ASK1 mediates the teratogenicity of diabetes in the developing heart by inducing ER stress and inhibiting critical factors essential for cardiac development

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Submitted 13 March 2015; accepted in final form 13 July 2015

Wang F, Wu Y, Quon MJ, Li X, Yang P. ASK1 mediates the teratogenicity of diabetes in the developing heart by inducing ER stress and inhibiting critical factors essential for cardiac development. Am J Physiol Endocrinol Metab 309: E487–E499, 2015. First published July 14, 2015; doi:10.1152/ajpendo.00121.2015.—Maternal diabetes in mice induces heart defects similar to those observed in human diabetic pregnancies. Diabetes enhances apoptosis and suppresses cell proliferation in the developing heart, yet the underlying mechanism remains elusive. Apoptosis signal-regulating kinase 1 (ASK1) activates the proapoptotic c-Jun NH2-terminal kinase 1/2 (JNK1/2) leading to apoptosis, suggesting a possible role of ASK1 in diabetes-induced heart defects. We aimed to investigate whether ASK1 is activated in the heart and whether deleting the Ask1 gene blocks diabetes-induced adverse events and heart defect formation. The ASK1-JNK1/2 pathway was activated by diabetes. Deleting Ask1 gene significantly reduced the rate of heart defects, including ventricular septal defects (VSDs) and persistent truncus arteriosus (PTA). Additionally, Ask1 deletion diminished diabetes-induced JNK1/2 phosphorylation and its downstream transcription factors and endoplasmic reticulum (ER) stress markers. Consistent with this, caspase activation and apoptosis were blunted. Ask1 deletion blocked the increase in cell cycle inhibitors (p21 and p27) and the decrease in cyclin D1 and D3 and reversed diabetes-repressed cell proliferation. Ask1 deletion also restored the expression of BMP4, NKX2.5, and GATA5, Smad1/5/8 phosphorylation, whose mutations or deletion result in reduced cell proliferation, VSD, and PTA formation. We conclude that ASK1 may mediate the teratogenicity of diabetes through activating the JNK1/2-ER stress pathway and inhibiting cell cycle progression, thereby impeding the cardiogenesis pathways essential for ventricular septation and outflow tract development.

maternal diabetes; heart defects; apoptosis signal-regulating kinase 1; apoptosis; endoplasmic reticulum stress

CONGENITAL HEART DEFECTS (CHDs) are the most common birth defects, with an incidence of four to 10 per 1,000 live births (12, 29). Although major progress has been made in establishing the genetic basis of CHDs (29), clinical evidence has suggested that nongenetic factors are major contributors to the induction of CHDs (12, 21, 49, 56). Preexisting maternal diabetes is one such nongenetic factor that significantly increases the risk of CHDs (21, 49, 56). Offspring from diabetic mothers have a fivefold increase of major types of heart defects, including ventricular septal defects (VSDs) (21, 39, 56) and outflow tract defects (21, 49). Studies in diabetic animal models reveal the same types of CHDs (15, 23, 40) as those in human diabetic pregnancies, and apoptosis (25, 26), impaired cell proliferation (3), and gene dysregulation (15, 32, 46, 48) are observed in the mouse hearts exposed to maternal diabetes. However, the mechanisms governing diabetes-induced defective heart formation remain elusive.

Maternal diabetes induces oxidative stress in the developing embryo (6, 9, 26, 45, 48, 53, 58, 59) and activates apoptosis signal-regulating kinase 1 (ASK1), a mitogen-activated protein kinase (MAPK) kinase, in neurulation stage embryos (47, 57). ASK1 responds to oxidative stress and induces apoptosis via the activation of c-Jun NH2-terminal kinase 1/2 (JNK) and/or p38 MAPK (42). ASK1 also is a key component of the proapoptotic inositol-requiring enzyme 1α (IRE1α) complex (28), whose activation triggers prolonged unfolded protein response (UPR), leading to endoplasmic reticulum (ER) stress (44). We hypothesize that the ASK1-JNK1/2-ER pathway is activated in the developing heart exposed to maternal diabetes, and Ask1 mediates the teratogenicity of maternal diabetes, leading to heart defects.

Both apoptosis (25, 26) and impaired cell proliferation (3) are implicated in the etiology of heart defect formation in diabetic pregnancies. Cell proliferation is controlled by cyclins and their catalytic partners, cyclin-dependent kinases (CDKs) (14). D-type cyclins (cyclin1 D1, D2, and D3) and their associated CDKs (CDK4 and CDK6) are of particular importance to embryonic heart development because the cyclin D/CDK complexes initiate the progression from G1 phase into the S phase (14). Moreover, the cyclin D/CDK complexes remove the cell cycle inhibitors p27Kip1 and p21Cip1 from the cyclin E/CDK2 and cyclin A/CDK2 complexes (14). Compound deletion of all three cyclin Ds results in VSD (14), a common type of CHD in diabetic pregnancies (15). A recent study reports that high glucose suppresses cyclin D1 and increases p21Cip1 in the chick embryo (38). Because the ASK1-JNK1/2 or p38 MAPK pathway critically regulates cell proliferation, it is possible that ASK1 mediates the inhibitory effect of maternal diabetes on cell proliferation by modulating cyclin Ds and cell cycle inhibitors.

Studies in gene deletion mouse models and human mutations have revealed a group of factors that are essential for cardiac septation and outflow tract (OFT) development. VSDs and OFT defects are observed in embryos having the bone morphogenetic protein 4 (BMP4) gene deleted (24). Human mutations of transcription factors, NK2 homeobox 5 (Nkx2.5) (37), GATA binding protein 4 (GATA4) (7), and T-box transcription factor 5 (Tbx5) (2) manifest cardiac septation defects. These three transcription factors interact with each other to

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form complexes in driving cardiac gene expression during cardiogenesis, and they also promote cell proliferation by inducing cyclin D2 and CKD4 transcription (33). We hypothesize that maternal diabetes impedes the BMP4 pathway and suppresses the expression of cardiogenesis factors.

In the present study, we investigated the role of ASK1 in maternal diabetes-induced heart defects. We found that the ASK1-JNK1/2 pathway was activated in the developing heart by maternal diabetes. Moreover, inactivation of this pathway by deleting Ask1 ameliorated the incidence of heart defects by suppressing the ER stress-induced apoptotic response. Ask1 deletion also restored cell cycle progression by reversing the alteration in cardiac-specific transcription factors cyclin D and cell cycle inhibitors. Our studies support a critical role for ASK1 in maternal diabetes-induced VSDs and OFT defects.

METHODS

Mice and reagents. Wild-type (WT) C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Ask1−/− mice on the C57BL/6J background were provided by Dr. Hidenori Ichijo at the University of Tokyo. Streptozotocin (STZ) from Sigma (St. Louis, MO) was dissolved in sterile 0.1 M citrate buffer (pH 4.5). The procedures for animal use were approved by the University of Maryland School of Medicine Institutional Animal Care and Use Committee.

Mouse models of diabetic embryopathy. Mouse models of maternal diabetes-induced embryonic malformations were described previously (18, 57). Briefly, 10-wk-old WT and Ask1−/− female mice were intravenously injected daily with 75 mg/kg STZ over 2 days to induce diabetes. Using STZ to induce diabetes is not a complicating factor because STZ is cleared from the bloodstream rapidly (serum half-life, 15 min) (36), and pregnancy is not established until 1–2 wk after STZ injection (59). Diabetes was defined as a 12-h fasting blood glucose level of ≥14 mM. Male and female mice were paired at 3 PM, and a vaginal plug was present. Nondiabetic controls were established by injecting WT female mice with vehicle. Embryonic hearts were harvested for analyses on E12.5 and E17.5.

India ink injection and hematoxylin-eosin staining. After the pregnant dams were euthanized on E17.5, fetuses were excised from the uteri and decapitated. Then, the rib cage was removed, and the hearts were decapitated. The heart was flushed with phosphate-buffered saline (pH 7.4), and a small piece of tissue was taken from the apex of the heart (as an anatomic landmark). Hearts were then fixed in methacarn (60% methanol, 30% chloroform, and 10% glacial acetic acid), embedded in paraffin, and cut into 8-μm sections. After deparaffinization and rehydration, all specimens then underwent hematoxylin and eosin (H & E) staining in a standard procedure. All heart sections were photographed and examined for heart defects.

Western blotting. Western blotting was performed as described previously (17, 57). Briefly, E12.5 embryonic hearts were sonicated in ice-cold RIPA lysis buffer (Millipore, Bedford, MA). Proteins were separated by SDS-PAGE and immunoblotted using primary antibodies for β- actin (monoclonal, heavy-chain binding protein, C219/C/EBP homologous protein), phospho- (p)-ERK1/2 (PKR-like ER kinase), p-eIF2α (eukaryotic translation initiation factor 2α), p-IRE1 (eukaryotic translation initiation factor 2α), p-JNK1/2, p-Elk1 (ETS domain-containing protein 1), p-ATF2 (activating transcription factor 2), p-smad1/5/8, CDK2, CDK4, cyclinD1, cyclinD2, cyclin D3, p21, p27, Ask1, p-ASK1, caspase 3, caspase 8 and p-histone H3. The intensity of the protein bands was determined by densitometry and normalized by the densities of β-actin. Signals were detected using the SuperSignal West Femto kit (Thermo Scientific, Rockford, IL).

Measurement of oxidized thioredoxin levels. Oxidized thioredoxin (Trx) levels were assessed using the OxyBlot protein detection kit (Millipore). After Trx immunoprecipitation, immunoprecipitated protein was released from the protein G beads using 12% SDS solution. Protein was derivatized by 1 × 2,4-dinitrophenylhydrazine at room temperature for 15 min. The reaction was stopped by adding the neutralization solution and separated by a 12% SDS-PAGE gel. Membranes were incubated with a rabbit anti-dinitrophenol antibody, and signals were detected using the SuperSignal West Femto kit (Thermo Scientific).

Real-time PCR. Total RNA was isolated from hearts using an RNeasy Mini Kit (Qiagen, Valencia, CA) and reverse transcribed using the high-capacity cDNA archive kit (Applied Biosystems, Grand Island, NY). RT-PCR for NXX2.5, Gata4, Tbx5, P21, P27, CDK2, CDK4, cyclin D1, cyclin D2, cyclin D3, and β-actin was performed using Maxima SYBR Green/ROX qPCR Master Mix assay (Thermo Scientific). RT-PCR was performed using the StepOnePlus system (Applied Biosystems).

Immunofluorescence. E12.5 hearts were fixed in 4% paraformaldehyde overnight, followed by embedding in optimal cutting temperature compound (Sakura Finetek, Torrance, CA). Ten-micrometer heart cryosections were antigen-unmasked using citrate buffer and blocked in 5% bovine serum albumin in PBST (0.1% Triton X-100 in PBS) for 1 h. The following antibodies were used as primary antibodies: p-ASK1 (1:200) abd p-histone H3 (1:100) (Millipore). Normal rabbit or mouse IgG at the same dilution as those for antibodies was used as control. 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 from embryos of three different dams per group.

TUNEL assay. The TUNEL assay was performed using the In Situ Cell Death Detection Kit (Millipore) (57). Ten-micrometer serial heart frozen sections were fixed with 4% paraformaldehyde and incubated with TUNEL reagent. Sections were counterstained with DAPI. TUNEL-positive cells in each section were counted. Heart sections from three embryos of different dams per group and three sections per heart were analyzed. The percentage of apoptotic cells was calculated as the number of apoptotic cells divided by 200 cells in a selected area and then multiplied by 100.

Statistical analyses. Data on heart defect rates were analyzed by chi-square test. Data on protein and mRNA expression are presented as means ± SE. Two-way ANOVA and one-way ANOVA were performed using the SigmaStat 3.5 software, followed by Holm-Sidak and Tukey test to estimate significance. Statistical significance was accepted when P < 0.05.

RESULTS

Maternal diabetes activates ASK1 in the developing heart. To assess whether ASK1 is activated by maternal diabetes in the developing heart, we measured the levels of p-ASK1 at threonine 845 (Thr845). Phosphorylation of ASK1 at Thr845 correlates with enhanced ASK1 activity and increased apoptosis (43). Maternal diabetes increased the levels of p-ASK1 significantly in E12.5 hearts (Fig. 1A), a critical timing of embryonic cardiogenesis. Immunofluorescent staining of p-ASK1 confirmed that embryonic hearts exposed to maternal diabetes had a higher signal of p-ASK1 staining than that in embryonic hearts from nondiabetic dams (Fig. 1B). Under the inactive state, ASK1 binds to its endogenous inhibitor Trx (34). To further ascertain ASK1 activation by maternal diabetes, the levels of ASK1-Trx complexes were determined by immunoprecipitation (IP). The amount of Trx in ASK1 immunoprecipitates in embryonic hearts of diabetic dams was significantly
Fig. 1. Maternal diabetes activates apoptosis signal-regulating kinase 1 (ASK1) in the developing heart. A: phospho (p)-ASK1 protein levels in E12.5 (day 12.5 of pregnancy) hearts. B: images of p-ASK1 immunostaining. Rabbit normal IgG served as control. Red signal was p-ASK1, and cell nuclei were stained by DAPI (blue). Three embryonic hearts from 3 different dams (n = 3) per group and similar results were obtained. Bars, 150 μm. C: representative images of immunoprecipitation (IP) using an ASK1 antibody. In the bar graphs, levels of thioredoxin (Trx) were assessed in ASK1 immunoprecipitates and normalized by Trx levels in 2% input. Normal rabbit IgG was used as the IP negative control. D: oxidized Trx levels in E12.5 hearts determined by Trx IP followed by immunoblotting of oxidized proteins. E: ASK1 mRNA levels in E12.5 hearts and other embryonic tissues from embryos of 3 dams (n = 3). F: p-ASK1 immunostaining in E15.5 hearts with or without defects from diabetic dams; normal hearts are from nondiabetic (ND) dams (n = 3). In A and B, experiments were repeated 3 times using three E12.5 embryonic hearts of 3 dams (n = 3) per group. In C and D, E12.5 hearts from 5 to 6 embryos (1 litter) per group were used per IP run, and 3 litters (n = 3) per group were used. *Significant differences (P < 0.05) compared with the other groups. WB, Western blot; DM, diabetes mellitus; WT, wild type; DNP, dinitrophenol.
less than that in embryonic hearts of nondiabetic dams (Fig. 1C), indicating that maternal diabetes dissociates the inactive ASK1/Trx complexes and thus activates ASK1.

Formation of the Trx-ASK1 complex occurs only with the reduced form of Trx. Upon oxidation by reactive oxygen species, oxidized Trx is released from the inactive ASK1/Trx complex and thus enables ASK1 activation (34). Because it has been demonstrated that maternal diabetes induces oxidative stress in the developing embryo, we determined whether diabetes increases Trx oxidation. Indeed, maternal diabetes significantly increased the amount of oxidized Trx in the developing heart (Fig. 1D).

E12.5 embryonic hearts had the highest levels of ASK1 mRNA compared with those in other embryonic tissues (Fig. 1E), suggesting that embryonic hearts are more susceptible to ASK1 activation than other tissues. At E15.5, defective hearts from embryos of diabetic dams had higher p-ASK1 immunostaining signals compared with those in normal hearts of embryos from nondiabetic and diabetic dams (Fig. 1F), indicating that ASK1 activation correlates with heart defect formation.

Ask1 gene deletion prevents septation and OFT defects in diabetic pregnancies. Maternal diabetes induced 25.5% overall heart defects (Table 1). India ink injections revealed two major types of OFT defects, persistent truncus arteriosus (PTA) and heart defects (Table 1). India ink injections revealed two major types of OFT defects, persistent truncus arteriosus (PTA) and muscular septum (Fig. 2A). Posterior VSDs were large and located in the cardiac outlet and associated with an overriding aorta (Fig. 2A). The VSDs in anterior positions (Fig. 2A) were located in the cardiac outlet and associated with an overriding aorta (Fig. 2A). Posterior VSDs were large and located in the muscular septum (Fig. 2A).

To test whether Ask1 gene deletion ameliorates maternal diabetes-induced heart defects, we examined the incidence of heart defects in WT and Ask1−/− embryos from WT parents and Ask1−/− parents, respectively. Targeted Ask1 gene deletion did not affect heart development under nondiabetic conditions (Table 1). Under diabetic conditions, only one of the 54 Ask1−/− embryos exhibited heart defects (Table 1), whereas 14 of 55 WT embryos manifested heart defects (Table 1). Ask1 deletion did not affect elevated blood glucose levels in diabetic dams (Table 1). These findings support our hypothesis that ASK1 mediates the teratogenic effect of maternal diabetes on the developing heart.

Caspase activation and apoptosis are diminished in the absence of ASK1. We next evaluated the impact of Ask1 deletion in maternal diabetes-induced apoptosis in the developing heart. The abundance of cleaved caspase 3 and caspase 8, indices of cell apoptosis, was significantly higher in WT embryonic hearts of diabetic dams than in WT embryonic hearts from nondiabetic dams (Fig. 2B), and Ask1 gene deletion blocked the cleavage of these two caspases induced by maternal diabetes (Fig. 2B). TUNEL assay detected excess apoptotic cells in the atrioventricular cushion, the ventricular myocardium, and the OFT in embryonic hearts from diabetic dams (Fig. 2, C and D). The number of apoptotic cells was significantly higher in WT embryonic hearts from diabetic dams than in embryos from nondiabetic dams and in Ask1−/− embryonic hearts under diabetic conditions (Fig. 2, C and D).

Targeted deletion of Ask1 suppresses the proapoptotic JNK1/2 pathway. ASK1 activation triggers JNK1/2 and/or p38 MAPK (42). To test whether maternal diabetes-induced ASK1 activation leads to the activation of JNK1/2 and/or p38 MAPK, we assessed the phosphorylation of these two kinases. Maternal diabetes increased the levels of p-JNK1/2 but not p38 MAPK (Fig. 3A), and Ask1 deletion abolished JNK1/2 phosphorylation (Fig. 3A). To further confirm whether Ask1 deletion inactivates the JNK1/2 pathway, we measured the phosphorylation of c-Jun, ATF2, Elk1, and FoxO3a (forkhead transcription factor 3a), four transcription factors downstream of JNK1/2. Levels of p-c-Jun, p-ATF2, and p-Elk1 were significantly higher in embryonic hearts of diabetic dams than in embryonic hearts of nondiabetic dams. Ask1 deletion suppressed maternal diabetes-increased phosphorylation of these three transcription factors (Fig. 3B). Levels of p-FoxO3a, an inactive form, were higher in embryonic hearts of nondiabetic dams than in embryonic hearts of diabetic dams. Ask1 deletion abrogated diabetes-reduced FoxO3a phosphorylation (activation) (Fig. 3B).

Both JNK1/2 activation and ER stress lead to apoptosis in diabetic embryopathy (18). In addition, JNK1/2 activation causes ER stress (18). To investigate whether maternal diabetes induces ER stress in the developing heart, we measured the levels of ER markers. Levels of ER stress markers p-ERK, p-eIF2α, p-IRE1α, BiP, and CHOP were increased significantly in hearts of embryos from diabetic dams compared with hearts of embryos from nondiabetic dams (Fig. 4A). Ask1 gene deletion abrogated the increase in ER stress marker expression (Fig. 4A). In addition, robust XBPI (X-box binding protein 1) splicing, another indicator of ER stress, was observed in WT embryonic hearts exposed to maternal diabetes, whereas there was no XBPI splicing in WT embryonic hearts under nondiabetic conditions or in Ask1−/− embryonic hearts under diabetic conditions (Fig. 4B).

Table 1. Heart defect rates in E17.5 embryos of nondiabetic and diabetic dams

<table>
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<tr>
<th>Experimental Group</th>
<th>Glucose Level, mM</th>
<th>Total Embryos</th>
<th>Total Heart Defect Embryos</th>
<th>VSD Embryos</th>
<th>Outflow Tract Defect Embryos</th>
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<tr>
<td>ND WT ♂ × WT ♀</td>
<td>6.32 ± 0.58</td>
<td>57</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>ND Ask1−/−♂ × Ask1−/− ♀</td>
<td>7.57 ± 0.84</td>
<td>55</td>
<td>0 (0%)</td>
<td>0</td>
<td>0</td>
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<tr>
<td>DM WT ♂ × WT ♀</td>
<td>24.3 ± 1.12</td>
<td>55</td>
<td>14 (25.5%)*</td>
<td>7 (12.7%)*</td>
<td>7 (12.7%; 3 PTA, 2 malalign defects, 2 right-sided aortic arches)*</td>
</tr>
<tr>
<td>DM Ask1−/−♂ × Ask1−/− ♀</td>
<td>23.4 ± 1.04</td>
<td>54</td>
<td>1 (1.9%)</td>
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Values are means ± SE (9 litters/experimental group). ND, nondiabetic; DM, diabetes mellitus; WT, wild type; Ask1−/−, apoptosis signal-regulating kinase 1-knockout; ♂, male dams; ♀, female dams; VSD, ventricular septal defects; PTA, persistent truncus arteriosus. Blood glucose levels were determined at E8.5–E17.5 (days of pregnancy) and averaged for each dam. *Significant difference (P < 0.05) compared with other groups analyzed by the chi-square test.
Fig. 2. Ask1 gene deletion suppresses maternal diabetes-induced heart defects, caspase activation, and cell apoptosis in the developing heart. A: hematoxylin and eosin-stained E17.5 hearts from anterior to posterior. A morphologically normal heart (left) from WT embryos of ND dam, a typical VSD heart (middle), and aorta-overriding ventricular septal defect (VSD) heart (right) from WT embryos of DM dams. E17.5 heart and blood vessels were imaged in whole mount after an injection of india ink: a normal heart from WT embryos of ND dam, a heart with persistent truncus arteriosus (PTA), and a heart with right-sided aortic arch (RAA) from WT embryos of diabetic dams. Scale bars, 300 μm. AO, aorta; PA, pulmonary artery; LA, left atrium; RA, right atrium; LV, left ventricle; RV, right ventricle. Dotted lines highlight the blood vessels.

B: representative immunoblotting for detection of cleaved caspase 3, caspase 8, and the quantification normalized to β-actin. Experiments were repeated 3 times using embryonic hearts of 3 different dams (n = 3) per group.

C and D: representative images of the TUNEL assay showing apoptotic cells (red signals) and quantified as TUNEL-positive cells/section (3 sections from 3 different hearts of each group). Cell nuclei were stained with DAPI (blue). TUNEL-positive cells in the atrioventricular and outflow tract endocardial cushion are shown in C and D, respectively. In C, TUNEL positive cells are also seen in the epicardium lining the ventricle. Bars, 150 μm. *Significant difference (P < 0.05) compared with other groups.
Ask1 gene deletion restores cell proliferation by diminishing alterations in cell cycle regulators. p-Histone H3 staining was used to detect proliferating cells in the embryonic heart (Fig. 5A). Maternal diabetes significantly reduced the number of p-histone H3-positive cells in the embryonic hearts, and Ask1 gene deletion prevented the reduction of proliferating cells (Fig. 5A). Cell cycle progression is controlled by cyclin Ds and their partners, CDKs. Protein and mRNA levels of cyclin D1 and D3 were repressed by maternal diabetes (Fig. 5, B and C), whereas the expression of CDK2 and CDK4 remained unchanged (data not included). Maternal diabetes did not affect cyclin D2 expression (data not included). Furthermore, the expression of two cell cycle inhibitors, p21 and p27, was increased by maternal diabetes (Fig. 5, D and E). Ask1 gene deletion abrogated the decrease in cyclin D1 and D3 (Fig. 5, B and C) and the increase in p21 and p27 (Fig. 5, D and E) under maternal diabetic conditions.

Deleting the Ask1 gene removes the inhibitory effect of maternal diabetes on the BMP4/Smad pathway. BMP4 is critical for embryonic cardiogenesis, and its deletion causes heart defects (24) that are similar to the defects observed in diabetic pregnancies. To determine whether maternal diabetes
influences BMP4 expression, mRNA and protein levels of BMP4 were measured. Maternal diabetes suppressed BMP4 mRNA and protein expression (Fig. 6A). In Ask1−/− embryonic hearts, maternal diabetes-suppressed BMP4 was diminished (Fig. 6A). BMP4 binds to its receptors, leading to phosphorylation of Smad1/5/8. Consistent with the repression of BMP4 expression, p-Smad1/5/8 levels were decreased significantly by maternal diabetes (Fig. 6B), and Ask1 gene deletion restored the phosphorylation of Smad1/5/8 (Fig. 6B).

Expression of transcription factors essential for ventricular septation is restored by Ask1 gene deletion. Mutations of the transcription factors Nkx2.5, GATA4, and Tbx5 result in VSD formation (2, 7, 37). To reveal whether maternal diabetes affects the expression of these transcription factors, mRNA levels of Nkx2.5, GATA4, and Tbx5 were assessed. Maternal diabetes suppressed the expression of Nkx2.5 and GATA4 but did not affect the expression of Tbx5 (Fig. 7). Ask1 gene deletion restored the expression of Nkx2.5 and GATA4 (Fig. 7).

DISCUSSION

ASK1 is activated in the developing heart by maternal diabetes. The mechanism underlying ASK1 activation is likely...
triggered by oxidative stress, because overexpression of the antioxidant superoxide dismutase 1 suppresses ASK1 activation (57). The present study further supports the hypothesis that maternal diabetes-induced oxidative stress causes ASK1 activation. Trx acts as an endogenous ASK1 inhibitor through a direct interaction with ASK1 (34). Consistent with the fact that Trx negatively regulates ASK1 activation, maternal diabetes reduces the amount of ASK1-Trx complexes in the developing heart and increases the levels of oxidized Trx. The interaction between Trx and ASK1 depends on the redox status of Trx.

Fig. 5. Maternal diabetes-inhibited cell proliferation and altered cell cycle regulator expression are abrogated by Ask1 gene deletion. A: representative images of p-histone H3 immunostaining, which labels proliferating cells. p-Histone H3-positive cells are labeled by red signals, and cell nuclei are stained by DAPI (blue). In the bar graph, p-histone H3-positive cells were quantified by National Institutes of Health ImageJ software and expressed as no. of cells/section. Embryonic hearts from 3 dams (n = 3) per group and 3 serial sections/heart were analyzed. Bars, 150 μm. B: protein levels of cyclin D1 and D3. C: mRNA levels of cyclin D1 and D3. D: protein levels of p21 and p27. E: mRNA levels of p21 and p27. Experiments were repeated 3 times using three E12.5 hearts from 3 dams (n = 3) per group. *Significant difference (P < 0.05) compared with other groups.
(34). The redox property of Trx relies on its redox regulatory domain containing the active cysteine residues 32 and 35 (10). ROS oxidizes the cysteine residues of Trx, thereby inducing the dissociation of Trx from ASK1, leading to ASK1 phosphorylation (Fig. 7B) (34).

JNK1/2 and p38 MAPK are downstream kinases of ASK1 and transmit the proapoptotic signaling cascade initiated by ASK1 to the nucleus (42). Diabetes activates JNK1/2 but not p38 MAPK in the developing heart. Ask1 deletion abrogates JNK1/2 activation and the activation of three transcription factors downstream of JNK1/2. Our previous evidence supports a reciprocal causative relationship between the JNK1/2 pathway and ER stress (18). Indeed, along with JNK1/2 activation, ER stress is manifested in the developing heart exposed to diabetes. ER stress is the consequence of ASK1 activation, suggesting that the two interrelated proapoptotic pathways, the JNK1/2 pathway and the ER stress pathway, act cooperatively in mediating the teratogenic effect of ASK1.

Because Ask1 deletion abolishes maternal diabetes-induced apoptosis and impaired cell proliferation in the developing heart, ASK1 exerts both proapoptotic and antiproliferative effects. Deleting either the Jnk1 or the Jnk2 gene or blocking ER stress by the chemical chaperone 4-phenylbutyrate (18) inhibits maternal diabetes-induced embryonic cell apoptosis. Therefore, the ER pathway appears to mediate the proapoptotic response of ASK1, and the JNK1/2 pathway, particularly JNK2 (18), may be responsible for the antiproliferative effect of ASK1.

Animal studies have demonstrated that excess apoptosis and impaired cell proliferation in the developing heart because ASK1 mediates the inhibitory effect of maternal diabetes on the expression of cardiac transcription factors Nkx2.5 and GATA4. The two transcription factors form the membranous portion of the ventricular septum and is responsible for septation of the common OFT (5, 20). The importance of BMP4 in heart development is further supported by a study showing that Bmp4 deletion in the anterior heart field, which contributes to the major portion of the OFT and the interventricular septum, results in VSD and PTA (24). BMPs bind to their cell membrane receptors and affect the development of other major organs (14), supporting the causal role of impaired cell proliferation in defective heart formation. We found that diabetes differentially regulates the three cyclin Ds. This differential regulation has been reported in the liver (31). Downregulation of cyclin D1 by diabetes is in agreement with a recent study in the chick model (38). Cyclin D2 is a GATA4 cofactor in cardiogenesis (54); however, we did not observe any changes in cyclin D2 expression. In the neonatal heart, GATA4 induces cyclin D2 transcription (55). In our study, reduced GATA4 expression does not cause cyclin D2 downregulation. This discrepancy may be due to differences in developmental timing or other tissue-specific differences. Thus, our study reveals that maternal diabetes impedes embryonic heart cell cycle progression by suppressing cyclin D1 and D3 expression and concomitantly increasing expression of the cell cycle inhibitors p21 and p27 (Fig. 7B).

BMP4 is essential for cardiomyocyte specification and is critically involved in the development of endocardial cushions that form the membranous portion of the ventricular septum and is responsible for septation of the common OFT (5, 20). The importance of BMP4 in heart development is further supported by a study showing that Bmp4 deletion in the anterior heart field, which contributes to the major portion of the OFT and the interventricular septum, results in VSD and PTA (24). BMPs bind to their cell membrane receptors and exert their effects through receptor-mediated activation of Smad transcription factors (24). BMP4 expression is repressed, and the activation of its downstream effectors, Smad1/5/8, is blunted in the heart by maternal diabetes. Ask1 deletion restores BMP4 signaling, suggesting that ASK1 activation leads to BMP4 repression. The mechanism whereby ASK1 represses BMP4 warrants further investigation.

ASK1 activation may also alter cell differentiation in the developing heart because ASK1 mediates the inhibitory effect of maternal diabetes on the expression of cardiac transcription factors Nkx2.5 and GATA4. The two transcription factors form...
a complex with another transcription factor, Tbx5, activating cardiac gene expression that is essential for embryonic cardiogenesis (2, 7, 37). Mutations in these three transcription factors produce VSD exclusively, suggesting their crucial role in the development of interventricular septum and endocardial cushions. Besides their function in cardiac gene expression, they positively regulate cell proliferation through increasing cyclin D2 and CKD4 expression (33). It is not clear how ASK1 suppresses Nkx2.5 and GATA4. Ask1 Studies have suggested a negative feedback loop between ASK1 activation and hypoxia-inducible factor-1α (HIF-1α) (16, 61), a critical factor for cardiogenesis (27). HIF-1α is required for Nkx2.5 expression in the Xenopus heart (27), and DNA binding sites of HIF-1α for positive transcription regulation are identified in the BMP4 promoter (50). Therefore, HIF-1α may be involved in the regulation of Nkx2.5, BMP4, and possibly Gata4 in the developing heart (Fig. 7B).

Inducible overexpressing ASK1 in adult cardiomyocytes does not regulate cardiac hypertrophic response but induces cardiomyocyte apoptosis (19), which is in agreement with our
finding that ASK1 activation is linked to apoptosis. Overexpressing ASK1 may enhance diabetes-induced heart defects. However, we found that embryonic hearts had the highest ASK1 levels compared with other embryonic tissues, questioning whether ASK1 overexpression would result in additive effects. Our study reveals that ASK1 activation is responsible for diabetes-induced heart defects. One reverse experiment may use a constitutively active ASK1 transgenic line to determine whether ASK1 activation mimics maternal diabetes to induce heart defects. Additionally, the inducible ASK1 transgenic mice (19) may not be suitable for our study because the α-myosin heavy chain promoter-driven transgene expression does not cover the key period of cardiogenesis, and being given doxycycline may cause adverse effects on embryonic development. Our future studies may use the gain-of-function approach by creating a constitutively active ASK1 transgenic line.

Hyperglycemia, but not the types of diabetes, determines the incidence of birth defects. Clinical studies have revealed a strong correlation between the degree of maternal hyperglycemia and the rate and severity of birth defects (8, 22, 41). When whole rodent embryos are cultured in high concentrations of glucose, it induces embryonic malformations similar to the defects observed in human fetuses exposed to hyperglycemia (30, 60). We found that modest hyperglycemia in a type 2 diabetic embryopathy model had lower birth defect incidence than that of type 1 diabetic embryopathy, which has a high degree of hyperglycemia (52). In contrast, insulin may have minimal roles in embryonic development because maternal insulin does not cross the human placenta (1, 4, 13). Thus, our findings in type 1 diabetes may be extrapolated to conditions of type 2 diabetes.

Although the use of ASK1 inhibitors is far from being a clinical application, and giving drugs during pregnancy represents a challenge, the findings in our study do provide a mechanistic basis for developing screening tests in assessing the risk of defects. Because oxidative stress causes ASK1 activation, maternal serum antioxidant enzyme activities, including the activities of superoxide dismutase 1 and glutathione peroxidase, lipid peroxidation markers such as malondialdehyde, and the oxidized DNA marker 8-hydroxydeoxyguanosine, may be good indicators of defect risks in diabetic pregnancies. These markers have been used for prediction of diabetic complications (11, 51). Measuring maternal serum levels of oxidized Trx may be a reliable marker for diabetes-induced heart defects.

In summary, ASK1 activation has multiple profound effects on the developing heart. ASK1 activates the interlinked JNK1/2-ER stress proapoptotic pathway, leading to excess apoptosis (Fig. 7B), and concomitantly suppresses cell cycle progression by repressing cyclin D expression and inducing cell cycle inhibitors (Fig. 7B). Maternal diabetes induces mainly septation defects in the ventricular septum and in the OFT. Thus, ASK1 gene deletion restores the BMP4 signaling and the expression of cardiac transcription factors Nkx2.5 and GATA4 (Fig. 7B). Our study reveals the causal role of ASK1 activation in maternal diabetes-induced heart defects and the signaling network downstream of ASK1 that transmits the proapoptotic, antiproliferative, and altered cell differentiation signals.

ACKNOWLEDGMENTS

We thank the Office of Dietary Supplements, National Institutes of Health, for its support. We thank Dr. Hidenori Ichijo at the Graduate School of Pharmaceutical Sciences, University of Tokyo, for providing us with the ASK1-knockout mice. We thank Dr. Julie Wu at the University of Maryland School of Medicine for critical reading and editing assistance.

GRANTS

This research was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grants R01-DK-083243, R01-DK-101972, and R01-DK-103024 and a Basic Science Award (1-13-BS-220) from the American Diabetes Association.

DISCLOSURES

None of the authors have any conflicts of interest, financial or otherwise.

AUTHOR CONTRIBUTIONS

F.W., Y.W., and X.L. performed experiments; F.W., M.J.Q., and P.Y. analyzed data; M.J.Q. and P.Y. interpreted results of experiments; P.Y. conception and design of research; P.Y. prepared figures; P.Y. drafted manuscript; P.Y. edited and revised manuscript; P.Y. approved final version of manuscript.

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