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Maternal Hyperglycemia Activates an ASK1–FoxO3a–Caspase 8 Pathway That Leads to Embryonic Neural Tube Defects

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Abstract

Neural tube defects result from failure to completely close neural tubes during development. Maternal diabetes is a substantial risk factor for neural tube defects, and available evidence suggests that the mechanism that links hyperglycemia to neural tube defects involves oxidative stress and apoptosis. We demonstrated that maternal hyperglycemia correlated with activation of the apoptosis signal–regulating kinase 1 (ASK1) in the developing neural tube, and *Ask1* gene deletion was associated with reduced neuroepithelial cell apoptosis and development of neural tube defects. ASK1 activation stimulated the activity of the transcription factor FoxO3a, which increased the abundance of the apoptosis-promoting adaptor protein TRADD, leading to activation of caspase 8. Hyperglycemia-induced apoptosis and the development of neural tube defects were reduced with genetic ablation of either *FoxO3a* or *Casp8* or inhibition of ASK1 by thioredoxin. Examination of human neural tissues affected by neural tube defects revealed increased activation or abundance of ASK1, FoxO3a, TRADD, and caspase 8. Thus, activation of an ASK1–FoxO3a–TRADD–caspase 8 pathway participates in the development of neural tube defects, which could be prevented by inhibiting intermediates in this cascade.

INTRODUCTION

Neurulation, a key process during embryogenesis, leads to formation of the neural tube, which eventually develops into the central nervous system. The failure of neurulation or neural tube closure results in neural tube defects (NTDs), with anencephaly and spina bifida

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SUPPLEMENTARY MATERIALS

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being the two most common types in humans. NTDs are the second most common birth defects in humans and occur in 0.2 to 3.5 per 1000 pregnancies (1).

Studies from teratogen-induced NTDs such as maternal diabetes (2–7) and NTDs induced in mice by gene disruption (8–10) have implicated stress-induced aberrant signaling and excessive neuroepithelial cell apoptosis as potential causes of NTD formation. Maternal diabetes–induced NTDs are associated with increased oxidative stress and apoptosis (2, 4, 6, 7, 11–14) through an unknown mechanism. Maternal hyperglycemia increases glucose flux to embryonic cells, leading to enhanced production of reactive oxygen species and impairment of endogenous antioxidant capacity (15, 16). Maternal hyperglycemia–induced oxidative stress triggers aberrant mitogen-activated protein kinase (MAPK) activity through an unknown mechanism, resulting in increased proapoptotic signaling (4, 12).

The MAPK signaling pathway is a three-component module with the MAPK kinase kinase (MAP3K) as the initiator, MAPK kinase (MAPKK), and MAPK. The MAP3K apoptosis signal–regulating kinase 1 (ASK1) is activated by reactive oxygen species in vitro, and its activation in cells is associated with apoptosis (17). Phosphorylation of Thr^{845} in the activation loop of ASK1 correlates with enhanced ASK1 activity and increased apoptosis (18). ASK1 may induce apoptosis through nontranscriptional or transcriptional dependent mechanisms (17). Because apoptotic gene induction is implicated in maternal diabetes–induced embryonic cell apoptosis (19), we reasoned that ASK1 activation may induce apoptotic gene expression by enhancing the activity of a group of transcription factors or one particular transcription factor.

The Forkhead O (FoxO) subfamily of Forkhead transcription factors is composed of three functionally related members: FoxO1, FoxO3a, and FoxO4 (20). FoxO transcription factors are key regulators of cell fate, and they drive apoptotic responses in stress-exposed cells (21, 22) by regulating expression of apoptosis-relevant target genes (23). The transcriptional activity of FoxO factors is controlled by their subcellular localization and phosphorylation state (24). Phosphorylation of FoxO factors at Thr^{24} and Thr^{32} prevents nuclear translocation, thereby inhibiting FoxO-dependent transcription (25). Because maternal diabetes deregulates the activity of Akt (26), a key regulator of FoxO activity, FoxO proteins may be critically involved in neuroepithelial cell apoptosis and NTD formation.

Caspase activation also is associated with NTD formation in diabetic pregnancies (4, 12). Caspases are classified as either executive or initiator caspases, which drive the caspase cascade leading to cell demise. To identify the initiator caspase and how it is activated is a key to preventing unwanted apoptosis. Although there is ongoing controversy whether ASK1-driven apoptosis involves the initiator caspase caspase 8 (27, 28), its cleavage (activation) occurs in NTDs that are associated with maternal diabetes (4, 12). Thus, it is of interest to determine whether caspase 8 activation is a causal event or a secondary consequence in maternal diabetes–induced NTD formation.

Here, we demonstrated that ASK1, FoxO3a, and caspase 8 are activated, and the apoptotic factor *TRADD* is increased in abundance in the developing neural tube of embryos of diabetic dams. Germline deletion of either the *Ask1* or the *FoxO3a* gene, or conditional

deletion of either the *FoxO3a* or the *Casp8* gene, in the neural tube was associated with attenuation of maternal diabetes–induced apoptosis and NTD formation. Treatment of diabetic mice or cultured embryos with the ASK1 inhibitor thioredoxin (Trx) resulted in reduced hyperglycemia-induced apoptosis and NTD formation. Furthermore, activation of this ASK1–FoxO3a–TRADD–caspase 8 pathway was detected in human NTD–affected tissues from late-stage pregnancies. Our studies therefore provide evidence for a molecular pathway that could mediate the pathogenesis of NTDs resulting from maternal diabetes.

RESULTS

Maternal diabetes activates ASK1 in the developing neuroepithelium through oxidative stress

Neural tube closure takes place very early in pregnancy, before day 28 of gestation in humans. Because human embryonic neural tubes are not readily accessible for study, mouse embryos at neurulation stages [from embryonic day 8 (E8.0) to E10.5] typically have been used for studies attempting to unravel the mechanism underlying NTD formation. Here, we used a mouse model of diabetic embryopathy in which NTDs were increased in the embryos of diabetic dams (4).

We determined the effect of maternal diabetes on ASK1 activation in hyperglycemia-induced NTD in this mouse model by monitoring the phosphorylation of ASK1 in E8.75 embryos from nondiabetic mice and mice with diabetic mellitus. Phosphorylation of Thr^{845} is essential for the catalytic activity of ASK1 and the induction of apoptosis downstream of ASK1 (18). We assessed phosphorylation of ASK1 at this site with a specific antibody (fig. S1, A and B) that has been extensively validated by assaying ASK1 catalytic activity downstream from oxidative stress using T845A mutants and antigen blocking (18). Phosphorylation of ASK1 at Thr^{845} was increased in embryos of diabetic mice compared to those of nondiabetic mice (Fig. 1, A and B), although phosphorylation of the related MAP3Ks TAK1 (transforming growth factor β–activated kinase) and MLK3 did not differ between the groups (Fig. 1, C and D). The phosphorylation of the kinase JNK1/2 (c-Jun N-terminal kinase 1/2) (Fig. 1, A and E), a downstream target of ASK1, was greater in the embryos of diabetic mice than in those of nondiabetic mice. In addition, immunofluorescence for phosphorylated ASK1 was detected in neural tubes in embryos in diabetic mice but not in those of nondiabetic mice (Fig. 1F). The tissues adjacent to the neural tube in the embryos of the diabetic mice did not show a detectable signal for phosphorylated ASK1 (Fig. 1F), suggesting that diabetes-induced ASK1 activation specifically occurs in the neuroepithelium.

To confirm that diabetes activates ASK1, we assessed the interaction between ASK1 and Trx, an inhibitor of ASK1 that is constitutively associated with nonactive ASK1 under basal conditions (29). Reactive oxygen species oxidize Trx, resulting in its dissociation from ASK1 and allowing subsequent phosphorylation and activation of ASK1 (29). The amount of Trx in ASK1 immunoprecipitates from E8.75 embryos of diabetic mice was significantly lower than that in embryos of nondiabetic mice (Fig. 1G), suggesting that maternal diabetes results in dissociation of ASK1-Trx complexes, leading to ASK1 activation. The decreased association of Trx and ASK1 was not due to reduced abundance of endogenous ASK1 or

Trx (Fig. 1G). Oxidative stress is thought to be an important initiator of NTD formation; however, there is no in vivo evidence that oxidative stress directly activates ASK1. To determine whether oxidative stress is associated with maternal diabetes–induced ASK1 activation, we assessed phosphorylation of ASK1 in wild-type embryos from nondiabetic mice and in wild-type and SOD1 (superoxide dismutase 1)–overexpressing embryos from wild-type diabetic female mice mated with SOD1transgenic males. Overexpression of human SOD1, which suppresses diabetes-induced oxidative stress (30), was associated with attenuation of increased phosphorylation of ASK1 in embryos from diabetic dams (fig. S1C), suggesting that maternal diabetes could induce activation of ASK1 in the embryo through oxidative stress.

Deletion of the *Ask1* gene ameliorates maternal diabetes–induced neuroepithelial cell apoptosis and NTD formation

We assessed whether the absence of ASK1 reduces the NTD incidences in diabetic pregnancies. The rate of NTD in *Ask1*$^{-/-}$ embryos of diabetic *Ask1*$^{-/-}$ dams was 2.8 ± 1.9%, which was significantly lower than that in wild-type embryos of diabetic wild-type dams (27.4 ± 3.2%) but not significantly different when compared to that in wild-type embryos of nondiabetic wild-type dams (1.0 ± 1.0%) and *Ask1*$^{-/-}$ embryos of nondiabetic *Ask1*$^{-/-}$ dams (1.0 ± 1.0%) (Fig. 1, H and I). Furthermore, diabetic *Ask1*$^{-/-}$ and diabetic wild-type mice had similar high blood glucose concentrations, indicating that deletion of *Ask1* did not affect blood glucose concentrations during hyperglycemia (Fig. 1J). NTDs occurred in 19 of 71 wild-type embryos exposed to diabetes, whereas only 1 in 70 wild-type embryos under nondiabetic conditions and 2 of 68 *Ask1*$^{-/-}$ embryos from diabetic *Ask1*$^{-/-}$ mice had NTDs (table S1). As previously reported (17), *Ask1* deletion did not adversely affect embryonic development based on NTD examination at E10.5 in *Ask1*$^{-/-}$ embryos of nondiabetic *Ask1*$^{-/-}$ mice (Fig. 1H and table S1) or embryo resorption rates (table S1).

We previously have shown that caspase 8, an initiator caspase that triggers activation of multiple apoptosis executors, is activated in diabetic embryopathy (31). Therefore, we assessed whether ASK1 promotes in caspase 8 activation. Generation of the cleaved 18-kD form of caspase 8, the most active form, was detected in wild-type E8.75 embryos of diabetic dams but not in wild-type embryos of nondiabetic wild-type dams or *Ask1*$^{-/-}$ embryos of diabetic *Ask1*$^{-/-}$ dams (Fig. 2A). In addition, *Ask1* gene deletion abolished maternal diabetes–induced caspase 3 cleavage, a direct index of apoptosis (Fig. 2A). As previously reported (4, 12), the number of apoptotic neuroepithelial cells was significantly higher in wild-type embryos of wild-type diabetic mice than in wild-type embryos of wild-type nondiabetic mice (Fig. 2, B and C), a phenotype that was attenuated by *Ask1* deletion (Fig. 2, B and C). These results demonstrate that ASK1 plays an essential role in the induction of apoptosis leading to NTD formation.

Deletion of the *Ask1* gene abrogates maternal diabetes–increased TRADD abundance

Microarray studies have demonstrated increased expression of various genes encoding apoptotic factors in embryos exposed to maternal diabetes (19). Our screen indicated that the expression of the mRNA for the adaptor protein TRADD, which activates caspase 8 (32),

was increased in E10 to E11 neural tubes and E8.75 embryos of diabetic dams (fig. S2, A and B).

To test the effect of *Ask1* deletion on diabetes-increased TRADD abundance, we measured TRADD protein and mRNA. The abundance of both TRADD protein (Fig. 2D) and mRNA (Fig. 2E) in *Ask1*$^{-/-}$ embryos of diabetic *Ask1*$^{-/-}$ dams was similar to that in wild-type embryos of nondiabetic wild-type dams and was significantly lower than that in wild-type embryos of diabetic wild-type mice. Thus, *Ask1* deletion abrogated diabetes-increased abundance of TRADD.

Casp8 gene deletion prevents maternal diabetes–induced neuroepithelial cell apoptosis and NTD formation

Because maternal diabetes–induced caspase 8 cleavage was diminished in the absence of ASK1 (Fig. 2A), we conditionally deleted *Casp8* in the neural tube from E8 onward to determine its contribution to NTD formation using the floxed *Casp8* mice and the nestin promoter–driven Cre mice. The abundance of protein and mRNA for endogenous nestin, a neural stem cell marker, was not affected by maternal diabetes (fig. S3, A and B). NTDs were observed in *Casp8*-positive embryos and in embryos without *Casp8* deletion or displaying incomplete *Casp8* deletion, but not in embryos with conditional deletion of *Casp8* (Fig. 2, F and G, and fig. S4). Furthermore, conditional deletion of the *Casp8* gene in the neural tube was associated with reduced cleavage of caspase 3 (Fig. 2H) and decreased numbers of apoptotic cells in the neural tube (Fig. 2, I and J) in response to diabetes. Specifically, under diabetic conditions, apoptosis was diminished in neural tubes with *Casp8* deletion but remained significantly higher in littermates without *Casp8* deletion or incomplete *Casp8* deletion (Fig. 2, I and J). Thus, caspase 8 activation downstream of ASK1 appears to contribute to diabetes-dependent apoptosis and NTD formation.

ASK1 triggers activation of FoxO3a

TRADD is encoded by a gene that is responsive to the FoxO family of transcription factors (33), which are activated when dephosphorylated and inhibited when phosphorylated by kinases, such as Akt (25). Maternal diabetes was associated with decreased phosphorylation of FoxO3a at Thr^{32} in embryos (Fig. 3, A and B) but did not alter the phosphorylation of FoxO1 and FoxO4 (Fig. 3, A, C, and D). Furthermore, phosphorylation and activation of Akt in embryos were significantly reduced by maternal diabetes (Fig. 3, A and E). Immunostaining revealed that FoxO3a dephosphorylation specifically occurred in the neuroepithelium of embryos of diabetic dams (Fig. 3F). Thus, ASK1 activation may relay its apoptotic signal to the nucleus through FoxO3a, thereby inducing the expression of genes encoding apoptotic factors and leading to NTDs.

To test whether *Ask1* deletion abrogates hyperglycemia-induced FoxO3a activation, we measured the amount of nuclear FoxO3a and phosphorylation of FoxO3a at Thr^{32} (25). Deletion of the *Ask1* gene abrogated diabetes-increased nuclear localization of FoxO3a (Fig. 3G). Phosphorylation of FoxO3a at Thr^{32} in *Ask1*$^{-/-}$ embryos of diabetic *Ask1*$^{-/-}$ mice was similar to that in wild-type embryos from nondiabetic mice (Fig. 3H). In contrast, phosphorylation of FoxO3a at Thr^{32} in wild-type embryos of diabetic dams was

significantly reduced (Fig. 3H). Thus, *Ask1* deletion was correlated with reduced hyperglycemia-induced activation of FoxO3a.

FoxO3a gene deletion reduces maternal diabetes–induced apoptosis and NTD formation

We used both germline deletion and conditional deletion of the *FoxO3a* gene in the neural tube from E8 onward to test the effect of FoxO3a activation in the induction of NTDs by diabetes. The NTD rate of $FoxO3a^{-/-}$ embryos from diabetic mice was 3.1%, which was significantly lower than that of wild-type embryos (25.6%) from diabetic wild-type mice and was similar to that of embryos from nondiabetic wild-type dams (Fig. 3I and table S2). Moreover, *FoxO3a* deletion was associated with a reduction in the maternal diabetes–induced increase in mRNA and protein abundance for TRADD (Fig.3, J and K), cleavage of caspases 3 and 8 (Fig.3L), and neuroepithelial cell apoptosis (Fig. 3, M and N).

The incidence of NTDs was significantly lower in embryos with conditional deletion of the *FoxO3a* gene ($FoxO3a^{L/L};Nes8Cre^{+}$) in the neural tube than in embryos without *FoxO3a* deletion from the same group of diabetic dams as the $FoxO3a^{L/L};Nes8Cre^{+}$ embryos (Fig. 3O and fig. S5). Thus, these results suggest that FoxO3a activation in the neural tube promotes the induction of apoptosis and NTD formation in diabetic embryopathy.

Thioredoxin reduces NTD formation by suppressing ASK1 activation

After having identified the potential cascade leading from ASK1 activation to apoptosis due to hyperglycemia, we assessed whether blocking the initial step in the cascade by an endogenous factor (Trx) could prevent diabetes-induced NTDs. NTD incidence was reduced in embryos from diabetic mice treated with human Trx (hTrx) (Fig. 4A and table S3). Recombinant Trx has been reported to enter cells (34), and we found that treatment of cultured conceptuses with recombinant hTrx produced a dose-dependent reduction in high glucose–induced NTDs (Fig. 4B and table S4). hTrx treatment of cultured conceptuses attenuated high glucose–induced activation of ASK1 (Fig. 4C), activation of FoxO3a (Fig. 4, D and E), increase in the abundance of TRADD (Fig. 4, D and E), and activation of caspase 8 (Fig. 4D). Injection of dams with hTrx resulted in detectable amounts of hTrx in embryos (fig. S6A) and decreased NTD formation in embryos (Fig. 4A) without affecting hyperglycemia in the diabetic dams (Fig. 4A). Furthermore, maternal hTrx injections were associated with restoration of the interaction between Trx and ASK1 and blockade of activation of caspases 3 and 8 in embryos from diabetic dams (fig. S6B and Fig. 4F). These data suggest that Trx can prevent hyperglycemia-induced NTDs in embryos by suppressing the ASK1-initiated apoptotic pathway.

The ASK1–FoxO3a–TRADD–caspase 8 pathway is present in human NTD–affected tissues

We assessed phosphorylation of ASK1 and cleavage of caspase 8 in neural tissues of human fetuses with NTDs and age-matched controls. Phosphorylation of ASK1 as assessed by Western blotting and immunostaining was increased in NTD-affected tissues compared to controls (Fig. 4, G and H, and table S5). In addition, cleaved caspase 8 was detectable in three of four cases of human NTD but not control cases (Fig. 4H). Because our mouse studies indicated that FoxO3a and TRADD are downstream of ASK1, we assessed the activation status of FoxO3a and presence of TRADD in NTD-affected tissues. Consistent

with activation of ASK1 and caspase 8 observed in the three NTD cases, phosphorylation of FoxO3a was reduced, and TRADD abundance was increased in the three NTD cases compared to their age-matched control neural tissues (Fig. 4I, fig. S7, and table S5). These findings suggest that the ASK1–FoxO3a–TRADD–caspase 8 cascade (fig. S8) may be involved in the induction of NTDs in humans.

DISCUSSION

Mouse diabetic embryopathy is a suitable model for studying the mechanisms of NTD formation because the incidence of NTDs is substantially increased in both the embryos of diabetic dams (35) as well as the fetuses of pregnant women with diabetes (36, 37). NTD rates are correlated not with the chronicity or the type of diabetes but instead with maternal hyperglycemia (38). Here, we used a mouse model of streptozotocin (STZ)–induced diabetes to induce sustained maternal hyperglycemia (glucose, >250 mg/dl). In our studies (11, 39) and those of others (35, 40), this mouse model on a C57BL/6J background has consistently produced an incidence rate of more than 22% in embryos exposed to hyperglycemia. In contrast, embryos from nondiabetic control mice display a rate of 0 to 1% NTDs. This rate of spontaneous NTD formation is lower than previously reported (41). The use of two complementary models, maternal diabetes–induced embryopathy and high glucose–induced embryopathy in vitro, suggests that the activation of ASK1, FoxO3a, TRADD, and caspase 8 in embryos could result from maternal hyperglycemia in vivo or high glucose in vitro (fig. S8).

Our results showed activation of ASK1, FoxO3a, and caspase 8 during neurulation by maternal diabetes and suggest that FoxO3a is downstream of ASK1 in the apoptotic pathway that leads to neuroepithelial cell apoptosis and NTD formation. The kinases JNK1/2, which are downstream of ASK1, can phosphorylate and activate FoxO proteins such as FoxO4 (42). We previously have demonstrated that JNK1/2 promotes the development of embryonic NTDs by maternal diabetes (4, 12). Thus, ASK1 may increase the activity of FoxO3a through its downstream kinases, or ASK1 may directly phosphorylate FoxO3a to modulate its activity.

Phosphorylation both stimulates and suppresses FoxO3a transcriptional activity. Because the phosphorylation sites in FoxO3a by ASK1 are unknown, we are not able to directly assess ASK1-induced phosphorylation. An oxidative stress–responsive kinase, Ste20-like kinase 1 (MST1), phosphorylates FoxO3a on Ser^{207}, leading to FoxO3a activation (43). It is possible that oxidative stress–activated ASK1 not only may phosphorylate FoxO3a at Ser^{207} but could also phosphorylate FoxO3a at other serine and/or threonine residues. Because an antibody that recognizes FoxO3a phosphorylated at Ser^{207} is not yet available, we are unable to assess the phosphorylation status of FoxO3a at this site. Because of this limitation in available reagents, we used an antibody that recognized FoxO3a phosphorylated at Thr^{32} to detect the cytosolic form of FoxO3a, which is a validated index of FoxO3a activation (25). In addition, we assessed the nuclear form of FoxO3a. Our findings using these two complementary methods support the hypothesis that maternal diabetes induces FoxO3a activation and that ASK1 deletion abolishes FoxO3a activation. Reduced phosphorylation of Thr^{32} in FoxO3a does not necessarily mean that the total phosphorylation of FoxO3a is

reduced. It is possible that, under maternal diabetic conditions, FoxO3a is phosphorylated at threonine and serine residues other than Thr^{32} by ASK1. ASK1 may directly inhibit Akt activity, or its association with FoxO3a may prevent Akt binding to FoxO3a. These events collectively result in reduced phosphorylation at Thr^{32}. Because our current study focuses on the relationship between ASK1 and FoxO3a, investigating the relationship between ASK1 and Akt may be included in future studies.

We showed that *Ask1* gene deletion abolishes caspase 8 cleavage in embryos exposed to maternal hyperglycemia. However, E10.5 and E15.5 $Casp8^{-/-}$ embryonic fibroblasts that overexpress ASK1 still commit to cell death (27), suggesting a dispensable role of caspase 8 in ASK1-induced apoptosis. E10.5 and E15.5 cells are beyond the neurulation stage and therefore respond to apoptotic insults in a different manner than embryos at the neurulation stage (44) in the present study. In addition, the mechanism underlying apoptosis induced by ectopic expression of ASK1 may not be the same as those induced by external insults such as maternal diabetes.

Excessive apoptosis may result from imbalanced proapoptotic and prosurvival signaling. In addition to the proapoptotic ASK1 activation, the activity of Akt, a prosurvival kinase, is suppressed in embryos by maternal diabetes (26). Because Akt inhibits the transcriptional activity of FoxO proteins (25), we investigated the possible roles of FoxO proteins in maternal diabetes–induced apoptosis. Although both germline and conditional *Foxo3a* gene deletion significantly reduced NTD formation, a small number of embryos with *Foxo3a* deletion in the neural tube still exhibited NTDs under diabetic conditions. However, when we deleted the *Casp8* gene in the neural tube, we completely blocked NTD formation. These observations suggest that other transcription factors may be involved in the pathway between ASK1 and caspase 8, such as c-Jun, ATF2 (activating transcription factor 2), and Elk1, all of which are activated in embryos by maternal diabetes (4, 12).

Treatment with hTrx resulted in suppression of the activation of ASK1, FoxO3a, and caspase 8 and of NTD formation. Because hTrx treatment attenuated the maternal diabetes–induced reduction in the Trx-ASK1 interaction, we believe that the hTrx inhibitory effect is specific to ASK1. However, we cannot rule out the possibility that Trx may simply act as an antioxidant that blocks diabetes-induced oxidative stress and, thus, blocks the ASK1-intiating apoptotic pathway. We showed that hTrx entered the embryo. Although it has been difficult to demonstrate whether hTrx enters embryonic cells, a recombinant form of hTrx rapidly enters cultured endothelial cells through lipid rafts (45). Therefore, hTrx may elicit its beneficial effects by entering into embryonic cells or function as an antioxidant extracellularly.

In humans, oxidative stress and apoptosis are implicated in NTDs caused by factors other than diabetes, such as valproic acid (46) and alcohol (47). Three of the four NTD cases that we examined demonstrated activation of the ASK1–FoxO3a–TRADD–caspase 8 pathway. However, because these tissues were collected long after the development of NTDs, it is possible that tissue degeneration occurring after NTD formation contributed to the activation of the proapoptotic intermediates.

In summary, we report here that deletion of the *Ask1* gene was associated with decreased NTD formation and that the transcription factor FoxO3a, the apoptotic factor TRADD, and caspase 8 are involved in NTD formation under conditions of maternal diabetes. In addition, we provide evidence for the therapeutic potential of the ASK1 physiological inhibitor Trx in preventing maternal diabetes–induced NTDs.

MATERIALS AND METHODS

Mice

The procedures for animal use were approved by the University of Maryland School of Medicine Institutional Animal Care and Use Committee. Wild-type C57BL/6J mice were purchased from The Jackson Laboratory. *Ask1*$^{-/-}$ mice on a C57BL/6J background (17) and *FoxO3a*$^{L/L}$ mice on an FVB background (48) were provided by H. Ichijo at the University of Tokyo and R. DePinho at the M.D. Anderson Cancer Center, respectively. *Casp8*$^{L/L}$ mice on a C57BL/6J background were provided by S. M. Stephen (49) at the University of California at San Diego. *FoxO3a*$^{+/-}$ mice on an FVB background (50) were obtained from the Mutant Mouse Regional Resource Centers and crossed back to C57BL/6J mice for 10 generations to generate *FoxO3a*$^{-/-}$ mice on a C57BL/6J background, which were used before age 12 weeks. The SOD1-Tg mice on a C57BL/6J background were revived from frozen embryos by The Jackson Laboratory (stock no. 002298) (51). The *Nes8Cre* mice were provided by W. Zhong and expressed Cre specifically in the developing neural tube at E8 onward (52), and it has been confirmed that Cre-Lox recombination occurs at E8 using the reporter line ROSA26 (53). *Nes8Cre* mice were originally on an FVB background, and we crossed them with C57BL/6J mice for 10 generations to generate *Nes8Cre* mice on a C57BL/6J background. Germline deletion of *FoxO3a* was done in the C57BL/6J background, and the results were confirmed in conditional deletion of *FoxO3a* experiment, which was performed on the FVB background with FVB *Nes8Cre* mice crossed with FVB *FoxO3a*$^{L/L}$ mice.

FoxO3a$^{L/L}$ (or *Casp8*$^{L/L}$) mice were crossed with *Nes8Cre* mice to generate *FoxO3a*$^{L/+}$*;Nes8Cre* (*Casp8*$^{L/+}$*;Nes8Cre*) male mice. *FoxO3a*$^{L/+}$*; Nes8Cre* mice were mated with nondiabetic or diabetic female *FoxO3a*$^{L/L}$ mice to generate *FoxO3a*$^{L/L}$*;Nes8Cre*$^{+}$, *FoxO3a*$^{L/+}$*;Nes8Cre*$^{+}$, *FoxO3a*$^{L/L}$*; Nes8Cre*$^{-}$, and *FoxO3a*$^{L/+}$*;Nes8Cre*$^{-}$ embryos. Likewise, *Casp8*$^{L/+}$*; Nes8Cre* mice were mated with nondiabetic or diabetic female *Casp8*$^{L/L}$ mice to generate *Casp8*$^{L/L}$*;Nes8Cre*$^{+}$, *Casp8*$^{L/+}$*;Nes8Cre*$^{+}$, *Casp8*$^{L/L}$*; Nes8Cre*$^{-}$, and *Casp8*$^{L/+}$*;Nes8Cre*$^{-}$ embryos.

Model of maternal diabetes–induced NTDs

We (4, 12) and others (19, 35, 40) have used a rodent model of STZ-induced diabetes in research of diabetic embryopathy. Briefly, 6- to 8-week-old mice were intravenously injected daily with STZ (75 mg/kg) in the tail vein over 2 days to induce diabetes. We used the U-100 insulin syringe (Becton Dickinson) with 28 gauge × ½ inch needles for injection. About 140-μl volume of STZ solution was injected per mouse. Diabetes was defined as 12-hour fasting blood glucose concentrations greater than or equal to 250 mg/dl, which usually occurred at 3 to 5 days after STZ injections. STZ has a very short (30 min) half-life (54), and pregnancies in our study were established after 1 to 2 weeks of STZ injections. We did

not detect differences in embryonic development between STZ/insulin-treated and non–STZ-treated mice (11), suggesting a lack of residual toxic effect of STZ in our animal model.

Insulin pellets were implanted subcutaneously in diabetic mice to restore euglycemia (glucose concentrations, 80 to 100 mg/dl) before mating (4, 12). Sustained-release insulin pellets, Linplant, were purchased from LinShin. Insulin-implanted diabetic mice were then mated with male mice of the respective genotypes. As done previously (44), male and female mice were paired at 3:00 p.m., and we designated next morning (8:00 a.m.) as E0.5 if vaginal plugs were present. On day 5.5 of pregnancy (E5.5), insulin pellets were removed to permit frank hyperglycemia (glucose, ≥250 mg/dl), so that the developing embryos were exposed to hyperglycemia during neurulation (E8 to E10.5). On the basis of our previous studies (11, 39), insulin treatment from E0.5 to E5.5 is essential for successful implantation establishment and, thus, prevents early embryonic lethality (resorption) caused by hyperglycemic exposure at early embryonic stages (<E5.5). Control nondiabetic pregnant mice were sham-operated for insulin implantation and removal.

Embryos were harvested at E8.75 (2:00 p.m. at E8.5) for biochemical and molecular analysis. At E10.5, embryos were examined under a Leica MZ16F stereomicroscope to identify NTDs. Images of embryos were captured by a DFC420 5-megapixel digital camera with software (Leica) and processed with Adobe Photoshop CS2. Normal embryos were classified as having completely closed neural tube and no evidence of other malformations. Malformed embryos were classified as showing evidence of failed closure of the anterior neural tubes resulting in exencephaly, a major type of NTD. Exencephaly is a lethal type of NTD characterized by the absence of a major portion of the brain, skull, and scalp. At E15.5, we still observed embryos with NTDs with no other sign of delayed embryonic development. At later embryonic stages, anencephalic embryos lacked skulls and consequently had uncovered brains. Spina bifida was not observed in our model. The open neural tube structure in embryos with NTDs was verified by serial sections through the anterior neural tubes. Because our model induces NTDs at E10.5, we did not examine other major structural malformations such as cardiovascular defects, which do not occur until later embryonic stages (E15.5).

Immunoprecipitation and immunoblot

We performed immunoprecipitation and immunoblot assays as previously described (39). For immunoprecipitation, 300 μg of protein from about five to six embryos of one dam was used, and for immunblotting, 30 to 50 μg of protein from one embryo were used and embryos were collected from different dams. Protease inhibitor cocktail (Sigma), lysis buffer (Cell Signaling Technology, #9803), Protein A Magnetic Bead slurry (New England Biolabs), rabbit anti-ASK1 antibody at a dilution of 1:100 (catalog no. sc-7931, Santa Cruz Biotechnology), and rabbit anti-Trx at a dilution of 1:100 (Cell Signaling Technology, #2298) were used for immunoprecipitations. Nuclear and cytoplasmic proteins were extracted by a NE-PER kit from Pierce (#78833). Immunobilon-P or Immunobilon-P^{SQ} (Milllipore) membranes were used for immunoblot detection of cleaved caspases. Membranes were exposed to goat anti-rabbit or goat anti-mouse (Jackson ImmunoResearch

Laboratories) or goat anti-rat (Chemicon) secondary antibodies. Signals were detected with an Amersham ECL Advance Detection Kit (GE Healthcare), and chemiluminescence emitted from the bands was directly captured with a UVP BioImaging EC3 system. Densitometric analysis of chemiluminescence signals was performed with VisionWorks LS Software (UVP). The following were primary antibodies from Cell Signaling Technology: anti-SOD1 (human-specific, #4266); rabbit anti–phospho-TAK1 (#9339), rabbit anti–phospho-MLK3 (#2811), rabbit anti–phospho-JNK1/2 (#9251), anti-JNK (#9252), rabbit anti–phospho-FoxO1 (Thr^{24})/FoxO3a (Thr^{32}) (#9464), anti-FoxO3a (#9467), anti-FoxO1 (#9462), anti-FoxO4 (#9472), anti–phospho-p38MAPK (#9913), and anti-p38MAPK (#9212). All Cell Signaling Technology antibodies were used at a dilution of 1:1000. The following antibodies were also used: anti–caspase 3 (Millipore, #AB3623, used at a dilution of 1:500), rabbit anti-TRADD (BioVision, #3561, used at a dilution of 1:2000), rat anti–caspase 8 (mouse-specific) (Alexis Biochemicals, #ALX-804-448, used at a dilution of 1:500), anti-Oct1 (Chemicon, #MAB5434, used at a dilution of 1:1000), and anti–β-actin (Abcam, #ab8224, used at a dilution of 1:2500). Phosphorylated ASK1 antibody used at a dilution of 1:1000 was provided by H. Ichijo of the University of Tokyo.

TUNEL assay

TUNEL assay was performed with the ApopTag Fluorescein In Situ Apoptosis Detection kit (Chemicon). Briefly, 10-μm frozen embryonic sections were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) and incubated with TUNEL reaction agents. TUNEL-positive cells in areas (about 200 cells) of neural tubes were counted. The proportion of TUNEL-positive cells was calculated as a fraction of the total cells and analyzed statistically.

Whole-conceptus cultures

E8 C57BL/6J conceptuses were cultured as previously described (39). Cultured conceptuses were treated with 0.1, 0.5, or 2 μg of hTrx per milliliter of culture medium (EMD Chemicals, #598101).

Collection of human fetal neural tissues

Human neural tissues affected by anencephaly and age-matched normal control cases without NTDs as characterized in table S2 were collected by the NICHD Brain and Tissue Bank for Developmental Disorders. The causes of these NTD cases were unknown. Tissues from the defective regions in cases with NTD and frontal cortexes of control cases were used for our experiments. Fetuses with anencephaly were born alive and died in a few hours. Fetal demise in controls was due to factors other than NTDs. Tissues were collected within 6 hours after fetal demise and frozen in isopentane–dry ice. The procedures were approved by the University of Maryland Baltimore Institutional Review Board committee. Frozen sections (10 μm) were used for immunofluorescence, and about 1 g of tissue was used for Western blotting.

hTrx treatment

Diabetic pregnant mice were injected subcutaneously daily with vehicle (PBS) or 0.45 μg of hTrx per gram of body weight (55) (EMD Chemicals, #598101) from E7 to E9. Embryos were dissected at E9 or E11 for analysis.

Statistical analyses

Data were presented as means ± SE. In animal studies, experiments were repeated at least three times, and embryonic samples from each replicate were from different dams. Statistical differences were determined by Student's *t* test for two-group comparisons and by one-way analysis of variance (ANOVA) for comparisons of more than two groups using SigmaStat 3.5 software. In ANOVA analysis, Tukey test was used to estimate the significance of the results. For germline gene deletion experiments, NTD rates were expressed in two ways: (i) by litter: number of NTD embryos in a litter divided by total number of embryos in the litter; (ii) by experimental group: number of NTD embryos from a group of dams compared to number of normally developed embryos from the group. The latter was used in conditional gene deletion experiments because NTD embryos and normally developed embryos were from the same group of diabetic dams. Significant differences between groups in NTD incidence expressed as percentage were assessed by the Wilcoxon rank sum test. Significant difference between groups in NTD rates expressed by number of embryos was analyzed by χ^2 test or Fisher's exact test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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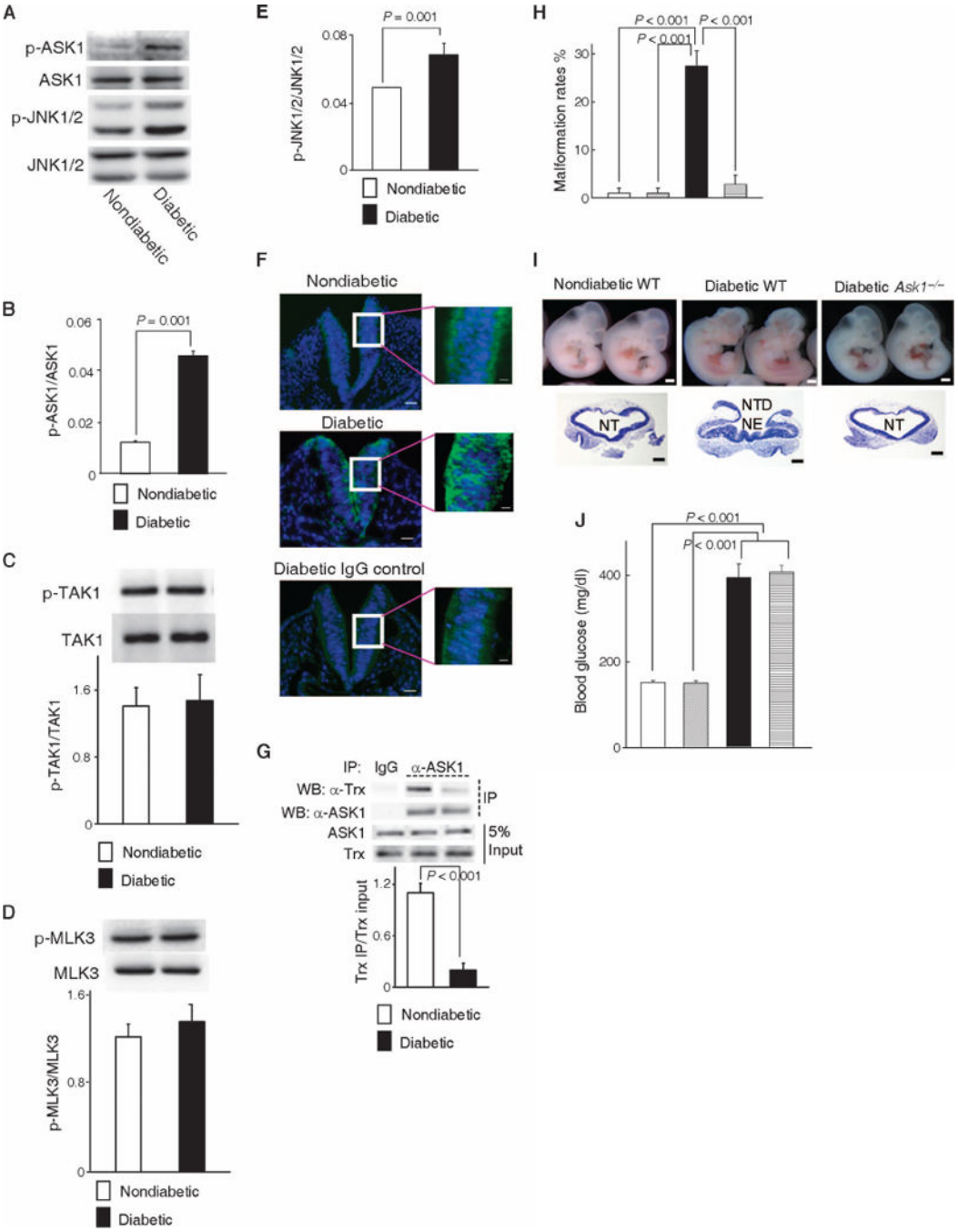

Fig. 1. Maternal diabetes induces ASK1 activation in the developing neural tube, and *Ask1* deletion ameliorates diabetes-induced NTD

(**A**) Phosphorylation of ASK1 and JNK1/2 in E8.75 embryos from nondiabetic and diabetic dams. (**B**) Quantification of phosphorylation of ASK1 from (A). (**C** and **D**) Phosphorylation of TAK1 (C) and MLK3 (D). (**E**) Quantification of phosphorylation of JNK1/2 from (A). (**F**) Immunostaining of phosphorylated ASK1 in the neural tube (the dense blue V-shaped structure). Scale bars, 30 μm (left) and 10 μm (right, magnified insets). IgG, immunoglobulin G. (**G**) Trx coprecipitation with anti-ASK1 in E8.75 embryos. IP,

immunoprecipitation; WB, Western blotting. (**H**) NTD rates. (**I**) Morphology of E10.5 embryos from nondiabetic wild-type (WT), diabetic WT, and diabetic *Ask1*$^{-/-}$ mice. Scale bars, 1 mm. White arrows indicate NTDs. Lower panels: frontal sections of embryos with NTDs in the upper panels showing open neural tubes (NTs). NE, neuroepithelium. (**J**) Blood glucose concentrations. *n* for (H) and (J) is >60 embryos per condition; specific *n* values are indicated in table S1. All other experiments were performed independently three times (one embryo from three different dams per experimental group), and graphs in (B), (C), (D), (E), and (G) show summaries of all data.

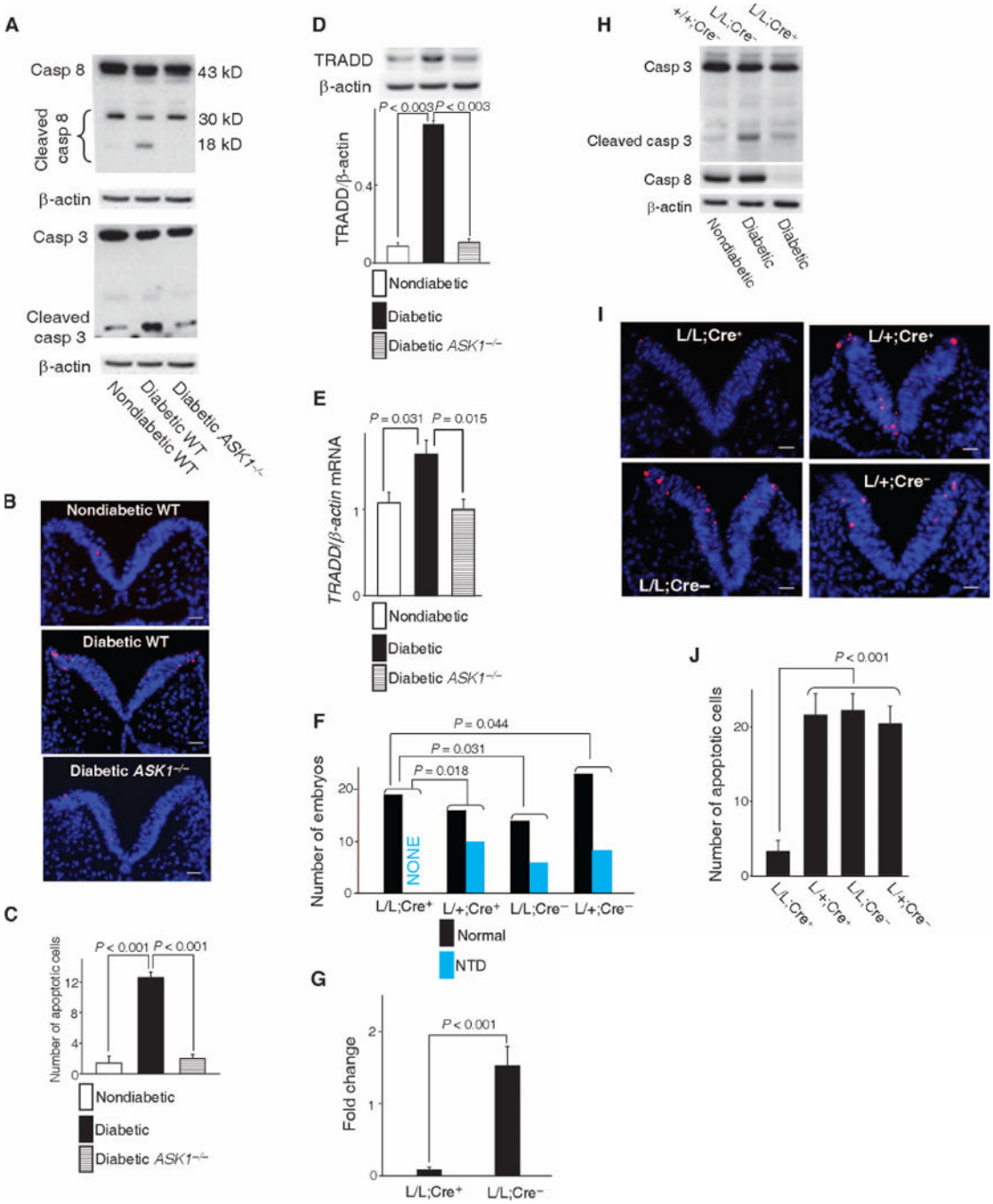

Fig. 2. Deletion of the *Ask1* gene blocks maternal diabetes–induced caspase activation and apoptosis, and conditional deletion of the *Casp8* gene reduces maternal diabetes–induced apoptosis and NTD formation

(**A**) Caspase 3 and 8 cleavage in E8.75 embryos. Results are representative of three independent experiments (one embryo from three different dams for each experimental group). (**B** and **C**) Representative images of the TUNEL (terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick end labeling) assays (B). Apoptotic cells are red, and nuclei were labeled in blue. The dense blue V-shaped areas are the neural tubes. Experiments were performed independently five times (one embryo from five

different dams), and five images were obtained from each embryo. Scale bars, 30 μm. (C) Quantification of apoptotic cell numbers in the whole neuroepithelium including the dorsal and the ventral domains. Five serial coronal sections though the anterior neural tubes of each embryo were analyzed. The numbers of apoptotic cells were counted in the neural tube area of each section and were averaged for each embryo. (**D** and **E**) Effect of *Ask1* gene deletion on TRADD protein abundance (D) and *TRADD* mRNA abundance (E) in E8.75 embryos, which were performed independently three times (one embryo from three different dams per experimental group). (**F**) Numbers of normally developed embryos and embryos with NTDs at E10.5 from diabetic dams. L/L; Cre^{+}(*Casp8*$^{L/L}$;*Nes8Cre*$^{+}$) indicates *Casp8* conditional deletion in the neural tube. Specific *n* values are given in fig.S4. (**G**)*Casp8* mRN Aabundance in E8.75 neural tubes of embryos from nondiabetic dams. (**H**) Cleaved caspase 3 in E8.75 embryos. WT mice denoted as+/+;Cre^{-}. (**I**) Representative images of the TUNEL assays in embryos of diabetic dams. Apoptotic cells were labeled in red, and nuclei were labeled in blue. Experiments were performed independently five times (one embryo from five different dams), and five images were obtained from each embryo. Scale bars, 30 μm. (**J**) Quantification of apoptotic cell numbers.

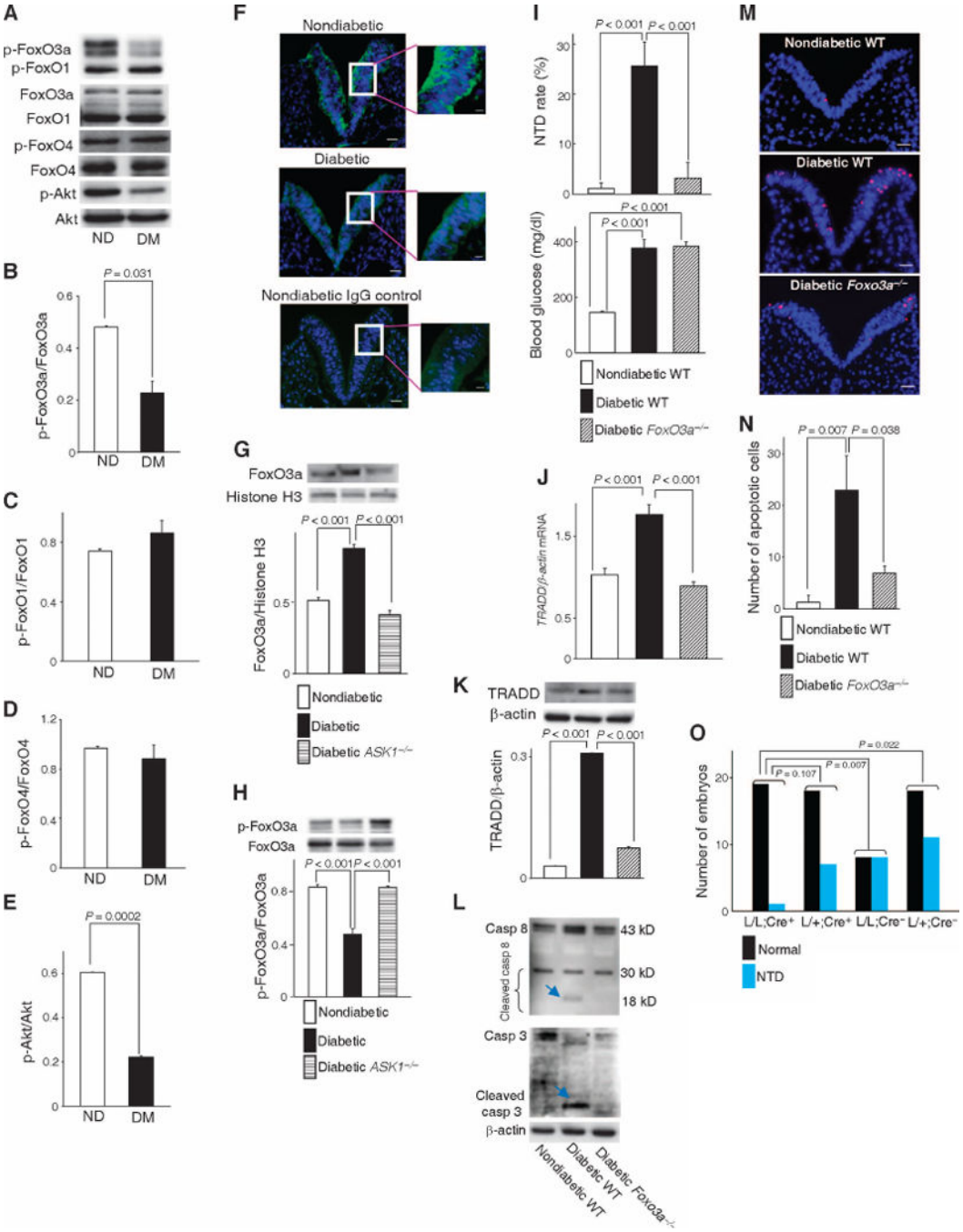

Fig. 3. FoxO3a is activated in the neural tube by maternal diabetes, and deletion of the *FoxO3a* gene ameliorates maternal diabetes–induced increase in TRADD abundance, caspase activation, apoptosis, and NTD formation

(**A** to **E**) Phosphorylation of FoxO3a, FoxO1, FoxO4, and Akt in E8.75 embryos from nondiabetic (ND) and diabetic mellitus (DM) dams (A). Quantification of phosphorylation of FoxO3a (B), FoxO1 (C), FoxO4 (D), and Akt (E). (**F**) Immunostaining of phosphorylated FoxO3a in the E8.75 neural tube (the dense blue V-shaped structure). Scale bars, 30 μm (left) and 10 mm (right, magnified insets). (**G** and **H**) Effect of *Ask1* gene deletion on nuclear amounts of FoxO3a (G) and phosphorylated FoxO3a (H) in E8.75 embryos. All

experiments were performed independently three times using one embryo from three different dams. (**I**) NTD rates and blood glucose concentrations. (**J** to **L**) Effect of *Foxo3a* gene deletion on *TRADD* mRNA (J), TRADD protein (K), caspase 8 (arrow indicates 18-kD cleaved product), and caspase 3 (arrow indicates cleaved caspase 3) (L) in E8.75 embryos. (**M** and **N**) Representative images of the TUNEL assays (M). Apoptotic cells were labeled in red, and nuclei were labeled in blue. The dense blue V-shaped areas are the neural tubes. Experiments were performed independently five times (one embryo from five different dams), and five images were obtained from each embryo. Scale bars, 30 μm. (N) Quantification of apoptotic cell numbers in the whole neuroepithelium including the dorsal and the ventral domains. (**O**) Numbers of normal embryos and embryos with NTDs at E10.5 from diabetic dams. L/L; Cre^+ (*FoxO3a*$^{L/L}$;*Nes8Cre*$^+$, full deletion) embryos had conditional deletion of caspase 8 in the neural tube. L/+;Cre^+ embryos had partial deletion, and L/L;Cre^- and L/+;Cre^- embryos had no deletion. Immunoblotting was performed independently three times (one embryo from three different dams), and data were summarized from these three experiments. RT-PCR (reverse transcription polymerase chain reaction) was performed independently five times (one embryo from five different dams). *n* is ≥24 embryos for (I) and ≥6 embryos for (O); specific *n* values are given in table S2 and fig. S5, respectively.

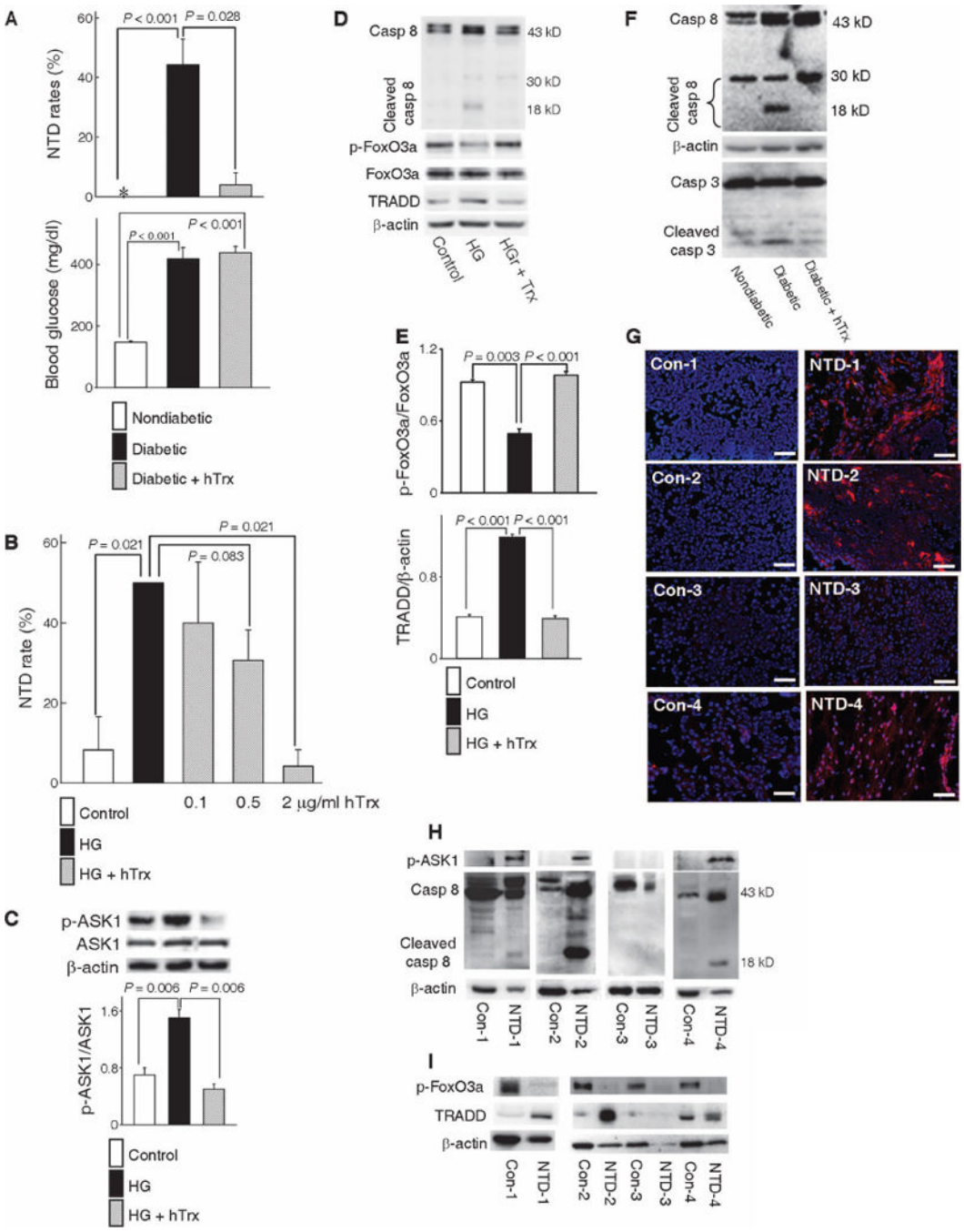

Fig. 4. Trx treatment blocks maternal diabetes in vivo and high glucose in vitro–induced ASK1 phosphorylation, apoptosis, and NTDformation,andASK1,FoxO3a, TRADD, and caspase 8 are activated in human fetal NTD tissues

(**A**) Effect of maternal hTrx injections on the incidence of NTDs at E10.5 and blood glucose concentrations. (**B**) Dose-dependent effect of hTrx on high glucose (HG)–induced NTDs. (**C**) Phosphorylation of ASK1 in cultured embryos. (**D**) Representative images of cleaved caspase 8, phosphorylated FoxO3a, and TRADD immunoblotting in cultured embryos. (**E**) Quantification of phosphorylated FoxO3a and TRADD immunoblotting in cultured embryos. (**F**) Effect of maternal hTrx injections on cleavage of caspases 3 and 8 in E8.75

embryos. Immunoblotting was performed independently three times (one embryo from three different dams), and data were summarized from these three experiments. (**G**) Immunostaining for phosphorylated ASK1 (red). Nuclei were stained in blue. Scale bars, 50 μm. (**H**) Immunoblots of phosphorylated ASK1 and caspase 8 cleavage in neural tissues of human fetuses with NTDs (NTD-1 to NTD-4) and age-matched controls (Con-1 to Con-4). (**I**) Immunoblots of phosphorylated FoxO3a and TRADD in neural tissues of human fetuses with NTDs (NTD-1 to NTD-4) and age-matched controls. *n* for (A) is ≥27 embryos per condition, and *n* for (B) is ≥11 embryos per condition; specific *n* values are indicated in tables S3 and S4.