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Research Paper

Differential Expression of CircRNAs in Embryonic Heart Tissue Associated with Ventricular Septal Defect

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Abstract

Objectives: To explore and validate the differential expression of circRNAs in the myocardium of congenital ventricular septal defect (VSD) and to explore a new avenue of research regarding the pathological mechanisms of VSD.

Methods: We detected circRNAs expression profiles in heart tissues taken from six aborted fetuses with VSD and normal group using circRNA microarray. Some differentially expressed circRNAs were studied by bioinformatics analysis. Finally, quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed to confirm these results.

Results: This study found abundant circRNAs in the myocardium taken from individuals in the normal group and the VSD group. After that, totally 6234 differentially expressed circRNAs between the normal group and the VSD group were confirmed (Fold change ≥ 2.0; $p < 0.05$). Then, this research carried out bioinformatics analysis and predicted the potential biological functions of circRNAs. Finally, the over-expression of *hsa_circRNA_002086* and under-expression of *hsa_circRNA_007878*, *hsa_circRNA_100709*, *hsa_circRNA_101965*, *hsa_circRNA_402565* were further validated by qRT-PCR.

Conclusions: There is a significant difference in expression of the circRNA in cardiac tissue from VSD group compared to the normal group. Combined with the microarray results and previous researches, circRNAs may contribute to the occurrence of VSD by acting as miRNA sponges or by binding proteins, these possible roles for circRNAs in VSD require elucidation in additional studies.

Key words: Congenital Heart Disease (CHD), fetation, heart development, miRNA sponges

Introduction

Congenital heart disease (CHD) is the most common birth defect and a leading cause of morbidity and mortality in patients with congenital malformations[1]. Moreover, the VSDs are the most common congenital cardiac abnormalities. The isolated incidence of VSD was 2.62 in 1000 births[2]. In addition to imaging, there was little laboratory tests were performed to confirm the diagnosis of VSD. Present interventions show little effect in early prevention or treatment of VSD, for its unclear pathogenesis.

With the developing of transcriptomic, previous studies have demonstrated that non-coding RNAs, such as miRNAs and lncRNAs play important roles in cardiac development [3]. As early as 1980s, the circRNA had been discovered[4], but the circRNA did not receive much attention when receiving the research technology at that time. With the development of the study, the researchers found that, unlike the previously studied linear RNA, circRNA forms a covalently closed continuous loop, there is no linear RNA molecule in the 3'and 5' ends connected together[5]. This feature confers the insensitivity of cyclic RNA to nuclease[4], and thus is more stable than linear RNA, which makes circRNAs more obvious advantage in the development and application of potential new clinical diagnostic markers than other types of RNA[6-8].

With the development of high-throughput sequencing technology, a large number of circRNA have now been identified spanning a wide variety of organisms. In 1993, it was discovered that there is a circular transcript in the mouse sperm-determining gene Sry[9]; this indicated that circRNAs are not a product of false shear in the transcription process. With the deepening of the research, circular RNAs (circRNAs) are discovered class of evolutionarily conserved endogenous non-coding RNA that play important roles in the regulation of gene expression[10-12], so as to participate in the occurrence and development of various diseases. In subsequent studies, it was reported that cirs-7 acts as a miRNA sponge in brain cells [12], and circTCF-25 was shown to inhibit the miRNA function[13]. Recently abundant circRNAs were found in the amphicytulas before implantation[14]. It is suggested that the circRNA plays an important role not only in the process of disease, but also in the process of embryonic development.

In previous studies, little research is about the role of circRNAs in the regulation of congenital heart disease. In this study, through the application of microarray, we determine the differences expression of circRNAs between the VSD group and the control group in the embryo abortion. Specifically, we identified a total of 6234 differentially expressed circRNAs, then predicted the functions of circRNA. At length, this work provided a new direction which can access to the pathological mechanisms of VSD.

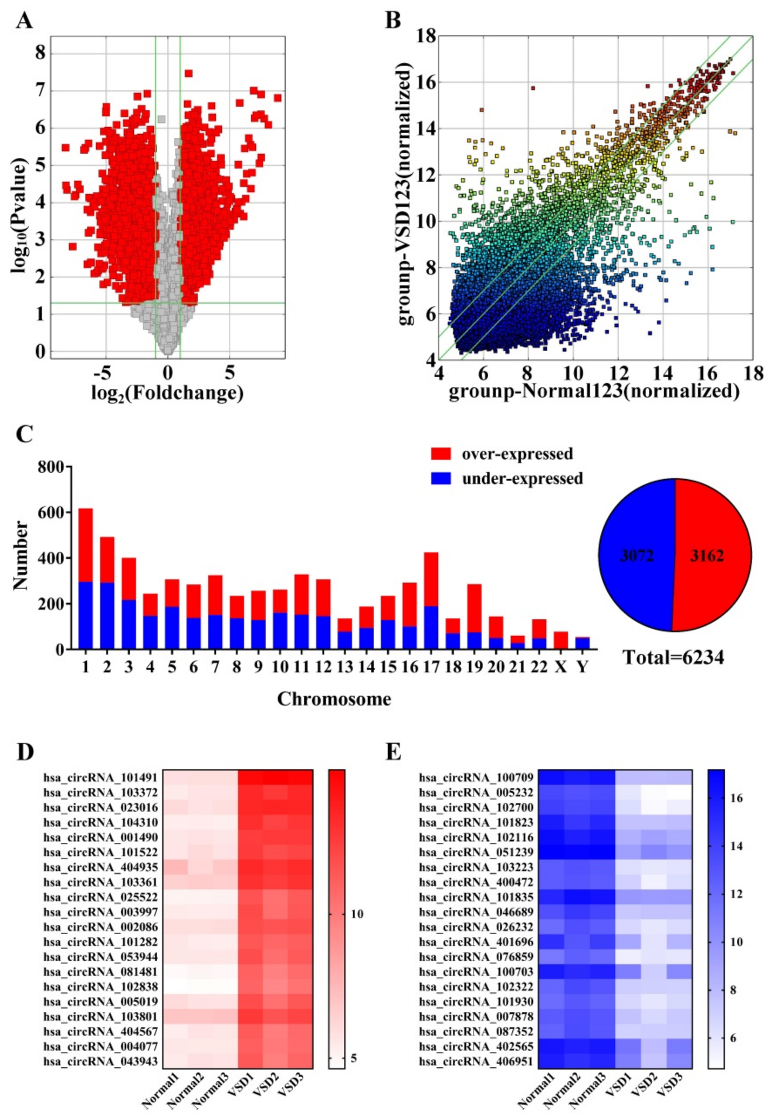

Figure 2. Detection of all circRNAs by microarray. A. Volcano Plot: The red points in the plot represent differentially expressed circRNAs that were statistically significant between the two groups. **B.** Scatter Plot: The values for the X and Y axes are normalized signal values (log2 scaled). The green lines represent fold change. CircRNAs above the top line and below the bottom green line exhibit more than a 2.0-fold change of circRNAs between the two groups. **C.** The histogram and fan diagram shows the distribution of all the differentially expressed circRNAs on human chromosomes: red represents over-expressed and blue represents under-expressed circRNAs. **D.** The thermal map revealed that the top 20 over-expressed circRNAs in CHD group. **E.** The thermal map revealed that the top 20 under-expressed circRNA in CHD group.

Results

Identification of differentially expressed circRNA profiles

We performed microarray assays on circRNA to identify circRNA expression signatures in CHD. A total of 12842 circRNA targets were detected by microarray probes in three pairs of samples. Comparing VSD cardiac tissue (n=3) to normal cardiac tissue (n=3), we identified 6234 differentially expressed circRNAs: 3162 circRNAs were over-expressed, and 3072 circRNAs were under-expressed at fold change (Fold change ≥ 2.0 and $p < 0.05$, **Figure 2c right, Supplementary Table 1**).

Volcanic maps (**Figure 2a**) and scatter plots (**Figure 2b**) show all of the detected circRNAs. Moreover, in volcano plot filtering identified significantly differentially expressed circRNAs between the two groups. The left red points mean the under-expressed circRNAs and the right red points represent the over-expressed circRNAs. The points out of two green lines near the middle of the scatter plots mean over 2.0-fold change. The histogram depicts the distribution of circRNAs on human chromosomes (**Figure 2C left**). Furthermore, the thermal map revealed that the top 20 over-expressed and under-expressed circRNAs between the VSD group and normal group (**Figure 2D and 2E**), and the top 20 differentially expressed circRNAs are shown in Table 1.

Table 1. Top 20 Over- and under- expressed circRNAs derived from VSD group

CircRNA	Host Gene Name	Fold Change	*p-value*
Over-expressed			
hsa_circRNA_101491	MAPKBP1	469.87	1.52110E-07
hsa_circRNA_103372	IP6K2	186.88	6.12402E-06
hsa_circRNA_023016	RBM4	185.63	1.22810E-07
hsa_circRNA_104310	ZDHHC4	168.79	8.76757E-06
hsa_circRNA_001490	KIF2A	122.80	9.69000E-08
hsa_circRNA_101522	DMXL2	82.44	1.66974E-05
hsa_circRNA_404935	ZBTB16	78.26	7.78875E-05
hsa_circRNA_103361	SMARCC1	74.09	3.47999E-06
hsa_circRNA_025522	ARHGDIB	62.48	9.51537E-05
hsa_circRNA_003997	CLMP	58.13	1.96476E-04
hsa_circRNA_002086	LOC401320	55.98	2.08650E-07
hsa_circRNA_101282	ABCC4	51.74	9.45923E-06
hsa_circRNA_053944	FAM98A	48.27	1.56638E-04
hsa_circRNA_081481	FBXO24	47.01	1.42946E-04
hsa_circRNA_102838	ITGB6	46.62	1.86864E-04
hsa_circRNA_005019	CHSY1	40.75	2.87632E-04
hsa_circRNA_103801	MYO10	35.02	7.82817E-05
hsa_circRNA_404567	PHTF1	33.33	3.20992E-04
hsa_circRNA_004077	VAT1L	33.02	6.97139E-05
hsa_circRNA_043943	VAT1	32.63	4.21995E-04
Under-expressed			
hsa_circRNA_100709	FAM53B	312.08	3.33090E-06
hsa_circRNA_005232	SLC8A1	305.53	3.43120E-05
hsa_circRNA_102700	SLC8A1	291.22	4.54877E-05
hsa_circRNA_101823	CNOT1	177.22	6.10264E-05
hsa_circRNA_102116	ZNF652	158.09	3.24855E-05
hsa_circRNA_051239	ATP5SL	140.01	4.31932E-05
hsa_circRNA_103223	DDX17	110.65	3.81112E-06
hsa_circRNA_400472	RYR2	93.16	1.54288E-04
hsa_circRNA_101835	NFATC3	83.73	7.31852E-05
hsa_circRNA_046689	ENOSF1	76.66	7.56315E-05
hsa_circRNA_026232	LARP4	76.64	1.78835E-04
hsa_circRNA_401696	ANKFY1	74.42	4.03502E-03
hsa_circRNA_076859	DST	67.12	9.68140E-05
hsa_circRNA_100703	CHST15	62.61	8.07226E-03
hsa_circRNA_102322	TMEM241	61.19	1.29360E-04
hsa_circRNA_101930	YWHAE	59.74	9.22908E-05
hsa_circRNA_007878	MAP4	56.33	9.76382E-05
hsa_circRNA_087352	UBQLN1	54.63	6.71974E-05
hsa_circRNA_402565	EDEM2	53.33	7.63520E-03
hsa_circRNA_406951	LOC493754	50.48	6.65697E-03

Validation of differentially expressed circRNAs

In order to verify the reliability of our microarray results. we randomly selected 5 circRNAs, *hsa_circRNA_002086, hsa_circRNA_007878, hsa_circRNA_100709, hsa_circRNA_101965, hsa_circRNA_402565*, from the top 20 differentially expressed circRNAs. *GAPDH* was used as a normalization control for qRT-PCR analysis. The results of the qRT-PCR indicated significant over-expression of *hsa_circRNA_002086* and under-expression of *hsa_circRNA_007878, hsa_circRNA_100709, hsa_circRNA_101965, hsa_circ RNA_402565* (**Figure 4**). Because microarray and qRT-PCR belong to two difference genetic tests, there are some errors between them. We confirmed the trend of 5 circRNAs differential expression were the same with chip, though the results of fold changes form qRT-PCR were different from those of microarray. This indicated that the results of qRT-PCR were well consistent with microarray results, demonstrating the high reliability of the microarray expression results.

Prediction of the function for the circRNAs host genes

According to previous researches, many circRNAs functions are related to their host genes[15, 16]. To eliminate some low variance multiples which may belongs to interference information, and convenient data analysis. We selected 282 differentially expressed circRNAs (Fold change ≥ 15.0; $p < 0.05$), of which 88 were over-expressed and 194 were under-expressed in CHD cardiac tissue were analyzed (**Supplementary Table 1**). The host genes of 282 differentially expressed circRNAs were input into DAVID (https://david.ncifcrf.gov), an online gene ontology (GO) analysis tool, and the number of target genes in each GO term was counted. Enrichment score was used to test and calculate the significance of the target gene enrichment in each GO term, and a p-value was acquired to describe the significance of the target gene GO term. Target genes were classified and analyzed according to cellular component, molecular function, biological process, and KEGG pathway (**Figure 4**).

We can find out from bioinformatics analysis, in biological process category (**Figure 4a**) parts of these genes are involved in the transcriptional regulation of RNA and cell differentiation. Then we also found the Protein Serine, Threonine Kinase Activity and Actin Binding that are involved in the regulation of cardiac cell activity and function in the molecular functions category (**Figure 4b**). In the cellular component category (**Figure 4c**), there are same peculiar structures of heart cells, such as Z Disc, T-Tubule, or some of the cellular structures, Actin Filament, Intercalated Disc, that are involved in cardiac cell function. In the final KEGG-pathway analysis (**Figure 4d**), we also found some signal pathways similar to Hypertrophic Cardiomyopathy, Arrhythmogenic Right Ventricular Cardiomyopathy, the heart disease related diseases. Therefore, from the results of bioinformatics analysis, these circRNAs we screened were largely related to the development of the heart.

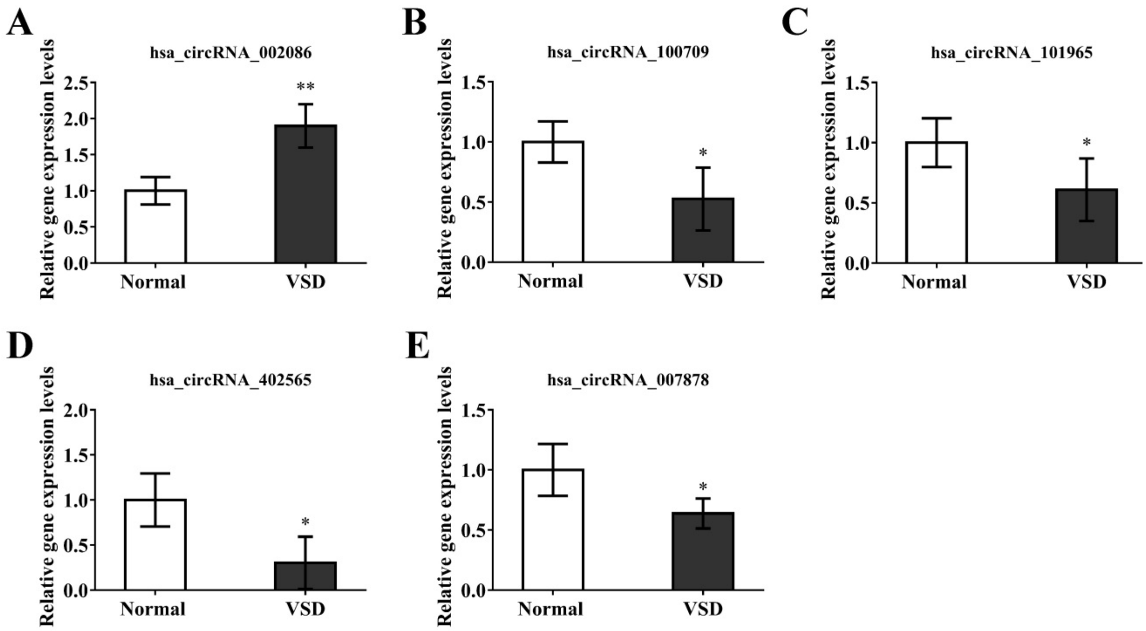

Figure 3. Validation of circRNA microarray data using real-time quantitative-PCR. The real-time RT-PCR reactions were repeated three times for *hsa_circRNA_002086, hsa_circRNA_007878, hsa_circRNA_100709, hsa_circRNA_101965, hsa_circRNA_402565*.

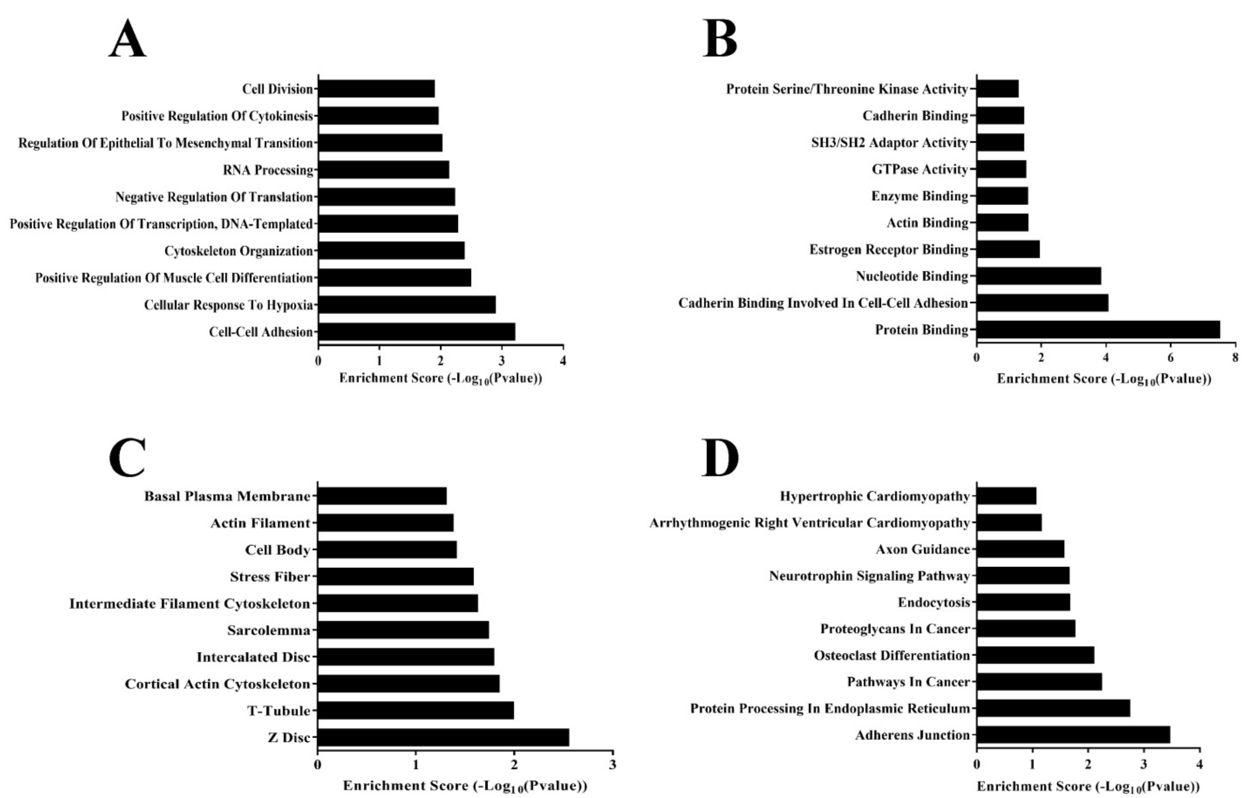

Figure 4. Gene ontology and Pathway-Express analysis about 282 differentially expressed circRNAs. a. Predicted target genes ontology terms in the biological process category. b. Predicted target genes ontology terms in the molecular functions category. c. Predicted target genes ontology terms in the cellular component category. d. Predicted target genes identified by Pathway-Express analysis using the DAVID online analysis tools.

Construction of the circRNA/miRNA interaction network.

In the early researches for circRNA functions, most investigator paid close attention to the function of miRNA sponges[12, 13, 17-19]. In order to evaluate the target miRNAs of circRNAs, this study used TargetScan and miRanda database to theoretically predict, based on conserved seed-matching sequence. The 6234 differentially expressed circRNAs are theoretically bound to miRNAs (**Supplementary Table 1**). The relationship between circRNA and miRNA in the first 20 sites of differential expression has been sorted out as network (**Figure 5a**). Reviewed previous studies, we have identified a number of miRNA which can take participation in regulation of cardiac development. The relationship between the miRNA, which had been researched, and their potentially combined circRNAs (**Figure 5b**). The relationship between them deserves further exploration.

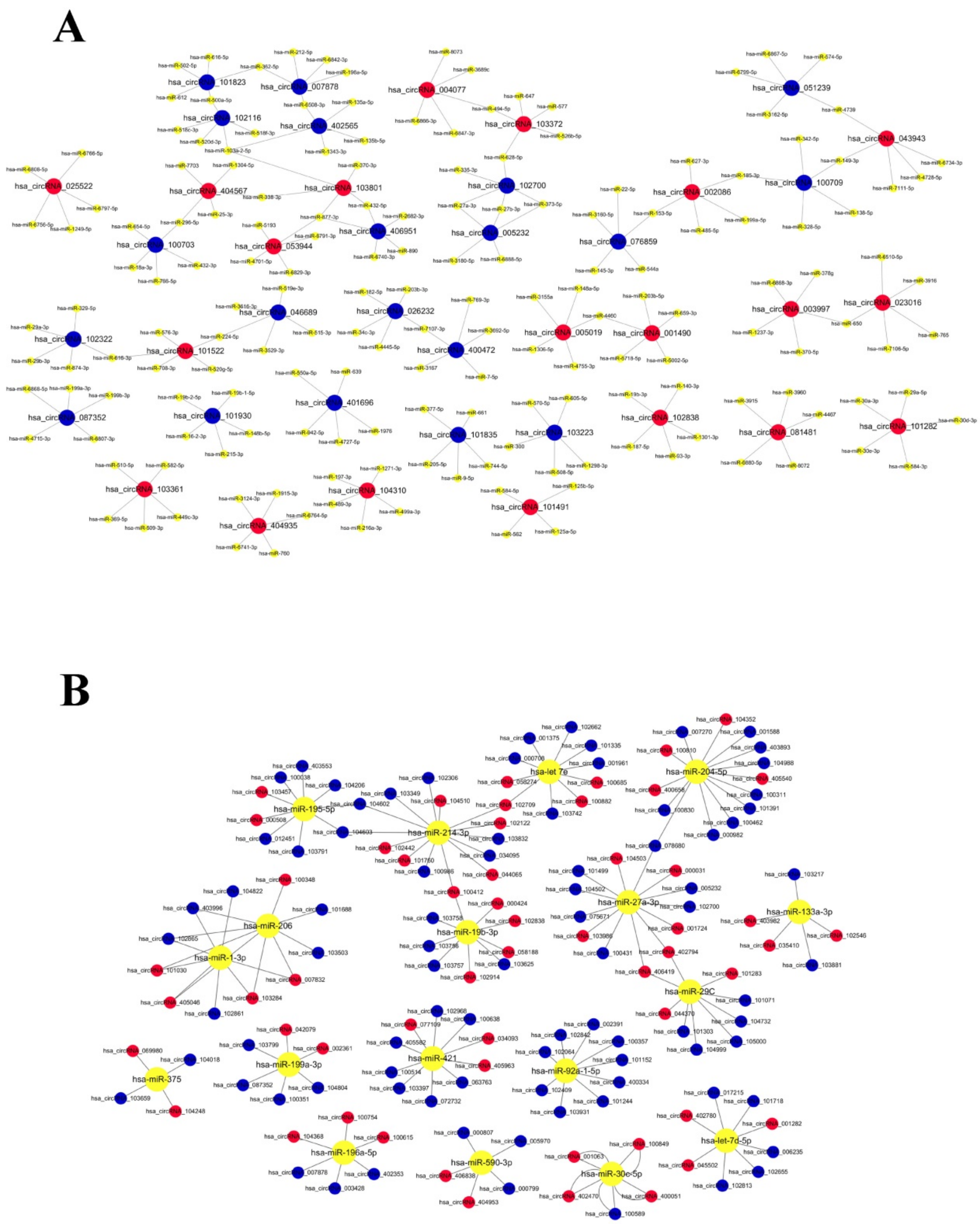

Figure 5. The network of the relationship between circRNAs and miRNAs**. A.** The relationship between Top 20 circRNAs and their potentially combined miRNAs **B.** The relationship between the miRNA, which had been researched, and their potentially combined circRNAs

Discussion

Ventricular septal defect (VSD) is the most common major congenital malformation, accounting for approximately 20% of neonatal deaths[2]. Although investigators have been throw themselves to explore the development and progression of congenital heart disease, including the identification of mutations in genes associated with VSD abnormalities, the detailed mechanisms of VSD remain a mystery[20]. In our study, it investigated the role of circRNAs in the development of human embryonic heart through miRNA sponges, based on high-throughput microarray screening.

It will be contributed to the diagnosis and rescue of abnormal embryos during pregnancy, for studying the circRNAs involved in embryonic development and the functional mechanisms of circRNAs. The researchers used high-throughput sequencing to explore the expression changes of circRNAs at different time points in the development of the rat retina, and tried to combine apoptosis to explore the role of circRNA in the development of neural cells. Han, J., et al. have researched that affluent differential expression of circRNAs among 3 different developmental time points, and 15 of which are related to apoptosis of circRNA[21]. From their study, we concluded that circRNA also plays a very important role in the process of embryonic development. Thus, the role of circRNAs in the development of embryonic heart is a direction worthy of further study.

Presently, circRNAs are at the forefront of research in cancer and cardiovascular disease, and these studies will further explore the mechanisms of development and progression of these diseases[22, 23]. MiRNA sponges function is the main research direction of investigators in existing researched. A latest study in 2017, circRNA-MYLK could serve as a sponge for miR-29a to abolish the endogenous suppressive effect on target gene VEGFA which promoted bladder cancer growth[24]. Through the complete pathway axis of the circRNA-miRNA-downstream target, the researchers elaborated the mechanism of circRNAs in the course of bladder cancer. Legnini, I. et al provided us with the latest research model of miRNA sponges. In 2016, the first study about angiocardiopathy found that the circRNA HRCR can regulate miR-223 by inhibiting the expression of ARC, which inhibits the development of cardiac hypertrophy and heart failure, thus confirming that circRNAs participate in the regulation of protein expression by effecting the biological function of miRNA[25]. Thus, in the development of cardiovascular system, circRNA may also affect the biological function of miRNA, which has an impact on the downstream target, and this mode of action is worthy of further exploration.

In the past studies, miRNA has been authenticated to be involved in the regulation of cardiac development. These preliminary work laid the foundation for us to further explore the function of miRNA sponges. In this study, through the software analysis and prediction, it found many miRNA binding sites in the circRNAs. As shown in Table 3, many circRNAs contain miR-30c binding sites; the high differential expression suggests that these circRNAs might be involved in the onset and development of CHD by regulating miRNA expression. Liu et al. reported that up-regulated miR-30c can act through the sonic hedgehog signal pathway, a signaling pathway associated with embryonic development and differentiation of P19 cells, and influences the balance between proliferation and apoptosis[26]. In another early research, miR-29c regulates the proliferation and apoptosis of P19 cells by regulating WNT 4 signaling molecules and regulates its differentiation into cardio myocytes[27]. Combined with the above researches suggest that one role for these differentially expressed circRNAs may be to function as a miR-30c or miR-29c sponge, which may affect heart development. We can further study the regulatory mechanism of upstream circRNAs through the discovered miRNAs. Of course, we can also further analyze and tap our chip results, then select the circRNA that we're interested in, and discover a new circRNA-miRNA-Target regulatory signaling pathway for heart development.

Table 3. Network analysis between miRNAs and circRNAs

MiRNA Binding Sites	Fold change	Regulation	CircRNA
hsa-let-7d-5p[31, 32]	38.3150488	down	hsa_circRNA_006235
	18.6883554	down	hsa_circRNA_101718
	7.6873022	down	hsa_circRNA_017215
	6.649988	down	hsa_circRNA_102655
	5.662462	down	hsa_circRNA_102813
	3.362295	up	hsa_circRNA_001282
	3.207422	up	hsa_circRNA_045502
	3.040495	up	hsa_circRNA_402780
hsa-let-7e[31, 32]	11.1323253	down	hsa_circRNA_101335
	6.9339121	down	hsa_circRNA_102662
	6.626608	down	hsa_circRNA_001375
	6.139229	down	hsa_circRNA_103742
	5.694939	down	hsa_circRNA_000708
	5.286563	down	hsa_circRNA_001961
	12.48349	up	hsa_circRNA_100882
	7.884907	up	hsa_circRNA_102709
	5.058363	up	hsa_circRNA_100685
	4.504873	up	hsa_circRNA_058274
hsa-miR-1-3p[33, 34]	41.9001898	down	hsa_circRNA_403996
	6.9060521	down	hsa_circRNA_102865
	4.007695	down	hsa_circRNA_102861
	3.283393	down	hsa_circRNA_104822
	6.540579	up	hsa_circRNA_101030
	3.882837	up	hsa_circRNA_405046
	3.864211	up	hsa_circRNA_103284

MiRNA Binding Sites	Fold change	Regulation	CircRNA
	3.21382	up	hsa_circRNA_007832
hsa-miR-133a-3p[33, 34]	10.9918076	down	hsa_circRNA_103881
	8.0107769	down	hsa_circRNA_103217
	7.158794	up	hsa_circRNA_102546
	4.499071	up	hsa_circRNA_403982
	4.148428	up	hsa_circRNA_035410
hsa-miR-19b-3p[33, 35]	6.3395835	down	hsa_circRNA_103625
	5.2972091	down	hsa_circRNA_103756
	5.1675127	down	hsa_circRNA_103757
	4.5267012	down	hsa_circRNA_103758
	46.61967	up	hsa_circRNA_102838
	9.424552	up	hsa_circRNA_100412
	6.843182	up	hsa_circRNA_000424
	5.656437	up	hsa_circRNA_102914
	5.311273	up	hsa_circRNA_058188
hsa-miR-195-5p[33, 34]	12.1003825	down	hsa_circRNA_012451
	11.4621834	down	hsa_circRNA_104603
	9.1727498	down	hsa_circRNA_103791
	8.3094564	down	hsa_circRNA_104602
	5.2359807	down	hsa_circRNA_104206
	4.9777722	down	hsa_circRNA_100038
	4.4409499	down	hsa_circRNA_403553
	7.55831	up	hsa_circRNA_103457
	6.324974	up	hsa_circRNA_000508
hsa-miR-196a-5p[3]	56.3330834	down	hsa_circRNA_007878
	4.3106251	down	hsa_circRNA_402353
	3.0475854	down	hsa_circRNA_003428
	7.735672	up	hsa_circRNA_104368
	4.679547	up	hsa_circRNA_100754
	3.397228	up	hsa_circRNA_100615
hsa-miR-199a-3p[33]	54.63029	down	hsa_circRNA_087352
	11.80673	down	hsa_circRNA_104804
	7.568176	down	hsa_circRNA_100351
	5.618868	down	hsa_circRNA_103799
	4.55836	up	hsa_circRNA_042079
	3.594391	up	hsa_circRNA_002361
hsa-miR-204-5p[36]	15.59543	down	hsa_circRNA_001588
	11.19295	down	hsa_circRNA_000982
	7.635485	down	hsa_circRNA_007270
	7.612147	down	hsa_circRNA_104988
	7.220489	down	hsa_circRNA_100462
	7.004912	down	hsa_circRNA_100311
	5.825728	down	hsa_circRNA_403893
	5.723085	down	hsa_circRNA_100830
	5.461927	down	hsa_circRNA_078680
	4.036292	down	hsa_circRNA_101391
	4.163626	up	hsa_circRNA_100810
	3.937716	up	hsa_circRNA_104352
	3.810963	up	hsa_circRNA_400658
	3.283699	up	hsa_circRNA_405540
hsa-miR-206[37]	41.90019	down	hsa_circRNA_403996
	14.206515	down	hsa_circRNA_103503
	6.9060521	down	hsa_circRNA_102865
	5.44311	down	hsa_circRNA_101688
	6.540579	up	hsa_circRNA_101030
	4.634693	up	hsa_circRNA_100348
	3.882837	up	hsa_circRNA_405046
	3.864211	up	hsa_circRNA_103284
	3.21382	up	hsa_circRNA_007832
hsa-miR-214-3p[33]	12.52012	down	hsa_circRNA_103349
	11.46218	down	hsa_circRNA_104603
	10.3626	down	hsa_circRNA_034095
	8.309456	down	hsa_circRNA_104602
	5.554202	down	hsa_circRNA_100986
	5.368699	down	hsa_circRNA_103832
	4.821045	down	hsa_circRNA_102306
	18.58771	up	hsa_circRNA_102442
	9.424552	up	hsa_circRNA_100412
	7.884907	up	hsa_circRNA_102709

MiRNA Binding Sites	Fold change	Regulation	CircRNA
	7.453939	up	hsa_circRNA_104510
	6.999214	up	hsa_circRNA_102122
	6.680745	up	hsa_circRNA_044065
	5.141421	up	hsa_circRNA_101760
hsa-miR-27a-3p[38, 39]	305.5269	down	hsa_circRNA_005232
	291.2235	down	hsa_circRNA_102700
	14.14891	down	hsa_circRNA_075671
	10.77599	down	hsa_circRNA_104502
	10.18955	down	hsa_circRNA_100431
	6.716819	down	hsa_circRNA_101499
	5.461927	down	hsa_circRNA_078680
	8.634449	up	hsa_circRNA_000031
	6.265159	up	hsa_circRNA_104503
	5.785755	up	hsa_circRNA_001724
	5.440395	up	hsa_circRNA_103986
	5.099158	up	hsa_circRNA_402794
	4.149106	up	hsa_circRNA_406419
hsa-miR-29c[27]	13.852547	down	hsa_circRNA_101071
	10.17929	down	hsa_circRNA_104999
	6.6941486	down	hsa_circRNA_101303
	4.6987668	down	hsa_circRNA_104732
	3.2086528	down	hsa_circRNA_105000
	5.099158	up	hsa_circRNA_402794
	4.280459	up	hsa_circRNA_101283
	4.149106	up	hsa_circRNA_406419
	4.008815	up	hsa_circRNA_044370
hsa-miR-30c-5p[26, 33]	14.99199	down	hsa_circRNA_100589
	25.32313	up	hsa_circRNA_001063
	3.875839	up	hsa_circRNA_402470
	3.640062	up	hsa_circRNA_100849
	3.079875	up	hsa_circRNA_400051
hsa-miR-375[40]	3.1354057	down	hsa_circRNA_104018
	2.0539568	down	hsa_circRNA_103659
	3.688859	up	hsa_circRNA_104248
	2.205257	up	hsa_circRNA_069980
hsa-miR-421[33]	11.004075	down	hsa_circRNA_063763
	8.3824519	down	hsa_circRNA_103397
	7.5803979	down	hsa_circRNA_100638
	7.3394522	down	hsa_circRNA_072732
	5.2343335	down	hsa_circRNA_102968
	3.9867779	down	hsa_circRNA_405582
	3.7947983	down	hsa_circRNA_100514
	31.05648	up	hsa_circRNA_034093
	26.61819	up	hsa_circRNA_405963
	3.100921	up	hsa_circRNA_077109
hsa-miR-590-3p[35]	77.197715	down	hsa_circRNA_000799
	11.605572	down	hsa_circRNA_000807
	5.6559582	down	hsa_circRNA_005970
	4.906641	up	hsa_circRNA_406838
	3.586507	up	hsa_circRNA_404953
hsa-miR-92a-1-5p[33]	16.27706	down	hsa_circRNA_102842
	8.83981	down	hsa_circRNA_103931
	5.648434	down	hsa_circRNA_100357
	5.005185	down	hsa_circRNA_002391
	4.601707	down	hsa_circRNA_102409
	3.924155	down	hsa_circRNA_400334
	3.521439	down	hsa_circRNA_101244
	3.465761	down	hsa_circRNA_102064
	3.369293	down	hsa_circRNA_101152

Furthermore, circRNAs are involved in many important regulatory functions, not just through the miRNAs sponges function. RNA binding motifs (RBM), and even the translation of synthetic proteins may be the pathway for circRNA to function[9, 28, 29]. As in bioinformatics analysis, molecular functions

(**Figure 4c**) show that the circRNAs we detected may be similar to its host genes, give full play to its functions through protein binding. For example, it has been found that circ-Foxo3 can affect protein cell localization by binding proteins. Circ-Foxo3 is expressed mainly in the cytoplasm, where it is associated with aging related proteins Id1 and E2F1, as well as the stress proteins HIF1 alpha and FAK. Circ-Foxo3 can reduce the expression of Id1 and E2F1 in the nucleus, but also reduce the stress response by regulating the expression of FAK and HIF1 alpha in mitochondria, and accelerating myocardial aging[30]. This suggests that in our microarray results, there may be a non-miRNA-sponges involved in the regulation of cardiac development, but this mode of action needs further analysis and screening.

This study still has some limitations. We had neither proved these circRNAs could directly regulate heart development, nor detected the dynamic expression of these circRNAs during heart development. Furthermore, we have no expression pattern analysis of the host genes of candidate circRNAs and their effects on mRNAs or miRNAs. It calls for further validations. Additionally, combined with the bioinformatics analysis and miRNA target prediction, prediction function, provides fertile areas for further research.

In conclusion, this study demonstrated the significant differentially circRNAs in myocardial tissue between VSD and normal group. These circRNAs might involve in the regulation of myocardial development. Our study provides some fundamental data for the follow-up studies of diagnostic markers and potential mechanisms of heart development. To our knowledge, this study is the preliminary exploration of circRNAs as a mechanism for heart development. Our data suggest that circRNAs might play an important role in heart development, and establish rationale to investigate the role of circRNA involved in heart development in additional studies that will elucidate mechanisms of heart development and development of VSD.

Materials and methods

Ethical statement

All human fetal heart tissues were obtained from Obstetrics and Gynecology Hospital affiliated of Nanjing Medical University from deceased donors as approved by the medical ethics committee. And it complies with The Population and Family Planning Law of the People's Republic of China. We followed established procedures for written informed parental consent. We conducted basic research in accordance with national institutes of health guidelines.

Experimental design

This experiment adopts a case-control study design. To examine the different expression of circRNA, we conducted high-throughput microarray technology to detect heart tissue divided into two different groups: VSD and normal (n=3 tissues per groups). We collected cardiac tissue from aborted fetus at 24-28 weeks of gestation depending on embryos diagnosed by ultrasonography (**Figure 1**). In order to exclude the interference of non-research purposes related factors, we excluded the tissues collected from whose mother had other diseases, and the embryos had genetic disorders, such as 21-trisomy syndrome. To validate the microarray, we randomly selected 5 circRNAs (*hsa_circRNA_002086, hsa_circRNA_007878, hsa_circRNA_100709, hsa_circRNA_101965, hsa_circRNA_402565*) and examined its expression in 12 pairs of fetal heart tissue samples at 24-28 week of gestation by quantitative reverse transcription-polymerase chain reaction (qRT-PCR).

Patients and sample collection

Enrollment occurred from January to June 2016 at the Obstetrics and Gynecology Hospital affiliated of Nanjing Medical University Department of Family Planning. Prenatal ultrasound diagnosis of VSD for aborted fetuses, and fetal abortion with VSD were confirmed by anatomy, and are not associated with other malformations. Controls included aborted fetuses whose prenatal diagnosis were no abnormal genotype and were confirmed to lack VSD or other cardiac malformation. The results of imaging diagnosis are shown in **Figure 1**.

Microarray analysis

Arraystar circRNA Microarray Technology (KANGCHEN, Shanghai, China) was used to analyze the differential expression of circRNAs.

Total RNA extraction and reverse transcription

Total RNA was extracted from the samples using TRIzol Reagent (Invitrogen, Carlsbad CA, USA), according to manufacturer's instructions. The RNA prep pure tissue kit (TIANGEN, DP431) was also used for subsequent RNA preparation. Based on the concentration of each sample, 1000 ng total RNA was input into the 20ul reverse transcription reaction. cDNA synthesis was performed on each sample using reverse transcription with random primers following the recommendations of the TaKaRa Prime Script™ RT Master Mix kit.

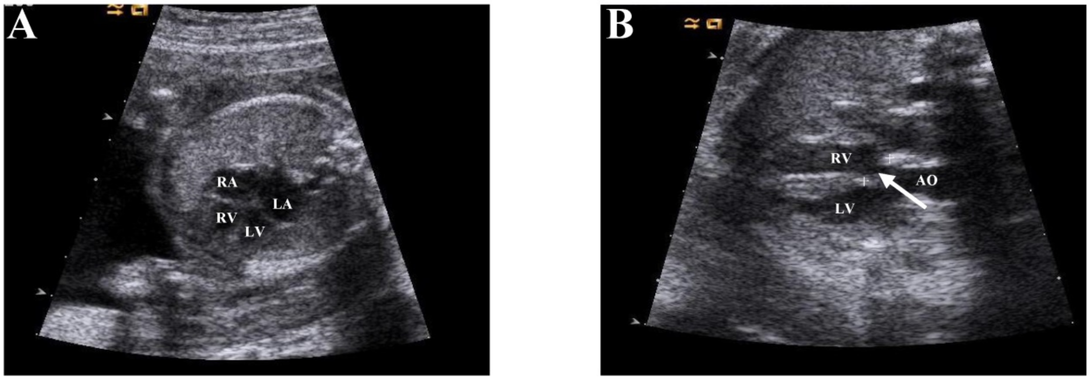

Figure 1. Echocardiographic diagnosis of fetal heart. a. The four chambers of the heart of a normal fetus (Arrow position). b: Resting diagram of Ventricular Septal Defect (VSD) (Arrow position). Note RV, right ventricle; LV, left ventricle; RA, right atrium; LA, left atrium; AO, aorta.

qRT-PCR detection of target genes

We used SYBR for qRT-PCR to evaluation results of chip. The experimental data were analyzed using the $2^{-\Delta\Delta CT}$ method. All data are the average of three independent experiments. Primer sequences are shown in **Table 2**.

GO analysis and Bioinformatics pathway

We retrieved the genes encoded by the circRNAs source region from the circBase (http://www.circbase.org) and predicted their target genes. Target genes were input into the DAVID (https://david.ncifcrf.gov) online GO analysis tool.

Statistical analysis

SPSS statistical software was used for data analysis. Data are given as mean ± standard deviation. Significant differences between groups were evaluated by the t test. A difference with $p < 0.05$ was considered statistically significant.

Table 2. Primers used in present study

Primer name	Primer sequences
Gapdh-F	TCGACAGTCAGCCGCATCTTCTTT
Gapdh-R	ACCAAATCCGTTGACTCCGACCTT
hsa_circRNA_402565-F	CAATCCCTCACATTCTCCA
hsa_circRNA_402565-R	GTTGCCACAGTAACCACATC
hsa_circRNA_101965-F	TAGAGGGTCGGCAGCA
hsa_circRNA_101965-R	TGTGGATAGTCCGTTCGT
hsa_circRNA_100709-F	GTGACACCTGGAGCCCT
hsa_circRNA_100709-R	CCTTGACTCATCTTCTTTGG
hsa_circRNA_007878-F	AGCCAAAGATGTTCCACC
hsa_circRNA_007878-R	GCTTCCACAGACCACCC
hsa_circRNA_002086-F	CTGGTGTCTGTCCTCCTTAC
hsa_circRNA_002086-R	GGGTGACCTGGTTGTGA

Supplementary Material

Supplementary table S1.
http://www.medsci.org/v15p0703s1.xlsx

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Competing Interests

The authors have declared that no competing interest exists.

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