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Analyzing exosomal miRNA profiles in tetralogy of fallot fetuses' amniotic fluid

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Amniotic fluid (AF)-derived exosomal miRNA have been explored as potential contributors to the pathogenesis of Tetralogy of Fallot (TOF). This study aimed to investigate the expression profiles of AF-derived exosomal miRNAs and their potential contribution to TOF development. Exosomes were isolated from AF samples obtained from pregnant women carrying fetuses diagnosed with TOF. AF-derived exosomal miRNAs expression profiles were generated using the Agilent human miRNA Array V21.0, comparing 5 TOF samples with 5 healthy controls. Differential expression analysis identified 257 significantly dysregulated miRNAs in the TOF group. KEGG pathway enrichment analysis revealed that the predicted targets of these differentially expressed miRNAs were enriched in pathways associated with congenital disorders. Notably, 25 of these miRNAs were previously reported to be regulated by both Notch and Wnt signaling pathways, which are critical to heart development. Further investigation using mouse embryonal carcinoma P19 cells revealed that miR-10a-5p overexpression inhibited cardiomyogenic differentiation, as evidenced by the suppression of cardiomyocyte marker genes like *TBX5*. A dual-luciferase reporter assay confirmed *TBX5* as a direct target of miR-10a-5p, suggesting a regulatory mechanism involving their interaction. In summary, our study demonstrates that miR-10a-5p may contribute to the pathogenesis of TOF by impairing cardiomyocyte differentiation through direct targeting of *TBX5*. These findings enhance our understanding of TOF and its molecular underpinnings.

Congenital heart disease (CHD) is the prevalent form of birth defect, occurring in approximately 5–10 per 1,000 live births[1]. Among CHDs, Tetralogy of Fallot (TOF) is the most common cyanotic subtype, affecting around 2 to 3 per 10,000 children and accounting for 10–15% of infants with CHDs[2]. The pathophysiology of TOF involves four primary components: ventricular septal defects, obstruction of the right ventricular outflow tract, aortic dextroposition, and right ventricular hypertrophy. These abnormalities manifest during the initial eight weeks of fetal development. Prenatal detection of TOF is increasingly achieved through obstetric scanning[3]. However, our understanding of the intricate genetic basis underlying TOF remains incomplete. Several genes encoding transcription modulators, signal transduction molecules, and structural proteins have been implicated in TOF pathogenesis[4]. Notably, mutations in genes such as PTPN11, TBX5, and JAG1 have been strongly associated with an increased risk of TOF, highlighting their significant roles in the disease process. Variations within the *PTPN11*gene's linkage disequilibrium block can disrupt Ras/MAPK signaling, contributing to congenital heart disease[5]. A specific *TBX5*mutation, E128X, produces a truncated protein, potentially impacting its function and associating with CHD[6]. Additionally, *JAG1*mutations, encompassing both protein-truncating and missense variations, have been observed in some non-syndromic TOF cases[7]. Moreover, whole exome sequencing by Page et al. identified NOTCH1 and FLT4 as commonly harboring genetic variants associated with TOF[8]. These findings underscore the genetic complexity underlying TOF development. Despite these advancements, the precise molecular mechanisms contributing to fetal TOF development are still not fully elucidated.

Amniotic fluid (AF), a complex biological fluid, consists of secretions from fetal organs such as the lungs, gastrointestinal tract, placenta, and amniocytes[9]. It also contains cells from organs like the heart, kidney, liver, and hematopoietic lineages, providing a reflection of fetal health and serving as a valuable source of biomarkers for assessing fetal structural maturation or degradation[10]. Recent studies have highlighted the significance of AF in providing insights into the process of fetal organogenesis, particularly regarding the respiratory,

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cardiovascular, and excretory systems[11,12]. Amniocentesis, a commonly employed clinical procedure during the second trimester, allows for the collection of AF to identify fetal malformations and evaluate certain pregnancy-related complications, including fetal infections and chromosomal abnormalities[13]. It is important to note that amniocentesis is a safe and feasible procedure with a very low complication rate[14,15].

Exosomes are specialized extracellular vesicles (EVs) that measure between 30 and 200 nm in size and are derived from various cell types[16,17]. These membranous vesicles are found in almost all biological fluids, including AF[18]. AF-derived extracellular vesicles serve as representatives of their parent cells and are believed to play crucial roles in regulating important biological processes[19]. Growing evidence suggests that EVs, particularly exosomes, contain potential biomarkers that could enhance clinical risk assessment and fetal management[20,21]. Notably, exosomes are rich in miRNAs, mRNAs, and proteins[22]. miRNAs, a class of small noncoding RNA molecules approximately 18 to 22 nucleotides long in their mature form, have garnered significant attention due to their evolutionary conservation and their vital roles in translational repression or mRNA degradation. Numerous miRNAs have been identified as being implicated in the progression, pathogenesis, and remodeling of cardiovascular diseases[23].

In recent investigations, miRNAs have been explored as potential etiological factors in the development of TOF. Zhang et al. employed microarray analysis to identify 18 miRNAs displaying significant alterations in expression within the right ventricular outflow tract tissue of infants afflicted with TOF[24]. Similarly, Grunert et al. demonstrated that several miRNAs exhibited differing levels of target gene expression associated with TOF[25]. Exosomal miRNAs, a type of small noncoding RNA found within exosomes, are known to possess greater stability compared to free miRNAs, exerting prolonged effects on the expression of disease-related genes[26]. Recent studies have indicated that exosomal miRNAs are actively involved in intercellular communication and paracrine signaling across a range of biological processes[27]. These findings have sparked interest in exploring the potential of exosomal miRNAs derived from amniotic fluid as valuable candidates for directly investigating the etiology of TOF.

To date, no studies have explored the potential correlation between exosomal miRNAs derived from AF and the prenatal development of TOF. Therefore, the objective of this study was to investigate the expression patterns of exosomal miRNAs in AF and elucidate their involvement in TOF development. Our findings revealed that 25 exosomal miRNAs derived from AF exhibited the potential to regulate the expression of genes associated with congenital heart disease, thus representing promising candidate miRNAs implicated in TOF. Among these miRNAs, miR-10a-5p emerged as a suppressor of cardiomyocyte marker gene expression, suggesting its ability to modulate cardiomyogenic differentiation by targeting TBX5. Considering that AF-derived exosomal miRNAs can provide direct insights into the maturation and degradation of fetal structures, our study contributes to a better understanding of the etiology of fetal TOF and offers the potential for early diagnosis, facilitating timely intervention.

Methods

Ethics statement and AF samples

A total of eighteen pregnant women, aged between 21 and 37 years, were recruited for this study during the gestational period of 19 to 22^{+6} weeks. Among the participants, nine women were carrying fetuses diagnosed with TOF through fetal echocardiography **(Figure S1)**, while the remaining nine were carrying healthy fetuses, but had indications for prenatal diagnosis during the second trimester of pregnancy. These women in the control group underwent the procedure for standard prenatal diagnostic indications, such as advanced maternal age, family history of genetic disorders, or other relevant clinical factors. Fetal echocardiographic examinations were conducted on all pregnant women by two independent operators (G.R.L and Q.Y.C) in accordance with the guidelines provided by the American Society of Echocardiography. Nonsmoking status and absence of genetic or chronic disorders were inclusion criteria for all participants in this study. The clinical characteristics of the pregnant women are presented in Table 1. Maternal age, gestational age, number of deliveries, and body mass index (BMI) did not show significant differences between the TOF group and the control group ($P > 0.05$). The gestational week was determined based on the last menstrual period and an ultrasound scan conducted between gestational weeks 11 and 13^{+6}. Fetuses with abnormal karyotypes were excluded from the study. AF samples were collected by amniocentesis at the Second Affiliated Hospital of Fujian Medical University from January 2019 to June 2020, following the acquisition of informed consent from the pregnant women before any study procedures were performed. This study received approval from the Ethics Committee of the Second Affiliated Hospital of Fujian Medical University (NO. 2019 − 233). All experimental procedures adhered to the principles outlined in the World Medical Association Declaration of Helsinki.

Characteristics	Control group ($n = 9$)	TOF group ($n = 9$)
Maternal age (years)	29.8 ± 5.5	28.0 ± 6.7
Gestational age (weeks)	20.5 ± 0.9	21.2 ± 2.1
Number of deliveries	2.4 ± 0.9	2.0 ± 1.2
Body mass index (kg/m^2)	21.9 ± 1.7	22.1 ± 2.8

Table 1. Clinical characteristics of healthy pregnant women (control group) and pregnant women carrying fetuses with TOF (TOF group). Values are the mean ± SD.

AF-derived exosomes isolation and characterization

Exosomes were obtained from AF samples (10 ml) using a differential ultracentrifugation method[28]. Initially, the AF was subjected to centrifugation at 300×g to eliminate cells, followed by centrifugation at 2,000×g for 30 min to remove cellular debris. Subsequently, the resulting supernatant underwent centrifugation at 12,000×g for 45 min. The resulting supernatant was carefully transferred to an ultracentrifuge tube and subjected to centrifugation at 110,000×g for 2 h. The resulting pellet was resuspended in 10 ml of phosphate-buffered saline (PBS), filtered through a 0.22-μm filter (Millipore, USA), and subjected to centrifugation at 110,000×g for 70 min. This step is repeated twice. Finally, the resulting pellet, containing the concentrated exosome population, was resuspended in 50 μl of PBS and stored at −80 °C until further use.

The morphology of exosomes was characterized using transmission electron microscopy (TEM). Approximately 20 μl of exosomes was fixed with 2% glutaraldehyde, followed by counterstaining with 4% uranyl acetate, and stored at 4 °C until TEM analysis. Fixed exosomes were then placed onto formvar-coated 200-mesh copper grids and allowed to air-dry at room temperature for 2 min. Subsequently, the grids were negatively stained with phosphotungstic acid. Images were captured using a transmission electron microscope (FEI, USA) operated at 60 kV.

The size distribution of particles within the range consistent with exosomes was determined using nanoparticle tracking analysis (NTA). Each diluted exosome sample was subjected to three 60-second video recordings using a nanoparticle tracking analyzer (Nanosight, UK). Prior to the nanoparticle analysis, the Nanosight system was calibrated and the automatic measurement settings were configured. The samples were initially diluted at a ratio of approximately 1,000-fold, and if necessary, the concentration was adjusted to ensure that it fell within the optimal working range (20–40 particles per frame) of the instrument.

Exosomal miRNA microarray and data analysis

The microarray analysis of the isolated exosomes was conducted by CapitalBio Corporation (Beijing, China, Report NO.: JD-YX-2019-0856-JSFU-01) using the Agilent (Santa Clara, CA, USA) human miRNA Array V21.0. The Agilent array design consisted of eight identical arrays per slide (8 × 60 K format), with each array containing probes targeting 2,549 human mature miRNAs based on miRBase R21.0. Each miRNA was probed 30 times for accurate detection. The microarray experiments were performed following the manufacturer's instructions. Briefly, miRNAs were labeled using the miRNA labeling reagent (Agilent, USA). Total RNA was dephosphorylated and ligated with pCp-Cy3, and the labeled RNA was subsequently purified and hybridized to the miRNA arrays. The resulting images were scanned using a microarray scanner (Agilent, USA), and the data were gridded and analyzed using Agilent feature extraction software version 10.10. The miRNA array data obtained were processed using GeneSpring software V13 (Agilent) for tasks such as summarization, normalization, and quality control. For data preprocessing, the default 90th percentile normalization method was employed. Differentially expressed (DE) miRNAs were identified by applying threshold criteria of P-value < 0.05 and $|\log2(\text{fold change})| \geq 1$, which are considered statistically significant. Further analysis involved log2 transformation and median-centering of the data based on miRNA expression, utilizing the Adjust Data function in CLUSTER 3.0 software. To create a hierarchical clustering, average linkage was applied. Finally, the resulting clustering tree was visualized using Java Treeview (Stanford University School of Medicine, Stanford, CA, USA).

Bioinformatics analysis

The bioinformatics analysis workflow is illustrated in **Figure S2**. To identify the target genes of differentially expressed (DE) miRNAs, we utilized the miRWalk 3.0 database, which incorporates two predictive algorithms: TargetScan and miRDB. From the miRWalk 3.0 database, we obtained a comprehensive list of 74 miRNAs regulated by Notch signaling and 210 miRNAs regulated by Wnt signaling[29]. Subsequently, we conducted gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses on the miRNA target genes[30]. To refine the list of genes, the KEGG DISEASE and OMIM databases were used to identify genes with established roles or predicted associations with TOF and CHD.

P19 cells culture and cardiogenic differentiation

P19 cells were acquired from the American Type Culture Collection (ATCC, Manassas, USA) (Lot NO. 63084358). The cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (Corning, USA), 100 μg/ml streptomycin, and 100 U/ml penicillin. The cell culture was maintained in a humidified incubator at 37 °C with 5% CO_2. To initiate cardiogenic differentiation, P19 cells were grown as cell aggregates on bacteriologic dishes using DMEM containing 10% FBS and 1% dimethyl sulfoxide (Sigma-Aldrich, USA) for 4 days. Subsequently, the cell aggregates were transferred to culture flasks containing DMEM with 10% FBS. The P19 cells were harvested after 0, 5, or 10 days of differentiation.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated from the isolated exosomes using TRIzol reagent (Invitrogen, USA), and subsequently, cDNA was synthesized from the RNA using the miRNA First Strand cDNA Synthesis Kit (Sangon Biotech, China) following the manufacturer's instructions. The qRT-PCR was performed using the SYBR® Prime Script™ RT-PCR Kit (Invitrogen, USA). The primer sequences can be found in **Table S1**. All reactions were carried out in triplicate. The relative expression levels of the miRNAs were normalized to the levels of U6 small nuclear RNA, and the fold change was calculated using the 2-ΔΔCt method. The results were represented as the fold change relative to the control.

Transfection of the miRNA mimic and inhibitor

Prior to transfection, cells were cultured in 12-well or 6-well plates for a duration of 24 h. The transfection was carried out using Lipofectamine 3000 (Invitrogen, USA) with a final concentration of 50 nM for both the miRNA mimic and negative control (Sangon Biotech, China). Twenty-four hours after transfection, the cells were used for subsequent experiments. The sequences for the miRNA mimic and negative control used in the transfection are provided in **Table S2**.

Dual-luciferase reporter assay

To identify miR-10a-5p target binding sites within the 3'-untranslated region (UTR) of TBX5 mRNA, HEK293 cells were plated in 24-well plates and co-transfected with either the wild-type or mutant (carrying a single point mutation) TBX5 3'-UTR reporter plasmids using Lipofectamine 3000. After 24 h, the cells were further transfected with the miR-10a-5p mimic or inhibitor (Sangon Biotech, China). Following an additional 24-hour incubation period, the cells were collected for the luciferase activity assay, which was conducted using the Dual-Luciferase Reporter Assay System (Promega, Germany).

Statistical analysis

Statistical analyses were conducted using version 3.5.2 of the R statistical package. All experiments were independently performed in triplicate to ensure reproducibility, and the quantitative data are presented as mean ± standard deviation (SD). Statistical analyses were carried out utilizing Prism 7 software (GraphPad, Canada). To compare two groups, a two-tailed Student's t-test was employed. Statistical significance was defined as a P-value less than 0.05 ($P < 0.05$). Hierarchical clustering was carried out to assess the expression levels of differentially expressed miRNAs (DE-miRNAs). The resulting clusters were visualized through the use of a heatmap.

Results

Extraction and identification of AF-derived exosomes by TEM and NTA

AF-derived exosomes were isolated using a differential ultracentrifugation method **(Fig. S3)**. TEM analysis revealed the presence of cup-shaped particles with a size range of approximately 50–200 nm, confirming their characteristic exosomal morphology (Fig. 1A). NTA demonstrated a high concentration of vesicles within the typical exosomal size range of 50–200 nm, while larger vesicles were notably absent (Fig. 1B). Collectively, our findings provide definitive evidence for the existence of exosomes in AF. Moreover, we have successfully verified an optimized exosome isolation method specifically tailored for AF samples.

Screening of DE-miRNAs

To ascertain the distinct expression patterns of AF-derived miRNAs between TOF and control groups, we compared exosome samples from 5 fetuses with TOF and 5 normal control fetuses. Employing a cut-off filter with criteria of fold change ≥ 2 and adjusted P-value < 0.05, we identified a total of 257 differentially expressed miRNAs (DE-miRNAs). Among these, 104 miRNAs exhibited up-regulation, while 153 miRNAs displayed down-regulation in the TOF group (Fig. 2A). The clustering analysis demonstrated clear separation of miRNA expression between the TOF (T1-T5) and control (N1-N5) groups. Notably, the genomic locations of the DE-miRNAs were randomly dispersed across various human chromosomes, without the presence of any discernible clusters (Fig. 2B).

DE-miRNAs identified in our study were compared with previous studies on TOF. Upon comparison with two other studies that analyzed miRNAs in the right ventricular myocardia of TOF patients compared to healthy hearts, we found that approximately 13.6% (35 out of 257) of the DE-miRNAs overlapped. Notably, miR-193b-3p, miR-204-5p, miR-10a-5p, and miR-222-3p exhibited altered expression in all three studies[25,31] (Fig. 2C). Furthermore, we conducted a comparison between our DE-miRNAs and the altered circulating miRNAs in the maternal sera of pregnant women carrying fetuses with TOF, as well as the circulating miRNAs in the sera of TOF patients from two other studies[32,33]. It was found that approximately 6.6% (17 out of 257) of the DE-miRNAs overlapped between these two studies (Fig. 2D). Specifically, miR-15b-5p and miR-26a-5p exhibited altered expression in all three studies.

Pathway enrichment analysis of DE-miRNAs

To explore the potential functions of the 257 DE-miRNAs, we utilized the miRWalk 3.0 database to predict their target genes. This analysis yielded a total of 3,234 genes that were identified as putative target genes (Table S2). Subsequently, we assessed these target genes through gene enrichment and KEGG pathway analysis. The KEGG disease and pathway enrichment analysis revealed that these target genes were associated with signaling pathways implicated in congenital disorders (Fig. 3A). Notably, among the top 20 enriched pathways, the *Wnt*signaling pathway was prominently featured. This pathway has been widely recognized for its crucial role in regulating vertebrate heart development[8,34,35] (Fig. 3B). Further analysis using GO revealed that these genes were primarily enriched in the *Wnt* signaling pathway (GO-BP), glutamatergic synapse (GO-CC), and protein kinase binding (GO-MF) (Fig. 3C and E). These findings suggest that the identified pathways play an important role in the development of TOF.

Expression of 25 DE-miRNAs and construction of the miRNA-target genes network involved in heart development

The pivotal roles of the *Notch* and *Wnt*signaling pathways in governing vertebrate heart development, particularly in the context of TOF, have been extensively documented[8,35]. In alignment with this knowledge, our study

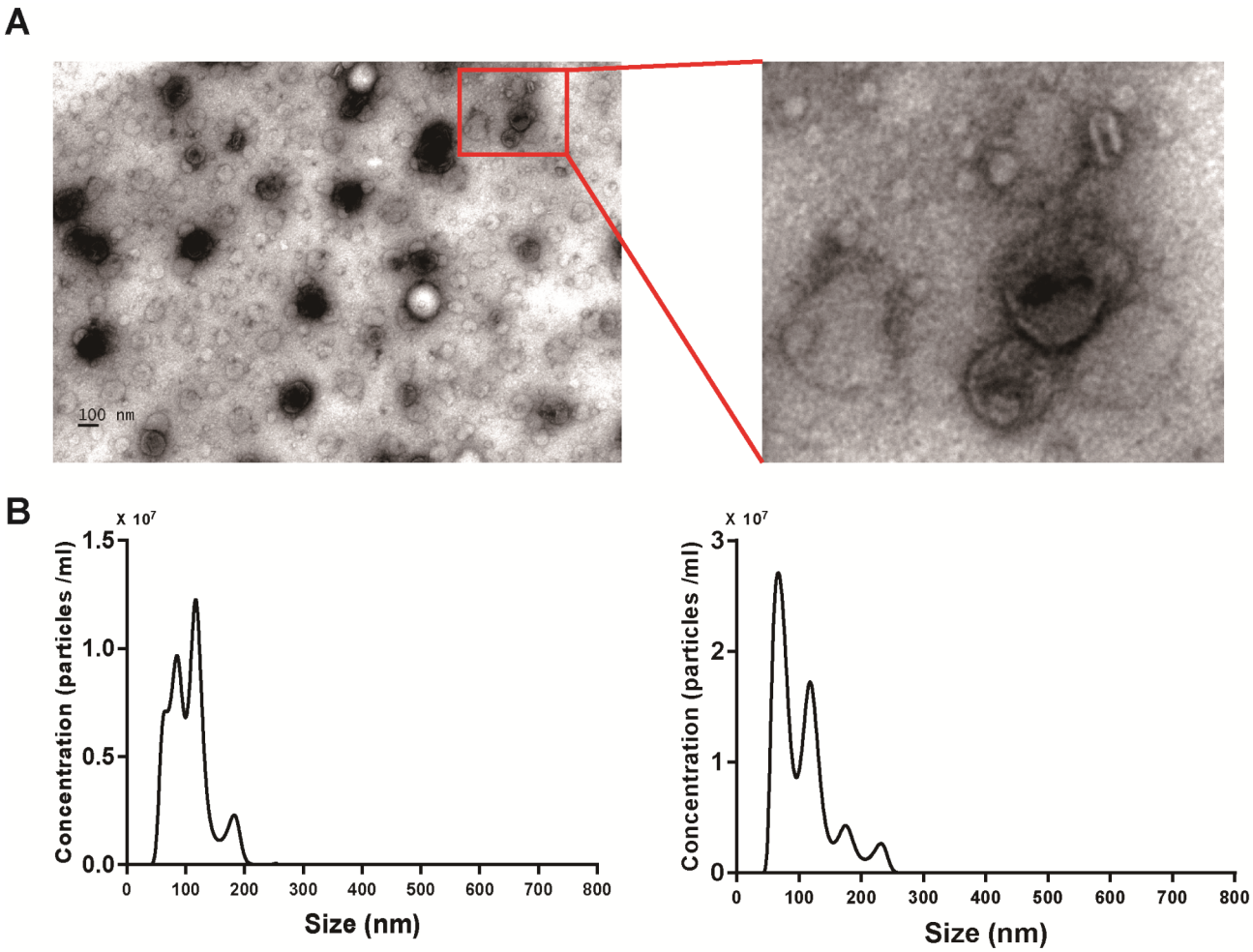

Fig. 1. Identification of human AF-derived exosomes by TEM, NTA and western blot. (**A**) Representative TEM image of AF-derived exosomes. (**B**) Representative results of NTA analysis of exosomes from human AF.

focused on elucidating the involvement of miRNAs in these pathways. Intriguingly, among the 257 DE-miRNAs identified in our investigation, a subset of 25 DE-miRNAs emerged as participants in both the *Notch* and *Wnt* signaling pathways (Fig. 4A). Among these, 23 miRNAs exhibited up-regulation and 2 miRNAs exhibited down-regulation (Fig. 4B). This compelling evidence suggests that these 25 DE-miRNAs may play a crucial role in the aberrant heart development observed in fetuses with TOF.

To investigate the potential mechanisms by which these 25 DE-miRNAs may contribute to the pathogenesis of TOF, we employed the miRWalk3.0 database to predict the target genes of these DE-miRNAs. Our comprehensive analysis revealed that 2,760 genes could potentially be targeted by these 25 DE-miRNAs (**Table S3**). Subsequently, we subjected the predicted target genes to further analysis to elucidate their functional relevance in the context of congenital heart defects. To accomplish this, we utilized the KEGG DISEASE and OMIM databases to identify a curated list of 82 genes with validated associations with TOF and CHD (**Table S4-S5**). Finally, from the pool of 82 genes, we identified 24 genes as the target genes of 18 out of the 25 DE-miRNAs (Fig. 4C). The regulatory influence of these miRNAs on the expression of these genes holds significant potential in terms of modulating heart development and contributing to CHD.

Impact of miRNAs on cardiomyogenic differentiation of P19 cells

To validate the findings obtained through microarray analysis, a total of six miRNAs (miR-200a-3p, miR-10a-5p, miR-30b-5p, miR-16-5p, miR-193b-3p, and miR-205-5p) were chosen for further analysis, encompassing those exhibiting the highest fold changes in expression between TOF and healthy tissues, as well as those demonstrating the greatest consistency among the samples. To authenticate the expression levels of these six miRNAs, we performed qRT-PCR analysis. Initially, we tested 5 TOF samples and 5 healthy samples that had been used in the microarray analysis. Additionally, an additional set of four TOF samples and four healthy samples was examined. The qRT-PCR results were consistent with the observations made through microarray analysis, reinforcing the reliability of our findings (Fig. 5A).

Subsequently, we proceeded to investigate the potential involvement of the DE-miRNAs in cardiomyogenic differentiation. Among the 25 DE-miRNAs, two miRNAs, namely miR-200a-3p and miR-10a-5p, exhibited

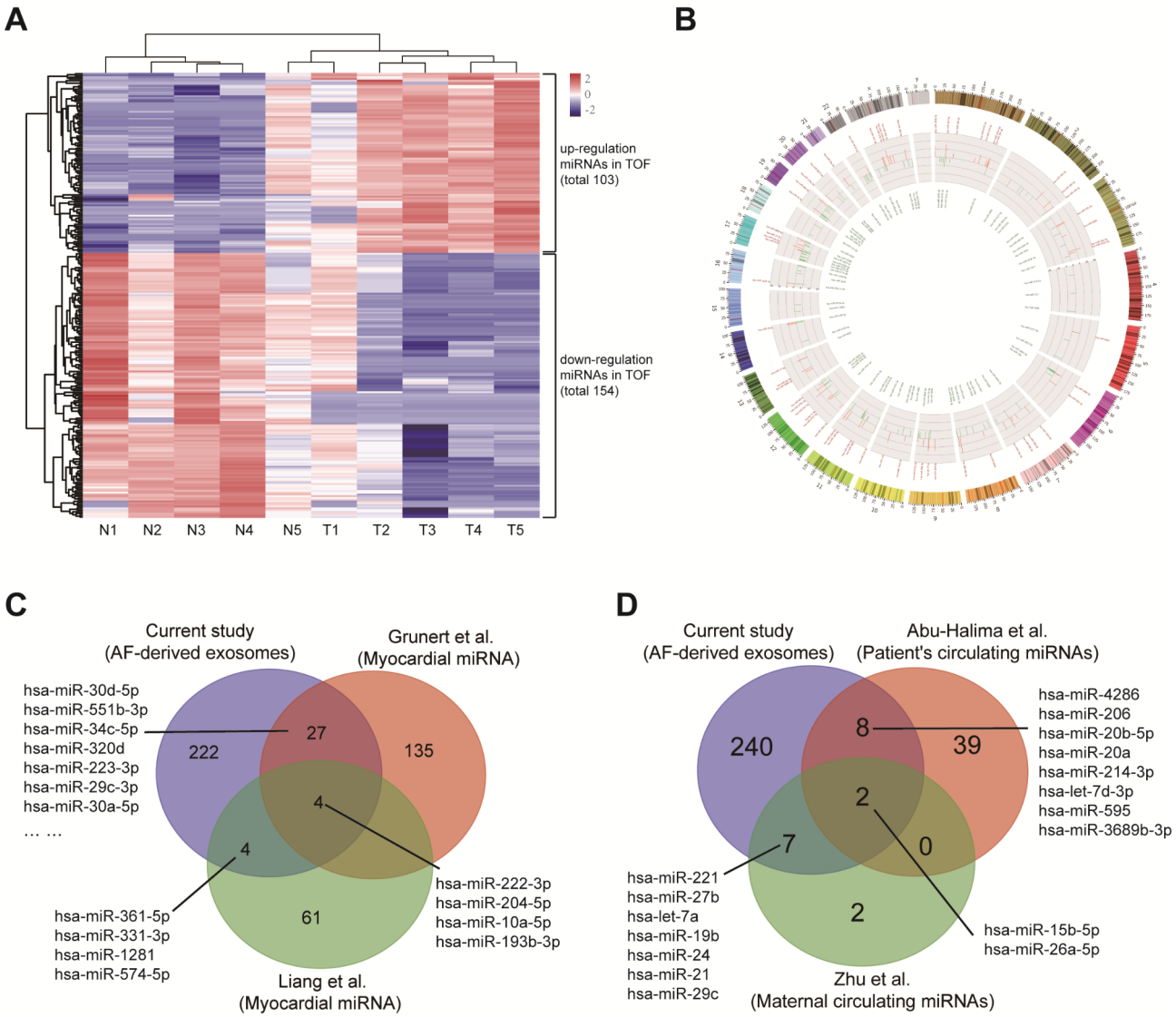

Fig. 2. Differentially expressed miRNAs in TOF AF-derived exosomes compared to AF-derived exosomes from healthy controls. (**A**) Heatmap based on the 257 DE-miRNAs in AF-derived exosomes from healthy controls (N1-N5) and fetuses with TOF (T1-T5). (**B**) The genomic locations of DE-miRNAs are distributed throughout the human chromosomes. (**C**) Overlap of DE-miRNAs in AF-derived exosomes from fetuses with TOF identified in the present study and differentially expressed myocardial miRNAs in TOF patients identified in previous studies. (**D**) Overlap of DE-miRNAs in AF-derived exosomes from fetuses with TOF identified in the present study and differentially expressed circulating miRNAs in TOF patients and circulating miRNAs in women carrying fetuses with TOF identified in previous studies.

the most substantial fold changes and were found to interact with several known genes crucial for heart development[36]. Consequently, miR-200a-3p and miR-10a-5p were selected for further functional analysis. To assess the impact of miR-200a-3p and miR-10a-5p on cardiomyogenic differentiation, we conducted transfection experiments in P19 cells using miRNA mimics or mimic controls. Subsequently, these transfected cells were induced to differentiate into cardiomyocytes. We then examined the expression of mRNA markers specific to terminally differentiated cardiomyocytes, such as *NKX2.5*, *TBX5*, and *GATA4*, which are known to play key roles in cardiomyocyte development. It is worth mentioning that the expression of these cardiomyocyte marker genes was not detectable in undifferentiated P19 cells. However, their expression significantly increased on day 10 of differentiation. Intriguingly, the overexpression of miR-10a-5p resulted in the negative regulation of cardiomyocyte marker gene expression during the differentiation process of P19 cells into cardiomyocytes (Fig. 5B). Conversely, the overexpression of miR-200a-3p only suppressed the expression of *GATA4* on day 10 (Fig. 5C). These findings provide compelling evidence that the upregulation of miR-10a-5p impedes the expression of cardiomyocyte marker genes during the cardiomyogenic differentiation of P19 cells.

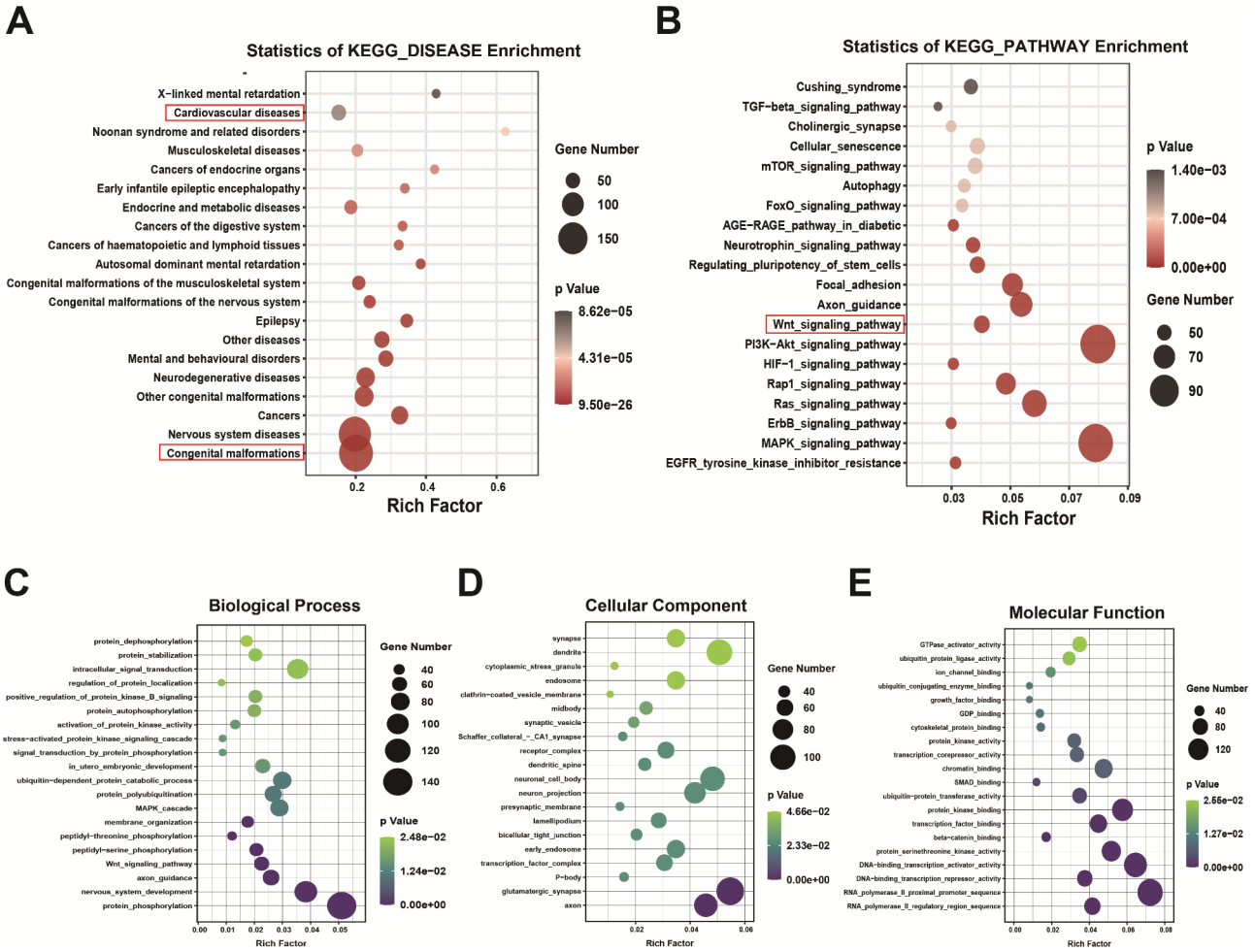

Fig. 3. Target prediction of differentially expressed miRNAs. (**A**) KEGG disease enrichment analysis of the target genes of DE-miRNAs. (**B**) The top 20 KEGG pathways based on gene enrichment of DE-miRNAs, including the *Wnt* pathway. GO-based gene enrichment analysis of the DE-miRNAs including biological process (**C**), cellular component (**D**) and molecular function (**E**).

TBX5 as a target of miR-10a-5p

To elucidate the molecular mechanism underlying the role of miR-10a-5p in fetal heart development, we utilized miRBase and TargetScan databases to predict potential target genes. Among the predictions generated by these algorithms, TBX5 emerged as the most probable target of miR-10a-5p during cardiomyogenic differentiation. Our bioinformatics analysis indicated that the 3'-untranslated region (3'-UTR) of TBX5 mRNA contains a putative binding site for miR-10a-5p (Fig. 6A).

To confirm the functional interaction between miR-10a-5p and TBX5, we cloned the segment of TBX5-3' UTR-WT containing the complementary site for miR-10a-5p, as well as a mutated version (TBX5-3' UTR-Mut), into a dual luciferase reporter system (Fig. 6B). The results of the assay revealed a significant decrease in relative luciferase activity in cells expressing TBX5-WT when transfected with the miR-10a-5p mimic, while no significant change was observed in cells expressing the mutant TBX5. Moreover, co-transfection of the miR-10a-5p inhibitor with the TBX5-WT reporter led to an increase in luciferase activity, further supporting the specific interaction between miR-10a-5p and the 3' UTR of TBX5 mRNA (Fig. 6C). Collectively, these findings provide compelling evidence that TBX5 is indeed targeted by miR-10a-5p, and the interaction between miR-10a-5p and the 3'-UTR of TBX5 mRNA plays a role in the regulation of TBX5 protein expression.

Discussion

TOF is the most prevalent cyanotic heart defect. While the etiology of idiopathic TOF (cases without 22q11.2 deletion) remains uncertain, it is likely influenced by a combination of subtle genetic, structural genomic, or epigenetic alterations interplaying with environmental stimuli[1]. Noncoding RNAs, which regulate gene expression at the posttranslational level, have emerged as key players in various cellular pathways and disease processes, including normal and abnormal heart development[37]. Specifically, miRNAs have been implicated in a range of biological processes and diseases, including TOF[38]. AF contains crucial factors for fetal development and provides valuable insights into the maturation and degeneration of fetal structures. In our study, we utilized

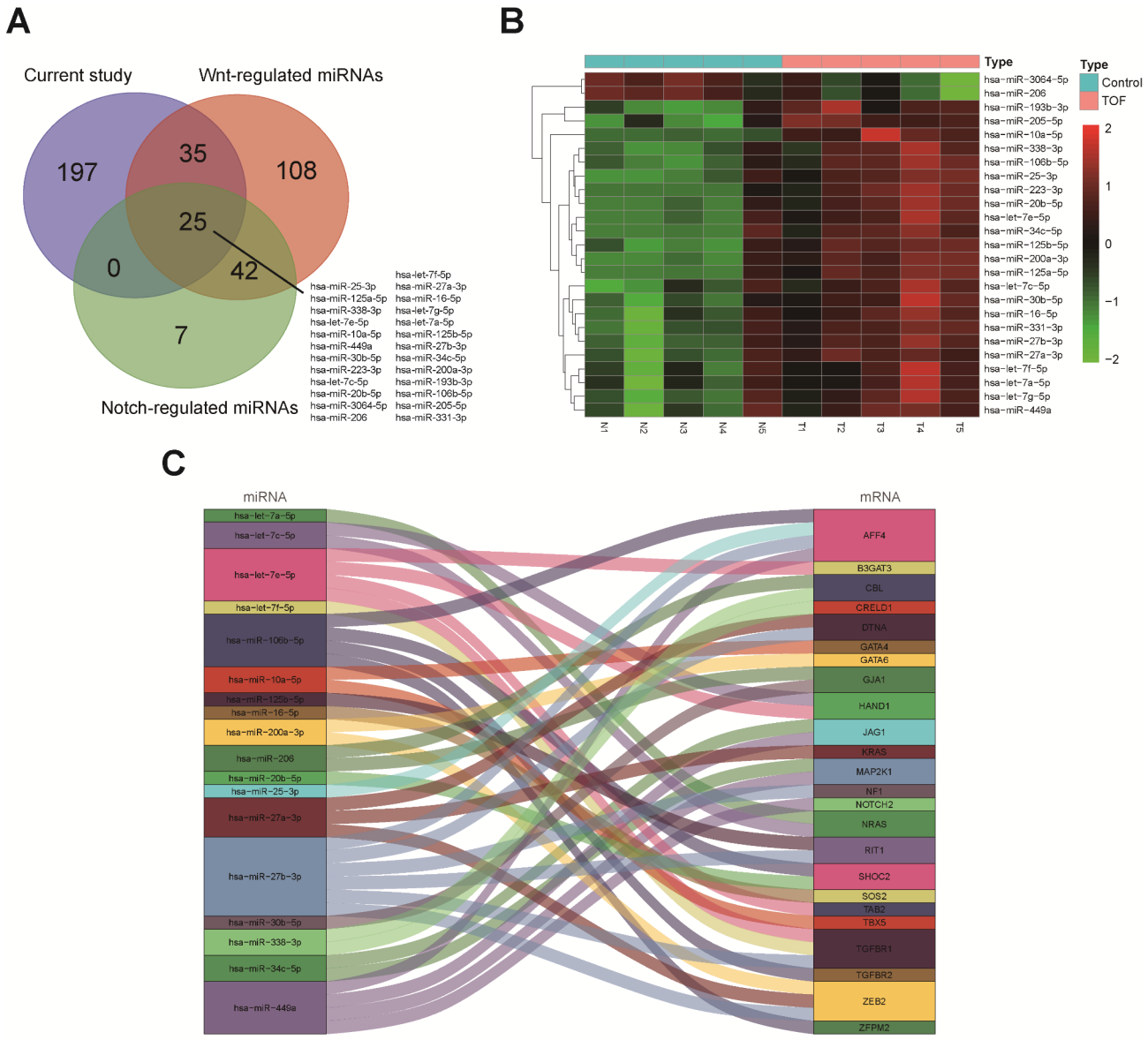

Fig. 4. Expression of 25 DE-miRNAs and Construction of the miRNAs-mRNAs Interaction Network. (**A**) Overlap of DE-miRNAs in AF-derived exosomes from fetuses with TOF identified in the present study versus *Notch*-regulated and *Wnt*-regulated miRNAs. (**B**) Heatmap of the 25 of 257 miRNAs in this study overlapped with *Notch*-regulated and *Wnt*-regulated miRNAs simultaneously. (**C**) The predicted target genes of the 25 DE-miRNAs were identified by screening the miRWalk3.0 database. The network map indicated that 18 DE-miRNAs targeted 24 genes involving in tetralogy of Fallot and congenital heart diseases.

TEM, NTA, and western blotting techniques to confirm the presence of exosomes in AF. Subsequently, we conducted a microarray analysis to compare the miRNA profiles of exosomes derived from AF samples obtained from fetuses diagnosed with TOF and healthy fetuses. Through this analysis, we identified a panel of 25 miRNAs present in AF-derived exosomes that are likely to play significant roles in the embryonic development of the heart.

miRNAs have been extensively studied in various pathophysiological processes and cardiovascular diseases, including TOF[39]. Previous research has highlighted the critical roles of specific miRNAs in heart development and function. For instance, Bittel et al. demonstrated the overexpression of miR-421 in the right ventricle tissues of infants with TOF, and its influence on the expression of *SOX4*, a key regulator in the Notch pathway[40]. Additionally, Liang et al. reported the significant downregulation of miR-940 in TOF patients, potentially due to its interaction with *JARID2*, thus implicating miR-940 in the development of TOF[31]. Additionally, aberrant expression of miR-1, miR-133[41], and miR-29[32] has been reported in TOF hearts. However, most of these investigations have focused on the analysis of miRNA expression profiles in the peripheral blood or cardiac tissues of TOF patients.

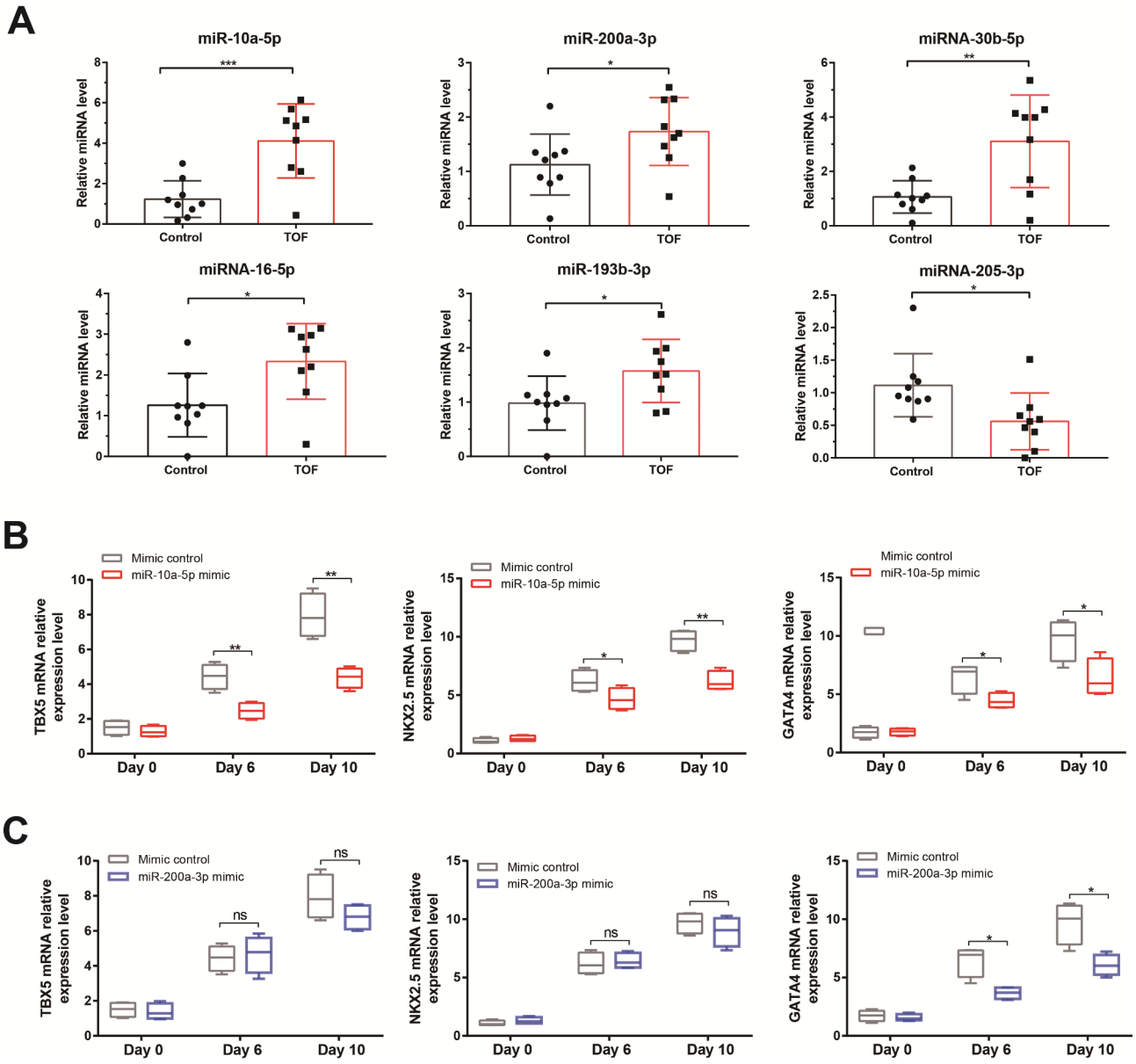

Fig. 5. Effect of miR-10a-5p and miR-200a-3p on P19 cell differentiation. (**A**) Six miRNAs with the largest fold changes in expression between TOF and control group were validated in all 5 TOF and 5 healthy samples used for array analysis and in 4 additional paired samples by qRT-PCR analysis. The data were representative of at least three independent experiments. (**B**) P19 embryonic carcinoma stem cells were transfected with miR-10a-5p mimic, and the cells were stimulated with 1% DMSO to induce differentiation into cardiomyocytes. qRT-PCR was used to analyze the expression of cardiomyocyte marker gene transcripts (*TBX5*, *NKX2.5*, and *GATA4*) during differentiation. The results show that upregulation of miR-10a-5p inhibited the expression of cardiomyocyte marker genes. (**C**) Overexpression of miR-200a-3p inhibited the expression of *GATA4*, but had no effect on the expression of *TBX5* and *NKX2.5*. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

In our study, we focused on miRNAs present in exosomes isolated from AF of TOF fetuses. AF-derived exosomes, released by fetal cells, carry valuable information about the overall physiological state of the fetus and offer potential insights into fetal organogenesis and degeneration, including cardiovascular development[42]. By utilizing microarray analysis, we identified 257 differentially expressed miRNAs in AF-derived exosomes from TOF fetuses compared to healthy fetuses. Interestingly, despite previous reports associating specific miRNAs (miR-1, miR-133, miR-499, miR-940, and miR-421) with TOF, we did not observe significant alterations in their expression profiles in our study. This discrepancy could be attributed to the different sources of the miRNAs, which may reflect distinct aspects of TOF pathogenesis. It is plausible that exosomal miRNAs derived from AF provide a more accurate representation of the genetic causes underlying TOF[43].

Several recent studies have highlighted the decreased expression of genes associated with the Notch and Wnt signaling pathways in individuals with TOF[8,34,35]. Additionally, altered expression of several miRNAs predicted

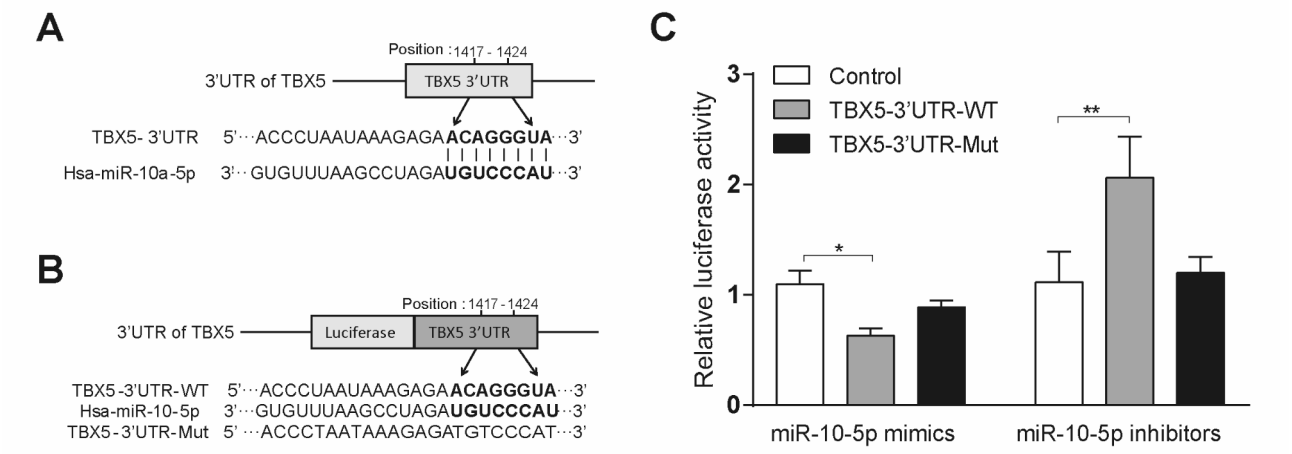

Fig. 6. TBX5 is a direct target of miR-10a-5p. (**A**) The binding site of miR-10a-5p in the 3′-UTR of TBX5 was predicted by bioinformatics analyses. (**B**) Luciferase reporters expressing the wild-type or mutated 3′-UTR of TBX5 were designed according to miR-10a-5p seed sequences. (**C**) A dual-luciferase reporter assay was performed to verify that miR-10a-5p regulated the luciferase activity of TBX5 3'UTR- wild-type but did not influence that of Tbx5 3'UTR-mutated. $^{**}P < 0.01$.

to interact with these pathways has been observed in TOF patients[24,25,40]. In our study, we aimed to identify DE-miRNAs that have a direct impact on gene expression in TOF by comparing them to *Notch*- and *Wnt*-regulated miRNAs. Out of the 257 miRNAs analyzed, 25 overlapped with miRNAs regulated by Notch and Wnt signaling, including miR-19b[32]and let-7e-5p[44], which have been implicated in TOF. Furthermore, the expression of several well-established TOF-associated genes, such as *GATA4*, *NKX2.5*, *JAG1*, *FOXC2*, *TBX5*, and *TBX1*, has been found to be correlated with TOF[2,8]. Cardiac development is a complex process, and even minor disruptions can have catastrophic consequences. Therefore, it is not surprising that numerous transcription factors and signaling pathways involved in cardiogenesis have been implicated in TOF[2]. These well-established TOF-related genes and pathways provide a solid foundation for comprehending the role of miRNAs in TOF development and have the potential to uncover valuable biomarkers for prenatal TOF diagnosis.

The transcription factors *NKX2.5*, *GATA4*, and *TBX5*are widely recognized genetic markers of cardiomyocyte differentiation[45]. In our experiments, we found that miR-10a-5p was significantly upregulated in AF-derived exosomes from TOF fetuses and had inhibitory effects on the expression of cardiomyocyte marker genes, particularly *TBX5*. *TBX5*, a member of the T-box transcription factor family, is a well-established regulator of tissue development[46]. To validate the direct interaction between miR-10a-5p and *TBX5*, we constructed a luciferase reporter plasmid and confirmed their interaction. Nevertheless, it is important to note that miR-10a-5p possesses numerous downstream target genes, and some of these genes may indirectly modulate the expression of *TBX5*[47,48]. Therefore, solely relying on our in vitro experiments, we were unable to establish a definitive direct inverse relationship between miR-10a-5p and *TBX5*. Notably, previous studies have demonstrated that miR-10a-5p directly targets TBX5 by binding to its 3'-untranslated region, which leads to the downregulation of TBX5 expression, thereby promoting apoptosis and reducing cell proliferation[49]. This interaction has significant implications for cardiac development, as TBX5 is a critical transcription factor involved in cardiomyocyte differentiation and heart morphogenesis[50]. The negative regulation of TBX5 by miR-10a-5p suggests a mechanism by which miR-10a-5p modulates cell proliferation and apoptosis in cardiac cells. These findings align with our results, indicating that the dysregulation of the miR-10a-5p/TBX5 axis may play a significant role in the pathogenesis of TOF.

Hence, based on our in vitro experiments, we were unable to establish a direct inverse relationship between miR-10a-5p and *TBX5*. However, recent advancements in induced pluripotent stem cells (iPSCs) have provided a valuable tool for in vitro modeling of CHD and have enabled a better understanding of the underlying mechanisms involved in different forms of CHD. CHD often arises from disturbances in cardiovascular cell differentiation and migration in vivo, and iPSC-derived cardiomyocytes have the potential to replicate the genetic and epigenetic alterations that contribute to the cellular basis of CHD. Collaborative efforts with the Pediatric Cardiac Genomics Consortium have sparked a multitude of ongoing research projects focused on utilizing hiPSC cardiomyocyte models to investigate various forms of CHD[51]. In line with this, our study utilized pluripotent P19 cells as a myocardial cell model to explore the influence of miR-10a-5p and *TBX5* on the developmental mechanisms leading to cardiac structural malformations.

Our study provides novel insights into the role of AF-derived exosomal miRNAs in the pathogenesis of TOF. However, several limitations must be acknowledged. First, our sample size was relatively small, which may limit the generalizability of our findings. Larger cohort studies are necessary to validate these results. Second, while our control group consisted of pregnancies undergoing amniocentesis for standard prenatal diagnostic indications unrelated to TOF (such as advanced maternal age or family history of genetic disorders), the presence of other underlying conditions in these pregnancies cannot be entirely ruled out and may influence exosomal miRNA profiles. Finally, while we demonstrated the impact of miR-10a-5p on cardiomyocyte marker gene expression

in vitro, further investigation is necessary to fully elucidate the phenotypic effects of miR-10a-5p upregulation on cardiomyocyte differentiation in vivo, including direct assessment of differentiation kinetics and potential broader developmental consequences.

Conclusion

In conclusion, our study successfully confirmed the presence of exosomes in AF and provided initial insights into the dysregulated expression of miRNAs in exosomes derived from the AF of fetuses with TOF. Through our analysis, we identified 25 miRNAs that exhibited differential expression between AF-derived exosomes from TOF fetuses and those from healthy fetuses. Moreover, our findings demonstrated that miR-10a-5p exerts inhibitory effects on the expression of cardiomyocyte marker genes and may play a role in modulating cardiomyogenic differentiation by directly targeting *TBX5*. However, it is important to note that our understanding of the genetic mechanisms underlying TOF remains incomplete, and the outcomes presented in this study highlight the need for further investigations in this field.

Data availability

The data presented in this study is available on the GEO database (accession numbers: GSE186059).

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References

1. Hill, M. C. et al. Integrated multi-omic characterization of congenital heart disease. *Nature* **608**, 181–191. https://doi.org/10.1038/s41586-022-04989-3 (2022).
2. Morgenthau, A. & Frishman, W. H. Genetic Origins of Tetralogy of Fallot. *Cardiol. Rev.* **26**, 86–92. https://doi.org/10.1097/CRD.0000000000000170 (2018).
3. Mamalis, M. et al. Comparison of the Results of Prenatal and Postnatal Echocardiography and Postnatal Cardiac MRI in Children with a Congenital Heart Defect. *J. Clin. Med.* **12** https://doi.org/10.3390/jcm12103508 (2023).
4. Gou, Z. et al. Prenatal diagnosis and mRNA profiles of fetal tetralogy of Fallot. *BMC pregnancy childbirth*. **22**, 853. https://doi.org/10.1186/s12884-022-05190-0 (2022).
5. Goodship, J. A. et al. A common variant in the PTPN11 gene contributes to the risk of tetralogy of Fallot. *Circ. Cardiovasc. Genet.* **5**, 287–292. https://doi.org/10.1161/circgenetics.111.962035 (2012).
6. Khatami, M., Heidari, M. M., Kazeminasab, F. & Zare Bidaki, R. Identification of a novel non-sense mutation in TBX5 gene in pediatric patients with congenital heart defects. *J. Cardiovasc. Thorac. Res.* **10**, 41–45. https://doi.org/10.15171/jcvtr.2018.07 (2018).
7. Safari-Arababadi, A., Behjati-Ardakani, M., Kalantar, S. M. & Jaafarinia, M. Silencing mutations in JAG1 gene may play crucial roles in the pathogenesis of Tetralogy of Fallot. *Cell. Mol. Biol. (Noisy-le-grand)*. **64**, 103–107 (2018).
8. Page, D. J. et al. Whole Exome Sequencing Reveals the Major Genetic Contributors to Nonsyndromic Tetralogy of Fallot. *Circ. Res.* **124**, 553–563. https://doi.org/10.1161/circresaha.118.313250 (2019).
9. Bowen, C. M., Ditmars, F. S., Gupta, A., Reems, J. A. & Fagg, W. S. Cell-Free Amniotic Fluid and Regenerative Medicine: Current Applications and Future Opportunities. *Biomedicines* **10** https://doi.org/10.3390/biomedicines10112960 (2022).
10. Bhatti, G. et al. The amniotic fluid proteome changes with term labor and informs biomarker discovery in maternal plasma. *Sci. Rep.* **13**, 3136. https://doi.org/10.1038/s41598-023-28157-3 (2023).
11. Zwemer, L. M. & Bianchi, D. W. The amniotic fluid transcriptome as a guide to understanding fetal disease. *Cold Spring Harb Perspect. Med.* **5** https://doi.org/10.1101/cshperspect.a023101 (2015).
12. Goumy, C. et al. Reduced telomere length in amniocytes: an early biomarker of abnormal fetal development? *Hum. Mol. Genet.* **31**, 2669–2677. https://doi.org/10.1093/hmg/ddac054 (2022).
13. Evans, M. I., Andriole, S. & Evans, S. M. Genetics: update on prenatal screening and diagnosis. *Obstet. Gynecol. Clin. North. Am.* **42**, 193–208. https://doi.org/10.1016/j.ogc.2015.01.011 (2015).
14. Li, Y. et al. The application of late amniocentesis: a retrospective study in a tertiary fetal medicine center in China. *BMC pregnancy childbirth*. **21**, 266. https://doi.org/10.1186/s12884-021-03723-7 (2021).
15. Short, D. J. et al. The safety, acceptability, and success rates of amniocentesis in the context of preterm prelabor rupture of membranes and threatened preterm labor: a systematic review and meta-analysis. *J. Maternal-Fetal Neonatal Med.* **37**, 2332784. https://doi.org/10.1080/14767058.2024.2332784 (2024).
16. Jeppesen, D. K. et al. Reassessment of Exosome Composition. *Cell* **177**, 428–445e418. https://doi.org/10.1016/j.cell.2019.02.029 (2019).
17. Pegtel, D. M., Gould, S. J. & Exosomes *Annu. Rev. Biochem.* **88**, 487–514 https://doi.org/10.1146/annurev-biochem-013118-111902 (2019).
18. Ebert, B. & Rai, A. J. Isolation and Characterization of Amniotic Fluid-Derived Extracellular Vesicles for Biomarker Discovery. *Methods Mol. Biol.* **1885**, 287–294. https://doi.org/10.1007/978-1-4939-8889-1_19 (2019).
19. Stremersch, S., De Smedt, S. C. & Raemdonck, K. Therapeutic and diagnostic applications of extracellular vesicles. *J. Control Release*. **244**, 167–183 (2016).
20. Dixon, C. L. et al. Amniotic fluid exosome proteomic profile exhibits unique pathways of term and preterm labor. *Endocrinology* **159**, 2229–2240. https://doi.org/10.1210/en.2018-00073 (2018).
21. Nguyen, C. M. et al. Placental Exosomes as Biomarkers for Maternal Diseases: Current Advances in Isolation, Characterization, and Detection. *ACS Sens.* https://doi.org/10.1021/acssensors.3c00689 (2023).
22. Rezaie, J., Feghhi, M. & Etemadi, T. A review on exosomes application in clinical trials: perspective, questions, and challenges. *Cell. Commun. Signal.* **20**, 145. https://doi.org/10.1186/s12964-022-00959-4 (2022).
23. Davidson, S. M. et al. Methods for the identification and characterization of extracellular vesicles in cardiovascular studies: from exosomes to microvesicles. *Cardiovasc. Res.* **119**, 45–63. https://doi.org/10.1093/cvr/cvac031 (2023).
24. Zhang, J. et al. MicroRNA deregulation in right ventricular outflow tract myocardium in nonsyndromic tetralogy of fallot. *Can. J. Cardiol.* **29**, 1695–1703. https://doi.org/10.1016/j.cjca.2013.07.002 (2013).
25. Grunert, M., Appelt, S., Dunkel, I., Berger, F. & Sperling, S. R. Altered microRNA and target gene expression related to Tetralogy of Fallot. *Sci. Rep.* **9**, 19063. https://doi.org/10.1038/s41598-019-55570-4 (2019).
26. Xu, J. et al. Progress in research on the role of exosomal miRNAs in the diagnosis and treatment of cardiovascular diseases. *Front. Genet.* **13**, 929231. https://doi.org/10.3389/fgene.2022.929231 (2022).

27. Tian, C., Gao, L., Zimmerman, M. C. & Zucker, I. H. Myocardial infarction-induced microRNA-enriched exosomes contribute to cardiac Nrf2 dysregulation in chronic heart failure. *Am. J. Physiol. Heart Circ. Physiol.* **314**, H928–h939. https://doi.org/10.1152/ajpheart.00602.2017 (2018).
28. Théry, C., Amigorena, S., Raposo, G. & Clayton, A. Isolation and characterization of exosomes from cell culture supernatants and biological fluids. *Curr. Protoc. Cell. Biol.* **30**, 32221–232229 (2006).
29. Dweep, H. & Gretz, N. miRWalk2.0: a comprehensive atlas of microRNA-target interactions. *Nat. Methods.* **12**, 697. https://doi.org/10.1038/nmeth.3485 (2015).
30. Kanehisa, M. & Goto, S. KEGG: kyoto encyclopedia of genes and genomes. *Nucleic Acids Res.* **28**, 27–30. https://doi.org/10.1093/nar/28.1.27 (2000).
31. Liang, D. et al. miRNA-940 reduction contributes to human Tetralogy of Fallot development. *J. Cell. Mol. Med.* **18**, 1830–1839. https://doi.org/10.1111/jcmm.12309 (2014).
32. Zhu, S. et al. Identification of maternal serum microRNAs as novel non-invasive biomarkers for prenatal detection of fetal congenital heart defects. *Clin. Chim. Acta.* **424**, 66–72. https://doi.org/10.1016/j.cca.2013.05.010 (2013).
33. Abu-Halima, M., Meese, E., Keller, A., Abdul-Khaliq, H. & Radle-Hurst, T. Analysis of circulating microRNAs in patients with repaired Tetralogy of Fallot with and without heart failure. *J. Transl Med.* **15**, 156. https://doi.org/10.1186/s12967-017-1255-z (2017).
34. Kozyrev, I. et al. Dysregulation of Notch signaling in cardiac mesenchymal cells of patients with tetralogy of Fallot. *Pediatr. Res.* **88**, 38–47. https://doi.org/10.1038/s41390-020-0760-6 (2020).
35. Matos-Nieves, A., Yasuhara, J. & Garg, V. Another Notch in the genetic puzzle of tetralogy of Fallot. *Circ. Res.* **1244**, 462–464. https://doi.org/10.1161/CIR.0000000000000606 (2019).
36. Hussain, N. et al. Down-regulation of miR-10a-5p in synoviocytes contributes to TBX5-controlled joint inflammation. *J. Cell. Mol. Med.* **22**, 241–250. https://doi.org/10.1111/jcmm.13312 (2018).
37. Yu, H., Wang, X. & Cao, H. Construction and investigation of a circRNA-associated ceRNA regulatory network in Tetralogy of Fallot. *BMC Cardiovasc. Disord.* **21**, 437. https://doi.org/10.1186/s12872-021-02217-w (2021).
38. Smith, T., Rajakaruna, C., Caputo, M. & Emanueli, C. MicroRNAs in congenital heart disease. *Ann. Transl Med.* **3**, 333. https://doi.org/10.3978/j.issn.2305-5839.2015.12.25 (2015).
39. Yang, Q. et al. Aberrant expression of miR-29b-3p influences heart development and cardiomyocyte proliferation by targeting NOTCH2. *Cell Prolif*, e12764 (2020). https://doi.org/10.1111/cpr.12764
40. Bittel, D. C., Kibiryeva, N., Marshall, J. A. & O'Brien, J. E. MicroRNA-421 Dysregulation is Associated with Tetralogy of Fallot. *Cells* **3**, 713–723. https://doi.org/10.3390/cells3030713 (2014).
41. Condorelli, G., Latronico, M. & Cavarretta, E. microRNAs in cardiovascular diseases: current knowledge and the road ahead. *J. Am. Coll. Cardiol.* **63**, 2177–2187. https://doi.org/10.1016/j.jacc.2014.01.050 (2014).
42. Shi, R. et al. Maternal exosomes in diabetes contribute to the cardiac development deficiency. *Biochem. Biophys. Res. Commun.* **483**, 602–608. https://doi.org/10.1016/j.bbrc.2016.12.097 (2017).
43. Juntao Xie et al. The relationship between amniotic fluid miRNAs and congenital obstructive nephropathy. *Am. J. Transl Res.* **9**, 1754–1763 (2017).
44. Li, D. et al. Characterization of circulating microRNA expression in patients with a ventricular septal defect. *PLoS One.* **9**, e106318. https://doi.org/10.1371/journal.pone.0106318 (2014).
45. Anderson, D. J. et al. NKX2-5 regulates human cardiomyogenesis via a HEY2 dependent transcriptional network. *Nat. Commun.* **9**, 1373. https://doi.org/10.1038/s41467-018-03714-x (2018).
46. Zhang, Y. et al. A new TBX5 loss-of-function mutation contributes to congenital heart defect and atrioventricular block. *Int. Heart J.* https://doi.org/10.1536/ihj.19-650 (2020).
47. Akerman, M. et al. Differential connectivity of splicing activators and repressors to the human spliceosome. *Genome Biol.* **16**, 119. https://doi.org/10.1186/s13059-015-0682-5 (2015).
48. Wang, D. & Zhai, G. Yangfei Ji & Haiyun Jing. microRNA-10a targets T-box 5 to inhibit the development of cardiac hypertrophy. *Int. Heart J.* **581**, 100–106 (2017).
49. Hussain, N. et al. Down-regulation of miR-10a-5p promotes proliferation and restricts apoptosis via targeting T-box transcription factor 5 in inflamed synoviocytes. *Biosci. Rep.* **38** https://doi.org/10.1042/BSR20180003 (2018).
50. Zhang, X. et al. Gestational Leucylation Suppresses Embryonic T-Box Transcription Factor 5 Signal and Causes Congenital Heart Disease. *Advanced science (Weinheim, Baden-Wurttemberg, Germany)* 9, e2201034 (2022). https://doi.org/10.1002/advs.202201034
51. Gelb, B. et al. The Congenital Heart Disease Genetic Network Study: rationale, design, and early results. *Circ. Res.* **112**, 698–706. https://doi.org/10.1161/circresaha.111.300297 (2013).

Author contributions

H.N.Y, Q.Y.C and G.R.L designed the study. H.N.Y, Y.D.L and S.Q.L performed the experiment. H.N.Y and Y.R.Y analyzed the data. H.N.Y and G.R.L wrote the manuscript. All authors read and approved the final manuscript.

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Declarations

Ethics approval and consent to participate

This study was approved by the Ethics Committee of the Second Affiliated Hospital of Fujian Medical University (NO. 2019 – 233) and conducted according to the principles of the Declaration of Helsinki.

Competing interests

The authors declare no competing interests.

Additional information

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