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Pregestational type 2 diabetes mellitus induces cardiac hypertrophy in the murine embryo through cardiac remodeling and fibrosis

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Abstract

Background—Cardiac hypertrophy is highly prevalent in patients with type 2 diabetes mellitus (T2DM). Experimental evidence has implied that pregnant women with T2DM and their children are at an increased risk of cardiovascular diseases. Our previous mouse model study has revealed that maternal T2DM induces structural heart defects in their offspring.

Objective—The present study aims to determine whether maternal T2DM induces embryonic heart hypertrophy in a murine model of diabetic embryopathy.

Study design—The T2DM embryopathy model was established by feeding 4-week-old female C57BL/6J mice with a high-fat diet (HFD) for 15 weeks. Cardiac hypertrophy in embryos at embryonic day 17.5 was characterized by measuring heart size and thickness of the right and left ventricle walls and the interventricular septum, as well as the expression of β-myosin heavy chain (β-MHC), atrial natriuretic peptide (ANP), insulin-like growth factor 1 (IGF1), desmin (DES), and adrenomedullin (ADM). Cardiac remodeling was determined by collagen synthesis and fibronectin synthesis. Fibrosis was evaluated by Masson staining and determining the expression of connective tissue growth factor (CTGF), osteopontin (OPN), and Galectin 3 (GAL3) genes. Cell apoptosis also was measured in the developing heart.

Results—The thicknesses of the left ventricle walls and the interventricular septum of embryonic hearts exposed to maternal diabetes were significantly thicker than those in the nondiabetic (ND)

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Authors' contributions

XL, Peng Y, and Pei Y designed the study. XL and Peng Y performed the experiment and analyzed the data. XL, Peng Y, and Pei Y wrote the manuscript. All authors revised the manuscript critically and approved the final version.

group. Maternal diabetes significantly increased β-MHC, ANP, IGF1 and DES expression, but decreased expression of ADM. Moreover, collagen synthesis was significantly elevated, whereas fibronectin synthesis was suppressed, in embryonic hearts from diabetic dams, suggesting that cardiac remodeling is a contributing factor to cardiac hypertrophy. The cardiac fibrosis marker, GAL3, was induced by maternal diabetes. Furthermore, maternal T2DM activated the pro-apoptotic c-Jun-N-terminal kinase (JNK1/2) stress signaling and triggered cell apoptosis by increasing the number of TUNEL positive cells (10.4 ± 2.2% of the T2DM group vs. 3.8 ± 0.7% of the ND group, $P < 0.05$).

Conclusions—Maternal T2DM induces cardiac hypertrophy in embryonic hearts. Adverse cardiac remodeling, including elevated collagen synthesis, suppressed fibronectin synthesis, profibrosis and apoptosis, is implicated as the etiology of cardiac hypertrophy.

Keywords

pregestational type 2 diabetes; hypertrophy; cardiac remodeling; fibrosis; diabetic embryopathy

Introduction

The mechanism of “early life programming” has been well accepted, linking early growth and subsequent risk of diseases including obesity, type 2 diabetes mellitus (T2DM), and ischemic heart disease[1–6]. Pregestational diabetes is the leading cause for fetal intrauterine growth retardation[7]. It is well known that a pregnant woman with T2DM and her unborn child are both at increased risk of pregnancy complications such as pre-eclampsia, preterm births, stillbirths, macrosomia, miscarriage, intrauterine growth retardation, and congenital anomalies[8–12]. Specifically, heart hypertrophy was highly prevalent in asymptomatic patients with T2DM[13]. However, it is unknown whether pregestational type 2 maternal diabetes influences the heart of the fetus.

Neonatal echocardiographic data indicate that cardiac septal overgrowth affects 10% to 40% of neonates born to mothers with pregestational diabetes[14–16]. The functional impact of neonatal cardiac septal hypertrophy can range from clinically asymptomatic to potentially fatal congestive heart failure stemming from left ventricular outflow tract obstruction. The underlying mechanism has been widely investigated by using a streptozocin-induced diabetic rat or mouse model of type 1 diabetes[17–19]. However, there are few reports about cardiac hypertrophy in embryos from a T2DM model.

Cardiac remodeling resulting in cardiac rupture occurs in acute streptozocin-induced diabetes mouse models[20]. Further evidence indicated that cardiac remodeling in patients with T2DM is associated with endothelial dysfunction, collagen turnover, and a late onset of heart failure[21, 22]. Diabetes affects cardiac remodeling through a variety of mechanisms, including fibrosis[23]. However, it is unknown whether cardiac remodeling occurs in the embryo of a mother with pregestational T2DM. Therefore, it is important to investigate the effect of cardiac remodeling and fibrosis on heart of offspring whose mother suffered from T2DM.

In this study, hearts from fetuses of high-fat diet (HFD)-induced T2DM dams were investigated[24]. Heart hypertrophy was significantly increased in the fetus from a T2DM mother, and cardiac remodeling occurred in the embryo from a diabetic mother. Regulation of collagen and fibronectin synthesis was disturbed in these fetal hearts by the imbalance of matrix metalloproteinases (MMPs) and the tissue inhibitors of matrix metalloproteinases (TIMPs). The subsequent increased fibrosis and apoptosis caused by maternal T2DM during pregnancy may provide a direct rationale for the emergence of heart failure in a later life stage of a neonate.

Methods and Materials

Study design

In this study, two experimental groups, the T2DM dams and the nondiabetic dams, were used to determine the effect of T2DM on cardiac hypertrophy in E17.5 embryos. Cardiac hypertrophy was morphologically evaluated and further confirmed by the expression of hypertrophy markers using quantitative PCR. To elucidate the underlying mechanism of cardiac hypertrophy, cardiac remodeling and fibrosis were examined via measurement of the collagen/fibronectin synthesis markers in embryonic hearts from T2DM and nondiabetic dams. H9C2 cell culture model was used to confirm these changes in high glucose *in vitro*-induced cardiac hypertrophy.

Animals and the T2DM model

To investigate the effect of T2DM on embryonic hearts, T2DM mice model was established as previously described[24]. Briefly, 4-week-old female C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). The mice were fed an HFD (Research Diets, New Brunswick, NJ, USA) for 15 weeks. The HFD contained 20% protein, 20% carbohydrates, and 60% fat. HFD-fed mice were mated with lean male mice. Embryonic day 0.5 (E0.5) of pregnancy was established at noon on the day when a vaginal plug was observed. Blood glucose measurement, a glucose tolerance test, and an insulin tolerance test proved that the HFD mice have the T2DM phenotype. Fetus' hearts at 17.5-day pregnancy were collected and kept in −80°C for further use.

Evaluation of hypertrophy with hematoxylin and eosin staining and calculation of hearts dimensions

To determine hypertrophy in embryonic heart from T2DM dams, hematoxylin and eosin (H&E) staining was performed using heart sections. The procedures of heart hematoxylin and eosin (H&E) staining have been described[25]. Briefly, the pregnant dams were euthanized on E17.5, and fetuses were excised from the uteri and decapitated. Fetus' hearts were then fixed in methacarn (60% methanol, 30% chloroform, and 10% glacial acetic acid), embedded in paraffin, and cut into 10-μm sections. After deparaffinization and rehydration, all specimens then underwent H & E staining through a standard procedure. All heart sections were photographed and examined for heart dimensions using Photoshop CS6 (Adobe, San Jose, California, USA). We measured the thickest dimension of the left ventricle, interventricular septum, and right ventricle.

mRNA changes in cardiac hypertrophy and remodeling markers

To demonstrate the expression of hypertrophy and cardiac remodeling markers in messenger RNA level, mRNA was isolated from E17.5-day hearts and H9C2 cells using the RNeasy Mini Kit (Qiagen, Hilden, Germany), and reversed transcribed using the High-Capacity cDNA Archive Kit (Applied Biosystems, Grand Island, NY, USA). Real-time qPCR for β-myosin heavy chain (β-MHC), atrial natriuretic peptide (ANP), insulin-like growth factor 1 (IGF1), desmin (DES), adrenomedullin (ADM), connective tissue growth factor (CTGF), osteopontin (OPN), galectin-3 (GAL)3, MMP1, MMP13, TIMP1, TIMP2, TIMP3, TIMP4 COL1a, COL4a, FN1 and β-Actin were performed using the Maxima SYBR Green/ROX qPCR Master Mix Assay (Thermo Fisher Scientific, Waltham, MA, USA) in the StepOnePlus system (Applied Biosystems, Foster City, CA, USA). The primer sequences used are listed in Supplementary Table 1.

Measurement of cardiac remodeling markers

To determine the molecular changes of cardiac remodeling markers, Western blotting was performed as previously described[26]. Briefly, hearts from the different experimental groups were sonicated in lysis buffer containing a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). Equal amounts of protein and the Precision Plus Protein standards (Bio-Rad Laboratories, Hercules, CA, USA) were resolved by SDS-PAGE and transferred onto Immobilon-P membranes (Millipore, Billerica, MA, USA). Membranes were incubated in 5% nonfat milk for 45 minutes and then incubated for 18 hours at 4°C with the primary antibodies. To determine whether equivalent amounts of protein were loaded among all samples, membranes were stripped and incubated with a mouse antibody against β-actin (Abcam, Cambridge, MA, USA) to generate a signal used as a loading control. Signals were detected using the SuperSignal West Femto Maximum Sensitivity Substrate kit (Thermo Scientific, Waltham, MA, USA). The sources and dilutions of antibodies used in each experiment are listed in Supplementary Table 2.

Fibrosis in embryonic heart from T2DM dams

To evaluate fibrosis extent, collagen content was evaluated by the masson's trichrome staining with a heart section of nondiabetes (ND) and T2DM offspring by Masson's trichrome stain kit (Sigma-Aldrich, St. Louis, MO, USA). One hundred photographs were taken for each group. We randomly took 500×400 pixel area on each photography, and measured the collagen area using ImageJ 1.49v (NIH, Bethesda, MD, USA) to 100 photography, and then the average collagen area was calculated. To measure the nuclear density of cells on each paraffin section, we randomly counted nuclear on 500×400 pixel area on 50 pictures, and then calculated the average nuclear density. The cardiomyocyte area were measured by adjusting the color threshold on 500×400 pixels, which covered only the cell area, and then each cell area was divided by the amount of nuclear in this area.

Cell apoptosis in embryonic heart from T2DM dams

To investigate the effect of T2DM on cardiac cell apoptosis, the TUNEL assay was performed using the ApopTag Fluorescein Direct In Situ Apoptosis Detection Kit (Millipore, Billericam, MA, USA) as previously described[26]. Briefly, 10-μm paraffin heart sections

were incubated with TUNEL reaction agents. Three hearts from three different dams per group were used. TUNEL-positive cells in a randomly selected area (~200 cells) of hearts were counted. The percentage of TUNEL-positive cells was calculated as a fraction of the total cell number multiplied by 100.

Cell culture

H9C2 rat cardiomyoblasts (Sigma-Aldrich, St. Louis, MO, USA) were maintained in DMEM supplemented with 10% fetal bovine serum, 90 mg/dl glucose (Invitrogen, Carlsbad, CA, USA), 100U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2. Sub-confluent cells were treated with high glucose (HG, 200 mg/dl) DMEM for 3 days. The cell surface area was determined with image analysis software ImageJ 1.49v (NIH, Bethesda, MD, USA).

Statistical Analyses

Data are presented as mean ± SE. Each set of experiments were repeated independently at least three times with comparable results, and embryonic samples from each replicate were taken from different dams. Statistical differences were evaluated using one-way analysis of variance (ANOVA) with Sigmaplot 12.5 software. For one-way ANOVA analysis, a Tukey test was used to estimate the significance of the results, with $P < 0.05$ indicating statistical significance.

Results

Maternal T2DM induces hypertrophy in the fetal heart

After feeding with a HFD for 15 weeks, dams had a blood glucose level that was significantly higher than that of ND dams (Fig. 1A), suggesting a successful establishment of the T2DM mouse model with modest hyperglycemia, which mimics T2DM in humans and is consistent with our previous findings[24]. A total of 76 fetal hearts from 16 ND dams and 40 fetal hearts from 9 T2DM dams were analyzed for heart dimension by measuring thickness of the left and right ventricle, and interventricular septum in a four-chamber view. As shown in Fig. 1B, the fetal heart size from T2DM dams was significantly larger than the size of fetal hearts from ND dams. Further investigation revealed that the thickness of the interventricular septum was higher in the T2DM group than that in the ND group (0.54 ± 0.02 mm vs. 0.42 ± 0.01 mm, $P < 0.001$), and the left ventricular walls were thicker (0.36 ± 0.01 mm vs. 0.28 ± 0.01 mm, $P < 0.05$) in the T2DM group than in the ND group (Fig. 1C). However, there was no difference in the right ventricular wall thickness between the two groups (0.26 ± 0.01 mm vs. 0.27 ± 0.01 mm) (Fig. 1C). The cardiomyocyte size in the T2DM group was significantly larger than that of the ND group ($p < 0.05$) (Fig. 1D).

Maternal T2DM increases the expression of hypertrophic markers in the developing heart

Re-expression of the early developmental gene, β-MHC, is well documented in contributing to the pathogenesis of cardiac hypertrophy in adults with T2DM[27]. Therefore, it is of interest to determine the effect of maternal diabetes on the expression of β-MHC and ANP, another cardiac hypertrophic marker, in the developing heart. The expression levels of β-MHC and ANP were significantly higher in the fetal hearts of the T2DM group compared with the ND

group ($p < 0.01$ and $p < 0.05$ respectively) (Fig. 2A and 2B). It has been reported that IGF1[28, 29], DES[30, 31], and ADM[32–34] are dysregulated along with progression of cardiac hypertrophy. Consistent with findings of others, the expression of IGF1 and DES were significantly increased in the fetal hearts of T2DM dams compared with those from ND dams ($p < 0.001$ and $p < 0.05$ respectively) (Fig. 2C and 2D). ADM attenuates hypertrophic remodeling[33], and maternal T2DM suppressed the expression of ADM (Fig. 2E).

Collagen synthesis genes were partially induced by T2DM

Cardiac hypertrophy and remodeling are pathological features of many cardiac diseases, including diabetic cardiomyopathy[35]. Cardiac remodeling and stiffness are the consequence of alteration in key components of the extracellular matrix, including collagen and fibronectin[36]. Numerous reports have demonstrated the effect of collagen concentrations and collagenolysis on hypertrophic cardiomyopathies[37]. However, the mechanism is still unclear. Because collagen dynamics are maintained by MMPs and TIMPs, we determined the expression of MMP1, MMP2, MMP8, MMP9, MMP13, and four genes of the TIMPs family. Two out of the five MMPs, MMP1 and MMP13, were significantly decreased in the fetal hearts from the DM group compared with those of the ND group ($p < 0.01$) (Fig. 3A and 3B). The expression of TIMP1 was significantly increased by maternal diabetes ($p < 0.001$) (Fig. 3C), whereas the expression of TIMP2, TIMP3, and TIMP4 was decreased by maternal diabetes ($p < 0.05$, $p < 0.01$ and $p < 0.05$ respectively) (Fig. 3D, 3E, and 3F). Furthermore, the collagen synthesis genes, COL1 and COL4, were up-regulated in the fetal hearts by maternal diabetes (Fig. 3G and 3H). All of the data demonstrated that the collagen synthesis genes, *Col1* and *Col4*, were induced by maternal T2DM.

Maternal T2DM suppresses fibronectin synthesis

Fibronectin is another important component of extracellular matrix and contributes to the pathogenesis of cardiac hypertrophy[38]. MMP24, which degrades fibronectin but not collagen, was significantly increased at both the messenger RNA level and the protein level in fetal hearts by maternal diabetes ($p < 0.05$) (Fig. 4A and 4C). MMP24 was mainly expressed in the peripheral areas of the heart in the ND group. However, in the DM group, MMP24 was overexpressed not only in the peripheral tissue, but also inside of the myocardium of the fetal heart (Fig. 4E). The overall expression of MMP24 in the DM group was higher than that of the ND group. Correlated with MM24 up-regulation, messenger RNA and protein levels of fibronectin were significantly down-regulated by maternal diabetes ($p < 0.05$) (Fig. 4B and 4D).

Maternal T2DM induces pro-fibrosis in cardiomyocytes of the developing heart

Hyperglycemia induces progressive cardiomyocyte fibrosis in diabetic patients[23]; however, little is known regarding the effect of maternal diabetes on fetal cardiomyocyte fibrosis. Fibrosis was evaluated by Masson's trichrome staining in fetal heart sections. No fibrosis was observed in the fetal hearts of either group (Fig. 5A); however, collagen staining was much higher in the fetal hearts from DM dams compared with those from ND dams ($p < 0.01$) (Fig. 5A), confirming our previous findings that T2DM increased collagen synthesis, but did not induce fibrosis. Furthermore, the expression of pro-fibrosis markers, CTGF,

OPN, and GAL3 was detected. Among them, only GAL3 expression in the DM group was significantly higher than that in the ND group ($p < 0.05$) (Fig. 5B, 5C, and 5D).

Maternal T2DM induces cell apoptosis in the developing heart

Cell apoptosis is involved in the induction of diabetic embryopathy[24, 39]. We hypothesized that maternal diabetes-induced cardiac hypertrophy was associated with enhanced cell apoptosis. Indeed, the percentage of TUNEL positive cells was significantly higher in the DM group comparing with that of the ND group (10% vs. 4%) (Fig. 6A and 6B). Increased levels of BAX, a pro-apoptotic factor, and decreased levels of Bcl-2, a pro-survival factor, were observed in the fetal hearts from the DM group (Fig. 4C). The activation of the pro-apoptotic Jun-N terminal kinase (JNK) signaling pathway is a central mechanism in diabetic embryopathy[40]. The levels of phosphorylated JNK and c-Jun were significantly increased in the DM group compared with the ND group (Fig. 4D).

Cardiac hypertrophy was induced in H9C2 cell line by high glucose in vitro

The H9C2 cell line, a cardiomyoblast cell line, shows similar hypertrophic responses to those shown by primary neonatal cardiomyocyte cells[41]. To confirm our *in vivo* findings, H9C2 cells were treated with modestly high glucose stimulation (200 mg/dl) for 3 days *in vitro*. Consistent with our *in vivo* findings, H9C2 cells showed a hypertrophic phenotype (i.e., manifesting larger cell size) after treatment with 200 mg/dl glucose compared with cells cultured in normal glucose (90 mg/dl) (Fig. 7A and 7B). Furthermore, the expression of hypertrophy genes, ANP, BNP, and IGF1, were all significantly increased by modestly high glucose stimulation ($p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively) (Fig. 7C, 7D, and 7E). ADM expression, a cardiac hypertrophy attenuator, was suppressed by modest high glucose (Fig. 7G). However, no difference of DES expression was observed between the normal and the modest high glucose groups (Fig. 7F).

Comment

Previous studies have shown that mild elevation of glucose seen in pregestational diabetes has a profound influence on the development of the offspring heart[16, 42, 43]. Our hypothesis is that chronic mild hyperglycemia during pregnancy is associated with cardiomyocyte hypertrophy of the offspring. Here, we used a T2DM mouse model with mild hyperglycemia induced by a HFD to investigate the effect of modest maternal glucose elevation during pregnancy on the developing embryonic heart. The average fasting glucose level in this model was 169 ± 38 mg/dl, which is comparable to the blood glucose level of a patient with T2DM[44]. Our results indicate that the fetuses of T2DM dams have hypertrophic hearts, supporting the clinical observations of babies of women with pregestational T2DM[15, 16, 45, 46]. It has been shown that transient hyperglycemic exposure is sufficient to induce septal overgrowth[47]. Thus, chronic mild hyperglycemia in T2DM is the major cause for fetal cardiac hypertrophy.

It is worth noting that our results suggest that maternal pregestational T2DM induces a significant hypertrophy in the fetus's interventricular septum and left ventricle, but not in the right ventricle wall. The underlying mechanism for this observation needs further

investigation. In adults, more blood volume/pressure loads into the left heart than the right heart[48, 49]. However, in fetuses, the blood volume in the right ventricle is higher than that in the left ventricle[50]. Therefore, the differences in responses to maternal diabetes between the right and left ventricles were probably not caused by blood volume/overload, but rather the different organogenesis of the left and right ventricles[50–52]. The two heart ventricles derive from different progenitors. The left ventricle is from the first heart field cells (FHF), whereas the right ventricle derives from the second heart field cells (SHF)[53]. Thus, it is possible that the effects of maternal diabetes on FHF and SHF are different. The impact of maternal diabetes on SHF cell differentiation is less than that of FHF cells, and, thus, there is no difference in the right ventricular wall thickness between the nondiabetic and the diabetic groups.

The pathological remodeling response of the adult heart to T2DM is characterized by hypertrophy, ventricular dilatation, and fibrosis[54]. Moreover, the imbalance between MMPs and TIMPs is usually associated with adverse LV remodeling[55]. However, there is little evidence of the effect of the imbalance of MMPs/TIMPs on cardiac remodeling in the embryonic heart from a mother with pregestation T2DM. In our animal model system, embryonic hearts from the diabetic group expressed more collagen, but downregulated fibronectin synthesis, which may contribute to developing heart hypertrophy. In addition, maternal T2DM induced an imbalance of MMP1/13 to TIMP1 and activated collagen genes in the embryonic heart, which may have contributed to the observed increase in collagen synthesis. Meanwhile, our findings that maternal T2DM elevated MMP24 and suppressed fibronectin might be further evidence that modest hyperglycemia during pregnancy induces cardiac remodeling. In this study, we found that imbalance between MMPs and TIMPs, alterations of collagen/fibronectin synthesis, pro-fibrosis and elevated cardiac cell apoptosis are involved in the development of fetal heart hypertrophy under maternal type 2 diabetes. It is of interest to prevent cardiac hypertrophy by blocking these changes. For example, TIMP1 is a potential target in restoring the balance of MMPs and TIMPs. If diabetes-increased TIMP1 expression is decreased by a specific pharmaceutical agent, it may result in rebalance of MMPs and TIMPs, and, thus, prevent abnormal cardiac remodeling. Similarly, cell apoptosis inhibitors are possible candidates in improving fetal heart function and suppression of heart hypertrophy.

Cardiac hypertrophy from pathological stimuli often results in heart failure[58]. Previous work indicates that hypertrophic cardiomyocytes are more sensitive to cell stress, which will increase cell apoptosis[59–63]. In this study we observed an increased level of apoptosis in the fetal heart cells of the T2DM group compared with the nondiabetic group. Moreover, the proapoptotic marker, BAX, was upregulated and the antiapoptotic marker, Bcl2, was downregulated in the embryonic hearts from diabetic dams. This increased apoptosis, along with proapoptotic changes, may contribute to the development of heart failure for the offspring later in life. The most profound finding in this study is the interventricular septum hypertrophy in embryos of diabetic dams. Therefore, it is pivotal to determine which signaling pathway is involved in cardiac cell apoptosis. Because the JNK signaling pathway implicates in the dysmorphogenesis of the outflow tract of the developing heart[64–66], we hypothesized that, in the fetal heart from a T2DM mother, JNK and its downstream signaling are activated. Indeed, phosphorylation of JNK and c-JUN are increased in the

T2DM group. JNKs belong to the superfamily of MAP kinases, which regulate cell proliferation, differentiation, and apoptosis[67]. Moreover, the JNK pathway is involved in the pathological process of fibrosis[68]. Therefore, the JNK pathway may attribute to apoptosis and increased collagen synthesis in the heart of embryos from T2DM dams.

According to the World Health Organization, cardiovascular disease is a major contributor to the growing public health epidemic in chronic diseases, such as chronic lung disease, chronic kidney disease and diabetes[69]. These chronic diseases are responsible for nearly two-thirds of all global deaths[70]. Therefore, to prevent the epidemic of chronic diseases, it is important to recognize who may be at high-risk for developing cardiovascular disease. Maternal diabetes during pregnancy is clinically associated with a higher risk of cardiovascular disease in the offspring[71–77], which suggests that prenatal exposure to hyperglycemia may cause cardiac changes which might increase a person's risk of developing cardiovascular disease in adulthood. Therefore, it is important to determine whether maternal diabetes in pregnancy has any long-term influence on the postnatal heart. Generally, hypertrophic cardiomyopathy observed in the infant of a mother with pregestational diabetes is regarded as a relatively benign phenomenon[43, 78–80]. Most infants are clinically asymptomatic and have resolution of the hypertrophy within months after birth. However, the infant heart is relatively stiff and sensitive to excessive stress from hypertension and diabetes at adult stage, which ultimately leads to cardiovascular diseases[46, 71, 81]. Therefore, periodically evaluating the heart of a person whose mother had T2DM during pregnancy should be performed, just as it should be performed for the children suffering from intrauterine growth restriction due to maternal diabetes in pregnancy[82, 83], which would help to recognize the high-risk people and has prevention significance.

The clinical implications of this study particularly relate to adverse pregnancy outcomes under various maternal conditions. In addition to maternal diabetes and obesity[84, 85], alcohol intake[86], tobacco exposure[87], and maternal medication use[88] can also lead to fetal cardiac dysfunction. Current preconception care for women with diabetes can reduce cardiac defects in the offspring[10]; however, the birth defect rates associated with maternal diabetes are still several times higher than that in the general population[46, 89]. Based on the findings from animal studies[75], antioxidants and multi-vitamins have been used in the prevention of adverse pregnancy outcomes[90, 91]. Mechanistic studies including the current study inform the molecular changes in the pathogenesis of cardiac hypertrophy in diabetic pregnancy, and such studies may not be feasible in humans because of the inaccessibility of human fetal cardiac tissues. Once animal studies reveal the detailed morphological changes, molecular signaling pathways and epigenetic alterations, clinical applications using advanced imaging technologies, peripheral miRNA determinations, metabolomic and proteomic approaches[92–95] can be designed for early diagnosis, monitoring prognosis after interventions, and the development of effective treatments. High glucose is the major teratogen in diabetic pregnancy[75, 96]. Future studies may focus on the downstream molecular targets of glucose and recapitulate the key findings of animal studies in possible vascular tissues such as blood vessels in the placenta. The animal model described in this study allows deep mechanistic studies; however, it may not faithfully reflect the human conditions.

This concern is significantly alleviated because both diabetic animal models and human pregestational diabetes induce a same set of structural birth defects[62, 63, 72, 73, 75–77, 97–99].

Conclusions

Pregestational type 2 maternal diabetes appears to be a major cause of cardiac hypertrophy in the embryonic heart. We observed that cardiac remodeling and profibrosis are involved in the underlying mechanism. Our findings indicate that an imbalance of MMPs/TIMPs contributes to the reorganization of collagen, and, when combined with the alteration of fibronectin, results in cardiac remodeling. Furthermore, we showed that pregestational type 2 maternal diabetes induces profibrosis and apoptosis, which may be responsible for the dysfunction of the embryonic heart under modest hyperglycemic conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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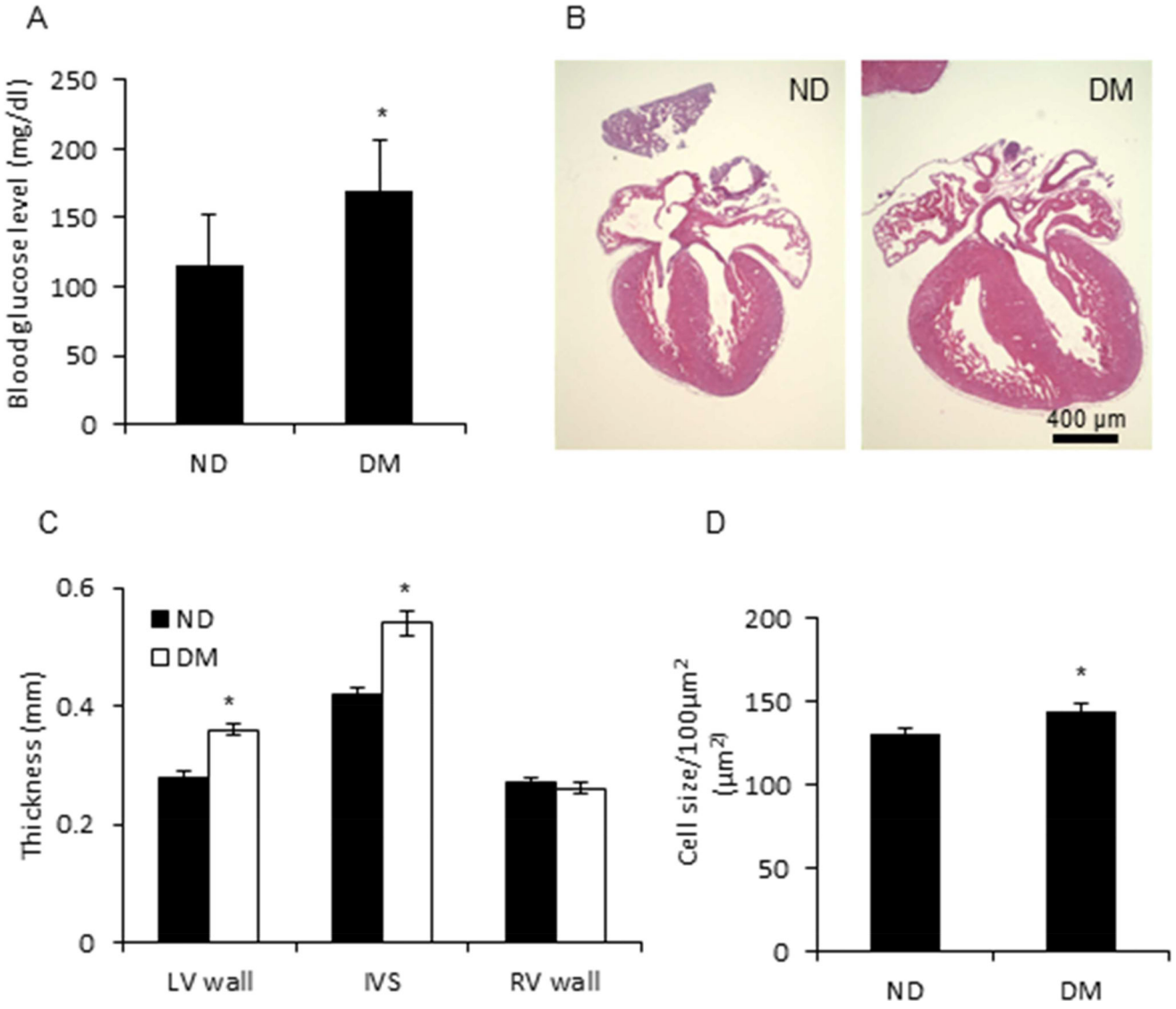

Figure 1. Cardiac hypertrophy in embryos from a T2DM dam

A, Blood glucose level of T2DM mouse model. ND, Nondiabetes, DM, type 2 diabetes mellitus. **B**, Enlarged heart in embryos from ND and DM dam. **C**, Thickness of left ventricle (LV) wall, interventricular septum (IVS), and right ventricle (RV) wall of embryo from ND and DM dam. **D**, Cardiomyocyte size in heart of embryo from ND and DM dam. 76 and 40 hearts of embryos from 16 ND dams and 40 DM dams, respectively, were removed at E17.5. * indicates significant difference ($P < 0.05$) when compared to ND group.

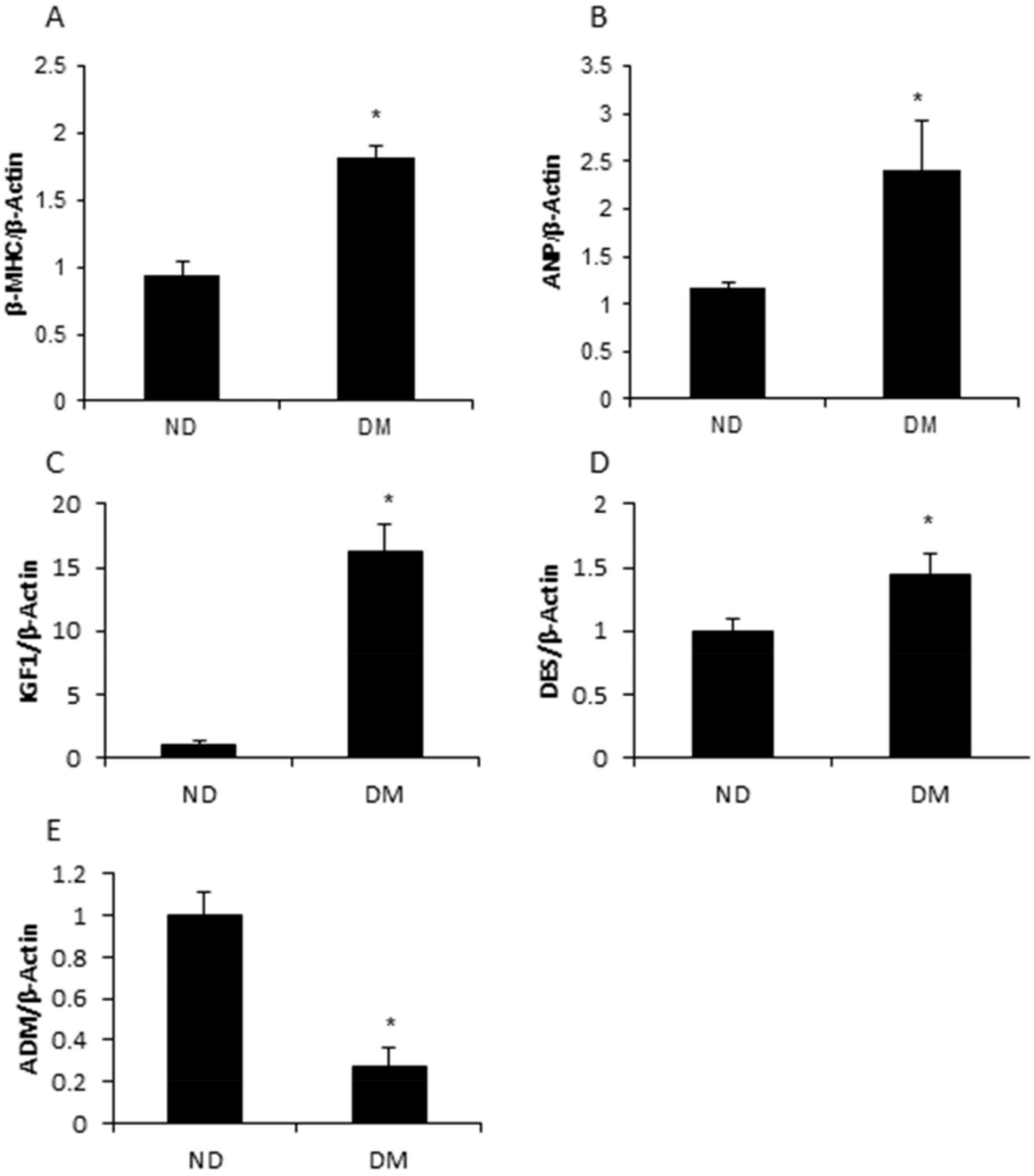

Figure 2. Alteration of hypertrophy-associated genes in embryonic heart in T2DM pregnancy

Hyperglycemia increased the expression of fetal genes, β-MHC (**A**), and ANP (**B**). Hypertrophy-associated genes, IGF1 (**C**), DES (**D**), and ADM (**E**), were influenced by T2DM in the embryonic heart. Each experiment was repeated three times. * indicates significant difference ($P < 0.05$) when compared to ND group.

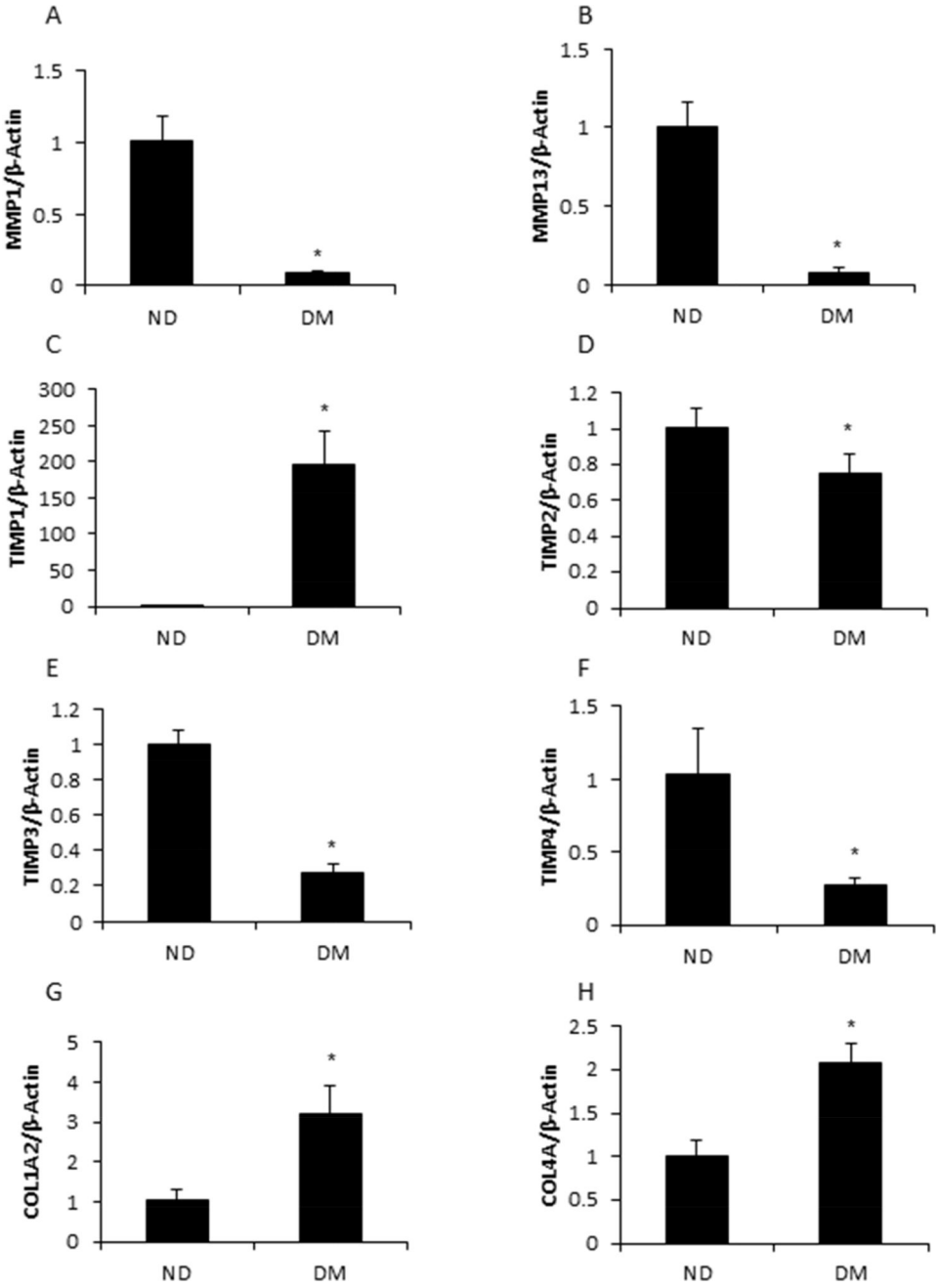

Figure 3. Collagen synthesis was increased by hyperglycemia

Hyperglycemia suppressed the expression of matrix metalloproteinases, MMP1 (**A**) and MMP13 (**B**). T2DM influenced the expression of TIMP1 (**C**), TIMP2 (**D**), TIMP3 (**E**), and TIMP4 (**F**). Hyperglycemia activated collagen genes, COL1A2 (**G**) and COL4A (**H**). Each experiment was repeated three times. * indicates significant difference ($P < 0.05$) when compared with the ND group.

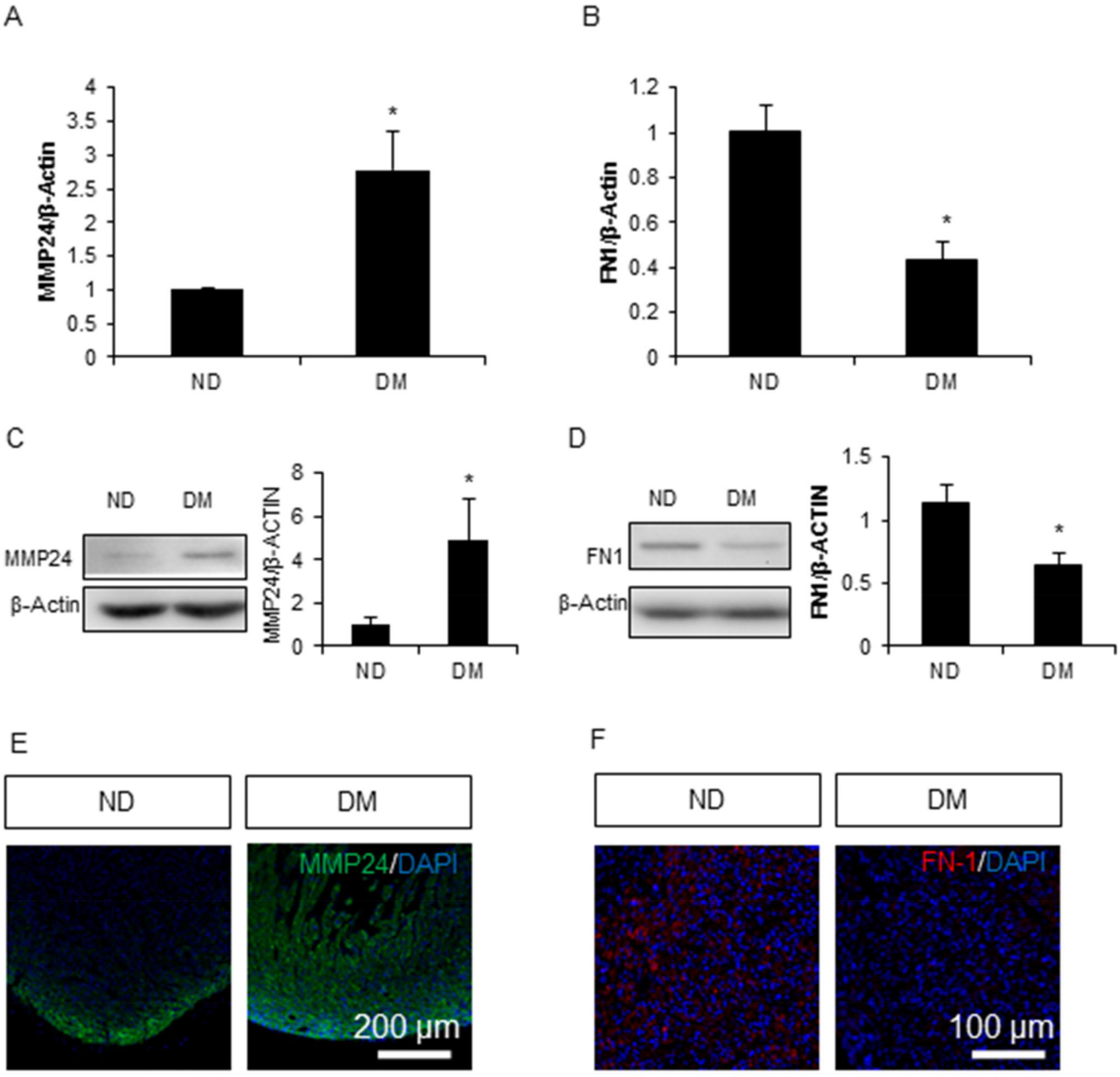

Figure 4. Fibronectin synthesis was suppressed by hyperglycemia

mRNA expression of MMP24 (**A**) and FN1 (**B**) in the embryonic heart. Western blot of MMP24 (**C**) and FN1 (**D**) in embryonic heart. Immunofluorescence of MMP24 (**E**) and FN1 (**F**) in embryonic heart. Each group contained three samples, * indicates significant difference ($P < 0.05$) when compared with the ND group.

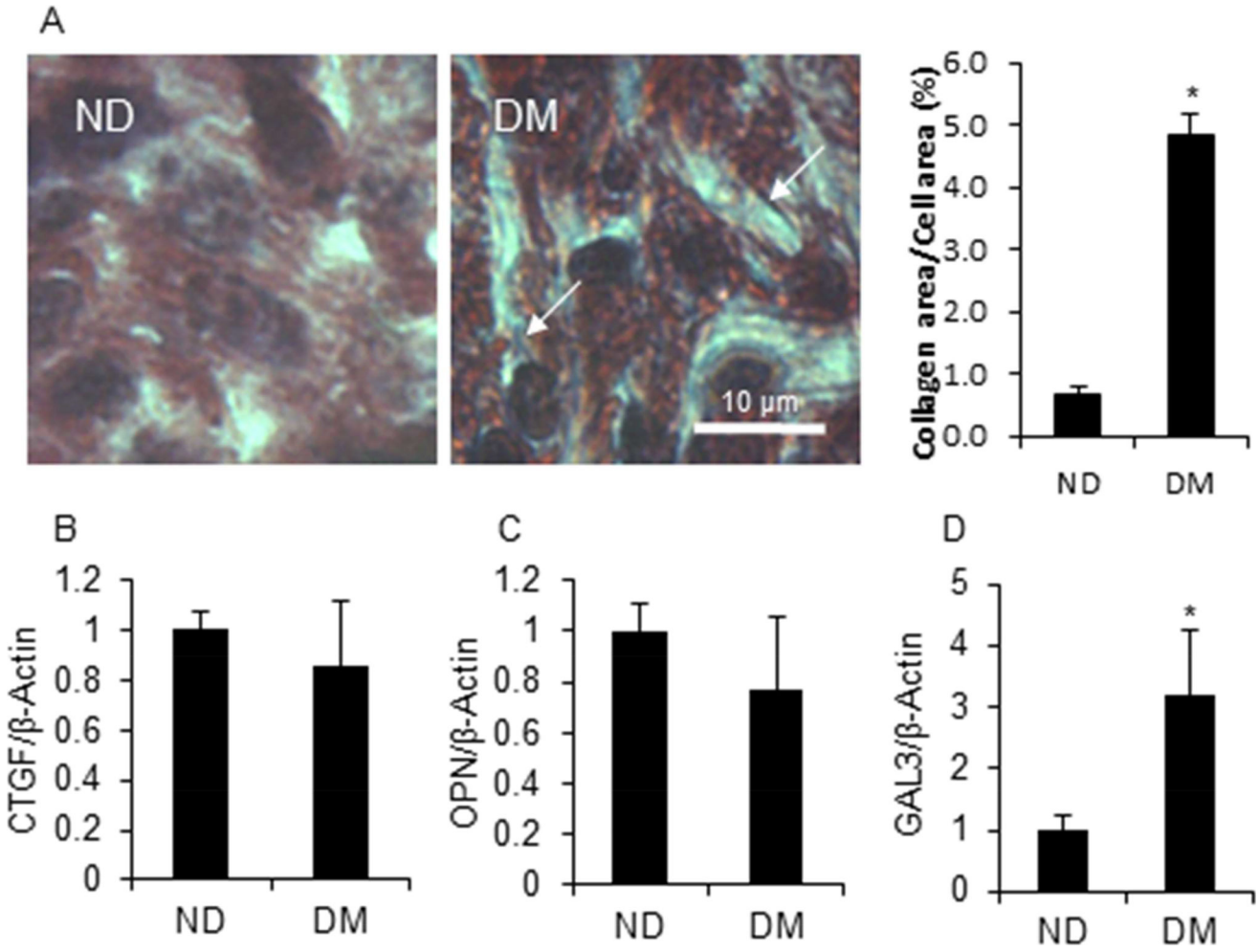

Figure 5. T2DM induced pro-fibrosis in the embryo heart

A. Masson staining of the embryo heart section. Arrow represents collagen staining (blue line). mRNA expression of pro-fibrosis markers, CTGF (**B**), OPN (**C**), and GAL3 (**D**). Each group contained three samples. * indicates significant difference ($P < 0.05$) when compared with the ND group.

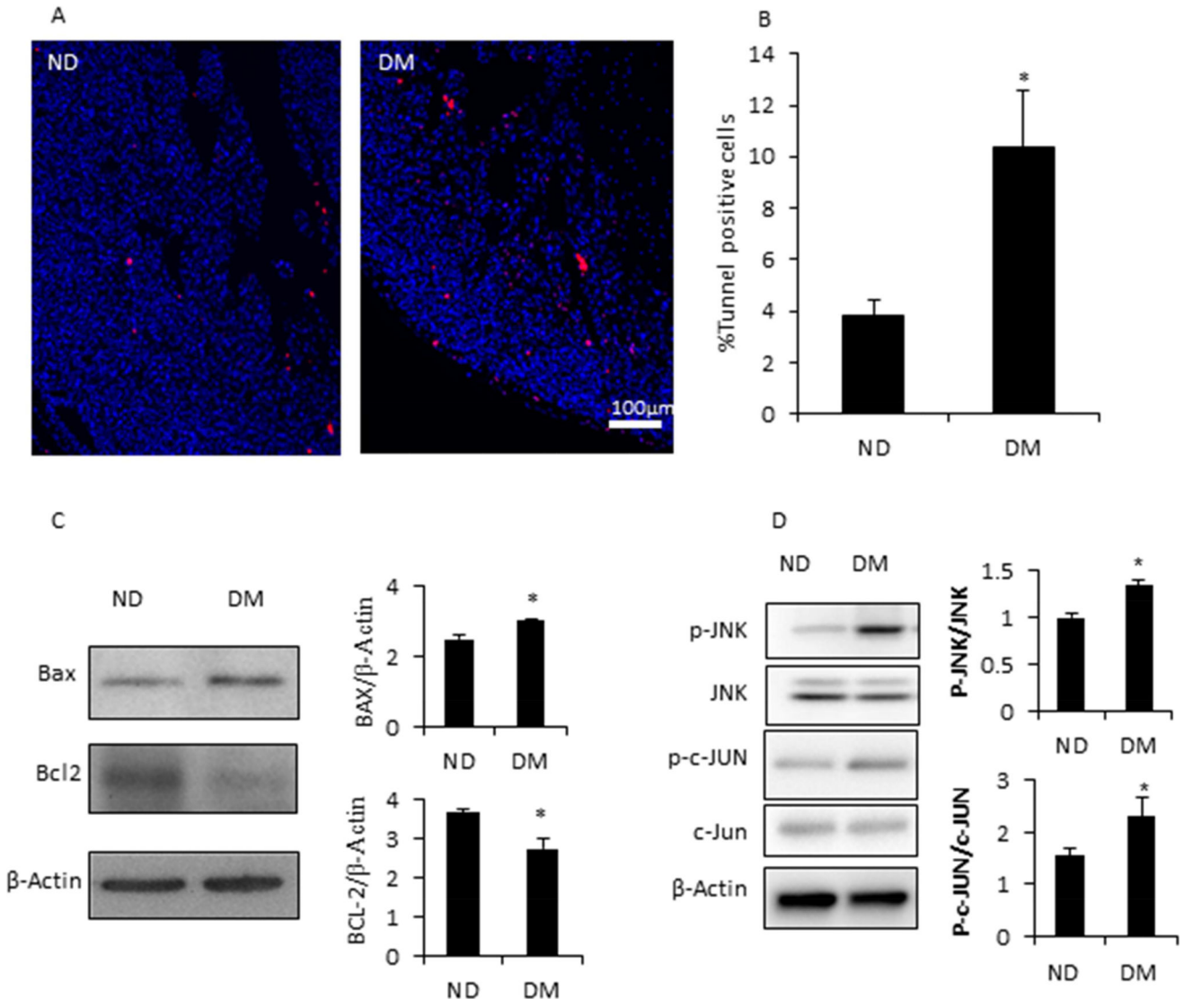

Figure 6. T2DM induced apoptosis of cardiomyocytes in embryo heart

A, TUNEL assay of embryo heart from ND and DM dams. **B**, Quantification of TUNEL positive cells based on panel A. **C**, Western blot of pro-apoptotic factor, BAX, and anti-apoptotic factor, BCL-2, in embryo hearts from ND and DM dams. **D**, Activation of JNK and c-JUN in heart of embryos from ND and DM dams. * indicates significant difference ($P < 0.05$) when compared with the ND group.

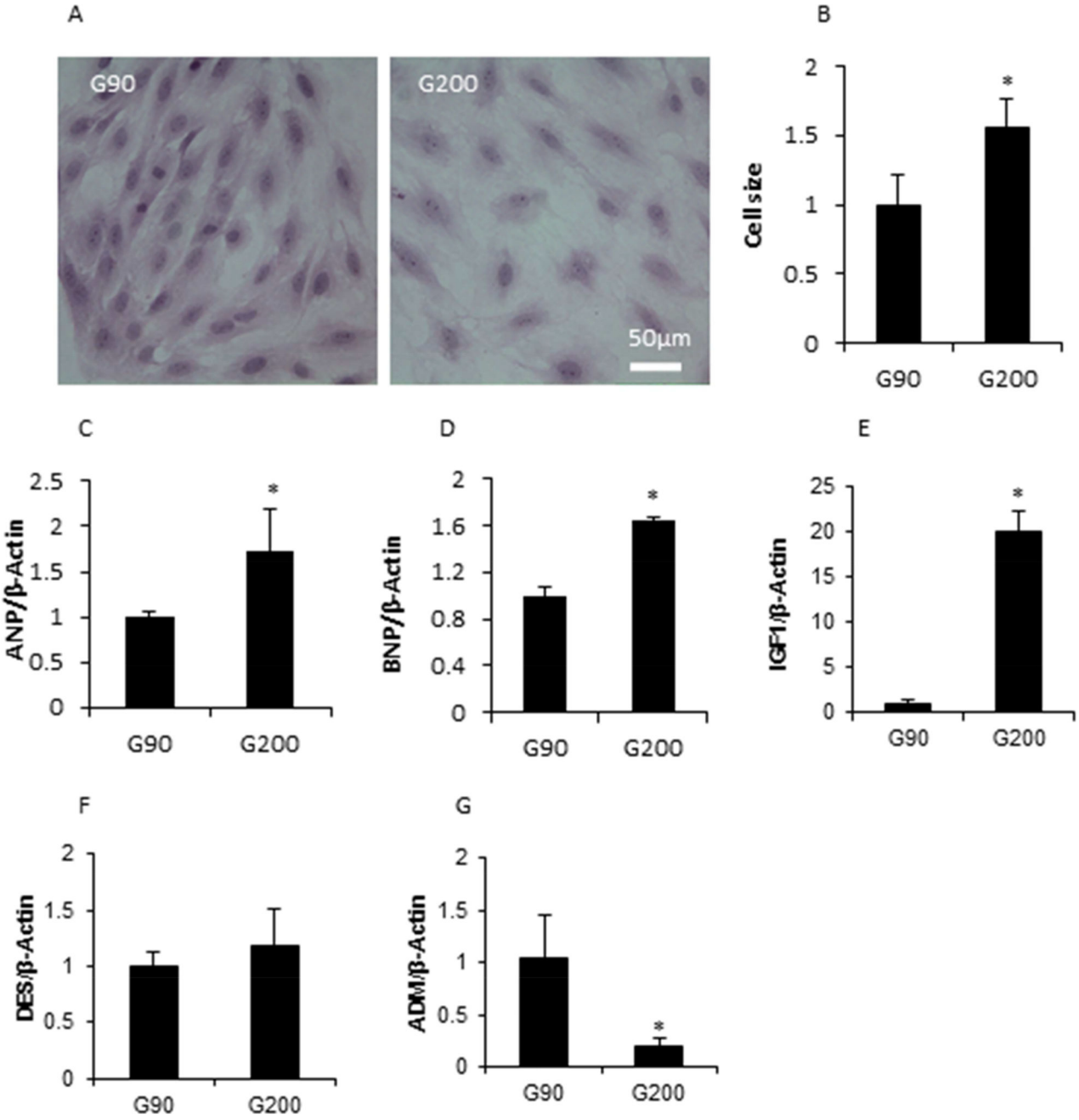

Figure 7. Cardiac hypertrophy was induced by high glucose in vitro

A, The cell size was increased by high glucose (200 mg/dl) in H9C2 cell line. **B**, Quantification of cell hypertrophy based on panel A. High glucose influenced hypertrophy associated markers (**C–G**). * indicates significant difference ($P < 0.05$) when compared with the low glucose group.