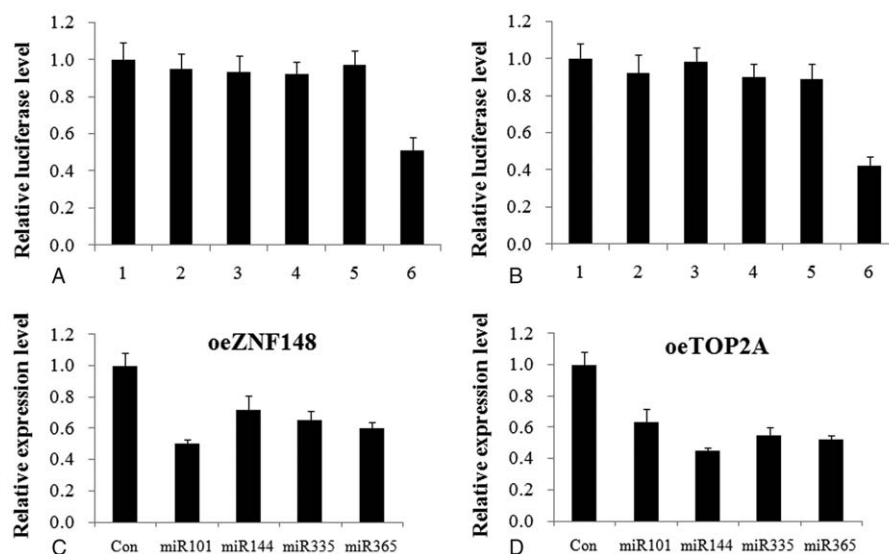

Figure 5. Mechanism of ceRNA regulation of ZNF148 and TOP2A expression. (A) Luciferase assays showed Luciferase activity decreased significantly in the "psiCHECK-2 + TOP2A 3′UTR + ZNF148 shRNA" group, indicating that ZNF148 regulates TOP2A expression through TOP2A 3′UTR. 1: psiCHECK-2 + Con; 2: psiCHECK-2 + shCon; 3: psiCHECK-2 + ZNF148 shRNA; 4: psiCHECK-2 + TOP2A 3′UTR + Con; 5: psiCHECK-2 + TOP2A 3′UTR + shCon; 6: psiCHECK-2 + TOP2A 3′UTR + ZNF148 shRNA. (B) Luciferase assays showed Luciferase activity decreased significantly in the "psiCHECK-2 + ZNF148 3′UTR + TOP2A shRNA" group, indicating that TOP2A regulates ZNF148 expression through ZNF148 3′UTR. 1: psiCHECK-2 + Con; 2: psiCHECK-2 + shCon; 3: psiCHECK-2 + TOP2A shRNA; 4: psiCHECK-2 + ZNF148 3′UTR + Con; 5: psiCHECK-2 + ZNF148 3′UTR + shCon; 6: psiCHECK-2 + ZNF148 3′UTR + TOP2A shRNA. (C) Overexpression of ZNF148 mRNA caused significant downregulation of miR101, miR144, miR335, and miR365 in the HCT116 cells. (D) Overexpression of TOP2A mRNA caused significant downregulation of miR101, miR144, miR335, and miR365. $^{*}P < 0.05$; $^{**}P < 0.01$.
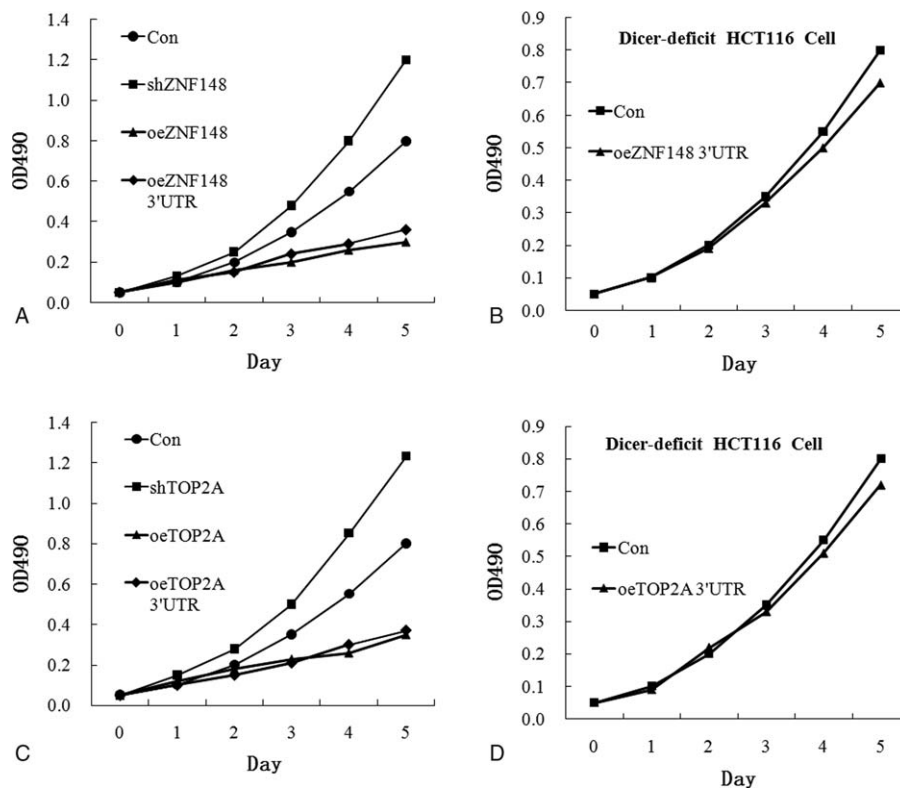
Figure 6. ZNF148 and TOP2A regulate cell proliferation by ceRNA regulation. (A) Cell proliferation curve of HCT116 cells transfected with shZNF148, oeZNF148, and oeZNF148 3′UTR. (B) Cell proliferation curve of Dicer deficit HCT116 cells transfected with oeZNF148 3′UTR. (C) Cell proliferation curve of HCT116 cells transfected with shTOP2A, oeTOP2A, and oeTOP2A 3′UTR. (D) Cell proliferation curve of Dicer deficit HCT116 cells transfected with oeTOP2A 3′UTR.

ZNF148 3′UTR inhibited cell proliferation. However, over-expression of ZNF148 3′UTR in Dicer-deficit HCT116 cells did not inhibit cell proliferation. We also found that the alteration of TOP2A and TOP2A 3′UTR affected the proliferation in HCT116 cells. Therefore, we could conclude that ZNF148 and TOP2A regulate each other via the ceRNA mechanism, which plays an important role in cell proliferation in CRC.

5. Conclusion

In summary, our study suggested that ZNF148 and TOP2A regulate each other in CRC through the ceRNA mechanism via miR101, miR144, miR335, and miR365. This mechanism also plays an important role in the regulation of cell proliferation in CRC. The ceRNA regulation between ZNF148 and TOP2A contributes to a better understanding of the pathogenesis of colorectal carcinoma and may facilitate the development of targeted therapies.

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