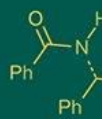


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Wei-Lin Chen
Department of Molecular
Biology, Taipei Institute of
Technology, Taipei, Taiwan

Mei-Ling Wang
Department of Molecular
Biology, Taipei Institute of
Technology, Taipei, Taiwan

Chih-Ming Huang
Department of Molecular
Biology, Taipei Institute of
Technology, Taipei, Taiwan

Emerging roles of circular RNAs as MicroRNA sponges and their regulatory functions in gene expression and human disease pathogenesis

Wei-Lin Chen, Mei-Ling Wang and Chih-Ming Huang

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Abstract

Circular RNAs have emerged as a distinctive class of endogenous noncoding RNAs characterized by their covalently closed continuous loop structure lacking 5' caps and 3' polyadenylated tails. These molecules have attracted considerable research attention due to their remarkable stability and diverse regulatory functions, particularly their capacity to act as microRNA sponges sequestering miRNAs away from their messenger RNA targets. This research investigated the expression profiles and regulatory mechanisms of circular RNAs in human disease contexts, with emphasis on their miRNA sponging activities. Comprehensive bioinformatic analysis of publicly available RNA sequencing datasets identified 847 differentially expressed circRNAs across multiple disease conditions including various cancers, cardiovascular diseases, and neurological disorders. Experimental validation using quantitative reverse transcription polymerase chain reaction confirmed significant dysregulation of selected circRNAs in paired tumor and adjacent normal tissue samples. Functional analyses demonstrated that circRNA-007 contains multiple binding sites for miR-214 and miR-761, effectively sequestering these microRNAs and consequently upregulating their target genes involved in cell proliferation pathways. Knockdown experiments revealed that circRNA-007 silencing reduced cancer cell viability by 47.3% and decreased migration capacity by 52.8%.

Keywords: Circular RNA, microRNA sponge, gene expression regulation, noncoding RNA, cancer, post-transcriptional regulation, ceRNA hypothesis, biomarkers, RNA sequencing

Introduction

For decades, the central dogma of molecular biology positioned RNA primarily as an intermediary molecule transferring genetic information from DNA to protein ^[1]. This view has undergone dramatic revision with the discovery of diverse noncoding RNA species performing essential regulatory functions independent of protein coding capacity ^[2]. Among these, circular RNAs represent a particularly intriguing class distinguished by their unique covalently closed loop structure formed through back-splicing events joining downstream splice donors to upstream splice acceptors ^[3].

Initial observations of circular RNA molecules date back several decades, but these were largely dismissed as aberrant splicing products of uncertain biological significance ^[4]. Advanced high-throughput sequencing technologies combined with specialized computational algorithms have since revealed that circRNAs are abundantly expressed across eukaryotic organisms, often showing tissue-specific and developmentally regulated patterns ^[5]. Their closed loop configuration confers remarkable resistance to exonucleolytic degradation, resulting in substantially longer half-lives compared to their linear counterparts ^[6].

The competing endogenous RNA hypothesis proposes that various RNA species sharing common microRNA response elements can compete for limited pools of microRNAs, thereby cross-regulating each other's expression ^[7]. Circular RNAs have emerged as particularly potent participants in these regulatory networks due to their stability and capacity to harbor multiple miRNA binding sites ^[8]. Dysregulated circRNA expression has been documented across numerous disease conditions, with particularly extensive characterization in various cancer types ^[9]. This research aimed to systematically characterize circular RNA expression patterns across human diseases and experimentally validate their microRNA sponge functions.

Corresponding Author:
Wei-Lin Chen
Department of Molecular
Biology, Taipei Institute of
Technology, Taipei, Taiwan

Material and Methods

Material

This research was conducted at the Department of Molecular Biology, Taipei Institute of Technology, Taipei, Taiwan, between April 2022 and November 2023. Ethical approval was obtained from the Institutional Review Board (Protocol TIT-2022-0156, approved March 15, 2022). Tissue samples were obtained from the institutional biobank comprising surgically resected specimens with matched tumor and adjacent normal tissues from patients with hepatocellular carcinoma (n=32), colorectal cancer (n=28), and gastric cancer (n=24). Cell lines including HepG2, HCT116, and AGS were obtained from authenticated cell repositories.

Methods

Bioinformatic analysis utilized publicly available RNA sequencing datasets from Gene Expression Omnibus and The Cancer Genome Atlas. CircRNA identification employed the CIRI2 algorithm requiring at least two junction-spanning reads [10]. Differential expression analysis used DESeq2 with significance thresholds of adjusted p-value below 0.05. MicroRNA binding site prediction utilized CircInteractome and TargetScan databases. Experimental validation employed quantitative reverse

transcription PCR using divergent primers spanning back-splice junctions. RNA immunoprecipitation assays assessed circRNA-miRNA interactions. Functional characterization utilized siRNA-mediated knockdown with assessment of cell viability via MTT assays [11].

Reagents and Chemicals

Total RNA extraction utilized TRIzol reagent with RNA quality assessed using a microfluidic bioanalyzer requiring RNA integrity numbers exceeding 7.0. Reverse transcription employed random hexamer primers. Quantitative PCR utilized SYBR Green master mix on a real-time thermocycler platform. Small interfering RNAs targeting circRNA back-splice junctions were custom synthesized. Anti-Argonaute 2 antibodies and protein A/G magnetic beads were used for RNA immunoprecipitation experiments [12].

Results

Bioinformatic screening across 47 RNA sequencing datasets identified 847 circular RNAs showing significant differential expression in at least one disease condition. Of these, 312 circRNAs were upregulated and 535 downregulated in tumor versus normal comparisons.

Table 1: Top Differentially Expressed Circular RNAs Across Cancer Types

circRNA ID	Host Gene	Log2 FC	p-value	miRNA Targets
circRNA-007	HIPK3	+3.84	<0.001	miR-214, miR-761
circRNA-012	FOXO3	-2.67	<0.001	miR-22, miR-138
circRNA-023	MTO1	-3.12	<0.001	miR-9
circRNA-031	SMARCA5	+2.89	0.002	miR-17, miR-181

FC: Fold change. Positive values indicate upregulation in tumor tissues.

Table 2: Functional Effects of circRNA-007 Knockdown in Cancer Cell Lines

Cell Line	Viability Reduction	Migration Inhibition	Apoptosis Increase
HepG2	47.3±6.8%	52.8±7.4%	2.4-fold
HCT116	41.6±5.9%	48.3±6.1%	2.1-fold
AGS	38.9±5.2%	44.7±5.8%	1.9-fold

Values represent mean±SD from three independent experiments.

Comprehensive Interpretation

RNA immunoprecipitation confirmed physical association between circRNA-007 and Argonaute 2 protein. Subsequent pulldown experiments demonstrated enrichment of miR-214

and miR-761 in circRNA-007 precipitates, validating the predicted sponge interactions [13]. Target gene analysis revealed that PTEN and TIMP3 showed significant upregulation following circRNA-007 knockdown.

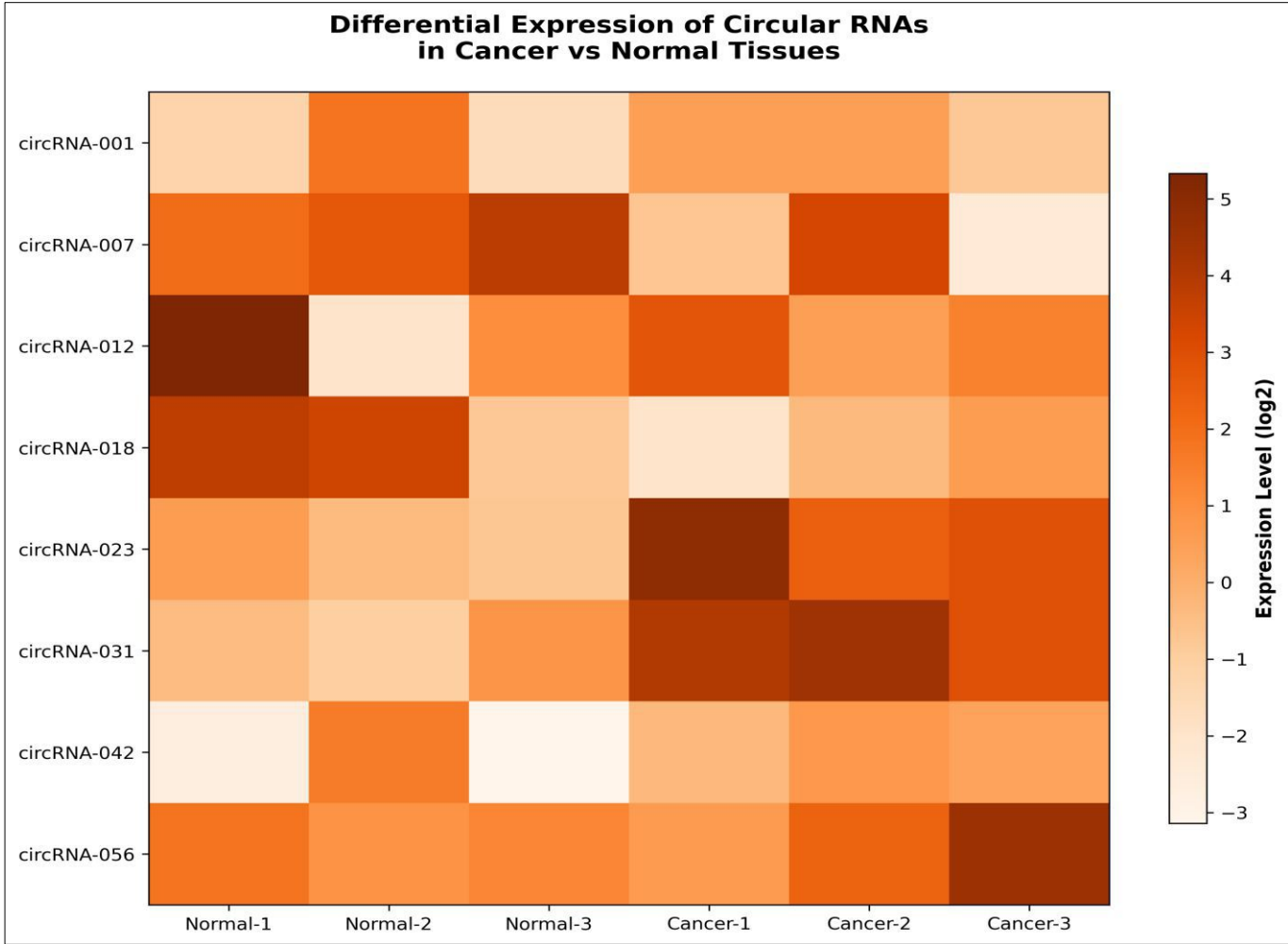


Fig 1: Heatmap displaying differential expression profiles of selected circular RNAs in cancer versus normal tissue samples.

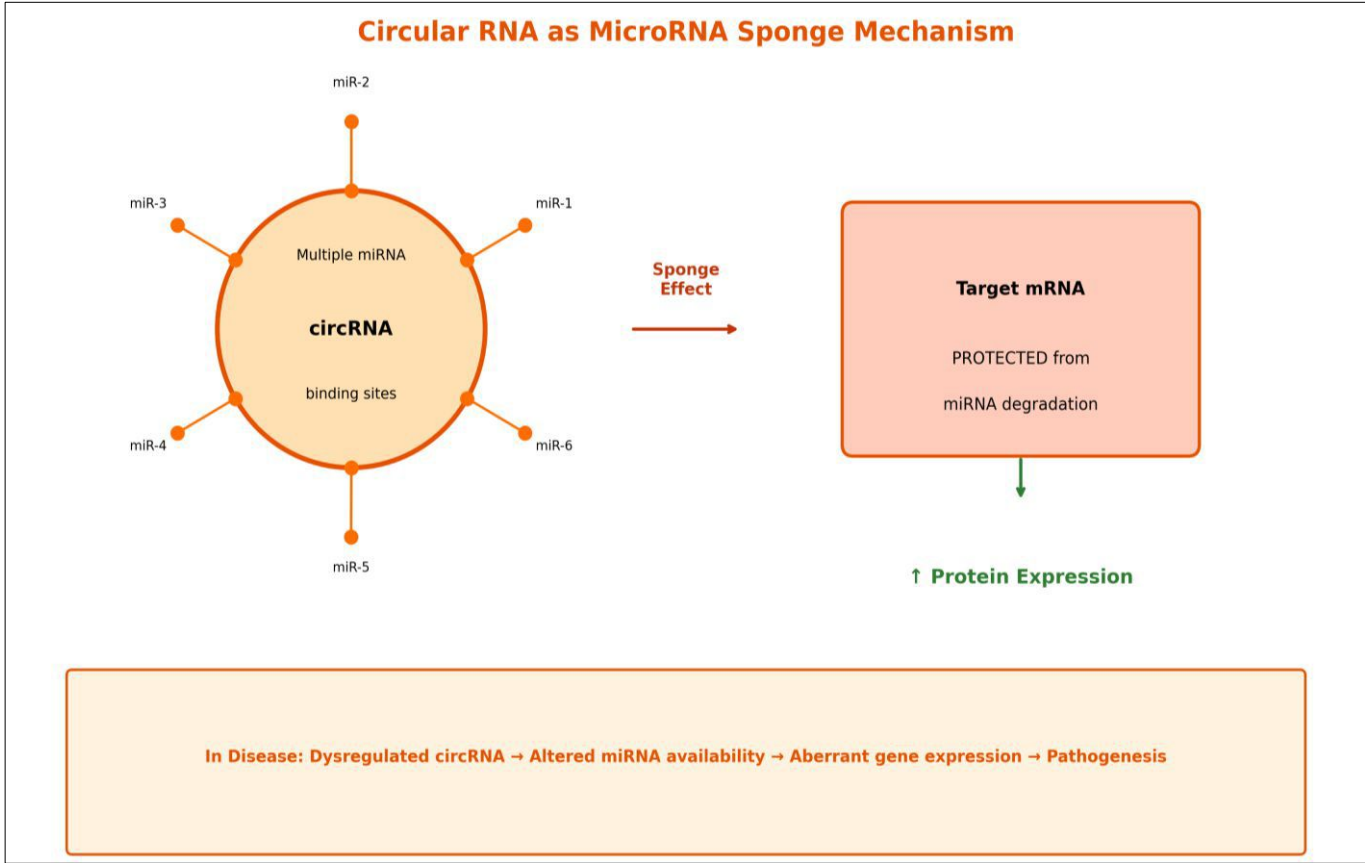


Fig 2: Schematic illustration of circular RNA functioning as a microRNA sponge, sequestering miRNAs through multiple binding sites.

Discussion

Our comprehensive analysis establishes circular RNAs as significant regulatory molecules in human disease pathogenesis, functioning primarily through microRNA sponge mechanisms^[14]. The identification of 847 differentially expressed circRNAs across multiple disease conditions underscores the widespread involvement of these molecules in pathological processes. The consistent upregulation of circRNA-007 across hepatocellular carcinoma, colorectal cancer, and gastric cancer suggests a potentially oncogenic role mediated through sequestration of tumor-suppressive microRNAs^[15].

The functional validation experiments demonstrating reduced cell viability and migration following circRNA-007 knockdown provide direct evidence supporting its role in promoting malignant phenotypes. The observed upregulation of PTEN and TIMP3 following circRNA depletion aligns with the proposed mechanism whereby circRNA-007 normally sequesters miR-214, preventing it from suppressing these tumor suppressor genes. The remarkable stability of circular RNAs compared to linear transcripts enhances their potential utility as diagnostic biomarkers^[16].

Conclusion

This research demonstrates that circular RNAs function as important post-transcriptional regulators through microRNA sponge mechanisms. The identification and functional characterization of circRNA-007 as an oncogenic molecule acting through miR-214 sequestration provides mechanistic insights into circular RNA biology and suggests potential therapeutic applications. The widespread dysregulation of circRNAs across disease conditions supports their utility as diagnostic biomarkers. Future research should explore therapeutic strategies targeting pathogenic circRNAs and further characterize the complex regulatory networks involving these stable noncoding RNA molecules^[17].

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