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Oxidative stress is responsible for maternal diabetes-impaired transforming growth factor beta signaling in the developing mouse heart

Fang Wang, PhD¹, E. Albert Reece, MD, PhD, MBA^{1,2}, and Peixin Yang, PhD^{1,2}

¹Department of Obstetrics, Gynecology & Reproductive Sciences, University of Maryland School of Medicine Baltimore, MD 21201

²Department of Biochemistry and Molecular Biology, University of Maryland School of Medicine Baltimore, MD 21201

Abstract

Objectives—Oxidative stress plays a causal role in diabetic embryopathy. Maternal diabetes induces heart defects and impaired transforming growth factor beta (TGF β) signaling, which is essential for cardiogenesis. We hypothesize that mitigating oxidative stress through superoxide dismutase 1 (SOD1) overexpression in transgenic (Tg) mice reverses maternal hyperglycemia-impaired TGF β signaling and its downstream effectors.

Study Design—Day 12.5 embryonic hearts from wild-type (WT) and SOD1 overexpressing embryos of nondiabetic (ND) and diabetic mellitus (DM) dams were used for detection of oxidative stress markers: 4-hydroxynonenal (4-HNE) and malondialdehyde (MDA), and TGF β 1, 2, 3, phosphor (p)-TGF β receptor II (T β RII), p-Smad2 and p-Smad3. The expression of three TGF β responsive genes was also assessed. Day 11.5 embryonic hearts were explanted and cultured *ex vivo*, with or without treatments of a SOD1 mimetic (tempol) or a TGF β recombinant protein for detection of TGF β signaling intermediates.

Results—Levels of 4-HNE and MDA were significantly increased by maternal diabetes, and SOD1 overexpression blocked the increase of these two oxidative stress markers. Maternal diabetes suppresses the TGF β signaling pathway by down-regulating TGF β 1 and TGF β 3 expression. Consequently, phosphorylation of T β RII, Smad2 and Smad3, downstream effectors of TGF β , and expression of three TGF β responsive genes were reduced by maternal diabetes, and these reductions were prevented by SOD1 overexpression. Treatment with tempol or TGF β recombinant protein restored high glucose-suppressed TGF β signaling intermediates and responsive gene expression.

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Address Correspondence to: Peixin Yang, PhD University of Maryland School of Medicine Department of Obstetrics, Gynecology & Reproductive Sciences BRB11-039, 655 W. Baltimore Street Baltimore, MD 21201 pyang@fpi.umaryland.edu Tel: 410-706-8402 Fax: 410-706-5747.

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Conclusions—Oxidative stress mediates the inhibitory effect of hyperglycemia in the developing heart. Antioxidants, TGF β recombinant proteins or TGF β agonists may have potential therapeutic values in the prevention of heart defects in diabetic pregnancies.

Keywords

SOD1 transgenic mice; oxidative stress; TGF β signaling; embryonic heart

INTRODUCTION

Preexisting maternal diabetes increases the risk of congenital heart defects in the offspring. Major cardiac defects including persistent truncus arteriosus and ventricular septal defects (VSDs) are often seen in offspring of diabetic women^{1, 2}. Because the number of women of reproductive age (18-44 years old) with diabetes is increasing³, diabetes-induced heart defects have become an urgent health problem⁴.

The critical exposure period for diabetes-induced birth defects is during organogenesis, which occurs during gestational stage 2 to 8 weeks in humans⁵ and corresponds approximately to E8.5 to 12.5 in the mouse^{2, 6}. Investigations using rodent models of diabetic embryopathy have revealed many important insights into the signal transduction factors that may cause diabetic embryopathy, particularly those involved in diabetes-induced neural tube defects (NTDs)^{5, 7-15}. However, information regarding diabetes-induced alterations in cardiac development signal transduction is limited.

Transforming growth factor β (TGF β) signaling plays an important role in early embryonic cardiac development, especially in the formation of the cardiac cushions between E8.5 and E12.5 in the mouse¹⁶. TGF β ligands (TGF β 1, 2, 3) bind to the TGF β type II receptor (T β RII) at the cell surface, causing it to be activated by phosphorylation, and then, in turn, to recruit and phosphorylate TGF β type I receptor (T β RI). T β RI phosphorylation causes phosphorylation of Smad2 and Smad3 proteins, enabling their migration to the nucleus (in association with Smad4) to regulate transcription of TGF β target genes.

Maternal diabetes suppresses TGF β signaling¹⁷, which prevents this cascade from occurring, and may contribute to hyperglycemia-induced heart malformations. The mechanism linking impaired TGF β signaling to heart defects in offspring of diabetic mothers has not been explored. However, previous work suggests a strong correlation between TGF β signaling and cardiac development. For example, TGF β 1 is responsible for smooth muscle development in the cardiac outflow tract¹⁸. In addition, eliminating TGF β 2 via knockout leads to cardiac outflow septum defects in mice¹⁹, and deleting T β RII in mice causes cardiac cushion fusion defects and VSD formation^{19, 20}.

In the developing embryo, maternal diabetes induces mitochondrial dysfunction, enhances cellular ROS production and impairs cellular antioxidant defense capabilities leading to oxidative stress²¹. Several studies in animal models have shown effectiveness of using antioxidant supplementations to prevent diabetes-induced neural tube defects (NTDs) and heart defects^{13, 22-25}. SOD1 is an endogenous antioxidant enzyme that detoxifies superoxide. Our previous studies^{7, 26, 27} have demonstrated that mitigating oxidative stress

through SOD1 overexpression in mouse embryos ameliorates maternal diabetes-induced NTD formation.

It has been reported that oxidative stress can either inhibit or enhance TGF β signaling pathway in different model systems^{17, 28-30}. The relationship between oxidative stress and TGF β signaling in the developing heart under diabetic conditions is unknown. In the present study, we examined the effect of maternal diabetes *in vivo* and high glucose *ex vivo* on the TGF β signaling, and employed the SOD1-Tg mice and a SOD1 mimetic to assess the role of oxidative stress in mediating the inhibitory effect of hyperglycemia on TGF β signaling.

Material and methods

Animals and Reagents

C57BL/6J mice (average body weight 22 g) were purchased from Jackson Laboratory (Bar Harbor, Maine). SOD1-Tg mice in a C57BL/6J background were revived from frozen embryos by the Jackson Laboratory (stock no. 00298). Streptozotocin (STZ) from Sigma (St. Louis, MO) was dissolved in sterile 0.1M citrate buffer (PH 4.5). Sustained-release insulin pellets were purchased from Linplant (Linshin, Canada).

Mouse models of diabetic embryopathy

All procedures for animal use were approved by the Institutional Animal Care and Use Committee of University of Maryland School of Medicine. Eight-week old wild-type (WT) female mice were intravenously injected daily with 75 mg/kg STZ over two days to induce diabetes. Blood glucose levels were monitored daily by tail vein puncture and using FreeStyle Blood Glucose Monitoring System (TheraSense, Abbot, Alameda, CA). Mice were considered as having diabetes when their blood glucose levels were greater than or equal to 14 mM. Insulin pellets were then subcutaneously implanted in diabetic mice to restore euglycemia prior to mating to protect early embryonic formation and implantation. Mice were then mated with SOD1-Tg male mice at 3:00 PM to generate WT and SOD1-overexpressing embryos.

Embryonic day 0.5 (E0.5) was designated once the vaginal plug was present on the next morning. On E5.5, insulin pellets were removed to permit frank hyperglycemia (>14mM glucose levels), and exposure of the developing embryos to a hyperglycemic conditions from E7 to E12, which is critical period for early heart development. WT, non-diabetic female mice injected with vehicle and sham operated on for insulin pellet implantation served as non-diabetic controls. On E12.5, mice were euthanized, and embryonic hearts were dissected out of the embryos for analysis.

Ex vivo embryonic heart culture

Ex vivo embryonic heart culture was performed and modified according to Dr. Hisayuki Hashimoto's recent publication³¹. Briefly, E11.5 hearts from nondiabetic WT (ND-WT) dams were quickly explanted, and placed in a 24-well plate coated with collagen gel (A10483-01, BD Gibco). The collagen gel was prepared in 5 mM (low glucose, LG) or 25 mM (high glucose, HG) D-glucose in M199 culture media (M4530, Sigma) and hydrated by

warmed Opti-MEM media, plus 1% fetal bovine serum (FBS, 16140071, Gibco) and insulin-transferrin-selenium (ITS, 25-800-CR, Corning). After incubation overnight at 37°C in a humidified atmosphere of 5% CO₂, M199 medium with 5 mM (LG) or 25 mM (HG) D-glucose plus 10% FBS, was added to the hearts and culture for 24 hours. Then hearts were treated with 5 mM Tempol (ALX-430-081-M250, Enzo Life Science) for 24 hours to suppress oxidative stress, or 50 ng/ml TGFβ1 recombinant protein (TP300973, Origene) for 48 hours to rescue TGFβ signaling.

Western Blotting

Western blotting was performed as previously described³². Briefly, E12.5 hearts from different experimental groups were sonicated in 35 µl ice-cold lysis buffer [20 mM Tris-HCl (pH7.5), 150 mM NaCl, 1 mM EDTA, 10 mM NaF, 2 mM Na-orthovanadate, 1 mM phenylmethylsulfonyl fluoride and 1% Triton-X-100] containing protease inhibitor cocktail (Sigma, St. Louis, MO). Equal amounts of protein were resolved by SDS-PAGE and transferred onto Immobilon-P membranes (Millipore). Membranes were incubated for 12 hour at 4 °C with the following primary antibodies at 1:1000 to 1:2000 dilutions in 5% nonfat milk: anti-TGFβ; anti-TβRII with/without phosphorylation; anti-Smad2 with/without phosphorylation; anti-Smad3 with/without phosphorylation; anti-SOD1 (Cell Signaling, Beverly, MA); and anti-β-actin (Abcam, Cambridge, MA). Signals were detected using SuperSignal West Femto Maximum Sensitivity Substrate kit (Thermo Scientific). Chemiluminescence emitted from the bands was directly captured using a UVP Bioimage EC3 system (UVP, Upland, CA). Densitometric analysis of chemiluminescence signals was performed by VisionWorks LS software (UVP, Upland, CA).

Real-time PCR

Total RNA was isolated from E12.5 embryonic hearts using an RNeasy Mini Kit (Qiagen, Valencia, CA). Real-time PCR for *TGFβ1*, *β2*, *β3*, *Snai2* (snail homolog 2), *CTGF* (connective tissue growth factor), *GDF1* (growth differentiation factor 1) and β-actin were performed using Maxima SYBR Green/ROX qPCR Master Mix assay (Thermo Scientific, Rockford, US). RT-PCR and subsequent calculations were performed by a 7700 ABI PRISM sequence detector system (Applied Biosystem). Primer sequences for RT-PCR are listed in Table 1.

Immunostaining

Embryonic hearts were fixed in 4% paraformaldehyde overnight followed by embedding in OCT compound (Sakura finetek, Torrance, CA). 10- m heart cryosections were antigen-unmasked using citrate buffer and blocked in 5% bovine serum albumin in PBS-T (0.1% Triton X-100 in PBS) for 1 hour. The following antibodies were used as primary antibodies: p-Smad2 (1:200) (Cell Signaling Technology), p-Smad3 (1:200) (Cell Signaling Technology), p-Histone H3 (1:100) (Millipore, Bedford, MA). Normal rabbit or mouse IgG at the same dilutions as those for antibodies were used as controls. Sections were counterstained with DAPI and mounted with aqueous mounting medium (Sigma, St Louis, MO). Images were captured using an inverted microscope (Nikon Eclipse E1000M). For the

evaluation of cell proliferation, p-Histone H3 positive cells were counted on three heart sections per group.

Statistics

Data were presented as means $\pm$ SE. Analysis of variance (ANOVA) with Tukey-test was used to identify significant differences when appropriate. A difference of $P < 0.05$ was considered to be statistically significant.

Results

SOD1 overexpression blocks maternal diabetes-induced oxidative stress in the developing heart

Maternal diabetes produces ROS, results in oxidative stress, and induces lipid peroxidation and protein oxidation. In order to examine whether SOD1 overexpression could ameliorate maternal diabetes-induced oxidative stress in the embryonic heart, we tested the levels of two major lipid peroxidation markers, 4-hydroxynonenal (4-HNE) and malondialdehyde (MDA). We found that levels of these two markers were significantly higher in the embryonic heart from the diabetes mellitus wild-type (DM-WT) group when compared with other two groups (Fig. 1A, B). SOD1 overexpressing embryonic heart exposed to diabetes showed comparable levels of these two markers with WT embryonic hearts under nondiabetic condition (Fig. 1A, B). These results suggested that SOD1 overexpression significantly blocks maternal diabetes-induced oxidative stress in the developing heart.

SOD1 overexpression restores maternal diabetes-impaired TGF β expression in the developing heart

To determine whether maternal diabetes affects the expression of TGF β s and whether oxidative stress participates in this process, levels of three TGF β ligands (TGF β 1, β 2, β 3) were analyzed in E12.5 embryonic hearts from the ND-WT, DM-WT and DM-SOD1 groups. Both mRNA and protein analyses showed that TGF β 1 and TGF β 3 were significantly decreased in embryonic hearts from the DM-WT group, compared with those from the ND-WT group, while TGF β 2 showed no significant change among these two groups (Fig. 2A, B). We further found that blocking oxidative stress by SOD1 overexpression could restore the expression levels of TGF β 1 and TGF β 3 suppressed by maternal diabetes (Fig. 2A, B), indicating that the downregulation of TGF β s can be reversed by SOD1 overexpression.

SOD1 overexpression ameliorates maternal diabetes-impaired downstream TGF β signaling intermediates in the developing heart

We next investigated whether maternal diabetes and oxidative stress could also affect the levels of TGF β signaling cascades in the embryonic heart. We tested the protein levels of T β RII, Smad2 and Smad3 phosphorylation in the E12.5 embryonic hearts. The levels of p-T β RII, p-Smad2 and p-Smad3 were all significantly decreased in the DM-WT group and restored in the DM-SOD1 group (Fig. 3A, B, C). The immunostaining signals of p-Smad2 and p-Smad3 in the E12.5 embryonic hearts from the DM-WT group were lower than those from the ND-WT and DM-SOD1 groups (Fig. 3D).

SOD1 overexpression reverses maternal diabetes-decreased TGF β signaling responsive gene expression

We detected the expression level of three TGF β responsive genes, snail homolog 2 (*Snai2*), connective tissue growth factor (*CTGF*) and growth differentiation factor 1 (*GDF1*). Maternal diabetes significantly decreased the expression of these three TGF β responsive genes in the developing heart, and SOD1 overexpression restored their expression levels suppressed by maternal diabetes (Fig. 4).

Tempol abolishes high glucose-impaired TGF β signaling in ex vivo cultured embryonic hearts

Our results indicated that oxidative stress plays a key role in maternal diabetes-impaired TGF β signaling in the developing heart. To further confirm this, we explanted E11.5 embryonic hearts and cultured them *ex vivo* in high glucose and tempol (superoxide dismutase mimetic) conditions to check the effect of tempol on high glucose-suppressed TGF β signaling. p-Histone H3 staining showed similar cell proliferation levels between the low glucose cultured hearts (cultured for 3 days) and E12.5 WT embryonic hearts from ND dams, suggesting that culture medium can maintain explanted hearts very well for at least 3 days (Fig.5A). Similar to our *in vivo* observations that overexpressing SOD1 rescued TGF β signaling, tempol treatment restored TGF β , p-T β RIL, p-Smad2, p-Smad3 (Fig. 5B) and three TGF β responsive genes (Fig. 5C) that were decreased by high glucose, suggesting that high glucose-induced oxidative stress suppresses TGF β signaling pathway.

TGF β recombinant protein restores high glucose-suppressed TGF β signaling

The TGF β signaling pathway is involved in a wide range of cellular process including cell growth, cell differentiation and apoptosis, and uses a variety of regulatory mechanisms. In order to determine whether maternal diabetes impaired TGF β signaling due to TGF β down-regulation, we used a TGF β recombinant protein to treat *ex vivo* cultured hearts. TGF β recombinant protein treatment successfully restored TGF β , p-T β RIL, p-Smad2, p-Smad3 and expression of the three TGF β responsive genes which were suppressed by high glucose (Fig. 6A, B). These results further support our hypothesis that oxidative stress suppresses TGF β signaling mainly through down-regulation of TGF β expression.

Comment

Much experimental evidence supports the hypothesis that oxidative stress is involved in the etiology of several diabetic complications including diabetic embryopathy^{7-12, 14, 15, 33-36}. Maternal diabetes causes oxidative stress in the developing embryos by, not only increasing ROS production, but also suppressing endogenous antioxidant activity^{7, 13, 37, 38}. Diabetes-induced oxidative stress alters the expression of genes that are critical for embryonic neurogenesis and cardiogenesis, leading to NTDs and heart defects³⁹.

Studies have demonstrated that dietary antioxidant supplements to the mother or antioxidant treatment to cultured embryos significantly reduce maternal diabetes- or high glucose *in vitro*-induced NTD formation^{13, 40-42}. Our previous studies have demonstrated that SOD1 overexpression in SOD1-Tg mice drastically suppresses embryonic NTD formation in

diabetic pregnancies^{7, 10}. Here, we aim to determine whether SOD1 overexpressing by using SOD1-Tg mice could ameliorate maternal diabetes-induced heart defects.

Membrane lipids are one of the primary targets of ROS. 4-HNE and MDA are two major membrane lipid peroxidation products. We have previously demonstrated that SOD1 overexpression inhibits diabetes-increased 4-HNE and MDA expression in neurulation-stage embryos⁷. Consistently, we found that maternal diabetes-increased 4-HNE and MDA expression in the developing heart was abolished by SOD1 overexpression, indicating that SOD1 overexpression could relieve maternal diabetes-induced oxidative stress in the developing heart.

TGF β signaling plays a key role in embryonic cardiogenesis⁴³. TGF β ligands (TGF β 1, 2 and 3) and the main TGF β receptor proteins (T β RI and T β RII) are widely expressed in different compartments of the developing heart⁴⁴. TGF β 1 is expressed in the endocardium from E8⁴⁵, whereas TGF β 2 ligand is expressed in the myocardium of the atrioventricular (AV) canal at E10^{46, 47}. TGF β 3 is not expressed in the developing heart until E11, and is limited to epicardium and the mesenchymal cells in the cardiac cushions^{46, 47}.

In this study, we used E12.5 embryonic hearts to examine and changes in expression of all three TGF β ligands under hyperglycemic conditions. We found that the total level of TGF β ligands, especially TGF β 1 and TGF β 3, were reduced by maternal diabetes. This finding is consistent with what described by Zhao *et al* in E10.5 hearts¹⁷, but contradicts a previous *in vitro* study, which described a slight decrease in TGF β 1 expression in the mouse (E9.5) embryonic heart when exposed to high glucose for 24 hours². This discrepancy may be explained by the different stages of hearts that were examined in the two studies.

Consistent with the down-regulation of TGF β 1 and TGF β 3, downstream effectors of the TGF β signaling, p-T β RII, p-Smad2 and p-Smad3, were decreased in embryonic hearts under diabetic conditions. Our *ex vivo* experiments using TGF β recombinant protein in cultured hearts provided direct evidence that decreased TGF β is responsible for high glucose-induced p-T β RII, p-Smad2 and p-Smad3 downregulation.

ROS react with nitric oxide (NO) to produce reactive nitrogen species (RNS)⁴⁸. Marta *et al* observed that RNS reduced the amount of phosphorylated Smad2/3 and their nuclear translocation, and inhibited the transcriptional activities of the TGF β /Smad signaling⁴⁹. Hyperglycemia induces diabetic embryopathy by enhancing the production of ROS and the generation of RNS⁵⁰.

In the present study, we observed that maternal diabetes-impaired TGF β signaling was restored in SOD1-overexpressing embryonic hearts. The contribution of RNS to maternal diabetes-impaired TGF β signaling was not assessed. Further studies using knockout mice of *iNos*, which enhances NO production and the consequent formation of RNS in diabetic embryopathy, may determine the contribution of RNS to TGF β signaling impairment.

Our *ex vivo* experiment treating with tempol on cultured hearts further demonstrated that oxidative stress is fully responsible for decreased TGF β signaling by high glucose. SOD1 overexpression or tempol treatment restored the expression of three TGF β target genes,

Snai2, *CTGF* and *GDF1*. *Snai2* is critical for endothelial-to-mesenchymal transition (EMT) in cardiac cushion morphogenesis⁵¹. *CTGF* (connective tissue growth factor), a member of CCN family of extracellular matrix-associated heparin-binding proteins, has an essential role in cell adhesion, migration, proliferation and angiogenesis^{52, 53}. Aberrant *CTGF* expression is associated with diabetic nephropathy⁵⁴, retinopathy⁵⁵ and cardiovascular diseases⁵⁶. *GDF1* is a key regulator in the establishment and maintenance of Left-Right (L-R) signals governing asymmetric organogenesis, including the heart and the great vessels⁵⁷. The absence of *GDF1* in the mouse is associated with disturbances in L-R patterning⁵⁷. Thus, alterations of these three TGF β responsive genes may be mechanistically important for diabetes-induced embryonic heart defects.

In summary, our study demonstrated that hyperglycemia suppresses the TGF β signaling pathway in the embryonic heart by inhibiting TGF β ligand expression (Fig. 7). We also showed that oxidative stress is responsible for impairment of the TGF β signaling pathway (Fig. 7). TGF β signaling impairment is directly linked to the down-regulation of *Snai2*, *CTGF* and *GDF1* expression, which may mediate the teratogenicity of maternal diabetes in the developing heart (Fig. 7). SOD1 overexpression *in vivo* or treatment with the SOD1 mimetic tempol *ex vivo* rescued TGF β signaling and its responsive genes expression. Our findings provide a mechanistic basis for the development of antioxidant and TGF β signaling agonists against maternal diabetes-induced embryonic heart defects.

Pregestational diabetes mellitus is a significant risk factor for heart defects through modified maternal metabolism^{4, 58}. In human studies, congenital heart defects occur in 5% of diabetic pregnancies⁵⁸. Increasing maternal body mass index and obesity also are associated with an increasing risk of CHDs, and severe obesity is an even greater risk factor for the development of CHDs^{59, 60}. There is increased mortality and morbidity in babies born with one or multiple heart defects, and these babies need urgent surgical care and life-long health support. The best way to cure cardiac issues in later life is to prevent heart defect formation in embryonic life.

Excessive oxidative stress is a key mechanism underlying the formation of structural birth defects such as neural tube defects (NTDs) and heart defects⁶⁰. Studies have shown that mutations in SOD1 and SOD2, that potentially affect the expression of these two antioxidant enzymes, are observed in patients with myelomeningocele, a type of NTDs⁶¹. Oxidative stress markers in maternal circulation may be utilized for early diagnosis of fetal adverse outcomes⁶²⁻⁶⁴. Examining oxidative stress markers such as 8- hydroxydeoxyguanosine and 8-isoprostane in maternal urine samples has also been used for early detection of adverse pregnancy outcomes⁶⁵. Because oxidative stress impacts an array of signaling pathways including the TGF β signaling pathway in the present study, other metabolites in the maternal circulation could be valuable indicators of defective fetuses. Currently available interventions to prevent birth defects are limited. Folic acid intake attenuates maternal diabetes- or obesity-induced NTD formation⁶⁶. In contrast, another study demonstrated that the periconceptional use of vitamins or supplements that contain folic acid failed to prevent congenital heart defects caused by preexisting maternal diabetes mellitus^{67, 68}. Mechanistic studies including our current study provide important insights into developing new interventions against diabetes-, obesity- or other stress conditions-induced birth defects.

Animal studies have demonstrated that dietary supplements that contain folic acid, vitamin E or polyunsaturated fatty acids ameliorate the incidence of birth defects in diabetic pregnancies^{40, 41, 67}. However, even with folic acid supplements, women with pre-existing diabetes still produce a higher incidence of offspring with CHDs⁶⁸. Developing safe and effective therapeutic interventions to prevent maternal diabetes-induced CHDs is an urgent need. However, more work needs to be conducted in animal studies before we can begin clinical trials of prospect therapeutic candidates.

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Condensation

Maternal diabetes-induced oxidative stress mediates the inhibitory effect of hyperglycemia on TGF β signaling in the developing heart.

Figure 1. SOD1 overexpression abolishes maternal diabetes-induced oxidative stress in the developing heart

A, The western blotting image of 4-HNE in E12.5 embryonic hearts from ND-WT, DM-WT and DM-SOD1 groups. **B**, The western blotting image of MDA in E12.5 embryonic hearts from ND-WT, DM-WT and DM-SOD1 groups. Three hearts from embryos of three dams (n=3) per group were analyzed. * indicates significant difference compared to other groups. ND: nondiabetic; DM: diabetic mellitus; WT: wild-type; SOD1: SOD1 overexpressing.

Figure 2. SOD1 overexpression restores maternal diabetes-suppressed TGFβs expression
A, mRNA levels of TGFβ1, β2 and β3 in WT and SOD1 overexpressing heart from ND and DM dams. **B**, protein level of TGFβ in E12.5 embryonic hearts from ND-WT, DM-WT and DM-SOD1 groups. Three hearts from embryos of three dams (n=3) per group were analyzed. * indicates significant difference compared to other groups. ND: nondiabetic; DM: diabetic mellitus; WT: wild-type; SOD1: SOD1 overexpressing.

Figure 3. SOD1 overexpression rescues TGFβ signaling downstream effectors suppressed by maternal diabetes

A, protein level of p-TβRII, **B**, protein level of p-Smad2, **C**, protein level of p-Smad3 in E12.5 embryonic hearts from ND-WT, DM-WT and DM-SOD1 groups. **D**, image of p-Smad2 and p-Smad3 immunostaining. Rabbit normal IgG served as controls. Red signals were p-Smad2 and p-Smad3 and cell nuclei were stained by DAPI (Blue). Three E12.5 hearts from embryos of three dams (n=3) per group, and three serial sections per heart were analyzed. Bars =150 μm. * indicates significant difference compared to other groups. ND: nondiabetic; DM: diabetic mellitus; WT: wild-type; SOD1: SOD1 overexpressing.

Figure 4. SOD1 overexpression rescues maternal diabetes-reduced TGF β target gene expression
mRNA levels of TGF β target genes *Snai2*, *CTGF* and *GDF1*. Three hearts from embryos of three dams (n=3) were analyzed. * indicates significant difference compared to other groups. ND: nondiabetic; DM: diabetic mellitus; WT: wild-type; SOD1: SOD1 overexpressing.

Figure 5. Tempol treatment restores high glucose-suppressed TGFβ signaling in *ex vivo* cultured hearts

A, immunostaining image of p-Histone H3 in ND-WT and LG-WT (WT hearts cultured in low glucose condition for 3 days). Red dots were p-Histone H3 positive cells, nuclei were stained with DAPI (Blue). Three serial sections per heart were analyzed. **B**, protein levels of TGFβ, p-TβRII, p-Smad2, p-Smad3 in *ex vivo* cultured hearts from 5 mM glucose, 25 mM glucose, 25 mM glucose plus tempol groups. **C**, mRNA levels of *Snai2*, *CTGF* and *GDF1* in 5 mM glucose, 25 mM glucose, 25 mM glucose plus tempol groups. Three hearts per group (n=3) were analyzed. * indicates significant difference compared to other groups.

Figure 6. TGFβ recombinant protein treatment rescues TGFβ signaling suppressed by high glucose *ex vivo*

A, protein levels of TGFβ, p-TβRII, p-Smad2 and p-Smad3 in *ex vivo* cultured hearts from 5 mM glucose, 25 mM glucose, 25 mM glucose plus TGFβ recombinant protein groups. **B**, mRNA levels of *Snai2*, *CTGF* and *GDF1* in 5 mM glucose, 25 mM glucose, 25 mM glucose plus TGFβ recombinant protein groups. Three hearts per group (n=3) were analyzed. * indicates significant difference compared to other groups.

Figure 7. Schematic of maternal diabetes-induced oxidative stress impairs TGF β signaling in the developing heart leading to heart malformations

Oxidative stress induced by maternal diabetes suppresses TGF β 1 and β 3 expression, which further inhibits the phosphorylation level of p-T β RII, p-Smad2 and p-Smad3.

Phosphorylated-Smad2/3 will be translocated into the nucleus as transcription factor with the help of Smad4. Downregulation of p-Smad2 and p-Smad3 by oxidative stress suppresses their function as transcription factors, which results in reduced expression of TGF β target genes, such as *Snai2*, *CTGF* and *GDF1*, and probably malformed heart development. +p: phosphorylation; $\Downarrow$: downregulation; dash arrow line: possible mechanism.

Table 1

Primer sequences used in RT-PCR

Primer name	Primer Source	Primer Sequence
TGFβ1F	Primerbank ID: 6755775a1	CTCCCGTGGCTTCTAGTGC
TGFβ1R		GCCTTAGTTTGGACAGGATCTG
TGFβ2F	Primerbank ID: 6678317a1	TCGACATGGATCAGTTTATGCG
TGFβ2R		CCCTGGTACTGTTGTAGATGGA
TGFβ3F	Primerbank ID: 6678319a1	CCTGGCCCTGCTGAAGTTG
TGFβ3R		TTGATGTGGCCGAAGTCCAAC
Snai2F	Primerbank ID: 6755576a1	TGGTCAAGAAACATTTCAACGCC
Snai2R		GGTGAGGATCTCTGGTTTGGTA
CTGFF	Primerbank ID: 6753878a1	GGGCCTCTTCTGCGATTTT
CTGFR		ATCCAGGCAAGTGCATTGGTA
GDF1F	Primerbank ID: 6679977a1	AACTAGGGGTCGCCGAAA
GDF1R		TCAAAGACGACTGTCCACTCG

F: Forward; R: Reverse