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Novel circRNA-miRNA-mRNA networks regulated by maternal exercise in fetal hearts of pregestational diabetes

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ABSTRACT

Background: Maternal exercise lowers the incidence of congenital heart defects (CHDs) induced by pregestational diabetes. However, the molecular mechanisms underlying the beneficial effects of maternal exercise remain unclear. The present study aimed to identify circular RNA (circRNA), microRNA (miRNA) and mRNA networks that are regulated by maternal exercise in fetal hearts of pregestational diabetes.

Methods: Pregestational diabetes was induced in adult C57BL/6 female mice by streptozotocin. The expression profiles of circRNAs, miRNAs and mRNAs in E10.5 fetal hearts of offspring of control and diabetic mothers with or without exercise were analyzed using next generation sequencing. circRNA-miRNA-mRNA networks in fetal hearts were mapped and key candidate transcripts were verified by qPCR analysis.

Results: Pregestational diabetes dysregulated the expression of 206 circRNAs, 66 miRNAs and 391 mRNAs in fetal hearts. Maternal exercise differentially regulated 188 circRNAs, 57 miRNAs and 506 mRNAs in fetal hearts of offspring of pregestational diabetes. A total of 5 circRNAs, 12 miRNAs, and 28 mRNAs were incorporated into a final maternal exercise-associated regulatory network in fetal hearts of offspring of maternal diabetes. Notably, maternal exercise normalized the dysregulated circ_0003226/circ_0015638/miR-351-5p and circ_0002768/miR-3102-3p.2-3p pairs in fetal hearts of pregestational diabetes.

Conclusion: Maternal exercise reverses the dysregulated circ_0003226/circ_0015638/miR-351-5p and circ_0002768/miR-3102-3p.2-3p pairs, and partially normalizes circRNA, miRNA, and mRNA expression profiles in fetal hearts of pregestational diabetes. These findings shed new light on the potential mechanisms of the beneficial effects of maternal exercise on the developing heart in diabetic pregnancies.

1. Introduction

Congenital heart defects (CHDs) are the most common birth defects, affecting ~1 % of newborns. Overall, between 1970 and 2017, the prevalence of CHDs globally increased by 10 % [1]. Although genetic factors have been widely investigated, only about 15 % of cases can be attributed to a specific genetic cause [2]. Recently, studies have focused on identifying environmental teratogens which promote CHDs. Clinical and epidemiological studies have established a strong link between maternal pregestational diabetes mellitus and a higher risk of CHDs [3]. The global diabetes prevalence in 2019 was estimated to be 9.3 % (463 million people), rising to 10.2 % (578 million) by 2030 and 10.9 % (700 million) by 2045 [4]. The prevalence of pregestational diabetes mellitus accounts for nearly 1.5 % of all pregnancies following the emerging increase of diabetes in adolescents, which results in a 3–5 fold increased risk for fetal cardiac and valve malformations compared with the general population [5–7]. Despite increased clinical efforts to improve glycemic control during diabetic pregnancy, the rate of congenital

Abbreviations: AMPK, AMP-activated protein kinase; CHDs, congenital heart defects; circRNA, circular RNA; CON, control; ECM, extracellular matrix; EX, exercise; GO, gene ontology; IGFR, insulin-like growth factor receptor; IP, intraperitoneal; KEGG, Kyoto encyclopedia of genes and genomes; MAPK, mitogen-activated protein kinase; miRNA, microRNA; mTOR, mammalian target of rapamycin; pre-RNA, precursor RNA; RNA Pol II, RNA polymerase II; RNA-seq, RNA sequencing; qPCR, quantitative polymerase chain reaction; STZ, streptozotocin; TGF-β, Transforming growth factor beta; VEGF, vascular endothelial growth factor.

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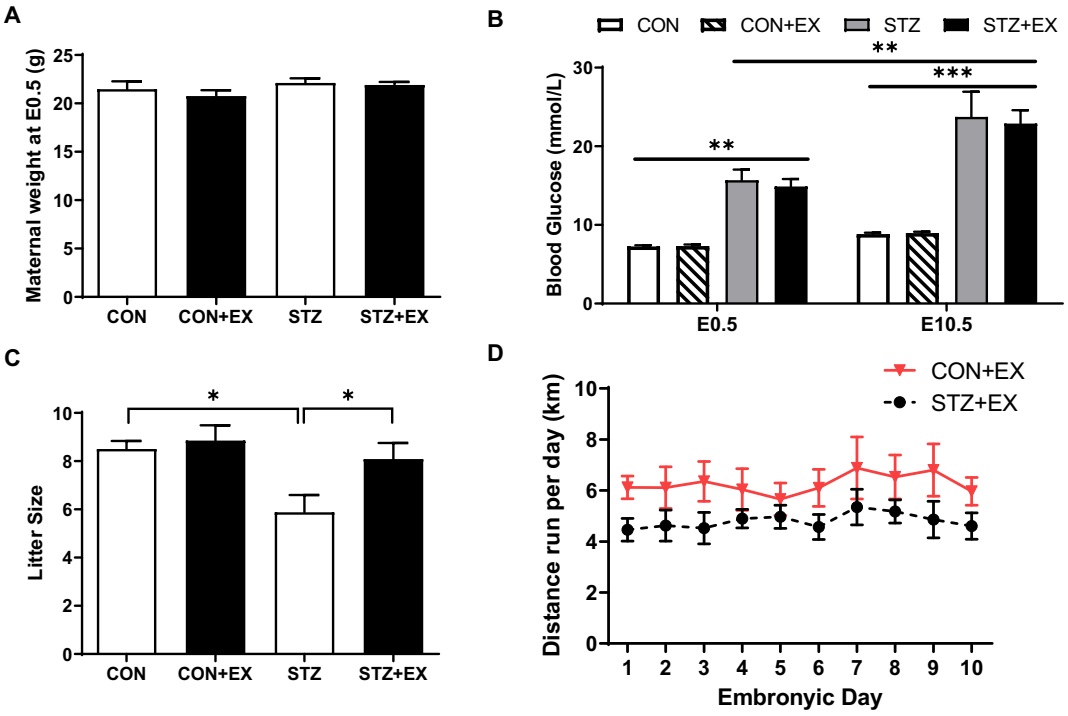

Fig. 1. Effects of maternal exercise on blood glucose and litter size in the offspring of pregestational diabetes. (A) Maternal body weight at E0.5. (B) Non-fasting blood glucose of control and diabetic pregnant dams with or without exercise at E0.5 and E10.5. (C) Litter size of offspring in each group at E10.5. (D) Running distance per day during gestation. Data are means ± SEM of 6–10 mice per group. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. CON, control; EX, exercise; STZ, streptozotocin.

malformations remains high in studies of diabetic gestation of type 1 and type 2 diabetes [8]. Therefore, finding an effective treatment to control the incidence CHDs of offspring in pregestational diabetes is essential. Based on our previous study, maternal voluntary exercise lowers the incidence of CHDs induced by pregestational diabetes in mice [9]. However, the molecular mechanisms underlying maternal exercise's protection are not well understood.

Circular RNAs (circRNAs), a novel class of non-coding RNAs, are formed by precursor mRNA (pre-mRNA) through a back-splicing process [10]. Based on a covalently closed-loop structure without 3′ and 5′ ends, circRNAs are much more stable than linear RNAs and can resist degradation by RNA exonuclease or RNase R [11]. Of note, circRNAs exhibit cell type, tissue- and developmental stage-specific expression [12,13]. These characteristics make circRNAs a promising candidate for the development and application of potential clinical diagnostic markers [14]. Recent research demonstrated that circRNAs act as biomarkers in several human diseases [15]. circRNAs may contribute to CHD pathogenesis and may serve as novel non-invasive biomarkers for the diagnosis of CHDs in children [16]. Dysregulated circRNAs were expressed higher in myocardial tissue of those with ventricular septal defects [17]. Although the functions of most circRNAs are unknown, ongoing studies have shown that some circRNAs can regulate gene expression by acting as miRNA sponges, transcribers and interacting with RNA binding proteins (RBPs) [18]. circRNAs can directly bind to miRNAs to inhibit combinations between miRNAs and the 3′-untranslated region of target genes, which abolishes the effects of miRNAs on gene expression [19]. The recent discovery of the circRNA–miRNA interaction, in which the two types of RNA molecules interact to determine the regulation of gene expression, is a promising field of study for early detection and prognosis of disease [20]. In addition, an increasing number of studies have demonstrated that miRNAs control various stages of embryonic heart development [21]. Therefore, circRNA-miRNA-mRNA networks might contribute to embryonic heart development. However, few genome-wide studies have identified the roles of dysregulated interactions between circRNAs, miRNAs and mRNAs in embryonic heart development exposed to pregestational diabetes.

The present study tested the hypothesis that maternal exercise rescues gene expression and circRNA-miRNA-mRNA networks in fetal hearts of pregestational diabetes induced by streptozotocin (STZ) in mice. The expression profiles of circRNAs, miRNAs and mRNAs in fetal hearts of diabetic and non-diabetic mothers with or without exercise were analyzed using RNAseq analysis. Functional annotations of the differentially expressed transcripts and circRNA-associated networks were performed using Gene Ontology (GO) and Kyoto encyclopedia of genes and genomes (KEGG) enrichment analysis. Our study provides novel insights into potential mechanisms of maternal exercise on fetal heart development in pregestational diabetes.

2. Methods

2.1. Animals

This study used mice in accordance with the Guide to Care and Use of Animals of the Canadian Council of Animal Care and was approved by the Animal Care Committee at Western University, Canada. Adult C57BL/6 mice were purchased from Charles River Laboratories, Quebec, Canada. They were fed a standard rodent diet (Cat. #2018, Teklad Global Diets®, Envigo, Boyertown, PA). Diabetes was induced in female mice at 11–20 weeks of age by intraperitoneal (IP) injections of STZ (Sigma, Canada, 75 mg/kg) for 3 days. Female mice with non-fasting blood glucose >11 mmol/L, regarded as diabetic, were bred with normal males (12–16 weeks old). The presence of a vaginal plug indicated embryonic day 0.5 (E0.5). Non-fasting blood glucose measurements were taken in the morning at E0.5 and E10.5 to monitor glycemic levels using a glucose meter (One Touch Ultra2; LifeScan, Burnaby, BC, Canada). Running wheels were installed in cages to allow voluntary exercise of female mice. Daily running distance was recorded based on the number of rotations of the running wheel using the Multi-Device

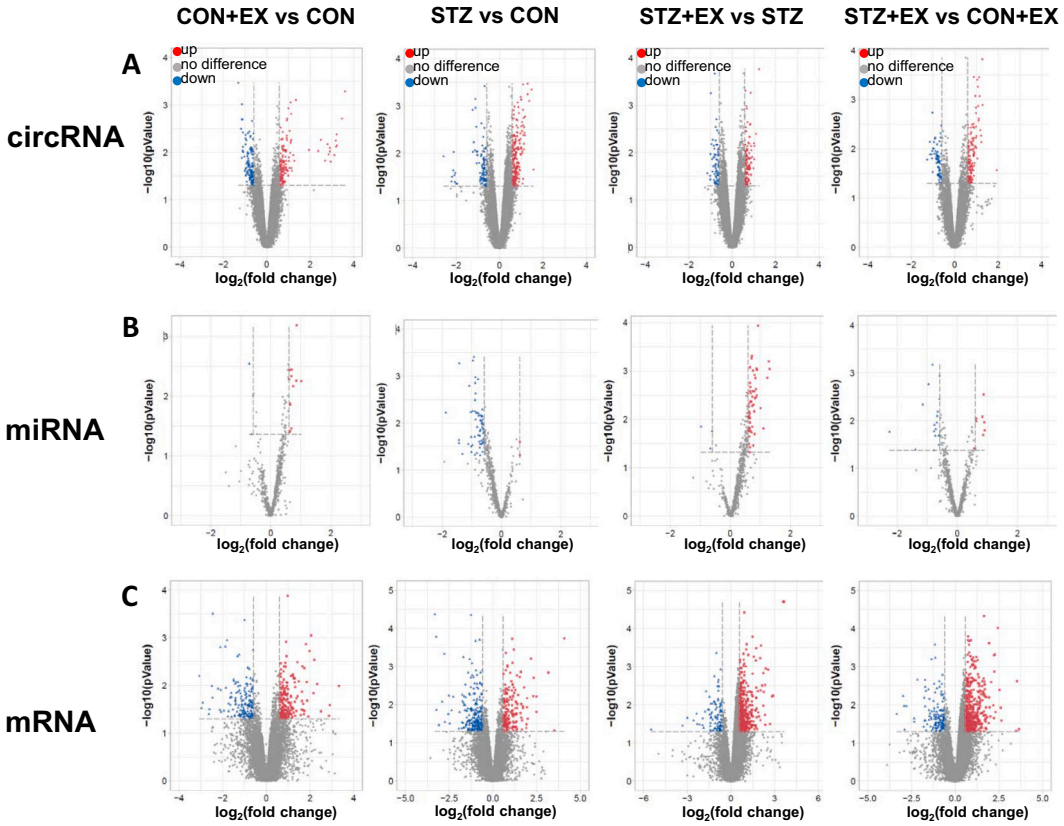

Fig. 2. Expression profiles of circRNA, miRNA and mRNA in E10.5 fetal hearts. Volcano plots of circRNAs (A), miRNAs (B) and mRNAs (C). Red, blue and gray points represent genes that were upregulated, downregulated and no difference between two groups, respectively. The vertical lines correspond to 1.5-fold upregulation and downregulation, respectively, and the horizontal line represents $P = 0.05$. CON, control; EX, exercise; STZ, streptozotocin. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Channel Interface software (Columbus Instruments, Columbus, USA). Mice were randomly assigned to the following 4 groups (n = 8–10 mice per group): controls (CON); control with maternal exercise (CON + EX); pregestational diabetes (STZ); pregestational diabetes with maternal exercise (STZ + EX).

2.2. The workflow of RNA-Seq

Three or four E10.5 hearts from the same mother were pooled into one sample. Then the whole transcriptome from CON, CON + EX, STZ and STZ + EX groups (n = 3 samples in each group) was profiled. Total RNA was isolated from E10.5 hearts at using the Qiagen miRNeasy mini kit (Cat #217004). High throughput RNA sequencing was done after removing ribosomal RNA and building a library. The quality of RNA samples was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies). The library products were sequenced on the Illumina HiSeq™ 3000 system at the Robarts Research Institute, Western University.

2.3. Differentially expressed circRNAs, miRNAs, mRNAs analysis

Differentially expressed transcripts were analyzed by comparing CON + EX versus CON, STZ versus CON, STZ + EX versus STZ and STZ + EX versus CON + EX with the threshold of absolute fold-change >1.5 and P-value < 0.05 using Partek Flow Software (Partek Inc., Finland). Volcano plots were adopted to visualize these differentially expressed circRNAs, miRNAs and mRNAs.

2.4. Construction of circRNA-miRNA-mRNA regulatory networks

circRNA-miRNA-mRNA regulatory networks were mapped based on bioinformatics predictions and transcriptome data to understand the potential associations between differentially expressed circRNAs, miRNAs and mRNAs using miRNA binding-site prediction of circRNAs, miRNA target gene prediction, and correlation analysis. Information about circRNAs is available on the circBase website (http://www.circbase.org/) (see Supplementary Fig. 1 for a flowchart of bioinformatic data analyses). Some differentially expressed circRNAs in embryonic hearts from pregestational mothers were normalized by maternal exercise, which were then chosen as candidates for subsequent analysis. Predicting the target genes of the promising circRNAs was performed by circBase and RegRNA2.0 (http://regrna2.mbc.nctu.edu.tw/) [22]. The putative RNA-RNA interactions were confirmed by Clustal Omega and RNAhybrid [23,24]. Then, the potential circRNAs targets were intersected with miRNAs that were differentially expressed in the STZ or STZ + EX groups. Then, the miRNA-mRNA pairs were established using 4 different databases (Targetscan, miRTarBase, miRWalk, miRDB). To improve the prediction reliability, 3 or more databases showing the same prediction were selected for subsequent analysis. Then, the potential miRNAs targets were intersected with differentially expressed mRNAs from mRNA sequencing data, to identify mRNA targeted by differentially expressed miRNAs. Pregestational diabetes and maternal exercise associated circRNA-miRNA-mRNA regulatory networks based on common target miRNAs of the circRNAs and mRNAs, and the negative correlation between the expression of circRNAs and miRNAs and between the expression of miRNAs and mRNAs were constructed. Finally, circRNA-associated networks were visualized by Cytoscape software [25].

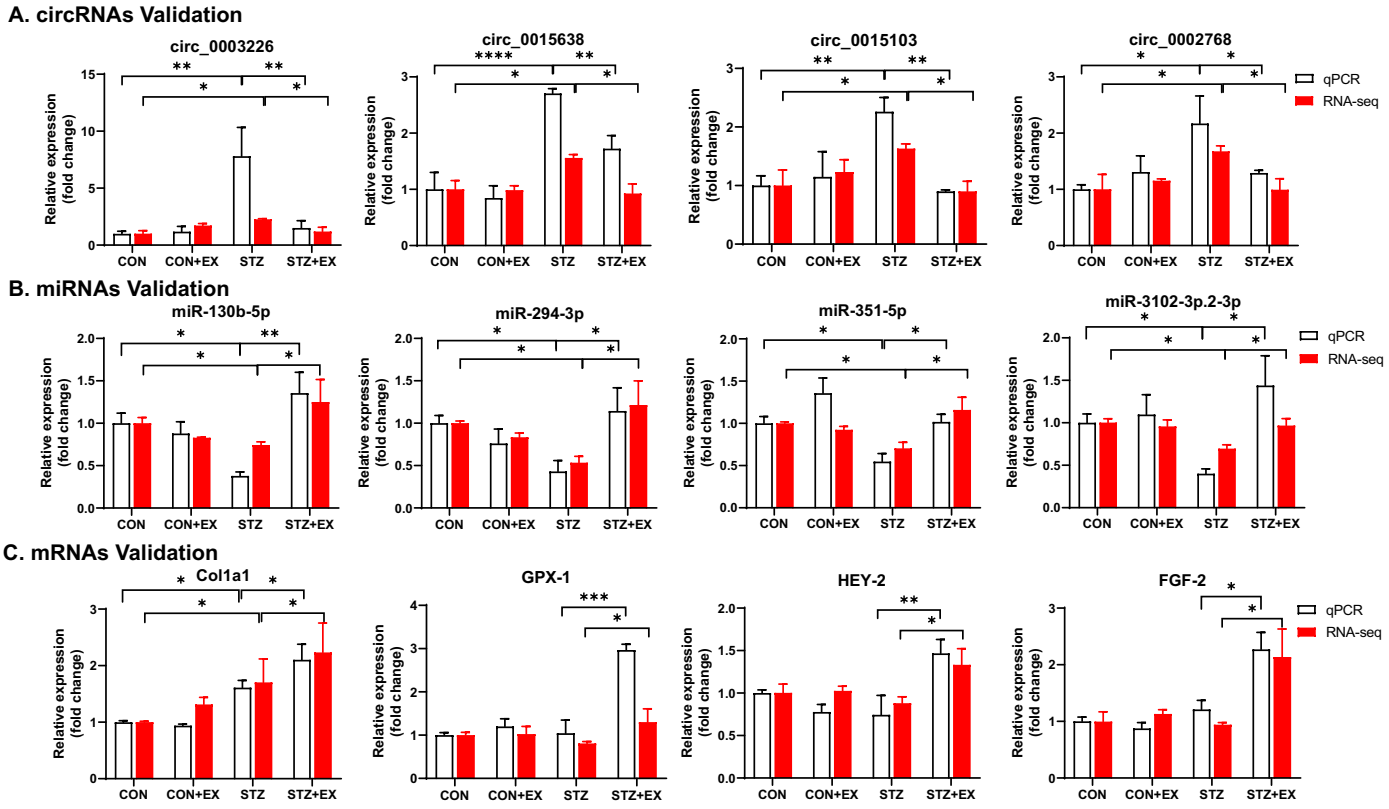

Fig. 3. Validation of circRNA, miRNA and mRNA expression using qPCR in E10.5 fetal hearts. Validation of selected circRNA (A), miRNA (B), and mRNA (C) expression using qPCR. Data from both qPCR and RNAseq analyses were plotted in the same graph for side-by-side comparisons. Data are presented as means ± SEM. $N = 4$–6 and 3 samples per group for qPCR and RNAseq analysis, respectively. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. CON, control; EX, exercise; STZ, streptozotocin.

2.5. Real-time qPCR analysis

Differentially expressed circRNAs, miRNAs and mRNAs related to embryonic heart development were selected to verify the reliability of the RNA-Seq results using qPCR. Total RNA was isolated from E10.5 hearts at using the Qiagen miRNeasy mini kit (Cat #217004). Two E10.5 hearts were pooled for one sample ($n = 4$ samples per group). For circRNAs and mRNAs, first-strand cDNA was synthesized from 250 ng of RNA in a total volume of 20 μL [26]. In addition, divergent primers were designed to amplify the junction sites of circRNAs. We followed previously published procedures to quantify mRNA expression [27]. For miRNAs, the specific stem-loop reverse transcription primers were designed according to previous published procedures [26]. Reverse transcription for mRNA was done using random primers. Next, the qPCR product was amplified using a miRNA-specific forward primer, the universal reverse primer and SYBR Green Mastermix in a total volume of 10 μL. circRNA and mRNA levels were extrapolated using the comparative CT method normalized to 28S-Ribosomal RNA. miRNAs expressions were normalized to that of Snord 47. qPCR primers are listed in Supplementary Table 1.

2.6. GO and KEGG pathway enrichment analysts

To determine the possible biological roles and signaling pathways of the differentially expressed transcripts, as well as the circRNA-associated regulatory networks mapped, gene ontology (GO) and Kyoto encyclopedia of genes and genomes (KEGG) pathway analyses were done using the online program, DAVID (https://david.ncifcrf.gov/home.jsp). The biological process was selected for the GO enrichment analysis.

2.7. Statistics

All data are presented as means ± SEM. Statistical analysis was performed using two-way ANOVA followed by Tukey's multiple comparison test in SPSS Statistics (version 26.0, IBM, USA). Two-way repeated-measures ANOVA, followed by the Bonferroni post hoc test, was used to analyze the running distance differences over time between control and diabetic mothers. A $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effects of maternal exercise on blood glucose and litter size in pregestational diabetes

Our recent studies have shown that pregestational diabetes induces a wide spectrum of CHDs including atrial septal defects, ventricular septal defects, atrioventricular canal defects, double outlet right ventricle, transposition of great arteries and tetralogy of Fallot [9,27,28]. Notably, maternal voluntary exercise lowers the incidence of CHDs induced by pregestational diabetes without any significant changes in blood glucose levels of the diabetic dams [9].

In the present study, prior to pregnancy, there was no difference in female body weight among the 4 groups (Fig. 1A). Non-fasting blood glucose levels were similar at E0.5 in diabetic mothers with or without exercise during pregnancy (Fig. 1B). By E10.5, non-fasting blood glucose levels of diabetic mothers with or without exercise were not statistically different from each other but both were significantly increased over their respective values at E0.5 (Fig. 1B, $P < 0.05$). There was no difference in litter size between non-diabetic controls with or without exercise during pregnancy (Fig. 1C). However, smaller litter sizes were observed from diabetic dams, which was improved by maternal exercise.

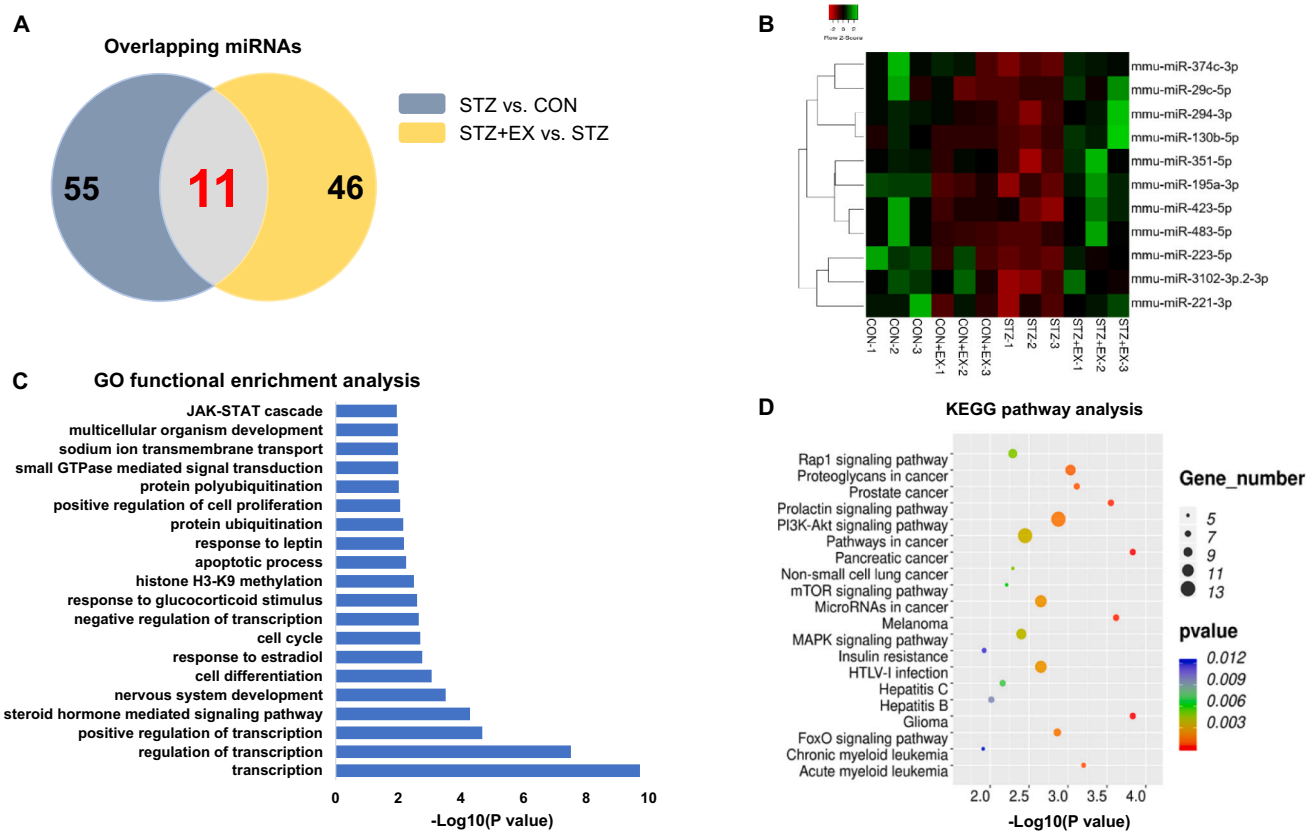

Fig. 4. Biological function analysis of the differentially expressed miRNAs in E10.5 fetal hearts. (A) Overlapping and differentially expressed miRNAs between two comparisons: STZ vs. CON and STZ + EX vs. STZ with >1.5-fold change and $P < 0.05$ on a venn diagram. (B) The heatmap of 11 overlapping and differentially expressed miRNAs from A. (C–D) GO term enrichment and KEGG pathway analyses of 11 overlapping and differentially expressed miRNAs from A. CON, control; EX, exercise; STZ, streptozotocin.

Diabetic mothers ran approximately 3 to 6 km/day, and there was no significant difference in running distance per day between diabetic and non-diabetic mothers (Fig. 1D).

3.2. Identification of differentially expressed circRNAs, miRNAs and mRNAs

A total of 13,584 circRNAs were identified in E10.5 hearts. Volcano plots of differentially expressed circRNAs in fetal hearts between paired groups are displayed in Fig. 2A. Maternal exercise upregulated 142 circRNAs and downregulated 122 circRNAs in fetal hearts of control mice (CON + EX vs. CON). A total of 138 circRNAs were upregulated and 68 circRNAs were downregulated in fetal hearts of offspring of maternal diabetes compared to controls (STZ vs. CON). Notably, maternal exercise upregulated 120 circRNAs and downregulated 68 circRNAs in fetal hearts of offspring of maternal diabetes (STZ + EX vs. STZ), and upregulated 210 circRNAs and downregulated 98 circRNAs in fetal hearts of offspring of maternal diabetes compared to controls (STZ + EX vs. CON + EX, Fig. 2A).

A total of 724 miRNAs were identified in E10.5 hearts (Fig. 2B). Maternal exercise upregulated 13 miRNAs and downregulated 3 miRNAs in fetal hearts of control mice (CON + EX vs. CON). Maternal diabetes upregulated 2 miRNAs and downregulated 64 miRNAs in fetal hearts of offspring of maternal diabetes compared to controls (STZ vs. CON). Of note, maternal exercise upregulated 54 miRNAs and downregulated 3 miRNAs in fetal hearts of offspring of maternal diabetes (STZ + EX vs. STZ). Additionally, maternal exercise upregulated 8 miRNAs and downregulated 18 miRNAs in fetal hearts of offspring of maternal diabetes compared to those of control mice (STZ + EX vs. CON + EX, Fig. 2B).

A total of 19,385 mRNAs were identified E10.5 hearts (Fig. 2C). Of those mRNA, 179 were upregulated and 164 were downregulated in fetal hearts of controls by maternal exercise (CON + EX vs. CON). Maternal diabetes upregulated 164 mRNAs and downregulated 227 mRNAs in fetal hearts compared to controls (STZ vs. CON). Maternal exercise upregulated 409 mRNAs and downregulated 97 mRNAs in fetal hearts of offspring of maternal diabetes (STZ + EX vs. STZ). Additionally, 413 and 145 mRNAs were upregulated and downregulated, respectively by maternal exercise in fetal hearts of offspring of maternal diabetes compared to those of controls (STZ + EX vs. CON + EX, Fig. 2C). Notably, 70 overlapping mRNAs were dysregulated in fetal hearts of offspring of maternal diabetes but restored to normal levels by maternal exercise (Supplementary Fig. 2, Supplementary Table 2). All differentially expressed transcripts (cirRNA, miRNA and mRNA) shown in the volcano plots in Fig. 2 are listed in a supplementary Excel file "differentially expressed genes".

3.3. qPCR validation

To validate the RNA-seq results, qPCR analysis was performed on 4 circRNAs (circ_0003226, circ_0015638, circ_0015103 and circ_0002768, Fig. 3A), 4 miRNAs (miR-130b-5p, miR-294-3p, miR-351-5p and miR-3102-3p.2-3p, Fig. 3B) and 4 mRNAs (Cola1a1, Gpx-1, Hey-2 and Fgf-2, Fig. 3C) that are associated with heart development and were differentially expressed from RNA-seq analysis in E10.5 hearts. Our results show that the levels of these 12 transcripts analyzed by qPCR were highly consistent with those of RNA-seq analysis in control and diabetic groups with and without maternal exercise (Fig. 3).

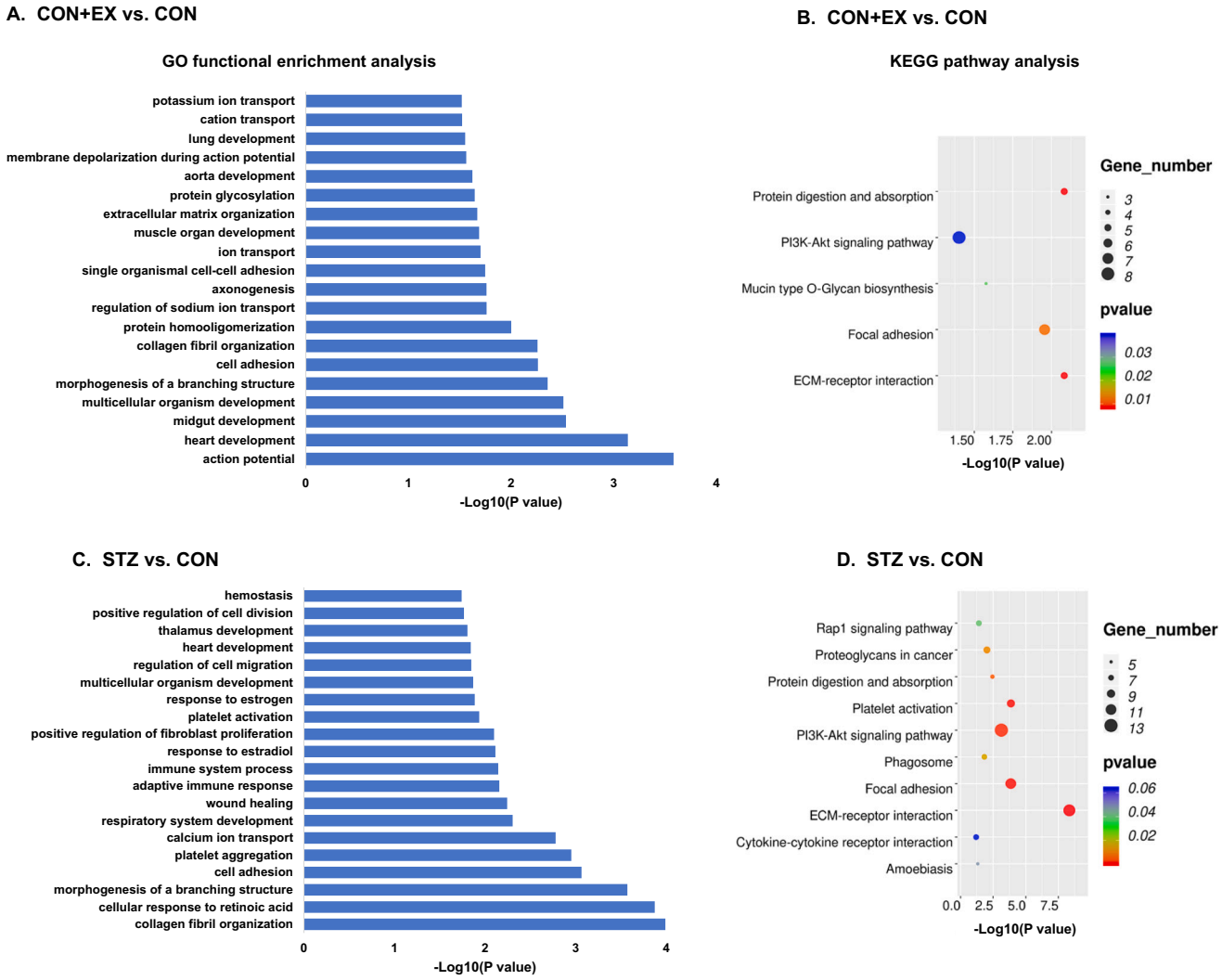

Fig. 5. Biological function of the differentially expressed mRNAs from RNA-seq. (A and B) GO and KEGG analysis of the differentially expressed mRNAs in CON + EX compared with CON group. (C–D) GO and KEGG analysts of the differentially expressed mRNAs in STZ compared with CON group. (E–F) GO and KEGG analysis of the differentially expressed mRNAs in STZ + EX compared with STZ group. (G–H) GO and KEGG analysis of the differentially expressed mRNAs in STZ + EX compared with CON + EX group.

3.4. Biological function of overlapping and differentially expressed miRNAs

Pregestational diabetes resulted in differential expression of 66 miRNAs in E10.5 fetal hearts compared to controls (Fig. 4A). In response to maternal exercise, 57 miRNAs were differentially expressed in fetal hearts of offspring of maternal diabetes. Notably, 11 overlapping miRNAs were downregulated in fetal hearts of offspring of maternal diabetes, but restored to normal levels by maternal exercise as shown in a venn diagram and a heatmap (Fig. 4A, B, Supplementary Table 3).

The GO functional enrichment analysis showed that the target genes of 11 overlapping miRNAs were highly enriched in biological processes, including “transcription”, “regulation of transcription”, “cell differentiation”, “cell cycle”, “apoptotic process”, “cell proliferation” and “multicellular organism development”, which are associated with embryonic heart development (Fig. 4C). The KEGG pathway enrichment analysis showed that target genes of overlapping miRNAs were also highly enriched in pathways associated with heart and embryo development, including Rap1, PI3K-AKT, mTOR, MAPK, insulin resistance, prolactin, and FoxO signaling pathways (Fig. 4D).

Biological function of differentially expressed mRNAs

GO enrichment analysis showed that the differentially expressed mRNAs in E10.5 fetal hearts by maternal exercise (CON + EX vs. CON) and pregestational diabetes (STZ vs. CON) were enriched in “heart development”, “multicellular organism development”, “collagen fibril organization” and “aorta development” (Fig. 5A, C). KEGG pathway analysis showed that maternal exercise and pregestational diabetes significantly altered mRNAs enriched in ECM-receptor interaction, Rap1 and PI3K-AKT signaling pathways (Fig. 5B, D). Maternal exercise of diabetic mothers (STZ + EX vs. STZ) resulted in altered mRNAs in fetal hearts, which were highly enriched in biological processes including “cell migration” “collagen fibril organization”, “apoptotic process”, “protein processing in ER”, and “arginine and proline metabolism” (Fig. 5E, F). Compared to maternal exercise in controls, exercise of diabetic mothers (STZ + EX vs. CON + EX) modified mRNAs in fetal hearts, which were enriched in “cell adhesion”, “collagen fibril organization”, “Wnt signaling pathway”, “fibroblast growth factor receptor signaling pathway”, “positive regulation of cell migration”, “ECM-receptor interaction”, and cAMP, PI3K-Akt and TGF-β signaling pathways (Fig. 5G, H).

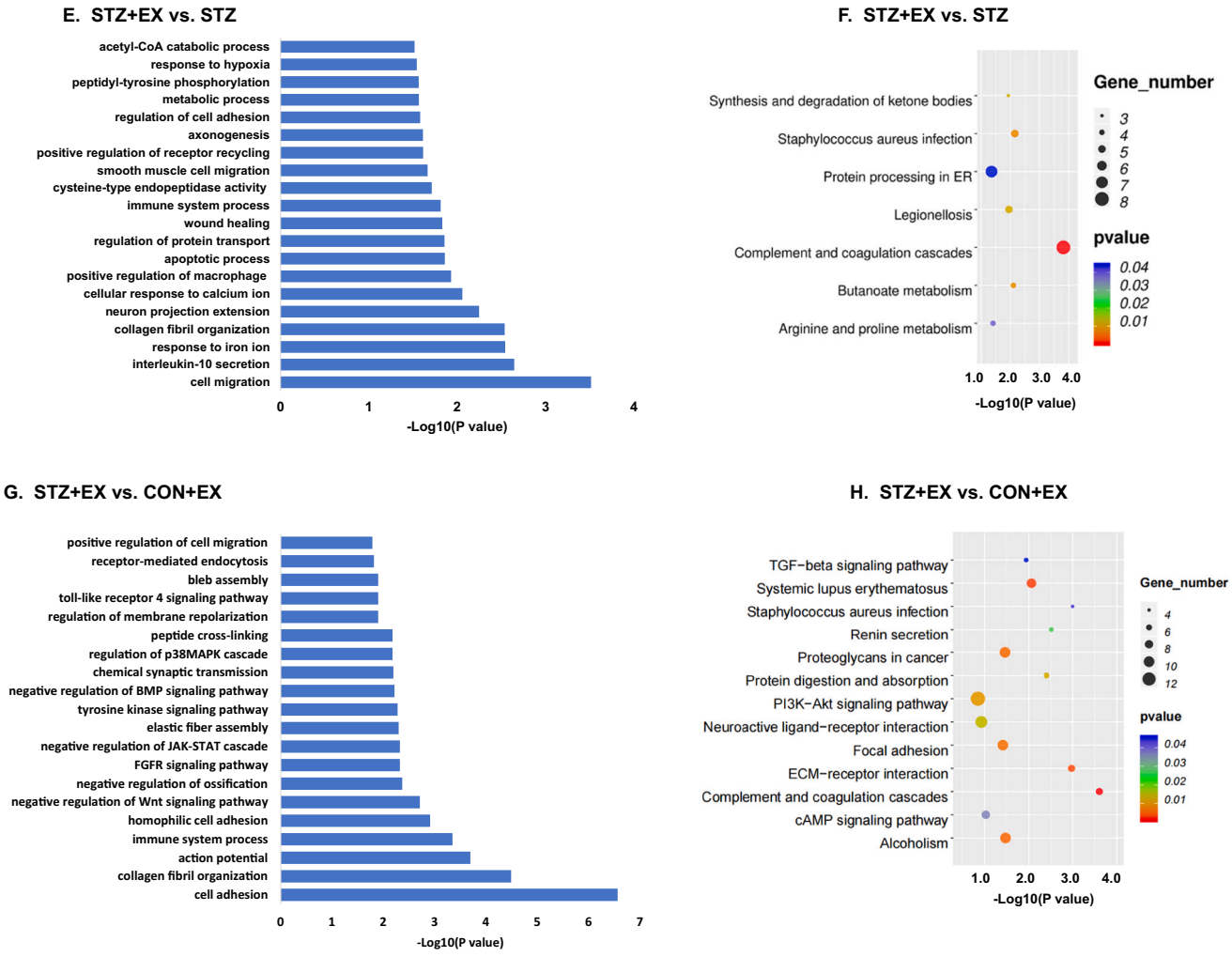

Fig. 5. (*continued*).

3.5. Construction of circRNA-miRNA-mRNA regulatory network

There were 24 overlapping and differentially expressed circRNAs in fetal hearts between two comparisons (STZ vs. CON and STZ + EX vs. STZ) as shown in a venn diagram and a heatmap (Fig. 6A, B). Among them, 16 circRNAs were downregulated while 7 circRNAs were upregulated in fetal hearts of offspring of maternal diabetes compared to controls (STZ vs. CON). Notably, these circRNAs were normalized by maternal exercise (STZ + EX vs. STZ) (Supplementary Table 4). The differentially expressed circRNAs were enriched in "coronary vascular morphogenesis", "positive regulation of GTPase activity", "cell migration", "positive regulation of transcription", "apoptotic process", "cell differentiation" and "cardiac muscle tissue development" (Supplementary Fig. 3).

Maternal exercise induced 264 differentially expressed circRNAs in fetal hearts of controls. The 10 top-ranked differentially expressed circRNAs were selected, including 5 up-regulated and 5 down-regulated circRNAs. The miRNA target analysis of these 10 circRNAs was performed. The potential circRNAs targets were intersected with miRNAs that were differentially expressed between fetal hearts of offspring of control mice with or without exercise. After circRNA-miRNA pairs were constructed, miRNAs in this network were selected and their potential miRNAs targets were intersected with differentially expressed mRNAs from mRNA sequencing data. In total, 6 circRNAs, 3 miRNAs, and 40 mRNAs were incorporated into a maternal exercise-associated regulatory network, consisting of 47 nodes and 50 interactions in fetal hearts of control mice (Fig. 6C). Maternal exercise upregulated 3 circRNAs (circ_0001217; circ_0004656; circ_0003023), 2 miRNAs (miR_31-5p; miR_19a-3p) and 17 mRNAs, and downregulated 3 circRNAs (circ_0005577; circ_0004822; circ_0004978); 1 miRNAs (miR_5510) and 23 mRNAs in fetal hearts of controls.

Pregestational diabetes-associated circRNA-miRNA-mRNA regulatory network in fetal hearts was constructed with 5 circRNAs, 17 miRNAs, and 61 mRNAs. The network had 83 nodes and 219 interactions (Fig. 6D). Furthermore, in this network, circRNAs, miRNAs and mRNAs were upregulated, downregulated, and upregulated, respectively in fetal hearts of offspring of maternal diabetes compared controls (STZ vs. CON), consistent with the inhibitory function of circRNAs on miRNA expression, and miRNA inhibition on mRNA expression.

As shown in Fig. 6E, 5 circRNAs, 12 miRNAs, and 28 mRNAs were incorporated into a final maternal exercise-associated regulatory network consisting of 45 nodes and 76 interactions in fetal hearts of offspring of maternal diabetes. Consistently, expressions of circRNAs, miRNAs and mRNAs in the network were downregulated, upregulated, and downregulated, respectively in fetal hearts of offspring of maternal diabetes with maternal exercise compared to those without exercise (STZ + EX vs. STZ).

Pregestational diabetes induced differential expression of circRNAs in fetal hearts with maternal exercise. The 10 top-ranked differentially expressed circRNAs were selected, including 5 up-regulated and 5 down-regulated circRNAs. In total, 4 circRNAs, 6 miRNAs, and 12 mRNAs were incorporated into a pregestational diabetes-associated regulatory

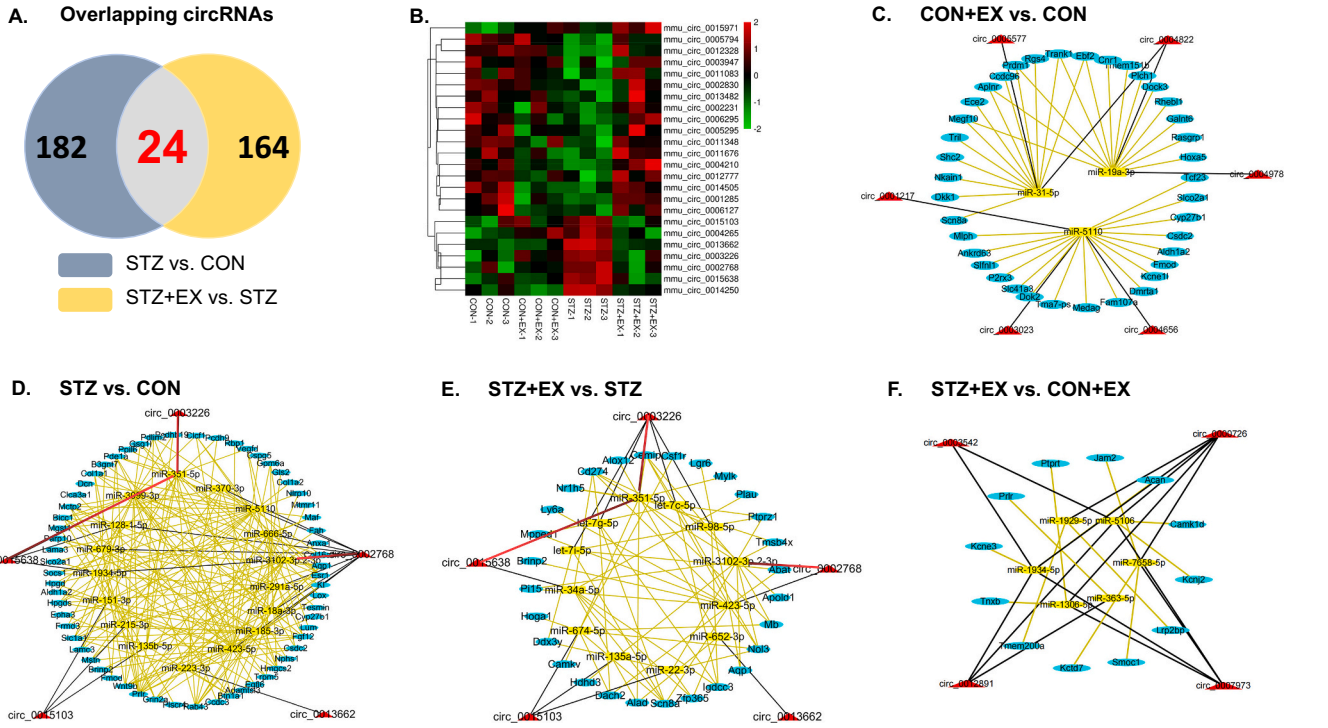

Fig. 6. circRNA-miRNA-mRNA regulatory networks in embryonic hearts of healthy and diabetic mothers with or without exercise during pregnancy. (A) Venn diagram analysis of the dysregulated circRNAs between two comparisons: STZ vs. CON and STZ + EX vs. STZ. (B) Overlapping differentially expressed circRNAs between two comparisons (STZ vs. CON and STZ + EX vs. STZ) were visualized on a heatmap with fold change >1.5 and $P < 0.05$. (C) Maternal exercise-associated regulatory network in embryonic hearts of offspring of non-diabetic mothers. (D) Pregestational diabetes-associated regulatory network in embryonic hearts of offspring of diabetic mothers. (E) Maternal exercise-associated regulatory network in embryonic hearts of offspring of diabetic mothers. (F) Pregestational diabetes-associated regulatory network in embryonic hearts of offspring of maternal exercise. All transcripts were differentially expressed between the indicated groups. circRNAs, miRNAs and mRNAs are indicated by red, yellow, blue colors, respectively. Black lines indicate circRNA-miRNA interactions. Yellow lines indicate miRNA-mRNA interactions. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

network in fetal hearts of offspring of maternal exercise (STZ + EX vs. CON + EX), consisting of 22 nodes and 26 interactions (Fig. 6F).

Biological function of differentially expressed mRNAs in the circRNA-associated network.

The differentially expressed mRNAs in maternal exercise-associated regulatory network in fetal hearts of control mice were mainly enriched in “cardiac muscle cell differentiation”, “muscle organ development” and “heart development” (Fig. 7A).

The differentially regulated mRNAs in the pregestational diabetes-associated circRNA-miRNA-mRNA network were enriched in essential biological processes associated with embryonic heart development, including “blood vessel morphogenesis”, “vascular endothelial growth factor signaling pathway”, and “cardiovascular system development” (Fig. 7B).

Differentially regulated mRNAs in the maternal exercise-associated regulatory network in fetal hearts of offspring of pregestational diabetes were highly enriched in essential biological processes, such as “cell migration”, “cardiovascular system development” and “angiogenesis” (Fig. 7C).

Differentially regulated mRNAs in the pregestational diabetes-associated regulatory network in fetal hearts of offspring of maternal exercise were highly enriched in essential biological processes, such as “regulation of heart rate by cardiac conduction” (Fig. 7D).

Since both circ_0003226 and circ_0015638 have the same putative target, miR-351-5p, the potential functions of miR-351-5p were assessed and found to be highly enriched in regulation of “transcription”, “cell differentiation”, “apoptotic process”, “cell migration” and “organism development”, as well as heart development related signaling pathways, including VEGF, MAPK, AMPK, insulin signaling pathways (Fig. 8A, B).

To assess the potential functional roles of the circ_0002768-miR-3102-3p.2-3p pair, GO analysis was performed and showed that miR-3102-3p.2-3p targets were highly associated with embryonic heart development-associated biological processes, including “transcription”, “insulin-like growth factor receptor”, “embryo development”, “apoptotic process”, “Notch signaling pathway” and “cardiac muscle cell differentiation”. KEGG pathway analysis showed miR-3102-3p.2-3p were significantly enriched in AMPK, Rap1, insulin, FoxO signaling pathways (Fig. 8C, D).

4. Discussion

In this study, RNA-seq was used to systematically analyze circRNA, miRNA and mRNA profiles in embryonic heart samples of offspring from non-diabetic and pregestational diabetic mothers, with or without exercise during pregnancy. To obtain a comprehensive view of the transcriptional landscape during embryonic heart development within these different environments, circRNA, miRNA, and mRNA transcriptomic data were integrated to construct circRNA-miRNA-mRNA regulatory networks. Differentially expressed transcripts induced by maternal pregestational diabetes were identified in embryonic hearts, which are likely involved in the pathogenesis of CHDs. Notably, maternal exercise normalized some transcript levels in embryonic hearts of offspring from pregestational diabetic dams. These transcripts provide a rich set of embryonic heart development-associated gene candidates for use as biomarkers or targets of novel CHD prevention strategies. In addition, promising circRNAs that mimic the benefits of maternal exercise were identified based on circRNA-associated regulatory networks in embryonic hearts. Our integrative analysis provided new insights into the roles of circRNAs and other transcripts in embryonic heart development of offspring during pregestational diabetes that may serve as reference

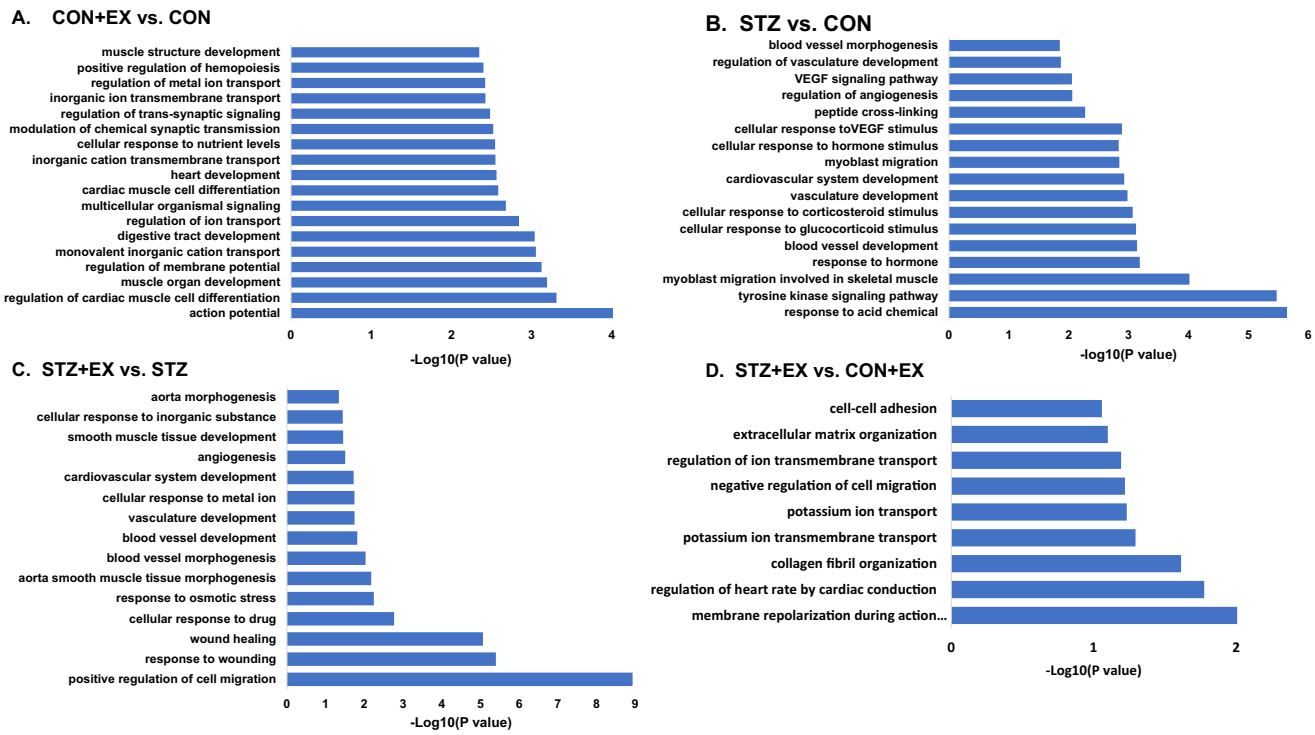

Fig. 7. Biological function of the differentially expressed mRNAs in the circRNA-associated regulatory network. (A) Biological functions of the differentially expressed mRNAs from maternal exercise-associated regulatory network in embryonic hearts of offspring of non-diabetic dams. (B) Biological functions of the differentially expressed mRNAs from pregestational diabetes-associated regulatory network in embryonic hearts of offspring of diabetic dams. (C) Biological functions of the differentially expressed mRNAs of maternal exercise-associated regulatory network in embryonic hearts of offspring of diabetic dams. (D) Biological functions of the differentially expressed mRNAs between STZ + EX and CON + EX groups.

values for developing novel therapeutic strategies.

Maternal exercise did not lower non-fasting blood glucose of pregestational diabetic mice at E0.5 or E10.5, despite normalizing pup size, which is consistent with our previous findings using the same model [9]. E10.5 is a critical period of time where cardiac abnormalities and other developmental defects associated with maternal diabetes evolve. Insults during this window can interfere with the temporal expression patterns of genes encoding molecules involved in heart development and tissue remodeling [29,30]. Therefore, the expression profiles of circRNA, miRNA, and mRNA in embryonic hearts at E10.5 from non-diabetic and diabetic mothers with or without maternal exercise were analyzed using whole-transcriptome sequencing. Our results show that pregestational diabetes alone altered 206 circRNAs in embryonic hearts. Maternal exercise modified 188 circRNAs, of which 23 circRNAs were normalized in embryonic hearts from diabetic mothers. GO enrichment analysis shows that host genes of the differentially expressed circRNAs were enriched in regulation of gene transcription. Transcription factors play an important role in embryonic heart development. In humans, mutations of several transcription factors, such as NKX2–5, GATA4, GATA6 and TBX5, have been associated with a variety of congenital heart diseases including atrial septal defects, ventricular septal defects, tricuspid valve abnormalities and atrioventricular block [31]. In the present study, although these transcript factors were not differentially expressed, other genes and signaling pathways including VEGF, FGF6, retinoic acid, MAPK and notch signaling pathways that regulate the functions of NKX2–5, GATA4, GATA6 and TBX5 were differentially expressed [32–34]. Maternal exercise may regulate the functions of cardiac transcription factors through these genes and signaling pathways.

In the present study, pregestational diabetic mice subjected to exercise produced embryonic hearts with differential circRNA expression involved in regulation of transport. This agrees with a previous microarray analysis which showed that dysregulated genes in murine hearts of offspring of diabetic mothers are mainly enriched in transcription factors, transport, metabolism, metal-ion homeostasis and cell cycle/apoptosis [35]. We found differentially expressed circRNAs in embryonic hearts from non-diabetic and diabetic mothers under exercise intervention were both involved in "positive regulation of GTPase activity". Rho GTPases are implicated in cardiogenesis [36]. Acting as downstream effectors of Rho kinases, Rho GTPases control endocardial cell differentiation and migration leading to proper heart formation. The inhibition of Rho kinases could lead to the impairment of endocardial cushion formation which are responsible for forming the septa, leading to CHDs [36]. Exercise's pro-Rho GTPase effects may limit these consequences of pregestational diabetes.

miRNAs regulate gene expression involved in embryonic heart development, and have been shown to serve as diagnostic biomarkers and treatments for CHDs [21,37,38]. In our study, 11 miRNAs were differentially expressed in embryonic hearts of diabetic mothers and normalized by maternal exercise. Among them, miR-29, miR-483, miR-221, miR-195, miR-423-5p, miR-130b-5p, miR-351-5p and miR-374 are involved in cardiac performance and remodeling in pathological conditions [38–45]. miR-223 protects neonatal rat cardiomyocytes from hypoxia-induced apoptosis and excessive autophagy [46]. miR-294 promotes cardiomyocyte cell cycle reentry [47]. Furthermore, a novel miRNA, miR-3102-3p.2-3p, was identified among these overlapping miRNAs, and its downregulation by pregestational diabetes and upregulation by maternal exercise were validated by qPCR analysis. Functional analysis shows that its targets mRNAs include FOXO1, Bcl2, IGF1, IGF1R, IGFBP, and IRS1, which are involved in heart development [48]. However, the specific role of miR-3102-3p.2-3p in heart development during pregestational diabetes remains to be investigated.

Normal embryonic heart development is tightly controlled by mRNAs and their dysregulation result in CHDs. In our study, 70 overlapping mRNAs were dysregulated in fetal hearts of offspring of maternal diabetes but restored to normal levels by maternal exercise. The dysregulated mRNAs were associated with embryonic heart

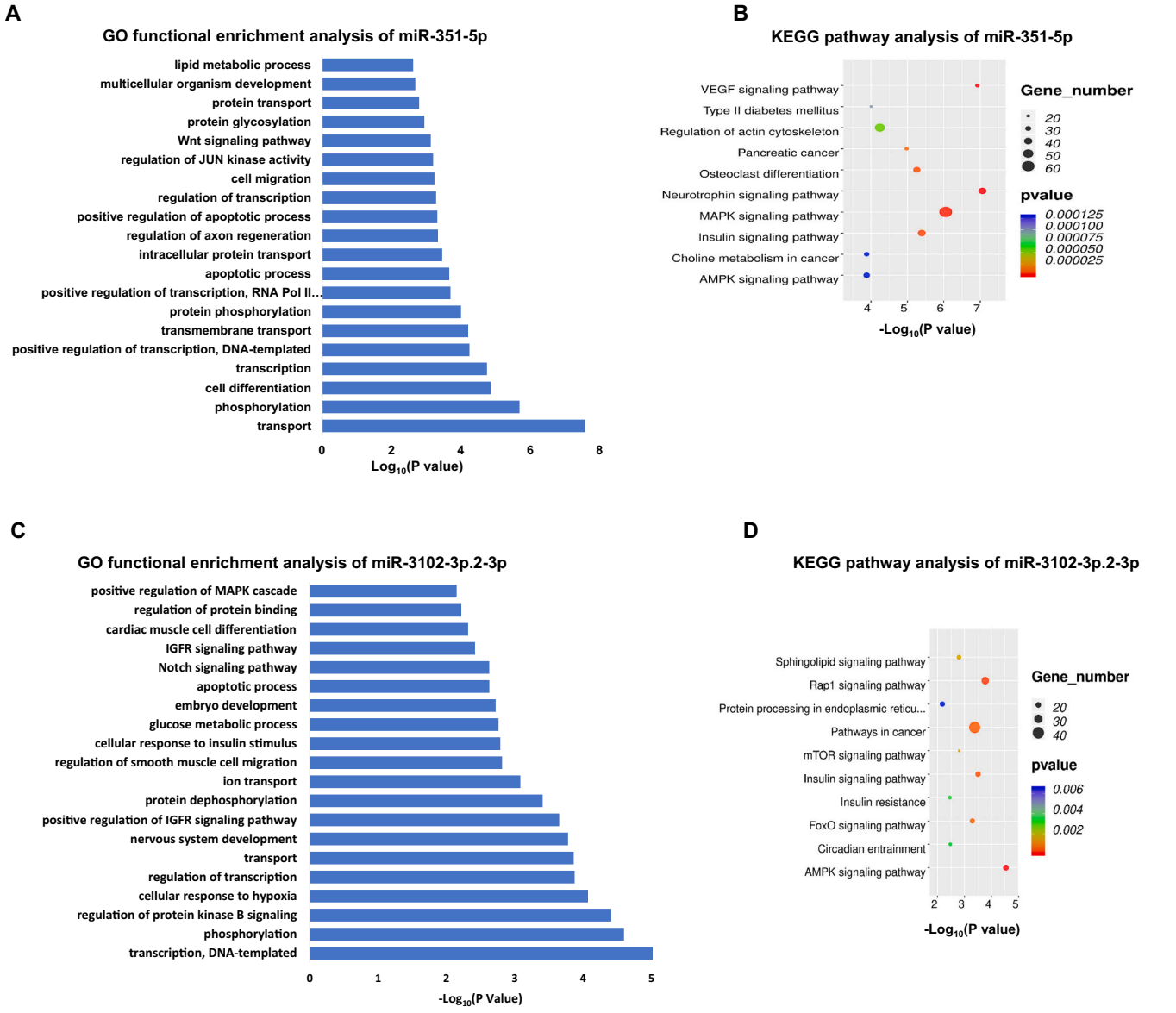

Fig. 8. Functional analysis of circ_0006127/circ_0015103-miR-351-5p and circ_0002768-miR-3102-3p.2-3p pairs. (A and B) GO function and KEGG pathway analyses of miR-351-5p, a putative target of circ_0006127 and circ_0015103. (C and D) GO function and KEGG pathway analyses of miR-3102-3p.2-3p, a putative target of circ_0002768.

development in fetal hearts exposed to diabetic pregnancy. Maternal exercise normalized the fetal heart expression of a number of novel genes including *Hmgn2* (High mobility group nucleosomal binding domain 2), *Zfp985* (Zinc finger protein 985), *Tox2* (TOX high mobility group box family member 2), *Brinp2* (bone morphogenic protein/retinoic acid inducible neural-specific 2) and *Nrros* (Negative regulator of reactive oxygen species), which are involved in development, regulation of transcription by RNA polymerase II, cellular response to retinoic acid, and TGF-beta activation [49,50].

A novel finding of the present study is the identification of circRNA-miRNA-mRNA regulatory networks that may play critical roles in heart development in diabetic pregnancy with and without maternal exercise. Of note, circRNAs (circ_0003226 and circ_0015638), and their target miR-351-5p, and circ_0002768, which targets miR-3102-3p.2-3p, were present in pregestational diabetes- and maternal exercise-related regulatory networks in fetal hearts and validated by qPCR analysis. Previous studies have shown cardioprotective effects of miR-351-5p/Sirt6 signaling through reducing oxidative stress [51]. Thus, normalization of miR-351-5p may lower oxidative stress, which is implicated in CHDs induced by maternal diabetes [52].

In conclusion, the present study analyzed the transcriptomic profiles of circRNAs, miRNAs, and mRNAs in fetal hearts of offspring of pregestational diabetes with and without maternal exercise. Differentially expressed transcripts induced by pregestational diabetes were identified. Notably, maternal exercise normalized the dysregulated expression of circ_0003226/circ_0015638/miR-351-5p and circ_0002768/miR-3102-3p.2-3p pairs in fetal hearts of offspring of pregestational diabetes. These changes may provide new insight into the potential mechanisms of the beneficial effects of maternal exercise on the developing heart in diabetic pregnancies. Our data offer exciting opportunities to further investigate the physiological and pharmacological roles of these transcripts regulated by maternal exercise in lowering the incidence of CHDs during pregestational diabetes.

CRediT authorship contribution statement

FY, XL and QF conceived and designed the experiments. FY and XL performed the experiments and data analyses. FY and QF wrote the

manuscript. FY, XL, RVN, DLJ, and QF revised the manuscript. All authors contributed to the interpretation of results and proofreading of the manuscript.

Declaration of competing interest

The authors declare that there is no conflict of interest.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2022.121308.

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