OBSTETRICS

The increased activity of a transcription factor inhibits autophagy in diabetic embryopathy

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BACKGROUND: Maternal diabetes induces neural tube defects and stimulates the activity of the forkhead box O3 (Fox)O3a in the embryonic neuroepithelium. We previously demonstrated that deleting the *FOXO3a* gene ameliorates maternal diabetes-induced neural tube defects. Macroautophagy (hereafter referred to as "autophagy") is essential for neurulation. Rescuing autophagy suppressed by maternal diabetes in the developing neuroepithelium inhibits neural tube defect formation in diabetic pregnancy. This evidence suggests a possible link between FoxO3a and impaired autophagy in diabetic embryopathy.

OBJECTIVE: We aimed to determine whether maternal diabetes suppresses autophagy through FoxO3a, and if the transcriptional activity of FoxO3a is required for the induction of diabetic embryopathy.

STUDY DESIGN: We used a well-established type 1 diabetic embryopathy mouse model, in which diabetes was induced by streptozotocin, for our in vivo studies. To determine if FoxO3a mediates the inhibitory effect of maternal diabetes on autophagy in the developing neuroepithelium, we induced diabetic embryopathy in *FOXO3a* gene knockout mice and FoxO3a dominant negative transgenic mice. Embryos were harvested at embryonic day 8.5 to determine FoxO3a and autophagy activity and at embryonic day 10.5 for the presence of neural tube defects. We also examined the expression of autophagy-related genes. C17.2 neural stem cells were used for in vitro examination of the potential effects of FoxO3a on autophagy.

RESULTS: Deletion of the *FOXO3a* gene restored the autophagy markers, lipidation of microtubule-associated protein 1A/1B-light chain 3I to light chain 3II, in neurulation stage embryos. Maternal diabetes decreased light chain 3I-positive puncta number in the neuroepithelium, which was restored by deleting FoxO3a. Maternal diabetes also decreased the expression of positive regulators of autophagy (Unc-51 like autophagy activating kinase 1, Coiled-coil myosin-like BCL2-interacting protein, and autophagy-related gene 5) and the negative regulator of autophagy, p62. *FOXO3a* gene deletion abrogated the dysregulation of autophagy genes. In vitro data showed that the constitutively active form of FoxO3a mimicked high glucose in repressing autophagy. In cells cultured under high-glucose conditions, overexpression of the dominant negative FoxO3a mutant blocked autophagy impairment. Dominant negative FoxO3a overexpression in the developing neuroepithelium restored autophagy and significantly reduced maternal diabetes-induced apoptosis and neural tube defects.

CONCLUSION: Our study revealed that diabetes-induced FoxO3a activation inhibited autophagy in the embryonic neuroepithelium. We also observed that FoxO3a transcriptional activity mediated the teratogenic effect of maternal diabetes because dominant negative FoxO3a prevents maternal diabetes-induced autophagy impairment and neural tube defect formation. Our findings suggest that autophagy activators could be therapeutically effective in treating maternal diabetes-induced neural tube defects.

Key words: congenital anomalies, diabetic embryopathy, impaired autophagy, maternal diabetes, teratogenesis

Introduction

Pregestational maternal diabetes, both type 1 and type 2, significantly increases the risk of structural embryonic anomalies, with neural tube defects (NTDs) and cardiovascular defects as the 2 major types.[1] Clinical data show that approximately 8000 babies born each year in the United States have structural birth defects due to pregestational maternal diabetes.[2] Diabetes in the general population is on the rise and, proportionally, the number of women of reproductive age (18−44 years) with diagnosed diabetes is rapidly increasing. Currently, there are close to 3 million women in the United States and 60 million women worldwide of reproductive age with diabetes,[3] and these numbers are expected to double in the next 10−15 years. Therefore, maternal diabetes-induced birth defects have become a serious public health problem. Despite modern preconception care and rigorous diabetes control, the rates of structural birth defects are still 3−10 times greater in mothers with diabetes than those without diabetes.[1] Mechanistic studies using animal models have revealed the involvement of several signaling pathways and transcriptional factors in the etiology of diabetic embryopathy, which may help to identify potential therapeutics.[4−10]

Intracellular homeostasis is essential for normal embryonic development and any cellular imbalance resulting from cellular organelle stress may lead to abnormal embryogenesis.[5] Cellular organelle stress, including proapoptotic kinase activation, endoplasmic reticulum stress, and autophagy impairment, has been shown to occur in the developing neuroepithelium exposed to maternal diabetes.[5,11−14] Autophagy, an intracellular catabolic process, removes dysfunctional protein aggregates and damaged cellular organelles, and thus maintains intracellular homeostasis. The autophagy process starts with the formation of double membrane vacuoles, called "autophagosomes," which requires a group of proteins derived from autophagy-related genes (Atgs).[15] The lipidation of microtubule-associated protein l light chain (LC)3I is essential

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AJOG at a Glance

Why was this study conducted?

Previous studies have shown that autophagy is suppressed in diabetic embryopathy; however, the mechanism underlying maternal diabetes-repressed autophagy in the developing embryo is unknown.

Key findings

Using gene deletion mouse models of the transcription factor FoxO3a, we found that the transcription activity of FoxO3a is required for autophagy impairment in maternal diabetes-induced neural tube defects.

What does this add to what is known?

The findings provide mechanistic insights for the transcriptional regulation whereby maternal diabetes represses autophagy gene expression in diabetic teratogenesis.

for the elongation, curvature, and closure of autophagosome membranes.[16] Autophagosomes engulf dysfunctional proteins and organelles, and eventually fuse with lysosomes for substrate degradation and recycling. Impaired autophagy leads to intracellular imbalance and ultimately results in cell apoptosis.[5]

Our previous studies have demonstrated that maternal diabetes represses autophagosome formation in neuroepithelial cells of neurulation stage embryos.[5,14] Impaired autophagy causes neuroepithelial cell apoptosis,[5] a critical event in the induction of diabetic embryopathy.[11,17] The natural compound, trehalose, restores autophagic activity in the developing neuroepithelium suppressed by maternal diabetes leading to restoration of intracellular homeostasis, diminished endoplasmic reticulum stress and cell apoptosis, and reduction of NTD formation.[14] Further studies reveal that the proapoptotic kinase, protein kinase C alpha, induces microRNA-129-2 expression, which in turn suppresses the activity of the transcription coactivator, Peroxisome proliferator-activated receptor gamma coactivator 1α (PGC-1α). This evidence suggests that maternal diabetes impairs autophagy using a transcriptional mechanism.

Members of the class O of forkhead box transcription factors (Fox), specifically FoxO1, FoxO3a, and FoxO4, are key regulators of cellular longevity and cell fate.[18] The transcriptional activity of FoxO3a is regulated by phosphorylation and subcellular localization.[18] Phosphorylated FoxO3a remains in the cytoplasm, whereas dephosphorylated FoxO3a translocates to the nucleus and initiates transcription of dependent genes via a transactivation/chromatin remodeling domain (TAD).[19] We have previously reported that maternal diabetes dephosphorylates FoxO3a, but not FoxO1 or FoxO4.[11] We have also demonstrated that deleting the *FOXO3a* gene ameliorates maternal diabetes-induced neuroepithelial cell apoptosis and NTD formation.[11] These findings suggest the involvement of FoxO3a in the intracellular imbalance seen in diabetic embryopathy.

Because both FoxO3a and autophagy impairment are critically involved in the etiology of diabetic embryopathy, and because autophagy appears to be transcriptionally regulated by maternal diabetes, we hypothesize that maternal diabetes-activated FoxO3a suppresses autophagy by repressing Atg expression. We further postulate that the TAD of FoxO3a regulates autophagy inhibition and NTD formation in diabetic embryopathy.

In the present study, we used FoxO3a knockout (KO) mice and generated a neuroepithelium-specific dominant negative (DN) FoxO3a transgenic mouse line that lacks the TAD. FoxO3a deletion or DN FoxO3a overexpression abrogated the inhibitory effect of maternal diabetes on Atg expression and autophagy, leading to amelioration of diabetes-induced neuroepithelial cell apoptosis and NTDs. Besides its role in diabetic embryopathy, alteration of autophagy likely contributes to the pathogenesis of other pregnancy complications such as placental insufficiency, fetal growth restriction, preterm birth, and stillbirth.[20–24] Therefore, investigating the mechanism underlying maternal diabetes-impaired autophagy will provide a broader perspective of the causes of pregnancy complications.

Materials and Methods

Experimental design

Streptozotocin (STZ)-induced type 1 diabetes mouse model was used for our in vivo studies to assess the numbers of autophagosomes using LC3 immunofluorescent staining, gene expression levels using real-time quantitative polymerase chain reaction (RT-qPCR), and LC3 protein levels using Western blotting. To determine if FoxO3a mediates the inhibitory effect of maternal diabetes on autophagy in the developing neuroepithelium, we induced diabetic embryopathy in *FOXO3a* gene KO mice and FoxO3a DN transgenic mice. Embryos were harvested at embryonic day (E) 8.5 for analysis of FoxO3a and autophagy activity and at E10.5 for NTDs. C17.2 neural stem cells were used for in vitro examination of the potential effects of FoxO3a on autophagy.

Animals

All procedures for animal use were approved by the Institutional Animal Care and Use Committee of University of Maryland School of Medicine. Wild-type (WT) C57BL/6J and FoxO3a KO mice were purchased from the Jackson Laboratory (Bar Harbor, ME). We generated the Nestin promoter driven FoxO3a-DN construct. Pronuclei microinjection in the C57BL/6J background was carried out in the Genome Modification Facility of Harvard University (Cambridge, MA).

Model of diabetic embryopathy and morphological assessment of NTDs

We utilized the widely accepted streptozotocin (STZ)-induced diabetes rodent model. Briefly, 10-week-old WT female mice were intravenously injected daily

with 75 mg/kg STZ (Sigma, St Louis, MO) for 2 days. Diabetes was defined as a fasting blood glucose level ≥16.7 mmol/L. STZ-treated females were mated with WT males. E0.5 was counted once a vaginal plug was observed on the morning after the mice were paired. E8.5 embryos were harvested for biochemical and molecular analyses, and E10.5 embryos were collected for morphological studies.

Cell culture and transfection

C17.2 mouse neural stem cells (European Collection of Cell Culture, Salisbury United Kingdom) were maintained in Dulbecco's Modified Eagle's Medium (5 mmol/L glucose) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in a humidified atmosphere of 5% carbon dioxide. C17.2 cells were transfected with plasmids containing constitutively activated FoxO3a (Addgene plasmid no. 1788 Addgene, Teddington, United Kingdom) and DN FoxO3a (Addgene plasmid no. 1796 Addgene, Teddington, United Kingdom)—both plasmids were gifts from Dr Michael Greenberg—using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Transfected cells were cultured for 48 hours under low- (5 mmol/L) or high- (25 mmol/L) glucose conditions, and then were examined.

Western blotting

Equal amounts of protein (30 μg) from cultured cells or embryos were loaded on a sodium dodecyl sulfate—polyacrylamide (SDS-PAGE) gel and transferred onto Immunobilon-P membranes (Millipore, Billerica, MA) after electrophoresis. Membranes were blocked in 5% nonfat milk for 1 hour, and then incubated overnight at 4°C with the following primary antibodies diluted 1:1000 in 5% nonfat milk: FoxO3a (no. 2497), phosphor-FoxO3a (no. 9464), and LC3 (no. 2775) antibodies (Cell Signaling Technology, Danvers, MA). Membranes were then exposed to horseradish peroxidase (HPR)-conjugated goat antirabbit secondary antibodies. To calibrate protein amounts, membranes were stripped and probed with a mouse anti-β-actin antibody, 1:10,000 (ab8224; Abcam, Cambridge, United Kingdom). Signals were detected using the Super Signal West Femto maximum sensitivity substrate kit (Thermo Scientific, Waltham, MA). Quantification of blots was performed using VisionWorksLS software (UVP Company, Upland, CA).

TABLE 1
Primers for real-time polymerase chain reaction

Gene names		Primer sequences
Ulk1 (Unc-51 like autophagy activating kinase)	Forward	5'-ACATCCGAGTCAAGATTGCTG-3'
	Reverse	5'-GCTGGGACATAATGACCTCAGG-3'
Beclin1 (Coiled-coil myosin-like BCL2-interacting protein)	Forward	5'-ATGGAGGGGTCTAAGGCGTC-3'
	Reverse	5'-TCCTCTCCTGAGTTAGCCTCT-3'
Atg5	Forward	5'-TGTGCTTCGAGATGTGTGGTT-3'
	Reverse	5'-GTCAAATAGCTGACTCTTGGCAA-3'
p62	Forward	5'-AGGATGGGGACTTGGTTGC-3'
	Reverse	5'-TCACAGATCACATTGGGGTGC-3'

Atg, autophagy-related gene.
Xu et al. Impaired autophagy in maternal diabetes-induced neural tube defects. Am J Obstet Gynecol 2019.

RNA extraction and RT-qPCR

Total RNA was isolated from cells or embryonic tissue using the RNeasy mini kit and reverse transcribed using the QuantiTect reverse transcription kit (Qiagen, Hilden, Germany). Real-time polymerase chain reaction (RT-qPCR) was performed using the Maxima SYBR Green/ROX qPCR Master Mix assay (Thermo Scientific Danvers, MA). The primers for RT-qPCR are listed in Table 1. RT-qPCR and subsequent calculations were performed by a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA).

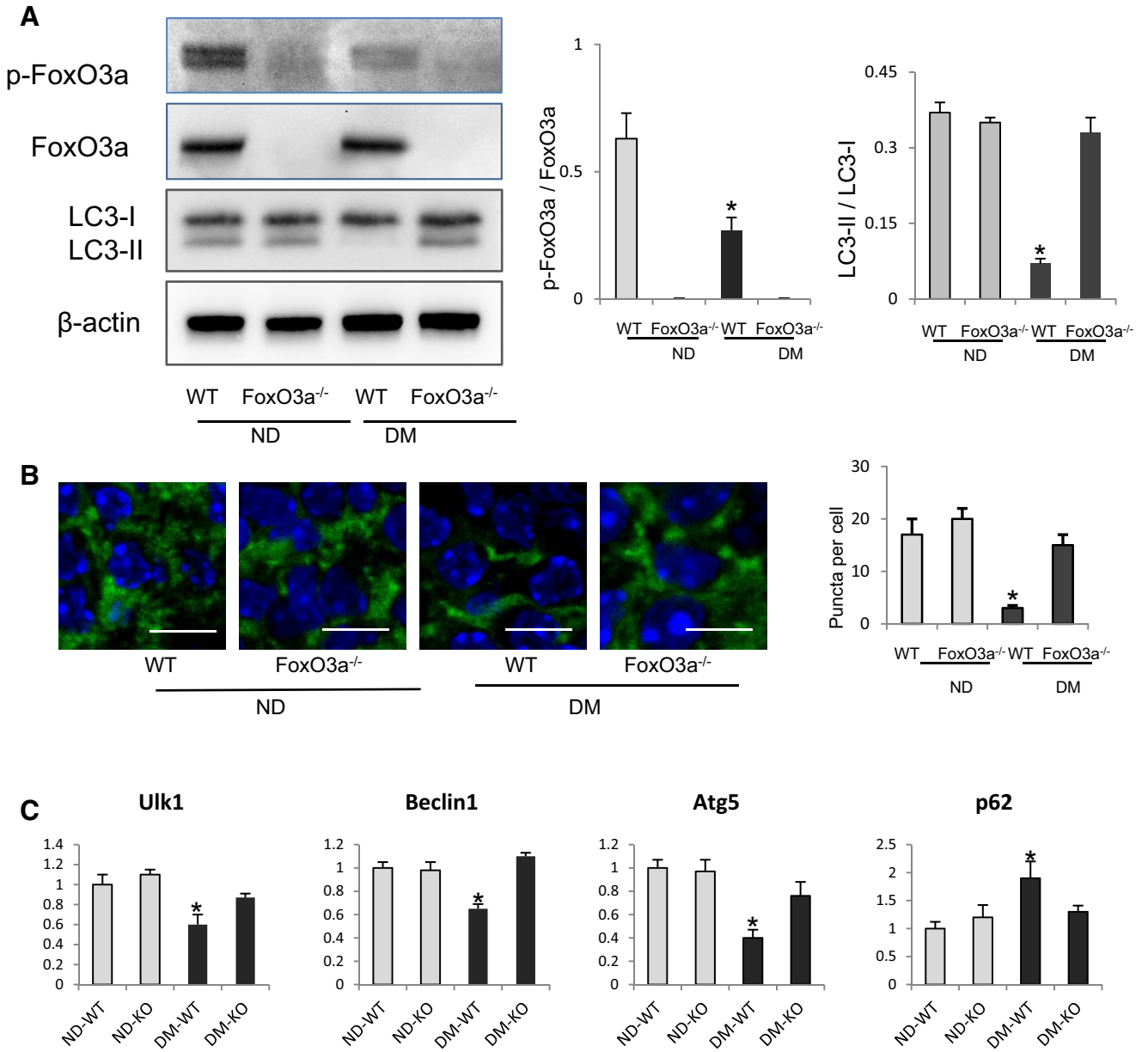
Immunofluorescent staining and microscopy

E8.5 embryos were fixed in the methanol-chloroform-glacial acetic acid solution (6:3:1), embedded in paraffin, and sectioned at 5 μm through the anterior neural tube. Deparaffinized sections were incubated with LC3 antibody (no. 2775; Cell Signaling Technology) at 1:200 dilution or green fluorescent protein (GFP) antibody (no. TP401; Torrey Pines Biolabs East Orange, NJ) at 1:400 dilution in phosphate-buffered saline with 10% heat-inactivated donkey serum overnight at 4°C. After washes in PBS, sections were incubated with Alexa Fluor 488-conjugated donkey antirabbit IgG secondary antibody (Eugene, OR). Cell nuclei were counter-stained with 4',6-diamidino-2-phenylindole (Cat.28718-90-3, Sigma-Aldrich, St. Louis, MO).

The ApopTag Red in situ apoptosis detection kit (S7165; Millipore Billerica, MA) was used to detect apoptosis in E8.5 embryos. Cyto-ID (ENZ-51031-0050; Enzo Life Sciences Farmingdale, NY, USA) was used to observe autophagosomes in C17.2 cells according to the user's manual.

All fluorescent staining, except anti-LC3I, were analyzed by a Nikon Ni-U microscope (Nikon, Melville, NY) with a Plan Apo 20X objective lens (Nikon, Melville, NY) and Iplab software (Scientific Instrument Company, CA). For LC3 staining, images were recorded using a laser scanning

FIGURE 1
***FOXO3a* deletion restores autophagy suppressed by maternal diabetes**



A, Protein abundance of phosphorylated (p)-FoxO3a, FoxO3a, and light chain (LC)3 in embryonic day (E) 8.5 wild-type (WT) and *Foxo3a* knockout (KO) ($FoxO3a^{-/-}$) embryos from nondiabetic or diabetic dams. Quantification of relative expression levels of p-FoxO3a vs total FoxO3a and LC3II vs LC3I were shown in 2 graphs. **B**, Confocal images of immunostaining for LC3 in neuroepithelial cells of E8.5 embryos. LC3 punctate foci with diameter ≥20 pixels were quantified by ImageJ software. Bars = 5 μm. **C**, Messenger RNA levels of autophagy related genes. mRNA relative fold change is normalized by β-actin. Experiments were performed using 3 embryos from 3 different dams per group (n = 3). *Significant difference compared to other groups ($P < .05$).

DM, diabetes mellitus; *KO*, knock out; *ND*, non-diabetes.

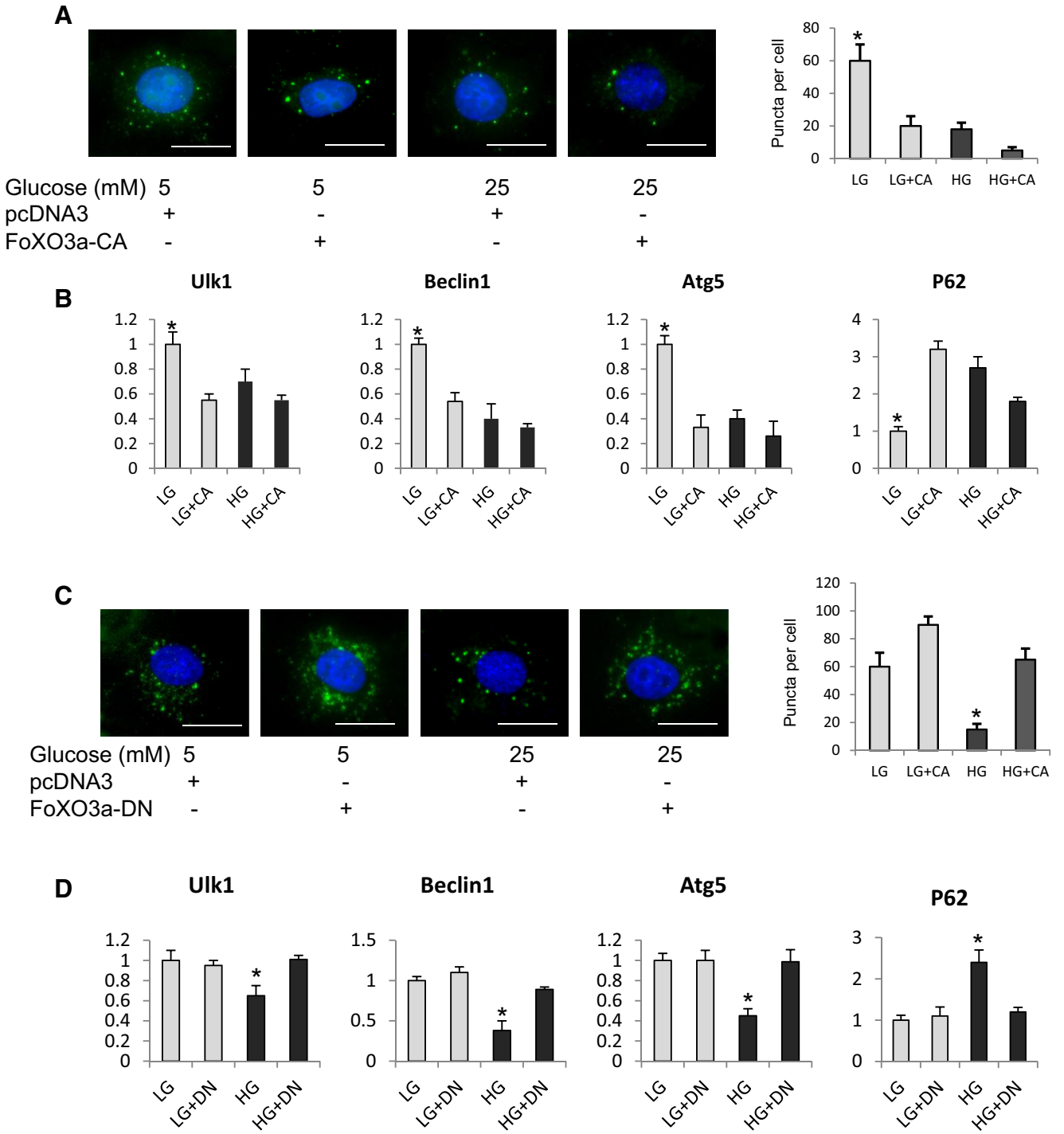
Xu et al. Impaired autophagy in maternal diabetes-induced neural tube defects. Am J Obstet Gynecol 2019.

confocal microscope (LSM 710; Zeiss, Oberkochen, Germany). All images in a given figure were taken with the same setting.

Statistical analyses

All experiments were repeated in triplicate. Data are presented as means ± SE. Student's t test was used for comparisons between 2 groups. One-way ANOVA or two-way ANOVA analysis of variance was performed for >2 group comparisons using the software (SigmaPlot 12.5;

FIGURE 2
Dominant negative (DN) FoxO3a reverts high glucose (HG)-suppressed autophagy

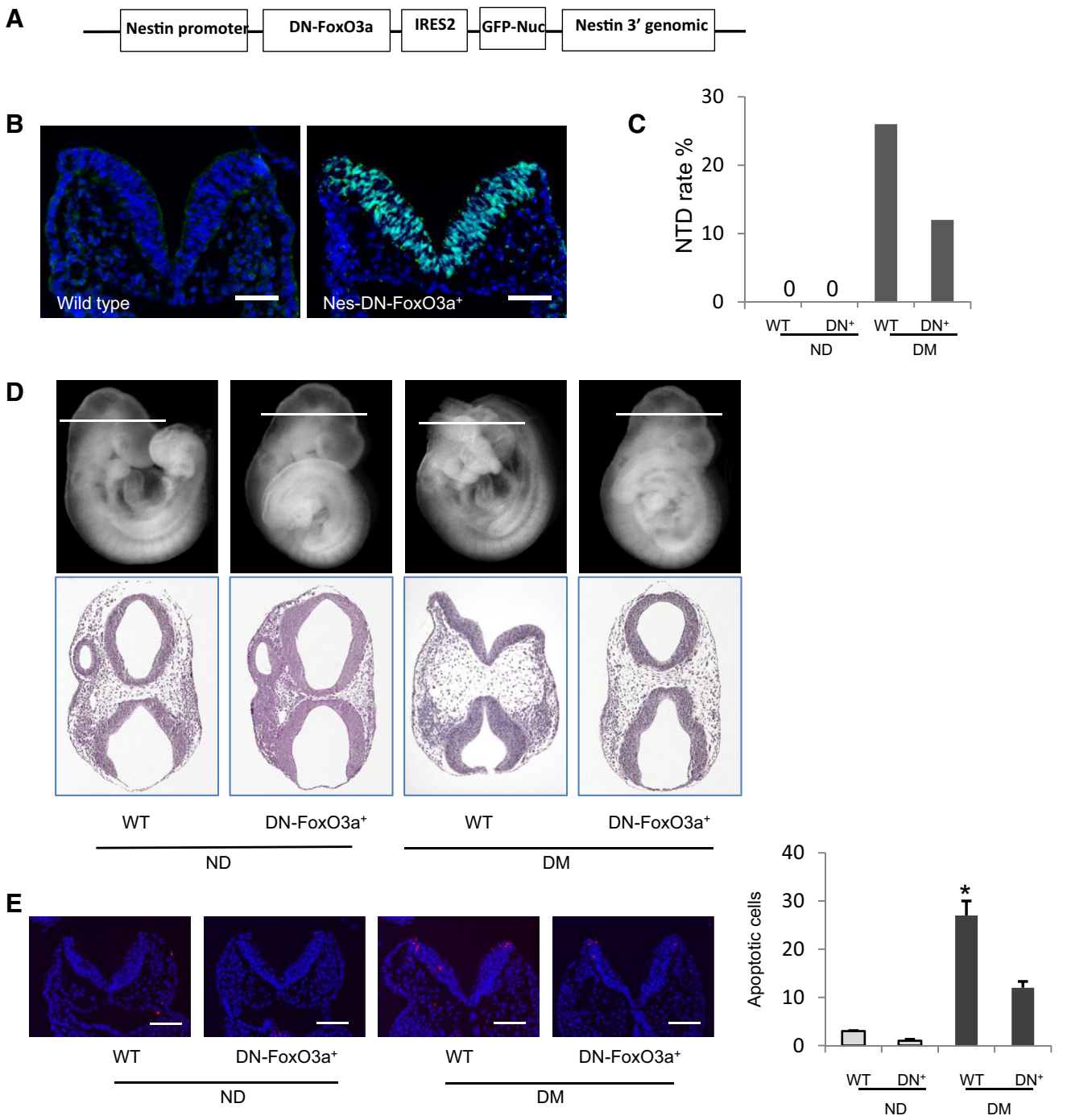


A and **C**, Representative Cyto-ID staining (green) images and quantification of autophagosomes in C17.2 neural stem cells. Blank pcDNA3 plasmids were used as control. Cell nuclei counter-stained with 4',6-diamidino-2-phenylindole, bars = 15 μm. **A**, Low glucose (LG) (5 mmol/L) or HG (25 mmol/L) culture conditions. C17.2 cells were transfected with constitutively active (CA) FoxO3a plasmids. **C**, C17.2 cells were transfected with DN FoxO3a. Messenger RNA levels of autophagy related genes in 4 groups of those shown in **B**, A; and in **D**, C. mRNA relative fold change is normalized by β-actin. Experiments were repeated 3 times (n = 3). *Significant difference compared to other groups ($P < .05$).

CA, constitutively active; *DN*, dominant negative; *LG*, low glucose.

Xu et al. Impaired autophagy in maternal diabetes-induced neural tube defects. Am J Obstet Gynecol 2019.

FIGURE 3
Dominant negative (DN) FoxO3a overexpression ameliorates apoptosis and neural tube defects (NTDs) in diabetic pregnancy



A, Schematic of DN FoxO3a transgenic construct. **B**, Green fluorescent protein (GFP) antibody staining on embryonic day (E) 8.5 wild-type (WT) and transgenic positive embryos. **C**, Neural tube defect (NTD) rates in E9.5 WT and DN FoxO3a transgenic embryos from nondiabetic or diabetic dams. **D**, Representative images of E9.5 embryos; positions of sections used for hematoxylin-eosin staining (white lines of whole embryos). **E**, Representative images and quantifications of terminal deoxynucleotidyl transferase 2′-deoxyuridine 5′-triphosphate nick end labeling assays. Apoptotic cells in V-shape of neuroepithelium (red). **B** and **E**, Cell nuclei counter-stained with 4',6-diamidino-2-phenylindole, bars = 30 μm. *Significant difference compared to other groups ($P < .05$).

CA, constitutively active; *DM*, diabetes mellitus; *DN*, dominant negative; *IRES*, internal ribosome entry site; *ND*, nondiabetes.

Xu et al. Impaired autophagy in maternal diabetes-induced neural tube defects. Am J Obstet Gynecol 2019.

SigmaStat, San Jose, CA). Statistical significance was indicated when $P < .05$.

Results

FOXO3a gene deletion abrogates the inhibition of maternal diabetes on autophagy

We have previously reported that maternal diabetes activates FoxO3a through dephosphorylation, and that deletion of the *FOXO3a* gene ameliorates maternal diabetes-induced NTDs.[11] Using FoxO3a KO mice, we found that the ratio of phosphorylated FoxO3a to total FoxO3a was significantly reduced in E8.5 embryos from diabetic dams, compared with embryos from nondiabetic dams. We also observed that phosphorylated FoxO3a was diminished in embryos of *FOXO3a* KO mice (Figure 1, A).

To determine the impact of FoxO3a on autophagosome formation in neurulation stage embryos, the abundance of LC3II, an index of autophagosome formation, was assessed (Figure 1, A). Maternal diabetes significantly decreased LC3II protein expression in E8.5 embryos, whereas expression levels of LC3II in embryos of FoxO3a KO diabetic dams was comparable to expression levels in embryos of nondiabetic dams (Figure 1, A). Under nondiabetic conditions, the expression level of LC3II in embryos from FoxO3a KO dams showed no difference when compared to WT embryos (Figure 1, A). Similarly, the number of LC3-positive puncta, an index of autophagosomes, in neuroepithelial cells from embryos of diabetic dams were significantly reduced by maternal diabetes, and *FOXO3a* deletion restored the number of LC3-positive puncta to that of the nondiabetic groups (Figure 1, B). Maternal diabetes also suppressed the expression of genes that promote autophagic activity, including Ulk1, Beclin1, and Atg5, and up-regulated the negative autophagy regulator p62 (Figure 1, C). *FOXO3a* deletion rescued the expression of Ulk1, Beclin1, and Atg5 and abrogated the increase of p62 induced by maternal diabetes (Figure 1, C).

FoxO3a activation mimics high glucose in inhibiting autophagy

High glucose significantly decreased the number of autophagic puncta in C17.2 neural stem cells (Figure 2, A). Constitutively activated FoxO3a mimicked the inhibitory effect of high glucose on autophagosome formation in cells cultured under normal glucose conditions (Figure 2, A). Both high glucose and constitutively activated FoxO3 suppressed the expression of Ulk1, Beclin1, and Atg5, and increased the expression of p62 (Figure 2, B). There was no synergistic effect on autophagy inhibition between high glucose and constitutively activated FoxO3a (Figure 2, A and B). DN FoxO3a blocked the inhibitory effect of high glucose on autophagosome formation (Figure 2, C). Cells transfected with DN-FoxO3a cultured under high-glucose conditions showed comparable expression levels of Ulk1, Beclin1, Atg5, and p62 to those in cells cultured under normal-glucose conditions (Figure 2, D).

Transgenic mice of the DN FoxO3a mutant is resistant to diabetic embryopathy

To further determine whether the TAD of FoxO3a is necessary to elicit the effects of FoxO3a activation on NTD formation and autophagy suppression under maternal diabetic conditions, we generated a mouse line that specifically overexpressed DN FoxO3a, which lacks the TAD, in the developing neuroepithelium (Figure 3, A). The transgene DN FoxO3a, along with a separate cell nucleus localized GFP, were driven by the Nestin promoter from E8.0 onward.[5] GFP localized in the nucleus confirmed neuroepithelium-specific expression of the transgenes (Figure 3, B). The NTD rate in DN FoxO3a embryos was significantly lower than in WT embryos under maternal diabetic conditions (Figure 2, C and D, and Table 2). Blood glucose levels in diabetic dams were 3 times greater than those in nondiabetic dams (Table 1). The DN FoxO3a embryos were morphologically indistinguishable from their WT litter mates (Figure 2, D). The number of terminal deoxynucleotidyl transferase 2′-deoxyuridine 5′-triphosphate nick end labeling positive apoptotic cells in the developing neuroepithelium was significantly higher in WT embryos of diabetic dams compared to that in embryos from nondiabetic dams (Figure 3, E). The increase in neuroepithelial cell apoptosis by maternal diabetes was significantly blunted by DN FOxO3a overexpression (Figure 3, E).

DN FoxO3a rescues autophagy in the developing neuroepithelium exposed to diabetes

To investigate whether DN FoxO3a expression in the developing neuroepithelium restored autophagy, indices of autophagic activity were assessed in neurulation stage embryos. Immunoblotting data showed that total FoxO3a protein was significantly increased in DN FoxO3a embryos (Figure 4, A). The ratio of phosphorylated-FoxO3a to total FoxO3a was significantly decreased in DN FoxO3a embryos exposed to diabetes (Figure 4, A), indicating that FoxO3a activity was increased by maternal

TABLE 2
Dominant negative FoxO3a overexpression in neuroepithelium ameliorates diabetes-induced neural tube defects

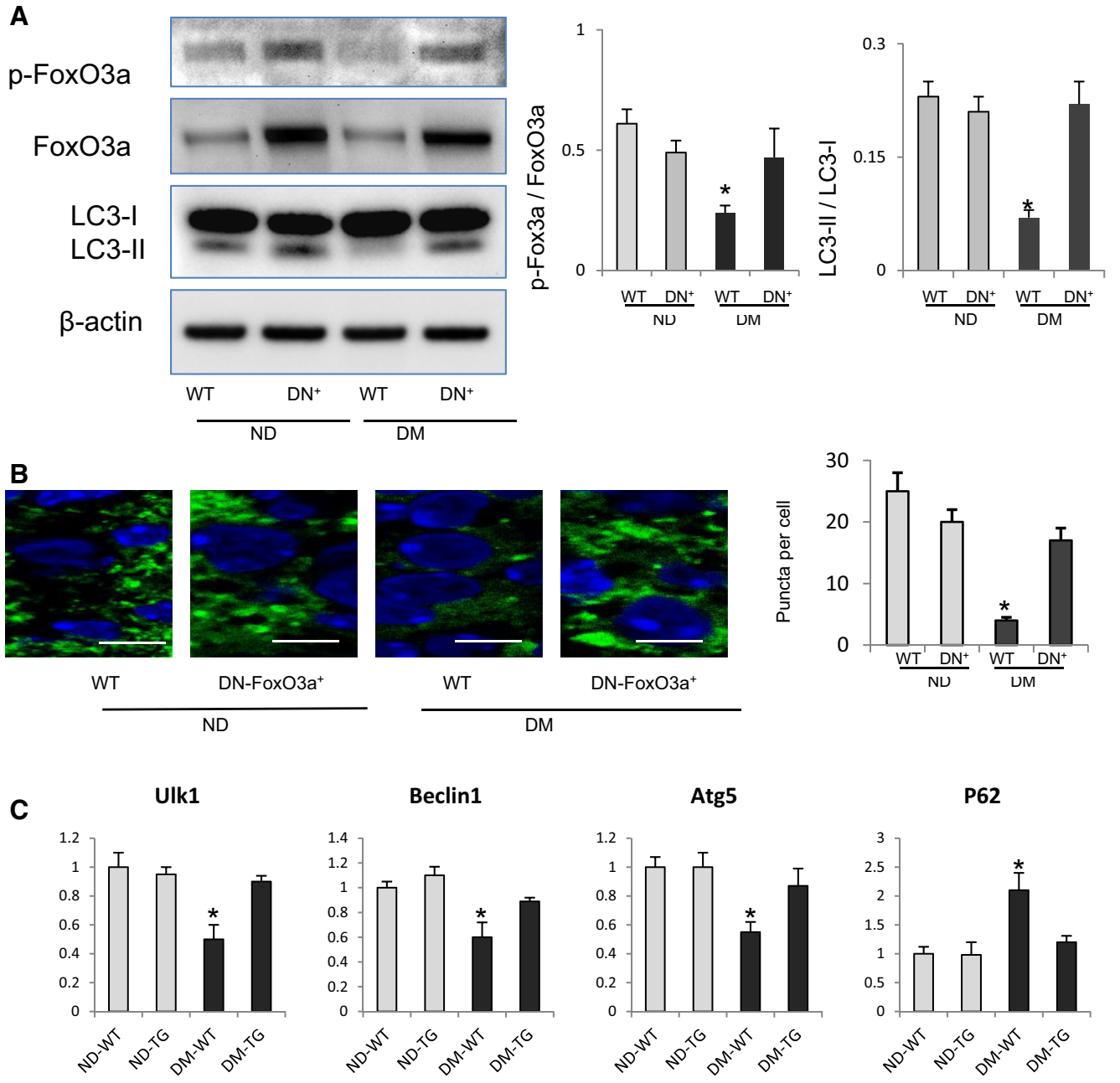
Group (dams)	Blood glucose, mg/dL	Total embryos	Embryos with NTD	NTD rate, %
Nondiabetic (n = 10)	139.5 ± 21.9	WT 43	0	0
		DN FoxO3a 42	0	0
Diabetic (n = 10)	444.5 ± 46.2	WT 41	11	26.8[a]
		DN FoxO3a 42	5	11.9

DN, dominant negative; *NTD*, neural tube defect; *WT*, wild-type.

[a] Significant difference compared with other groups in Fisher exact tests ($P < .05$).

Xu et al. Impaired autophagy in maternal diabetes-induced neural tube defects. Am J Obstet Gynecol 2019.

FIGURE 4
Dominant negative (DN) FOxO3a overexpression rescues autophagy in diabetic pregnancy



A, Protein levels of p-FoxO3a, FoxO3a, and light chain (LC)3 in embryonic day (E)8.5 wild-type (WT) and DN FoxO3a transgenic embryos from nondiabetic or diabetic dams. Quantification of relative expression levels of p-FoxO3a vs total FoxO3a and LC3II vs LC3I shown in 2 graphs. **B**, Confocal images of immunostaining for LC3 in neuroepithelial cells of E8.5 embryos. LC3 punctate foci with diameter ≥20 pixels were quantified by ImageJ, bars = 5 μm. **C**, Messenger RNA levels of autophagy related genes. mRNA relative fold change is normalized by β-actin. Experiments were performed using 3 embryos from 3 different dams per group (n = 3). *Significant difference compared to other groups ($P < .05$).

CA, constitutively active; *DM*, diabetes mellitus; *DN*, dominant negative; *ND*, nondiabetes; *TG*, transgenic.

Xu et al. Impaired autophagy in maternal diabetes-induced neural tube defects. Am J Obstet Gynecol 2019.

diabetes. The ratio of phosphorylated FoxO3a to total FoxO3a in DN FoxO3a embryos was restored to the level in embryos of nondiabetic dams (Figure 4, A).

In addition, in DN FoxO3a embryos LC3II protein abundance was significantly decreased by maternal diabetes, whereas LC3II protein abundance was comparable to that found in embryos of nondiabetic dams (Figure 4, A). The number of LC3 puncta in neuroepithelial cells was significantly reduced by

maternal diabetes, and DN FoxO3a expression rescued LC3 punta number (Figure 4, B).

Maternal diabetes-suppressed expression of Ulk1, Becn1, and Atg5 were reverted back to the level in embryos of nondiabetic dams by DN FoxO3a overexpression (Figure 4, C). Maternal diabetes-increased p62 expression was abrogated by DN FoxO3a overexpression (Figure 4, C).

Comment

The principal findings of the present study are that the transcription factor FoxO3a mediates the inhibitory effect of high glucose in vitro and maternal diabetes in vivo on autophagy by altering autophagy gene expression. Deleting the TAD of FoxO3a restores autophagy suppressed by maternal diabetes and, thus, ameliorates NTDs in diabetic pregnancy. In the present study, the autophagy positive regulators, Ulk1, Becn1, and Atg5, and the autophagy inhibitor, p62, were uncovered as FoxO3a-responsive genes. We showed that FoxO3a repressed the expression of Ulk1, Becn1, and Atg5, and stimulated p62 expression. Deleting FoxO3a or inactivating WT FoxO3a under diabetic conditions restored autophagy. Therefore, our results indicate that FoxO3a regulates diabetes-induced NTDs via suppression of autophagy. Therefore, the transcription activity of FoxO3a is required for high glucose in vitro and maternal diabetes in vivo-suppressed autophagy.

The present study revealed the importance of FoxO3a in autophagy impairment of diabetic embryopathy. Previous has been shown that FoxO3a is critically involved in the induction of diabetic embryopathy. It has been demonstrated that FoxO3a upregulates proapoptotic gene expression in diabetic embryopathy.[11] In contrast, the present study revealed that FoxO3a repressed Atg expression. These findings increased our understanding in the gene regulation program controlled by FoxO3a in diabetic embryopathy. It has been known that FoxO3a is regulated by oxidative stress-induced kinase signaling. Oxidative stress is the central mechanism in the induction of diabetic embryopathy. It has been shown that maternal diabetes induces oxidative stress by enhancing the production of endogenous reactive oxygen species and decreasing cellular antioxidant defense enzyme activity.[8,9,17,25–27] Studies using the transgenic mouse model that overexpresses the antioxidant enzyme, superoxide dismutase 1, have demonstrated that eliminating reactive oxygen species caused by maternal diabetes can effectively resolve cellular stress and proapoptotic signaling.[28–30] In complementary with the previous findings that FoxO3a mediates the proapoptotic effect of maternal diabetes, the present study further discovered that the transcription activity of FoxO3a is essential for cell apoptosis because deletion of the FoxO3a transactivation domain abolished maternal diabetes-induced neuroepithelial cell apoptosis and NTD formation. Supplements containing natural antioxidants, such as curcumin (found in the spice turmeric) and epigallocatechin-3-gallate (EGCG) (found in green tea), also effectively suppress cellular stress, apoptosis, and NTD formation in diabetic pregnancy.[9,26,31] Specifically, EGCG can block maternal diabetes-induced FoxO3a activation.[31] This experimental evidence supports the hypothesis that FoxO3a is a true downstream effector of oxidative stress in diabetic embryopathy. While the current study extends the function of FoxO3a as an autophagy negative regulator, the FoxO3a upstream intermediators, oxidative stress and its activated kinases, are also presumptive negative autophagy regulators.

We have shown previously that FoxO3a is regulated by the proapoptotic kinase, apoptosis signal-regulating kinase 1 (ASK1), and its downstream c-Jun N-terminal kinases 1/2 (JNK1/JNK2) in diabetic embryopathy, and that deletion of *Ask1*, *Jnk1*, or *Jnk2* abrogates the increase of FoxO3a activity in neurulation stage embryos exposed to maternal diabetes.[11,32] The prosurvival kinase, Akt (also known as protein kinase B), negatively regulates FoxO3a by phosphorylation.[33] Under diabetic or high-glucose conditions, Akt activity is significantly down-regulated, thereby increasing the amount of dephosphorylated (activated) FoxO3a in the cell. The regulation of FoxO3a in the context of autophagy regulation is not investigated in the current study because the focus of this study is to reveal whether FoxO3a suppresses autophagy. However, the findings indicate that FoxO3a is multifunctional in mediating the teratogenesis of maternal diabetes.

Clinical implications

Alterations in autophagy are observed in many pregnancy complications, including placental insufficiency, fetal growth restriction, preterm birth, and stillbirth.[20–24] Thus, findings in the present study may further simulate research into the role of autophagy impairment in the cause of maternal-fetal complications. Many components of the upstream pathway that negatively regulate autophagy in diabetic pregnancy are implicated in the pathogenesis of many maternal-fetal complications. These components include oxidative stress,[19,31–35] proapoptotic kinases,[20,34] and the transcription factor FoxO3a.[17,31] Linking autophagy regulation with the proposed oxidative stress-ASK1/JNK1/2-FoxO3a pathway may be a future direction in uncovering the causes of pregnancy complications. We have previously reported that the naturally occurring compound trehalose is a potent autophagy activator and prevents diabetes-induced NTDs.[14] Testing the beneficial effects of natural compounds, such as trehalose, as autophagy activators will be an important step in the development of possible therapeutics in treating pregnancy complications related to diabetes. Trehalose, a natural disaccharide, is produced in bacteria, yeast, insects, fungi, and plants but not in vertebrates.[35] It consists of 2 glucose molecules joined by the α, α-1, 1-glycosidic bond. Currently, it is being evaluated in several clinical trials

including trials on reversal of arterial aging (ClinicalTrials.gov identifier: NCT01575288), oculopharyngeal muscular dystrophy (ClinicalTrials.gov identifier: NCT02015481), and spinocerebellar ataxia 3 (ClinicalTrials.gov identifier: NCT02147886). Trehalose has been used as a food stabilizer and the Food and Drug Administration characterizes trehalose as the Generally Regarded as Safe category. Further studies need evaluate the safety profile of trehalose in pregnancy.

Research implications

Autophagy is a relatively new intracellular organelle process and elucidating the role of autophagy dysregulation in maternal-fetal complications is an important area. Because oxidative stress is involved in many maternal-fetal complications,[21,36–40] investigating oxidative stress-altered autophagy will provide new mechanistic insights on the pathogenesis of these maternal-fetal complications. Immediate future study should also determine if mitigating oxidative stress by either superoxide dismutase 1 in vivo expression or dietary supplements of EGCG or curcumin would restore autophagy in the developing neuroepithelium through inactivation of FoxO3a.

Because ASK1 and JNK1/2 are upstream of FoxO3a, altered activity of these kinases may also impact autophagy in the developing neuroepithelium under diabetic or high-glucose conditions. Future studies may evaluate the outcome of deleting *Ask1*, *Jnk1*, or *Jnk2* on maternal diabetes-suppressed autophagy. Likewise, using a constitutively active Akt mouse model may alleviate autophagy inhibition by maternal diabetes. Altered kinase activity is involved in the etiology of an array of maternal and fetal complications including in utero growth restriction and preterm birth.[20,34] Studying the potential relationship between kinase activity and autophagy regulation may reveal new mechanistic insights underlying the cause of these pregnancy complications.

Previous studies have supported the notion that FoxO3a differentially regulates gene expression, and that FoxO3a repression of gene transcription relies on histone deacetylases.[33] We have found that maternal diabetes alters the expression of histone deacetylases in neurulation stage embryos.[41] Therefore, it is possible that the cross-talk between FoxO3a and histone modifiers may be required for autophagy inhibition in diabetic embryopathy and warrants further study.

One FoxO3a-responsive gene, tumor necrosis factor receptor type 1-associated death domain (TRADD), has been identified as playing a role in diabetic embryopathy.[11,42] TRADD is a proapoptotic factor leading to caspase 8-dependent apoptosis in neuroepithelial cells.[11,41] The role of TRADD in autophagy regulation is unknown. Therefore, future studies may elucidate whether TRADD participates in autophagy impairment in diabetic embryopathy.

Strengths and weaknesses

The strengthens of our study are the use of genetically modified mouse models in determining the negative regulation of autophagy by FoxO3a, the delicate design of the animal studies in the cause of diabetic embryopathy, and the direct mechanistic insights gaining from animal studies. One of the weaknesses of our study is the lack of immediate clinical impact. The study is not a human study due to the inaccessible human fetal tissues. Nevertheless, diabetic embryopathy is a significant health problem and the current study has potential translational value. ■

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Glossary of Terms

Diabetic embryopathy: Embryonic developmental deficiency in the prenatal or postnatal fetus induced by maternal diabetes in pregnancy.

Neural tube defects (NTDs): Congenital abnormalities resulting from the failure of neurulation, a process involving the formation of the primitive brain and spinal cord during embryonic development.

Autophagy: Autophagy (or autophagocytosis) is the natural, regulated, destructive mechanism of the cell that disassembles unnecessary or dysfunctional components. Autophagy allows the orderly degradation and recycling of cellular components. In macroautophagy, targeted cytoplasmic constituents are isolated from the rest of the cell within a double-membraned vesicle known as an autophagosome. The autophagosome eventually fuses with lysosomes and the contents are degraded and recycled. Three forms of autophagy are commonly described: macroautophagy, microautophagy, and chaperone-mediated autophagy. In disease, autophagy has been seen as an adaptive response to stress that promotes survival, whereas in other cases it appears to promote cell death and morbidity. In the extreme case of starvation, the breakdown of cellular components promotes cellular survival by maintaining cellular energy levels.

Autophagy process-autophagosome: During the autophagy process, a double-membrane vacuole is formed, which engulfs dysfunctional cellular components and eventually fuses to lysosome for the degradation of such components.

FoxO3a: Forkhead box transcription factor O3a is a human protein encoded by gene *Foxo3a.* As a transcription factor characterized by a distinct forkhead DNA-binding domain, FOXO3a is involved in many important biological processes. For example, in the PI3 kinase signaling pathway, FoxO3a can be translocated out the nucleus in its phosphorylation form by Akt. FoxO3a plays an important role in triggering apoptosis by up-regulating genes involved in the cell death pathways.

Dominant negative (DN): A mutation leading to a gene product that adversely affects the normal product (wild-type gene product) in a cell. Usually, a DN mutation leads to the loss of function of a specific protein.

LC3: Microtubule-associated protein 1 light chain 3, also called autophagy-related gene 8. The convention of cytosolic LC3I (18 kDa) to autophagosome membrane bounded LC3II (16 kDa) is a critical event in autophagy.

LC3 lipidation: The covalent attachment of LC3 to the lipid phosphatidylethanolamine that is an essential step for autophagosome formation.

C17.2: The C17.2 cell line is a clone of neural stem cells isolated from the external germinal layer of the neonatal mouse cerebellum. C17.2 cells have a potential to differentiate into a variety of cell types, such as neurons, oligodendrocytes, and astrocytes, when they are transplanted into the appropriate part of the central nervous system in vivo or induced by neural factors in vitro.

miR-129-2: MicroRNA-129-2 is a member of the microRNA-129 precursor family. It was recognized as a tumor suppressor by affecting cell migration, proliferation, and apoptosis through down-regulating the Sox4 gene in tumor cells. In diabetic embryopathy, it is up-regulated by maternal diabetes and suppresses autophagy.

Endoplasmic reticulum stress: The endoplasmic reticulum is the cellular organelle that modifies and correctly folds newly synthesized proteins into 3-dimensional structures. Accumulation of misfolded proteins in the endoplasmic reticulum lumen causes endoplasmic reticulum stress, which, in turn, activates the unfolded protein response and leads to apoptosis.

Unfolded protein response: The accumulation of unfolded or misfolded proteins in the endoplasmic reticulum lumen triggers the activation of 3 signaling pathways: the kinase of inositol-requiring enzyme 1-α pathway, the protein kinase R-like endoplasmic reticulum kinase pathway, and the activating transcription factor 6 pathway. These 3 pathways are collectively called the "unfolded protein response." Prolonged unfolded protein response induces cell apoptosis.

Trehalose: Trehalose is a natural disaccharide consisting of 2 molecules of glucose. It is produced by bacteria, yeast, insects, fungi, and plants. Trehalose has been used as a stabilizer in food products and is designated as Generally Regarded as Safe by the Food and Drug Administration. Trehalose can activate autophagy and is being examined in several clinical trials in treating human diseases. There are several clinical trials using trehalose in treating the following diseases: arterial aging (ClinicalTrials.gov identifier: NCT01575288), oculopharyngeal muscular dystrophy (ClinicalTrials.gov identifier: NCT02015481), and spinocerebellar ataxia 3 (ClinicalTrials.gov identifier: NCT02147886).