

Original Article

Comprehensive analysis of differentially expressed non-coding RNAs and mRNAs in gastric cancer cells under hypoxic conditions

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Abstract: Hypoxia commonly occurs in solid cancers, especially in gastric cancer due to its rapid growth. The ability of gastric cancer cells to survive and progress under hypoxic conditions has been known for decades, but the mechanisms underlying this characteristic remain poorly understood. As cancer cells undergo changes in their genetic profile under certain conditions, we investigated the expression profile of non-coding RNAs (circRNAs, lncRNAs, and miRNAs) and mRNAs in gastric cancer MKN-28 cells under hypoxic conditions via sequencing and subsequent bioinformatic analyses. In addition, quantitative reverse transcription polymerase chain reaction (qRT-PCR) was used to validate the results. We identified a number of significantly differentially expressed circRNAs, lncRNAs, miRNAs, and mRNAs in hypoxia-exposed MKN-28 cells relative to the normoxia control, and results of qRT-PCR were consistent with sequencing data. Pathway enrichment analyses revealed the principal functions of the significantly deregulated genes. Furthermore, examination of co-expression and competing endogenous RNA (ceRNAs) networks illustrated the complex regulatory pathways among non-coding RNAs and mRNAs, implicating these pathways in gastric cancer. In conclusion, our findings provide a novel perspective on non-coding RNAs and mRNAs and lay the foundation for future research on the potential roles of non-coding RNAs in gastric cancer under hypoxic conditions.

Keywords: Non-coding RNAs, mRNAs, gastric cancer, hypoxia

Introduction

While hypoxia is technically defined as a reduced level of molecular oxygen, this condition can be viewed simplistically as an imbalance between oxygen supply and demand. Hypoxia is a common phenomenon in solid cancers, including gastric cancer due to its rapid growth [1-7]. Tumor hypoxia exerts several effects on tumor biology. Hypoxia-inducible factors (HIFs) are key transcription factors that allow cancer cells to survive under hypoxic conditions [7-9]. HIFs are composed of the oxygen-sensitive subunit HIF-1/2 α and the stable HIF-1 β subunit [10-13]. Clinically, hypoxia and the expression of HIF-1 and HIF-2 are associated with increased distant metastasis and poor survival in a variety of tumor types [14-16]. Metastatic disease is the leading cause of cancer-related deaths and requires critical interac-

tions between tumor cells and the microenvironment. Accumulating evidence demonstrates that hypoxia is a potent factor that promotes the metastatic progression of gastric cancer [2, 17, 18].

Previous studies have documented that only 10-20% of transcripts are protein-coding RNAs, and the majority of the human genome is thus transcribed into non-coding RNAs [1, 19]. MicroRNAs (miRNAs) are 22-25 nucleotide single-stranded non-coding RNAs that directly bind to the 3'-untranslated regions (3'-UTRs) of target mRNAs, leading to mRNA degradation or translational suppression. During tumor development, miRNAs play vital roles by modulating the expression of oncogenes and tumor suppressors or by directly exerting their functions as oncogenes or tumor suppressors. Long non-coding RNAs (lncRNA), which are longer than

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Table 1. PCR primers for selected genes

Gene name	Forward primer	Reverse primer
<i>ADM</i>	ATGAAGCTGGTTTCCGTCG	GACATCCGCAGTTCCTCTT
<i>AKAP12</i>	GAGATGGCTACTAAGTCAGCGG	CAGTGGGTTGTGTAGCTCTTC
<i>ANGPTL4</i>	GTCCACCGACCTCCCGTTA	CCTCATGGTCTAGGTGCTTGT
<i>BHLHE40</i>	ATCCAGCGGACTTTCGCTC	TAATTGCGCCGATCCTTTCTC
<i>CA9</i>	GGATCTACCTACTGTTGAGGCT	CATAGCGCCAATGACTCTGGT
<i>ERRFI1</i>	CTGGAGCAGTCGCAGTGAG	GCCATTCATCGGAGCAGATTTG
<i>FIBCD1</i>	GTCCTCCTAAGCGGACAGC	CCCCGGAAGAAGTTCACGG
<i>NDRG1</i>	CTCCTGCAAGAGTTTGATGTCC	TCATGCCGATGTCATGGTAGG
<i>PPFIA4</i>	CTCTGCGGATGTTGTCTCCC	ATGCTGCCACTGGTTACACG
<i>CALB2</i>	ACTTTGACGCAGACGGAAATG	GAAGTCTCTTCGGTTGGCAG
<i>SLC26A9</i>	CTGGCCCCAGAGTCGAAATTC	CCTGAGCACCGAAATCAGGAT
<i>IGFBP3</i>	AGAGCACAGATACCCAGAATC	GGTGATTCAGTGTCTTCCATT

200 nt, play an important role in tumorigenesis and tumor progression by regulating gene expression via cis and/or trans mechanisms [20, 21]. Recently, a novel class of RNAs, termed circular RNAs (circRNA), has been found to be widely expressed across eukaryotic organisms, including *Homo sapiens* and *Mus musculus* [22, 23] and to function as genetic regulatory molecules. Previously, the circRNA CiRS-7 was found to be highly expressed in neuronal tissues and to bind and inhibit the function of miR-7 [24-26].

Overall, the mechanisms underlying the role of non-coding RNAs in the development of gastric cancer cells in hypoxic microenvironments remain elusive. Accordingly, we investigated the differential expression of circRNA, lncRNA, miRNA, and mRNA in gastric cancer MKN-28 cells under hypoxic conditions. Subsequently, we not only determined the associated pathways and gene ontology of these molecules but also delineated the comprehensive functional landscape of the coding-noncoding co-expression and competing endogenous (ceRNAs) networks. Our findings will contribute to our understanding of the novel mechanism of gastric cancer development in hypoxic microenvironments and illuminate potential new therapeutic targets.

Materials and methods

Cell culture

The gastric cancer cell line MKN-28 obtained from the Shanghai Digestive Surgery Institute

(Shanghai, China) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) under hypoxic conditions (1% oxygen) in an incubator (Forma Series II 3110 Water Jacketed CO₂ Incubator, Thermo Scientific).

RNA sequencing

For RNA sequencing, total RNA was extracted from cells after hypoxia exposure (48 h) using TRIzol (Invitrogen) according to the manufacturer's instruc-

tions. The TruSeq RNA sample preparation kit (Illumina) was used to generate poly-A-selected, indexed cDNA libraries according to the manufacturer's instructions. Subsequently, sequencing libraries were generated according to the manufacturer's recommendations. The libraries were sequenced on an Illumina HiSeq 2500 platform, and 100-bp paired-end reads were generated. Quality control of the obtained reads was performed using the FastQC suite with default parameters.

Western blot analysis

Cells were collected for protein extraction after hypoxia/normoxia treatment. Total protein was extracted with RIPA buffer (Solarbio) in the presence of a protease inhibitor cocktail (Roche Applied Science). Western blotting was performed according to the standard protocol. The primary antibodies were GAPDH (Cell Signaling Technology), HIF-1 α (Abcam), CA9 (ProteinTech), AKAP12 (ProteinTech), CALB2 (ProteinTech), SLC26A9 (Abcam), and PPFIA4 (Abcam).

qRT-PCR

Total RNA was isolated using the Trizol reagent (Invitrogen) and converted to cDNA with 1 mg RNA using a reverse transcription kit (TOYOBO). SYBR Green reagent (Applied Biosystems) was used for qRT-PCR to analyze mRNA expression. GAPDH was used as an endogenous control for normalization. The $\Delta\Delta C_t$ method was used to quantify relative gene expression. The PCR primers that were designed for specific genes and used in our study are listed in **Table 1**.

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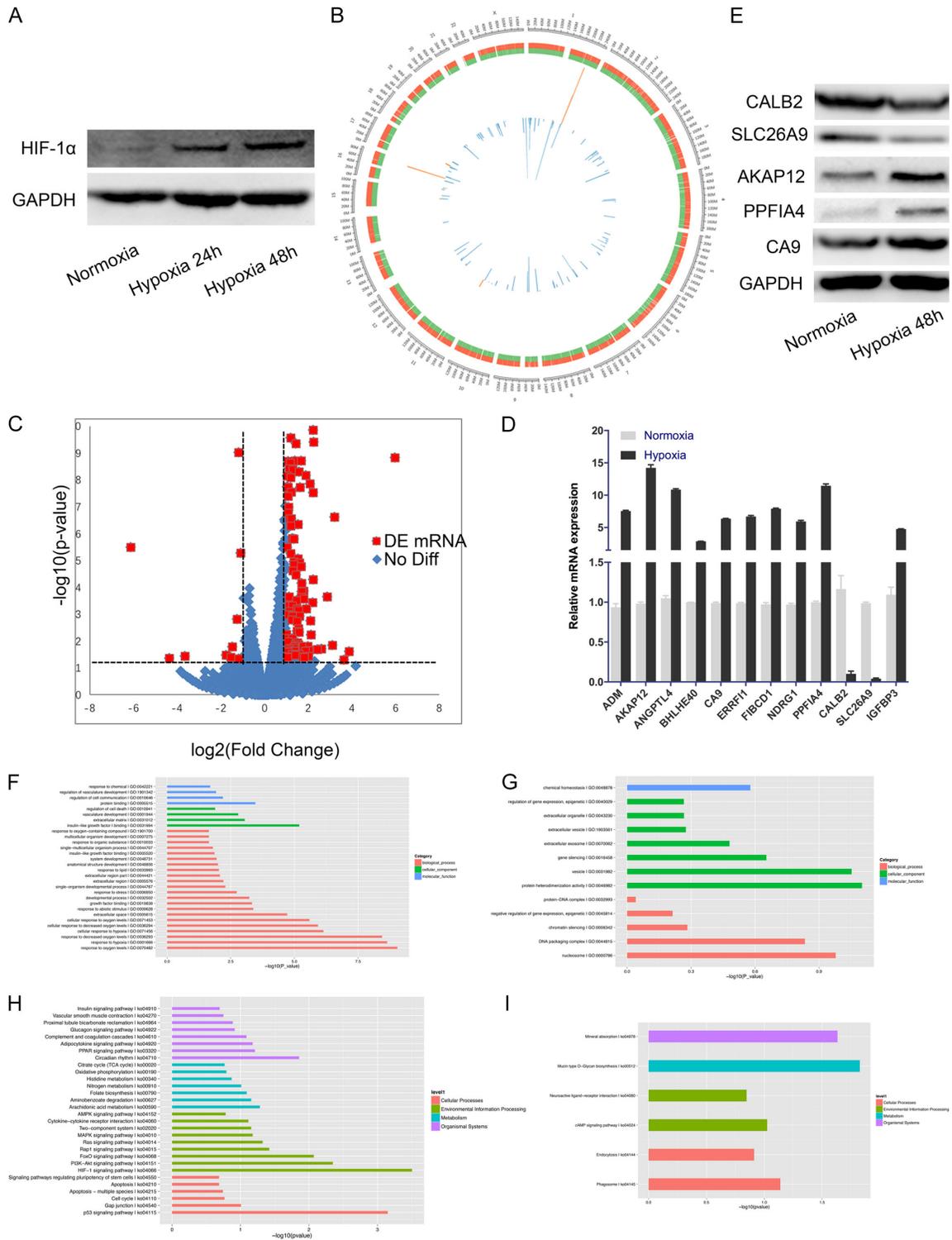


Figure 1. Analysis of differentially expressed RNAs, validation, and enrichment analysis of the genes induced by hypoxia. **A.** Western blotting of HIF-1 α expression following hypoxia treatment for either 24 or 48 h. GAPDH was used as a loading control. **B.** Circos plots show the mRNA distribution on human chromosomes. **C.** Volcano plot of differentially expressed (DE) mRNAs between normoxia- and hypoxia-treated cells. **D.** Expression of 12 differentially expressed genes (*ADM*, *AKAP12*, *ANGPTL4*, *BHLHE40*, *CA9*, *ERRF1*, *FIBCD1*, *NDRG1*, *PPFIA4*, *CALB2*, *SLC26A9*, and *IGFBP3*) after hypoxia treatment for 48 h as validated by qRT-PCR. **E.** Expression of five differentially expressed genes (*CA9*, *AKAP12*, *CALB2*, *SLC26A9*, and *PPFIA4*) after hypoxia treatment for 48 h as validated by western blot. **F.** GO analysis of hypoxia-induced genes. **G.** GO analysis of hypoxia-inhibited genes. **H.** KEGG pathway analysis of hypoxia-induced genes. **I.** KEGG pathway analysis of hypoxia-inhibited genes.

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Table 2. Number of differentially expressed mRNAs, circRNAs, lncRNAs, and miRNAs that were induced or inhibited by hypoxia

	Upregulated (Hypoxia-induced)	Downregulated (Hypoxia-inhibited)
mRNA	120	10
circRNA	24	21
lncRNA	29	40
miRNA	17	3

Table 3. Validated genes differentially expressed in hypoxia-treated MKN-28 cells vs. normoxia-treated MKN-28 cells

Gene name	Hypoxia-MKN-28 vs. normoxia-MKN-28			
	RNA-seq		qRT-PCR	
	Fold-change	<i>p</i> -value	Fold-change	<i>p</i> -value
<i>ADM</i>	4.322	0.000	7.539	0.013
<i>AKAP12</i>	3.669	0.000	14.212	0.005
<i>ANGPTL4</i>	3.880	0.000	10.840	0.019
<i>BHLHE40</i>	3.522	0.000	2.849	0.032
<i>CA9</i>	6.455	0.000	6.346	0.027
<i>ERRFI1</i>	3.694	0.000	6.687	0.031
<i>FIBCD1</i>	4.634	0.000	7.899	0.013
<i>NDRG1</i>	3.961	0.000	5.915	0.021
<i>PPFIA4</i>	8.544	0.000	11.430	0.007
<i>CALB2</i>	0.049	0.042	0.100	0.031
<i>SLC26A9</i>	0.080	0.036	0.039	0.004
<i>IGFBP3</i>	9.012	0.000	4.739	0.025

Bioinformatic analyses

Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were used to determine the roles of the differentially expressed genes. The lncRNAs/circRNAs/miRNAs and mRNAs with expression levels that shared a meaningful correlation were subjected to co-expression analysis. The potential miRNA response elements were compared to the sequences of lncRNAs/circRNAs and mRNAs as well as with the overlapping same miRNA seed sequence binding site on the lncRNAs/circRNAs and the mRNA predicted interactions.

Statistical analysis

A two-tailed Student's *t*-test was used to analyze differences between normoxic and hypoxic conditions. A *p*-value <0.05 was considered to be statistically significant.

Results

Differential expression, validation, and enrichment analysis of genes regulated by hypoxia

In order to confirm the response to hypoxia, we performed western blotting to examine the expression of the hypoxia-inducible transcription factor HIF-1 α . As **Figure 1A** shows, HIF-1 α was dramatically induced by hypoxia, especially after a 48-h incubation. The expression levels of the identified transcripts were then quantified, and differentially expressed genes between normoxia and hypoxia (48 h) conditions were determined. As shown in **Table 2**, a total of 130 mRNAs exhibited fold-changes >2.0 and *P*<0.05. Among them, 120 and 10 mRNAs were induced and inhibited by hypoxia treatment, respectively. Upregulated mRNAs (92.31%) were more common than downregulated mRNAs (7.69%) following hypoxia treatment. The hypoxia-induced mRNAs were distributed across all chromosomes, including the sex chromosomes X and Y (**Figure 1B**), while the hypoxia-inhibited mRNAs were observed only on chromosome 1, 11, 15, 16, and 17. Differentially expressed mRNAs with statistically significant differences between the two groups were identified using volcano plot filtering (**Figure 1C**). To validate the sequencing data, we selected a total of 12 differentially expressed genes (*ADM*, *AKAP12*, *ANGPTL4*, *BHLHE40*, *CA9*, *ERRFI1*, *FIBCD1*, *NDRG1*, *PPFIA4*, *CALB2*, *SLC26A9*, and *IGFBP3*) at random for qRT-PCR. Additionally, the expression of five differentially expressed genes (*CA9*, *AKAP12*, *CALB2*, *SLC26A9*, and *PPFIA4*) was further quantified by western blot. In both cases, the results were consistent with the sequencing data (**Figure 1D** and **1E**; **Table 3**).

Next, to identify the relevant biological functions of the differentially expressed genes, GO analysis of three categories, biological process, cellular component, and molecular function, were examined. Hypoxia-induced genes showed significant involvement primarily in categories related to response to hypoxia and extracellular space remodeling (**Figure 1F**), while hypoxia-inhibited genes exhibited significant enrichment among categories related to the nucleosome and DNA packaging (**Figure 1G**). Accordingly, KEGG pathway analyses revealed that the most significant pathways were p53 signaling for hypoxia-induced genes (**Figure 1H**) and the phagosome for hypoxia-inhibited genes (**Figure 1I**).

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Differentially expressed circRNAs regulated by hypoxia

Among the identified genes, 4505 transcripts are annotated as circRNAs. Of these, 45 circRNAs were differentially expressed (**Table 2**). These circRNAs were classified into the following six categories according to generation type: exons, antisense, intergenic, intron-exon, intronic, and one exon (**Figure 2A**). As shown in **Figure 2B**, the circRNAs were widely distributed on all chromosomes, including the sex chromosomes X and Y. Differentially expressed circRNAs with statistically significant differences between the hypoxia group and the normoxia group were identified using volcano plot filtering (**Figure 2C**). A total of 24 circRNAs were induced by hypoxia, while 21 circRNAs were inhibited.

Recent studies revealed that circRNAs are primarily generated from exons or introns of their parental genes and can regulate the expression of parental genes. To explore the potential function of circRNAs, we performed GO analysis and pathway analysis for circRNAs. The most highly significantly regulated biological functions were the nBAF complex for hypoxia-induced circRNAs (**Figure 2D**) and the nuclear lumen for hypoxia-inhibited circRNAs (**Figure 2E**). This pathway analysis indicated that numerous pathways related to cellular processes may be involved in the development of hypoxic gastric cancer cells (**Figure 2F, 2G**). Among these pathways, cell cycle was the most highly significant, consistent with the differentially expressed mRNA pathway analysis.

Differentially expressed lncRNAs regulated by hypoxia

As shown in **Table 2**, 69 lncRNAs were differentially expressed (fold-change >2; $P < 0.05$). Among these, 29 lncRNAs were upregulated, and 40 were downregulated by hypoxia. Volcano plot analysis was performed to visualize the fold change and determine the statistical significance of expression profiles for the hypoxia-treated MKN-28 cells after normalization to the normoxia-treated cells (**Figure 3A**). Circos plots clearly showed the expression of lncRNAs and their location on the chromosomes in hypoxia- and normoxia-treated MKN-28 cells (**Figure 3B**).

Many studies have demonstrated that lncRNAs play crucial roles in the regulation of gene expression at the epigenetic, transcriptional, and post-transcriptional levels. To investigate whether the differentially expressed lncRNAs were involved in the regulation of genes and associated signaling pathways related to gastric cancer, we predicted the potential targets of lncRNAs using the database and analyzed these targets using GO and KEGG. We found that for upregulated transcripts from the hypoxia group, the most highly enriched GOs were intracellular (biological process), cytoplasm (cellular component), and organelle (molecular function) (**Figure 3C**). In the downregulated transcripts, the most highly enriched GOs were the nucleus (biological process) and the regulation of cellular metabolic process (cellular component) (**Figure 3D**). Pathway analysis revealed a total of 30 pathways that were related to upregulated transcripts (**Figure 3E**), and the most highly enriched network was endocytosis (cellular process). Additionally, 30 pathways were identified to associate with the downregulated transcripts (**Figure 3F**). Among them, the most highly enriched network was apoptosis (cellular process). These enriched pathways may represent the key pathways regulated by lncRNAs in gastric cancer tumorigenesis in a hypoxic microenvironment.

Differentially expressed miRNAs regulated by hypoxia

Several studies have described important roles of miRNA in the progression of gastric cancer under hypoxia. Here, 20 differentially expressed miRNAs were identified between gastric cancer cells grown under hypoxic and normoxic conditions (**Table 2**). Of these, 17 lncRNAs were upregulated and only three lncRNAs were downregulated (**Table 2; Figure 4A**). The differentially expressed miRNAs are distributed on chromosomes 1, 2, 8, 11, 14, 18, and 22 (**Figure 4B**). In general, miRNAs play important roles in tumorigenesis by regulating target gene expression through mRNA degradation or translation repression. To elucidate the mechanisms that are involved in differentially expressed miRNAs under hypoxic conditions, we explored the potential targets of differentially expressed miRNAs using miRanda. Additionally, GO and KEGG pathway analyses were utilized to illustrate the biological functions associated with the significant pathways. We found that among upregu-

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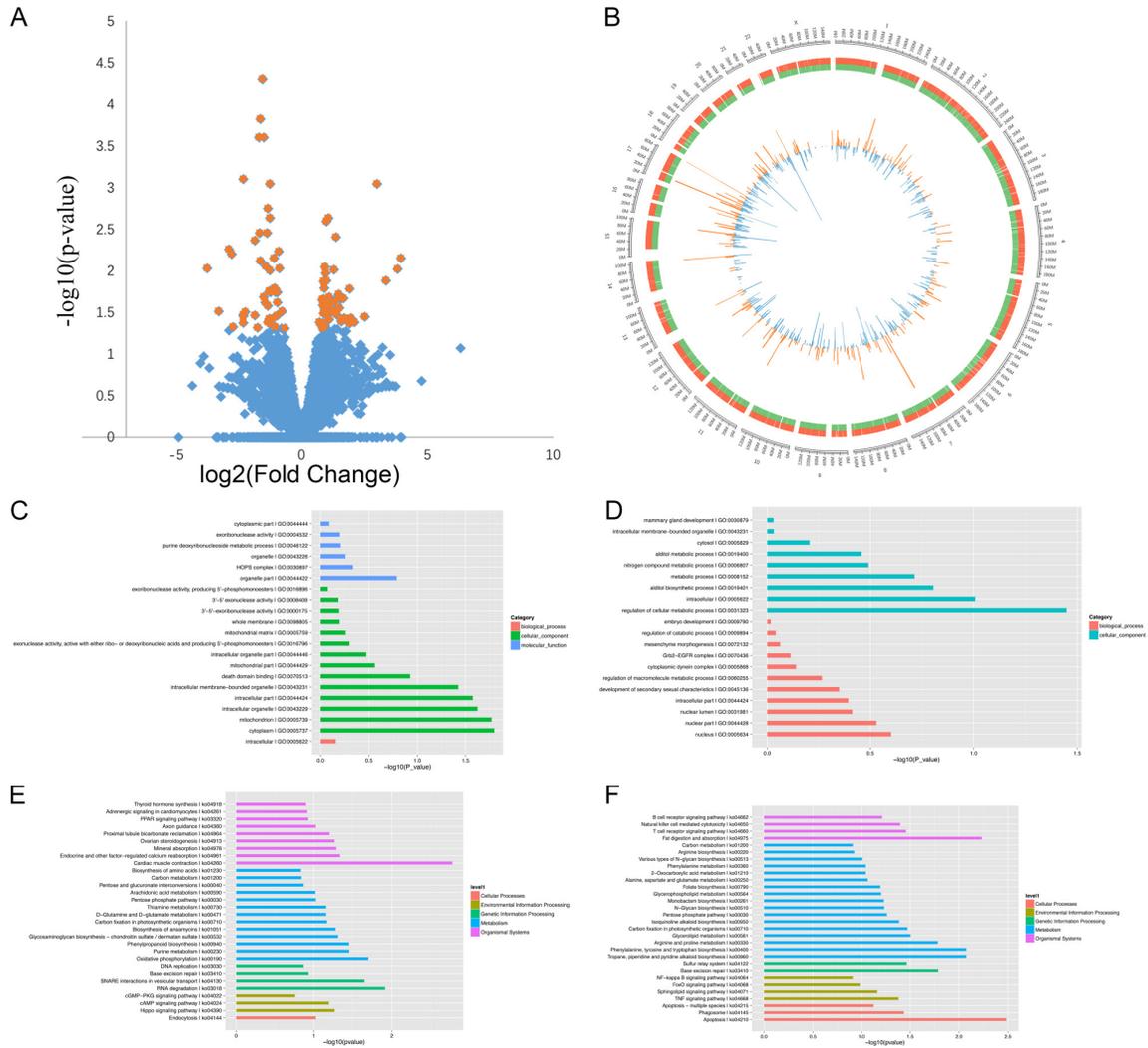


Figure 3. Differentially expressed lncRNAs regulated by hypoxia. **A.** Volcano plot of differentially expressed lncRNAs between the normoxia- and hypoxia-treated cells. **B.** Circos plot shows the lncRNAs distribution on human chromosomes. **C.** GO analysis of hypoxia-induced lncRNA targets. **D.** GO analysis of hypoxia-inhibited lncRNA targets. **E.** KEGG pathway analysis of hypoxia-induced lncRNA targets. **F.** KEGG pathway analysis of hypoxia-inhibited lncRNA targets.

lated and downregulated transcripts, the most highly enriched GO was binding (biological process) (Figure 4C and 4E). In the pathway analysis, the most enriched network for both upregulated and downregulated transcripts was endocytosis (cellular process) (Figure 4D and 4F).

Co-expression of circRNAs/miRNAs, lncRNAs/mRNAs, and miRNAs/mRNAs

Recent studies have reported that circRNAs bind cancer-associated miRNAs. To evaluate the potential functions of circRNAs, we investigated the co-expression of differentially expressed miRNAs and circRNAs (Figure 5A). The circRNA/miRNA interaction was predicted using miRNA target prediction software based on

miRanda. In addition, we built a co-expression network using correlation analysis between differentially expressed lncRNAs and mRNAs (Figure 5B). The network was complex and indicated that one lncRNA was associated with multiple mRNAs and vice versa. Meanwhile, we also generated a co-expression network of differentially expressed miRNAs and their potential targets (Figure 5C). The results show that one miRNA has the potential to regulate multiple targets.

Construction of ceRNA network

Accumulating evidence has highlighted roles of circRNAs and lncRNAs as ceRNAs in oncogenesis and cancer progression. Thus, we con-

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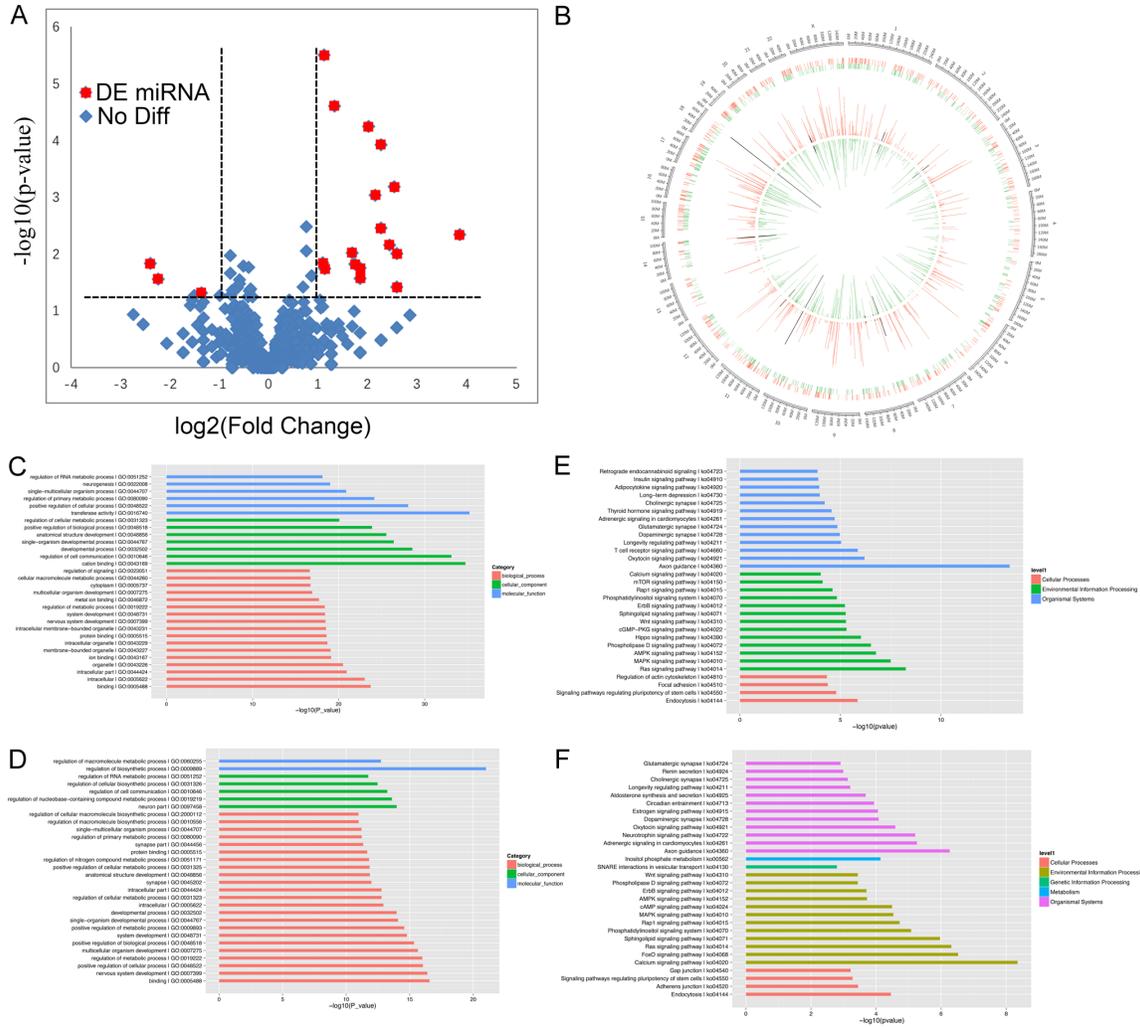


Figure 4. Differentially expressed miRNAs regulated by hypoxia. **A.** Volcano plot of differentially expressed (DE) miRNAs between the normoxia- and hypoxia-treated cells. **B.** Circos plot shows the miRNAs distribution on human chromosomes. **C.** GO analysis of hypoxia-induced miRNA targets. **D.** GO analysis of hypoxia-inhibited miRNA targets. **E.** KEGG pathway analysis of hypoxia-induced miRNA targets. **F.** KEGG pathway analysis of hypoxia-inhibited miRNA targets.

structured circRNA-miRNA-mRNA (**Figure 6A**) and lncRNA-miRNA-mRNA (**Figure 6B**) ceRNA networks for hypoxia-treated gastric cancer cells based on our sequencing data.

Discussion

Hypoxia is prevalent in solid tumors and is associated with a variety of biological functions [4-7]. Generally, expression of HIF-1 α is closely associated with hypoxia, and this factor is strongly expressed in a variety of malignant tumors, acting as an essential factor to regulate the adaption of tumor cells to hypoxia. Furthermore, HIF-1 α has been reported to be

closely associated with the chemo-resistance of gastric cancer [27]. Moreover, hypoxia in gastric cancer cells influences multiple steps within the metastatic progression [18, 28]. A number of studies have delineated potential biomarkers and therapeutic targets that regulated by hypoxia for incorporation into therapeutic strategies for gastric cancer. Roles for non-coding RNAs in the development of gastric cancer have also been reported in recent years. For example, circRNA_100269 was reported to be downregulated in gastric cancer and to suppress tumor cell growth via targeting miR-630 [29]. Additionally, dysregulation of lncRNA-UCA1 contributes to the progression of gastric

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C

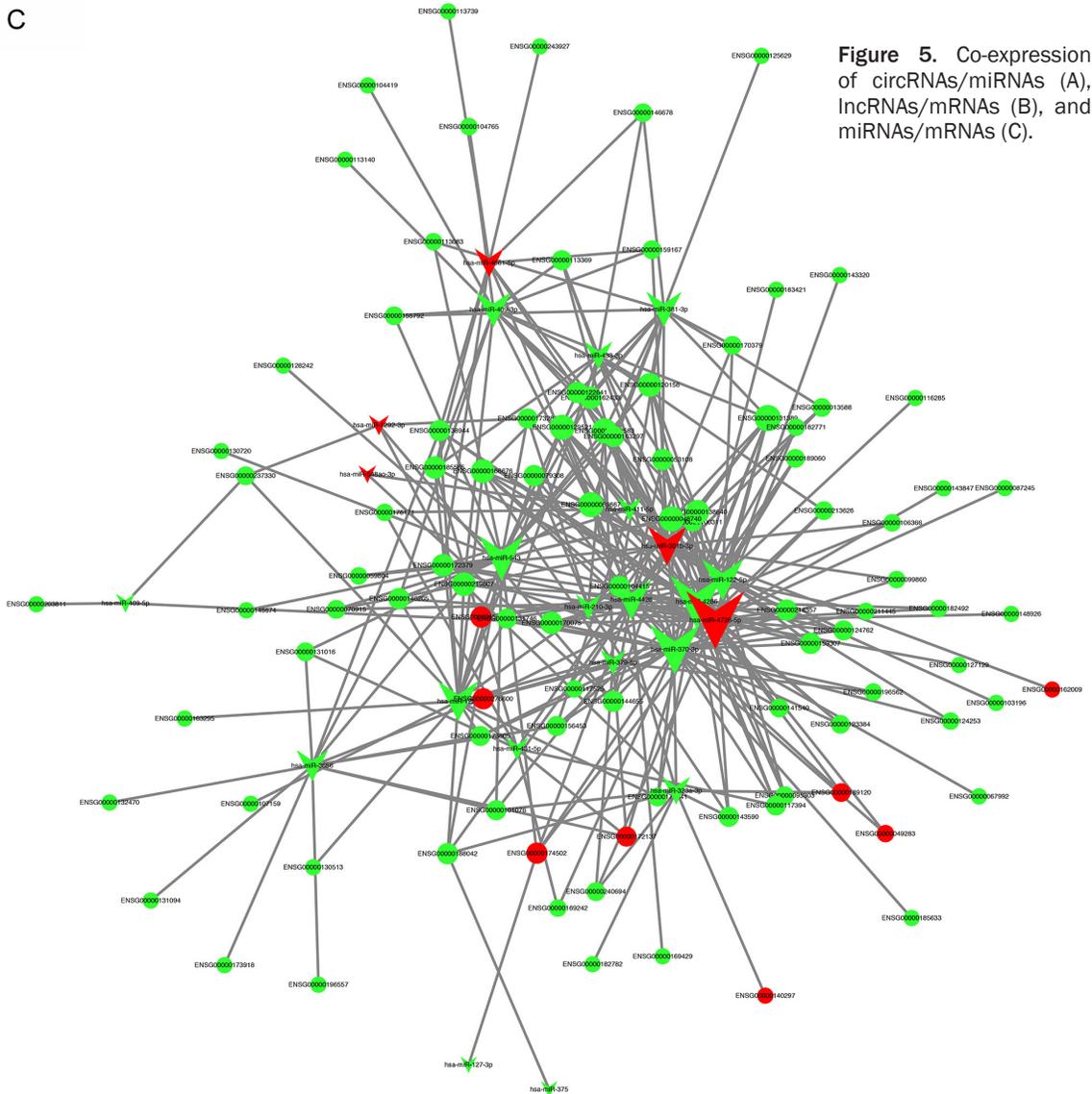


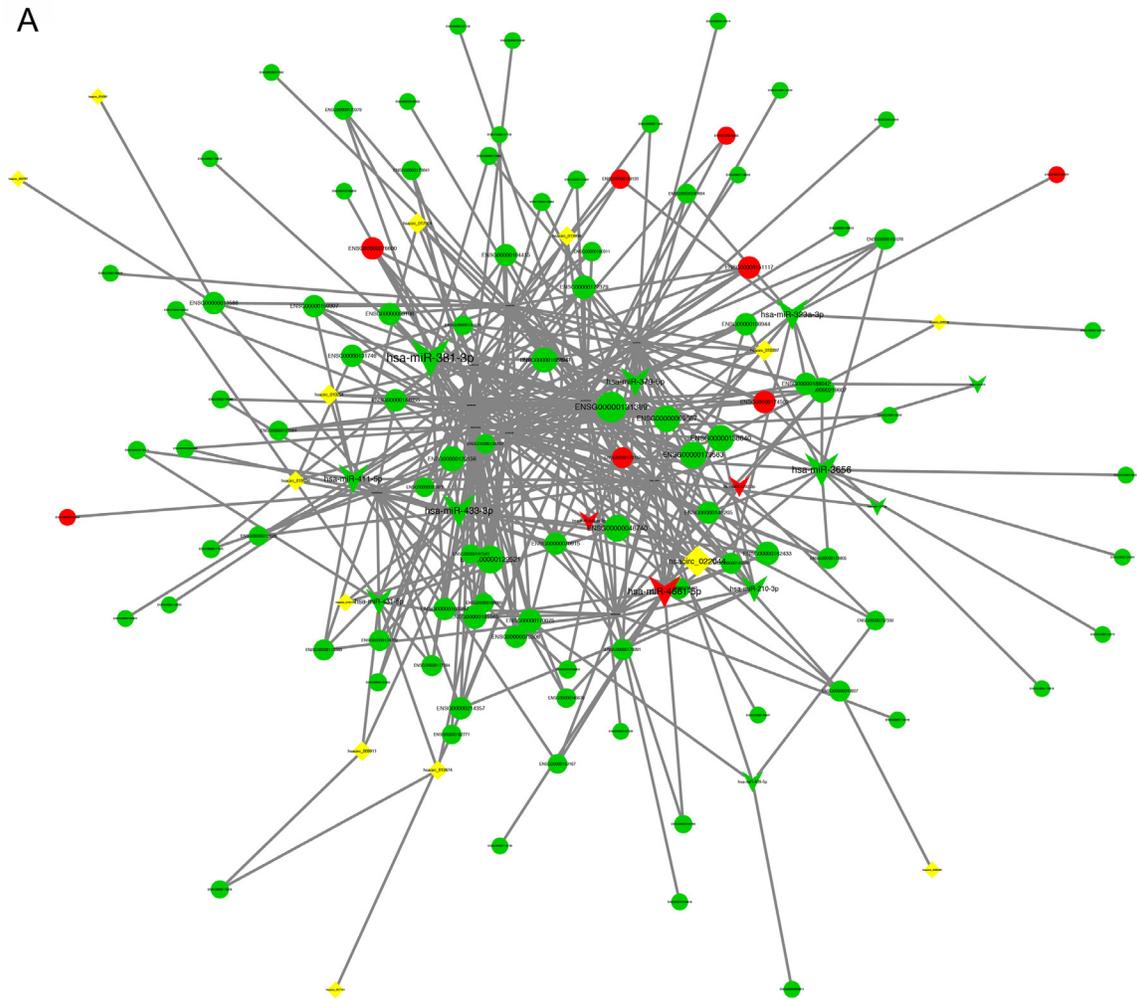
Figure 5. Co-expression of circRNAs/miRNAs (A), lncRNAs/mRNAs (B), and miRNAs/mRNAs (C).

cancer via regulation of the PI3K-Akt-mTOR signaling pathway [30], and miR-455 is downregulated in gastric cancer and inhibits cell proliferation, migration, and invasion via targeting insulin-like growth factor 1 receptor [31]. While these studies have provided molecular insight into gastric cancer, the mechanisms underlying development of gastric cancer under hypoxic conditions and the role of non-coding RNAs in this process remain unclear.

In the present study, we investigated the expression profile of non-coding RNAs and mRNAs in gastric cancer MKN-28 cells following hypoxia using sequencing. A total of 130 mRNAs were differentially expressed with fold

changes >2.0 and $P < 0.05$. We then used qPCR to validate the sequencing data. Among the detected genes, 4505 transcripts are annotated as circRNAs, and of these, 45 were differentially expressed. Additionally, 24 circRNAs were induced, while 21 circRNAs were inhibited by hypoxia. Among the differentially expressed lncRNAs, 29 were upregulated, and 40 were downregulated by hypoxia. Furthermore, 20 differentially expressed miRNAs were identified between the hypoxia- and normoxia-treated cells, and 17 of these were upregulated, while only three were downregulated.

Previous studies illustrated a crucial role for noncoding RNAs in the progression of gastric



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B

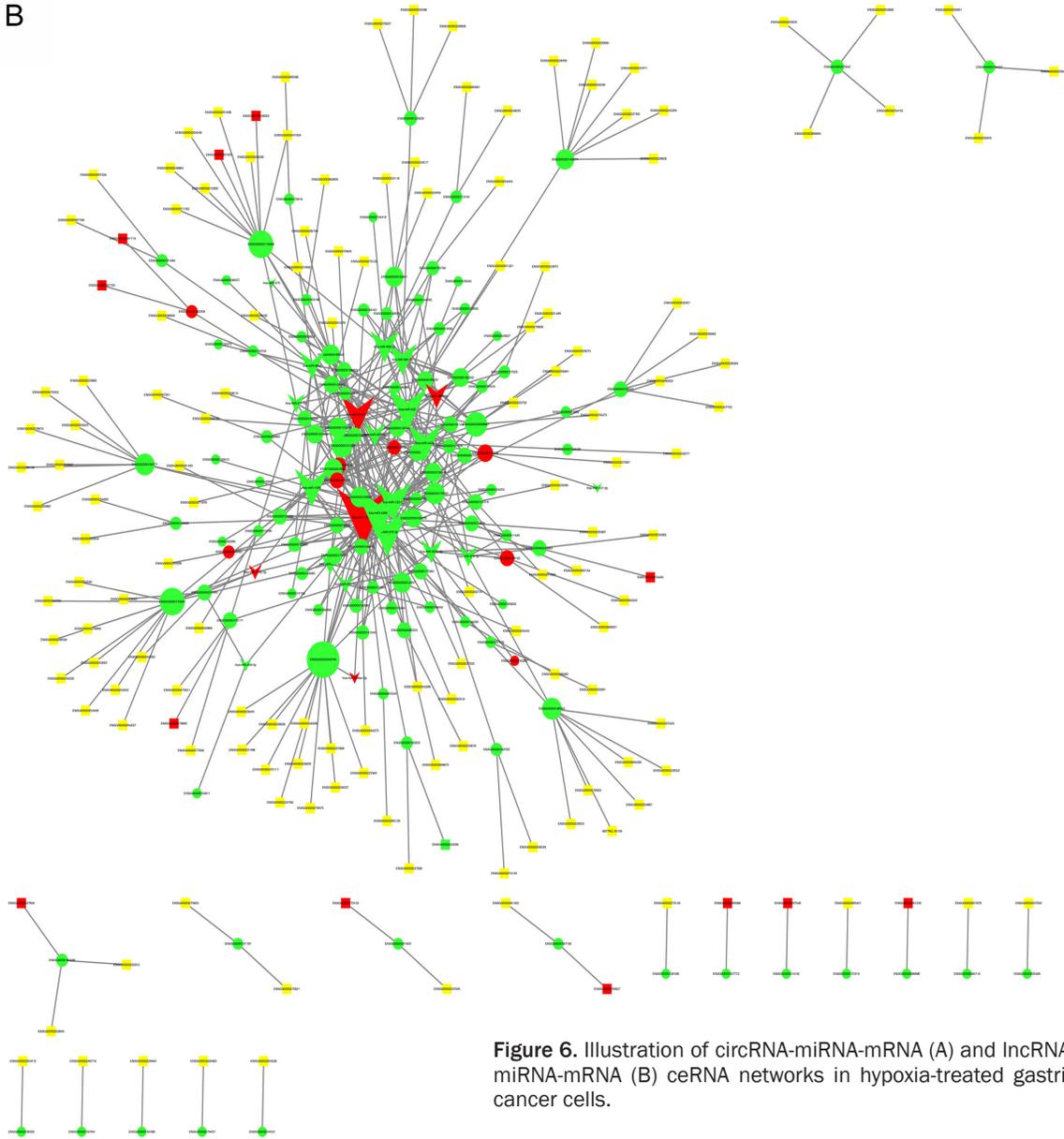


Figure 6. Illustration of circRNA-miRNA-mRNA (A) and lncRNA-miRNA-mRNA (B) ceRNA networks in hypoxia-treated gastric cancer cells.

cancer [32, 33], although the effects of hypoxia on these molecules has not been examined. For example, Shao described the global circRNA expression profile in human gastric cancer [34]. Moreover, some circRNAs persist in human body fluid, offering potential for use as novel biomarkers for the screening of high-risk gastric cancer patients [34]. Accumulating evidence has highlighted the crucial roles of circRNAs and lncRNAs acting as ceRNAs in oncogenesis and cancer progression. Therefore, we constructed circRNA-miRNA-mRNA and lncRNA-miRNA-mRNA ceRNA networks for

hypoxia-induced gastric cancer cells based on our sequencing data.

In conclusion, we employed an RNA-seq approach to identify a transcriptional signature describing protein-coding mRNAs and non-coding RNAs regulated by hypoxia in gastric cancer cells. This approach allowed us to delineate these molecules with high confidence. Ultimately, this study will provide novel insights into the biological functions and molecular mechanisms of non-coding RNAs on gastric cancer under hypoxic conditions.

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Disclosure of conflict of interest

None.

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