

Identification of key genes associated with congenital heart defects in embryos of diabetic mice

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Abstract. Maternal diabetes has been reported to be a critical factor for congenital heart defects (CHD) in offspring. The present study aimed to screen the key genes that may be involved in CHD in offspring of diabetic mothers. The present study obtained the gene expression profile of GSE32078, including three embryonic heart tissue samples at embryonic day 13.5 (E13.5), three embryonic heart tissue samples at embryonic day 15.5 (E15.5) from diabetic mice and their respective controls from normal mice. The cut-off criterion of $P < 0.08$ was set to screen differentially expressed genes (DEGs). Their enrichment functions were predicted by Gene Ontology. The enriched pathways were forecasted by Kyoto Encyclopedia of Genes and Genomes and Reactome analysis. Protein-protein interaction (PPI) networks for DEGs were constructed using Cytoscape. The present study identified 869 and 802 DEGs in E13.5 group and E15.5 group, respectively and 182 DEGs were shared by the two developmental stages. The pathway enrichment analysis results revealed that DEGs including intercellular adhesion molecule 1 (*Icam1*) and *H2-M9* were enriched in cell adhesion molecules; DEGs including bone morphogenetic protein receptor type 1A, transforming growth factor β receptor 1 and SMAD specific E3 ubiquitin protein ligase 1 were enriched in the tumor growth factor- β signaling pathway. In addition, DEGs including *Icam1*, *C1s* and Fc fragment of IgG receptor IIb were enriched in *Staphylococcus aureus* infection. Furthermore, the shared DEGs including *Icam1*, nuclear receptor corepressor 1 (*Ncor1*) and AKT serine/threonine kinase 3 (*Akt3*) had high connectivity degrees in the PPI network. The shared DEGs including *Icam1*, *Ncor1*

and *Akt3* may be important in the cardiogenesis of embryos. These genes may be involved in the development of CHD in the offspring of diabetic mothers.

Introduction

Diabetes mellitus is a chronic disease characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both (1). According to insulin-dependent and non-insulin-dependent, adult diabetes is classified: Type 1 and type 2 diabetes. A genetic defect on insulin was found in type 1 diabetes, whereas insulin resistance is the key metabolic abnormality in type 2 diabetes (2). In addition, maternal diabetes in pregnancy is defined as glucose intolerance or first recognition during pregnancy, who is easy to happen type 2 diabetes postpartum (3). Besides, maternal diabetes in pregnancy is related to an increased risk of congenital heart defects (CHD), macrosomia, miscarriage, and other birth defects in offspring (4,5). CHD was defined as deficits of the structure and function arising from cardiac embryogenesis stage (6). It has been reported that CHD is the major consequences in diabetic embryopathy (7).

An infant born to a diabetic mother has been shown to exhibit axial mesodermal dysplasia spectrum with atrioventricular septal defects (8). Many studies have reported that maternal diabetes altered expression of genes in developmental embryo (4,9,10). *Bmp4*, belongs to the TGF- β superfamily, is a myocardial signaling molecule which activated epithelial-mesenchymal transition (EMT) during cardiogenesis (11). The expression of *Bmp4* has been reported to be down-regulated by the *Msx1* that expressed in atrioventricular canal endocardial cells during EMT (12,13). *Pax3* is also essential for heart formation and outflow tract development in the mouse embryo (14). Study showed that the downregulation of *Bmp4*, *Msx1* and *Pax3* could contribute to the pathogenesis of maternal diabetes-induced CHD (15). In addition, it has been reported that hyperglycemia altered the expression of *eNOC* and *VEGF* that are involved in the regulation of vasculogenesis (16). Furthermore, a recent study on microarray analysis showed that several genes were altered in embryonic heart tissues from diabetic mother and were closely associated with CHD, such as *Smyd1*, *Tsc1* and *Gjal* (4). However, the exact pathogenesis of CHD in offspring of diabetic mother is still unknown.

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In this study, we explored the gene expression profiles of embryonic heart tissue samples at embryonic day 13.5 (E13.5) and embryonic day 15.5 (E15.5) from diabetic mice, and their respective controls. The differentially expressed genes (DEGs) were screened in E13.5 and E15.5 groups. Gene Ontology (GO) functions, Kyoto Encyclopedia of Genes and Genomes (KEGG) and Reactome pathway enrichment analyses were then performed to identify the DEGs. We also constructed the protein-protein interaction (PPI) networks for the DEGs and analyzed several important shared genes that were associated with CHD. Our study aimed to identify the critical genes that might be involved in CHD in offspring of diabetic mother and to provide evidence to further clarify the relationship between CHD and diabetes.

Materials and methods

Affymetrix microarray data. The gene expression profile of GSE32078 was obtained from the study of Vijaya *et al.* (4), which was deposited in Gene Expression Omnibus (GEO) database (<http://www.ncbi.nlm.nih.gov/geo/>) of National Center of Biotechnology Information (NCBI). A total of 12 samples were available, including three embryonic heart tissue samples at E13.5 and three embryonic heart tissue samples at E15.5 from streptozotocin-induced diabetic Swiss albino mice (8–10 weeks), as well as their respective controls from normal mice. The day when a copulation plug was observed was counted as E0.5. Raw data were collected with the Affymetrix Mouse Genome 430 2.0 Array (Affymetrix, CA, USA). All raw data files were pretreated by RMA method in Affy package (17).

Screening of DEGs. The Linear Models for Microarray Data (LIMMA) package (18) was used to identify DEGs. All genes were tested with *F*-test and the DEGs with the threshold *P*-value <0.08 were screened.

GO and KEGG pathway enrichment analysis. ClusterProfiler, a new ontology-based tool, offers three methods (group GO, enrich GO, enrich KEGG) for genes classification and enrichment analyses (19,20). ClusterProfiler package was used to identify the main biological processes and metabolic pathways in DEGs. Default parameters (organism, mouse; ont, BP; *P*-value cut-off, 0.05; *P*-adjust method, none; readable, T) were used as the cut-off criteria for GO function enrichment analysis. And the default parameters (organism, mouse; *P*-value cut-off, 1; *q*-value cut-off, 1; readable, 1) were used as the cut-off criteria for KEGG pathway enrichment analysis.

Reactome pathway enrichment analysis. ReactomePA package was used to identify the main biological processes and metabolic pathways in DEGs (21,22). Default parameters (organism, mouse; *P*-value cut-off, 1; *q*-value cut-off, 1; minGSSize, 1; readable, 1) were used as the cut-off criteria for Reactome pathway enrichment analysis.

PPI network construction. The Search Tool for the Retrieval of Interacting Genes (STRING) database was used to obtain PPI data. The PPI networks of up- and downregulated DEGs

were visualized by Cytoscape (<http://cytoscape.org/>) that is an open source software for visualizing complex networks and integrating these networks with any type of attribute data (23). The Required Confidence score >0.4 was chosen as the threshold.

Results

Screening of DEGs. The microarray dataset GSE32078 from GEO database was obtained to identify the DEGs in E13.5 and E15.5 hearts of embryos from diabetic mice compared with their respective controls. Totally, 869 DEGs were obtained in E13.5 group when compared with the control group, including 411 up- and 458 downregulated DEGs. Meanwhile, 802 genes were upregulated and 1,295 genes were downregulated in E15.5 group when compared with the control group. Finally, a total of 182 DEGs were shared between E13.5 and E15.5 groups, including 63 up- and 119 downregulated DEGs.

GO function enrichment analysis of DEGs. According to the GO enrichment analysis, we found that the upregulated DEGs in E13.5 group were mainly enriched in developmental process ($P=2.32E-08$) and the downregulated DEGs were mainly enriched in organic substance metabolic process ($P=2.93E-06$). In the E15.5 group, the upregulated DEGs were mainly enriched in cellular process ($P=2.64E-19$) and the downregulated DEGs were mainly enriched in cellular process ($P=2.01E-19$). Besides, we also performed the GO function enrichment analysis for the genes that were shared by E13.5 group and E15.5 group. The results showed that the upregulated DEGs were mainly related to cellular process ($P=0.00650$) and the downregulated DEGs were mainly related to organic substance metabolic process ($P=0.00031$) (Fig. 1).

KEGG and Reactome pathway analysis of DEGs. KEGG and Reactome enrichment pathway analysis were performed to explore the enriched pathways of the DEGs. The KEGG enrichment analysis results showed that the upregulated DEGs in E13.5 group were enriched in 64 pathways, such as *Icam1*, *H2-M9*, and *4930468A15Rik* were enriched in cell adhesion molecules (CAMs) ($P=1.56E-03$). The downregulated DEGs in E13.5 group were enriched in 84 pathways, such as *Bmpr1a*, *Tgfb1* and *Smurf1* were enriched in TGF- β signaling pathway ($P=4.03E-03$). The upregulated DEGs in E15.5 group were enriched in 129 pathways, such as *Icam1*, *C1s* and *Fcgr2b* were enriched in *Staphylococcus aureus* infection ($P=1.80E-04$). Meanwhile, the downregulated DEGs in E15.5 group were enriched in 156 pathways, such as *Erollb*, *Dnajc3* and *Dnajb11* were enriched in protein processing in endoplasmic reticulum ($P=1.27E-03$). Moreover, the shared DEGs that were upregulated such as *Cmah* and *Gmppa* were enriched in nucleotide sugar metabolism ($P=2.65E-03$). And the shared DEGs that were downregulated such as *Akt3*, *Pak3* and *Hgf* were enriched in renal cell carcinoma ($P=5.00E-04$); *Akt3*, *Pak3* and *Pak1* were enriched in T cell receptor signaling pathway ($P=2.60E-03$) (Table I). Reactome enrichment analysis results showed that the upregulated DEGs in E13.5 group were enriched in 154 pathways and the downregulated DEGs in E13.5 group were enriched in 192 pathways. The upregulated DEGs in E15.5 group were enriched in 27 pathways

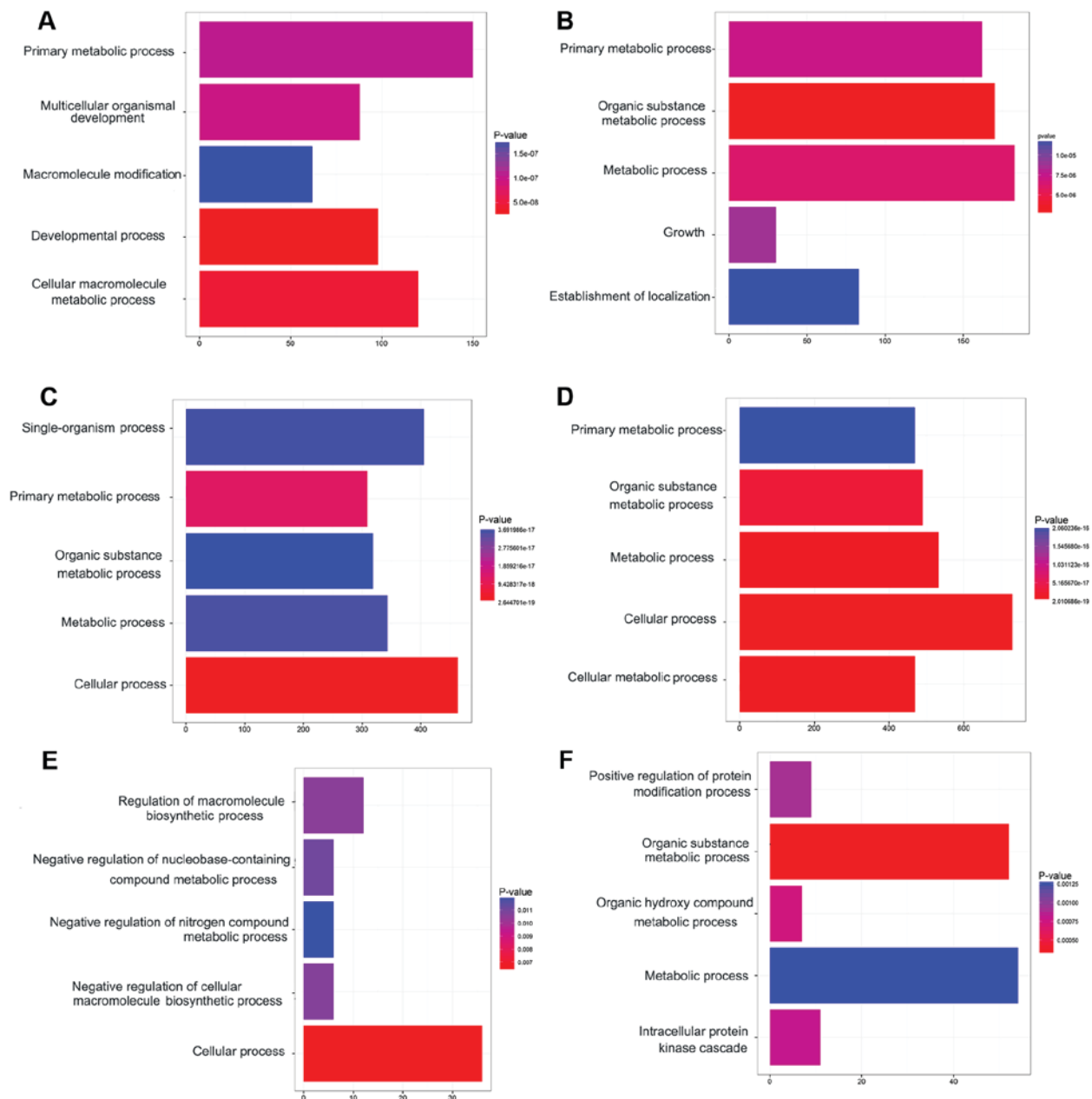


Figure 1. GO function analysis of DEGs in the developing hearts of embryos from diabetic mice. (A) GO function analysis of upregulated DEGs in E13.5 group; (B) GO function analysis of downregulated DEGs in E13.5 group; (C) GO function analysis of upregulated DEGs in E15.5 group; (D) GO function analysis of downregulated DEGs in E15.5 group; (E) GO function analysis of upregulated DEGs in E13.5 group and E15.5 group; and (F) GO function analysis of downregulated DEGs both in E13.5 group and E15.5 group. GO, gene ontology; DEGs, differentially expressed genes; E13.5, embryonic day 13.5; E15.5, embryonic day 15.5.

and the downregulated DEGs in E15.5 group were enriched in 24 pathways. In addition, the shared DEGs that were upregulated such as *Nr6a1* and *Esrrg* were enriched in nuclear receptor transcription pathway ($P=5.09E-03$) and the shared DEGs that were downregulated such as *Pak3* and *Pak1* were enriched in activation of Rac ($P=1.85E-03$) (Table II).

PPI network construction. STRING was used to construct the PPI networks for DEGs. The PPI networks for up- and downregulated DEGs in E13.5 group contained 114 and 112 nodes, respectively (Fig. 2); the PPI networks for up- and downregulated DEGs in E15.5 group contained 345 and 562 nodes, respectively (Fig. 3). The proteins such as *Icam1*,

Akp3, *Stat1* and *Bcr1* had high connectivity degrees in the PPI networks of E13.5 (Table III) and E15.5 (Table IV) groups. Additionally, the PPI network for shared DEGs contained 17 nodes and 12 PPI pairs (Fig. 4). The shared DEGs with top three connectivity degrees of the PPI network were *Icam1*, *Ncor1* and *Akt3* (Table V).

Discussion

Maternal diabetes is a relatively common disease that results in an increased incidence of congenital malformations such as neural tube defects and heart defects (9). CHD are the most common type of birth defects and a main cause of

Table I. Enrichment analysis of top five KEGG pathways for DEGs.

DEGs	Term	Description	P-value	Gene ID	Count
Upregulated DEGs in E13.5	mmu04514	Cell adhesion molecules (CAMs)	1.56x10 ⁻⁰³	<i>Icam1, H2-M9, 4930468A15Rik, H2-D1, Itga4, H2-Ob, Cntnap2, Nrxa2</i>	8
	mmu04672	Intestinal immune network for IgA production	3.77x10 ⁻⁰³	<i>Gm13306, Itga4, Il15, H2-Ob</i>	4
	mmu05330	Allograft rejection	8.24x10 ⁻⁰³	<i>H2-M9, H2-D1, H2-Ob, Il12b</i>	4
	mmu04940	Type I diabetes mellitus	1.24x10 ⁻⁰²	<i>H2-M9, H2-D1, H2-Ob, Il12b</i>	4
	mmu04146	Peroxisome	2.74x10 ⁻⁰²	<i>Agps, Nudt19, Slc25a17, Acsbg2</i>	4
Downregulated DEGs in E13.5	mmu04350	TGF- β signaling pathway	4.03x10 ⁻⁰³	<i>Bmpr1a, Tgfb1, Smurf1, Ltbp1, Lefty2, Thbs2</i>	6
	mmu05211	Renal cell carcinoma	9.14x10 ⁻⁰³	<i>Akt3, Pak3, Hgf, Pak1, Pik3ca</i>	5
	mmu04080	Neuroactive ligand-receptor interaction	1.05x10 ⁻⁰²	<i>Prlr, Gabra1, Tshr, Gabra2, Gabrb2, Crhr2...</i>	11
	mmu05223	Non-small cell lung cancer	1.66x10 ⁻⁰²	<i>Akt3, Pik3ca, Stk4, Rarb</i>	4
	mmu04210	Apoptosis	1.87x10 ⁻⁰²	<i>Irak3, Il3, Akt3, Endod1, Pik3ca</i>	5
Upregulated DEGs in E15.5	mmu05150	<i>Staphylococcus aureus</i> infection	1.80x10 ⁻⁰⁴	<i>Icam1, C1s, Fcgr2b, H2-Ab1, C2, Fpr2, Cfh, H2-Aa</i>	8
	mmu04514	Cell adhesion molecules (CAMs)	1.18x10 ⁻⁰³	<i>Icam1, Vcan, H2-K1, Cd80, Ncam1...</i>	13
	mmu04145	Phagosome	1.61x10 ⁻⁰³	<i>Lamp2, H2-K1, Tap2, Tuba4a, Thbs2, Fcgr2b...</i>	14
	mmu05219	Bladder cancer	2.20x10 ⁻⁰³	<i>Rb1, Mmp9, Myc, E2f3, Egfr, E2f2</i>	6
	mmu05220	Chronic myeloid leukemia	2.27x10 ⁻⁰³	<i>Bcl2l1, Rb1, Myc, Cblb, E2f3, Sos2, E2f2, Abl1</i>	8
Downregulated DEGs in E15.5	mmu04141	Protein processing in endoplasmic reticulum	1.27x10 ⁻⁰³	<i>Ero1lb, Dnajc3, Dnajb11, Sec62, Der1l, Mapk8...</i>	18
	mmu01100	Metabolic pathways	2.25x10 ⁻⁰³	<i>Phgdh, B3gnt5, Sucla2, Alad, B4galt6, Mmab...</i>	77
	mmu04977	Vitamin digestion and absorption	4.84x10 ⁻⁰³	<i>Tcn2, Rbp2, Plb1, Lrat, Slc5a6</i>	5
	mmu04010	MAPK signaling pathway	9.22x10 ⁻⁰³	<i>Cacna1a, Ptpn5, Mapk8, Cacna2d1, Akt3, Gna12...</i>	22
	mmu00512	Mucin type O-Glycan biosynthesis	9.57x10 ⁻⁰³	<i>Gcnt3, C1galt1, Galnt2, Galnt7, Galnt15</i>	5
Upregulated genes in shared DEGs	mmu00520	Amino sugar and nucleotide sugar metabolism	2.65x10 ⁻⁰³	<i>Cmah, Gmppa</i>	2
	mmu01100	Metabolic pathways	6.08x10 ⁻⁰¹	<i>Lias, Gmppa</i>	2
Downregulated genes in shared DEGs	mmu05211	Renal cell carcinoma	5.00x10 ⁻⁰⁴	<i>Akt3, Pak3, Hgf, Pak1,</i>	4
	mmu04660	T cell receptor signaling pathway	2.60x10 ⁻⁰³	<i>Akt3, Pak3, Pak1, Grap2</i>	4
	mmu04730	Long-term depression	6.09x10 ⁻⁰³	<i>Cacna1a, Gna12, Ppp1r17</i>	3
	mmu04210	Apoptosis	9.95x10 ⁻⁰³	<i>Akt3, Il3, Endod1</i>	3
	mmu04012	ErbB signaling pathway	1.03x10 ⁻⁰²	<i>Akt3, Pak3, Pak1</i>	3
	mmu04510	Focal adhesion	1.96x10 ⁻⁰²	<i>Akt3, Pak3, Hgf, Pak1,</i>	4
	mmu04010	MAPK signaling pathway	5.02x10 ⁻⁰²	<i>Akt3, Cacna1a, Gna12, Pak1</i>	4
	mmu04080	Neuroactive ligand-receptor interaction	5.48x10 ⁻⁰²	<i>Prlr, Tshr, Gabrb2, Crhr2</i>	4
	mmu04630	Jak-STAT signaling pathway	4.49x10 ⁻⁰²	<i>Akt3, Prlr, Il3</i>	3
	mmu00140	Steroid hormone biosynthesis	3.31x10 ⁻⁰²	<i>Hsd17b7, Hsd11b2</i>	2

E13.5, embryonic day 13.5; E15.5, embryonic day 15.5; DEGs, differentially expressed genes; KEGG, Kyoto Encyclopedia of Genes and Genomes; DEGs, differentially expressed genes.

Table II. Enrichment analysis of top five Reactome pathways for DEGs.

DEGs	Term	Description	P-value	Gene ID	Count
Upregulated DEGs in E13.5	4809882	Neuronal system	2.10x10 ⁻⁰³	<i>Gjd2, Braf, Kcnb1, Chrna3, Gabrg2, Gls...</i>	10
	4810204	Neurotransmitter receptor binding and downstream transmission in the post-synaptic cell	2.14x10 ⁻⁰³	<i>Braf, Chrna3, Gabrg2, Arhgef9, Ap2a2, Epb4.1l1, Rps6ka2</i>	7
	4810917	Interferon γ signaling	2.64x10 ⁻⁰³	<i>Icam1, Oas1h, Irf7, Sp100, H2-D1</i>	5
	4809881	Transmission across chemical synapses	3.10x10 ⁻⁰³	<i>Braf, Chrna3, Gabrg2, Gls, Arhgef9, Ap2a2, Epb4.1l1, Rps6ka2</i>	8
	4810848	Regulation of cytoskeletal remodeling and cell spreading by IPP complex components	5.26x10 ⁻⁰³	<i>Parvb, Pxn</i>	2
Downregulated DEGs in E13.5	4810408	GABA receptor activation	3.56x10 ⁻⁰⁴	<i>Gabra1, Gabra2, Gabrb2, Gngt2, Kcnj3, Kcnj9</i>	6
	4809881	Transmission across chemical synapses	8.56x10 ⁻⁰⁴	<i>Cacna1a, Gabra1, Gabra2, Gabrb2, Camk2b, Gngt2...</i>	10
	4809882	Neuronal system	9.71x10 ⁻⁰⁴	<i>Cacna1a, Gabra1, Gabra2, Gabrb2, Camk2b, Kcnq5...</i>	12
	4810957	GABA A receptor activation	1.01x10 ⁻⁰³	<i>Gabra1, Gabra2, Gabrb2</i>	3
	4810204	Neurotransmitter receptor binding and downstream transmission in the post-synaptic cell	1.68x10 ⁻⁰³	<i>Gabra1, Gabra2, Gabrb2, Camk2b, Gngt2, Kcnj3, Kcnj9, Chrb3</i>	8
Upregulated DEGs in E15.5	4810016	Immune system	1.13x10 ⁻⁰⁷	<i>Ifitm3, Ifitm6, Lgals3, H2-K1, Tap2, Bcl2l1...</i>	64
	4810918	Interferon signaling	5.69x10 ⁻⁰⁵	<i>Icam1, Stat1, Irf1, Oas1h, Ncam1, Ifnar2...</i>	13
	4810008	EGFR interacts with phospholipase C- γ	5.88x10 ⁻⁰⁵	<i>Adcy7, Plcg1, Adrbk1, Egfr, Creb1, Camk4, Adcy9</i>	7
	4810917	Interferon γ signaling	6.00x10 ⁻⁰⁵	<i>Icam1, Stat1, H2-K1, Irf1, Oas1h, Ncam1, Socs3...</i>	10
	4810011	PLCG1 events in ERBB2 signaling	7.26x10 ⁻⁰⁵	<i>Adcy7, Plcg1, Adrbk1, Egfr, Creb1, Camk4, Adcy9</i>	7
Downregulated DEGs in E15.5	4809896	Metabolism of water-soluble vitamins and cofactors	2.69x10 ⁻⁰³	<i>Amn, Mmaa, Mmab, Tcn2, Slc23a2, Mthfr, Rfk, Slc5a6, Mocs1, Ppcs</i>	10
	4809897	Metabolism of vitamins and cofactors	2.69x10 ⁻⁰³	<i>Amn, Mmaa, Mmab, Tcn2, Slc23a2, Mthfr, Rfk, Slc5a6, Mocs1, Ppcs</i>	10
	4809898	Defective TCN2 causes hereditary megaloblastic anemia	2.69x10 ⁻⁰³	<i>Amn, Mmaa, Mmab, Tcn2, Slc23a2, Mthfr, Rfk, Slc5a6, Mocs1, Ppcs</i>	10
	4809899	Defects in cobalamin (B12) metabolism	2.69x10 ⁻⁰³	<i>Amn, Mmaa, Mmab, Tcn2, Slc23a2, Mthfr, Rfk, Slc5a6, Mocs1, Ppcs</i>	10
	4809900	Defects in vitamin and cofactor metabolism	2.69x10 ⁻⁰³	<i>Amn, Mmaa, Mmab, Tcn2, Slc23a2, Mthfr, Rfk, Slc5a6, Mocs1, Ppcs</i>	10
Upregulated genes in shared DEGs	4810720	Nuclear receptor transcription pathway	5.09x10 ⁻⁰³	<i>Nr6a1, Esrrg</i>	2
	4810917	Interferon γ signaling	9.26x10 ⁻⁰³	<i>Icam1, Oas1h</i>	2
	4810918	Interferon signaling	2.35x10 ⁻⁰²	<i>Icam1, Oas1h</i>	2
	4810429	Generic transcription pathway	3.07x10 ⁻⁰²	<i>Nr6a1, Esrrg</i>	2
	4810016	Immune system	6.30x10 ⁻⁰²	<i>Icam1, Oas1h, C6, Fbxw11, Kif2a</i>	5

Table II. Continued.

DEGs	Term	Description	P-value	Gene ID	Count
Downregulated genes in shared DEGs	4810839	Activation of Rac	1.85x10 ⁻⁰³	<i>Pak3, Pak1</i>	2
	4810573	CD28 co-stimulation	5.02x10 ⁻⁰³	<i>Pak1, Grap2</i>	2
	4810613	Generation of second messenger molecules	5.02x10 ⁻⁰³	<i>Pak1, Grap2</i>	2
	4810625	Signaling by Robo receptor	9.02x10 ⁻⁰³	<i>Pak3, Pak1</i>	2
	4810145	FCERI mediated MAPK activation	1.55x10 ⁻⁰²	<i>Pak1, Grap2</i>	2

E13.5, embryonic day 13.5; E15.5, embryonic day 15.5; DEGs, differentially expressed genes.

Table III. DEGs with the top 10% connectivity degree in the PPI network in E13.5 group.

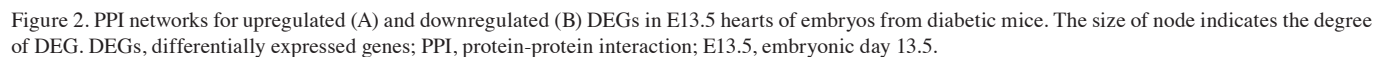
DEGs	ID	Degree	ID	Degree	ID	Degree	ID	Degree
Upregulated DEGs	Icam1	9	H2-M9	5	Ube2t	4	Calb1	3
	H2-L	8	Gabrg2	5	Sgip1	4	Itga4	3
	Irf7	7	Pxn	5	Ap2a2	3		
	Oas1h	5	Il12b	4	Rnf2	3		
Downregulated DEGs	Akp3	13	Ncor1	7	Pik3ca	6	Akt3	5
	Nr3c1	10	Stat2	6	Bmpr1a	6	Gna15	5
	Ar	9	Il3	6	Gngt2	5	Mut	4

DEGs, differentially expressed genes; PPI, protein-protein interaction; E13.5, embryonic day 13.5.

Table IV. DEGs with the top 10% connectivity degree in the PPI network in E15.5 group.

DEGs	ID	Degree	ID	Degree	ID	Degree	ID	Degree	ID	Degree
Upregulated DEGs	Stat1	38	Mmp9	19	Ifih1	16	Ezr	13	Lgals3bp	12
	Egfr	37	Irf1	18	Cxcl1	15	Mbp	13	Oasl2	12
	Icam1	27	Ccr2	18	Ifi35	14	Hnrnpa1	13	Rbm25	12
	Myc	27	Fpr2	18	Actb	14	Rmcs2	13	Ccr9	12
	Pten	25	Ncam1	17	Ccl6	14	Cpsf6	13	H2-Aa	12
	Stat2	21	Plcg1	17	Cblb	14	Adrbk1	12	Abl1	12
	Yes1	20	Creb1	16	H2-K1	14	Rsad2	12		
Downregulated DEGs	Brca1	33	Dlgap5	20	Tmem48	17	Rpn1	12	Hao1	10
	Chek1	30	Creb1	20	Hspa5	16	Kcnj11	12	Ugt2b5	10
	Bub1	29	Aspm	20	Lsm4	14	Med1	11	Abcb11	10
	Rrm2	26	Zwint	20	Akt3	14	Calr	11	Gnao1	10
	Sgol2	25	Cenpm	19	Sec61a1	13	Kalrn	11	Gabrg2	10
	Cep55	25	Spc24	19	Lpar3	13	Slc10a1	11	Cd40	10
	Plk4	24	Eme1	19	Hjurp	13	Rgn	10	Chrm4	10
	Tpx2	24	Foxm1	19	Nasp	13	Cfi	10	Ppp2r1a	9
	Kif23	23	Xpo1	18	Igf1r	13	Ddost	10	Nfatc2	9
	Ska1	23	Troap	18	Gucy1b3	13	Prkx	10	Hpd	9
	Prc1	23	Oip5	17	Ncor1	12	Rae1	10	Fanci	9

DEGs, differentially expressed genes; PPI, protein-protein interaction; E15.5, embryonic day 15.5.



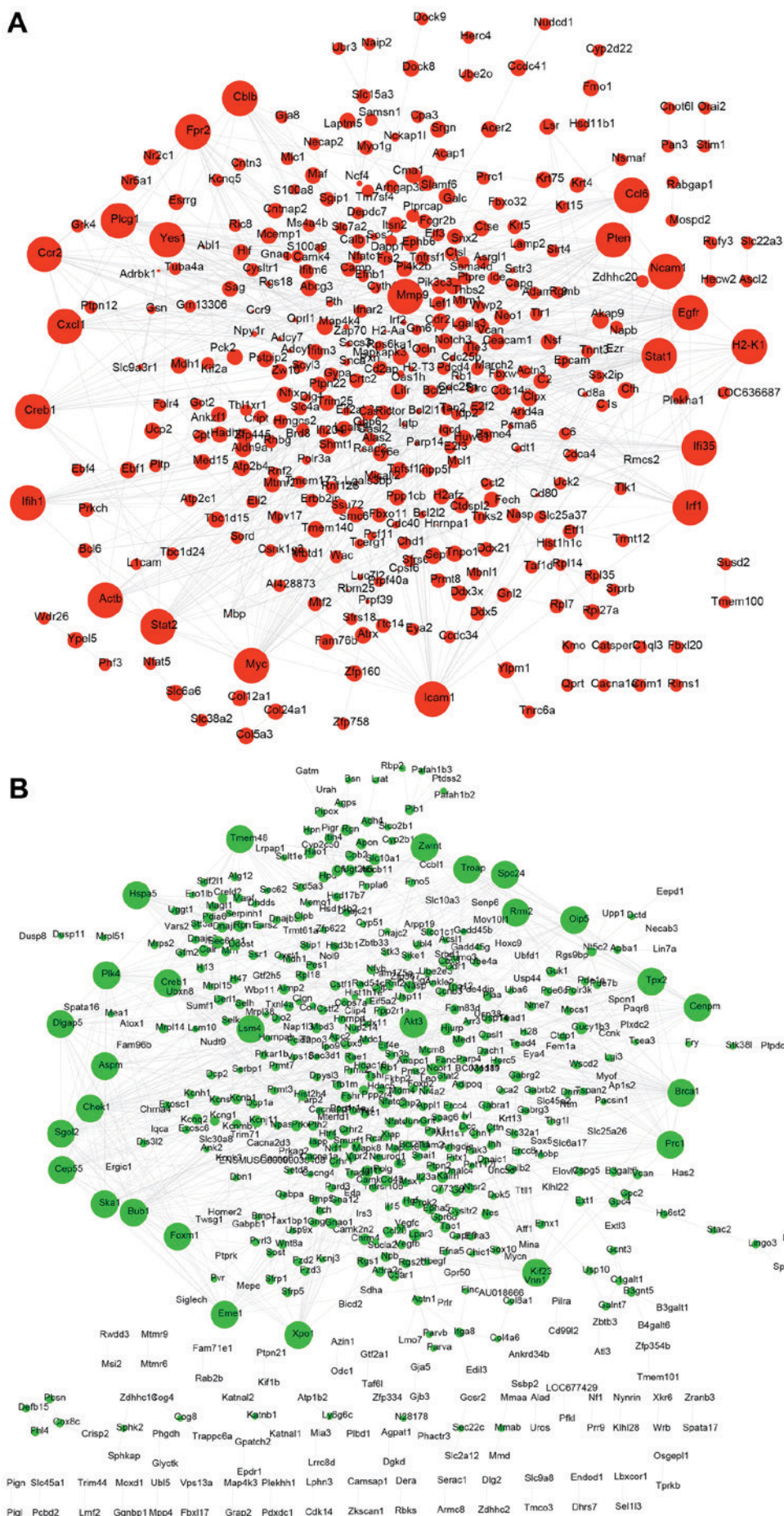


Figure 3. PPI networks for upregulated (A) and downregulated (B) DEGs in E15.5 hearts of embryos from diabetic mice. The size of node indicates the degree of DEG. DEGs, differentially expressed genes; PPI, protein-protein interaction; E15.5, embryonic day 15.5.

Table V. Connectivity degrees in the PPI network for shared DEGs in E13.5 and E15.5 groups.

ID	Degree	ID	Degree	ID	Degree	ID	Degree
Icam1	3	Oca2	1	Hgf	1	Tpx2	1
Ncor1	3	Slc45a2	1	Esrrg	1	Il3	1
Akt3	2	Appl1	1	Pak3	1		
Oas1h	2	Sgip1	1	Pak1	1		
Nr6a1	2	Calb1	1	Iqcd	1		

DEGs, differentially expressed genes; PPI, protein-protein interaction; E13.5, embryonic day 13.5; E15.5, embryonic day 15.5.

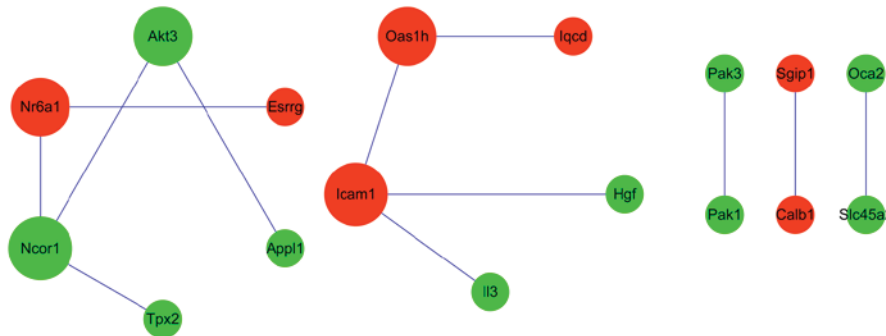


Figure 4. PPI networks for DEGs both in E13.5 and E15.5 hearts of embryos from diabetic mice. The red and green nodes respectively indicate up- and downregulated DEGs. The size of node indicates the degree of DEG. DEGs, differentially expressed genes; PPI, protein-protein interaction; E13.5, embryonic day 13.5; E15.5, embryonic day 15.5.

birth defects-related mortality and morbidity (24,25). It has been reported that CHD are closely associated with maternal diabetes (26). Previous study had screened and analyzed several DEGs in embryonic heart tissues from diabetic mother (4). However, the molecular mechanism between CHD and diabetes remains largely unknown. In this study, we have screened 869 and 1,295 in E13.5 and E15.5 groups, respectively and 182 DEGs were shared by two groups. Moreover, the DEGs such as *Icam1* and *H2-M9* were significantly enriched in cell adhesion molecules (CAMs); DEGs such as *Bmpr1a*, *Tgfb1* and *Smurf1* were enriched in TGF- β signaling pathway; DEGs such as *Icam1*, *C1s* and *Fcgr2b* were enriched in *Staphylococcus aureus* infection. Finally, several key shared DEGs that were the genes with the top three node degrees in the network were analyzed, including *Icam1*, *Ncor1* and *Akt3*.

Our results showed that *Icam1* had the highest connectivity degree not only in the PPI network of the upregulated DEGs in E13.5 group but also in the PPI network of the shared DEGs. *ICAM1*, also called CD54, is a cell surface glycoprotein which is typically expressed on endothelial cells and cells of the immune system (27) and the protein is considered to participate in atherogenesis by promoting monocyte accumulation in the arterial intima (28). An earlier study has found that the expression of *ICAM1* on endothelial cells and circulating monocytes may be critical for the adhesion of the cells on the vascular endothelium (29). And the expression of *ICAM1* has been also detected on cardiac myocytes both in adult humans with unexplained cardiac dysfunction (30) and animals with myocarditis (31,32). According to the

pathway enrichment results in our study, we found that *Icam1* were enriched in *Staphylococcus aureus* infection, CAMs and interferon γ signaling. Inflammation is showed to participate in the pathogenesis of type 2 diabetes (33). Upregulation of adhesion molecules such as *ICAM1* is pivotal in the development of inflammatory responses (34). Besides, autoimmune-associated congenital heart block (CHB) may result from pathogenic cross-talk between inflammatory and profibrosing pathways (35). Therefore, *Icam1* might be involved in the development of heart in mouse embryos.

Ncor1 also had a high node degree in the PPI network for the shared DEGs. It is well known that *Ncor1* is a transcriptional coregulator that controls the activity of many transcription factors (such as *MEF2*, *ERRs*) and has wide-ranging effects on gene expression patterns (36). *MEF2* family has been associated with regulation of myocardially-expressed genes within the heart, such as cardiac α -actin (37). *MEF2* is critical for normal heart development and mitochondrial integrity (38). There is a strict relationship between oxygen consumption and cardiac work (39). Oxidative stress is thought to play a particularly critical role in the development of cardiovascular pathology (40). *SIRT1* that is part of *Ncor1*/SMRT complex (36) could retard aging and confer oxidative stress resistance to the heart *in vivo* (41,42). *SMRT* (*Ncor2*), the homolog of *Ncor1*, is also considered to take part in heart formation (43). Thus, *Ncor1*, as a transcriptional coregulator, might regulate several genes that are related to the heart development through several different signaling pathways.

Akt3 was interacted with *Ncor1* in the PPI network for the shared DEGs. Study has showed that *Akt3* is a member of the

Akt subfamily that comprises three closely related isoforms *Akt1*, *Akt2* and *Akt3*. *Akt* regulates several cellular process including metabolism, cell growth, proliferation, survival and angiogenesis (44). Dysregulation of *Akt* leads to many diseases such as cancer, diabetes, cardiovascular and neurological diseases (44). *Akt* has an important role in the functional behavior in the cardiovascular system such as cardiomyocytes, thrombocytes and endothelial cells (44). *Akt1* is demonstrated to be essential for heart development and function (45). In addition, *Akt3* was found to be enriched in most of the KEGG pathways in shared DEGs, especially renal cell carcinoma. Renal insufficiency in patients with acquired heart failure and ischemic heart disease is related to higher mortality and morbidity (46). Therefore, *Akt3* might to be related to the heart development during embryogenesis.

Additionally, there are some limitations in the present study. For example, much more samples should be used to clarify the finding; the expression level of *Icam1*, *Ncor1*, and *Akt3* should be verified by RT-PCR in the maternal diabetes associated with CHD. However, the present study may provide a scientific guidance for future study to clarify the relationship between CHD and diabetes. It is helpful to explain the molecular mechanism of the CHD development in offspring of diabetic pregnancies. These DEGs may be the therapeutic target in the offspring of diabetic pregnancies with CHD.

In conclusion, we have identified many DEGs in embryonic heart tissue samples at E13.5 and E15.5 from diabetic mice using bioinformatics analysis. And we found that the shared DEGs such as *Icam1*, *Ncor1*, and *Akt3* had high connectivity degrees in the network. Our study implied that maternal diabetes could affect *Icam1*, *Ncor1*, and *Akt3* which are critical in the heart development during embryogenesis and might result in CHD. However, more research is needed to confirm these results and further explore the complex molecular mechanism.

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