Hsa_circ_0009910 knockdown in HeLa cells increases miR-198 expression levels and decreases c-Met expression levels and cell viability

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Abstract. Cervical cancer (CC) is considered a public health problem. Circular RNAs (circRNAs) serve important roles in different types of cancer, including CC. However, the mechanisms used by circRNAs to facilitate CC progression are currently unclear. The present study analyzed the effects of hsa_circ_0009910 knockdown on microRNA (miRNA/miR)-198 and mesenchymal-epithelial transition factor (c-Met) expression levels and its impact on apoptosis and the viability of HeLa cells. Differentially expressed circRNAs in CC were identified using analysis of circRNA microarray data. Bioinformatics analysis was performed to predict circRNA-microRNA (miRNA) and miRNA-mRNA interactions. The knockdown of hsa_circ_0009910 in HeLa cells was performed using small interfering RNA and the expression levels of hsa_circ_0009910, miR-198 and c-Met were assessed using reverse transcription-quantitative PCR. The viability and apoptosis of HeLa cells were evaluated using MTT, neutral red uptake and ApoLive-Glo™ multiplex assays. Hsa_circ_0009910 was significantly upregulated in HeLa cells and the knockdown of hsa_circ_0009910 increased miRNA-198 expression levels, reduced c-Met expression levels and decreased cellular viability, but not apoptosis, in HeLa cells. Overall, these results indicated that hsa_circ_0009910 could act as a molecular sponge of miRNA-198 and contribute to the upregulation of c-Met expression levels. The hsa_circ_0009910/miRNA-198/c-Met interaction network affects the viability, but not apoptosis, of HeLa cells. Based on this mechanism, the present study suggests that hsa_circ_0009910 may be a promising biomarker for CC.

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Introduction

Cervical cancer (CC) is the fourth most common cancer in women worldwide and is considered a public health problem in developing countries (1). Although progress has been made in therapeutic methods, the overall survival of patients with CC remains unsatisfactory due to recurrence and metastasis (2). Therefore, it is important to identify novel diagnostic and prognostic biomarkers that allow the adequate management of patients (3). Approximately 90% of cases of CC are caused by long-term infection of high-risk human papillomavirus (HPV), including the HPV-16 and HPV-18 genotypes (4). In addition to viral infection, epigenetic factors (DNA methylation, non-coding RNAs and post-translational modifications of histones) are also related to the development of CC (5).

In terms of epigenetic contribution, non-coding RNAs (ncRNAs) serve key roles in CC (6). Circular RNAs (circRNAs) are the most recent addition to the group of endogenous ncRNAs (7). CircRNA transcripts are generated through the back-splicing of the precursor mRNA (8). Structurally, they present a covalently closed circle shape as the 5' and 3' ends are joined by a 3',5'-phosphodiester bond (9). CircRNAs exhibit tissue-specific expression (10) and are resistant to digestion by Ribonuclease R, therefore, they are more stable compared with their linear isoforms (11). CircRNAs are considered to be master regulators of gene expression because of their ability to modulate different mechanisms in cells, although these are currently not fully understood (12). Using high-throughput sequencing technologies and bioinformatics, numerous novel differentially expressed circRNAs (DECs) in CC cell lines and tissues have been identified (13). Previous

research indicates that these circular transcripts participate in the initiation and progression of CC through modulation of biological processes such as cell proliferation, migration, invasion, epithelial-mesenchymal transition (EMT), metastasis and apoptosis (14,15).

In all types of cancer, circRNAs act as competing endogenous RNAs (ceRNAs) by competitively binding microRNA (miRNA/miR) response elements (MREs) that are present at the 5' end of miRNAs (16). Mature miRNAs can regulate gene expression at the post-transcriptional level by targeting specific mRNAs. miRNAs can bind to the 3'-untranslated region of the mRNA, which consequently leads to mRNA downregulation mediated by the RNA-induced silencing complex (17). CircRNAs mainly function as miRNA sponges and inhibit the regulatory effects of the miRNA on their target mRNA (18), thus forming a circRNA/miRNA/mRNA regulatory network (19). This mechanism has been previously described in CC (15). For example, hsa_circ_0031288 exhibits high expression levels in cervical cancer cells, and it has been reported that it acts as a sponge for hsa-miR-139-3p and promotes increased expression levels of B cell lymphoma 6 (Bcl-6) mRNA. Hsa_circ_0031288/hsa-miR-139-3p/Bcl-6 affects the proliferation, migration and invasion of HeLa cells (20). Hsa_circ_0071474 expression levels are increased in cervical cancer cells and hsa_circ_0071474 has been reported to bind to miR-137 to promote Kruppel-like factor 12 (KLF12) mRNA upregulation. The hsa_circ_0071474/miR-137/KLF12 network is important in tumor proliferation (21).

Despite the aforementioned studies, the expression profiles and biological functions of numerous other circRNAs and their roles in the initiation and progression of CC are still unknown and need to be investigated to improve the current understanding of their underlying mechanisms in CC. The present study analyzed the effects of hsa_circ_0009910 knockdown on miR-198 and c-Met expressions levels, cell viability and apoptosis in HeLa cells.

Materials and methods

Differential expression analysis. The expression microarray data (accession no. GSE113696) were obtained from Gene Expression Omnibus (GEO; https://www.ncbi.nlm.nih.gov/geo/). The expression levels of circRNAs in five CC cell lines (HeLa, CaSki, SiHa, C-33A and SW756) were compared with circRNA expression in human cervical epithelial cells (HCerEpiC) to identify DECs. The circRNA microarray had been performed using the Arraystar Human circRNA Array (8x15K; Arraystar Inc.). The interactive online tool GEO2R (https://www.ncbi.nlm.nih.gov/geo/geo2r/) was used to perform differential expression analysis (22). The P-values were adjusted to reduce the false positive rate using the Benjamini and Hochberg's false discovery rate (FDR) method (23). Finally, the DECs were selected based on the cut-off values of logFC<-4.3 or >4.1 and FDR <0.1266.

Prediction of structure circRNAs. The genomic sequence and structural elements of hsa_circ_0009910 were predicted using the Circular RNA Interactome (https://circinteractome.nia.nih.gov/) (24) and Cancer-Specific CircRNA Database (https://gb.whu.edu.cn/CSCD/) (25).

Gene Expression Profiling Interactive Analysis (GEPIA). The GEPIA database (http://gepia.cancer-pku.cn/) (26) was utilized to analyze the mRNA expression level of mitofusin 2 (MFN2) and mesenchymal-epithelial transition factor (c-Met or MET) in 306 biopsies from patients with cervical squamous cell carcinoma and endocervical adenocarcinoma (CESC) and 13 with healthy tissue. Expression data were normalized and $\log_2$ transformed (TPM+1). $P<0.05$ was considered statistically significant.

Construction of endogenous competing RNA network. Due to potential circRNA-miRNA-mRNA-RNA-binding proteins (RBP) regulation, interaction networks were constructed for subsequent experimental analysis. The Circular RNA Interactome (24) was used to predict circRNA-miRNA and circRNA-RBP interactions; interactions with a score of <90% were excluded.

Interactions between miRNAs-mRNAs were established using the DIANA-TarBase v.8 software (version 8; http://carolina.imis.athena-innovation.gr/diana_tools/web/index.php?r=tarbasev8%2Findex) (27) and miRTarBase (http://mirtarbase.mbc.nctu.edu.tw/php/index.php) (28). To increase the prediction accuracy, target genes were selected using the following criteria: i) The prediction score was >0.443; ii) ≥1 original article must support the interaction; and iii) the article demonstrates the miRNA-mRNA interaction with experimental evidence from reporter assays, western blot or quantitative PCR. Finally, a circRNA-miRNA-mRNA interaction network was constructed by combining circRNA-miRNA, circRNA-RBP and miRNA-mRNA pairs.

Gene Ontology (GO) analysis. To predict the functional implications of mRNAs in the network, GO analysis was performed using PANTHER (http://www.pantherdb.org/) (29). According to the program, pathways with an FDR <0.05 were considered to indicate significantly enriched pathways.

Cell culture. The HaCaT immortalized human keratinocyte cell line and HPV-18 positive CC cell line (HeLa) were purchased from American Type Culture Collection. The cells were cultured in DMEM/F-12 (1:1) medium (Caisson Labs, Inc.) supplemented with 10% fetal bovine serum (PAA Laboratories GmbH; GE Healthcare), 100 U/ml penicillin and 100 μg/ml streptomycin (Caisson Labs, Inc.). The cells were cultured at 37˚C with 5% CO_2.

Transfection. To perform the hsa_circ_0009910 knockdown, a small interfering RNA (siRNA) targeting hsa_circ_0009910 (si-circ9910) and siRNA negative control (si-NC) were synthesized by Integrated DNA Technologies, Inc. (Table I). HeLa cells were seeded in 6-well plates at 80% confluence and subsequently transfected with si-circ9910 (50 nM) or siRNA negative control using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol, and harvested 48 h later for further analysis.

RNA extraction. Total RNA was extracted from HaCaT and HeLa cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The concentration of RNA was determined by spectrophotometry

Table I. Primer sequences.

A, RT-qPCR

Gene	Sequence (5'-3')
hsa_circ_0009910	F: AGGTTCTGGACGTCAAAGGTT
	R: TTGCATCGAGAGAAGAGCAGG
c-Met	F: TATTTCCCAGATCATCCATTGCA
	R: AATGTAGGACTGGTCCGTCAAAA
GAPDH	F: GACCCCTTCATTGACCTCAAC
	R: GTGGCAGTGATGGCATGGAC
miR-198	F: TCATTGGTCCAGAGGGGAGATAG
	R: GCAGGGTCCGAGGTATTC
RNU44	F: CCTGGATGATGATAAGCAAATG
	R: GTCAGTTAGAGCTAATTAAGACC

B, siRNA

Gene	Sequence (5'-3')
si-circ9910	AGCAGGGACAUUGCGCGGCCA
si-NC	CGUUAAUCGCGUAUAAUACGCGUA

F, forward; R, reverse; miR, microRNA; circ, circular; siRNA, small interference RNA; RNU44, small nucleolar RNA, C/D box 44; NC, negative control.

using a NanoDrop 2000c Spectrophotometer (NanoDrop Technologies; Thermo Fisher Scientific, Inc.).

Reverse transcription-qPCR (RT-qPCR). The expression levels of hsa_circ_0009910 and c-Met were analyzed using RT-qPCR using the CYBRFast™ 1-Step RT-qPCR Lo-ROX Kit (Tonbo™ Biosciences; Cytek® Biosciences) according to the manufacturer's protocol. The following thermocycling conditions were used for qPCR: Complementary DNA (cDNA) synthesis at 50˚C for 10 min, DNA polymerase activation at 95˚C for 2 min, 40 cycles of denaturation at 95˚C for 20 sec and annealing and extension at 60˚C for 30 sec. Reactions were performed using the QuantStudio™ 3 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.).

The expression level of miR-198 (accession no. MI0000240) was assessed using the TaqMan® MicroRNA Assay (Applied Biosystems; Thermo Fisher Scientific, Inc.). The miR-198 cDNA was obtained using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions and the reactions were performed using a BioRad T100™ Thermal Cycler (Bio-Rad Laboratories, Inc.). qPCR of miR-198 was performed using the TaqMan® Universal PCR Master Mix (Applied Biosystem; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions and were conducted using the QuantStudio 3 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). Data were normalized using GAPDH and small nucleolar RNA, C/D box 44 (RNU44; accession no. NR_002750) as internal controls and relative expression were calculated using the $2^{-\Delta\Delta Cq}$ method (30). Primer sequences are shown in Table I.

MTT assay. The MTT reduction assay was used to assess cell viability (31). HeLa cells (10,000 cells/well) were seeded in 96-well plates. The next day, cells were transfected with si-circ9910 for 48 h as aforementioned. After transfection, 20 μl of MTT solution (5 mg MTT/ml PBS) was added to each well and left to incubate for 3 h at 37˚C. At termination of the experiment, MTT was removed by aspiration and the cells were treated with 100 μl of dimethyl sulfoxide (DMSO) to dissolve the formazan crystals followed by gentle shaking of the microplate for 15 min. Absorbance was recorded at 570 nm using an Epoch Spectrophotometer (BioTek; Agilent Technologies, Inc.). The percentage of viability was calculated as follows: (Absorbance of experimental group/absorbance of NT group) x100%. The DMSO 5% and A23187 10 μM groups served as positive controls for all viability and apoptosis assays.

Neutral red uptake (NRU) assay. The NRU assay (cat. no. N4638; Sigma-Aldrich; Merck KGaA) was used to determine the accumulation of the neutral red dye in the lysosomes of viable cells (32). HeLa cells (10,000 cells/well) were seeded in 96-well plates. The next day, cells were transfected with si-circ9910 for 48 h as aforementioned. After transfection, cells were incubated for 2 h at 37˚C with 100 μl neutral red (40 μg neutral red/ml DMEM). At termination of the experiment, the neutral red solution was removed by aspiration and the cells were washed twice with 150 μl PBS. Then, 150 μl of destain solution was added to each well followed by gentle shaking of the microplate for 10 min. Absorbance was recorded at 540 nm using an Epoch Spectrophotometer (BioTek; Agilent Technologies, Inc.). Results were presented as a percentage of viability (calculated as aforementioned in the MTT assay).

Viability and apoptosis assay. To assess cell viability and apoptosis, the ApoLive-Glo™ Multiplex Assay (Promega Corporation) was used according to the manufacturer's instructions. HeLa cells were seeded in 96-well plates (10,000 cells/well). The next day, cells were transfected with si-circ9910 for 48 h as aforementioned. After transfection, 20 μl viability reagent was added to each well and mixed by orbital shaking at 300-500 rpm for ~30 sec at room temperature. Plates were incubated for 3 h at 37˚C and fluorescence measured at $400_{excitation}/505_{emission}$ nm to determine viability. Subsequently, 100 μl Caspase-Glo® 3/7 reagent was added to the wells and mixed briefly by orbital shaking at 300-500 rpm for ~30 sec at room temperature. Plates were then left for 3 h at 37˚C and luminescence was measured to determine apoptosis levels. Fluorescence and luminescence were measured using an Infinite M200 (Tecan Group, Ltd.) plate reader. Results were presented as a percentage of viability and apoptosis (calculated as aforementioned in the MTT assay).

Statistical analysis. All data were analyzed using the SigmaPlot (version 10.0; Systat Software Inc.) software. The results are

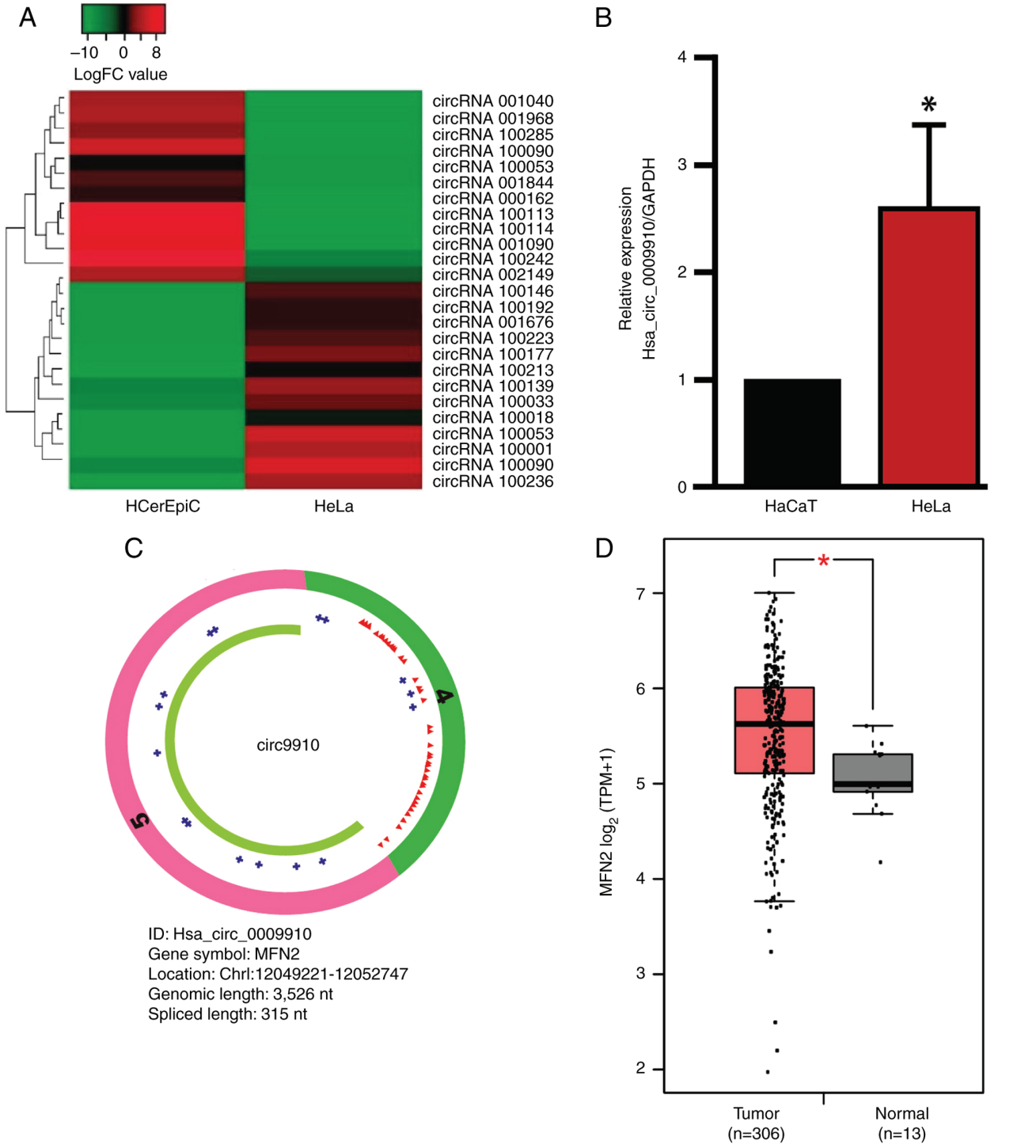

Figure 1. Hsa_circ_0009910 is upregulated in cervical cancer cells. (A) The heat map shows the expression of downregulated and upregulated circRNAs in HeLa cells and HCerEpiC. (B) Hsa_circ_0009910 expression levels in HaCaT and HeLa cells. (C) The hsa_circ_0009910 schematic shows exon 4 (green), exon 5 (pink), miRNA response elements (red triangles), RBP-binding sites (blue crosses) and an open reading frame (green line). (D) Comparison of the MFN2 expression in CESC compared with normal tissue using data retrieved from TCGA. The data are presented as the mean ± standard deviation and from at least three independent experiments where applicable. *P<0.05. circRNA, circular RNA; miRNA, microRNA; RBP, RNA-binding protein; HCerEpiC, human cervical epithelial cells; MFN2, mitofusin 2; nt, nucleotide; CESC, cervical squamous cell carcinoma and endocervical adenocarcinoma; TMP, transcripts per million.

presented as the mean ± standard deviation. Unpaired Student's t-test was used for comparative analysis between two groups and one-way analysis of variance followed by Dunnett's post hoc test was used for comparison among multiple groups. P<0.05 was considered to indicate a statistically significant difference.

Results

DECs in CC. To identify novel circRNAs involved in the initiation and progression of CC, the expression profiles of circRNAs in HCerEpiC compared with HeLa cells in a previously published circRNAs microarray dataset (accession no.

Table II. Basic characteristics of the 25 differentially expressed circRNAs in cervical cancer.

circRNA	logFC	False discovery rate	Expression	Type	Chromosome	Strand	Gene symbol
Hsa_circ_0000379	-10.45	0.044	Down	Intronic	12	+	PLBD1
Hsa_circRNA_001968	-10.45	0.044	Down	/	/	/	/
Ha_circ_100285	-10.81	0.044	Down	/	/	/	/
Hsa_circ_0008563	7.79	0.063	Up	Exonic	1	-	ECE1
Hsa_circ_0009910	7.22	0.063	Up	Exonic	1	+	MFN2
Hsa_circ_0000263	-7.8	0.063	Down	Exonic	10	+	TCONS_00017720
Hsa_circ_0012634	7.76	0.063	Up	Exonic	1	-	TMEM59
Hsa_circ_0013222	-7.23	0.063	Down	Exonic	1	-	GCLM
Hsa_circ_0000488	-7.67	0.078	Down	Intronic	13	-	DLEU2
Hsa_circ_100113	-6.02	0.078	Down	/	/	/	/
Hsa_circ_0011279	5.86	0.078	Up	Exonic	1	+	SERINC2
Hsa_circ_0011692	5.61	0.078	Up	Exonic	1	-	STK40
Hsa_circ_0009581	5.45	0.078	Up	Exonic	1	-	RERE
Hsa_circ_0005866	-5.6	0.078	Down	Exonic	1	-	RPA2
Hsa_circRNA_001844	-8.37	0.078	Down	/	/	/	/
Hsa_circ_0009189	6.65	0.078	Up	Exonic	1	+	SAMD11
Hsa_circ_0012417	7.09	0.082	Up	Exonic	1	-	EPS15
Hsa_circ_0011385	5.7	0.082	Up	Exonic	1	+	EIF3I
Hsa_circRNA_001090	-10.53	0.088	Down	/	/	/	/
Hsa_circ_0000069	5.65	0.116	Up	Exonic	1	-	STIL
Hsa_circ_0012107	6.01	0.116	Up	Exonic	1	+	ST3GAL3
Hsa_circ_0000423	6.04	0.116	Up	Exonic	12	-	PPP1R12A
Hsa_circRNA_100242	-4.77	0.123	Down	/	/	/	/
Hsa_circ_0001627	-4.21	0.126	Down	Intronic	6	-	BACH2
Hsa_circ_0009361	4.17	0.126	Up	Exonic	1	-	GNB1

circRNA, circular RNA; logFC, log-fold change; Down, downregulated; Up, upregulated; +, positive; -, negative; /, not applicable.

GSE113696) were analyzed. This analysis identified 25 DECs in HeLa cells with potential roles in CC. A heat map was used to demonstrate the expression patterns of the circRNAs identified in the HCerEpiC and HeLa cell lines (Fig. 1A). Within the top 25 DECs, 13 circRNAs were upregulated and 12 downregulated, based on the cut-off values of logFC <-4.3 or >4.1 and FDR <0.1266. The information of each circRNA was recorded (Table II) as follows: The parental gene from which each circRNA was derived, circRNA type, expression level and chromosomal location.

Hsa_circ_0009910 is upregulated in CC cells. In the circRNA microarray data, hsa_circ_0009910 (also known as circ9910) was identified as a DEC. To confirm this finding, the expression levels of hsa_circ_0009910 in HaCaT and HeLa cells were evaluated using RT-qPCR. The expression levels of hsa_circ_0009910 were significantly upregulated in HeLa cells compared with the HaCaT control cells (Fig. 1B), consistent with the data obtained from the circRNAs microarray. Hsa_circ_0009910 has 315 nucleotides and is generated from exon 4 and exon 5 of the MFN2 gene through back-splicing (33). Structurally, hsa_circ_0009910 contains multiple MREs and interacts with several RBPs (Fig. 1C). Additionally, the expression levels of the parental gene MFN2 in TCGA-retrieved data were assessed. MFN2 is significantly upregulated in CESC samples compared with normal cervical tissue (Fig. 1D). These results indicate that hsa_circ_0009910 is upregulated in CC cells.

Construction of the ceRNA regulatory network for hsa_circ_0009910. To identify potential functions of hsa_circ_0009910 in cancer, a circRNA-miRNA-mRNA interaction network mediated by hsa_circ_0009910 was constructed. CircRNA-miRNA interactions were predicted using the Circular RNA Interactome platform and miRNA-mRNA interactions were identified using the DIANA-TarBase v.8 and miRTarBase software. The prediction software demonstrated that hsa_circ_0009910 harbored 10 motifs that could interact with miRNAs, which could in turn regulate 135 mRNAs. In addition, hsa_circ_0009910 could interact with seven RBPs (Fig. S1).

As hsa_circ_0009910 could indirectly regulate 135 mRNAs, GO analysis was performed using the PANTHER classification system to gain insight into the biological processes that may be altered by hsa_circ_0009910 (Fig. S2). Hsa_miR_198 (miR-198) is a notable miRNA regulated by

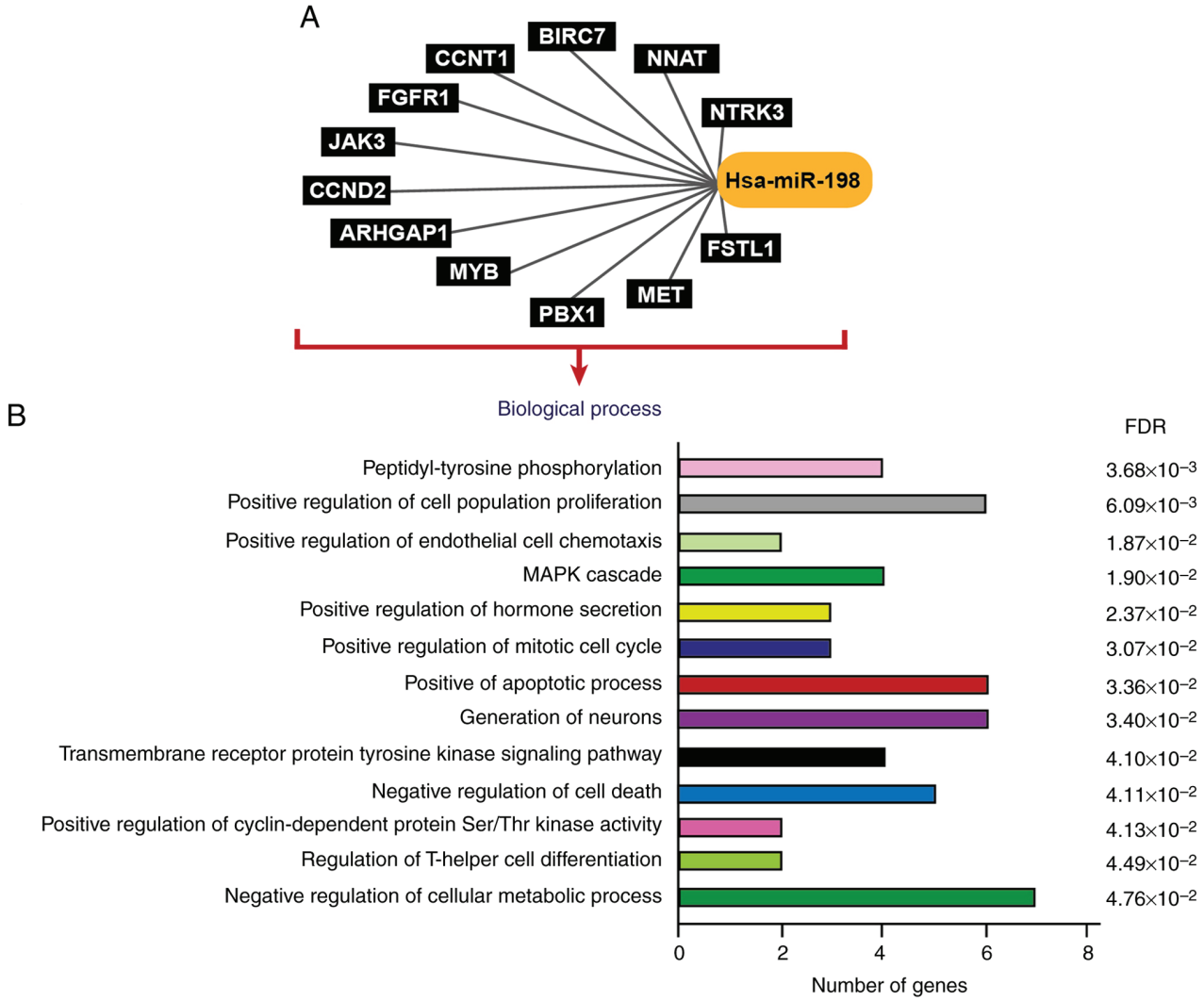

Figure 2. Probable target genes for miR-198 and the Gene Ontology biological process. (A) The interaction node with the 12 mRNAs potentially regulated by miR-198 and (B) the pathway enrichment analysis of these 12 mRNAs are shown. FDR <0.05 was considered to indicate a statistically significant difference. miR, microRNA; FDR, false discovery rate.

hsa_circ_0009910, due to the roles of miR-198 reported in prostate cancer, oral squamous cell carcinoma, colorectal cancer and glioblastoma (34-37). Numerous mRNAs were shown to be potentially regulated by hsa-miR-198 (Fig. 2A) which participated in several biological processes (Fig. 2B), including the 'positive regulation of cell population proliferation', the 'positive regulation of mitotic cell cycle' and the 'negative regulation of cell death' as well as other processes involved in the development of cancer. Thus, miR-198 was selected as a biologically relevant target of hsa_circ_0009910 for further analysis.

Hsa_circ_0009910 can act as a sponge for miR-198. To investigate the role of hsa_circ_0009910 in downstream gene expression and if it could serve as a miRNA sponge, hsa_circ_0009910 knockdown in HeLa cells with si-circ9910 was performed. The expression levels of hsa_circ_0009910 were reduced by ~95% compared with the siRNA negative control in HeLa cells (Fig. 3A). The region potential of binding between hsa_circ_0009910 and miR-198 was identified (Fig. 3B). Subsequently, the expression levels of miR-198 in HeLa cells with hsa_circ_0009910 knockdown were evaluated. Knockdown of hsa_circ_0009910 led to a notable increase in miR-198 expression levels in HeLa cells compared with the siRNA negative control (Fig. 3C). Additionally, the expression profiles of miR-198 in numerous normal tissues were evaluated using data from the miRTarBase database, which demonstrated that cervical tissue had the third highest expression levels of miR-198 of the 18 tissues assessed (Fig. S3).

c-Met is a potential target of hsa_circ_0009910/miR-198. c-Met was chosen as a target gene from those identified in the present study as previous studies have demonstrated its molecular interactions with miR-198 (38-40) and that it serves an important role in the development of CC (41). Rho GTPase activating protein 1 (ARHGAP1) and follistatin-like 1 (FSTL1) were not chosen for further investigation primarily as, to the best of our knowledge, there is no experimental evidence of its ARHGAP1 interaction with miR-198 and a relationship of mutual transcriptional regulation between FSTL1 and miR-198 has been reported (42,43). Therefore, only c-Met was further investigated in the present study. c-Met was shown to

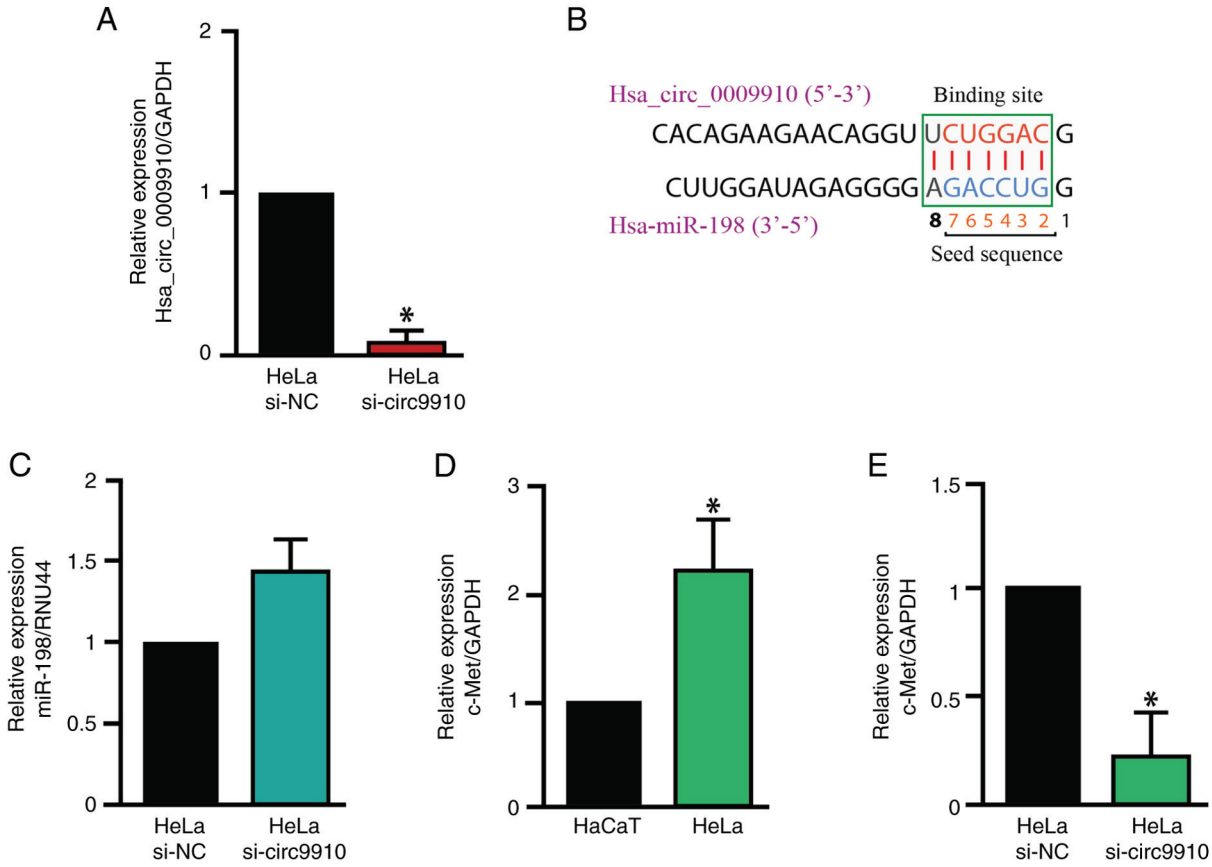

Figure 3. Hsa_circ_0009910 knockdown increases miR-198 and decreases c-Met mRNA expression levels. (A) Relative expression of hsa_circ_0009910 in HeLa cells in the HeLa si-circ9910 and HeLa si-NC groups. (B) Binding site of hsa_circ_0009910/miR-198, depicting the sequence of hsa_circ_0009910 (red letters) and the sequence of miR-198 (blue letters). The binding type is 7mer-m8. (C) Expression levels of miR-198 in HeLa si-circ9910 and HeLa si-NC. (D) Basal expression levels of c-Met in HaCaT and HeLa cells. (E) Expression levels of c-Met in HeLa si-circ9910 and HeLa si-NC. Data presented are from at least three independent experiments and expressed as the mean ± standard deviation. *P<0.05. circ, circular; miR, microRNA; si-circ9910, small interfering RNA against hsa_circ_0009910; NC, negative control.

be upregulated in CC using the TCGA database (Fig. S4) and the expression levels of c-Met were significantly increased in HeLa cells compared with that in HaCaT cells (Fig. 3D). Therefore, the influence of the hsa_circ_0009910/miR-198 axis on c-Met expression levels was evaluated. In this regard, hsa_circ_0009910 knockdown significantly decreased the expression levels of c-Met in HeLa cells (Fig. 3E).

Knockdown of hsa_circ_0009910 decreases viability and does not affect apoptosis in HeLa cells. To investigate the functional role of hsa_circ_0009910/miR-198/c-Met axis, hsa_circ_0009910 knockdown was performed in HeLa cells and the cell viability and apoptosis was analyzed. The MTT, NRU and ApoLive-Glo Multiplex assays indicated that the knockdown of hsa_circ_0009910 significantly decreased the viability of HeLa cells (Fig. 4A-C), meanwhile, apoptosis levels were not affected (Fig. 4D).

Knockdown of hsa_circ_0009910 does not affect the mRNA level of E6 and E7. Additionally, as HeLa cells were infected with HPV-18, and its E6 and E7 oncoproteins maintain the cancerous phenotype and prevent apoptosis, the effect of hsa_circ_0009910 knockdown on E6 and E7 expression was analyzed. Knockdown of hsa_circ_0009910 did not affect the mRNA level of E6 and E7 in HeLa cells (Fig. S5).

Discussion

CircRNAs are important molecules in the initiation and progression of different types of human cancer (44). CircRNAs regulate gene expression through different mechanisms, as they function as sponges for miRNAs, interact with RBPs, act as scaffolds for protein complexes, regulate gene transcription and certain circRNAs are translated into small proteins (45). In CC, a set of abnormally expressed circRNAs have been identified that may function as oncogenes (46,47) or tumor suppressors (48,49). However, research on the circRNAs in CC is currently in the early stages and the understanding of mechanisms used by circRNAs to promote cancer is limited.

In the present study, a potential regulatory network in HeLa constituting hsa_circ_0009910, miR-198 and c-Met cells was identified. Previous studies have reported several similar regulatory networks in certain types of cancer (50,51) including CC (52,53). A regulatory network in CC controlled by hsa_circ_0001400 has been previously described, which participates in the pathogenesis of CC by sponging hsa-miR-326, resulting in Akt mRNA upregulation, a key molecule in the PI3K-Akt signaling pathway. The hsa_circ_0001400/miR-326/Akt network exerts its oncogenic effect by maintaining the cell cycle active, promoting migration and inhibiting cancer cell apoptosis (54). By contrast, hsa_circ_0132980, also known

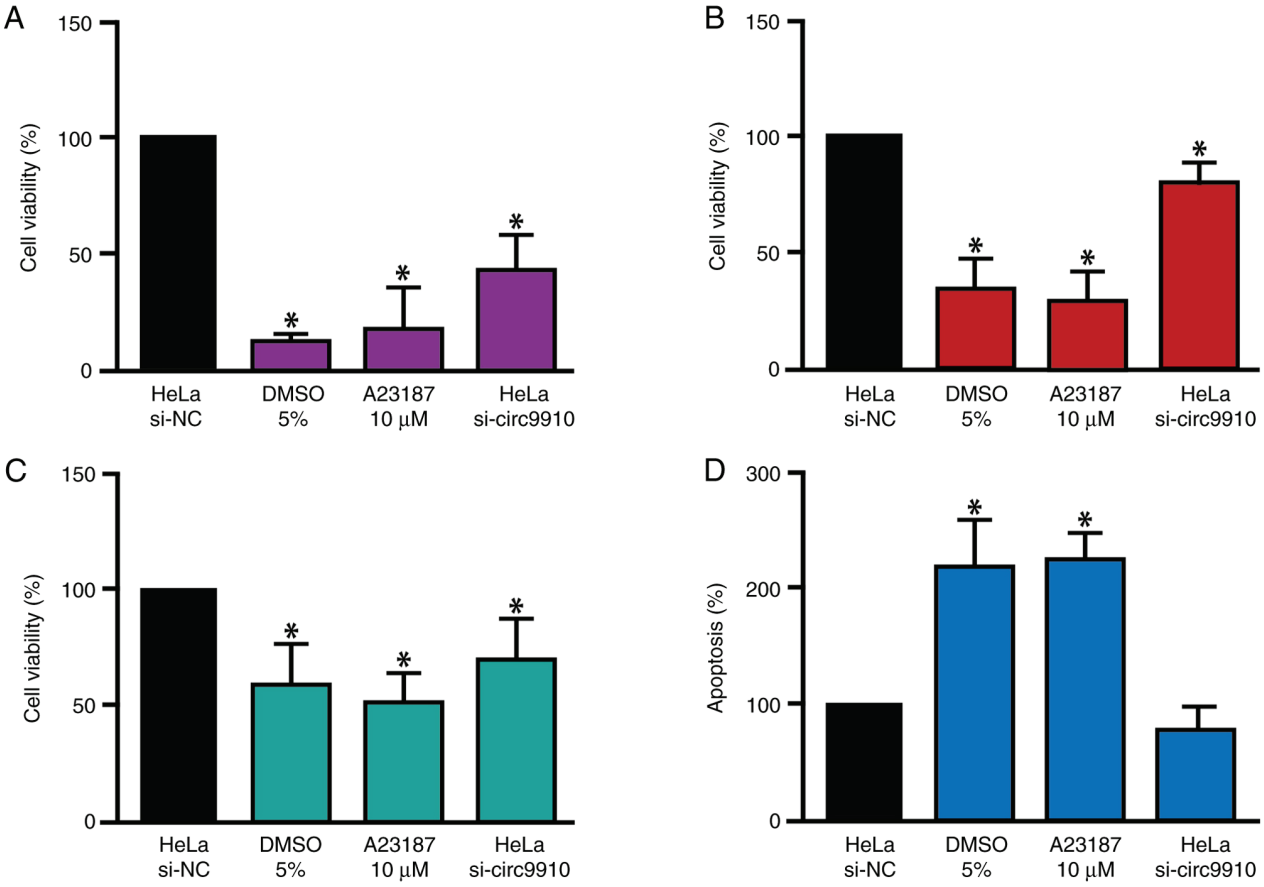

Figure 4. Knockdown of hsa_circ_0009910 affects HeLa cell viability but not apoptosis. The viability of HeLa cells with hsa_circ_0009910 knockdown was assessed with the (A) MTT, (B) neutral red uptake and (C) ApoLive™ assay. (D) Apoptosis of HeLa cells were assessed using the ApoLive assay. The DMSO 5% and A23187 10 μM groups served as positive controls. All data presented are from at least three independent experiments and are expressed as the mean ± standard deviation. *P<0.05. circ, circular; DMSO, dimethyl sulfoxide.

as circSLC26A4, is an oncogenic circRNA that acts as a molecular sponge of miR-1287-5p to promote an increase in homeobox genes A7 (HOXA7) mRNA levels. The hsa_circ_0132980/miR-1287-5p/HOXA7 network facilitates CC progression by modulating the proliferation, invasion and tumor growth of cancer cells (55). Another example of a regulatory network includes hsa_circ_0058514, also known as circAGFG1, which functions as a miR-370-3p sponge and promotes an increase in raf-1 proto-oncogene, serine/threonine kinase (RAF1) mRNA expression levels. RAF1 serves a key role in the phosphorylation and activation of MEK1/2 and ERK1/2 proteins. The hsa_circ_0058514/miR-370-3p/RAF1 network promotes the proliferation and migration of CC cells (46). Furthermore, as hsa_circ_0009910 has binding sites for numerous miRNAs, it participates in other regulatory networks. In osteosarcoma, hsa_circ_0009910 functions as a sponge of miR-449a and consequently promotes the upregulation of IL-6 receptor mRNA, which is involved in the regulation of JAK1/STAT3 signaling pathway (56). In acute myeloid leukemia, hsa_circ_0009910 can bind to miR-5195-3p to promote growth factor receptor-bound protein 10 mRNA upregulation, and consequently influences the proliferation and apoptosis of cancer cells (57). Over the last decade, the importance of regulatory networks between circRNAs, miRNAs and their target mRNAs has been evidenced by an increasing role in numerous human diseases and cellular processes.

The present study demonstrated that hsa_circ_0009910 knockdown increased the expression levels of miR-198 and decreased the expression levels of c-Met mRNA in HeLa cells. Hsa_circ_0009910 may function as a sponge of miR-198, which results in the decreased of c-Met mRNA expression levels. To the best of our knowledge, there has been no evidence previously that miR-198 is sponged by hsa_circ_0009910, although it has been reported that hsa_circ_0009910 functions as a sponge for miR-145 (33,58), miR-335-5p (59), miR-34a-5p (60) and miR-20a-5p (61). In addition, this exonic circRNA is primarily localized in the cytoplasm of cancer cells, which supports its function as a sponge for miRNAs (60). By contrast, it has been reported that c-Met mRNA is a target of miR-198 in esophageal cancer (38), hepatocellular carcinoma (39), ovarian cancer (40) and osteosarcoma (62). c-Met, also known as mesenchymal-epithelial transition factor or hepatocyte growth factor receptor, is a receptor tyrosine kinase located on the cell membrane of epithelial and endothelial cells (63). c-Met activation promotes the activation of signaling pathways that are related to biological processes such as cell proliferation, survival, apoptosis, migration and invasion (64).

In addition to the potential regulation of miR-198 by hsa_circ_0009910, the previous research suggests that the regulation of this miRNA is more complex. Studies have reported that lncSChLAP1 (65), circ0004390 (40), circRNA LPAR3 (38), circ_0002060 (66), circ_ERBB2 (67), circRNA

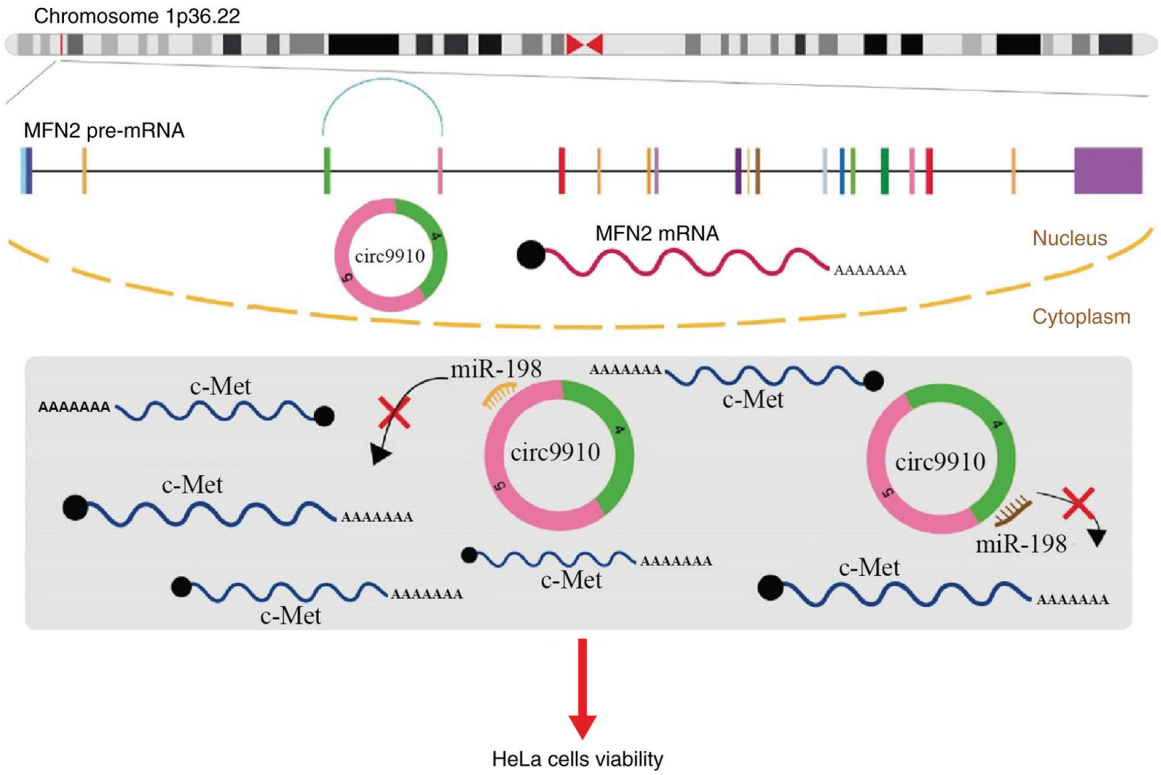

Figure 5. Proposed hsa_circ_0009910/miR-198/c-Met regulatory network. Hsa_circ_0009910 promotes cell viability by directly regulating miR-198 and indirectly c-Met mRNA expression levels. circ, circular; MFN2, mitofusin 2; miR, microRNA; circ9910, hsa_circ_0009910.

AKT3 (68) and circ_0005198 (69) can regulate miR-198 expression levels in cancer.

Similarly, post-transcriptional regulation of c-Met mRNA is complex. In addition to the miR-198/c-Met interaction, it has been reported that c-Met can be regulated by other miRNAs in CC, including to miR-1 (70), miR-23b-3p (71), miR-454-3p (72) and miR-876-5p (73). This may explain why c-Met expression levels in hsa_circ_0009910 knockdown cells were notably decreased. Therefore, the abnormal expression and function of c-Met results from dysregulation of a set of miRNAs, and these miRNAs in turn may be regulated by long ncRNAs or circRNAs. This highlights the importance of further research into regulatory networks in CC.

The knockdown of hsa_circ_0009910 decreases cell viability, potentially through the modulation of the miR-198/c-Met axis. This result could be explained based by two hypotheses. First, hsa_circ_0009910 knockdown increases expression levels of miR-198, which may allow this miR-198 to function as a negative regulator of its target mRNAs, including the c-Met oncogene. Second, the decrease of c-Met expression levels mRNA may result in the disruption of certain signaling pathways that control cellular proliferation and cell viability such as the PI3K/Akt, Ras/MAPK, JAK/STAT, Wnt/β-catenin, FAK/Src and NF-κB pathways (63).

By contrast, hsa_circ_0009910 knockdown did not increase in HeLa cell apoptosis. However, it has been reported in acute myeloid leukemia and osteosarcoma that the knockdown of hsa_circ_0009910 decreases Bcl-2 expression levels and increases Bax expression levels, which are anti- and pro-apoptosis proteins, respectively (56,57,61). Furthermore, in chronic myeloid leukemia, the knockdown of hsa_circ_0009910 promotes the activation of caspase-3 (60). By contrast, the circRNA_0000285 (74), circRNA_0001400 (54) and circ-ATAD1 (75) positively regulate apoptosis in CC cells. These data suggest that hsa_circ_0009910-mediated regulation of apoptosis may be cell-type specific and that in HeLa cells, apoptosis is modulated by mechanisms that do not include hsa_circ_0009910, or at least not under the experimental conditions used in the present study. Additionally, HeLa cells are infected with HPV-18, a highly oncogenic HPV (76). The E6 and E7 oncoproteins maintain the cancerous phenotype in HeLa cells and experimental evidence suggests that the elimination of E6 and E7 are necessary events to induce apoptosis in HeLa cells (77). Certain apoptosis-inducing agents, such as N-benzylcinnamide (PT-3), in HeLa indirectly cause a decrease in E6 or E7 oncoprotein expression levels (78). This may explain why apoptosis did not increase in the present model as, although the expression levels of hsa_circ_0009910 were decreased, E6 and E7 of HPV-18 expression and activity may have persisted.

Due to their function and regulatory activity in different types of cancer, including CC, hsa_circ_0009910 (58), miR-198 (79) and c-Met (41,71) may be considered biomarkers or therapeutic targets. A challenge in CC continues to be the identification of biomarkers for early diagnosis in precancerous lesions (80). In this sense, hsa_circ_0009910, miR-198 and c-Met may be markers for the early diagnosis of CC in the future. However, further investigations using precancerous cervical lesion samples and animal models are needed to assess their potential use as biomarkers. A limitation of the present study is that the interaction between hsa_circ_0009910 and miR-198 was not demonstrated, and this would be necessary to confirm that miR-198 is sponged by hsa_circ_0009910.

To summarize the present findings, hsa_circ_0009910 functioned as a sponge for miR-198 and promoted an increase in c-Met expression levels, and as this regulatory network decreased the cell viability of HeLa cells (Fig. 5). Taken together, these results indicate that hsa_circ_0009910 could be a molecular sponge of miR-198 and contribute to the upregulation of c-Met expression levels. The hsa_circ_0009910/miR-198/c-Met interaction network affects cell viability but not apoptosis in HeLa cells. Based on this mechanism, hsa_circ_0009910 may be a promising biomarker for CC.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

BXTM and JLL designed and performed the experiments. AOS, GFT, YCC and DHS conceived and designed the study. BXTM and DHS confirm the authenticity of all the raw data. BXTM, YCC, JLL and DHS wrote the manuscript. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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