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Nkx2–5 Regulates the Proliferation and Migration of H9c2 Cells

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Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

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Background: The protein NKX2–5 affects mammalian heart development. In mice, the disruption of *Nkx2–5* has been associated with arrhythmias, abnormal myocardial contraction, abnormal cardiac morphogenesis, and death. However, the details of the mechanisms are unclear. This study was designed to investigate them.

Material/Methods: Rat cardiomyocytes from the H9c2 cell line were used in our study. First, we knocked down *Nkx2–5* in the H9c2 cells and then validated consequent changes in cell proliferation and migration. We then used RNA sequencing to determine the changes in transcripts. Finally, we validated these results by quantitative reverse transcription-polymerase chain reaction.

Results: We confirmed that *Nkx2–5* regulates the proliferation and migration of H9c2 cells. In our experiments, *Nkx2–5* regulated the expression of genes related to proliferation, migration, heart development, and disease. Based on bioinformatics analysis, knockdown of *Nkx2–5* caused differential expression of genes involved in cardiac development, calcium ion-related biological activity, the transforming growth factor (TGF)-β signaling pathway, pathways related to heart diseases, the MAPK signaling pathway, and other biological processes and signaling pathways.

Conclusions: *Nkx2–5* may regulate proliferation and migration of the H9c2 cells through the genes *Tgfb-2*, *Bmp10*, *Id2*, *Wt1*, *Hey1*, and *Cacna1g*; rno-miR-1-3p; the TGF-β signaling pathway; the MAPK signaling pathway; as well as other genes and pathways.

MeSH Keywords: Heart Defects, Congenital • MAP Kinase Signaling System • Signal Transduction

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Background

Congenital heart disease (CHD), which arises from defective cardiac structure, has a global incidence of approximately 1% [1]. Individuals with CHD are at risk for heart failure and arrhythmias [2]. Common types of CHD include atrial septal defect (ASD), ventricular septal defect (VSD), patent ductus arteriosus, and tetralogy of Fallot [3], with ASD and VSD being the most common types [4]. ASD is a continuous interruption of the cardiac atrial septum, which can lead to heart failure, arrhythmia, and pulmonary hypertension. Among possible pathogenic factors leading to CHD [5, 6], genetic factors, especially abnormalities of *NKX2–5* [4], are considered to be associated with the disease, especially with ASD [7].

Human *NKX2–5*, located at chromosome 5q35.1, consists of 3213 bases, including 3 exons. Through alternative splicing, 3 isoforms are possible [8]. All 3 isoforms are expressed in the heart, with isoform 1, which contains the homeodomain, being the most abundant [8]. Isoforms 2 and 3 lack the homeodomain [8]. Human NKX2–5 consists of 324 amino acids and contains several functional domains, including the HD domain, which has a role in DNA binding and activation of transcription [9,10]; the NK2-specific domain, which helps to regulate the transcriptional activation of NK-2-class proteins [11]; and the nuclear localization signal, which is involved in the phosphorylation of NKX2–5 [12]. NKX2–5 is conserved in mammals [13] and functions in the morphogenesis of the heart [14,15]. In mice, disruption *of Nkx2–5* has been associated with arrhythmias, abnormal myocardial contraction, abnormal cardiac morphogenesis, and death [16]. Notably, the structural abnormalities of the hearts of these mice are highly similar to those of patients with CHD [16,17]. Therefore, we hypothesized that mutations in *NKX2–5* are related to CHD.

Our study relied in part on miRNAs, which are noncoding RNAs that are highly evolutionarily conserved [18]. miRNAs can bind to mRNA to inhibit the expression of a target gene [19,20].

In our study, we knocked down the *Nkx2–5* gene in H9c2 cells and investigated the changes in cell proliferation, migration, and the transcripts to clarify the function and mechanisms of NKX2–5 in the heart.

Material and Methods

The shRNA lentiviral vector used for *Nkx2–5 knockdown*

The shRNA lentiviral vector GV493 was designed and made by Shanghai GeneChem. GV493 contained the following elements: hU6 (promoter), MCS (polyclonal restriction site), CBh (promoter of the enhanced green fluorescent protein [GFP] gcGFP gene), gcGFP gene, internal ribosomal entry site, and puromycin resistance gene. The inserted sequence used for knocking down *Nkx2–5* was TCTCAACGCCTACGGCTACAA, and the inserted sequence for the negative control was TTCTCCGAACGTGTCACGT.

Rat cardiomyocyte cells and infection by shRNA lentiviral vector

The rat cardiomyocyte cells from the H9c2(2-1) cell line were cultured with high-glucose Dulbecco's modified Eagle's medium. H9c2 cells were infected by shRNA lentiviral vector with a multiplicity of infection equal to 10. Seventy-two hours after infection, puromycin at a concentration of 0.4 μg/mL was used to kill uninfected cells.

Quantitative reverse transcription-polymerase chain reaction

After the concentration of RNA from cells of the different groups was measured, the GoScript™ Reverse Transcription Mix Oligo(dT) (Promega) was used to obtain cDNA. In total, 2000 ng of RNA was used in the 20-μL reaction system. The cDNA was diluted to 40 ng/μL with nucleic acid-free water for quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Eastep®qPCRMaster MixKit (Promega) was used to complete the qRT-PCR for genes according to the manufacturer's instructions. All-in-One™ miRNA qRT-PCR Detection Kit (GeneCopoeia) was used to complete the qRT-PCR for miRNAs according to the manufacturer's instructions. Some qRT-PCR primers for genes were designed by Tsingke Biological Technology. Primer sequence are listed in Table 1. The qRT-PCR primers for miRNAs and some genes were purchased from GeneCopoeia. Due to the trade secrets involved, the sequence information cannot be provided. The protocols for qRT-PCR of mRNAs and miRNAs are listed individually in Tables 2 and 3. Beta-actin and U6 genes were used as internal standards. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression levels of genes, according to the cycle threshold values of the target mRNAs, miRNAs, and internal standards, respectively.

Western blot

Equal amounts of protein obtained from cells in the control group and the *Nkx2–5* knockdown group were separated through 10% SDS-PAGE and transferred onto a membrane. Tris-buffered saline containing 0.1% Tween-20 (TBST) was used to dissolve 5% nonfat dry milk to block the membrane for 1 h at room temperature. The membrane was then incubated with antibodies specific for NKX2–5 (Proteintech,1: 500) and β-tubulin (Proteintech, 1: 1000) at 4°C overnight. The next day, the membrane was washed 3 times with TBST and then incubated with secondary antibody (Proteintech, 1: 2000) for 1 h at room temperature. Afterward, the membrane was again

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Table 1. Sequence information of all quantitative reverse transcription-polymerase chain reaction (qRT-PCR) primers.

Genes	Sequence of prime	Tm
Nkx2–5-F	GTAAGCGACAGCGGCAGGAC	58.7˚
Nkx2–5-R	CGACGCCAAAGTTCACGAAG	58.7˚
Beta-actin-F	TGAGAGGGAAATCGTGCGTGAC	53.9˚C
Beta-actin-R	ATCTGCTGGAAGGTGGACAGTGAG	53.9˚C
Cacna1g-F	GACCAAACAGCGGGAGAGTC	60.27
Cacna1g-R	CTGCCCACTACGGGCCAC	62.78
Cited1-F	GCCACCCCTTCTACCAAACC	60.61
Cited1-R	GATCCCAGTGCCCCAGTTTA	59.38
Emp2-F	GACAATGCCTGGTGGGTAGG	60.39
Emp2-R	ATAGACGGAAGCCCCGATCA	60.47
Fos-F	GGAGGGAGCTGACAGATACG	59.33
Fos-R	CAGACCCCCAGTCAAGTCCA	60.13
Heyl-F	CTCTTCTCCCCCTTACCCGA	60.03
Heyl-R	ACCAGACGCACTGTCATCAG	60.04
Id2-F	GGTCCGTTAGGAAAAACAGCC	60.5
Id2-R	CTGACGATAGTGGGGTGCGAG	62.8
Itga7-F	CATGGATCTGGATGGGACCG	59.96
Itga7-R	AGGCATTCTCGTTGGACAGG	60.04
Nectin3-F	ATCGCTGTCTTTGTGACTGTG	58.86
Nectin3-R	TGAGTCCCATCTTCTTGCACT	59.02
Olfm1-F	CAAACCAGAACGCAGGCAAC	60.59
Olfm1-R	CGAGGCGTTGGTTTGGTAGG	61.3
Pou5f1-F	TGGCTTCAGACTTCGCCTTC	60.32
Pou5f1-R	GATCCCCAGCACCTCTGAAC	60.11
Ptk2b-F	TTGACCACCCTCACATCGTC	59.68
Ptk2b-R	GAGCGTACAGGACCAGAGTG	59.83
S1pr1-F	CCCAGTGGTTAAGGCTCTCC	59.75
S1pr1-R	CTGTGTAAGCCACTCCTGCT	59.68
Syt1-F	GGATGACGATGCTGAAACCG	59.35
Syt1-R	TCTTTTTGTCAGGCAGCAGGA	60.13
Syt13-F	TTCATCCTCCCTCAGAACGGT	60.8
Syt13-R	GGTCATAGTCCAGGCGAAAGTG	61.2
Tenm4-F	TGTGGATGTGGAAGAGCGTG	60.32
Tenm4-R	CCCAGTCCCAGTTCTCGAAG	59.75
Tgfb2-F	TTGGATGCCGCCTATTGCTT	60.4
Tgfb2-R	TACAGGCTGAGGACTTTGGTG	59.65
Wnt4-F	CTCGTCTTCGCCGTGTTCTC	61.07
Wnt4-R	GCACTGAGTCCATCACCTCG	60.46
Wt1-F	CTTCTCCGGCCAGTTCACC	60.38
Wt1-R	TATCCTTGGTTGCGGATGGAG	59.86
Xdh-F	CTGTTGACCCATGGAGGGAC	60.04
Xdh-R	CGTATAGGCGTCCATCACCC	60.04

Table 2. The protocol of quantitative reverse transcription-polymerase chain reaction (qRT-PCR) of mRNAs.

	Temperature	Times	Cycle
Amplification stage			
Pre-denature	95°C	2 min	1 cycle
Amplification	95°C	15 s	40 cycles
	60°C	50 s	
Dissociation stage			
Melting curves	95°C	5 s	1 cycle
	65°C	5 s	1 cycle
	95°C	Hold	1 cycle

Table 3. The protocol of quantitative reverse transcription-polymerase chain reaction (qRT-PCR) of miRNAs.

	Temperature	Times	Cycle
Amplification stage			
Pre-denature	95°C	10 min	1 cycle
Denature	95°C	10 s	
Amplification	Tm-2°C	20 s	40 cycles
	72°C	10 s	
	Temperature range	**Heat ratio**	**Temperature**
Dissociation stage			
Melting curves	65–95°C	0.5°C/time	6 s/time
	30°C		30 s

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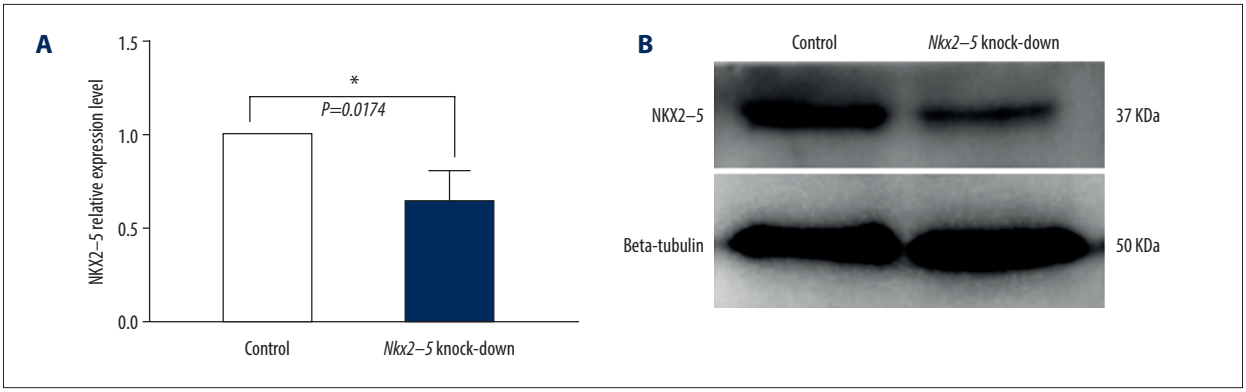

Figure 1. Verification of the knockdown effect of shRNA lentivirus on *Nkx2–5* in H9c2 cells by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and western blot analysis, respectively. (**A**) qRT-PCR detection of the expression of *Nkx2–5* in the control group and the *Nkx2–5* knockdown group. (**B**) Western blot detection of the expression of *Nkx2–5* in the control group and the *Nkx2–5* knockdown group.

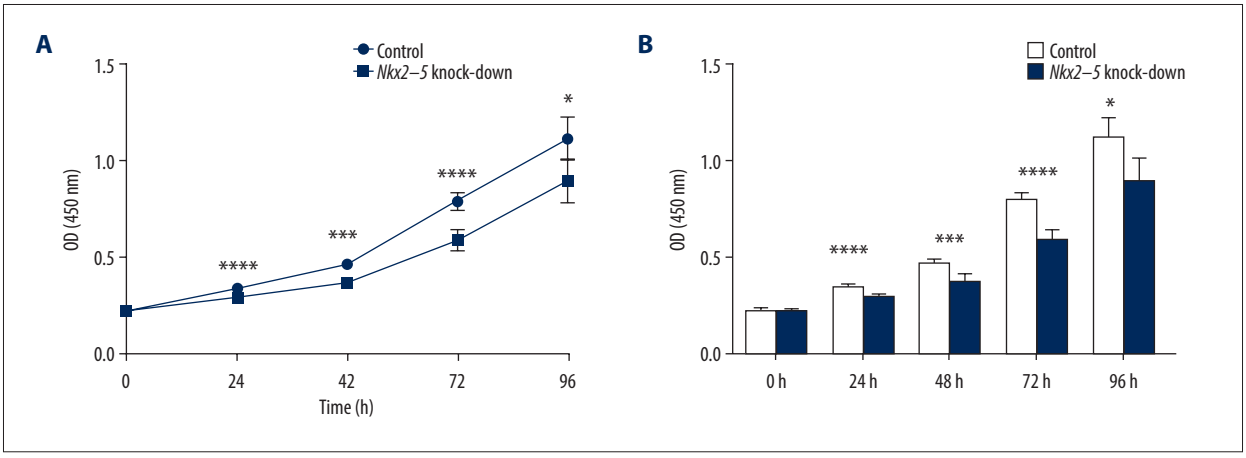

Figure 2. Knockdown of *Nkx2–5* inhibited the proliferative capacity of H9c2 cells. The CCK8 method was used to detect the effect of *Nkx2–5* on the proliferative capacity of H9c2 cells. (**A**) Cell growth was detected with the CCK8 method for 4 consecutive days. (**B**) Statistics of the CCK8 curve are shown.

washed 3 times with TBST and substrate was added for enhanced chemiluminescence.

Cell proliferation test

The CCK8 method was used to detect the cells' ability to proliferate. Cells from each group were seeded into 96-well plates at the same concentration and tested every 24 h. First, we removed the old culture medium and added 100 µL of fresh culture medium and 10 µL of CCK8 solution to each well. We then continued the cell cultures for 2 h. Finally, we measured the absorbance at 450 nm with a microplate reader and constructed the CCK8 cell proliferation curve according to the numerical values.

RNA sequencing

RNA was extracted from cells using Trizol reagent (Invitrogen), and the quantity and purity of RNA were validated. A chain-specific library was constructed by removing ribosomal RNA, and this library was sequenced using Illumina Novaseq™ 6000.

The small RNA-sequencing (RNA-seq) library was completed by using the TruSeq Small RNA Sample Prep Kits (Illumina), and this library was sequenced using Illumina Hiseq2000/2500 with a single-end read length of 50 bp. R package "ballgown" was used to screen the genes with a *P*-value <.05. TargetScan and Miranda were used to predict the target genes of miRNAs.

Statistical analysis

The numerical results are described as the mean±standard deviation. GraphPad Prism (version 8.3.0) was used for statistical analysis and making statistical charts according to data (mean±standard deviation). The differences between the 2 groups were analyzed using *t* tests (and nonparametric tests), and *P*<.05 indicated statistical significance.

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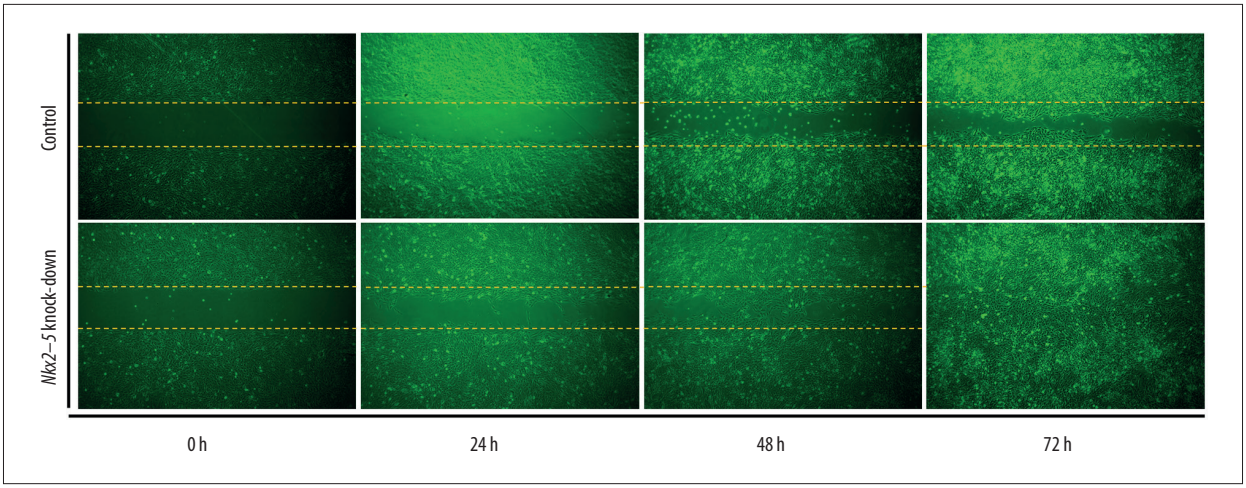

Figure 3. Knockdown of *Nkx2–5* increase the migration ability of H9c2 cells. The cell scratch test was used to detect the effect of *Nkx2–5* on the migration ability of H9c2 cells (0–72 h). Cells were seeded in the culture-insert, which was removed after 24 h, and cells were then continuously observed for 72 h.

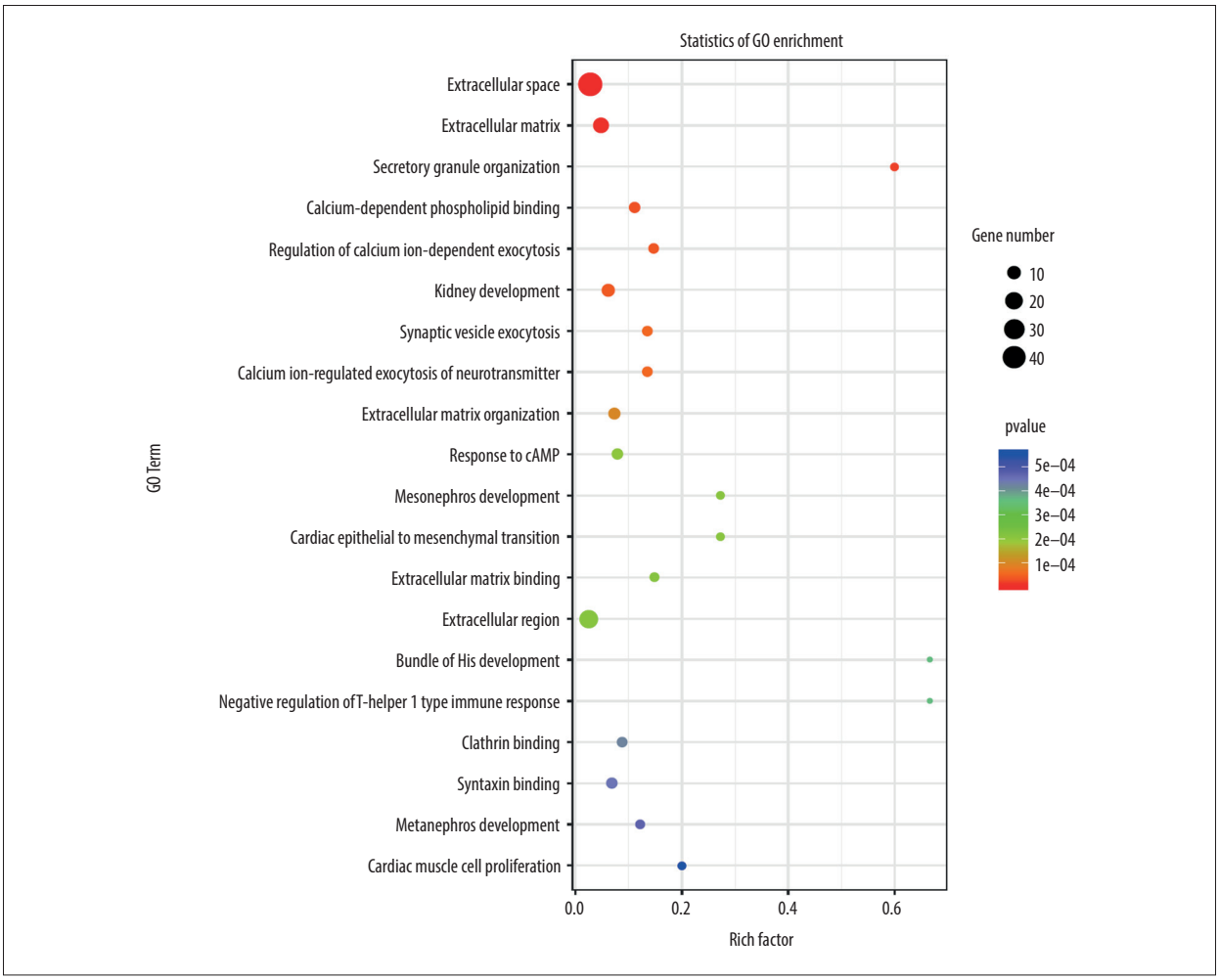

Figure 4. Gene Ontology (GO) analysis of the differential expression of genes caused by knockdown of *Nkx2–5* indicated that the genes are enriched in many biological processes, including cardiac epithelial to mesenchymal transition, development of the cardiac bundle of His, and cardiac muscle cell proliferation.

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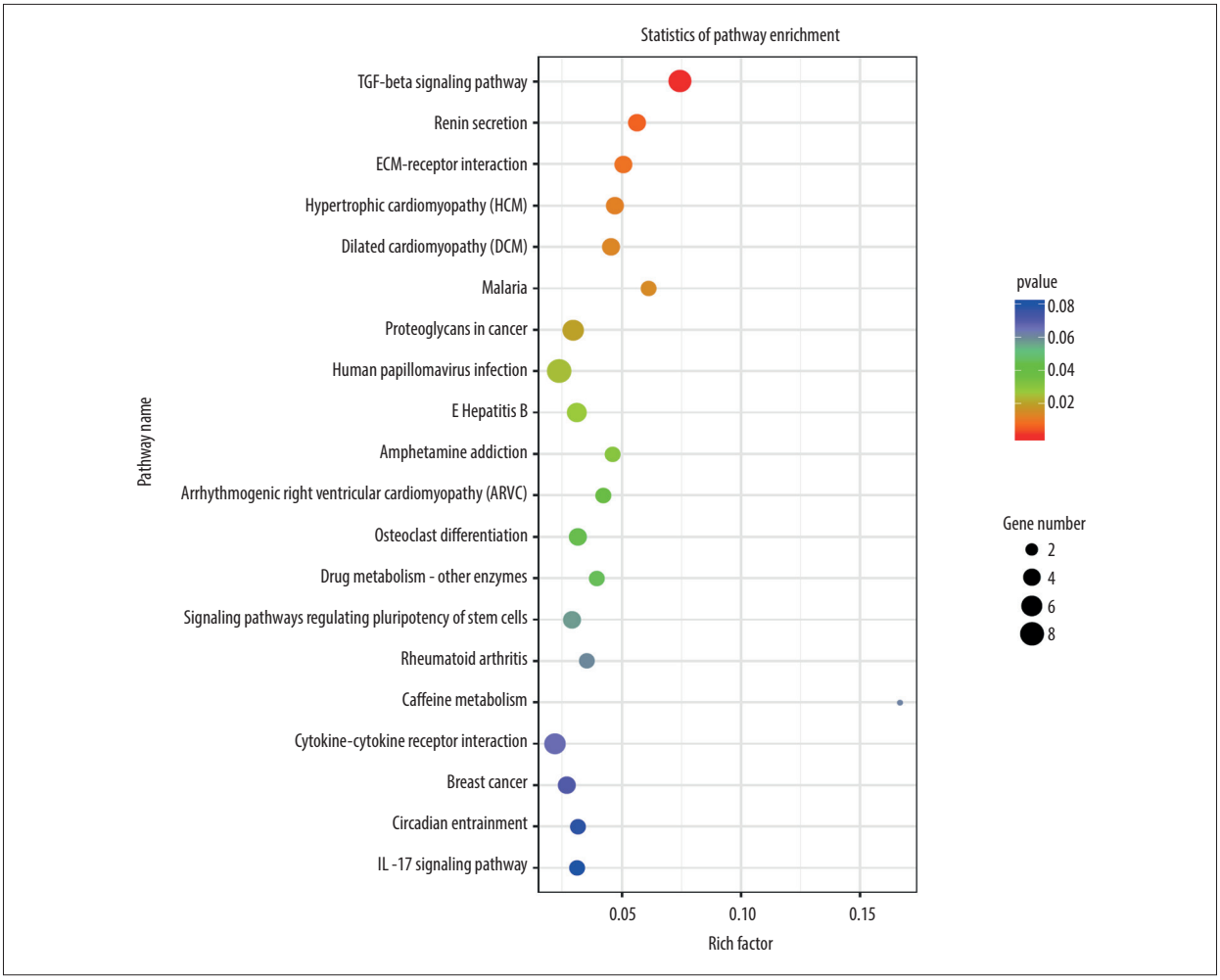

Figure 5. Pathway analysis of the differential expression of genes caused by knockdown of *Nkx2–5* indicated that the genes are enriched in the transforming growth factor (TGF)-β signaling pathway, and pathways related to hypertrophic cardiomyopathy, dilated cardiomyopathy, and arrhythmogenic right ventricular cardiomyopathy.

Results

Generation of *Nkx2–5* knockdown H9c2 cells

H9c2 cells were infected with lentivirus and amplified after puromycin selection, and qRT-PCR and western blot analysis were used to validate the effect of shRNA on *Nkx2–5*. The qRT-PCR and western blot results (Figure 1) indicated that the shRNA knocked down the expression of *Nkx2–5*.

Knockdown of *Nkx2–5* inhibited the proliferation of H9c2

The CCK8 test was used to validate the effect of *Nkx2–5* on the proliferative capacity of H9c2 cells. The results indicated that the knockdown of *Nkx2–5* decreased the proliferative capacity of the H9c2 cells (Figure 2).

Knockdown of *Nkx2–5* increased the migration of H9c2

The cell scratch test was used to validate the effect of *Nkx2–5* on the migration of H9c2 cells. The results indicated that the knockdown of *Nkx2–5* increased the migration ability of the H9c2 cells (Figure 3).

Knockdown of *Nkx2–5* changed the transcripts

To investigate the mechanisms leading to changes in the proliferation and migration of H9c2 cells, we used RNA-seq on the transcripts of cells. *P*-value <0.05 was used to identify the differentially expressed genes, and the results indicated that the knockdown of *Nkx2–5* changed the expression levels of several genes. Gene Ontology (GO) enrichment analysis (Figure 4) suggested that enriched genes involved the extracellular space, extracellular matrix, calcium-dependent phospholipid binding, regulation of calcium ion-dependent exocytosis, calcium

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Table 4. The expression level of the differentially expressed genes related to cell proliferation according to FPKM value.

Gene name	Up/down	Knock-down group	Control group	Fold change	P-value
Bche	Up	0.354186667	0.127371333	2.780740826	0.028197175
Cd81	Up	3.180889	1.060321333	2.999929267	0.037666133
Col18a1	Up	1.622587377	0.805795694	2.013646124	0.037666133
Crlf1	Up	1.371389526	0.381314621	3.596477685	0.037666133
Ednra	Up	7.472959951	2.16629906	3.449643722	0.037666133
Emp2	Up	0.398951333	0.114417	3.486818684	0.037666133
Hmga1	Up	11.72277317	5.04543594	2.323441088	0.028197175
Ptk2b	Up	1.624791991	0.688358574	2.360386072	0.037666133
Rxfp2	Up	0.95118	0.338642	2.808806941	0.028197175
Serpine2	Up	14.57077164	7.18728464	2.027298537	0.028197175
Cenpe	Down	2.752010696	5.505788696	0.498510584	0.043675528
Id2	Down	2.615604826	6.236435442	0.435260138	0.035903501
Il1rl1	Down	1.041805031	2.359768185	0.475813274	0.028197175
LOC100359539	Down	16.170679	46.241846	0.34092758	0.037666133
Ndrg1	Down	0.058952144	0.581966619	0.131365005	0.028197175
Nkx2–5	Down	0.811241	1.914524333	0.40684553	0.037666133
Ripor2	Down	0.862623412	4.835583656	0.141300869	0.035903501
Tgfb2	Down	10.51144076	22.92765142	0.468730695	0.02243198
Tnn	Down	0.975868592	2.531033413	0.378168426	0.02243198
Wt1	Down	0.1274315	0.345506667	0.367150658	0.028197175

Table 5. The expression level of the differentially expressed genes related to cell migration according to FPKM value.

Gene name	Up/down	Knock-down group	Control group	Fold change	P-value
Cemip	Down	0.299191667	0.636106333	0.470348511	0.028361462
Tgfb2	Down	10.74689399	22.92765142	0.468730695	0.028361462
Tnn	Down	0.957156921	2.531033413	0.378168426	0.011414219
Aqp1	Up	12.23365337	2.509491509	4.874953084	0.028361462
Col18a1	Up	1.622587377	0.805795694	2.013646124	0.028361462
Efna1	Up	7.933838	3.165927	2.506007877	0.011414219
Emp2	Up	0.398951333	0.114417	3.486818684	0.011414219
Itga7	Up	35.6172742	15.57743844	2.286465412	0.011414219
Lcp1	Up	0.530143013	0.17685835	2.997557156	0.011414219
Ptk2b	Up	1.624791991	0.688358574	2.360386072	0.028361462
S1pr1	Up	0.402147	0.115644	3.477456677	0.011414219

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Table 6. The expression level of the differentially expressed genes related to cardiovascular development, function and disease according to FPKM value.

Gene name	Up/down	Knock-down group	Control group	Fold change	P-value
Cited1	Down	0.137443766	0.688688943	0.199573069	0.032938043
Id2	Down	2.714471751	6.236435442	0.435260138	0.000365474
Lrp2	Down	0.017384	0.059996333	0.28975104	0.003245192
Nkx2–5	Down	0.778915667	1.914524333	0.40684553	0.006318441
Olfm1	Down	0.128097341	0.290353507	0.441177177	0.000208684
Olfm2	Down	1.721408393	3.702179558	0.464971611	0.011101623
Pou5f1	Down	0.308853095	0.694788528	0.444528202	0.011101623
Serpina3c	Down	1.586827	3.243215667	0.489275819	0.009289732
Tgfb2	Down	10.74689399	22.92765142	0.468730695	0.000208684
Wnt4	Down	1.074377465	3.383843595	0.317502105	0.002484204
Wt1	Down	0.126853	0.345506667	0.367150658	0.002484204
Xdh	Down	6.73928628	14.19602639	0.47473047	0.011101623
Aqp1	Up	12.23365337	2.509491509	4.874953084	0.02208052
Cacna1g	Up	2.314802229	0.980096727	2.361809977	0.006318441
Chrd	Up	2.940675	1.427321333	2.060275378	0.011101623
Dcn	Up	11.71709687	5.777820047	2.027944238	0.009289732
Ednra	Up	7.472959951	2.16629906	3.449643722	0.006318441
Efna1	Up	7.933838	3.165927	2.506007877	0.02208052
Eln	Up	4.81633855	0.152180345	31.64888704	0.038387993
Emp2	Up	0.398951333	0.114417	3.486818684	0.005207576
Heyl	Up	0.220188667	0.104787	2.101297553	0.000208684
Myo7a	Up	13.55489722	5.230017929	2.591749666	0.011101623
Nalcn	Up	1.496093741	0.738173261	2.026751469	0.035903501
Ptk2b	Up	1.624791991	0.688358574	2.360386072	0.005207576
Rap1gap	Up	3.104311744	0.954441805	3.252489287	0.011101623
Ren	Up	0.471765676	0.090147279	5.233276913	0.032938043
S1pr1	Up	0.402147	0.115644	3.477456677	0.011101623
Tenm4	Up	0.426035667	0.071534	5.955708707	0.000556863
Thbs2	Up	2.631674667	0.831391667	3.165384947	0.009289732

ion-regulated exocytosis of neurotransmitter, cardiac epithelial to mesenchymal transition, development of the cardiac bundle of His, and cardiac muscle cell proliferation. Pathway enrichment analysis (Figure 5) suggested that those genes are enriched in the transforming growth factor (TGF)-β signaling pathway, and pathways related to hypertrophic cardiomyopathy, dilated cardiomyopathy, and arrhythmogenic right ventricular cardiomyopathy.

Knockdown of *Nkx2–5* changed the expression of genes associated with proliferation

The CCK8 test suggested that the knockdown of *Nkx2–5* in H9c2 cells decreased cell proliferation. To investigate the mechanism, we selected and analyzed genes associated with cell proliferation based on their FPKM values. In the *Nkx2–5* knockdown group, the results indicated that the expression of genes related to cell proliferation was changed. Among these

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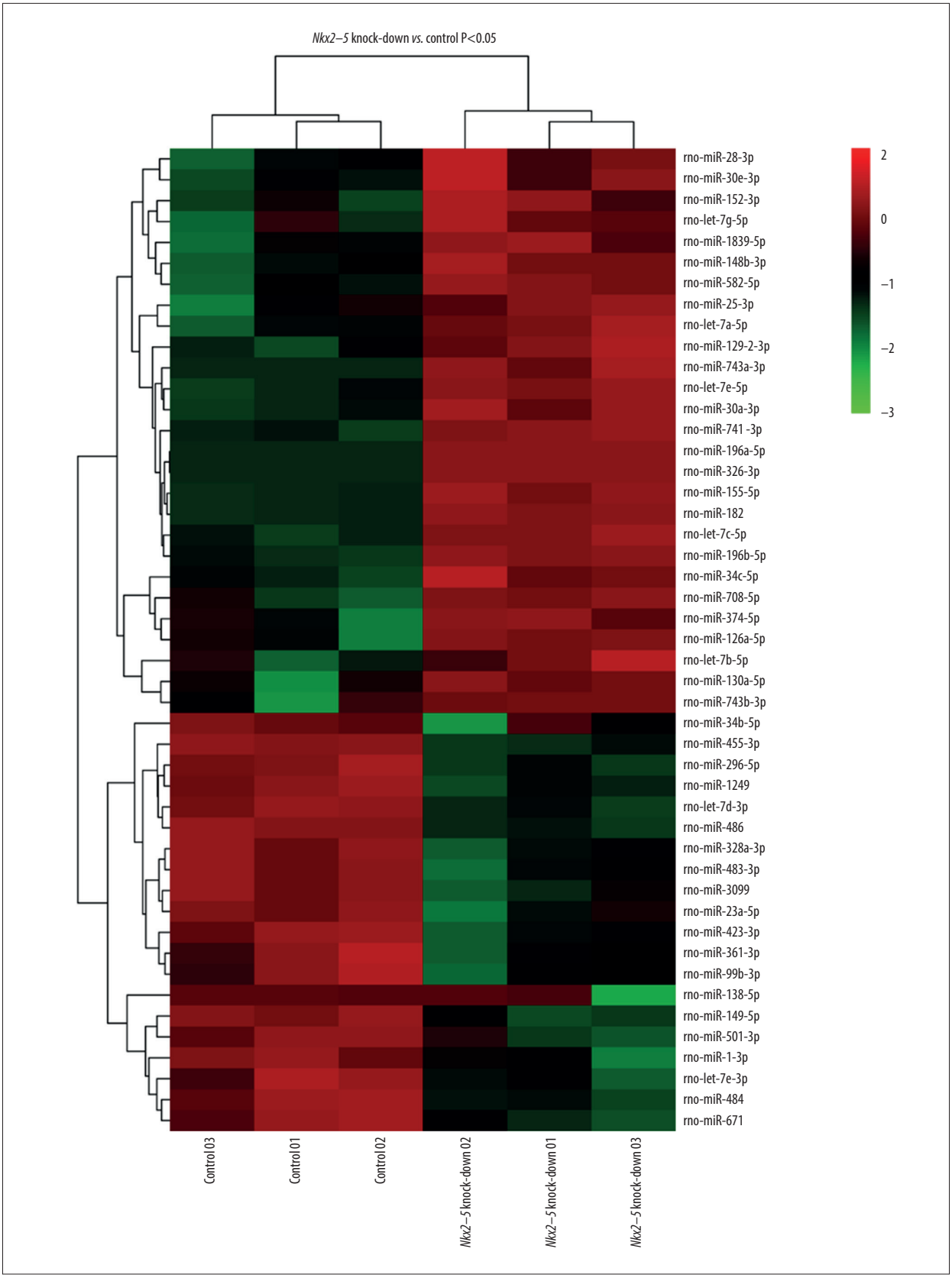

Figure 6. Heat map of the differentially expressed miRNAs following knockdown of *Nkx2–5*.

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Table 7. The expression level of the differentially expressed miRNAs.

miRNA name	Up/down	Knock-down group	Control group	Fold change	P-value
rno-let-7c-5p	Up	48915.11106	30241.59174	1.617478057	0.000585036
rno-miR-148b-3p	Up	13050.37614	8878.710132	1.469850456	0.004440698
rno-miR-34c-5p	Up	78966.93058	57614.25952	1.370614345	0.012571048
rno-let-7a-5p	Up	47559.38952	34788.86768	1.367086447	0.00358211
rno-miR-25-3p	Up	57776.68845	42623.04547	1.355526988	0.033953322
rno-miR-152-3p	Up	42183.6253	31396.35416	1.34358356	0.022837988
rno-miR-28-3p	Up	30385.28687	22978.91272	1.322311775	0.02820609
rno-let-7g-5p	Up	34278.14567	27082.60381	1.265688702	0.046242269
rno-let-7b-5p	Up	124070.9522	103767.0116	1.195668549	0.046844482
rno-let-7c-5p	Up	48915.11106	30241.59174	1.617478057	0.000585036
rno-miR-361-3p	Down	7844.3994	10147.88957	0.773007958	0.032547771
rno-miR-149-5p	Down	35717.84765	51835.03885	0.689067635	0.007257529
rno-miR-501-3p	Down	8354.323719	12609.30375	0.662552341	0.023377351
rno-miR-423-3p	Down	45506.30495	73698.63424	0.617464698	0.004694093
rno-miR-455-3p	Down	22803.13515	38129.14689	0.59804997	0.000814365
rno-let-7d-3p	Down	74365.97971	124461.4958	0.597501896	0.000455668
rno-miR-328a-3p	Down	15499.58264	28493.79288	0.543963477	0.001862949
rno-miR-296-5p	Down	6382.826735	12088.66725	0.528000863	0.004167743
rno-miR-484	Down	4410.345009	8941.350383	0.493252677	0.019675131
rno-miR-486	Down	17019.31937	55793.9353	0.305038877	0.000505176
rno-miR-1-3p	Down	56.20631293	64.31886677	0.873869764	0.049593776

genes, *Bche*, *Cd81*, *Col18a1*, *Crlf1*, *Ednra*, *Emp2*, *Hmga1*, *Ptk2b*, *Rxfp2*, and *Serpine2* were upregulated, and *Cenpe*, *Id2*, *Il1rl1*, *LOC100359539*, *Ndrg1*, *Nkx2–5*, *Ripor2*, *Tgfb2*, *Tnn*, and *Wt1* were downregulated. The expression of the genes is shown Table 4.

Knockdown of *Nkx2–5* changed the expression of genes related to migration

The knockdown of *Nkx2–5* was found to increase cell migration. To clarify the mechanisms, we selected and analyzed genes related to migration based on their FPKM values. In the *Nkx2–5* knockdown group, the results indicated that the expression of genes related to cell migration was changed. Among these genes, *Cemip*, *Tgfb2*, and *Tnn* were downregulated, and *Aqp1*, *Col18a1*, *Efna1*, *Emp2*, *Itga7*, *Lcp1*, *Ptk2b*, and *S1pr1* were upregulated. The expression of the genes is shown in Table 5.

Knockdown of *Nkx2–5* changed the expression of genes associated with cardiovascular morphogenesis, cardiovascular function, and disease

To clarify the functional mechanisms of NKX2–5 in the heart, we selected and analyzed genes associated with cardiovascular morphogenesis, function, and disease based on their FPKM values. In the *Nkx2–5* knockdown group, the results indicated that the expression of *Cited1*, *Id2*, *Lrp2*, *Olfm1*, *Olfm2*, *Pou5f1*, *Serpina3c*, *Tgfb2*, *Wnt4*, *Wt1*, and *Xdh* was downregulated, while the expression of *Aqp1*, *Cacna1g*, *Chrd*, *Dcn*, *Ednra*, *Efna1*, *Eln*, *Emp2*, *Heyl*, *Myo7a*, *Nalcn*, *Ptk2b*, *Rap1gap*, *Ren*, *S1pr1*, *Tenm4*, and *Thbs2* was upregulated. The expression of the genes is shown Table 6.

Knockdown of *Nkx2–5* changed the expression of miRNAs

To clarify the function of miRNAs in the heart, we used a *P*-value <.05, rat species, and an miRbase database to screen for differentially expressed miRNAs. The miRNAs selected through this process are depicted in the heat map shown in Figure 6. The miRNAs are listed in Table 7. The target genes

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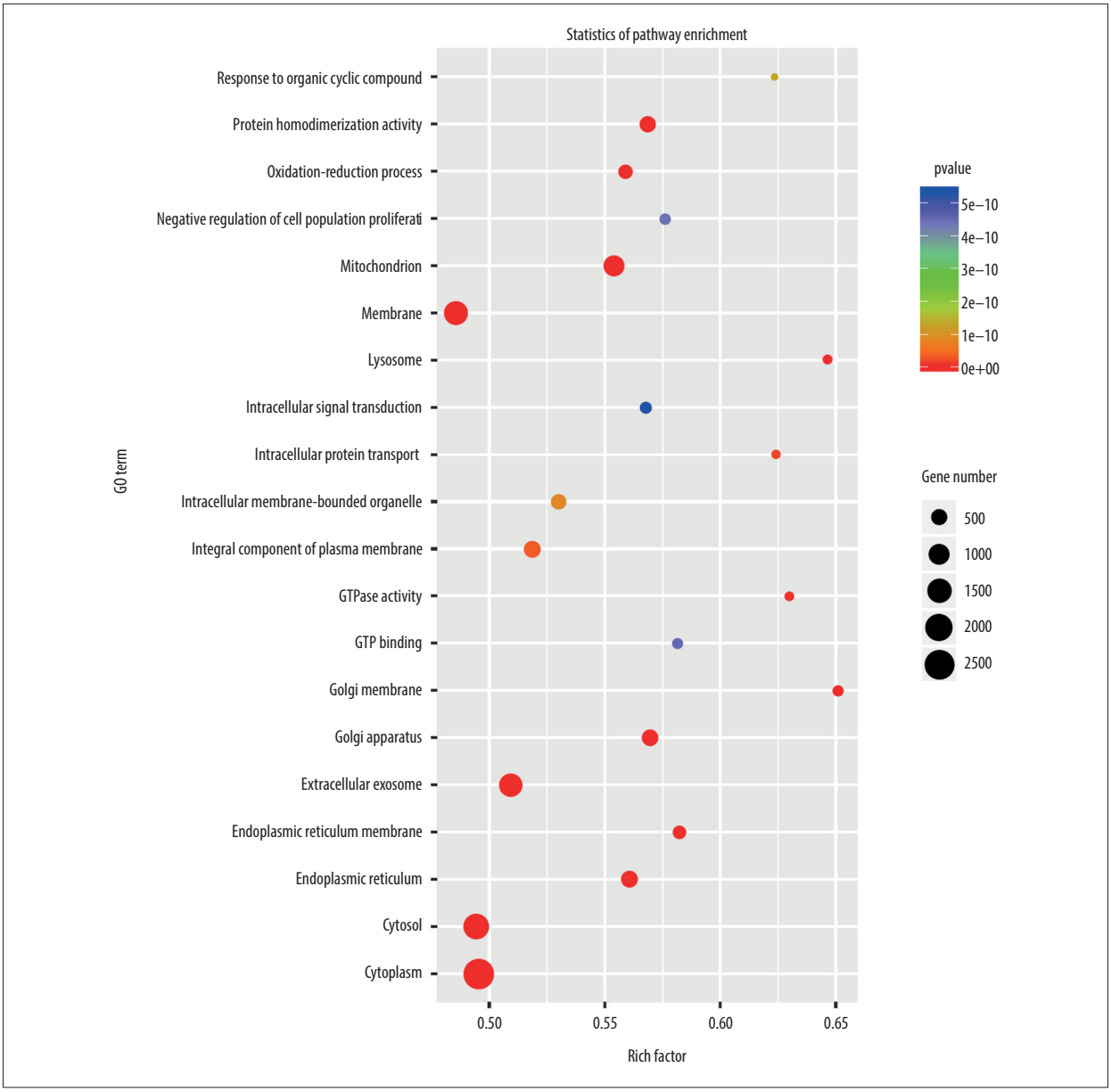

Figure 7. Gene Ontology (GO) enrichment analysis of the target genes of the differentially expressed miRNAs caused by knockdown of *Nkx2–5* indicated that those genes function in many biological processes.

of the miRNAs were analyzed after prediction, and GO analysis (Figure 7) indicated that they are related to transcriptional regulation, redox, signal transduction, apoptosis, cell differentiation, cell proliferation, protein phosphorylation, proteolysis, intracellular signal transduction, protein ubiquitin, gene expression, protein binding, metal ion binding, ATP binding, homologous protein binding, homologous domain protein dimerization body activity, DNA binding, RNA binding, zinc ion binding, and calcium ion binding. Pathway enrichment analysis (Figure 8) suggested that the target genes are enriched in the MAPK signaling pathway and other signaling pathways.

Validation of RNA-seq

We used qRT-PCR to validate the RNA-seq results. The results from the *Nkx2–5* knockdown group (Figures 9, 10) showed that the expression of *Tgfb-2* (0.62±0.03), *Wnt4* (0.56±0.19), *Xdh* (0.70±0.12), *Lrp2* (0.69±0.12), *Cited1* (0.64±0.28), *Syt1* (0.78±0.16), *Emp2* (0.15±0.06), *Pou5f* (0.75±0.11), *Itga7* (0.89±0.04), rno-miR-1-3p (0.14±0.04), rno-let-7a-5p (0.73±0.13), rno-miR-148b-3p (0.32±0.03), rno-miR-361-3p (0.09±0.01), and rno-miR-25-3p(0.45±0.03) was downregulated, and the expression of *Id2* (1.58±0.16), *Cacna1g* (1.64±0.41), *Wt1* (5.22±1.57), *Hey1* (3.01±1.62),

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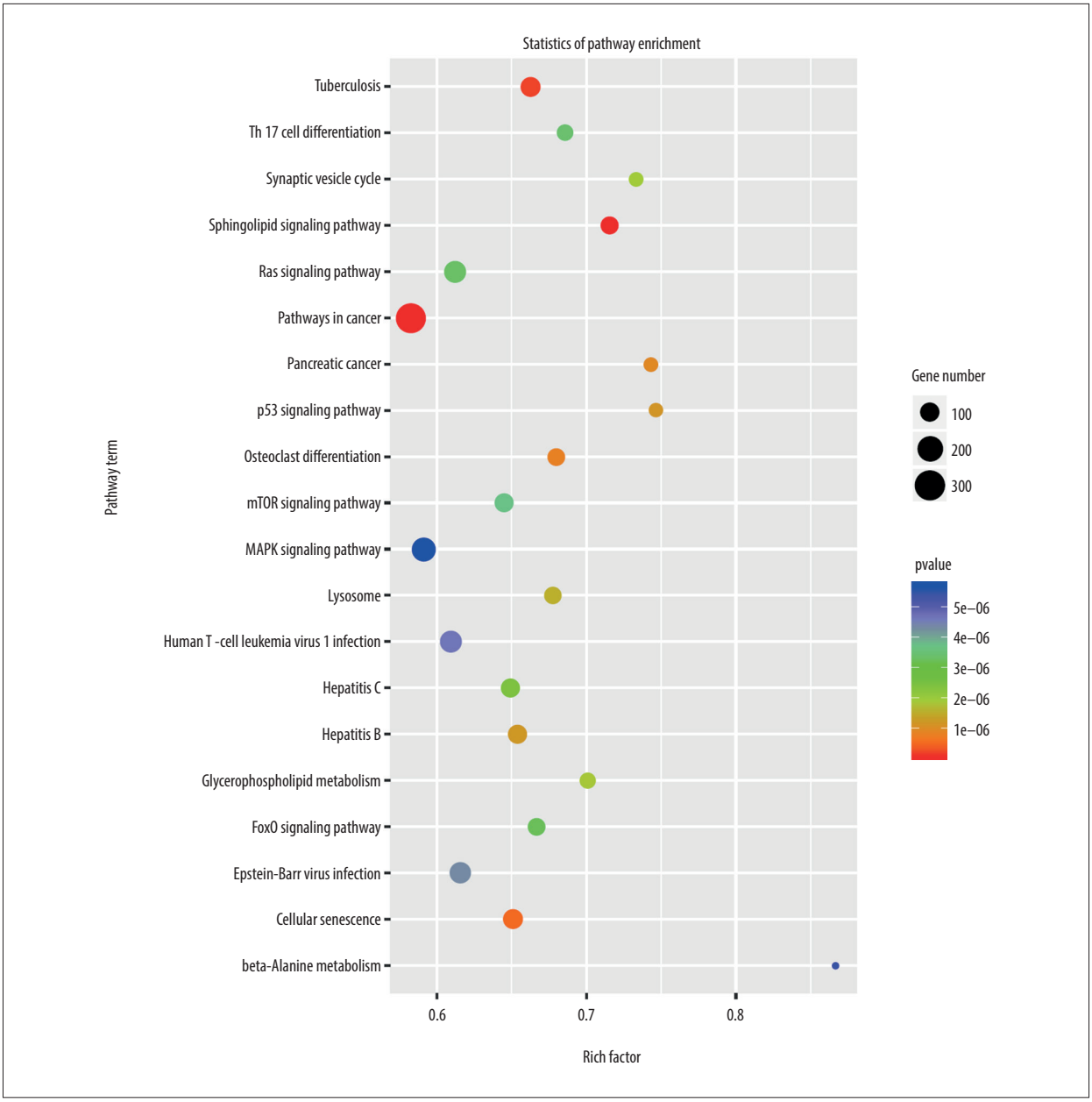

Figure 8. Pathway enrichment analysis of the target genes of miRNAs that were differentially expressed owing to knockdown of *Nkx2–5* indicated that the target genes are enriched in the MAPK signaling pathway and other signaling pathways.

Olfml (2.65±0.31), *Slrp1* (2.30±0.33), *Nectin3* (1.80±0.27), *Tenm4* (2.51±0.82), *Bmp10* (35.62±3.18), rno-miR-1-5p (1.31±0.09), rno-miR-149-5p (1.37±0.22), and rno-miR-455-3p (1.72±0.28) was upregulated.

Discussion

In this study, we found that *Nkx2–5* regulates the proliferation and migration of H9c2 cells, as well as the expression of genes associated with proliferation, migration, heart development, and heart disease. Bioinformatics analysis suggested that the genes that were differentially expressed following knockdown of *Nkx2–5* are enriched in cardiac development, calcium ion-related biological activity, the TGF-β signaling pathway, pathways related to heart diseases, the MAPK signaling pathway, and other biological processes and signaling pathways.

Cardiac development includes the proliferation, migration, and differentiation of heart precursor cells [21,22]. Cardiac development starts on both sides of the front of the mesoderm, with cells from the heart-forming regions migrating

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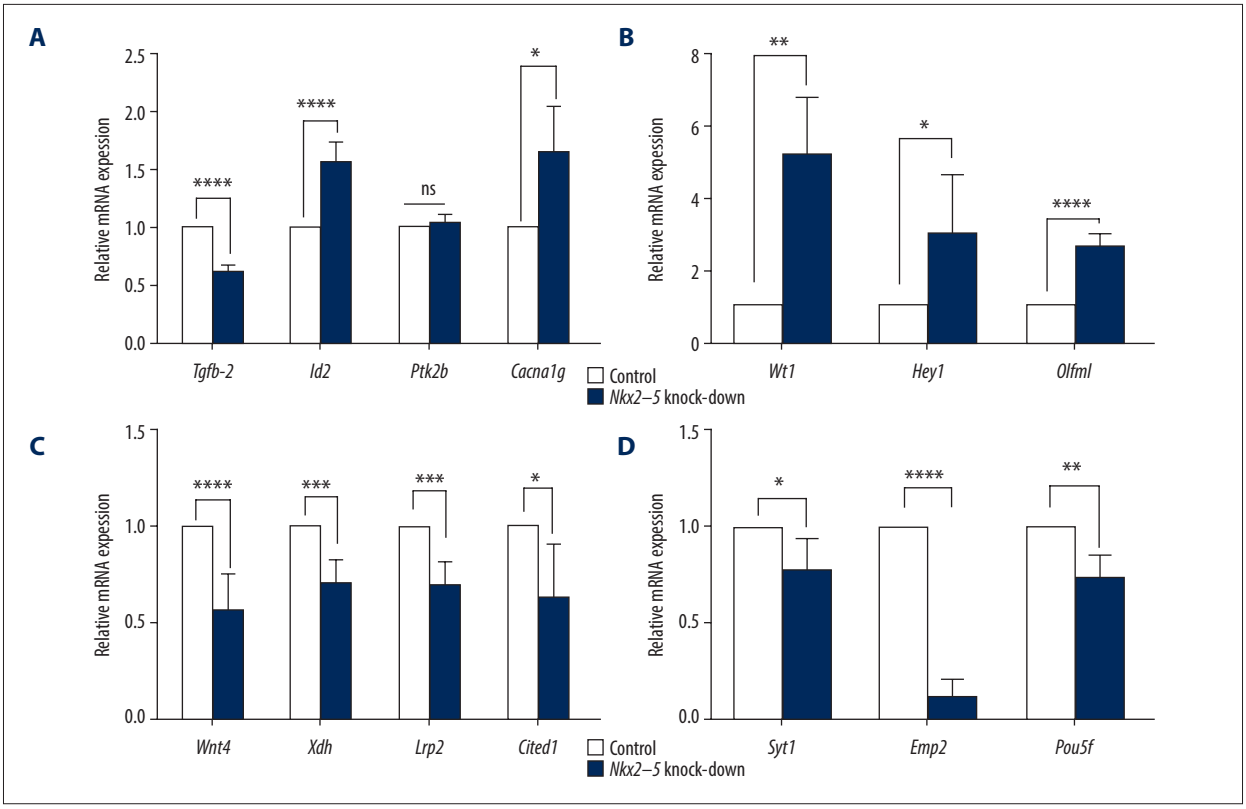

Figure 9. (**A–D**) Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis of the relative expression levels of *Tgfb-2*, *Id2*, *Ptk2b*, *Cacna1g*, *Wt1*, *Heyl*, *Olfml*, *Wnt4*, *Xdh*, *Lrp2*, *Cited1*, *Syt1*, *Emp2*, and *Pou5f* in the control group and in the *Nkx2–5* knockdown group. The expression level of the genes in the control group was calculated as 1. The bar represents the fold change of the genes in the *Nkx2–5* knockdown group compared with the control group. * *P*<.05; ** *P*<.01; *** *P*<.001; **** *P*<.0001.

and forming the heart tube [21,22]. The heart tube subsequently twists and cyclizes into a 3-dimensional heart structure [21,22]. With the differentiation of cardiac precursor cells, the heart gradually achieves contraction and relaxation related to the pumping function [23]. Therefore, the proliferation, migration, and differentiation of the heart precursor cells are necessary for cardiac morphogenesis and function. In a previous study, the knockout of *Nkx2–5* in mice resulted in abnormal heart development, growth arrest, and embryo death [24]. Histological examination revealed that the structure of the heart tube occurred normally, but the heart tube failed to twist and form the 3-dimensional heart structure [24]. In our study, the knockdown of *Nkx2–5* in H9c2 rat cardiomyocytes changed cell proliferation and migration, as well as gene expression. The results from qRT-PCR showed that in the *Nkx2–5* knockdown group, the expression of *Tgfb-2*, *Wnt4*, *Xdh*, *Lrp2*, *Cited1*, *Syt1*, *Emp2*, *Pou5f*, *Itga7*, rno-miR-1-3p, rno-let-7a-5p, rno-miR-148b-3p, rno-miR-361-3p, and rno-miR-25-3p was downregulated, and the expression of *Id2*, *Cacna1g*, *Wt1*, *Hey1*, *Olfml*, *Slrp1*, *Nectin3*, *Tenm4*, *Bmp10*, rno-miR-1-5p, rno-miR-149-5p, and rno-miR-455-3p was upregulated. Among the genes with altered expression, *Tgfb-2*, *Bmp10*, *Id2*, *Wt1*, *Hey1*, *Cacna1g*, and miR-1-3p are associated with cardiac morphogenesis and function.

TGF-β2 has functions in many biological activities [25]. Mice with dysfunction of TGF-β2 have been shown to have developmental defects of multiple organs leading to death at birth [26]. In addition, TGF-β2-deficient mice were found to develop outflow tract malformations, permanent arterial trunks, membrane peripheral VSD, aortic valve hypertrophy, tricuspid valve deformity, and complete atrioventricular septal defect [27]. These findings suggest that the disruption of TGF-β2 results in the incomplete twisting of the heart tube and abnormal development of the atrioventricular septum [27]. In our study, we found that *Nkx2–5* knockdown changed the expression of *Tgf-β2* and *Bmp10*. Further, our bioinformatics analysis indicated that the TGF-β signaling pathway was enriched. Together, these results suggest that NKX2–5 affects the TGF-β signaling pathway through *Tgf-β2*, *Bmp10*, and other genes and thereby influences the regulation of cardiac development.

Id2 is expressed in endocardial pads, inflow tracts, outflow tracts, and developing heart valves [28,29], as well as in the

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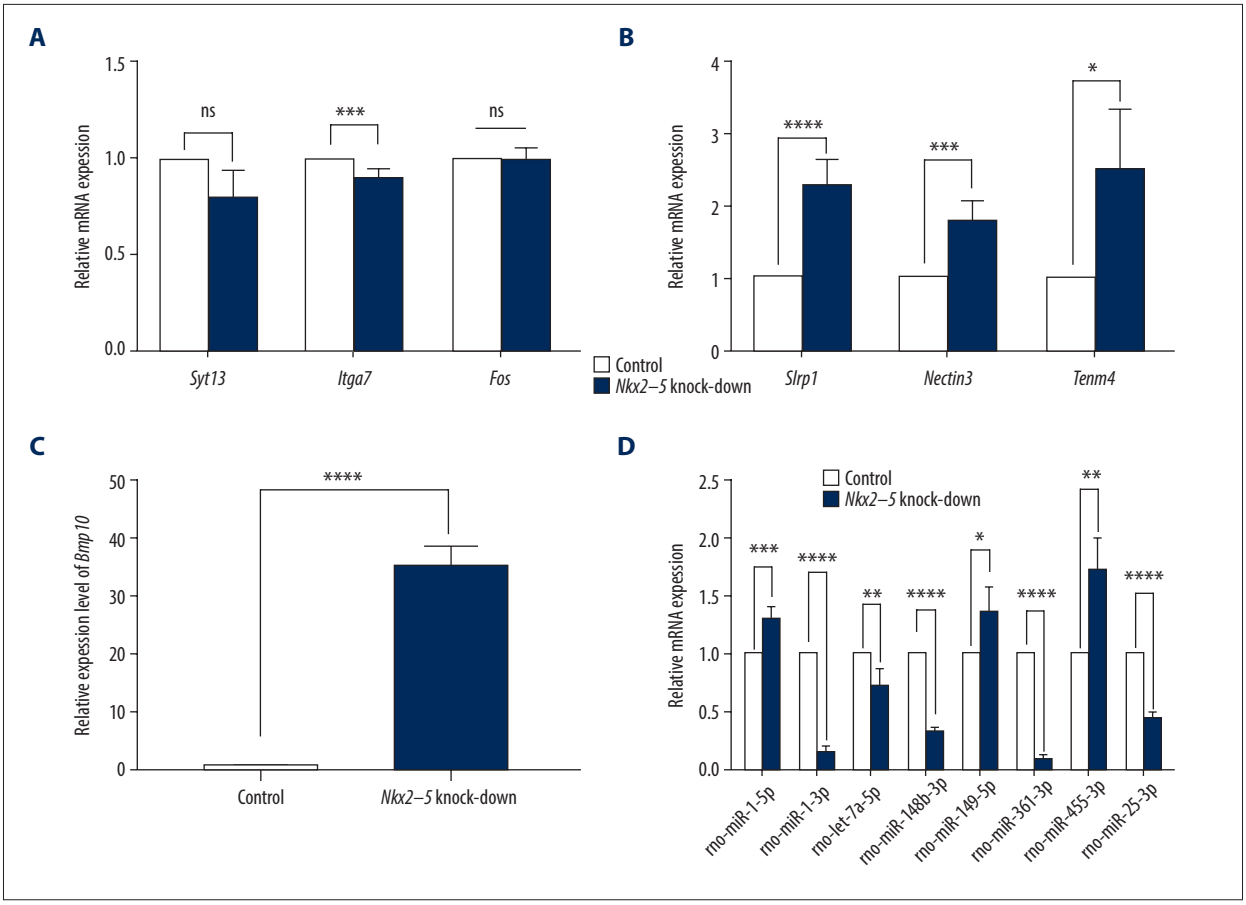

Figure 10. (**A–D**) Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis of the relative expression levels of *Syt13*, *Itga7*, *Fos*, *Slrp1*, *Nectin3*, *Tenm4*, *Bmp10*, rno-miR-1-5p, rno-miR-1-3p, rno-let-7a-5p, rno-miR-148b-3p, rno-miR-149-5p, rno-miR-361-3p, rno-miR-455-3p, and rno-miR-25-3p in the control group and in the *Nkx2–5* knockdown group. The expression level of the genes in the control group was calculated as 1. The bar represents the fold change of the genes in the *Nkx2–5* knockdown group compared with the control group. * *P*<.05; ** *P*<.01; *** *P*<.001; **** *P*<.0001.

cardiac neural crest [29,30]. The Id protein functions in myogenesis [31] and in cell growth, differentiation, and neurogenesis [32]. The disruption of *Id2* results in defects in the structures related to the cardiac neural crest [29,30]. In our study, we found that *Nkx2–5* knockdown changed the expression of *Id2*, which was similar to results reported by Lim et al. [33]. Therefore, we suggest that NKX2–5 regulates the formation of the structures related to the cardiac neural crest through *Id2*.

Wt1 functions in the epicardial epithelial-mesenchymal transition (EMT) process through *Snai1* and *Cdh1* [34]. The epicardial EMT process is thought to be involved in the development of the heart [34,35]. Knockout of *Wt1* resulted in decreased proliferation of dense myocardial cells, abnormal coronary artery formation, defects in the EMT process, and abnormal activation of the Wnt signaling pathway [34,36]. In the present study, the knockdown of *Nkx2–5* in H9c2 cells changed the expression of *Wt1*, suggesting that NKX2–5 may regulate the epicardial EMT process through *Wt1*.

Hey1 belongs to the *Hey* gene family [37], which includes *Hey1*, *Hey2*, and *HeyL* [38,39]. *Hey1* is expressed in the endocardial layer of the atrioventricular tube, which forms the membranous septum and valves of the heart [39]. Previously, knockout of *Hey1* and *HeyL* was shown to damage the endocardial EMT process and result in VSD and dysplastic valves [39,40]. The knockdown of *Nkx2–5* in H9c2 cells in the current study changed the expression of *Hey1*, suggesting that NKX2–5 may regulate the morphogenesis of the membranous septum and valves of the heart through *Hey1*.

Human *CACNA1G*, which is homologous to rat *Cacna1g*, is also called *Cav3.1*. Cav3.1 participates in the heart's electrophysiological activities [41,42]. After myocardial infarction in mice, knocking down Cav3.1 decreased the myocardial contractile function and increased the cardiac rhythm variation [43]. In our study, the knockdown of *Nkx2–5* changed the expression of *Cacna1g*, suggesting that NKX2–5 may regulate cardiac electrophysiological activity through *Cacna1g*.

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miRNA1 functions in the heart [44,45]. Previously, the knockout of miRNA1 resulted in a lack of the characteristic striped appearance in the mouse myocardium, and knockdown of miRNA1 resulted in VSD and cardiac dysfunction [46]. In our study, the knockdown of *Nkx2–5* altered the expression of rno-miR-1-3, suggesting that NKX2–5 may regulate cardiac development and function through miRNA1.

Conclusions

Nkx2–5 regulates cell proliferation and migration and the expression of genes associated with proliferation, migration, heart development, and disease in H9c2 cells. Genes associated with these activities include *Tgfb-2*, *Id2*, *Ptk2b*, *Cacna1g*, *Wt1*, *Heyl*, *Olfml*, *Wnt4*, *Xdh*, *Lrp2*, *Cited1*, *Syt1*, *Emp2*, *Pou5f*, *Syt13*, *Itga7*, *Fos*, *Slrp1*, *Nectin3*, *Tenm4*, *Bmp10*, rno-miR-1-5p, rno-miR-1-3p, rno-let-7a-5p, rno-miR-148b-3p, rno-miR-149-5p, rno-miR-361-3p, rno-miR-455-3p, and rno-miR-25-3p. Bioinformatics analysis suggested that genes that were differentially expressed because of *Nkx2–5* knockdown are enriched in cardiac development, calcium ion-related biological activity, the TGF-β signaling pathway, pathways related to heart diseases, the MAPK signaling pathway, and other biological processes and signaling pathways.

Conflict of interest

None

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